N- and C-terminal amino acids of [alpha]-actinin and studies on the effects of [alpha]-actinin on F-actin

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N- and C-terminal amino acids of α-actinin and studies on the effects of α-actinin on F-actin

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Inderjit Singh

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LIST OF ABBREVIATIONS

Å = angstrom = 10^-8 centimeters
ADP = adenosine 5'-diphosphate
ATP = adenosine 5'-triphosphate
deoxy ATP = deoxyadenosine 5'-triphosphate
ATPase = adenosine triphosphatase
AET = 2-amino ethyl-isothiouronium
°C = degree centigrade
CAF = calcium activated proteolytic factor
CPA = carboxypeptidase A
CPB = carboxypeptidase B
CTP = cytidine 5'-triphosphate
CNT = 3-carboxylate-4-nitrothiophenolate
dCTP = deoxycytidine 5'-triphosphate
DFP = diisopropyl fluoroephosphate
dl/gm = deciliter/gm
DNS-Cl = dimethyl aminonaphthalene-5-sulfonyl chloride
DTNB = 5,5'-dithiobis (2-nitrobenzoic acid)
DTT = dithiothreitol
EDTA = ethylenediaminetetraacetic acid
EGTA = 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane
g = acceleration due to gravity = 980 cm/sec
gm = gram
GTP = guanosine 5'-triphosphate
dGTP = deoxyguanosine 5'-triphosphate
HPT = hydroxypatite
ITP = inosine 5'-triphosphate
ITPase = inosine triphosphatase
M = molar
μM = micromolar
μ = micron
mM = millimolar
MCE = 2-mercaptoethanol
mg = milligram
NAD = nicotinamide adenine dinucleotide
NADH = nicotinamide adenine dinucleotide, reduced form
NEM = N-ethyl maleimide
nm = nanometer
NTPase = nucleoside triphosphatase
Pi = inorganic phosphate
S_20,w^0 = sedimentation coefficient
SDS = sodium dodecylsulfate
n_sp = specific viscosity
S.E. = standard error
TCA = trichloroacetic acid
TBS = 2,4,6 trinitrobenzene sulfonate
Tris = tris-(hydroxymethyl)-aminomethane
TAME = p-toluene sulfonyl-L-arginine methyl ester
UTP = uridine 5′-triphosphate

ι/2 = an ionic strength calculated on molarity basis
I. INTRODUCTION

Biological movement systems have attracted increasing research interest during the last several years, and it is becoming evident that movement in widely different systems involves proteins similar or identical to those found in skeletal muscle. Because skeletal muscle can be obtained in large amounts, it is the most thoroughly characterized movement system in biology, and findings in skeletal muscle have formed the foundation for many recent studies in other movement systems. Skeletal muscle movement, or contraction, has been shown to result from specific interactions of two proteins, myosin and actin, with Mg$^{2+}$ and ATP. The details of how these interactions transform chemical energy of ATP into mechanical movement, however, remain unclear. Although the actin-myosin-Mg-ATP system is sufficient for *in vitro* contractile responses, this system operates without control; i.e., contraction in this system begins upon addition of ATP and continues until ATP is exhausted. Consequently, the actin-myosin-Mg-ATP system contains no mechanism for relaxation or stopping contraction in the continued presence of ATP as is necessary in living organisms.

The search for a mechanism for skeletal muscle contraction has intensified in recent years with the availability of new and powerful techniques for assaying protein homogeneity and for studying protein-protein or protein-ligand interaction.
This search has both stimulated and been stimulated by the discovery of several new proteins that constitute the myofibril or the contractile threads in skeletal muscle. In addition to tropomyosin, which was discovered in 1948 but whose role remained enigmatic until the last several years, the proteins, troponin, α-actinin, β-actinin, C-protein, and M-line protein, have all been isolated from the myofibril in the last ten years. As the result of a large amount of experimental effort by many different laboratories, troponin and tropomyosin together have been shown to function directly in initiation and cessation of skeletal muscle contraction. M-line proteins and C-protein seem to have a role in assembly of myosin molecules into filaments, and β-actinin ostensibly functions to regulate assembly of actin into filaments. The role of α-actinin, however, has remained unclear. Because α-actinin has recently been shown to reside only in the Z-disk of skeletal muscle myofibrils, it might be suggested that α-actinin serves as an anchor to fasten actin filaments in the proper three dimensional array and thereby permit development of a directional force when actin interacts with myosin. This suggestion is supported by the finding that among the myofibrillar proteins, α-actinin binds only to the actin filament; it does not bind to unpolymerized actin.

On the other hand, when added to actin-myosin-Mg-ATP
systems in vitro, α-actinin clearly strengthens and accelerated the in vitro measures of contraction. Hence, α-actinin seems to affect the actin-myosin interaction in in vitro systems. The suggestion that α-actinin alters contractile response in skeletal muscle and therefore may be directly involved in the actin-myosin contractile interaction is supported by the finding that the effects of α-actinin on in vitro contractile responses are specific for the cations and nucleotides that are necessary for tension development. Because α-actinin is located only in the Z-disk in skeletal muscle and because the Z-disk is nearly 0.8 μm distant from the overlap of thick and thin filaments where the actin-myosin interaction occurs, it is clear that if α-actinin affects the actin-myosin interaction, it must do so by affecting the structure of the actin filament. Furthermore, this effect must be transmitted 0.8 μm along the actin filament to the region of actin-myosin overlap. Such a long propagation of conformational changes in protein structure have not yet been observed in biology, and it is necessary to establish that α-actinin can alter the structure of actin filaments before a direct role of α-actinin in the actin-myosin interaction can be considered seriously. Determining the biological role of α-actinin has become increasingly intriguing during the last two years when α-actinin has been discovered in every movement system that
has been examined for its presence.

This study, therefore, has been directed at the broad goal of ascertaining the biological function of α-actinin in movement systems. This objective has been approached in two ways. First, several studies were done to determine whether binding of α-actinin to actin filaments alters actin structure. Viscosity studies were done to insure that α-actinin bound to actin under the conditions used. Because tropomyosin also binds to actin filaments in vitro and constitutes part of the actin or thin filament in vivo, the effect of tropomyosin on α-actinin binding to actin and the ability of α-actinin to alter actin structure was also studied.

After ascertaining by viscometric studies that α-actinin bound to polymerized actin in the presence of ATP and tropomyosin, the effect of α-actinin on the ability of actin to cleave the terminal phosphate off ATP was studied. The ATPase activity of actin has been reported to result from transient perturbations in the structure of polymerized actin, and any effect of α-actinin on ATPase activity might, therefore, be presumed to result from an effect of α-actinin on these transient structural perturbations. Secondly, some clues to the precise structural relationship among α-actinin, the thin or actin filament, and the Z-disk were sought by studying the N- and C-terminal amino acids of highly purified α-actinin before and after treatment with an
enzyme that removes Z-disks.

The Z-disk, where α-actinin is located in skeletal muscle, is composed of two parts. One part is a lattice consisting of filaments that ostensibly join directly to actin or thin filaments. The other part is an amorphous matrix that seems to envelop the Z-lattice filaments. Purified α-actinin was treated with a recently discovered proteolytic enzyme that removes Z-disks without causing large changes in the size of either α-actinin or actin molecules. If α-actinin constitutes the Z-filaments that join directly to the actin filaments, it seems very unlikely that the enzyme could remove Z-disks without cleaving the peptide chain of either α-actinin or actin. If, on the other hand, α-actinin constitutes the amorphous matrix, then α-actinin could be released by proteolytic destruction of the Z-filaments. Because proteolytic removal of a small peptide from either the N- or C-terminal ends of α-actinin or actin could be sufficient to remove Z-disks without causing a large change in molecular size of the α-actinin or actin molecule, the N- and C-terminal amino acids of α-actinin and actin were studied before and after treatment with the enzyme.

It is obvious, of course, that because all the experiments described in this thesis were done with in vitro systems and purified proteins, the results of these
experiments can only indicate whether it is possible or impossible for α-actinin to have an active role in strengthening the actin-myosin interaction in vivo. Experiments on myofibrils are required to show whether α-actinin actually participates in the contractile interaction. Results from in vitro systems such as those used in this study, however, are needed to provide evidence on the mechanism of α-actinin's action and thereby provide guidance for the design of experiments using intact myofibrils.
II. LITERATURE REVIEW

Before reviewing the existing information on α-actinin and actin, and the possible role of the α-actinin-actin interaction in Z-disk structure and in contraction of skeletal muscle myofibrils, it is necessary to discuss very briefly some of the nomenclature and methodology that is unique to muscle biology, and to describe some of the subtle distinctions between contractile protein preparations and why these distinctions are important when considering the effects of α-actinin on actin.

First, although the two proteins, actin and myosin, are both necessary and sufficient for an in vitro contractile response, the actomyosin complex can be obtained experimentally in several forms that differ from one another in important ways. A 24-hour, high-ionic strength (\(I/2 = 0.6\) or more) extract of minced muscle yields a viscous, translucent solution called "natural actomyosin". Natural actomyosin, or myosin B as it sometimes is called, contains actin and myosin in some variable proportion (usually between three and four parts of myosin to one part of actin by weight) depending on length and pH of the extraction and on condition of muscle being extracted. Natural actomyosin contains the regulatory proteins, tropomyosin, troponin, α-actinin, β-actinin, C-protein, and M-line proteins in addition to actin and myosin, and these regulatory proteins
may also exist in different proportions depending on extracting conditions. Because of this variability in composition and because natural actomyosin already contains α-actinin, addition of more α-actinin produces only a very small response. For this reason, natural actomyosin is not useful as an in vitro assay system for α-actinin. Actomyosin can also be produced by preparing purified actin and purified myosin, and then mixing these two purified proteins together in a known ratio to produce "synthetic or reconstituted actomyosin". Reconstituted actomyosin contains no α-actinin, and therefore can be used to assay for the effects of added α-actinin on the actin-myosin interaction. Reconstituted actomyosin, however, has the disadvantage that two different protein preparations, which are both fairly long and complex, must be done simultaneously to prepare reconstituted actomyosin. When these two preparations are superimposed on the procedure required to make purified α-actinin (see later in this review), the preparative procedures required to study α-actinin become very onerous indeed.

Second, because this thesis involves only work done with in vitro systems, it is necessary to describe the two different assays used by muscle biochemists to measure in vitro contractile responses. The first of these assays is measurement of the specific activity of the Mg$^{2+}$-modified
ATPase of actomyosin suspensions. A number of experiments have shown that Mg$^{2+}$-modified ATPase activity is the only ATPase activity associated with shortening or tension development of myofibrils. It is presumed, therefore, that Mg$^{2+}$-modified ATPase activity of actomyosin suspensions is related to contractile activity in these suspensions and that a higher specific activity indicates a faster rate or greater extent of contractile activity. Because the actin-myosin complex is dissociated by ATP at ionic strengths above 0.3 (Eisenberg and Moos, 1967), assay of contractile activity by using Mg$^{2+}$ modified ATPase activity must be done at ionic strengths below 0.2, where the actomyosin complex is not soluble; hence these assays are done with suspensions. The second in vitro assay of contractile activity is based on Ebashi's finding (Ebashi, 1961) that, under proper conditions, turbidity of an actomyosin suspension increases dramatically after addition of ATP. The conditions required to cause this increase parallel the conditions required for tension development or shortening in isolated muscle fibers or myofibril. Consequently, it is widely accepted that this increase in turbidity of actomyosin suspensions upon addition of ATP reflects the amount of contractile activity in those suspensions. The nature of the physical phenomena causing the turbidity increase is unknown (Briskey, 1967b), but some loss of water from individual actomyosin particles
followed by clumping of these partly dehydrated particles to form larger particles which scatter more light must be involved. Comparison of the effects of different conditions on turbidity development of actomyosin suspensions and the effects of these same conditions on tension development or shortening of intact fibers suggests that the length of time required for turbidity development to begin after ATP has been added and the rate of increase of turbidity development after it has begun are related directly to rate of shortening. Some investigators have also suggested that the amount of turbidity increase is related to the extent of contraction, but because amount of turbidity increase is also obviously directly related to the amount of protein present, this latter assumption may not be valid in all instances (Arakawa et al., 1970b). These two assays of in vitro contractile activity are widely used by muscle biochemists, and they have been especially valuable in the study of α-actinin. Hereafter in this thesis, these two tests will simply be called the ATPase and the turbidity assays.

With this brief review of some of the nomenclature and methodology associated with the study of α-actinin, the properties of α-actinin, actin, and the Z-disk will be reviewed. The review will be divided into six major sections, but the discussion in each section will be based on material
covered in the preceding sections. The six major sections are: (1) discovery of α-actinin; (2) purification of α-actinin; (3) properties of α-actinin; (4) properties of actin; (5) Z-disk structure and enzymatic removal of Z-disks; and (6) the α-actinin/F-actin interaction and the role of α-actinin in muscle.

A. Discovery of α-Actinin

In 1964, Ebashi and coworkers (Ebashi et al., 1964) announced the discovery of a new protein that could be isolated from muscle and that increased the rate of turbidity development in reconstituted actomyosin suspensions. This new protein was subsequently named α-actinin (Ebashi and Ebashi, 1965). α-Actinin was discovered in low ionic strength extracts of the residue left after extraction of myosin from minced rabbit muscle. Ebashi and coworkers had been working with these low ionic strength extracts because one year earlier, they had discovered another new muscle protein, which they called native tropomyosin, in these extracts (Ebashi, 1963; Ebashi et al., 1964). Native tropomyosin had a remarkable ability to suppress the turbidity increase of reconstituted actomyosin in the absence of Ca^{2+}, but this suppression was completely relieved by the presence of 10^{-5} M Ca^{2+} in the turbidity assay. Native tropomyosin was subsequently shown to consist of two
components, tropomyosin, which had been known to be a component of myofibrils since 1948 (Bailey, 1948), and a new protein which Ebashi and coworkers named troponin (Ebashi and Kodama, 1965). Addition of native tropomyosin to reconstituted actomyosin, however, did not completely obviate the differences in turbidity response between synthetic actomyosin and natural actomyosin. In the presence of moderate concentrations of KCl (60-80 mM although Ebashi and coworkers could obtain the same effect at 30-50 mM) and ATP (1 mM), reconstituted actomyosin requires a long period of time after addition of ATP before giving a turbidity response, whereas natural actomyosin gives an immediate turbidity increase upon the addition of ATP (Ebashi et al., 1964). Because native tropomyosin suppresses the turbidity increase of actomyosin suspensions in the absence of Ca\(^{2+}\), addition of native tropomyosin did not remove the differences in the rate of turbidity development between natural and reconstituted actomyosin. On the other hand, when the crude, low ionic strength extract of a myosin-extracted residue was added to reconstituted actomyosin suspensions, these suspensions gave an immediate turbidity response upon addition of ATP, just as natural actomyosin suspensions did (Ebashi et al., 1964). This finding suggested that the crude, low ionic strength extracts contained another factor in addition to native tropomyosin, and that this second factor was
capable of enlarging the range of ionic strength and ATP concentration under which reconstituted actomyosin would give an immediate turbidity increase in response to ATP. Examination of the sedimentation pattern of the low ionic extracts in the analytical ultracentrifuge revealed the presence of a second component that sedimented more rapidly than native tropomyosin (Ebashi and Ebashi, 1965). This second component could be salted out of the low ionic strength extracts at low ammonium sulfate saturation (between 0 and 36% ammonium sulfate saturation), and when the component obtained by this salting out was added to reconstituted actomyosin suspensions, it greatly accelerated the rate at which these suspensions would give a turbidity increase in response to ATP. Ebashi et al. (1964), therefore, suggested that this new protein factor had an important role in contraction of living muscle because living muscle contracts at ionic strengths of 0.15 to 0.18 and at ATP concentrations of 3-5 mM, but it is impossible to obtain an in vitro turbidity increase from reconstituted actomyosin under these conditions. Ebashi and coworkers originally suggested (Ebashi et al., 1964) that α-actinin may be obligatory to obtain any turbidity response at all, regardless of ionic strength, and that all actin preparations made up to that time were contaminated with α-actinin. Ebashi et al. (1964) suggested that this contamination accounted for the ability
of muscle biochemists to obtain a turbidity increase with reconstituted actomyosin. Later, however, it was shown (Ebashi and Ebashi, 1965; Seraydarian et al., 1967) that under selected conditions, a turbidity response could be obtained with actomyosin especially prepared to contain no α-actinin. Consequently, α-actinin is not obligatory for an in vitro contractile response, but it greatly increases the rate of turbidity development of reconstituted actomyosin suspensions at high ionic strength and ATP concentration.

B. Purification of α-Actinin

All studies of α-actinin before 1969 used α-actinin prepared from low ionic strength extracts of myosin-extracted residue as originally described by Ebashi and co-workers (Ebashi and Ebashi, 1965; Ebashi et al., 1964). In this procedure, some of the myosin and sarcoplasmic proteins were removed from minced muscle by extraction with Guba-straub solution (0.3 M KCl, 0.15 M K-phosphate, pH 6.5) for 15 min. This extraction was followed by two washes of the myosin-extracted residue with 20 mM KCl, 2 mM NaHCO₃ and two more cold water washes. The washed residue was then extracted with water at room temperature for 4 hours, the extract was removed through filterpaper, and the residue was extracted for another 4 hours at room temperature with 1 mM NaHCO₃. This second extract was also removed through filter
paper, the two extracts were combined, and α-actinin was salted out between 0 and 36% ammonium sulfate saturation. The precipitate was dissolved in 1 mM NaHCO$_3$, and α-actinin was salted out of this solution between 0 and 18% ammonium sulfate saturation. After dissolving in 1 mM NaHCO$_3$ and dialyzing against 1 mM NaHCO$_3$ to remove the last traces of ammonium sulfate, this protein constituted the α-actinin used in all early experiments on α-actinin, although Seraydarian et al. (1967) later suggested that additional purification could be produced by salting this preparation out one or two additional times with 3.3 M KCl. Several claims were made that these preparations of α-actinin were "pure" (Briskey et al., 1967b; Nonomura, 1967; Seraydarian et al., 1967), but analytical centrifuge patterns (Briskey et al., 1967b; Seraydarian et al., 1967) showed that the protein in these preparations sedimented as a small 6S boundary with a very long leading edge indicative of the presence of heterogenous, large aggregates. The presence of these large aggregates was particularly evident early in the ultracentrifuge run. Although Seraydarian et al. (1967) stated that their α-actinin preparations purified by 3.3 M KCl precipitation sedimented primarily as a single boundary in the analytical ultracentrifuge, the ultracentrifuge patterns shown were of a preparation containing only 2.2 mg protein/ml, and it is
extremely difficult to detect heterogeneity in sedimentation diagrams done at such low protein concentration. Indeed, area under the 6 S boundary of these early α-actinin preparations was less than 30% of the area expected for the protein concentration in the ultracentrifuge, and Briskey et al. (1967b) mentioned that some rapidly sedimenting material was present in the ultracentrifuge patterns of more concentrated protein solutions, even in α-actinin preparations that had been purified by additional salting out with 3.3 M KCl. These early workers, however, believed that the 6 S α-actinin molecule had a pronounced tendency to aggregate, particularly in the presence of salt, and that any rapidly sedimenting material present in their α-actinin preparations was due simply to aggregation of the monomeric 6 S α-actinin species. This view is not too surprising because many of the myofibrillar proteins known at that time exhibited pronounced aggregating tendencies depending on conditions. For example, tropomyosin aggregates as salt concentration is lowered, actin aggregates dramatically to form long polymers when salt concentration is raised, and myosin's propensity to aggregate was just beginning to be understood at that time.

The view that the 6 S α-actinin species had pronounced aggregating tendencies was strengthened when Nonomura (1967) described the separation of an α-actinin
preparation made according to Ebashi and Ebashi (1965) into three different components by using prolonged differential centrifugation. The three components obtained were a 6 S component \((S_{20,w}^0 = 6.21)\), a 10 S component \((S_{20,w}^0 = 10.0)\), and a 25 S component \((S_{20,w}^0 = 25.6)\). Because all three of these components seemed approximately equally effective in accelerating the rate of turbidity development in reconstituted actomyosin suspensions, Nonomura (1967) concluded that all three components were \(\alpha\)-actinin and the larger components were simply aggregates of the 6 S component. This conclusion was supported by showing that in the presence of 4 M urea, the 6 S, 10 S, and 25 S fractions all dissociated to form a single 4 S species in the analytical ultracentrifuge. Removal of urea by dialysis caused reappearance of a 6 S boundary in the preparations that originally contained the 6 S species, and reappearance of a 10 S boundary in the preparations that originally contained 10 S species. Removal of urea from the preparations that originally contained 25 S species, however, resulted in reappearance of both 6 S and 10 S boundaries. Therefore, it seemed possible to convert the 25 S component into the 6 S and 10 S components by dissociation in urea followed by reconstitution, but the same procedure did not convert the 10 S component into the 6 S component. Although it was evident from the ultracentrifuge diagrams shown (Nonomura, 1967) that
Nonomura's three components were not homogenous after their separation by a single differential centrifugation. Nonomura (1967) nevertheless estimated the molecular weights of the 6 S and the 25 S components as 162,000 and 3,200,000 daltons, respectively, when determined by sedimentation equilibrium techniques, and as 156,000 and 3,3600,000 daltons, respectively, when determined by light scattering measurements. Based on Nonomura's assumption of spherical molecules, molecular diameters of 78 Å and 214 Å were calculated for the 6 S and the 25 S components, respectively (Nonomura, 1967). Both the 6 S and 25 S components were described as globular, although the reduced viscosity of 0.135 dl/g that Nonomura reported for both components is considerably greater than the 0.03 to 0.04 dl/g generally accepted as the reduced viscosity of completely spherical molecules. The accuracy of Nonomura's measurements was difficult to assess at the time of his report, not only because of the obvious heterogeneity of his preparations, but also because his sedimentation equilibrium and viscosity experiments were done in 1 mm NaHCO₃, an ionic strength that should cause considerable theoretical difficulties with these two procedures.

The early news that α-actinin was simply a myofibrillar protein with a propensity for aggregation that is normal for myofibrillar proteins soon encountered several
difficulties. First, Masaki et al. (1967) found that although the 6 S and 25 S components isolated by Nonomura seemed immunologically similar, both these components were immunologically different from 10 S component. Consequently, it seemed unlikely that the 10 S component could be an aggregate of the 6 S component. Second, Goll et al. (1967; 1969) showed that very brief tryptic digestion of myofibrils liberated a protein that exhibited potent \( \alpha \)-actinin-like activity in both the ATPase and turbidity assays and that sedimented primarily as a single 6.2 S boundary in the analytical ultracentrifuge. This protein, which Goll et al. (1969) called Z-protein, was three to five times more active in both the ATPase and turbidity assays of \( \alpha \)-actinin activity, and also contained a three to five-fold higher proportion of its protein as the 6 S species than Ebashi's \( \alpha \)-actinin preparations. Moreover, although Ebashi's \( \alpha \)-actinin preparations had an amino acid analysis remarkably similar to that of actin (indeed, many investigators at that time believed that \( \alpha \)-actinin was nothing more than denatured actin), the amino acid composition of Z-protein was completely different from that of actin. The possibility that Z-protein was a proteolytic fragment that was three to five times more active on a weight basis than Ebashi's \( \alpha \)-actinin because it was only one-third to one-fifth as large as Ebashi's 6 S \( \alpha \)-actinin species seemed very
unlikely because Z-protein also had a 6 S sedimentation coefficient, and it seemed impossible to cleave a 6 S \( \alpha \)-actinin molecule into another 6 S molecule having only one-third the mass of the original molecule. Therefore, discovery of Z-protein raised the possibility that only the 6 S species in Ebashi's \( \alpha \)-actinin preparations was active in the ATPase and turbidity assays and that Ebashi's \( \alpha \)-actinin preparations were not as active in the ATPase and turbidity assays as the Z-protein preparations because the 6 S species made up less than one-third of the protein in Ebashi's \( \alpha \)-actinin preparations. This possibility, of course, was in direct opposition to Nonomura's earlier conclusion (Nonomura, 1967) that three different active molecular species existed in Ebashi's \( \alpha \)-actinin preparations. Third, because they had found that trypsin would release a potent \( \alpha \)-actinin activity from myofibrils, Goll et al. (1969) treated Ebashi's \( \alpha \)-actinin preparations with trypsin to determine whether trypsin could also release a potent \( \alpha \)-actinin activity from these preparations. After 30-40 min. of proteolytic digestion, all \( \alpha \)-actinin activity originally present in Ebashi's \( \alpha \)-actinin preparations could be isolated in a fraction constituting only 6% of the protein in the original fraction (Goll et al., 1969). Moreover, only 15-30% of the protein in Ebashi's \( \alpha \)-actinin preparations would bind to actin (Ebashi had earlier reported
that \( \alpha \)-actinin bound to actin). Therefore, these findings suggested that Ebashi's \( \alpha \)-actinin preparations contained only 5-20% \( \alpha \)-actinin and that the remaining 80-90% of the protein in these preparations was inactive (Goll et al., 1969). Although the nature of the inactive protein was unknown, Goll et al. (1969) suggested that, on the basis of its sedimentation behavior and salting out characteristics, it may be denatured actin.

Because the three lines of evidence discussed in the preceding paragraph suggested that Ebashi's \( \alpha \)-actinin preparations may be grossly impure, and because of the uncertainty that this implication caused regarding the properties of \( \alpha \)-actinin, Arakawa et al. (1970b) devised an entirely new procedure for extraction and purification of \( \alpha \)-actinin in the anticipation that comparison of \( \alpha \)-actinin made by an entirely different procedure with Ebashi's \( \alpha \)-actinin (Ebashi and Ebashi, 1965) and with Z-protein (Goll et al., 1969) would provide additional information on the molecular nature of \( \alpha \)-actinin. Arakawa's procedure (Arakawa et al., 1970b) involved preparation of a crude \( \alpha \)-actinin fraction by extracting myofibrils at very low ionic strength (less than 0.001) and pH 8.5 at 0°C for 60-72 hours. This crude \( \alpha \)-actinin extract was carefully fractionated between 15 and 25% ammonium sulfate saturation to produce a \( P_{15-25} \) fraction. The \( P_{15-25} \)
fraction already contained 25-30% of its protein as the 6 S \( \alpha \)-actinin species and was five to six-fold more active in the ATPase and turbidity assays of \( \alpha \)-actinin activity than Ebashi's \( \alpha \)-actinin preparations. These properties of the \( P_{15-25} \) fraction supported the idea that only the 6 S species is capable of accelerating \textit{in vitro} measures of contraction, and that only the 6 S species should therefore be called \( \alpha \)-actinin. Additional purification of this 6 S \( \alpha \)-actinin species was achieved by chromatography on a DEAE-cellulose column at pH 7.5 (Robson et al., 1970). The 6 S \( \alpha \)-actinin eluted between 240 and 300 mM KCl, and rechromatography on a second DEAE-cellulose column produced a preparation that contained 80 to 85% of its protein as the 6 S species and another 10-15% of its protein as a 9 S species (Robson et al., 1970). Robson et al. (1970) have summarized several lines of indirect evidence that the 9 S material in these \( \alpha \)-actinin preparations purified by two successive passes through DEAE-cellulose column was an aggregate of the 6 S \( \alpha \)-actinin species. These chromatographically purified \( \alpha \)-actinin preparations were two to three times more active in the ATPase and turbidity assays of \( \alpha \)-actinin activity than the \( P_{15-25} \) fraction which was already five to six-fold more active than Ebashi's \( \alpha \)-actinin preparations. The successful purification of \( \alpha \)-actinin supported the conclusion that only the 6 S species
has the ability to accelerate \textit{in vitro} measurements of contraction. Nonomura's earlier report (Nonomura, 1967) that the 10 S and 25 S components could also accelerate rate of turbidity development of reconstituted actomyosin suspensions evidently was due to contamination of these components with 6 S component because Nonomura did not attempt any purification of his components beyond separation by simple differential centrifugation. Moreover, the efficiency of DEAE-cellulose chromatography in purifying the 6 S \(\alpha\)-actinin species from \(P_{15-25}\) crude \(\alpha\)-actinin fractions made it possible to use DEAE-cellulose chromatography to determine whether a highly active 6 S \(\alpha\)-actinin could also be purified from Ebashi's \(\alpha\)-actinin (Ebashi and Ebashi, 1965). Such an experiment showed that Ebashi's \(\alpha\)-actinin preparations contained a 6 S \(\alpha\)-actinin species almost equal in activity to \(\alpha\)-actinin prepared by the procedure of Arakawa \textit{et al.} (1970b) and Robson \textit{et al.} (1970), but that this 6 S \(\alpha\)-actinin constituted only 5% of the total protein in Ebashi's \(\alpha\)-actinin preparations. These results confirmed the earlier suggestion that only 5-20% of the protein in the early \(\alpha\)-actinin preparations was active \(\alpha\)-actinin (Goll \textit{et al.}, 1969), and it is now clear that only the 6 S species has \(\alpha\)-actinin activity and that only the 6 S species should be referred to as \(\alpha\)-actinin.

\(\alpha\)-Actinin purified by two passes through a DEAE-cellulose
column migrates either as a single band or as a single major band with a faint, more slowly migrating, minor band during polyacrylamide gel electrophoresis at pH 8.3 (Goll et al., 1972). It seems likely that the faint, more slowly migrating, minor band corresponds to the 9.1 S material seen in analytical ultracentrifuge patterns. During polyacrylamide gel electrophoresis in the presence of 0.1% SDS, doubly chromatographed α-actinin always migrates as a single electrophoretic band (Goll et al., 1972). Because 0.1% SDS dissociates proteins into their subunits (Shapiro et al., 1967; Weber and Osborn, 1969), the finding that doubly chromatographed α-actinin migrates as a single electrophoretic band in SDS-polyacrylamide gel electrophoresis supports the earlier suggestion of Robson et al. (1970) that the 9.1 S material may be an aggregate of the 6 S α-actinin species. The behavior of doubly chromatographed α-actinin in the analytical ultracentrifuge and in polyacrylamide gel electrophoresis clearly establishes it as the most homogeneous form of α-actinin described thus far, and hereafter in this review, α-actinin purified by two successive passes through DEAE-cellulose column will be referred to as purified α-actinin.
C. Properties of α-Actinin

1. Effect of α-actinin on Mg$^{2+}$-activated superprecipitation and ATPase activities of reconstituted actomyosin.

Shortly after the discovery that α-actinin increased the rate of turbidity development (Ebashi and Ebashi, 1965), Maruyama (1966) reported that α-actinin also increased the Mg$^{2+}$-modified ATPase activity of reconstituted actomyosin suspensions. Because turbidity development and Mg$^{2+}$-modified activity are widely accepted as two in vitro measures of contractile activity (see introduction to this review), it was suggested that α-actinin may be an important protein regulating the strength of the actin-myosin interaction. The early α-actinin preparations, however, exhibited several characteristics that argued against α-actinin having a physiological role of strengthening the actin-myosin interaction. First, ratios as high as 0.3 to 0.6 parts of α-actinin to 1 part of actomyosin by weight were required to cause clear increases in rate of turbidity development and Mg$^{2+}$ modified ATPase activity of reconstituted actomyosin suspensions (Ebashi and Ebashi, 1965; Maruyama, 1966). These ratios were higher than the amount of α-actinin thought to exist in the myofibril. Second, and even more important, these early studies showed that, although α-actinin accelerated the rate of turbidity development and Mg$^{2+}$-modified ATPase activities of
reconstituted actomyosin at ionic strengths between approximately 0.04 and 0.08, α-actinin was completely ineffective at ionic strengths above 0.08. Because the intracellular ionic strength in mammalian muscle cells is near 0.17 to 0.20, these early α-actinin preparations had no activity at in vivo conditions.

In an extensive reinvestigation of the effect of α-actinin on the turbidity development and Mg$^{2+}$-modified ATPase activity of reconstituted actomyosin, Seraydarian et al. (1967) confirmed these findings of Ebashi and his coworkers (Ebashi and Ebashi, 1965; Maruyama, 1966). Seraydarian et al. (1967), however, used essentially the same procedure to purify α-actinin as Ebashi and coworkers, and as has already been described, α-actinin produced by this procedure contains approximately only 5% of its protein as the active 6 S α-actinin species. When the more highly purified α-actinin preparations made according to Arakawa et al. (1970b) and purified according to Robson et al. (1970) were tested for ability to accelerate turbidity development or to increase Mg$^{2+}$-modified ATPase activity, it was found that these α-actinin preparations, which were extracted at 0°C instead at room temperature as Ebashi's preparations were, caused the greatest percent increase in rate of turbidity development and Mg$^{2+}$ modified ATPase activity at ionic strengths of approximately 0.13 to 0.15.
Moreover, addition of as little as 0.05 parts of purified a-actinin to 1 part of actomyosin increased the Mg\textsuperscript{2+}-modified ATPase activity and the rate of turbidity development of reconstituted actomyosin suspensions considerably. The effectiveness of a-actinin at ionic strengths above 0.1 has since been confirmed by Danker (1971), and it presently seems that the earlier results indicating that a-actinin had no effect on Mg\textsuperscript{2+}-modified ATPase activity and rate of turbidity increases of reconstituted actomyosin at ionic strengths above 0.09 were due either to the impurity of these early preparations or to the fact that these early preparations were extracted at room temperature, or both these circumstances.

Although the availability of purified a-actinin made it possible to show that a-actinin activated the \textit{in vitro} measures of contraction at ionic strengths near those existing \textit{in vivo} and at a-actinin to actin ratios approximately the same as those that seemed to exist \textit{in vivo}, these results did not prove that a-actinin actually had a physiological role of strengthening the actin-myosin interaction. Temple and Goll (1970) have reported that ability of a-actinin to increase rate of turbidity development and Mg\textsuperscript{2+}-modified ATPase activities of reconstituted actomyosin is specific for those nucleoside triphosphates and cations that support tension development of glycerinated
muscle strips. Thus, although actomyosin cleaves the
terminal phosphate of TTP, GTP, and CTP as well as ATP,
$\alpha$-actinin increases only the $\text{Mg}^{2+}$-modified CTPase and
ATPase activities of actomyosin, and only CTP and ATP
among these four nucleotides support tension development.
Moreover, although both $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ activate the ATPase
activity of actomyosin, $\alpha$-actinin increases only the
$\text{Mg}^{2+}$-modified ATPase activity, and only the $\text{Mg}^{2+}$-modified
ATPase activity seems coupled to tension development. It
was also observed that $\alpha$-actinin had no effect on the abil-
ity of purified myosin to hydrolyze ATP in the absence of
actin (Temple and Goll, 1970), and it is well known that
myosin has no contractile activity in the absence of actin.
Consequently, the effect of $\alpha$-actinin on reconstituted
actomyosin suspensions seems specific for those condi-
tions that support tension development or shortening of
glycerinated muscle strips.

2. Localization of $\alpha$-actinin

In their paper describing the discovery of $\alpha$-actinin,
Ebashi et al. (1964) indicated that most actin preparations
were contaminated by $\alpha$-actinin, and inferred that $\alpha$-actinin
bound to actin. This inference was subsequently con-
firmed when Maruyama and Ebashi (1965) reported that $\alpha$-
actinin-free actin differs considerably from ordinary
Straub-type actin prepared by the procedures usually used for actin purification. Straub F-actin is very viscous at protein concentration of 3 mg/ml or higher and tends to become turbid during storage at 0°C. If allowed to stand for several days at 0°C, Straub-F-actin eventually assumes a gel-like state. α-Actinin-free F-actin displays none of these characteristics, but addition of 1 part of Ebashi's α-actinin to 3 parts of F-actin by weight causes formation of the gel state after 1 hour at 0°C (Maruyama and Ebashi, 1965). It was also observed that addition of large amounts of Ebashi's α-actinin to Straub-type F-actin caused local precipitation of the actin. This precipitate could be dispersed by mixing but after such dispersion, part of the α-actinin-F-actin complex could be sedimented at forces too low to sediment normal F-actin. These observations, plus the fact that addition of α-actinin increased the viscosity of F-actin solutions, led Maruyama and Ebashi (1965) to suggest that α-actinin cross-linked actin filaments. These same observations were subsequently made by Briskey and coworkers (Briskey et al., 1967b; Seraydarian et al., 1967) who also concluded that α-actinin has a remarkable ability to cross-link F-actin strands.

The ability of α-actinin to cross-link F-actin filaments suggested that α-actinin might be located in the Z-disk of myofibrils, for only here does cross-linking of
F-actin filaments occur in vivo. Soon after the discovery of α-actinin, evidence accumulated from three independent methods (Goll et al., 1967, 1969; Masaki et al., 1967; Robson et al., 1970; Stromer et al., 1967b, 1969) that α-actinin was indeed located in the Z-disk. The first of these methods used the observation (Stromer et al., 1967a) that very brief trypsin treatment of myofibrils completely removes the Z-disk without causing other ultrastructurally observable deletions from the myofibril. It was discovered (Goll et al., 1967, 1969) that very limited tryptic digestion of myofibrils liberated a protein that possessed potent α-actinin activity in the ATPase and turbidity assays. The role of this protein, initially called Z-protein, in the development of new procedures to purify α-actinin has already been discussed. Liberation of Z-protein closely coincided with disappearance of the Z-disk, and it was concluded that the Z-protein, which was obviously a form of α-actinin, was therefore located in the Z-disk. Simultaneously, Masaki et al. (1967) made antibodies against Ebashi's α-actinin preparations, and found that after fluorescent labeling, these antibodies bound to both the Z-disk and the M-line. Absorption of this antibody fraction made against Ebashi's α-actinin preparation by a preparation of Nonomura's 6 S component (Nonomura, 1967) completely removed both Z-disk and M-line
binding. Another protein contaminant could be isolated from Ebashi's α-actinin preparations in very small amounts, and absorption of the crude anti-α-actinin antibodies with this contaminant protein removed M-line binding but did not affect Z-disk binding. Consequently, Masaki et al. (1967) concluded that the 6 S component of α-actinin was located at the Z-disk.

The third line of evidence suggesting that α-actinin might be located in the Z-disk involved the discovery of Stromer and coworkers (Stromer et al., 1967b, 1969) that Z-disks could be removed from skeletal muscle fibrils by prolonged (7-10 days) low ionic strength extraction, and that incubation of these Z-disk-extracted fibers with concentrated solutions of the extract resulted in reconstitution of Z-disks in these fibers. The Z-disk extracts contained α-actinin along with tropomyosin, troponin and other unidentified proteins (Stromer et al., 1969). Because the only ultrastructurally observable changes caused by low ionic strength extraction were removal of Z-disks and M-lines, it seemed likely that α-actinin was a constituent of one of these structures. No reconstitution of Z-disks, however, was observed after incubating Z-disk-extracted fibrils with a preparation of Ebashi's α-actinin (Stromer et al., 1969; Robson et al., 1970). A moderate reconstitution of Z-disks was later obtained by incubating
Z-disk-extracted fibers with the $P_{\text{15-25}} \alpha$-actinin preparation of Arakawa et al. (1970b).

Although tryptic release of $\alpha$-actinin from myofibrils, localization of binding of antibodies against Ebashi's $\alpha$-actinin preparations and partial reconstitution of extracted Z-disks by impure $\alpha$-actinin fractions provided suggestive evidence that $\alpha$-actinin was located in the Z-disk, all this evidence was very indirect. First, it was impossible to be certain that the $\alpha$-actinin liberated from myofibrils by tryptic digestion did not originate from some site other than the Z-disk but whose destruction could not be detected ultrastructurally. Second, the heterogeneity of the antibody fraction made against Ebashi's $\alpha$-actinin preparations precluded any direct conclusion that the anti-$\alpha$-actinin in this antibody fraction was the component that bound to the Z-disk. Third, heterogeneity of the $\alpha$-actinin fractions used to reconstitute extracted Z-disks made impossible to ascertain whether the $\alpha$-actinin in these fractions was responsible for the partial reconstitution observed.

The availability of highly purified $\alpha$-actinin prepared by DEAE-cellulose chromatography (Robson et al., 1970) enabled Schollmeyer et al. (1973) to use antibodies made against highly purified $\alpha$-actinin to determine directly, without any absorption of the antibody fraction,
that these antibodies bound only to the Z-disk in intact myofibrils. This procedure provided the first direct and conclusive evidence that α-actinin is probably localized in the Z-disk of skeletal muscle. Schollmeyer's experiments (Schollmeyer et al., 1973) were done at both the light and electron microscope level of resolution by using fluorescein and horseradish peroxidase-labeled antibodies. The experiments done at the electron microscope level have permitted some preliminary conclusions about the exact position of α-actinin in the molecular architecture of the Z-disk; these conclusions will be described later when Z-disk structure is reviewed. Because α-actinin can also be prepared from cardiac and smooth muscle, it was possible for Schollmeyer et al. (1973) to determine that α-actinin is located in both the intercalated disk and Z-disks of cardiac and muscle myofibrils, and in the dense bodies of smooth muscle. More recently, by using antibodies made against highly purified smooth muscle α-actinin, Schollmeyer et al. (1974) has shown that α-actinin is also found in a large number of nonmuscle motile systems including Limulus sperm, the brush borders of intestinal epithelial cells, and fibroblasts. Indeed, α-actinin has been located in every motile system that has been examined so far for its presence, and the physiological role of α-actinin becomes increasingly intriguing.
3. Physical properties of α-actinin

Some physical properties of α-actinin were reported already in the papers describing the discovery of α-actinin, and an extensive study of the physical parameters of the early α-actinin preparations was reported by Nonomura (1967). As discussed in the section on purification of α-actinin, however, virtually all these early studies on the nature of the α-actinin molecule were complicated by the extremely heterogeneous nature of early α-actinin preparations. Consequently, only the properties determined on highly purified preparation of α-actinin will be described in this section.

The ability to obtain highly purified α-actinin made it possible for the first time to estimate the amount of α-actinin in skeletal muscle myofibrils. Arakawa et al. (1970b) found that their P_{15-25} fraction constituted approximately 1 to 1.5% of total myofibrillar protein. Purification by DEAE-cellulose chromatography showed that approximately 25% of this fraction was the 6 S α-actinin species. Therefore, approximately 0.25 to 0.40% of total myofibrillar proteins could be isolated as α-actinin. Allowing for preparative losses of 50%, these data suggest that α-actinin constitutes approximately 1% of total myofibrillar proteins (Robson et al., 1970; Suzuki et al.,
1973). Recent preliminary studies by Tabatabai\(^1\) in which whole myofibrils are dissolved in SDS, the myofibrillar proteins separated by SDS-polyacrylamide gel electrophoresis, and the relative proportions of each of the myofibrillar proteins then determined by scanning the polyacrylamide gel suggest that \(\alpha\)-actinin may constitute as much as 2.5% of total myofibrillar protein. Whichever of these estimates of \(\alpha\)-actinin content is most nearly correct, it is clear that \(\alpha\)-actinin makes up only a small proportion of total myofibrillar protein, and the content of \(\alpha\)-actinin is certainly less than the 5 to 6% of total myofibrillar mass that Huxley and Hanson (1960) estimate is due to the Z-disk. Therefore, the Z-disk must contain proteins in addition to \(\alpha\)-actinin.

Amino acid composition of early \(\alpha\)-actinin preparations were very similar to the amino acid composition of actin (Ebashi and Ebashi, 1965), and many investigators, therefore, thought that \(\alpha\)-actinin may simply be an unusual form of denatured actin. Again, the availability of highly purified \(\alpha\)-actinin (Robson et al., 1970) made it possible to show conclusively that \(\alpha\)-actinin had an amino acid composition completely different from that of actin and that \(\alpha\)-actinin was therefore a new myofibrillar protein (Robson et al., 1970). Purified \(\alpha\)-actinin was clearly higher in

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histidine, arginine, aspartic acid, glutamic acid, alanine, and leucine and lower in threonine, serine, glycine, isoleucine, and tyrosine than actin. Moreover, purified α-actinin contained no 3-methyl histidine, an unusual amino acid found in both actin and myosin. It was possible to virtually duplicate the amino acid composition of Ebashi's early α-actinin preparations (Ebashi and Ebashi, 1965) by assuming that these preparations contained 15% of their protein as α-actinin and 85% of their protein as actin. This supports the suggestion of Goll et al. (1969) that Ebashi's early α-actinin preparations contained substantial amounts of denatured actin.

Purified α-actinin has been available only a relatively short period of time, and studies on the physical parameters of the α-actinin molecule are therefore relatively recent. Purified α-actinin from rabbit skeletal muscle has a sedimentation coefficient \( S_{20,w}^0 \) of 6.21 (Goll et al., 1972) to 6.23 (Robson et al., 1970) svedberg units in 100 mM KCl, 20 mM Tris-acetate, pH 7.5, and has a molecular weight of approximately 190,000 ± 10,000 as determined by sedimentation equilibrium studies in 100 mM KCl, 20 mM Tris-acetate, pH 7.5 (Goll et al., 1971). When sedimentation equilibrium studies were done in 5 M guanidine HCl, 20 mM Tris-acetate pH 7.5, 0.01 M MCE, a molecular weight of 75,000-90,000 daltons is obtained.
(Goll et al., 1971) for purified rabbit skeletal α-actinin. A molecular weight of 90,000-100,000 daltons is obtained by using calibrated 4% agarose columns in 6 M guanidine-HCl, pH 5.0 or by using polyacrylamide gel electrophoresis in the presence of 0.1% SDS (Goll et al., 1971). Because both guanidine-HCl and 0.1% SDS would be expected to dissociate α-actinin into its subunits, these results indicate that the 6.2 S α-actinin species in 100 mM KCl, 20 mM Tris-acetate, pH 7.5, is composed of two subunits of approximately equal mass. Purified rabbit skeletal muscle α-actinin also has an α-helical content of approximately 62% as estimated by circular dichroism at 222 nm, has a diffusion coefficient of approximately 2.71 x 10⁻⁷ cm²/sec, and has an intrinsic viscosity near 15 c.c/gm (Suzuki and Goll¹). It is possible to calculate from the diffusion coefficient and the intrinsic viscosity of α-actinin and by assuming a prolate ellipsoidal shape for the α-actinin molecule, that this molecule is approximately 30-40 Å in diameter and 400-570 Å long (Suzuki and Goll¹). Although extensive studies have not yet been done on the physical parameters of α-actinin purified from cardiac or smooth muscle, preliminary results

from SDS-polyacrylamide gel electrophoresis and sedimentation velocity experiments indicate that \( \alpha \)-actinin from these two types of muscle has physical parameters very similar to those determined for rabbit skeletal muscle \( \alpha \)-actinin.

4. \( \alpha \)-Actinin from white and red skeletal and cardiac muscles

Comparative studies of myofibrillar proteins isolated from predominantly red and white muscles have been useful in understanding the function of myosin and troponin. Although myosins prepared from red and white skeletal muscle have the same molecular size and shape and the same number of subunits, they differ in specific ATPase activity (Seidel et al., 1964; Gergely et al., 1965; Barany et al., 1965; Sreter et al., 1966; Samaha et al., 1970), in resistance to trypsin digestion (Gergely et al., 1965), in stability at alkaline and acidic pH values (Katz et al., 1966; Sreter et al., 1966; Barany et al., 1965) and in the type of light (Samaha et al., 1970; Locker and Hagyard, 1968; Weeds and Pope, 1971) and heavy subunits (Kuehl and Adelstein, 1970; Nakamura et al., 1971) present in the parent molecule. Barany and associates (Barany et al., 1967; Barany, 1967; Barany and Close, 1971) have shown that speed of contraction of a muscle is directly
related to the ATPase activity and rate of superprecipitation of actomyosin isolated from that muscle. Because the ATPase activity and rate of superprecipitation of actomyosin depends entirely on the myosin in the actomyosin, speed of muscle shortening is directly related to the speed that myosin from that muscle can cleave ATP. Therefore, myosin from rapidly contracting muscles has a high ATPase activity, whereas myosin from slowly contracting muscles has a low ATPase activity. Troponins from red and white muscle also differ in activity because Furukawa and Peter (1971) have reported that troponin from red muscle binds less Ca\(^{2+}\) than troponin from white muscle. Consequently, myosin and troponin, which both have a direct role in the contractile process, possess distinctly different properties in physiologically different kinds of muscle.

If the ability of \(\alpha\)-actinin to increase the rate of turbidity development and Mg\(^{2+}\) activated ATPase activity of reconstituted actomyosin portends some direct role of \(\alpha\)-actinin in muscle contraction, it might be supposed by reasoning analogously with the myosin and troponin situations, that \(\alpha\)-actinin from white muscle would have a greater effect on the \textit{in vitro} measures of muscle contraction than \(\alpha\)-actinin from red muscle. On the other hand, Padykula and Gauthier (1970) have shown that Z-lines in red rat skeletal muscle fibers are approximately twice as wide as Z-lines in
white rat muscle fibers; because α-actinin is located in the Z-line (Goll et al., 1967; Masaki et al., 1967; Schollmeyer et al., 1973), it may be expected that red muscle would contain approximately twice as much α-actinin as white muscle if α-actinin had a purely structural role in muscle. Detailed studies of α-actinins from porcine red, white, and cardiac muscles (Robson and Zeece, 1973; Suzuki et al., 1973) have shown that the myofibrils in these muscles contain approximately equal proportions of α-actinin even though Z-disks in red muscle are 2.25 times wider than Z-disks in white muscle. The α-actinins from these three kinds of muscle, however, also had identical activities in both the ATPase and turbidity assays. Consequently, these studies provided no clear indication as to the role of α-actinin in muscle. The α-actinins from white, red and cardiac muscles had almost identical sedimentation coefficients (about 6.1 to 6.3 S) and subunit molecular weights as measured by SDS-polyacrylamide gel electrophoresis (Robson and Zeece, 1973; Suzuki et al., 1973). Moreover, α-actinins from red and white muscle had virtually identical circular dichroism spectra (about 59% α-helical form circular dichroism at 222 nm) and exhibited the same sensitivity to trypsin digestion (Suzuki et al., 1973). Red and white α-actinins, therefore, have almost identical molecular mass and secondary structure. On the other hand, α-actinin from red or cardiac
muscle migrated faster than α-actinin from white muscle in polyacrylamide gel electrophoresis at pH 8.3 (Robson and Zeece, 1973; Suzuki et al., 1973), and required higher KCl concentrations for elution from DEAE-cellulose columns (250-255 mM) than α-actinin from white muscle (220-245 mM). These differences among white, red and cardiac α-actinins were found to originate from differences in amino acid composition. Cardiac and red α-actinin possessed 12-16 more aspartic acid residues per 1,000 total amino acid residues than white α-actinin, and this difference plus a small difference in glutamic acid content resulted in cardiac or red α-actinin having approximately 17-28 more negatively charged amino acids per 1,000 total amino acids than white α-actinin. Cardiac and red α-actinins also contained more isoleucine and less glycine and valine than white α-actinin (Robson and Zeece, 1973; Suzuki et al., 1973). In general, red α-actinin had the same amino-acid composition as cardiac α-actinin, although polyacrylamide gel electrophoresis at pH 8.3 showed that red α-actinin contained a minor band with a mobility intermediate between that of white α-actinin and cardiac α-actinin (Robson and Zeece, 1973). This intermediate band may represent an intermediate form of α-actinin or may originate from the presence of an α-actinin molecule containing one red or cardiac peptide chain and one white peptide chain per double-chained
α-actinin molecule. Because α-actinin content of different muscle types is evidently similar, and because the different α-actinins seem to have the same activity in the ATPase and turbidity tests, the significance of the differences in amino acid composition between red and white α-actinins remains unclear.

5. Interaction of α-actinin with other myofibrillar proteins

The observations that led Ebashi and coworkers (Ebashi et al., 1964; Maruyama and Ebashi, 1965) to conclude that α-actinin binds to F-actin have already been described in the section discussing localization of α-actinin. It was also recognized in these early studies (Ebashi et al., 1964; Ebashi and Ebashi, 1965) that α-actinin had no effect on viscosity or ATPase activity of myosin, and it was presumed, therefore, that α-actinin did not bind to myosin. These conclusions were confirmed by Briskey, Seraydarian and Mommaerts (Briskey et al., 1967b; Seraydarian et al., 1967) in their extensive investigation of the properties of α-actinin preparations made essentially according to Ebashi's original procedures (Ebashi and Ebashi, 1965). Briskey and coworkers (Briskey et al., 1967b) studied the interaction of α-actinin with actin and myosin directly by examining mixtures of α-actinin and myosin or actin in the analytical ultracentrifuge, and by precipitating myosin or sedimenting
actin from mixtures containing α-actinin and assaying the supernatant solution for α-actinin. The results of these studies were interpreted as indicating that α-actinin bound to G- or F-actin but not to myosin (Briskey et al., 1967b; Seraydarian et al., 1967). Subsequently, Drabikowski et al. (1968) reported that the 6 S component of α-actinin prepared by Nonomura's procedure (Nonomura, 1967) did not bind to tropomyosin but did bind to the tropomyosin-troponin complex.

Because these early studies on interaction of α-actinin with other myofibrillar proteins had been done with relatively heterogenous α-actinin preparations (see earlier section on purification of α-actinin for a discussion of purity of early α-actinin preparations), Holmes et al. (1971) reexamined the interaction properties of α-actinin by using analytical ultracentrifugation and highly purified α-actinin made according to Arakawa et al. (1970b) and Robson et al. (1970). These studies clearly showed that purified α-actinin bound to F-actin but not to myosin, tropomyosin, the tropomyosin-troponin complex, or G-actin. Consequently, earlier studies which reported that α-actinin bound to G-actin (Briskey et al., 1967b) or the tropomyosin-troponin complex (Drabikowski et al., 1968) had been complicated by heterogeneity of early α-actinin preparations. Moreover, although α-actinin made according to Ebashi's
original procedure (Ebashi and Ebashi, 1965) was reported to bind to F-actin polymerized by 100 mM KCl but not to F-actin polymerized in 1 mM MgCl₂ or 1 mM CaCl₂ (Drabikowski and Nowak, 1968). Holmes and coworkers (Holmes et al., 1971) found that purified α-actinin bound equally well to F-actin polymerized either in 100 mM KCl or 2 mM MgCl₂. This confirmed the finding of Goll et al. (1969) who found that Z-protein, a partly purified form of α-actinin, bound both to F-actin polymerized in 100 mM KCl and to F-actin polymerized in 1 mM MgCl₂. Evidently, Drabikowski and Nowok's results (Drabikowski and Nowak, 1968) were also complicated by heterogeneity of early α-actinin preparations.

The studies of Holmes and coworkers (Holmes et al., 1971) with purified α-actinin clearly established that α-actinin bound only to F-actin among the known myofibrillar proteins, but the nature of the α-actinin/F-actin binding and the effect of this binding on conformation of either α-actinin or F-actin remained unknown. Studies with early, heterogeneous preparations of α-actinin showed that α-actinin accelerated the rate of actin polymerization in KCl, but that α-actinin did not, by itself, induce actin polymerization (Maruyama and Ebashi, 1965). It was also shown that α-actinin increased the specific viscosity of F-actin solutions but decreased the mean particle length in these solutions from 5.6 to 4.5 μm (Maruyama and Ebashi,
1965). The observation (Ebashi and Ebashi, 1965; Maruyama
and Ebashi, 1965) that addition of \( \alpha \)-actinin first caused
gelation and then precipitation at higher \( \alpha \)-actinin to
F-actin ratios suggested that \( \alpha \)-actinin may cross-link
F-actin filaments (Briskey et al., 1967b; Maruyama and
Ebashi, 1965) and that intrafilament cross-linking may ac­
count for the \( \alpha \)-actinin-induced decrease in mean filament
lengths as measured by flow birefringence of F-actin solu­
tion (Maruyama and Ebashi, 1965). Direct electron micro­
scope examination of \( \alpha \)-actinin/F-actin mixtures confirmed
that \( \alpha \)-actinin cross-links actin filaments and showed that
the predominant form of cross-linking was a lateral, side­
to-side binding of actin filaments to form large bundles
of fibers (Kawamura et al., 1970). The side-to-side as­
sociation of F-actin filaments induced by \( \alpha \)-actinin, how­
ever, was looser and more irregular than the highly ordered
paracrystals of F-actin formed in response to high MgCl\(_2\)
concentrations (Hanson, 1967). It was also found (Drabikowski
and Nowak, 1968) that \( \alpha \)-actinin caused a larger increase
in specific viscosity of F-actin at 0°C than at 21°C; this
suggested that binding of \( \alpha \)-actinin to actin is stronger at
0°C than at 21°C but did not indicate whether binding of
\( \alpha \)-actinin to actin produce laterally associated bundles of
F-actin filaments at both temperatures. Indeed, simple
side-to-side binding of F-actin filaments lying
approximately in register would be expected to decrease the specific viscosity of F-actin solutions slightly rather than increase it because such lateral association would decrease the average length-to-diameter ratio of the actin filaments.

Subsequent viscometric studies purified α-actinin (Holmes et al., 1971) showed that as little as 0.02 to 0.05 parts of purified α-actinin to one part of actin by weight caused a four- to five-fold increase in specific viscosity at 0°C, but that 0.10 to 0.20 parts of purified α-actinin to one part of F-actin by weight produced only a two- to three-fold increase in viscosity at 37°C. Holmes and coworkers (Holmes et al., 1971) interpreted their results to indicate that α-actinin bound to actin in two different ways. At low temperatures, α-actinin bound all along the length of actin filaments and caused formation of the lateral bundles of F-actin filaments observed by Kawamura et al. (1970) and also produced a cross-linked, three-dimensional gel structure having high specific viscosity. At higher temperatures, α-actinin binding is at least partly restricted to one end of the F-actin filament, and binding along the length of the actin filament does not occur until high α-actinin to F-actin ratios are reached. Binding strictly at the end of the actin filament would have very little effect on viscosity of F-actin
solutions. A number of different lines of evidence have now accumulated to indicate that this duality in mechanism of α-actinin binding exists in much the form that Holmes and coworkers (Holmes et al., 1971) suggested, and the two mechanisms for binding of α-actinin to F-actin filaments will be discussed again later in this section when the effect of tropomyosin on the α-actinin/F-actin interaction is reviewed.

Maruyama and coworkers (Maruyama, 1965a,b; Maruyama, 1966; Maruyama and Kawamura, 1968) have published a number of papers describing the properties of the myofibrillar protein called β-actinin. β-actinin inhibits network formation of F-actin filaments and also prevents aggregation of short F-actin filaments. Because α-actinin, which crosslinks actin filaments, and β-actinin, which prevents network formation of actin filaments, seem to have opposing effects on F-actin, the influence of α-actinin on F-actin in the presence of β-actinin was studied under several different conditions (Kawamura et al., 1970; Maruyama and Ebashi, 1965; Maruyama, 1966). In every instance studied so far, however, α-actinin and β-actinin seem to act independently. Thus, α-actinin increased the Mg$^{2+}$-modified ATPase activity of reconstituted actomyosin in the presence of β-actinin (Maruyama, 1966), α-actinin caused lateral association of F-actin filaments in the presence of β-actinin
(Kawamura et al., 1970), and α-actinin caused the same flow birefringence changes in F-actin solutions in the presence or absence of β-actinin (Maruyama and Ebashi, 1965). On the other hand, β-actinin, in each of these instances, was also able to exert its characteristic effect of preventing reaggregation of short F-actin filaments following their rupture by shear. Because these studies on the simultaneous presence of α-actinin and β-actinin were done with very heterogeneous preparations of both α-actinin and β-actinin, the significance of these results is still unclear. It seems evident, however, that α- and β-actinin must bind to different sites on the actin filament if they are able to exert their characteristic effects simultaneously.

Because tropomyosin also binds to F-actin filaments (Drabikowski et al., 1968; Drabikowski and Nowak, 1968; Holmes et al., 1971; Laki et al., 1962) it was of interest to learn whether tropomyosin affects the α-actinin/F-actin interaction. Drabikowski and coworkers (Drabikowski and Nowak, 1968; Drabikowski et al., 1968) found that the presence of tropomyosin greatly retarded precipitation of F-actin by α-actinin at 25°C and also greatly reduced the α-actinin-induced increase in specific viscosity of F-actin at 25°C. The effects of tropomyosin were similar
whether tropomyosin was added before or after addition of α-actinin. At 0°C, however, the presence of tropomyosin had very little effect on the ability of α-actinin to increase F-actin viscosity (Drabikowski and Nowak, 1968). These findings on the effects of tropomyosin on the α-actinin/F-actin interaction were confirmed by Holmes et al. (1971) who used purified α-actinin preparations. Again, Holmes and coworkers (Holmes et al., 1971) interpreted their results to indicate that tropomyosin, which is bound along the length of the actin filament, was dislodged by α-actinin at 0°C, and that α-actinin was then able to bind adjacent F-actin filaments side-to-side to produce the characteristic α-actinin-induced increase in F-actin viscosity. At 37°C, however, the ability of α-actinin to bind along the length of the F-actin filament was very much reduced, and α-actinin was unable to compete successfully with tropomyosin for binding sites along the length of the F-actin filament. Therefore, at 37°C, α-actinin binding to F-actin in the presence of tropomyosin was limited strictly to the ends of the F-actin filament, and α-actinin caused no increase in F-actin viscosity.

The mechanism presented by Holmes and coworkers (Holmes et al., 1971) for binding of α-actinin to F-actin has been strongly supported by determination of the stoichiometry of α-actinin/F-actin interaction in the
presence and absence of tropomyosin at 0°C and 37°C (Goll et al., 1972), and by direct electron microscope observation of binding of purified α-actinin to Z-disk extracted fibrils (Stromer et al., 1967b, 1969) in the presence and absence of tropomyosin and at 0°C and 37°C (Stromer and Goll, 1972). Several early attempts were made in other laboratories to determine the stoichiometry of the α-actinin/F-actin interaction, but heterogeneity of the early α-actinin preparations made it impossible to ascertain whether any stoichiometry actually existed (Drabikowski and Nowak, 1968; Goll et al., 1969). Indeed, the early reports (Briskey et al., 1967b; Seraydarian et al., 1967) that stoichiometry of the α-actinin/F-actin interaction was 0.9 parts of α-actinin to one part actin by weight later were found to be totally incorrect, and it was not until Robson et al. (1970) purified α-actinin that it was possible to determine that the stoichiometry of the α-actinin/F-actin interaction was 0.41 parts of α-actinin to one part F-actin by weight at 0°C (Robson et al., 1970). This weight ratio of α-actinin binding corresponds to a molecular binding ratio of 1 molecule of α-actinin to every 10-12 G-actin monomers. Because each strand of the F-actin helix makes a complete turn once every 13 G-actin monomers, this molecular binding ratio suggests that, at 0°C, α-actinin may bind only once per strand per turn of G-actin helix (Robson et al., 1970).
It was also found that tropomyosin had little effect on stoichiometry of the α-actinin/F-actin interaction at 0°C, but that addition of α-actinin to F-actin preparations saturated with tropomyosin caused release of tropomyosin in amounts approximately equal to the α-actinin bound (Robson et al., 1970). These early results formed part of the basis for the suggestion that α-actinin binds in two different ways to F-actin filaments (Holmes et al., 1971).

Subsequently, study of the stoichiometry of the α-actinin/F-actin interaction was extended to a comparison of α-actinin binding at 0°C and 37°C (Goll et al., 1972). At 37°C, no stoichiometry was evident in the α-actinin/F-actin interaction because F-actin did not bind all added α-actinin even at very low α-actinin:F-actin ratios. The proportion of added α-actinin that was bound at 37°C became increasingly less as α-actinin:F-actin ratios rose above 0.1 to 1.0. Moreover, in the presence of tropomyosin, very little of the added α-actinin (less than 3% of the weight of F-actin present) was bound, and addition of α-actinin did not cause release of tropomyosin from the tropomyosin/F-actin complex, even at very high α-actinin to F-actin ratios. Consequently, in the presence of tropomyosin at 37°C, stoichiometry of the α-actinin/F-actin interaction was between 0.01 to 0.03 parts α-actinin to 1.0 part F-actin. This weight ratio corresponds to a molecular binding ratio
of one α-actinin molecule to every 150-220 G-actin molecules. Because an F-actin filament 1 μm long (the approximate in vivo length of F-actin filaments) contains approximately 370 G-actin monomers, a molecular binding ratio of 0.01 to 0.03 parts of α-actinin to 1 part F-actin by weight is just sufficient to put one α-actinin molecule on one end of each of the two strands in the double-stranded F-actin filament (Goll et al., 1972). This detailed hypothesis for the mechanism of α-actinin binding to F-action was supported directly by electron microscope observations showing that protein cross-bridges bound all along thin filaments in Z-disk-extracted fibrils from rabbit proas muscle when purified α-actinin was incubated with these fibrils at 2°C, but when tropomyosin and α-actinin were incubated simultaneously with the Z-disk-extracted fibrils at 2°C, numerous protein cross-bridges identical to those observed when only α-actinin was present were seen all along the thin filament. These observations directly substantiated the proposal that, at 0°C, α-actinin binds all along the F-actin filament and that this binding is not altered by the presence of tropomyosin. When purified α-actinin was incubated with Z-disk-extracted fibrils at 37°C, a very limited number of cross-bridges were observed along the thin filament, but the frequency of these cross-bridges increased near the Z-disk end of the thin filament. Incubation
of tropomyosin with Z-disk-extracted fibrils at 37°C resulted in tufts of protein bound along the thin filament. When purified α-actinin and tropomyosin were incubated simultaneously with the Z-disk-extracted fibers at 37°C, the same tufts of material observed in fibrils incubated with tropomyosin alone were present, but cross-bridges such as those seen in fibrils incubated with α-actinin were infrequent and were found only at the Z-disk end of the thin filaments. Again these electron microscope observations directly substantiated the suggestion that at 37°C, binding of α-actinin along the length of the actin filament is very limited, and that at 37°C, the presence of tropomyosin strictly limits α-actinin binding to one end (the Z-disk end) of the F-actin filament.

Consequently, viscometric studies, quantitative binding studies, and electron microscope observations of protein binding to Z-disk-extracted fibrils have produced a detailed mechanism for the binding of α-actinin to F-actin. The significance of this detailed information on the mechanism of α-actinin binding to F-actin filaments will be discussed later in this review when the role of α-actinin in muscle is considered.
6. Studies on chemically modified \( \alpha \)-actinin and the effect of chemical modification of actin and myosin on \( \alpha \)-actinin's effect on actomyosin

Studies on the effects of chemical modification of amino acid side chains on \( \alpha \)-actinin's ability to increase rate of turbidity development and Mg\( ^{2+} \)-modified ATPase activity of reconstituted actomyosin suspensions have involved three general kinds of side-chain modification: (1) modification of sulfhydryl groups; (2) modification of \( \varepsilon \)-amino group of lysine; and (3) modification of histidine side chains. All the experiments done to date have used the early, heterogenous \( \alpha \)-actinin preparations, and no side chain modification studies have yet been done using purified \( \alpha \)-actinin. This circumstance, plus the fact that some of the side-chain modification experiments have not included careful analysis of exactly how many amino acid side-chains have been modified and whether this modification affected the ability of \( \alpha \)-actinin to bind to actin, makes the published results of the side-chain modification studies difficult to interpret.

In the most extensive study done thus far on the effect of modification of amino acid side-chains on activity of \( \alpha \)-actinin in the ATPase and turbidity assays, Seraydarian et al. (1968) used AET (2-aminoethyl-isothiouronium bromide) to block cysteine side-chains of \( \alpha \)-actinin, myosin, and
actin individually and collectively, and then tested the effects of such treatment on the activity of α-actinin. AET forms disulfide bonds with the sulfhydryl group of cysteine, and treatment with AET, therefore, results in addition of the 2-aminoethyl-isothiouronium moiety to cysteine side chains. Modification of α-actinin with AET had no effect on the ability of α-actinin to increase rate of turbidity development or Mg\(^{2+}\)-modified ATPase activity of reconstituted actomyosin suspensions, although the number of sulfhydryls in α-actinin that were blocked by AET treatment was not given (Seraydarian et al., 1968).

Modification of myosin with AET blocked approximately 50% of the total sulfhydryl groups in myosin. The Ca\(^{2+}\)-modified ATPase activity of AET-myosin was not suppressed at ionic strengths above 0.1 as the Ca\(^{2+}\)-modified ATPase of untreated myosin was. Reconstituted acto-AET myosin made by mixing AET-myosin with untreated actin had an ATPase activity and rate of turbidity development similar to or slightly less than reconstituted actomyosin made from untreated myosin and untreated actin. However, α-actinin had no effect on either ATPase activity or rate of turbidity development of acto-AET myosin (Seraydarian et al., 1968). This result was complicated by the finding that α-actinin did not bind very well to acto-AET myosin and therefore could not have been expected to increase the ATPase activity
or rate of turbidity development. The inability of α-actinin to bind to acto-AET myosin is most surprising because, as the authors pointed out (Seraydarian et al., 1968), α-actinin binds to the actin moiety of actomyosin and the actin of acto-AET myosin was untreated.

AET modification of actin also blocked approximately 50% of the total sulfhydryl groups of actin (Seraydarian et al., 1968). AET-modified G-actin polymerized normally, and AET-treatment of F-actin did not cause depolymerization. Reconstituted AET-actomyosin made by combining AET-actin with untreated myosin had a Mg\(^{2+}\)-modified ATPase activity and a rate of turbidity development slightly greater than reconstituted actomyosin made from untreated actin and untreated myosin. Addition of α-actinin to AET-actomyosin produced an additional increase in Mg\(^{2+}\)-modified ATPase activity and rate of turbidity development (Seraydarian et al., 1968). Reconstituted AET-acto-AET-myosin made by mixing AET-actin with AET-myosin responded in the same way that acto-AET-myosin did; i.e., its Mg\(^{2+}\)-modified ATPase activity and rate of turbidity development were normal but were not affected by addition of α-actinin (Seraydarian et al., 1968). On the other hand, when reconstituted actomyosin was treated with AET, it responded in much the same way as AET-actomyosin did; i.e., its Mg\(^{2+}\)-modified ATPase activity and rate of turbidity development were increased slightly.
and increased still further upon addition of α-actinin (Seraydarian et al., 1968).

Danker and Hasselbach (Danker, 1971; Danker and Hasselbach, 1971) have also studied the effects of sulfhydryl modification of actomyosin on the ability of α-actinin to accelerate the Mg$^{2+}$-modified ATPase activity of reconstituted actomyosin suspensions. Danker's approach, however, was to study the ionic strength dependence of actomyosin ATPase or ITPase activity before and after sulfhydryl modification by DTNB or before and after addition of α-actinin. As discussed earlier in this review (section on effect of α-actinin on Mg$^{2+}$-activated superprecipitation and ATPase), purified α-actinin is particularly effective at increasing the Mg$^{2+}$-modified ATPase activity of reconstituted actomyosin in the ionic strength range of 0.12 to 0.15. Therefore, α-actinin prevents the marked decrease that is normally observed in Mg$^{2+}$-modified ATPase activity in this ionic strength range (Danker, 1971). Sulfhydryl modification of reconstituted actomyosin with DTNB also prevents the usual marked decrease in Mg$^{2+}$-modified ATPase activity above ionic strengths of 0.05 (Danker and Hasselbach, 1971). Therefore, α-actinin mimics the effect of sulfhydryl modification by DTNB by preventing the usual depression of Mg$^{2+}$-modified ATPase activity of reconstituted actomyosin at ionic strengths above 0.05.
Danker (1971). Danker (1971) extended this similarity between the effects of α-actinin addition and DTNB sulfhydryl modification of actomyosin by showing that neither α-actinin nor DTNB-sulfhydryl modification had any effect on the ionic strength dependence of Mg$^{2+}$-modified ATPase of reconstituted actomyosin (Danker, 1971). Furthermore, addition of DTNB after α-actinin caused no further increase in Mg$^{2+}$-modified ATPase activity at high ionic strength (Danker, 1971). Danker (1971), therefore, proposed that α-actinin may increase the Mg$^{2+}$-modified ATPase activity of reconstituted actomyosin suspensions by modifying sulfhydryl groups in some way. Additional work testing the effects of sulfhydryl modification on α-actinin binding to actomyosin and comparing the effects of sulfhydryl modification and α-actinin binding on actin structure are needed before Danker’s interesting hypothesis (Danker, 1971) can be confirmed.

Muhlrad and coworkers (Muhlrad, 1968; Muhlrad et al., 1968) have studied the effects of modification of lysine or histidine side chains of actin on the ability of α-actinin to accelerate the Mg$^{2+}$-modified ATPase activity or rate of superprecipitation of reconstituted actomyosin prepared from such modified actins. An 800-fold molar excess of succinic anhydride succinylated 30% of the ε-amino groups of lysine and approximately 40% of the sulfhydryl groups of
actin. The Mg\(^{2+}\)-modified ATPase activity of actomyosin reconstituted from such modified actin and untreated myosin was 77% of the control. When actin treated with a 200- to 600-fold molar excess of succinic anhydride was used to make actomyosin, the Mg\(^{2+}\)-modified ATPase activity of the resulting actomyosin was up to 40% greater than that of control actomyosin. However, α-actinin had no effect on the Mg\(^{2+}\)-modified ATPase activities of either of these modified actomyosins.

Treatment of actin with TBS (2,4,6 trinitrobenzene sulfonate) modified four to ten of the 31 ε-amino side chains of actin, depending on the pH and the presence or absence of ATP in the reaction medium. Polymerization of G-actin decreased to 50% of normal if five moles of ε-amino groups were trinitrophenylated per mole of G-actin. The Mg\(^{2+}\)-modified ATPase activity and superprecipitation of actomyosin reconstituted from trinitrophenylated actin and untreated myosin depended on the extent of trinitrophenylation and on whether actin was in the G- or F-actin form during trinitrophenylation. The extent of superprecipitation (measured by the maximum amount of absorption increase attained after addition of ATP) decreased to 50% of normal when 1.8 moles of ε-amino acid groups per mole of G-actin monomer were trinitrophenylated while actin was in the G-form. If trinitrophenylation was done while actin
was in the F-form, however, 4.5 moles of \( \varepsilon \)-amino groups per mole of G-actin had to be trinitrophenylated to cause a 50% decrease in extent of superprecipitation. Neither the presence of ATP nor varying the pH during trinitrophenylation had any effect on the decrease in extent of superprecipitation caused by trinitrophenylation of actin. Trinitrophenylation of 0.6 moles of \( \varepsilon \)-amino groups per mole of actin did not affect the extent of superprecipitation but greatly reduced the rate of superprecipitation (measured by time required for an increase in absorbance to occur after addition of ATP) of actomyosin reconstituted from such actin. On the other hand, trinitrophenylation of 4.9 moles of \( \varepsilon \)-amino groups per mole of actin decreased both the extent and rate of superprecipitation but enhanced the \( \text{Mg}^{2+} \)-modified ATPase activity of actomyosin reconstituted from such actin. The ability of \( \alpha \)-actinin to increase the \( \text{Mg}^{2+} \)-modified ATPase activity of reconstituted actomyosin was lost completely when only 0.5 moles of \( \varepsilon \)-amino groups per mole of actin were trinitrophenylated even though polymerizability or ability of actin to form a complex with myosin at high ionic strength was not affected by trinitrophenylation of 0.5 \( \varepsilon \)-amino groups per G-actin molecule.

The presence of \( \alpha \)-actinin had no effect on the increase in \( \text{Mg}^{2+} \)-modified ATPase caused by trinitrophenylation of 4.9
ε-amino groups per actin molecule. Hence, ability of α-actinin to accelerate the Mg^{2+}-modified ATPase activity of reconstituted actomyosin suspensions seems extremely labile to modification of the ε-amino groups of actin. Muhlrad (1968) did not assay the effects of α-actinin on the rate and extent of turbidity development of reconstituted actomyosin suspensions so it is not clear whether the ability of α-actinin to affect the turbidity development of reconstituted actomyosin is also very labile to modification of the ε-amino groups of actin.

Muhlrad and coworkers (Muhlrad et al., 1968) also studied the effect of photooxidation of histidine in purified actin on the ability of α-actinin to increase the Mg^{2+}-modified ATPase activity and rate of turbidity development of reconstituted actomyosin suspensions. Photooxidation of actin for two minutes caused destruction of two histidine residues per G-actin monomer. The Mg^{2+}-modified ATPase activity of actomyosin reconstituted from such photooxidized actin and untreated myosin was only 20% that of actomyosin reconstituted from untreated actin and untreated myosin, but the extent of turbidity development of actomyosin reconstituted from photooxidized actin was approximately 65% that of untreated actomyosin. If photooxidation of actin was done in the presence of 0.2 mM ATP, two histidine residues were still destroyed, but the ATPase
activity of actomyosin reconstituted from such actin was 50% of normal and the rate of turbidity development was 80% of normal. Photooxidation of actin for two minutes did not oxidize tyrosine and tryptophan side chains. Photooxidation can also destroy methionine and cysteine side chains, but cysteine side-chains were protected in Muhlrad's experiments (Muhlrad et al., 1968). Muhlrad and coworkers, however, did not assay for the loss of methionine and therefore could not eliminate the possibility that some of their results were due to small losses of methionine. Regardless of whether photooxidation was done in the presence of 0.2 mM ATP or not, α-actinin had no effect on the Mg²⁺-modified ATPase activity of actomyosin reconstituted from photooxidized actin and normal myosin. The effect of α-actinin on the rate of turbidity development of such actomyosin was not tested.

All the experiments of Muhlrad and coworkers (Muhlrad, 1968; Muhlrad et al., 1968) were done with impure α-actinin preparations made according to Ebashi's early procedure (Ebashi and Ebashi, 1965). Furthermore, it is not clear from the results presented whether the amino acid side-chain modifications used by Muhlrad and colleagues (Muhlrad, 1968; Muhlrad et al., 1968) disrupted actin structure so severely that α-actinin could no longer bind to actin, or that the modified actin could not respond to
any protein modifier, either ε-actinin or the troponin-
tropomyosin system, regardless of whether they were bound. Consequently, additional work with highly purified ε-
actinin and more extensive testing of the effects of side-
chain modification on actin's ability to bind ε-actinin or
to function in the contractile actomyosin system is needed
to confirm the interesting results of Muhlrud and co­
workers (Muhlrad, 1968; Muhlrad et al., 1968).

D. Structure and Properties of Actin

Actin was discovered about 30 years ago (Straub, 1942)
as a contaminating protein in existing myosin preparations.
It was soon established that interaction of actin with
myosin, coupled with the splitting of ATP, is the elementary
process of muscle contraction (Szent-Gyorgyi, 1951). In
salt-free solvents, actin exists as dispersed globular
molecules, called G- or globular actin. G-actin is a rel­
atively well-characterized protein that has a monomeric
molecular weight of 41,785 (Elzinga et al., 1973), and whose
complete amino acid sequence is known. As usually isolated,
each molecule of G-actin contains one molecule of bound ATP
and one molecule of bound cation, usually Mg$^{2+}$ or Ca$^{2+}$.
Addition of neutral salt solutions, such as KCl, to a final
concentration of 100 mM, or addition of divalent cations,
such as Mg$^{2+}$ or Ca$^{2+}$, to a final concentration of 2-3 mM
causes polymerization of G-actin into long fibrous polymers called F- or fibrous actin. In a classical study on the structure of F-actin, Hanson and Lowy (1963) showed by electron microscope examination of negatively stained F-actin preparations that F-actin consists of two strands of spherical G-actin monomers coiled around each other to produce a double-stranded filament. This same structure was observed in negatively stained thin filaments from partly extracted myofibrils; these observations confirmed that actin constitutes part of the thin filaments in muscle (Huxley and Hanson, 1954) and that actin in vivo exists largely in the F- or fibrous form. Subsequently, careful X-ray diffraction measurements (O'Brien et al., 1971) and electron microscope observations (Moore et al., 1970) showed that the distance between cross-over points in the double-stranded F-actin helix is 360 Å and that the individual spherical G-actin subunits are 55 Å in diameter.

Although the length of F-actin filaments in mammalian skeletal muscle is very uniform at 1.0 μm, the length of F-actin filaments produced by polymerizing G-actin preparations in vitro is very heterogenous and ranges from 0.2 to over 8 μm (number average of 1.5 μm and weight average of 3.1 μm) depending on conditions of polymerization (Kawamura and Maruyama, 1970). The mechanism controlling length of F-actin filaments in vivo is still unclear,
but Maruyama and coworkers (Kawamura and Maruyama, 1970; Kawamura and Maruyama, 1972; Maruyama, 1966) have suggested that a new myofibrillar protein, called \( \beta \)-actinin, may be involved in controlling F-actin filament length \textit{in vivo}. Because \( \beta \)-actinin has not yet been obtained in a purified form, the extent of its role in determination of F-actin filament length is still unknown.

\( G \)-actin contains one 3-methyl histidine and five sulfhydryl groups per molecule, and like the other myofibrillar proteins, it has no cystine (Asatoor and Armstrong, 1967; Lusty and Fasold, 1969). Also like other myofibrillar proteins, \( G \)-actin contains a high proportion of glutamic and aspartic acid residues (Elzinga, 1970) and has an acidic isoelectric point of 4.7-4.8 (Connell, 1964). Within the last three years, proteins nearly identical to skeletal muscle \( G \)-actin have been isolated from a wide variety of different cell types (Adelstein and Conti, 1972; Berl and Puszkin, 1970; Fine and Bray, 1970; Palevitz \textit{et al.}, 1974), and it now seems probable that almost every cell type from protozoa to liver cells contain actin. Moreover, actin is a very highly conserved protein in all these different cell types (Bray, 1972). These recent observations indicate a vital and important role for actin in many forms of biological movement other than muscle contraction.
The one molecule of bound nucleotide and the one molecule of cation (Mg\(^{2+}\) or Ca\(^{2+}\)) associated with each G-actin monomer have been very important in studying the conformational changes in F-actin that are induced by its interaction with myosin or other myofibrillar proteins. Therefore, the remainder of this section will be restricted to a discussion of the bound nucleotide and cation of actin. Soon after the isolation of actin (Straub, 1942), it was discovered that during polymerization, the molecule of ATP bound to G-actin was dephosphorylated to ADP, and that F-actin contained only ADP (Laki et al., 1950; Straub and Feuer, 1950). Furthermore, the number of inorganic phosphate molecules released by hydrolysis of the bound ATP of G-actin was exactly equal to the total number of G-actin molecules incorporated into the F-actin polymer (Laki et al., 1950; Straub and Feuer, 1950). The ADP formed by cleavage of ATP during polymerization remains tightly bound to F-actin (Mommaerts, 1954), but the inorganic phosphate formed is released to the surrounding medium (Ulbrecht et al., 1960). Although polymerization of actin is accompanied by dephosphorylation of the ATP bound to each G-actin monomer, depolymerization of F-actin in the presence of phosphate is not accompanied by re-phosphorylation of ADP to ATP. Therefore, to obtain G-actin having bound ATP again, it is necessary to depolymerize
F-actin in the presence of ATP. This type of polymerization-depolymerization cycle was exploited by Mommaerts (1951) in devising a scheme for purification of actin.

The discovery that the ATP bound to G-actin was dephosphorylated during polymerization initiated a large number of studies attempting to determine whether dephosphorylation of the bound nucleotide of actin had any direct role in muscle contraction. Although this question has not yet been completely resolved, a great deal has been learned about the properties of the bound nucleotide of actin. One of the earliest discoveries showed that both the bound nucleotide and cation of G-actin were freely exchangeable with nucleotide or cation in the medium, whereas neither the bound nucleotide nor cation of F-actin exchanged very rapidly with nucleotide or cation in the medium (Barany et al., 1962; Martonosi et al., 1960a; Strohman, 1959; Strohman and Samorodin, 1962; Moos and Eisenberg, 1970; Kasai and Oosawa, 1968). This finding, which has been extensively documented, shows that conformation of the G-actin monomer is not the same when these monomers are polymerized in F-actin strands as it is when the monomers are unpolymerized. Although several investigators have suggested that the slowly exchangeable nucleotide bound to F-actin serves as a connecting link between individual G-actin monomers in the F-actin filament (Asakura and Oosawa, 1960;
Tsao, 1953), the preparation of a G-actin containing no bound nucleotide (Barany et al., 1966; Kasai et al., 1965; Kasai and Oosawa, 1968; Tokiwa et al., 1967) and conversion of this G-actin into an F-actin filament also containing no nucleotide clearly showed that the bound nucleotide is not necessary for binding G-actin monomers together in the F-actin filament. Numerous other attempts were made (Moos, 1964; Strohman, 1959; Strohman and Samorodin, 1962; Yagi and Noda, 1960) to show that transphosphorylating systems such as creatine kinase and creatine phosphate could phosphorylate the ADP bound to a G-actin that was formed by depolymerization of F-ADP actin in the absence of ATP while this ADP remained bound to the protein. Recent results (West et al., 1967) however, have demonstrated that the ADP nucleotide of G-actin must be released from the protein and be free in the medium before it can be phosphorylated by the creatine kinase and creatine phosphate system.

Because of the highly birefringent nature of F-actin solutions, spectroscopic studies of actin conformation before and after polymerization have been very difficult. Difference spectra in the range of 210 to 310 nm have suggested that during polymerization, some tryptophan and tyrosine residues formerly exposed to the solvent are folded into the interior of the molecule (Higashi and
Oosawa, 1965). Murphy (1971) also found that polymerization of G-actin was accompanied by increases in the interaction among aromatic residues and between the nucleotide and the protein. Electron paramagnetic resonance spectra of actin labeled with a paramagnetic derivative of maleimide showed that a strong immobilization of the label occurred during polymerization (Stone et al., 1970); this suggests that a sulfhydryl group may be folded into the interior of the molecule during polymerization. Comparison of ultraviolet absorption spectra of G-ATP-actin, G-ADP-actin, and G-ITP-actin suggests that binding of nucleotide to G-actin causes a conformational change near some tyrosine and tryptophan residues (West, 1970). Higashi and Oosawa (1965) also found that binding of nucleotide alters the environment of tyrosine and tryptophan residues, but in contrast to West (1970), Higashi and Oosawa (1965) found no spectral differences among G-actins containing bound ATP, ADP or ITP. G- and F-actin also differ in the reactivities of their ε-amino side chains (Muhlrad, 1968; see preceding section of this review), in availability of side chain sulfhydryl groups (Lusty and Pasold, 1969) in ability to stimulate the Mg^{2+}-modified ATPase activity of myosin (G-actin stimulates this activity only fourfold whereas F-actin stimulates it almost 100-fold, Cooke and Morales, 1971; Offer et al., 1972), and in various other ways.
(Holmes et al., 1971; Martonosi et al., 1960a). In spite of this abundant evidence that structures of G-actin monomers in the unpolymerized and polymerized states differ markedly, the exact nature of these differences remains unclear.

Several studies have been done to determine whether G-actin can bind nucleotides other than ATP and what the effect of substitution of nucleotides other than ATP would be on the structure of G-actin. Martonosi and coworkers (Martonosi, 1962; Martonosi and Gouvea, 1961), and Ikehara et al. (1961) have shown that rate of exchange of $^{14}$C-ATP bound to G-actin with different medium nucleotides decreased in the order ATP $>$ TTP $>$ UTP $>$ dATP $>$ dGTP $>$ dCTP. This order of reactivity agrees with the magnitude of the binding constants found for association of these same nucleotides to G-actin (Iyenger and Weber, 1964). That these nucleotides di- and triphosphates all have different binding constants for G-actin plus the fact that d-ATP, d-GTP, d-CTP, ribose, or adenosine do not bind to G-actin at all suggests that the nitrogenous base, sugar, and phosphate side chain all participate in the binding of nucleotide to G-actin. Although substitution of different nucleotides for ATP in G-actin has no obvious immediate effect on conformation or ability of the G-actin molecule to polymerize, it was noted that G-actin which contained UDP, ADP or any nucleotide with less affinity for actin than ADP was not stable in the absence of
ATP and lost the ability to polymerize upon standing for several hours at 0° (Martonosi and Gouvea, 1961; Nakamura and Tonomura, 1967).

In addition to studying the effects of different nucleotides on G-actin, a few studies have been done on the effects of different cations on G-actin structure. Strzelecka-Golaszewska and Drabikowski (1968) and Kasai and Oosawa (1968) have found that affinity of different cations for actin depends to a large extent on ionic radius of the cation. Assuming an affinity constant of 1.0 for the binding of Ca\(^{2+}\) to G-actin, the other cations tested had affinity constants of 0.90 for Mn\(^{2+}\), 1.07 for Cd\(^{2+}\), 0.22 for zinc, 0.18 for Co\(^{2+}\) and 0.08 for Ni\(^{2+}\). Several studies have shown that rate of exchange of ATP bound to G-actin with ATP in the medium is inversely proportional to the concentration of free cation in solution and that high concentrations of free cation decrease rate of ATP exchange (Strzelecka-Golaszewska, 1973). Based on this result, Kuehl and Gergely (1969) have proposed that exchange of ATP in G-actin occurs through those molecules that momentarily are free of bound cation. Therefore, it was suggested that the effects of various cations on rate of ATP exchange in G-actin solutions should parallel the binding constants of the same cations for G-actin, with cations having a high affinity for actin also causing a large decrease in rate of
exchange. Recent results, however, have failed to show this parallel between affinity of a cation for G-actin and effect of that cation on ATP exchange in G-actin solutions (Strzelecka-Golaszewska, 1973). It is currently believed that the types of cation bound may have a specific effect on conformation of the G-actin molecule. Whatever this conformational effect is, it must be small because neither optical rotatory dispersion nor circular dichroism spectra reveal any structural differences between G-actin which contain Ca^{2+}, Mg^{2+} or Mn^{2+} as the bound cation (Nagy and Strzelecka-Golazewska, 1972). Furthermore, the electron spin resonance spectra of G-actins which contain bound Ca^{2+}, Mg^{2+}, or Mn^{2+} are the same (Burley et al., 1972). It is known, however, that G-actin gradually loses polymerizability in the total absence of bound cation (Asakura et al., 1963a,b).

The most extensive studies on the actin nucleotide have involved examination of the exchange of the nucleotide bound to F-actin (i.e., ADP) under a variety of conditions, because actin exists in the F- or fibrous form in living muscle (Hanson and Lowy, 1963), and any contraction-induced change in actin may be expected to alter the exchangeability of the F-actin bound nucleotide. As already mentioned, the exchange of ADP bound to F-actin is very much slower than the exchange of ATP bound to G-actin (Martonosi et al.,
Martonosi et al. (1960b) first reported that very little radioactivity was incorporated into the ADP bound to actin during in vivo contractions of rabbit, rat, or pigeon skeletal muscle that had been injected with $^32P$ prior to stimulation. Subsequently, however, Szent-Gyorgyi and Prior (1966) found that exchange of actin-bound ADP was greatly accelerated in the presence of myosin and during superprecipitation. Superprecipitation seemed to be required for this rapid exchange, because very little exchange occurred if superprecipitation was prevented by lowering the temperature to 0° or by reducing Mg$^{2+}$ concentration. Szent-Gyorgyi and Prior (1966) interpreted their results to indicate that the contractile interaction of myosin with actin changed the structure of actin so that its nucleotide momentarily became exchangeable. This report initiated a number of studies on the exchange of F-actin bound ADP during interaction with myosin. Moos, Eisenberg and coworkers (Moos and Eisenberg, 1970; Moos et al., 1967) pointed out that the increase in exchange of F-actin-bound ADP during superprecipitation of actomyosin is not quantitatively correlated with superprecipitation itself because 1) it does not occur at the same time that the turbidity increase occurs in superprecipitating actomyosin, 2) it ceases before exchanging 100% of the bound ADP even when ATP
hydrolysis by actomyosin is continuing, 3) the rate of ADP exchange does not change with the ATPase rate when ATP concentration is varied, and finally 4) myosin will also accelerate exchange of F-actin-bound ADP in the presence of ADP where no superprecipitation occurs. Consequently, although it is clear that interaction with myosin greatly accelerates the normally sluggish exchange of F-actin-bound ADP with medium ATP or ADP, it seems equally clear that this accelerated rate of exchange is not directly related to contraction, at least as contraction is presently measured in vitro. Therefore, the ability of myosin to accelerate exchange of ADP bound to actin remains unexplained.

The increase in rate of F-actin-ADP exchange induced by myosin is not an unspecific affect caused by any protein that binds to actin because binding of tropomyosin to F-actin causes the opposite effect; vis., a decrease in rate of exchange of F-actin-bound ADP (Kitagawa et al., 1968). Moreover, the presence of sulfhydryl protective agents like DTT, cation chelators like EDTA, or Mg^{2+} (but not Ca^{2+}) decrease the rate of exchange of F-actin-bound ADP (Hayashi and Rosenbluth, 1960; Kitagawa et al., 1968). On the other hand, sulfhydryl blocking agents such as NEM, or substitution of CDP in the F-actin polymer both increase the rate of exchange of F-actin nucleotide (Estes and Moos, 1969; Kakol and Weber, 1965; Kitagawa et al., 1968).
Because of the interest in learning whether the bound ADP of F-actin has a role in contraction, several studies were done to determine whether a F-actin containing no nucleotide could be prepared and whether such a nucleotide-deficient F-actin would increase the Mg\(^{2+}\)-modified ATPase activity of myosin and support superprecipitation. Mommaerts (1952) was the first to report that F-actin gradually lost its bound nucleotide during prolonged dialysis, and in 1965, Kasai et al. (1965) developed a procedure for preparing polymerizable but nucleotide-free G-actin by treatment with EDTA and Dowex-1 in sucrose solution. This treatment caused removal of both the bound nucleotide and bound cation of actin and resulted in a G-actin preparation that was extremely labile, and that could be stored for only short periods of time in sucrose solutions at 0° before losing polymerizability. Subsequently, Barany et al. (1966) produced a nucleotide-free F-actin directly by substituting AMP for the ADP normally bound to F-actin and then removing the weakly bound AMP by adsorption on charcoal. Substitution of AMP for ADP was done by subjecting F-ADP-actin to ultrasonic treatment in the presence of an excess of AMP. Substitution of nucleotide-free F-actin for normal ADP-containing F-actin in mixtures with myosin had no effect on the Mg\(^{2+}\)-modified deoxy-ATPase or CTPase activities of the resulting actin-myosin complex, or on
ability of the actin-myosin complex to superprecipitate
(Barany et al., 1966; Nakamura and Tonomura, 1967; Tokiwa
et al., 1967). These experiments could not be done by
using ATP to cause superprecipitation or as a substrate for
Mg$^{2+}$-modified enzymic activity because ATP was very rapidly
incorporated into the ADP-free F-actin. Consequently,
deoxy-ATP or CTP, both of which support superprecipitation
of actomyosin (Hasselbach, 1956; Ikehara et al., 1961) but
neither of which are incorporated into nucleotide-free F-
actin were used. These experiments, therefore, again
strongly suggested that the nucleotide of F-actin was not
required for contraction, at least as contraction is meas­
ured in in vitro systems.

During this same period of intensive study of the
bound nucleotide of F-actin, it was found that F-actin
solutions subjected to ultrasonic treatment rapidly ex­
changed their bound nucleotide with nucleotide in the
medium (Asakura, 1961; Asakura et al., 1963a, 1963b;
Barany and Finkelman, 1963), and that if the nucleotide in
the medium was ATP, this rapid exchange was accompanied by
liberation of inorganic phosphate. The inorganic phosphate
originated from cleavage of ATP as it was incorporated into
the F-actin polymer in exchange for ADP. Repetition of the
release of the newly bound ADP followed by incorporation
and then splitting of another molecule of ATP produced a
continuous release of inorganic phosphate. Consequently, during ultrasonic treatment, purified actin seems capable of acting as an ATPase, although its specific activity under ordinary conditions of pH and temperature is one to two hundred fold less than the specific activity of actomyosin. Subsequently, it was shown that high temperatures (50 to 60°C, Asai and Tawada, 1966) or high pressures (1000 atmospheres, Ikkai and Ooi, 1966) also markedly increased the ATPase activity of F-actin. Whenever the ATPase activity of F-actin is high, the rate of exchange of its bound nucleotide is also high. Consequently, F-actin ATPase activity seems to originate entirely from cleavage of ATP as it is incorporated into the F-actin polymer in exchange for ADP. Because sonic vibration did not seem to greatly decrease viscosity or flow birefringence of actin, it was concluded that vibration did not cause depolymerization of F-actin filaments to G-actin. On the other hand, the nucleotide of F-actin normally exchanges quite slowly, and it was unclear how ultrasonic treatment could cause large increases in rate of nucleotide exchange and hence ATPase activity of F-actin without first depolymerizing F- to G-actin in which the nucleotide is readily exchangeable. In an attempt to explain this seeming dichotomy, Asakura et al. (1963b) proposed that sonic vibration ruptured two of the four bonds that an actin monomer forms with its neighbors in a F-actin filament, and
that rupture of those two bounds produced an actin species which Asakura et al. (1963b) called "f-actin", whose nucleotide was readily exchangeable with medium nucleotide like the nucleotide of G-actin was, but which was still part of an F-actin filament because two of the four bonds binding it to the thin filament still existed. In Asakura's scheme, therefore, F-actin ATPase proceeds as shown in the following equation:

\[
\begin{align*}
F-\text{ADP-actin} & \quad \text{Pi} \\
& \quad f-\text{ATP-actin} \\
& \quad f-\text{ADP-actin} \\
& \quad \text{medium ATP} \\
& \quad \text{ADP}
\end{align*}
\]

It was subsequently found (Hama et al., 1969; Nakaoka and Kasai, 1969) that ultrasonic treatment ruptures some long F-actin filaments to short filaments that co-exist with G-actin monomers. Therefore, it was suggested that nucleotide exchange and ATPase activity of actin solutions in a sonic field originates from rapid cycles of depolymerization-repolymerization occurring exclusively at the ends of each of these short polymers produced by ultrasonic treatment rather than by formation of an "f-actin" species as originally suggested by Asakura et al. (1963b). More recently,
however, it has been argued that because tropomyosin de-
presses F-actin ATPase activity (much as it was earlier
shown that tropomyosin depresses rate of exchange of ADP
bound to F-actin (Kitagawa et al., 1968), but has no effect
on the rate of polymerization of actin, the ATPase activity
of F-actin cannot originate solely from rapid cycles of
depolymerization-repolymerization at the ends of the F-actin
filaments (Tanaka and Oosawa, 1971). Plasmodium actin forms
a "Mg\(^{2+}\) polymer" that has a high ATPase activity similar to
that of skeletal actin in a sonic field (Totsuka and Hatano,
1970). Comparison of the extent to which nucleotide bound
to "Mg\(^{2+}\) polymer" of plasmodium actin with the ATPase activ-
ity of this actin showed that ATPase activity in the Mg\(^{2+}\)
polymer could not occur through depolymerization-repolymer-
ization cycles at the ends of the polymer (Totsuka and
Hatano, 1970) but more likely proceeded through formation
of a "f-actin" species as postulated by Asakura et al.
(1963b). Skeletal F-actin has a high ATPase activity at acid
pH values (pH of 4.5 to 5.5), and again, comparison of the
extent of nucleotide exchange with amount of ATP hydrolysis
led to the conclusion that at acid pH values, F-actin ATPase
activity probably occurs through formation of a "f-actin"
species rather than through rapid cycles of depolymerization-
repolymerization at the ends of the filaments (Kuroda and
Consequently, it currently seems likely that nucleotide exchange and ATPase activity of F-actin filaments occur through similar if not identical mechanisms, and that both these phenomena are caused by structural alterations in actin monomers within an F-actin filament. The exact nature of these structural changes and their relation to muscle contraction, however, remain enigmatic. Moos and Eisenberg (1970) and Appenheimer et al. (1972) have suggested that the rapid exchange of F-actin nucleotide caused by addition of myosin is simply due to repair of defective sites in the actin filament caused by strain placed on the filament during its interaction with myosin. On the other hand, Murthy et al. (1968) have suggested that organic solvents normally used to prepare actin destroy its native ATPase activity, and have described the preparation of a G-actin that possesses a potent Mg\(^{2+}\) modified ATPase activity. Two recent studies on exchange of the ADP bound to F-actin during contraction (Cheesman and Priston, 1972; Cheesman et al., 1969) clearly show that ADP is incorporated into actin filaments during in vivo contraction and that this incorporation does not occur when contraction is prevented by curarization of the muscle. This incorporation occurs extremely rapidly with 25% of the total actin nucleotide incorporating labeled ATP in a single isometric twitch and with 40% or more of the total actin nucleotide incorporating labeled ATP during a
five-second tetanus. Consequently, it seems clear that exchange of F-actin nucleotide occurs during contraction in vivo, and as pointed out by Cheesman et al. (1969), this exchange is an energy-requiring reaction and would be an extremely wasteful process unless it were an essential feature of contraction. Therefore, additional studies using new approaches are needed to examine nucleotide-exchange of actin.

E. Z-Disk Structure and Enzymic Removal of Z-Disks

Z disks in striated muscle are situated at both ends of the sarcomere and form fixed points to which actin filaments from opposite sides of adjacent sarcomeres attach. This arrangement enables the relative motion or tension development between actin and myosin filaments in an individual sarcomere to be transmitted to the ends of the muscle cell and causes shortening or tension development of the entire cell. A number of ultrastructural studies have attempted to determine the molecular architecture of the Z disk (Franzini-Armstrong, 1973; Franzini-Armstrong and Porter, 1964; Katchburian et al., 1973; Kelly, 1967; Kelly and Cahill, 1972; Knappeis and Carlsen, 1962; Landon, 1970a, 1970b; Reedy, 1964; Saide and Ullrick, 1973), and several models of Z-disk structure have been proposed as a result of these studies.

Although no single model of Z-disk architecture has yet been proven correct, all models agree that thin filaments
are disposed to occupy the corners of squares as they enter the Z disk (Franzini-Armstrong, 1973). The square lattice formed by four actin filaments on one side of the Z disk are shifted both vertically and horizontally by a distance equal to one-half the length of the side of the square lattice relative to the square lattice formed by thin filaments on the opposite side of the Z disk. Therefore, each thin filament on one side of the Z disk occupies the center of the square lattice formed by four thin filaments on the opposite side of the Z disk. Several different models exist, however, to explain how this arrangement of thin filaments is joined across the Z disk. The model that has received the most support and that seems to account for most of the structural properties observed for Z disks (Franzini-Armstrong, 1973; Katchburian et al., 1973) is based on Knappeis and Carlsen's early description (Knappeis and Carlsen, 1962) of Z-disk structure. In the Knappeis and Carlsen model, the thin filament, as it enters the Z disk splits into four separate, smaller filaments, called Z filaments, that pass diagonally through the Z disk and join the four thin filaments forming the square lattice facing that filament on the opposite side of the Z disk. Sides of the square lattice formed by thin filaments as they enter the Z disk are approximately 24-26 nm long, the thin filaments are approximately 10 nm in diameter as they enter the
Z-disk, and the four Z filaments continuing through the Z disk from each thin filament are approximately 4 to 5 nm in diameter (Franzini-Armstrong, 1973). Kelly and Cahill (1972) have shown that Z disks contain an amorphous or matrix component in addition to Z filaments. This amorphous component surrounds and envelopes the Z filaments, and in the Z disks of some muscles, it seems to completely fill the lattice spaces. Because the amount of amorphous component varies among Z disks from different muscles and because it is structureless and stains extraordinarily densely with normal electron microscope stains, the presence of the amorphous component has probably contributed to the disagreement concerning Z-disk architecture (Kelly and Cahill, 1972).

The Knappesi and Carlsen model (Knappesi and Carlsen, 1962) for Z-line architecture provides no information on the chemical nature of the Z filaments and the amorphous component. The amorphous component is very resistant to high ionic strength extraction with Weber-Edsall's solution (in Garamvogyi, 1962), and it survives both myosin and actin extraction (Hanson and Huxley, 1955). Corsi and Perry (1958) were the first to observe that extraction up to 4-5 days with low ionic strength solvents (5 mM Trisbuffer, pH 7.7) removed the dense or amorphous material from Z-disks. The low ionic strength extracts were shown to
contain tropomyosin and denatured actin (although these extracts also almost certainly contained \( \alpha \)-actinin, \( \alpha \)-actinin was not discovered until seven years later).

Samosudova (1966) also noted that low ionic strength extraction removed the dense, amorphous material of \( Z \)-disks, but she did not attempt to determine the nature of the protein extracted by low ionic strength solvents. Stromer and coworkers (Stromer et al., 1967b, 1969) were the first to exploit the potential of low ionic strength extraction of \( Z \) disks by combining a careful ultrastructural study of the extraction with biochemical analysis of the extracts. Stromer et al. (1967b, 1969) found that both \( M \) lines and the dense material from \( Z \) disks were removed from glycerinated muscle fibrils by 10 days extraction at \( 0^\circ \) with 2 mM Tris, pH 7.6, 1 mM DTT, and that the dense material could then be reconstituted in \( Z \)-disks by incubating the extracted myofibrils with a fraction salted out from the extract between 0 and 40% ammonium sulfate saturation. The crude extract before ammonium sulfate fractionation always contained tropomyosin, troponin, \( \alpha \)-actinin and other unidentified components. Reconstitution of the dense material in low ionic strength extracted fibrils could also be achieved by incubating the extracted fibrils with a fraction obtained from a Bailey extract of natural actomyosin followed by salting out between 0 and 40% ammonium sulfate saturation.
(Stromer et al., 1969). If low ionic strength extraction was prolonged until a gap was produced in the Z-disk region, reconstitution of a dense Z-disk structure was not possible. Both extracts capable of reconstituting the dense material in Z disks of extracted myofibrils were demonstrated to contain no tropomyosin. Huxley (1963) had earlier pointed out that the ultrastructure of tropomyosin crystals was remarkably similar to the ultrastructure observed in cross-sections through Z disk, and Endo et al. (1966) had reported that tropomyosin antibodies bound to Z-disks. Consequently, there was a widespread belief that the Z-disk was composed of tropomyosin. The results of Stromer and coworkers (Stromer et al., 1969), however, showed that the dense material in Z-disk could not contain tropomyosin but must be composed of some substance that salts out between 0 and 40% ammonium sulfate saturation. As has been discussed earlier in the review (see section on localization of α-actinin, α-actinin is located somewhere in the Z disk, and α-actinin salts out between 0 and 30% ammonium sulfate saturation (Arakawa et al., 1970b). Very recently, Schollmeyer et al. (1973) have succeeded in showing by careful antibody localization observations at the electron microscope level that α-actinin is located in the amorphous or dense component of the Z disk.

Although the low ionic strength extraction studies and
Localization of antibody binding have shown that the amorphous or dense material of Z disks is at least partly composed of α-actinin, composition of the Z filaments remains unclear. As indicated previously in this review, the 2 to 2.5% α-actinin contained in myofibril (Robson et al., 1970) cannot constitute the 6% of myofibrillar mass that is made up by the Z disk (Huxley and Hanson, 1960). A wide variety of proteolytic enzymes, including trypsin (Ashley et al., 1951; Endo et al., 1966; Goll et al., 1969; Stromer et al., 1967a), papain (Chaudhry, 1969), chymotrypsin (Stromer and Goll1), pepsin (Kelly and Cahill, 1972), and pronase (Kelly and Cahill, 1972), will quickly remove Z disks from myofibrils without causing other obvious ultrastructural changes. This observation suggests that Z disks contain a proteolytically labile component, and because α-actinin and F-actin are not unusually susceptible to proteases but tropomyosin is, it has continued to be attractive to suppose that part of the muscle cells tropomyosin might be located in the Z disk. The ultrastructural resemblance of tropomyosin crystals and cross-sections through the Z disk (Huxley, 1963) has already been mentioned. Fawcett (1968) also suggested on the basis of the ultra-

structural resemblance of abnormal Z-disks to tropomyosin crystals that the Z disk contained tropomyosin. Rash et al. (1968) pointed out that every extraction procedure that extracts Z disks also extracts tropomyosin and theorized that tropomyosin was the only protein other than actin and myosin that was present in large enough quantities to constitute Z disks. In spite of all this circumstantial evidence, however, Pepe (1966) found that tropomyosin antibodies did not bind to Z disks. More recently, Caspar et al. (1969) concluded on the basis of X-ray diffraction and electron microscope studies that the tropomyosin crystal net has completely different symmetry than the Z-disk lattice, and that no distortion of the tropomyosin lattice could produce a net having the Z-disk symmetry without altering the basic pattern of connections. Consequently, it must be concluded that no direct evidence exists to indicate that tropomyosin is located in the Z disk, and the biochemical nature of the Z filaments remain unclear.

As indicated previously in this review (see section on interaction of α-actinin with other myofibrillar proteins), α-actinin binds only to F-actin among the known myofibrillar proteins. Therefore, α-actinin in the dense or amorphous component of the Z disk must be able to bind to an F-actin filament. Recently, Busch et al. (1972) discovered a Ca^{2+}-activated factor, which they called CAF, in muscle. This
factor very quickly removes Z disks from myofibrils without causing other ultrastructurally detectable deletions. CAF has now been purified (Dayton et al., 1973, 1974) and has been shown to be a proteolytic enzyme. Incubation of myofibrils with CAF destroys the Z disk and concomitantly releases α-actinin from the rest of the myofibrillar structure. α-actinin released by CAF treatment of myofibrils, however, seems identical to untreated α-actinin, and shows no obvious indications of being proteolytically degraded (Dayton et al., 1974). Treatment of purified myofibrillar proteins with CAF shows that it degrades tropomyosin, troponin T, troponin I, and C-protein, but has no obvious effects on myosin, actin, α-actinin, or troponin-C. On the other hand, Reddy et al. (1974), who used less highly purified preparations of CAF, have reported that CAF will degrade α-actinin. Resolution of these contradictory findings could have important implications to Z-disk structure, because if CAF can remove Z disks without altering either actin or α-actinin, it would suggest that these two proteins do not interact directly in the Z disk but that a third protein, which is degraded by CAF, must be involved in binding α-actinin into the Z disk. Finally, that CAF, an enzyme that removes Z disks, also degrades tropomyosin adds to the circumstantial evidence suggesting that the Z disk contains tropomyosin.
F. Role of α-Actinin in Muscle

Although this review has discussed the purification and properties of α-actinin in detail and has summarized the direct evidence that α-actinin is located in the Z disk of striated muscle, little has been said about physiological role of α-actinin in muscle. Unfortunately, in spite of the abundance of information that has accumulated on the properties and exact location of purified α-actinin, little direct evidence exists concerning the physiological role of α-actinin in movement systems. α-Actinin was discovered because of its ability to accelerate the rate of turbidity development in reconstituted actomyosin suspensions (Ebashi and Ebashi, 1965). Although purification of α-actinin has shown, contrary to the earlier findings (Briskey et al., 1967b; Maruyama, 1966; Seraydarian et al., 1967), that purified α-actinin can increase the Mg$^{2+}$-modified ATPase activity and rate of turbidity increase in reconstituted actomyosin suspensions at ionic strengths and α-actinin to actin ratios close to those found in vivo (Arakawa et al., 1970b; Robson et al., 1970), the mechanism whereby α-actinin affects these in vitro measures of contraction remains completely unknown. The early suggestion that α-actinin might be located in the Z disk has been confirmed by direct antibody localization (Schollmeyer et al., 1973). The finding that α-actinin is located in the amorphous or dense material
rather than the Z filaments themselves, however, has weakened rather than strengthened the argument that α-actinin might have a purely structural role in muscle because it is difficult to imagine how the dense material could be involved in cross-linking F-actin filaments across the Z-disk. The ostensibly ubiquitous presence of α-actinin in all movement systems that have been examined for its occurrence suggest a fundamental role for α-actinin in actin-based movement systems. The presence of α-actinin at the ends of actin filaments in these nonmuscle motile systems, and the fact that it is located in the Z disk, which is also at the ends of actin filaments in muscle, might suggest that α-actinin controls directionality of F-actin filaments, perhaps by serving as a nucleus for initiating polymerization of actin in vivo.

On the other hand, it has been shown that the ability of α-actinin to increase the Mg\(^{2+}\)-modified ATPase activity or rate of turbidity increase of reconstituted actomyosin suspensions is not a nonspecific effect but requires the same nucleotide substrate and cations that are required for tension development of muscle strips (Hasselbach, 1956). Moreover, Maruyama and Kimura (1971) have shown that addition of α-actinin to actomyosin threads made by spreading actomyosin on a water surface (Hayashi, 1952) more than doubles the tension developed by these threads. Because
reconstituted actomyosin cannot produce an *in vitro* contractile response at physiological ionic strengths and because addition of α-actinin increases the ionic strength at which reconstituted actomyosin will undergo an *in vitro* contractile response to near physiological ionic strength, it is attractive to suggest that α-actinin has a physiological role in strengthening the actin-myosin interaction so that this interaction can occur at the high ATP and Mg\(^{2+}\) levels and the high ionic strengths found *in vivo* (Arakawa *et al.*, 1970b; Danker, 1971; Robson *et al.*, 1970; Temple and Goll, 1970). It is clear, however, that in skeletal muscle, α-actinin is located at the Z disk which is nearly 0.5 μm distant from the actin-myosin overlap where the actin-myosin interaction occurs. Obviously, if α-actinin has a direct role in modifying the actin-myosin interaction, it must be capable of altering the structure of the F-actin filament all along the filament even though it is restricted to one end of the filament. If it can be shown that α-actinin has such a remarkable ability to alter F-actin structure, then a direct role of α-actinin in modification of the actin-myosin interaction could be seriously considered. It would still be necessary to show how the ability of α-actinin to cause such a structural modification of α-actinin could be controlled (for example, binding of Ca\(^{2+}\) might turn α-actinin "on") before it could be concluded
that α-actinin acts physiologically to alter the actin-myosin interaction. In the absence of such evidence, the suggestion that α-actinin has a physiological role in strengthening the actin-myosin interaction must remain a tentative one.
III. METHODS AND MATERIALS

Except where indicated, all protein preparations were done at 0 to 3°C and used precooled solutions. Double-deionized, distilled water that had been redistilled in glass and stored in polyethylene containers was used for preparation of all solutions. All reagents and enzymes used were of the highest purity obtainable.

Chicken gizzard, chicken breast muscle, and bovine hearts were obtained in frozen form from Pel-Freez Biochemicals, Inc., Rogers, Arkansas. The muscle samples were thawed just before use. **Semitendinosus**, **biceps-femoris**, and **semimembranous** muscles were removed from pigs immediately after exsanguination at the University Meat Laboratory. The muscles were transported at once to the Muscle Biology Laboratory, were trimmed free of fat and connective tissue, were chilled on ice, were then ground in a precooled meat grinder, and were used immediately for preparation of the different myofibrillar proteins. Trimming was done in the cold room at 2°C, so cooling of the muscles began within 15-20 minutes after exsanguination.
A. Preparative Procedures

1. Actin

An acetone-dried myofibrillar powder was obtained from porcine skeletal muscle by using the method of Seraydarian et al. (1967). Actin was extracted from this acetone-dried powder, and was purified by using several modifications of the method of Seraydarian et al. (1967). The modifications followed the suggestions of Spudich and Watt (1971) and were introduced to eliminate tropomyosin contamination. The acetone powder was extracted twice by using first ten volumes and then eight volumes of 0.5 mM ATP, 0.5 mM MCE, 0.2 mM CaCl$_2$, pH 7.5. The extracts were separated from the residue after each extraction by centrifugation at 10,000 rpm for 10 minutes. After the second extraction, the two extracts were combined and were filtered by using Whatman filter paper No. 541 (coarse). Sufficient solid KCl was added to the filtered supernatant to make the final KCl concentration 3.3 M. Addition of this much KCl caused the temperature of the solution to drop below 0°C, and the solution was stirred gently at room temperature until the KCl dissolved (about 15-20 min). The temperature of the solution rose to approximately 4°C by the time the KCl has dissolved, and the temperature should not be permitted to rise above 6°C to prevent polymerization of the G-actin. As soon as the KCl was dissolved, the solution was
centrifuged at 10,000 rpm for 10 min at 2°C to remove any α-actinin that had been precipitated by this treatment. Volume of supernatant was measured, and the supernatant was decanted into a dialysis bag and was dialyzed against 6 volumes of 0.3 mM KHCO₃ for 5 hr to give a final KCl concentration between 0.5 and 0.6 M.

The actin solution was removed from the dialysis bag, allowed to set for 2-3 hr at room temperature to insure complete polymerization, and was then centrifuged overnight in a Type 19 rotor at 19,000 rpm. The F-actin pallet was rinsed three times with cold water, and was dissolved in 5 volumes of 0.5 mM ATP, 0.5 mM MCE, 0.2 mM CaCl₂, pH 7.5, by using a glass homogenizer. The resulting G-actin solution was clarified at 30,000-35,000 rpm for 60 min, the clarified supernatant was made 0.5 M in KCl by adding 2 M KCl, and was allowed to polymerize at room temperature for 5-10 min. The polymerized actin was sedimented at 30,000-35,000 rpm for 3 hr, and the F-actin pallet was converted into either F-actin or G-actin as desired. For F-actin, the pellet was rinsed three times with 0.1 M KCl, was transferred to a glass homogenizer by using 0.1 M KCl, was homogenized, and was dialyzed against 0.1 M KCl overnight to free it from all traces of ATP and CaCl₂. For G-actin, the F-actin pallet was rinsed three times with cold water, was suspended in four volumes of 0.5 mM ATP, 0.5 mM MCE,
0.2 mM CaCl₂, pH 7.5, by homogenizing thoroughly, and was dialized overnight against 0.5 mM ATP, 0.5 mM MCE and 0.2 mM CaCl₂, pH 7.5, to remove the last traces of KCl. After dialysis, the purified G-actin was subjected to a final clarification at 30,000-35,000 rpm for 1 hr. It is difficult to depolymerize actin completely at concentration above 7-8 mg/ml, but the G-actin solution was kept as concentrated as possible (4-6 mg/ml). Actin prepared this way was free from tropomyosin and troponin as tested by polyacrylamide disc gel electrophoresis in the presence of SDS, but often contained 0.5 to 1% of its protein as α-actinin (Figure 1). This α-actinin could only be removed by chromatography on a gel permeation column.

2. Tropomyosin

Tropomyosin was prepared by using a slight modification of the procedure described by Arakawa et al. (1970a). The procedure involves ammonium sulfate precipitation of the tropomyosin-troponin fraction remaining in the supernatant of the low ionic strength α-actinin extract of myofibrils after α-actinin is precipitated at 30% ammonium sulfate saturation (see Figure 2). The tropomyosin-troponin fraction was precipitated from this supernatant between 30 and 75% ammonium sulfate saturation, was dissolved in 1 mM KHCO₃, 5 mM MCE, and was dialyzed for 12-16 hr against two changes
Figure 1. SDS-Polyacrylamide gel electrophoresis of purified actin and tropomyosin. Purified actin was electrophoresed on a 7.5% polyacrylamide gel and purified tropomyosin was electrophoresed on both a 7.5% (center gel) and a 10% (right gel) polyacrylamide gel. Electrophoresis was done in the presence of 0.1% SDS, 100 mM Na-phosphate, pH 7.0 (Weber and Osborn, 1969). The actin gel was loaded with 33 μg protein. A faint band migrating at the same rate as the subunits of α-actinin can be seen halfway between the major actin band and the top of the gel. The 5 μg of tropomyosin loaded on the 7.5% gel shows one band, but the 1.25 μg of tropomyosin loaded on 10% gel shows two bands.
of 1 mM KHCO₃, 5 mM MCE to produce a 30-75 fraction. The dialyzed P₃₀₋₇₅ fraction was made 1 M in KCl by addition of 3 M KCl and was clarified at 35,000 rpm for 1 hr. Tropomyosin and troponin were separated by isoelectric precipitation of the clarified P₃₀₋₇₅ fraction at pH 4.6 in 1 M KCl. The tropomyosin-rich precipitate was dissolved in 1 M KCl, the pH was adjusted to 7.2-7.7, and isoelectric precipitation was repeated. The precipitate from the second isoelectric precipitation was dissolved in 1 mM KHCO₃, 5 mM MCE by adjusting the pH to 7.2-7.7 and was dialyzed for 16-24 hr against at least two changes of 1 mM KHCO₃, 5 mM MCE. The viscous solution was emptied from the dialysis bag, the protein concentration was adjusted to approximately 5 mg/ml, and 32 gm of ammonium sulfate and 32.6 mg of potassium carbonate were added to every 100 ml of supernatant. Any tropomyosin-troponin complex contaminating this fraction was removed by centrifugation at 15,000 X g for 15 min and purified tropomyosin was precipitated by adding six grams of ammonium sulfate to every 100 ml of supernatant and allowing the suspension to set at 0°C for 60-90 min. The tropomyosin precipitate was collected by centrifugation at 15,000 X g for 45 min, was dissolved in 100 mM KCl, was dialyzed 48-60 hr against four to six changes of 100 mM KCl, 1 mM KHCO₃ and was then clarified at 27,000 rpm for 45 min. The resulting tropomyosin sedimented as a single
hypersharpen boundary in the analytical ultracentrifuge, contained no visible troponin contamination as assayed by SDS-polyacrylamide gel electrophoresis (Figure 1), and exhibited no or only slight Ca\(^{2+}\) sensitizing activity when tested with reconstituted actomyosin suspensions. Purified tropomyosin from porcine skeletal muscle exhibited two bands in SDS-polyacrylamide gel electrophoresis. The slower migrating of these two bands corresponded to a molecular weight of 37,000 and faster migrating band corresponded to a molecular weight of 33,000. Several workers (Bodwell, 1967; Cummins and Perry, 1974) have previously reported the existence of two kinds of subunits in highly purified skeletal muscle tropomyosin.

3. \(\alpha\)-Actinin

\(\alpha\)-Actinin was extracted from bovine cardiac, chicken gizzard muscle by a modification procedure of Arakawa et al. (1970b). These modified procedures for bovine cardiac and chicken gizzard \(\alpha\)-actinin extraction are outlined in Figures 2, 3, and 4. \(\alpha\)-Actinin extraction procedure for chicken breast muscle is slightly different from the one used for \(\alpha\)-actinin extraction from chicken gizzard muscle. The changes made for extraction of \(\alpha\)-actinin from chicken breast muscle are as follows: step I, centrifuge at 4,000 \(X\) g for 10 min; step II, centrifuge at 4,000 \(X\) g for 10 min; step III, centrifuge at 4,000 \(X\) g for 10 min; step IV, resuspend
Figure 2. Flow sheet showing preparation of "swollen" myofibrils and crude $\alpha$-actinin and tropomyosin-troponin-containing extracts from bovine cardiac muscle at 2°C. Muscle tissue was obtained from left ventricles of bovine hearts. All solution volumes are based on the wet weight of ground tissue used in step I.
I. Ground bovine cardiac muscle
(a) Coarsely ground muscle was homogenized with 6 vol(V/W) of standard salt solution (SSS, 100 mM KCl, 200 mM K-phosphate, 1 mM sodium azide, 2 mM EGTA, 2 mM MgCl₂, pH 6.8) in a Waring Blender for different times and speeds as described below.
(i) Homogenize for 10 sec at 12,000 rpm and wait for 15 sec.
(ii) Homogenize for 10 sec at 12,000 rpm and wait for 15 sec.
(iii) Homogenize for 10 sec at 14,000 rpm and wait for 15 sec.
(iv) Homogenize for 10 sec at 18,000 rpm
(b) Centrifuge at 2,000 x g for 10 min.

II. Sediment
(a) Resuspend in 5 vol of SSS by using a stirring rod
(b) Centrifuge at 2,000 x g for 10 min.

III. Sediment
(a) Resuspend in 5 vol of 50 mM Tris-HCl pH 7.6, 1 mM EDTA.
(b) Homogenize in Waring Blender for 10 sec at 18,000 rpm and filter through one layer of cheese cloth.
(c) Centrifuge at 2,000 x g for 10 min.

IV. Sediment
IV. Sediment
   (a) Resuspend in 5 vol of 0.15 M KCl by using a stirring rod.
   (b) Centrifuge at 1,250 x g for 10 min.

V. Sediment
   (a) Resuspend in 5 vol of 1 mM EDTA pH 7.0, by using a stirring rod.
   (b) Centrifuge at 1,500 x g for 10 min.

VI. Sediment
   (a) Resuspend in 5 vol of deionized, distilled water by using a stirring rod.
   (b) Centrifuge at 6,000 x g for 15 min.

VII. Sediment
   (a) Resuspend in 5 vol of deionized, distilled water
   (b) Homogenize in Waring Blender for 10 sec at 12,000 rpm
   (c) Centrifuge at 8,000 x g for 20 min.

VIII. Sediment
   (a) Resuspend in 5 vol of deionized, distilled water by using a stirring rod.
   (b) Centrifuge at 13,000 x g for 20 min.

IX. Sediment

Figure 2 (Continued)
IX. Sediment
   (a) Resuspend in 5 vol of deionized, distilled water by using a stirring rod.
   (b) Centrifuge at 13,000 x g for 20 min.

Supernatant
(Extract B)

X. Sediment
   (a) Adjust pH of "swollen" myofibrils to pH 8.5 with 1 M Tris, pH 10.95.
   (b) Add MCE to 5 mM final concentration.
   (c) Homogenize at 15,000 rpm for 30 sec.
   (d) Store at 2°C for 62-72 hr.
   (e) After 62-72 hr, add 1 M Tris-acetate, pH 8.5 to give final concentration of 15 mM.
   (f) Homogenize at 15,000 rpm for 15 sec.
   (g) Centrifuge at 14,000 x g for 45 min.

Supernatant
(Extract C)

XI. Sediment
   (a) Suspend in same vol of 15 mM Tris-acetate, pH 8.5, as obtained in extract C by use of a stirring rod.
   (b) Centrifuge at 14,000 x g for 45 min.

Supernatant
(Extract D)

Sediment
(Discarded)

Figure 2 (Continued)
Combined myofibril washes (Extract A and B) or pH 8.5 extracts (Extract C and D)

(a) Filter through 8 layers of cheese cloth.
(b) Add 18 gm ammonium sulfate per 100 ml and adjust to pH 7.0.
(c) Let stand at 0°C for 15 min.
(d) Centrifuge at 14,000 x g for 15 min.

Supernatant (used for $P_{30-70}$
Tropomyosin-Troponin)

Sediment

(a) Dissolve in 1 mM KHCO$_3$, 5 mM MCE.
(b) Dialyze with two changes against 1 mM KHCO$_3$ for 12-18 hr.
(c) Centrifuge at 35,000 rpm for 1 hr.

Supernatant (P$_{0-30}$ extract, crude $\alpha$-actinin)

Combine with P$_{0-30}$ wash crude $\alpha$-actinin to give a P$_{0-30}$ crude $\alpha$-actinin extract.

Figure 3. Flow sheet showing ammonium sulfate fractionation of extracts obtained from bovine cardiac myofibrils by water washing or pH 8.5 extraction.
Figure 4. Flow sheet showing preparation of a "swollen" muscle homogenate and crude α-actinin-containing extracts from chicken gizzard muscle. Muscle tissue was obtained from chicken gizzards. All solution volumes are based on the wet weight of ground tissue used in step I.
I. Ground chicken gizzard muscle
(a) Coarsely ground muscle was homogenized with 5-7 vol (V/W) of 0.25 M sucrose,
1 mM EDTA, 50 mM Tris-acetate, 1 mM sodium azide, pH 8.25, in Waring Blender for different times and speeds as described below.
(i) Homogenize for 10 sec at 12,000 rpm and wait for 15 sec.
(ii) Homogenize for 10 sec at 12,000 rpm and wait for 15 sec.
(iii) Homogenize for 10 sec at 14,000 rpm and wait for 15 sec.
(iv) Homogenize for 10 sec 18,000 rpm.
(b) Centrifuge at 13,000 x g for 20 min.

II. Sediment
(a) Resuspend the tissue in 5 vol of original extraction solution by using a stirring rod.
(b) Centrifuge at 13,000 x g for 15 min.

III. Sediment
(a) Resuspend in 5 vol of 50 mM Tris-HCl, 1 mM EDTA, pH 7.65.
(b) Homogenize in Waring Blender for 10 sec at 18,000 rpm.
(c) Centrifuge at 11,000 x g for 15 min.

IV. Sediment
(a) Resuspend in 5 vol of 50 mM KCl by using a stirring rod.
(b) Centrifuge at 5,000 x g for 12 min.

V. Sediment
V. Sediment
   (a) Resuspend in 5 vol of 1 mM EDTA pH 7.0 by using a stirring rod.
   (b) Centrifuge at 6,000 x g for 12 min.

VI. Sediment
   (a) Resuspend in 5 vol of de-ionized, distilled water by using a stirring rod.
   (b) Centrifuge at 13,000 x g for 12 min.

VII. Sediment
   (a) Resuspend in 5 vol of de-ionized distilled water.
   (b) Homogenize in Waring Blender for 10 sec at 12,000 rpm.
   (c) Centrifuge at 13,000 x g for 20 min.

VIII. Sediment
   (a) Resuspend in 5 vol of de-ionized, distilled water by using a stirring rod.
   (b) Centrifuge at 13,000 x g for 20 min.

IX. Sediment
   (a) Resuspend in 5 vol of de-ionized, distilled water by using a stirring rod.
   (b) Centrifuge at 13,000 x g for 30 min.

X. Sediment

Figure 4 (Continued)
X. Sediment
(a) Add MCE to 6 mM final concentration.
(b) Adjust pH of "swollen" myofibrils to pH 8.5 with 1 M Tris.
(c) Homogenize in Waring Blender two times at 15,000 rpm for 30 sec.
(d) Store at 2°C for 62-72 hr.
(e) After 62-72 hr, add 1 M Tris-acetate, pH 8.5, to give final concentration of 15 mM.
(f) Homogenize at 15,000 rpm for 15 sec.
(g) Centrifuge at 13,000 xg for 1 hr.

XI. Sediment
(a) Resuspend in same vol 15 mM Tris-HCl, pH 8.5, as obtained in extract D by use of stirring rod.
(b) Centrifuge at 13,000 xg for 1 hr.

Combined myofibril washes (Extract A and B) or pH 8.5 extracts (Extract C and D) were fractionated with ammonium sulfate as shown in Figure 3.

Figure 4 (Continued)
in 0.15 M KCl and centrifuge at 2,000 X g for 10 min; step V, centrifuge at 2,000 X g for 10 min; step VI, centrifuge at 6,000 X g for 15 min; step VII, centrifuge at 13,000 X g for 15 min; step VIII, centrifuge at 13,000 X g for 15 min; step IX, centrifuge at 13,000 X g for 20 min. Remainder of the procedure is same for extraction of α-actinin from chicken breast and chicken gizzard muscles (Figure 4). Porcine skeletal α-actinin was obtained from residue of porcine skeletal muscle that remained after extraction of CAF (a Ca$^{2+}$ activated proteolytic enzyme) as outlined in Figure 5. α-Actinin was purified from the crude PO-30 fraction by using a modification of the method of Robson et al. (1970). The crude PO-30 extract was subjected to DEAE-cellulose chromatography (Bio-Rad, Cellex D, 0.95 m.eq./gm exchange capacity) by using the procedure described by Robson et al. (1970). α-Actinin eluted from this column at a KCl concentration characteristic of the tissue from which the α-actinin originated (Table 1), was salted out between 0-30% ammonium sulfate saturation and was dialyzed for 12 to 16 hr against at least two changes of 1 mM KHCO$_3$. After clarification at 35,000 rpm for 1 hr, this partly purified α-actinin fraction was made 50 mM in K-phosphate, pH 7.1 and was loaded onto a hydroxyapatite column (Bio-Gel, HPT) equilibrated with the same solvent. The hydroxyapatite column was packed after removing fines from Bio-Gel HPT hydroxyapatite by suspending it in 50 mM K-phosphate, pH
Ground porcine skeletal muscle
(a) Suspend ground muscle in 2 1/2 vol of 4 mM EDTA, pH 7.0-7.6 by homogenizing in Waring Blender at 15,000 rpm three times for 30 sec each time with 30 sec intervals.
(b) Centrifuge at 8,000 rpm for 20 min.

Supernatant
(Extract used for CAF)

Sediment
Prepare crude α-actinin (P0-30) according to the procedure outlined in Figure 2 except in the first step, this residue was suspended in standard salt solution by homogenizing only one time for 30 sec.

Figure 5. Flow sheet showing extraction of CAF from porcine skeletal muscle before preparation of "swollen" myofibrils and crude α-actinin and tropomyosin-troponin-containing extracts. Porcine semitendinosus, biceps femoris, and semimembranous muscles were used. All solution volumes are based on the weight of ground tissue used in step I.
Table 1. Potassium chloride and potassium phosphate concentrations\textsuperscript{a} at which α-actinin from different muscles is eluted from Bio-Rad and Whatman DE-52 DEAE-cellulose columns and from hydroxyapatite columns

<table>
<thead>
<tr>
<th></th>
<th>Bio-Rad\textsuperscript{b} DEAE-cellulose (mM KCl)</th>
<th>Whatman DE-52 DEAE-cellulose (mM KCl)</th>
<th>Hydroxyapatite\textsuperscript{c} (mM K-phosphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine cardiac α-actinin</td>
<td>230 - 245</td>
<td>170 - 200</td>
<td>110 - 117</td>
</tr>
<tr>
<td>Porcine skeletal α-actinin</td>
<td>220 - 250</td>
<td>185 - 208</td>
<td>100 - 115</td>
</tr>
<tr>
<td>Chicken gizzard α-actinin</td>
<td>215 - 220</td>
<td>185 - 210</td>
<td>85 - 90</td>
</tr>
<tr>
<td>Chicken breast α-actinin</td>
<td>230 - 255</td>
<td>200 - 225</td>
<td>115 - 125</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Potassium chloride and potassium phosphate concentrations were measured by using a standardized conductlyzer.

\textsuperscript{b}Bio-Rad and Whatman DE-52 DEAE-cellulose columns were eluted with a continuous KCl gradient. Figures are mM KCl at which elution of the α-actinin-containing peak began.

\textsuperscript{c}Hydroxyapatite columns were eluted with continuous K-phosphate, pH 7.1, gradients. Figures are mM K-phosphate, pH 7.1, at which elution of the α-actinin-containing peak began.
7.1, and decanting three times. The washed hydroxyapatite was then suspended in 50 mM K-phosphate, pH 7.1, and was poured into a column half-filled with 50 mM K-phosphate buffer, pH 7.1. The hydroxyapatite was allowed to settle under gravity for 8-12 hr and was then flushed with two bed volumes of 50 mM K-phosphate, pH 7.1. The flow rate of a 2.5 cm diameter column was not allowed to exceed 22 ml/hr to prevent excessive packing of the fine hydroxyapatite, thereby causing very slow flow rate. After loading the α-actinin off the Bio-Rad DEAE-cellulose column, the hydroxyapatite column was eluted with a continuous gradient of 50 to 300 mM K-phosphate buffer, pH 7.1. Purified α-actinin eluted at a phosphate concentration characteristic of the tissue from which it originated (Table 1). The hydroxyapatite column was then eluted with 500 mM K-phosphate buffer pH 7.1 to remove all remaining bound protein and was regenerated by flushing with 50 mM K-phosphate, pH 7.1. Such a column could be used four or five times before a decreasing flow rate necessitated repacking the column.

Porcine skeletal, chicken breast, and chicken gizzard muscle α-actinins were frequently subjected to a second chromatographic purification on Whatman DE-52 DEAE-cellulose before hydroxyapatite chromatography. In these instances, the α-actinin containing peak off the first DEAE-cellulose (Bio-Rad) column was made 50 mM in KCl, 20 mM in Tris-acetate,
pH 7.5, and loaded onto a DEAE-cellulose (Whatman DE-52) column equilibrated with the same solution. The column was eluted with a continuous gradient from 50 mM KCl, 20 mM Tris-acetate, pH 7.5 to 350 mM KCl, 20 mM Tris-acetate, pH 7.5, followed by 0.15 N KOH to elute all remaining protein. α-Actinin also eluted from the Whatman DE-52, DEAE-cellulose column at a KCl concentration that was characteristic of the muscle from which the α-actinin originated but that was also consistently lower than the KCl concentration required to elute α-actinin from Bio-Rad DEAE-cellulose (Table 1).

B. Studies on α-Actinin-Actin Interactions

1. Viscometric studies

Viscosity measurements were done with Cannon viscometers having flow times of 175-185 seconds for 1 ml of water at 37°C. At the protein concentrations used in this study, density differences between protein solutions and their solvents were negligible, especially when compared with the other errors inherent in measuring viscosity of F-actin and its complexes. Therefore, densities were neglected, and specific viscosity was calculated by using the formula:

\[ \eta_{sp} = \frac{t_p}{t_s} - 1 \]
where $t_p$ is flow time of the protein solution in seconds, and $t_s$ is flow time of the solvent in seconds. Actin concentration was always 1.0 mg/ml, and when added, tropomyosin and $\alpha$-actinin concentrations were 0.25 mg/ml, and 0.10 mg/ml respectively. All experiments were run in 20 mM Tris-acetate, pH 7.5, at 37°C and with varying amounts of KCl, Mg$^{2+}$, or ATP. Specific details will be given with the description of the individual experiments.

Actin was added to the viscometer in the G-actin form, and was followed sequentially by tropomyosin (when added), $\alpha$-actinin (when added), and ATP (when added). These ingredients were mixed in the viscometer. Then the G-actin in the viscometer was polymerized by addition of KCl or Mg$^{2+}$ or both, and the flow times were recorded immediately after polymerization.

2. Actin ATPase measurements

ATPase activity of purified porcine skeletal actin and the effect of $\alpha$-actinin and tropomyosin, either alone or together, on this activity was determined by measuring the release of inorganic phosphate from ATP. Disodium ATP (Sigma Chemical Co.) was treated to remove contaminating Ca$^{2+}$ and to convert it to the Tris salt as described by Goll and Robson (1967). Assays were done at 37°C. The reaction was initiated by addition of the appropriate amount of
ATP and was stopped by adding TCA to a final concentration of 5%. Precipitated protein was removed by centrifugation at 2,000 rpm at 2°C for 10 min, and inorganic phosphate concentration in the supernatant was determined by using Taussky and Shorr's method (Taussky and Shorr, 1953). Because reaction times were long (up to eight hrs), control tubes containing ATP without actin and proteins without ATP were included in every experiment. These controls were always low and constant, and inorganic phosphate concentrations in these controls were subtracted from inorganic phosphate concentrations in the sample tubes to give the amount of inorganic phosphate released from ATP by actin. Specific details on protein and ionic composition of the incubation medium will be given with the description of individual experiments.

C. Studies on α-Actinin

1. Quantitation of tryptophan

Tryptophan content of purified α-actinin was measured by using the procedure of Gaitonde and Dovey (1970).

2. Quantitation of cysteine and carboxymethylation of α-actinin

Sulfhydryl groups content of purified α-actinin was measured by using the procedure of Robyt et al. (1971) on
native α-actinin and on α-actinin denatured with different concentrations of SDS.

Reduction and carboxymethylation of proteins was done according to the procedure of Crestfield et al. (1963). Five to 100 mg of protein was reduced in a 12-ml screw-cap vial by adding 3.61 gm urea, 0.3 ml of 0.15 M EDTA, 3.0 ml of 1 M Tris-HCl, pH 8.6, and 0.1 ml of MCE to the protein solution. The solution was made up to 7.5 ml with de-ionized, distilled water, and a solution of 8 M urea, 0.2% EDTA was used to fill the vial to 12 ml. The vial was flushed with nitrogen, was covered with a disc of polyethylene to exclude air, and was closed with the screw cap. After incubating 4 hr at room temperature (22°C), the contents of the vial were transferred to a 25 ml beaker while being flushed with nitrogen. The protein was carboxymethylated by adding 1.0 ml of freshly prepared solution of 1.34 M iodoacetic acid in 1.0 M NaOH. After 15 min in the dark at 22°C, the reaction was stopped by addition of a five-fold excess (over iodoacetate) of MCE. Urea was removed by dialysis against five to six changes of 1 mM KHCO₃.

3. C-terminal amino acid determination

Carboxypeptidase A and carboxypeptidase B, purchased from Sigma Chemical Co., were used for C-terminal amino
acid determination. DFP-treated CPA was purchased in water suspension (47 units of activity/mg). One-tenth ml of enzyme suspension (21 mg/ml) was diluted with 1 ml of cold water, the diluted suspension was centrifuged at 2,500 rpm for 5 min, and the supernatant was discarded. This cold water washing was repeated two more times to eliminate all contaminating free amino acids that might exist in the commercial enzyme suspension. After the final cold water wash, the sedimented 2.1 mg of enzyme was solubilized by suspending in 0.3 ml of 1% sodium bicarbonate and adding 0.1 N sodium hydroxide dropwise with thorough mixing until the protein dissolved. The pH of the solution was lowered to 8-9 by adding 0.1 N HCl, and the final volume was adjusted by adding 0.2 N N-ethylmorpholine-acetate, pH 8.5, to give a final enzyme concentration of 1.0 mg/ml. CPA activity of the final solution was assayed by using the method of Folk and Schirmer (1963).

Fifteen one-hundredth ml of DFP-treated CPB (5 mg/ml, activity of 135 units/mg) was incubated for one hr at 0°C with 0.045 ml of 0.1 M Tris-HCl buffer, pH 7.6, and the volume was adjusted to 0.75 ml by adding 0.2 N N-ethylmorpholine-acetate buffer, pH 8.5, to give a final enzyme concentration of 1.0 mg/ml. CPB activity of the final solution was assayed by using the procedure of Folk et al. (1960).
Carboxymethylated α-actinin or actin was dissolved in 0.05 M SDS, 0.2 N N-ethylmorpholine-acetate buffer, pH 8.5. CPA or CPB, or both, were added to the protein at 37°C, and the reaction was stopped after different times of incubation by addition of Dowex-50WX8 (H⁺ form) and immediate adjustment of supernatant pH to 2.5-3.0. The Dowex-protein mixture was shaken for 20 min, the supernatant was discarded, and Dowex residue was washed twice with two volumes of water. Amino acids bound to Dowex were eluted with 2 volumes of 5 N ammonium hydroxide and Dowex was treated two more times with 2 volumes of 5 N ammonium hydroxide. The combined eluates were evaporated to dryness, and the residue was dissolved in 3 ml of 0.2 M citrate buffer, pH 2.2, and was subjected to amino acid analysis with the Beckman automatic amino acid analyzer. Norleucine was used as an internal standard. Specific details on enzyme to protein ratios and reaction times will be given with the description of individual experiments. The hippuryl-L-arginine and hippuryl-L-phenylalanine used for assaying CPB and CPA activity, respectively, were purchased from Sigma Chemical Co., norleucine was obtained from Pierce Chemical Co., and Dowex-50WX8 was purchased from J. T. Baker Chemical Co., Philisburg, N.J.
4. **N-terminal amino acid determination**

The procedure of Woods and Wang (1967) was used to determine the N-terminal amino acid of α-actinin, actin, and several other purified proteins that were used as standards. The purified proteins were carboxymethylated as described previously, and were then dansylated with dimethylaminonaphthalene-5-sulfonyl chloride. Approximately 1-2 n moles of peptide or protein in 20 μl of 0.1 M NaHCO₃ were dansylated by adding 20 μl of dansyl chloride and allowing the reaction to proceed for 6-10 hr at room temperature.

The dansylated protein was hydrolyzed with constant boiling HCl for 10 hr at 110°C. In some experiments, proteins were denatured before dansylation by incubating them in 0.05 M SDS for 10 min in a boiling water bath rather than by placing them in 8 M urea and carboxymethylating. This procedure was done to insure that the carboxymethylation procedure was not causing carboxymethylation of the N-terminal α-amino group of the protein. The hydrolysate was dried over NaOH pellets, was dissolved in acetone: 1 N HCl (9:1), and the dansylated amino acids were separated according to the procedure of Gray (1972) by using thin-layer chromatography on polyamide coated plates (Brinkman Instruments, Inc., Westburg, New York). The solvent system used was the one described by Gray (1972): the first solvent was water-90% formic acid (200:3, v/v). The polyamide-coated plates were
dried with an air blower, turned through 90°, and run in the second solvent (benzene-acetic acid, 9:1, v/v). The fluorescent dansylated amino acids were located by examining the polyamide coated plate under an ultraviolet light. Use of the two solvent systems gave adequate separation of all the DNS-amino acids except: DNS-Asp/DNS-Glu; DNS-Cysteine/DNS-OH; DNS-Arg/DNS-Histidine/ε-DNS-Lysine. These three sets of DNS-amino acids were resolved by additional (third and fourth) runs with other solvents in the same direction as solvent 2. A third run in ethyl-acetate-methanol-acetic acid (20:1:1, v/v) resolved DNS-Thr/DNS-Ser and DNS-Glu/DNS-Asp. DNS-His, DNS-Arg and ε-DNS-Lys were resolved by using 1 M ammonia-ethanol (1:1 v/v) as the fourth solvent. Dimethylaminonaphtalene-5-sulfonyl chloride and standard dansylated amino acids were purchased from Pierce Chemical Co., Rockford, Ill.

Because the experiments with dansyl chloride indicated that the N-terminal amino acids of α-actinin were not available for reaction, several procedures were used to determine whether the N-terminal amino acid(s) of α-actinin were acetylated as they are in other myofibrillar proteins for which N-terminal amino acids are known. Acylase I (amino acylase, N-acetylamino acid amidohydrolase), an enzyme purchased from Sigma Chemical Co., hydrolyzes the amide bond of N-acetyl-L-methionine. This enzyme was used in an attempt
to remove any acetate blocking the N-terminal amino acids of α-actinin and preventing them from reacting with dansyl chloride. Acylase I was dissolved in 0.1 M potassium phosphate buffer, pH 7.0, to give an enzyme concentration of 1.0 mg/ml. Activity of this acylase solution was assayed in two ways. First, the method of Mitz and Schlueter (1958) was used. This method involves measuring the decrease in absorbance at 238 nm as acylase I removes acetate from N-acetyl-L-methionine. The second procedure involved measuring the amount of free methionine released from acetyl-L-methionine (Sigma Chemical Co.) by treatment with acylase I. One-tenth ml of 15 mM N-acetyl-L-methionine in 100 mM phosphate buffer, pH 7.0 was treated with 0.05 mg acylase I for six to eight hr at room temperature. The acylase I-treated N-acetyl-L-methionine was then dansylated as described previously for purified proteins. Any free methionine released from N-acetyl-L-methionine by acylase I was dansylated and was detected as DNS-met on polyamide coated plates. Because no attempt was made to quantitate the intensity of the DNS-met detected on the polyamide coated plates, this method was not used as a quantitative measure of acylase activity, but could only indicate whether the acylase I was active or not. Complete absence of DNS-met on these polyamide coated plates would, of course, indicate that the acylase I was not active. Intact N-acetyl-L-methionine (not incubated with acylase I) and acylase I alone were run as controls.
Analysis for the presence of acetate on purified α-actinin and purified actin was done according to the procedure of Kuo and Younathan (1973). Two-tenth μmoles of lyophilized protein was hydrolyzed with 0.7 ml of constant boiling HCl (5.7 N) for 24 hr at 110°C. The hydrolysates were extracted with 1.0 ml of diethylether to remove any acetic acid released during hydrolysis, and this ether extract was in turn extracted two times with 0.8 ml of 0.5 N KOH:1 M Tris-HCl, pH 8.7 (9:1, v/v) each time to remove acetic acid into the aqueous phase. This entire process of ether extraction of the hydrolysate followed by hydroxide extraction of the ether was repeated four times, but the fourth ether extract was extracted only once with 1.0 ml of 0.5 N KOH:1 M Tris-HCl, pH 8.7 (9:1, v/v). All four 0.5 N KOH, 1 M Tris-HCl, pH 8.7, extracts were pooled, and pH of the pooled extracts was adjusted to 7.5. Acetate concentration in the pH 7.5 solution was measured spectrophotometrically. One and one-half ml of the acetate extract was added to a reaction mixture to give the following final concentrations in a final volume of 3.0 ml: 50 mM Tris-HCl buffer, pH 7.5; 20 mM KCl; 6 mM MgCl₂; 0.5 mM DTT; 1 mM ATP; 1 mM phosphoenolpyruvate; 0.15 mM NADH; 60 μl of 1.0 mg/ml pyruvate kinase; 60 μl of 1.0 mg/ml lactate dehydrogenase. The reaction was initiated by addition of 60 μl of 1.0 mg/ml acetate kinase. This spectrophotometric assay is based on the following equations.
(1) Acetate + ATP  Acetyl phosphate + ADP
(2) ADP + Phosphoenol pyruvate  Pyruvate + ATP
(3) Pyruvate + NADH + H⁺  Lactate + NAD.

These reactions are catalyzed by acetate kinase
(acetate + ATP  acetyl phosphate + ADP), pyruvate kinase
(phosphoenol pyruvate + ADP  pyruvate + ATP), and lactate
dehydrogenase (pyruvate + NADH + H⁺  lactate + NAD). Be-
cause one mole of acetate will oxidize one mole of NADH in
this sequence of reactions, and because the amount of NADH
oxidized can be calculated from the decrease in absorbance
at 340 nm, the decrease in absorbance at 340 nm is directly
proportional to the amount of acetate in the sample. Acetate
kinase (170 units/mg), pyruvate kinase (350 units/mg),
lactate dehydrogenase (400 units/mg), phosphoenol pyruvate,
and NADH were purchased from Sigma Chemical Co., St. Louis,
Missouri.

Because the acetate analysis showed that α-actinin con-
tained approximately one mole of acetate per peptide chain
in the α-actinin molecule, and because the dansylation ex-
periments indicated that the N-terminal α-amino groups in
α-actinin were unavailable for reaction, it seemed very
likely that the N-terminal α-amino groups in α-actinin were
acetylated. To identify the acetylated amino acid, 180 mg
(10 mg/ml) α-actinin in 10 mM Tris-HCl, pH 7.5, were di-
gested with 3 mg of pronase (Cal Biochem) at room temperature
(approx. 22°C) with stirring for approximately 14-18 hr. Pronase proteolysis was terminated by lowering pH of the α-actinin digest to 3.0 to denature the pronase. After 30 min at pH 3.0, the pH of the digest was raised to 7.5, and 3 mg of purified trypsin (Sigma Chemical Co.) were added. Trypsin was allowed to react for 14-18 hr at approximately 22°C. Then, pH of the reaction mixture was raised to 8.0, and the mixture was incubated in an 80°C water bath for 1 hr. Finally, 3 mg of CPB (127 units/mg, Sigma Chemical Co.) were added and were allowed to react for 8 hr at 37°C. CPB proteolysis was stopped by lowering pH of the reaction mixture to 2.5. Any precipitate that formed after this acidification was sedimented by centrifuging at 20,000 rpm for 30 min. The supernatant (25 ml) was loaded onto a 0.9 x 10 cm Dowex-50X8 (H⁺) column that had been equilibrated with 0.01N HCl. The column was eluted with 0.01 N HCl and 15 fractions of 5 ml each were collected. Each fraction was assayed for free amino groups by using the Ninhydrin assay procedure of Spies (1957). The ninhydrin negative fraction that eluted with the void volume of the column (first 40 ml elute) was collected and lyophilized. This fraction was called the N-acetyl-X fraction. Another identical experiment was performed with enzyme only (without α-actinin), and this fraction was called the enzyme control. The amount of acetate in the N-acetyl-X fraction and the enzyme control
was measured by using the procedure of Kuo and Younathan (1973) as described previously. One portion of the N-acetyl-X fraction and the enzyme control fraction was hydrolyzed with constant boiling 5.7 N HCl for 24 hr at 110°C and analyzed for amino acid content with a Beckman automatic amino acid analyzer. Another aliquot of the N-acetyl-X fraction and the enzyme control fraction was analyzed for amino acid content without acid hydrolysis.

Two samples of α-actinin were digested with pronase, trypsin, CPB and chromatographed as described in the preceding paragraph, but the other sample was chromatographed a second time on a Dowex-50X2 (H⁺) column (a less highly cross-linked, finer particle size Dowex than the one used in first column). The ninhydrin negative fraction eluted in the void of the Dowex-50X2(H⁺) column were again collected and lyophilized. One part of N-acety-X fraction was analyzed for amino acid content without acid hydrolysis and other part was hydrolyzed with 5.7 N HCl for 24 hr and then analyzed for amino acid content with a Beckman automatic amino acid analyzer.

D. CAF-Treatment of α-Actinin and Actin

Purified actin and α-actinin were treated with one part of purified CAF to 200 parts of actin or α-actinin by weight in 100 mM KCl, 5 mM CaCl₂, 10 mM MCE, 100 mM Tris-HCl,
pH 7.5, for one hr at 25°C. CAF-digestion of α-actinin was stopped by adding 10 mM EDTA, and the CAF-treated α-actinin was passed through a 2.5 x 85 cm Sephadex G-200 column that had been equilibrated with 10 mM Tris-HCl, pH 7.5; this procedure removed any small peptides that might have been released by CAF and that would interfere with N- and C-terminal analysis of the bulk of the α-actinin molecule that remained after CAF treatment. CAF-treated actin was sedimented by centrifugation at 60,000 rpm for 2 hr. The residue was dissolved in 1 mM KHCO₃, and was dialyzed against 1 mM KHCO₃.

Protein concentrations were measured by using the biuret method of Gornall et al. (1949) as modified by Robson et al. (1968). Polyacrylamide gel electrophoresis in the presence of SDS was done according to the procedure of Weber and Osborn (1969). Turbidity measurements were made according to Arakawa et al. (1970b). Analytical ultracentrifugal studies were conducted on a Spinco Model E analytical ultracentrifuge.
IV. RESULTS

As indicated in the Introduction of this thesis, the goal of this investigation was to determine the physiological function of α-actinin in muscle and the relation of this function to the structure and function of the Z-disk. Because careful studies of protein function require that the protein be completely homogeneous, initial efforts in this investigation were directed at improving existing methods for purifying α-actinin to insure that the α-actinin used was of maximum possible purity. After ascertaining that the α-actinin preparations were of necessary purity, subsequent efforts were directed at determining whether binding of α-actinin changes actin structure and whether release of α-actinin from myofibrils by the recently discovered Ca$^{2+}$-activated protease can be used to determine the exact location of α-actinin in Z-disks of skeletal muscle. For clarity, the results of this investigation will be presented in four different sections: 1) purification of α-actinin by hydroxyapatite chromatography; 2) studies on the α-actinin-actin interaction; 3) determination of tryptophan and cysteine content and the C- and N-terminal amino acids of purified α-actinin from several sources; and 4) studies on the effects of a Ca$^{2+}$-activated proteolytic enzyme from muscle on the C- and N-terminal amino acids of purified α-actinin and actin.
A. Purification of α-Actinin

As described in the Review of Literature, a major advance in the information available about α-actinin was made in 1970 when Robson et al. (1970) developed a method for purifying the existing crude α-actinin preparations by using DEAE-cellulose chromatography. Even rabbit skeletal α-actinin purified by two passages through a DEAE-cellulose column, however, contained about 10-15% of its protein as a 9.1 S boundary that sedimented ahead of the main 6.2 S α-actinin boundary in the analytical ultracentrifuge (Robson et al., 1970). Although several different lines of evidence suggested that the 9.1 S species was a salt-induced aggregate of the 6.2 S α-actinin species, it was important, because of the confusion that contaminating proteins may cause in N- and C-terminal amino acid determinations, to ascertain that the 9.1 S species was not a contaminating protein rather than an α-actinin aggregate. Hydroxyapatite columns separate protein on the basis of the strength of their adsorption to hydroxyapatite (a form of Ca₃(PO₄)₂), and purification of proteins on hydroxyapatite columns therefore depends on different properties of the protein than purification on ion-exchange cellulose columns. It is very likely that a greater degree of purification will be achieved if two different physical properties of a protein are used to purify that protein than if only one physical property of
the protein (e.g., the strength of its binding to ion-exchange columns) is used twice in succession during purification. Consequently, an attempt was made to purify α-actinin by using hydroxyapatite chromatography subsequent to an initial purification by a DEAE-cellulose column.

Robson (1970), Robson and Zeece (1973), and Robson et al. (1974) have shown that only small modifications of the low-ionic-strength, high-pH extraction procedure originally developed for extraction of α-actinin from skeletal muscle myofibrils (Arakawa et al., 1970b) are required to also extract α-actinin from cardiac and smooth muscle fibrils. Consequently, crude α-actinin preparations were made from cardiac, skeletal, and smooth muscle fibrils by using their procedures outlined in Figures 2, 3, and 4 (See Materials and Methods). These crude P0-30 α-actinin preparations were subjected to an initial purification by using DEAE-cellulose chromatography according to the general procedures described by Robson et al. (1970). A typical elution profile of bovine cardiac α-actinin off a DEAE-cellulose column is shown in Figure 6. All the α-actinin activity applied to the DEAE-cellulose column eluted in a single, slightly asymmetrical peak that eluted between 235 and 310 mM KCl (peak shown between the vertical lines in Figure 6). Elution profiles of gizzard α-actinin (R. M. Robson, T. W. Huiatt, and M. G. Zeece, unpublished results, 1974), of porcine cardiac
Figure 6. Elution profile of bovine cardiac PO-30 crude γ-actinin fraction off a 5.0 x 29.7 cm Bio-Rad DEAE-cellulose column. A sample of 4,644 mg PO-30 crude γ-actinin was applied, and the column was eluted with a continuous gradient consisting of 2000 ml each of 20 mM Tris-acetate, pH 7.5 and 500 mM KCl, 20 mM Tris-acetate, pH 7.5. After completion of the gradient, tightly bound proteins were eluted by washing with 0.2N KOH. The flow rate was 87.7 ml/hr and 17.5 ml fractions were collected. KCl concentration in the eluant is shown by the diagonal line; only the protein in the peak between the vertical lines had γ-actinin activity, and this protein was used for further study.
α-actinin (Robson and Zeece, 1973), and of porcine skeletal α-actinin (Robson and Zeece, 1973; Suzuki et al., 1973) were similar to that shown in Figure 6 for bovine cardiac α-actinin, although porcine skeletal α-actinin typically exhibited a double peak in the α-actinin region because of the presence of both "red" and "white" muscle α-actinin in the porcine skeletal extract (Robson and Zeece, 1973; Suzuki et al., 1973). The KCl concentration at which α-actinin eluted from the DEAE-cellulose column also varied slightly for the different α-actinins (Table 1) and was characteristic of the tissue from which the α-actinin originated. In all these tissues, most of the protein applied to the column was not eluted by KCl concentrations up to 500 mM, and a very large amount of protein was eluted by 0.2 N KOH (Figure 6). This same phenomenon was described earlier for rabbit skeletal α-actinin by Robson et al. (1970).

Based on analytical ultracentrifugal assays, Robson et al. (1970) concluded that DEAE-cellulose chromatography produced a remarkable purification of P₀-30 crude α-actinin preparations. This conclusion is substantiated by SDS-polyacrylamide gel electrophoresis (Figure 7) of the P₀-30 crude α-actinin preparation and the protein in the α-actinin-containing peak off the Bio-Rad DEAE-cellulose column. DEAE-cellulose chromatography removed almost all proteins having subunit molecular weights heavier than the 100,000 dalton
Figure 7. SDS-Polyacrylamide gel electrophoresis of a bovine cardiac P0-30 crude α-actinin preparation and of α-actinin after a single DEAE-cellulose chromatographic purification. Both gels are 7.5% polyacrylamide. Gel a shows P0-30 crude α-actinin before application to a DEAE-cellulose column. Gel b shows DEAE-cellulose purified α-actinin (protein eluted between vertical lines in Figure 6). Both gels are loaded with 25 μg of protein per gel. The major contaminant in P0-30 crude α-actinin preparations is a protein having the same molecular weight as actin (43,000 daltons). This confirms the suggestion of Robson et al. (1970) that denatured actin is the major contaminant in P0-30 crude α-actinin preparations.
α-actinin species, and removed much of the 43,000 dalton protein that is the major contaminant in the P₀₋₃₀ crude α-actinin preparations (Figure 7). As shown in Figure 7, however, α-actinin after a single DEAE-cellulose chromatographic purification still contained considerable 43,000 dalton impurity. Preliminary studies (results not shown in detail here) showed that a second DEAE-cellulose chromatographic purification removed only part of this 43,000-dalton impurity. Consequently, hydroxyapatite chromatography was attempted to determine whether it would provide more efficient removal of this 43,000 dalton contaminant. Application of DEAE-cellulose-purified α-actinin to a hydroxyapatite column in 50 mM K-phosphate followed by elution with a continuous 50 to 300 mM K-phosphate gradient resulted in resolution of DEAE-cellulose-purified α-actinin into two major and two minor fractions (Figure 8). Protein in the first major fraction (Fr. I in Figure 8) did not bind to hydroxyapatite in 50 mM K-phosphate and eluted with the solvent front. SDS-Polyacrylamide gel electrophoresis showed that this fraction contained almost all the 43,000 dalton material that contaminated the DEAE-cellulose purified α-actinin (gel b in Figure 9). The protein in this peak contained no α-actinin activity in either the ATPase or the turbidity assays. The second major peak was divided into three parts (Fr. II, III, and IV) for analysis by SDS-polyacrylamide gel electrophoresis (gels c, d,
Figure 8. Elution profile of DEAE-cellulose-purified bovine cardiac α-actinin off a 2.5 x 35.8 cm hydroxyapatite column. A sample of 507.8 mg of α-actinin purified by a single pass through a Bio-Rad DEAE-cellulose column (protein in peak between vertical lines in Figure 6) was applied, and the column was eluted with a continuous gradient consisting of 600 ml each of 50 mM K-phosphate, pH 7.1, and 300 mM K-phosphate, pH 7.1. After completion of the gradient, tightly bound proteins were eluted by washing with 500 mM K-phosphate, pH 7.1. Flow rate was 22.7 ml per hour, and 7.6 ml fractions were collected. K-phosphate concentration in the eluant is shown by a diagonal line. Fraction III, which eluted between 110 and 155 mM K-phosphate was pure α-actinin and was used for further study.
ADDED 0.5M K-PHOSPHATE
Figure 9. SDS-Polyacrylamide gel electrophoresis of the DEAE-cellulose-purified bovine cardiac $\alpha$-actinin fraction applied to a hydroxyapatite column and of the fractions eluted from this column by a 50-300 mM continuous K-phosphate gradient. All gels are 7.5% polyacrylamide. Gel a shows the DEAE-cellulose-purified $\alpha$-actinin which was applied to the hydroxyapatite column. Gels b, c, d, e and f show the proteins in fractions I, II, III, IV and V, respectively, eluted from the hydroxyapatite column (see Figure 8). All gels are loaded with 25 $\mu$g of protein per gel.
and e in Figure 9). The protein in the leading edge and main part of the peak consisted almost entirely of material having a subunit molecular weight of 100,000 daltons (gels c and d in Figure 9). Protein in the trailing edge of this peak (Fr. IV, Figure 8) and in the first minor peak that eluted immediately after the second major peak (Fr. V, Figure 8) also consisted largely of the 100,000 dalton \( \alpha \)-actinin subunit species but in addition contained some contaminating proteins having a molecular weight less than 100,000 daltons (gels e and f, Figure 9). The protein eluting in the second minor peak during flushing of the hydroxyapatite column by 500 mM K-phosphate (Figure 8) consisted largely of a 100,000 dalton species together with some contaminating proteins having molecular weights smaller than 100,000 daltons, and was similar in composition to the protein eluting in Fr. V, Figure 8 (SDS gels of the protein eluting in the second minor peak are not shown here).

The protein eluting in the center of the second major peak off hydroxyapatite columns (Fr. III, Figure 8 and gel d, Figure 9) was the purest preparation of \( \alpha \)-actinin obtained in this study, and this fraction was therefore used in all the subsequent experiments involving \( \alpha \)-actinin. Henceforth in this thesis, this fraction will be referred to simply as purified \( \alpha \)-actinin. Purified \( \alpha \)-actinin eluted from hydroxyapatite columns between 85 and 125 mM K-phosphate,
pH 7.1, depending on the tissue from which the α-actinin originated (Table 1). Densitometric analysis of SDS-polyacrylamide gels indicated that over 95% of the protein in purified α-actinin fractions consisted of the 100,000-dalton α-actinin species; hence, this fraction is sufficiently pure to permit careful studies of the N- and C-terminal amino acids of α-actinin.

Hydroxyapatite-purified α-actinin was also assayed by analytical centrifugation and in the ATPase and turbidity tests to determine whether hydroxyapatite purification affected the sedimentation pattern or the ability of α-actinin to increase the Mg\(^{2+}\)-modified ATPase activity and rate of turbidity development of reconstituted actomyosin suspensions. Analytical ultracentrifugation showed that hydroxyapatite chromatography removed the small amount of rapidly sedimenting 9.1 S material that Robson et al. (1970) described in preparations of rabbit skeletal α-actinin purified by DEAE-cellulose chromatography (Figure 10). A consistent difference observed with the bovine cardiac α-actinin compared to rabbit skeletal α-actinin was a lesser amount (5-10% as compared to 10-15%) of faster (9.1 S) component present in the bovine samples (Figure 10a). Although four or five lines of indirect evidence had indicated that the 9.1 S species was a salt-induced aggregate of the 6 S α-actinin species (Goll et al., 1972; Robson et al., 1970),
Figure 10. Sedimentation patterns of bovine cardiac α-actinin purified by two passages through a DEAE-cellulose column (diagram a) or by DEAE-cellulose chromatography followed by a hydroxyapatite column (diagram b). Final concentrations: a) 5.0 mg of DEAE-cellulose-purified α-actinin/ml, 100 mM KCl, 20 mM Tris-acetate, pH 7.5; b) 5.0 mg of hydroxyapatite-purified α-actinin/ml, 100 mM KCl, 20 mM Tris-acetate, pH 7.5. Temperature = 20°C. The observed sedimentation coefficient of both major peaks is 6.1; the observed sedimentation coefficient of the minor rapidly sedimenting peak in diagram a is 9.8 S.
MINUTES AFTER REACHING 60,000 RPM.
removal of the 9.1 S species from α-actinin preparations by hydroxyapatite-chromatography indicates that the 9.1 S species is probably a contaminant rather than an α-actinin aggregate. Because the 43,000 dalton species is the principal contaminant removed from DEAE-cellulose-purified α-actinin by hydroxyapatite chromatography, it is tempting to suggest that the 9.1 S peak in DEAE-cellulose-purified α-actinin (Figure 10) is composed of the 43,000 dalton species observed in SDS-polyacrylamide gels of DEAE-cellulose-purified α-actinin (Figures 7 and 9). Analytical ultracentrifugation of the protein eluted in Fr. I off hydroxyapatite columns (Figure 8) showed that this protein was very heterogeneous, although some material having a sedimentation coefficient of 8.5 to 9 S could be detected in this fraction. Therefore, it seems likely that the 9.1 S species seen in sedimentation patterns of DEAE-cellulose-purified α-actinin and the 43,000 dalton material seen in SDS-polyacrylamide gels of these same preparations originate, at least in part, from the same protein.

Although removal of the contaminating 9.1 S species from α-actinin by hydroxyapatite chromatography suggests that hydroxyapatite-purified α-actinin should be more active in the ATPase and turbidity assays than α-actinin purified by two passages through DEAE-cellulose columns, these two α-actinin preparations had nearly equal activity in both these
assays (Figure 11, Table 2). The reason that the increased purification produced by hydroxyapatite α-actinin has no effect on α-actinin activity is not clear. It is known that α-actinin activity in the ATPase and turbidity assays is very difficult to quantitate precisely (Arakawa et al., 1970b; Goll et al., 1969), and it may be that the small 5-10% increase in purity produced by hydroxyapatite chromatography (because the 9.1 S material makes up only 5-10% of the protein in bovine cardiac α-actinin preparations purified by two passages through a DEAE-cellulose column) is too small to be detected in the ATPase and turbidity assays of α-actinin activity. Whatever the reason for the lack of effect of hydroxyapatite purification on activity of α-actinin in the ATPase and turbidity assays, it is clear that hydroxyapatite-purified α-actinin is at least as active as DEAE-cellulose-purified α-actinin in both the ATPase and turbidity assays. Furthermore, hydroxyapatite-purified α-actinin is more homogeneous than DEAE-cellulose-purified α-actinin as assayed by either SDS-polyacrylamide gel electrophoresis or analytical ultracentrifugation. Consequently, hydroxyapatite chromatography was routinely used to purify the α-actinin preparations used in this investigation.
Figure 11. Effect of purification by DEAE-cellulose or hydroxyapatite chromatography on ability of bovine cardiac α-actinin to accelerate super-precipitation of reconstituted actomyosin suspensions. α-Actinin was purified either by two passages through DEAE-cellulose columns or by DEAE-cellulose chromatography followed by a hydroxyapatite column. Purified actin and myosin were made from porcine skeletal muscle. Final concentrations for superprecipitation assay: 100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 1.0 mM ATP, 20 mM Tris-acetate, pH 7.0, 0.4 mg actomyosin/ml, 0.02 mg α-actinin/ml when added. Temperature = 25°C.
Table 2. Effect of bovine cardiac α-actinin purified by two passages through DEAE-cellulose columns or by DEAE-cellulose chromatography followed by a hydroxyapatite column on the ATPase activity of reconstituted porcine skeletal actomyosin

<table>
<thead>
<tr>
<th>Type of α-actinin</th>
<th>α-Actinin added as percent of actomyosin by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>DEAE-cellulose-purified α-actinin</td>
<td>0.083±0.006^b</td>
</tr>
<tr>
<td>Hydroxyapatite-purified α-actinin</td>
<td>0.075±0.012</td>
</tr>
</tbody>
</table>

^aConditions of ATPase assay: 0.2 mg reconstituted actomyosin/ml, 100 mM KCl, 1.0 mM MgCl₂, 0.1 mM CaCl₂, 1.0 mM ATP, 20 mM Tris-acetate, pH 7.0, and α-actinin as indicated. Figures are µ moles Pi per mg actomyosin per min.

^bFigures are means of four experiments plus or minus standard errors.

B. Studies on the α-Actinin-Actin Interaction

1. Viscometric studies of the α-actinin-actin interaction

The discovery of α-actinin as a new myofibrillar protein that accelerates in vitro contractile responses of reconstituted actomyosin suspensions (Ebashi et al., 1964; Ebashi and Ebashi, 1965), was soon followed by the finding that α-actinin exerts its influence on actomyosin suspensions by binding to actin in the actomyosin complex (Ebashi and Ebashi, 1965; Maruyama and Ebashi, 1965). Indeed, it is now known that
α-actinin binds only to F-actin among the known myofibrillar proteins (Holmes et al., 1971), and that binding of α-actinin to F-actin causes cross-linking of F-actin filaments (Kawamura et al., 1970). Consequently if α-actinin has a physiological role in modifying the actin-myosin interaction, it must exert its effects by binding to and altering the structure of F-actin filaments. Because binding of α-actinin increases F-actin viscosity, the initial studies in this investigation measured viscosity of α-actinin-F-actin mixtures under a wide variety of conditions to determine whether α-actinin would bind to F-actin under physiological conditions in the presence of ATP and to learn whether viscosity measurements could be used to detect α-actinin-induced alterations in F-actin structure.

The data in Table 3 show that viscosity of F-actin polymerized in 1 mM MgCl₂ is similar to viscosity of F-actin polymerized in either 100 mM KCl or 100 mM KCl plus 1 mM MgCl₂. Although addition of ATP had no detectable effect on viscosity of F-actin polymerized in 100 mM KCl or in 100 mM KCl plus 1 mM MgCl₂ (Table 3), addition of ATP slightly lowers the viscosity of F-actin polymerized only in 1 mM MgCl₂ (Table 3). The simplest and most direct explanation for this result is that the added ATP binds the available Mg²⁺ (K_D for Mg ATP is 10⁻⁴ M), and that this binding results in less Mg²⁺ being available to produce polymerization. It
Table 3. Effect of α-actinin and ATP on viscosity of F-actin polymerized by different agents

<table>
<thead>
<tr>
<th>Agents used to polymerize actin</th>
<th>100 mM KCl</th>
<th>1 mM MgCl₂</th>
<th>100 mM KCl plus 1 mM MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-Actin</td>
<td>1.07 ± 0.04 (8)</td>
<td>1.10 ± 0.03 (16)</td>
<td>1.11 ± 0.03 (9)</td>
</tr>
<tr>
<td>F-Actin plus ATP</td>
<td>1.13 ± 0.04 (8)</td>
<td>0.95 ± 0.02 (16)</td>
<td>1.15 ± 0.02 (9)</td>
</tr>
<tr>
<td>F-Actin plus 10% α-actinin</td>
<td>2.60 ± 0.35 (6)</td>
<td>2.32 ± 0.24 (13)</td>
<td>2.70 ± 0.23 (6)</td>
</tr>
<tr>
<td>Percent increase caused by addition of α-actinin</td>
<td>143</td>
<td>111</td>
<td>143</td>
</tr>
<tr>
<td>F-Actin plus 10% α-actinin plus ATP</td>
<td>2.06 ± 0.18 (6)</td>
<td>1.09 ± 0.06 (13)</td>
<td>2.12 ± 0.12 (6)</td>
</tr>
<tr>
<td>Percent decrease caused by addition of ATP</td>
<td>21</td>
<td>53</td>
<td>21</td>
</tr>
</tbody>
</table>

α-Conditions: 1.0 mg F-actin/ml, 20 mM Tris-acetate, pH 7.5. When added, 1 mM ATP, 0.10 mg α-actinin/ml, 37°C. Figures are specific viscosity plus or minus standard errors of the number of determinations given in parentheses.
is known that F-actin filament length grows very slowly if Mg$^{2+}$ concentrations are below 1 mM during polymerization (Kasai et al., 1960). Because the F-actin preparations made in this study were not examined in the electron microscope, however, it is not possible to eliminate other possible interpretations of the effect of ATP on viscosity of F-actin polymerized by 1 mM MgCl$_2$. For example, ATP might increase the flexibility of F-actin filaments polymerized in 1 mM MgCl$_2$ and thereby convert the relatively rigid F-actin filament into a random coil that has a shorter mean free length than the F-actin filament.

Addition of α-actinin increases the viscosity of all three types of F-actin considerably (Table 3). Although the viscosity of F-actin polymerized in 1 mM MgCl$_2$ seemed to be increased slightly less by α-actinin than viscosity of F-actin polymerized in 100 mM KCl or in 100 mM KCl plus 1 mM MgCl$_2$, this difference was not statistically significant. Addition of ATP to α-actinin-F-actin mixtures lowers the viscosity of those mixtures in which the actin was polymerized in 100 mM KCl or in 100 mM KCl plus 1 mM MgCl$_2$ slightly, but greatly lowers the viscosity of those mixtures in which the actin was polymerized by 1 mM MgCl$_2$ alone (Table 3). Again the reason for this effect of ATP on viscosity of α-actinin-F-actin mixtures is not clear. It is possible that presence of a positively charged cation such
as K⁺ or Mg²⁺ is necessary for the negatively charged α-actinin molecule (net excess of 318 negative charges—uncorrected for asparagine and glutamine—per mole α-actinin at pH 7.0 as calculated from its amino acid composition) to bind to the negatively charged actin molecule (net excess of 13 negative charges—corrected for asparagine and glutamine—per mole of actin at pH 7.0 as calculated from its amino acid composition). Addition of 1 mM ATP might chelate most of the added Mg²⁺ and thereby prevent much of the binding of α-actinin to F-actin when 1 mM Mg²⁺ is the only cation present (Table 3). Addition of ATP to α-actinin-F-actin mixtures in the presence of 100 mM KCl would then have a much smaller effect on binding of α-actinin to actin because 100 mM KCl provides a large excess of K⁺ cations over the 1 mM ATP added. The binding constant of α-actinin for Mg²⁺ and K⁺ is not yet known, so it is not certain whether ATP can compete successfully with α-actinin and actin for Mg²⁺ cations. Attempts to measure the amount of α-actinin binding to F-actin in the presence of 1 mM ATP and 1 mM Mg²⁺ by using the binding assay described by Goll et al. (1972) were thwarted by the high levels of unsedimentable protein in the F-actin controls. Therefore, although competition between α-actinin and ATP for Mg²⁺ cations seems to be the most logical explanation for the ATP-induced decrease in viscosity of α-actinin-F-actin mixtures
in the presence of 1 mM MgCl₂ alone, additional evidence is needed to establish this interpretation.

Because the results in Table 3 suggest that ATP and free Mg²⁺ levels had important effects on the α-actinin-F-actin interaction, a series of experiments were done in which Mg²⁺ and ATP were varied systematically in the absence of K⁺ or other cations. Increasing Mg²⁺ concentration from 1 mM to 6 mM increases the specific viscosity of the F-actin formed by approximately 50% (Figure 12, Table 4). Addition of 1 mM ATP had very little effect or slightly decreased the viscosity of these F-actin preparations in the absence of α-actinin (Figure 12, Table 4); the effect of ATP seems unaffected by Mg²⁺ concentrations in the range of 1 to 6 mM. Addition of α-actinin causes a large increase in specific viscosity. This increase becomes larger with increasing Mg²⁺ concentrations until the α-actinin-F-actin complex would not flow through the capillary of the Cannon viscometer at 6 mM Mg²⁺ (Figure 12, Table 4). These results substantiate the conclusion that Mg²⁺ is involved in complexing of negatively charged α-actinin and F-actin molecules, and that α-actinin cross-linking of F-actin strands is much more extensive at 6 mM Mg²⁺ than it is at 1 mM Mg²⁺. This conclusion is further supported by finding that although 1 mM ATP almost completely prevents the α-actinin-induced increase in F-actin viscosity at 1 and 2 mM Mg²⁺, it has much less
Figure 12. Effect of α-actinin and Mg$^{2+}$ on F-actin viscosity in the presence and absence of ATP. α-Actinin was mixed with G-actin at 37°C and actin in the mixture was polymerized by different concentrations of MgCl$_2$. Final concentrations: 1.0 mg actin/ml, 20 mM Tris-acetate, pH 7.5, MgCl$_2$ as indicated, 1 mM ATP when added and 0.10 mg α-actinin/ml when added. Temperature = 37°C.
WOULD NOT FLOW

F-ACTIN

F-ACTIN PLUS 1mM ATP

F-ACTIN PLUS 10% α-ACTININ

F-ACTIN PLUS 10% α-ACTININ PLUS 1mM ATP

$\eta_{sp}$

$\text{MgCl}_2 (\text{mM})$
Table 4. Effect of α-actinin and ATP on viscosity of F-actin polymerized by different concentrations of MgCl₂

<table>
<thead>
<tr>
<th>ATP and α-actinin concentrations</th>
<th>MgCl₂ Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM</td>
</tr>
<tr>
<td>F-Actin alone</td>
<td>0.86</td>
</tr>
<tr>
<td>F-Actin plus 10% α-Actinin</td>
<td>1.57</td>
</tr>
<tr>
<td>F-Actin plus 1 mM ATP</td>
<td>0.83</td>
</tr>
<tr>
<td>F-Actin plus 10%α-Actinin plus 1 mM ATP</td>
<td>0.86</td>
</tr>
<tr>
<td>F-Actin plus 2 mM ATP</td>
<td>0.80</td>
</tr>
<tr>
<td>F-Actin plus 10%α-Actinin plus 2 mM ATP</td>
<td>0.80</td>
</tr>
<tr>
<td>F-Actin plus 4 mM ATP</td>
<td>0.84</td>
</tr>
<tr>
<td>F-Actin plus 10% α-Actinin plus 4 mM ATP</td>
<td>0.84</td>
</tr>
<tr>
<td>F-Actin plus 6 mM ATP</td>
<td>0.90</td>
</tr>
<tr>
<td>F-Actin plus 10% Actinin plus 6 mM ATP</td>
<td>0.86</td>
</tr>
</tbody>
</table>

aConditions: 1.0 mg F-Actin/ml, 20 mM Tris-acetate, pH 7.5, ATP and MgCl₂ as indicated, 0.10 mg α-Actinin when added, 37°C. Figures are specific viscosity.
effect at 4 and 6 mM Mg$^{2+}$ (Figure 12, Table 4). Because the affinity of ATP for binding a second molecule of Mg$^{2+}$ is much smaller than its affinity for binding the first molecule of Mg$^{2+}$, the ability of Mg$^{2+}$ added in molar excess over ATP to enhance the binding of $\alpha$-actinin to F-actin indicates that in the absence of K$^+$, Mg$^{2+}$ is necessary for binding of $\alpha$-actinin to actin.

Increasing the concentration of added ATP from 0 to 6 mM while keeping the concentration of Mg$^{2+}$ constant at 1 mM has no effect on F-actin viscosity (Figure 13, Table 4). This result suggests that ATP does not act directly on F-actin filaments to cause partial depolymerization or increased flexibility of the F-actin filament strands. If ATP did affect F-actin filaments directly, it would be expected that its effects would increase at higher concentrations of added ATP. As shown previously in Figure 12, addition of 1 mM ATP in the presence of 1 mM MgCl$_2$ removes most of the $\alpha$-actinin-induced increase in F-actin viscosity (Figure 13, Table 4). Addition of greater amounts of ATP up to 6 mM, however, has no further effect on viscosity of $\alpha$-actinin-F-actin mixtures (Figure 13, Table 4). This result also supports the contention that ATP lowers the $\alpha$-actinin-induced increase in F-actin viscosity by chelating Mg$^{2+}$ rather than by a direct effect on the F-actin filament because 1 mM ATP is sufficient to chelate most of the 1 mM Mg$^{2+}$ present in these mixtures,
Figure 13. Effect of α-actinin and ATP on viscosity of F-actin polymerized by MgCl₂. α-Actinin was mixed with G-actin at 37°C and actin in the mixture was polymerized by 1 mM MgCl₂. Final concentrations: 1.0 mg F-actin/ml, 20 mM Tris-acetate, pH 7.5, 1 mM MgCl₂, ATP as indicated, 0.10 mg α-actinin/ml when added. Temperature = 37°C.
The graph shows the relationship between ATP concentration (mM) and the specific viscosity (η_s) for two conditions:

- **F-ACTIN**: Represented by circles.
- **F-ACTIN PLUS 10% α-ACTININ**: Represented by triangles.

The specific viscosity remains constant across the range of ATP concentrations tested, indicating no significant change in viscosity with varying ATP levels for both conditions.
and higher concentrations of ATP would have little additional effect on the level of free Mg$^{2+}$ present.

Additional evidence supporting the idea that ATP decreases the $\alpha$-actinin-induced increase in viscosity of Mg$^{2+}$-polymerized F-actin by chelating Mg$^{2+}$ was obtained by examining the effect of Mg$^{2+}$ and ATP in equal molar amounts on the viscosity of F-actin and $\alpha$-actinin-F-actin mixtures (Figure 14, Table 4). ATP and Mg$^{2+}$ in equal molar amounts between 1 and 6 mM had no effect on viscosity of F-actin alone (Figure 14, Table 4). Adding 1, 2 and 4 mM Mg$^{2+}$ and ATP in equal molar amounts removed most of the $\alpha$-actinin-induced increase in F-actin viscosity (Figure 14, Table 4). Again, this result is consistent with the conclusion that ATP acts on $\alpha$-actinin-F-actin mixtures by chelating Mg$^{2+}$ because very little free Mg$^{2+}$ would exist when Mg$^{2+}$ and ATP are present at equal molar concentrations of 1, 2 or 4 mM. At 6 mM Mg$^{2+}$ and 6 mM ATP, however, free Mg$^{2+}$ would be approximately 0.7 to 0.8 mM if a dissociation constant of $10^{-4}$ M is assumed for Mg ATP, and if the binding of Mg$^{2+}$ and ATP to actin and $\alpha$-actinin in these mixtures is ignored. Free Mg$^{2+}$ concentrations of 0.7 to 0.8 mM are sufficient to support some binding of $\alpha$-actinin to F-actin, and the results in Figure 14 and Table 4 show that $\alpha$-actinin causes an appreciable increase in F-actin viscosity in the presence of 6 mM Mg$^{2+}$ plus 6 mM ATP. Consequently, all results from this systematic survey of the
Figure 14. Effect of Mg$^{2+}$ and ATP in equal molar ratios and $\alpha$-actinin on viscosity of F-actin polymerized by MgCl$_2$. $\alpha$-Actinin was mixed with G-actin at 37°C and actin in the mixtures was polymerized by MgCl$_2$. Final concentrations: 1.0 mg F-actin/ml, 20 mM Tris-acetate, pH 7.5, ATP and MgCl$_2$ added in equal molar ratios at levels indicated, 0.10 mg $\alpha$-actinin/ml when added. Temperature = 37°C.
effects of Mg\textsuperscript{2+} and ATP on viscosity of F-actin and \(\alpha\)-actinin-F-actin mixtures are consistent with the conclusion that, in the absence of K\textsuperscript{+}, Mg\textsuperscript{2+} is necessary for binding of negatively charged \(\alpha\)-actinin to negatively charged actin molecules. Consequently, it seems that ATP removes the \(\alpha\)-actinin-induced increase in viscosity of Mg\textsuperscript{2+}-F-actin by chelating Mg\textsuperscript{2+} and thereby inhibiting binding of \(\alpha\)-actinin to F-actin rather than by directly affecting the structure of F-actin filaments.

In the presence of 100 mM KCl, addition of ATP has much less effect on binding of \(\alpha\)-actinin to F-actin because the large concentration of K\textsuperscript{+} swamps out the effects due to addition of 1 mM ATP. Because mammalian skeletal muscle cells contain approximately 100–120 mM KCl \textit{in vivo}, binding of \(\alpha\)-actinin to actin in living muscle should not be affected greatly by ATP.

Thin filaments in mammalian skeletal muscle contain tropomyosin in addition to actin. Moreover, Goll \textit{et al.} (1972) and Robson \textit{et al.} (1970) have shown that tropomyosin has important effects on the amount of \(\alpha\)-actinin bound by F-actin filaments. Because any effect that \(\alpha\)-actinin exerts on F-actin structure \textit{in vivo} must be wielded in the presence of tropomyosin, a series of experiments were done to test the effects of ATP and tropomyosin on binding of \(\alpha\)-actinin to F-actin as measured viscometrically. It was recognized when these experiments were initiated that tropomyosin may
not bind to F-actin in exactly the same way in vitro as it does in vivo, and that tropomyosin added in vitro may completely cover both ends of the F-actin strand and thereby prevent binding of α-actinin at these ends. It was hoped, however, that in vivo binding of tropomyosin to F-actin could be reproduced well enough under in vitro conditions to permit measurement of some binding of α-actinin to the tropomyosin-F-actin complex. Unfortunately, no α-actinin binding to the tropomyosin-F-actin complex could be detected by viscometric measurements at 37°C (Table 5). Indeed, viscometric measurements could not even detect whether tropomyosin was bound to the F-actin strands under the different conditions used in Table 5 because addition of tropomyosin had no detectable effect on F-actin viscosity under these conditions. Direct measurements of tropomyosin binding, however, has shown that most of the added tropomyosin is bound to F-actin in 100 mM KCl (Goll et al., 1972). Goll et al. (1972) have also shown by direct measurements that less than 3% by weight, and possibly no α-actinin at all, is bound to tropomyosin-F-actin complexes at 37°C. Because α-actinin is clearly bound in the Z-disk in living muscle, these results indicate that additional methodological improvements are needed to assemble α-actinin-tropomyosin-F-actin complexes similar to those that exist in vivo. As will become evident later, the inability to demonstrate that
Table 5. Effect of α-actinin, tropomyosin, and ATP on viscosity of F-actin polymerized by different agents$^a$

<table>
<thead>
<tr>
<th>Agents used to polymerize actin</th>
<th>F-Actin plus 25% Tropomyosin</th>
<th>F-Actin plus 25% Tropomyosin plus ATP</th>
<th>F-Actin plus 25% Tropomyosin plus 10% α-actinin</th>
<th>F-Actin plus 25% Tropomyosin plus 10% α-actinin plus ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM KCl</td>
<td>1.14 ± 0.05 (4)</td>
<td>1.17 ± 0.07 (4)</td>
<td>1.20 ± 0.06 (3)</td>
<td>1.22 ± 0.07 (3)</td>
</tr>
<tr>
<td>1 mM MgCl₂</td>
<td>1.11 ± 0.02 (5)</td>
<td>1.09 ± 0.04 (5)</td>
<td>1.16 ± 0.02 (4)</td>
<td>1.21 ± 0.02 (4)</td>
</tr>
<tr>
<td>100 mM KCl plus 1 mM MgCl₂</td>
<td>1.15 ± 0.04 (5)</td>
<td>1.13 ± 0.04 (5)</td>
<td>1.22 ± 0.04 (4)</td>
<td>1.21 ± 0.03 (4)</td>
</tr>
</tbody>
</table>

$^a$Conditions: 1.0 mg F-Actin/ml, 20 mM Tris-acetate, pH 7.5, 1 mM ATP when added, 0.10 mg α-actinin/ml when added, and 0.25 mg tropomyosin/ml when added, 37°C. Figures are specific viscosity plus or minus standard errors of the number of determinations given in parentheses.
α-actinin is bound to tropomyosin-F-actin complexes at 37°C makes it impossible to interpret the results of some F-actin ATPase experiments unequivocally.

2. Effect of α-actinin on F-actin ATPase

The unusual ability of F-actin to liberate inorganic phosphate from ATP was described in the Review of Literature in this thesis. Liberation of inorganic phosphate from ATP by F-actin evidently occurs as part of a cycle in which bound ADP contained by actin monomers when they are aggregated into a F-actin filament, is exchanged for ATP in the surrounding medium and the ATP is dephosphorylated to ADP and inorganic phosphate during its incorporation into the F-actin filament. Repetition of this cycle by release of the newly bound ADP and incorporation and dephosphorylation of another molecule of medium ATP produces a continuous release of inorganic phosphate in F-actin solutions containing ATP and accounts for the ATPase activity of F-actin. The Mg\(^{2+}\)-modified ATPase activity of F-actin is only 0.5 to 1% of the Mg\(^{2+}\)-modified ATPase activity of the actin-myosin complex, and it seems very unlikely that F-actin ATPase activity has a direct role in releasing the energy used to cause sliding of the interdigitating thick and thin filaments during muscle contraction. F-actin ATPase can, nevertheless, be used as a sensitive probe of the structure of actin monomers in a F-actin aggregate. Indeed, because of the extreme asymmetry and large size of the F-actin filament and the resulting
thixotropic and birefringent nature of F-actin solutions, it is very difficult to measure structural alterations in actin monomers while they are part of a F-actin filament by using the usual spectroscopic or physicochemical methods. ATPase activity, therefore, is one of the few measurements that can be used to monitor structure of actin monomers in a F-actin filament. Consequently, effect of α-actinin on F-actin ATPase activity was studied under a variety of conditions, including those that the viscosity measurements had shown would enhance or inhibit binding of α-actinin to F-actin, to determine whether α-actinin can alter the structure of actin monomers in F-actin filaments and whether α-actinin might thereby be able to affect the actin-myosin interaction. It was hoped that measurements of F-actin ATPase activity would show whether cross-linking of actin filaments as detected viscometrically is necessary for α-actinin to affect the structure of actin monomers in F-actin filaments.

The time-course of release of inorganic phosphate from ATP by F-actin and the influence of α-actinin and DTT on this time-course is shown in Figure 15. Addition of 10% α-actinin by weight increases F-actin ATPase by 70 to 80% in the absence of DTT and by 80-90% in the presence of DTT (Figure 15). Kitagawa et al. (1968) have shown earlier that the presence of a sulfhydryl reagent such as DTT decreases the rate of nucleotide exchange in F-actin solution, and because
Figure 15. Effect of DTT and α-actinin on F-actin ATPase.
Final concentrations for the ATPase assay:
100 mM KCl, 1.0 mM MgCl₂, 0.05 mM CaCl₂, 1.0 mM ATP, 20 mM Tris-acetate, pH 7.5, DTT as indicated, 1.0 mg F-actin/ml, 0.10 mg α-actinin/ml when added. Temperature = 37°C.
Points are averages of 6 determinations on 3 different actin and α-actinin preparations.
nucleotide exchange is presumably an integral part of the ATPase activity of F-actin, it was expected that DTT would also decrease ATPase activity of F-actin (Figure 15). It is important to note, however, that DTT did not decrease but instead actually enhanced the relative effect of α-actinin on F-actin ATPase activity. The greater percent activation of F-actin ATPase activity by α-actinin in the presence of DTT occurred because the presence of DTT produced a lower F-actin ATPase activity in the absence of α-actinin and not because the absolute amount of α-actinin-induced activation is greater in the presence of DTT (Figure 15). Other experiments in our laboratory (M. C. Schmidt, unpublished results, 1974) have shown that the presence of DTT is essential to show consistently that α-actinin increases the rate of nucleotide exchange of F-actin. Although α-actinin consistently increases F-actin ATPase activity, even in the absence of DTT (Figure 15), the presence of DTT increases the uniformity of specific ATPase activities of different preparations of actin. For this reason, and because DTT seems essential to show a consistent effect of α-actinin on nucleotide exchange of F-actin, DTT was routinely included in all subsequent assays of F-actin ATPase activity. It should also be noted that the α-actinin-induced increase of F-actin ATPase activity in Figure 15 occurs under conditions (i.e., in the presence of 100 mM KCl) where viscosity measurements
showed that α-actinin cross-linked F-actin filaments. Moreover, the best estimates available indicate that skeletal muscle contains approximately 10% as much α-actinin by weight as it does actin. Therefore, the amount of α-actinin that causes the increase in F-actin ATPase activity shown in Figure 15 is about equal to the amount of α-actinin existing in skeletal muscle in vivo.

Increasing KCl concentration from 0 to 500 mM while keeping Mg and ATP concentration constant at 1 mM each caused a slight decrease in specific activity of F-actin ATPase (Figure 16). Addition of 10% α-actinin by weight, however, increases the specific activity of F-actin ATPase at all KCl concentrations between 0 and 500 mM. Indeed, because F-actin ATPase in the absence of α-actinin is lower in 500 mM KCl than it is in 0 mM KCl, α-actinin produces a larger percent increase in F-actin ATPase activity at 500 mM KCl (71% increase) than it does at 0 mM (38% increase), although the absolute value of the α-actinin-induced increase in specific activity is almost the same at all KCl concentrations tested. It should also be noted that the α-actinin-induced increase in F-actin ATPase at 0 mM KCl occurs in the presence of 1 mM Mg and 1 mM ATP. The viscosity measurements in the preceding section showed that α-actinin has no detectable effect on F-actin viscosity under these conditions. Consequently, α-actinin evidently can increase F-actin ATPase
Figure 16. Effect of α-actinin on F-actin ATPase activity at different KCl concentrations. Final concentrations for ATP assay: KCl as indicated, 1.0 mM MgCl$_2$, 0.05 mM CaCl$_2$, 1 mM ATP, 20 mM Tris-acetate, pH 7.5, 0.5 mM DTT, 1.0 mg G-actin/ml, 0.10 mg α-actinin/ml when added. Temperature = 37°C. Points are average of 4 determinations on 2 different actin and α-actinin preparations.
activity without cross-linking F-actin strands. α-Actinin, however, must bind to F-actin under these conditions because it seems very unlikely that α-actinin would increase F-actin ATPase activity without binding to the F-actin filament.

F-actin ATPase activity in 100 mM KCl, 1 mM MgCl_2, and 1 mM ATP decreases as pH increases from 5.0 to 8.5 (Figure 17). Moreover, α-actinin has no effect on F-actin ATPase between pH 5.0 and 6.0, but the ability of α-actinin to increase the specific activity of F-actin ATPase increases steadily between pH 6.5 and 8.5 (Figure 17). Because α-actinin precipitates at pH values below 6.0, part of the lack of ability of α-actinin to accelerate F-actin ATPase activity in the pH range of 5.0 to 6.0 may be due to precipitation of α-actinin before it binds to F-actin. Because α-actinin is soluble at pH 6.0, the lack of effect of α-actinin on F-actin ATPase activity at pH 6.0 cannot be explained by precipitation. Neither can the increasing ability of α-actinin to accelerate F-actin ATPase activity as pH increases from 6.5 to 8.5 be explained by difference in solubility of α-actinin.

In 100 mM KCl, 1 mM Mg^{2+}, and 1 mM ATP at pH 7.5, adding increasing amounts of α-actinin between 5 and 20% as much α-actinin as F-actin by weight causes an increase in the effect of α-actinin on F-actin ATPase activity (Figure 18). The additional increment in α-actinin-induced increase
Figure 17. Effect of α-actinin and pH on F-actin ATPase activity. Final concentrations for ATPase assay: 100 mM KCl, 1.0 mM MgCl$_2$, 0.05 mM CaCl$_2$, 1.0 mM ATP, 20 mM Tris-acetate at pH indicated, 0.5 mM DTT, 1.0 mg F-actin/ml, 0.10 mg α-actinin/ml when added. Temperature = 37°C. Points are averages of 4 determinations on 2 different actin and α-actinin preparations.
Figure 18. Effect of varying levels of α-actinin on F-actin ATPase activity in the presence or absence of DTT. Final concentrations for ATPase assay: 100 mM KCl, 1.0 mM MgCl₂, 0.05 mM CaCl₂, 1.0 mM ATP, 20 mM Tris-acetate, pH 7.5, 1.0 mg F-actin/ml, α-actinin added as indicated. Temperature = 37°C. Points are averages of 6 determinations on 3 different actin and α-actinin preparations.
in ATPase activity as α-actinin is increased from 5 to 20% of the actin present occurs both in the presence and the absence of DTT (Figure 18). In the presence of DTT, 5% as much α-actinin as F-actin increases F-actin ATPase by 26% over the control; 10% α-actinin causes a 67% increase and 20% α-actinin causes a 95% increase in F-actin ATPase activity. Previous studies have shown that at 37°C F-actin binds additional α-actinin as the amount of added α-actinin increases from 5 to 20% of the F-actin present by weight (Goll et al., 1972). Presumably, the additional increment in α-actinin-induced increase in F-actin ATPase activity is due to the extra α-actinin bound to F-actin at higher levels of added α-actinin. Because 10% as much α-actinin as F-actin by weight corresponds to the best estimates of the amount of α-actinin that exists in vivo, 10% α-actinin was used in subsequent experiments.

Because the viscosity experiments that attempted to measure binding of α-actinin to actin indicated that Mg\(^{2+}\) and ATP ostensibly had important effects on binding of α-actinin to F-actin filaments, and because muscle contraction in vivo requires both Mg\(^{2+}\) and ATP, a number of experiments were done to determine the effects of different ATP and Mg\(^{2+}\) concentrations on F-actin ATPase activity and on the ability of α-actinin to affect F-actin ATPase activity. The results of these experiments are summarized in Figures 19-22. The
specific activities of both F-actin ATPase and the α-actinin-modified F-actin ATPase increases as ATP concentration increases from 0.5 to 10 mM while Mg$^{2+}$ concentration is maintained constant at 1 mM in the presence of 100 mM KCl (Figure 19). When ATP concentration exceeds Mg$^{2+}$ concentration, however, α-actinin loses its ability to increase the specific activity of F-actin ATPase (Figure 19). Indeed, α-actinin inhibits F-actin ATPase activity at 5 or 10 mM ATP and 1 mM Mg$^{2+}$ (Figure 19). Adding increasing amounts of Mg$^{2+}$ from 0 to 1 mM while holding ATP concentration constant at 1 mM decreases F-actin ATPase activity greatly, but increasing the amount of Mg$^{2+}$ from 1 to 10 mM in the presence of 1 mM ATP causes only a slight additional decrease in F-actin ATPase activity (Figure 20). At added Mg$^{2+}$ concentrations of 0 and 0.5 mM, α-actinin has very little effect on F-actin ATPase, and in some instances, α-actinin even seems to inhibit F-actin ATPase activity slightly at 0 or 0.5 mM Mg$^{2+}$ in the presence of 1 mM ATP (Figure 20). In the range of 1 to 10 mM added Mg$^{2+}$, however, α-actinin increases the specific activity of F-actin ATPase by 45 to 60% (Figure 20). It was noted in the preceding experiment that the ability of α-actinin to increase F-actin ATPase activity decreased whenever ATP concentration exceeded Mg$^{2+}$ concentration.

Because the two experiments assaying the effects of
Figure 19. Effect of α-actinin and ATP concentration on F-actin ATPase activity. Final concentrations for ATPase assay: 100 mM KCl, 1.0 mM MgCl₂, 0.05 mM CaCl₂, ATP as indicated, 20 mM Tris-acetate, pH 7.5, 0.5 mM DTT, 1.0 mg F-actin/ml, 0.10 mg α-actinin/ml when added. Temperature = 37°C. Points are averages of 5 determinations on 2 different actin and α-actinin preparations.
Figure 20. Effect of α-actinin and Mg$^{2+}$ concentration on F-actin ATPase activity. Final concentrations for ATPase assay: 100 mM KCl, MgCl$_2$ as indicated, 0.05 mM CaCl$_2$, 1.0 mM ATP, 20 mM Tris-acetate, pH 7.5, 0.5 mM DTT, 1.0 mg F-actin/ml, 0.10 mg α-actinin/ml when added. Temperature = 37°C. Points are average of 4 determinations on 2 different actin and α-actinin preparations.
The graph shows the relationship between Mg$^{2+}$ concentration (mM) and the rate of ATP hydrolysis (μ moles P$_i$/mg actin/8 hr) for F-actin and F-actin plus α-actinin. The rates decrease with increasing Mg$^{2+}$ concentration.
varying ATP and varying Mg$^{2+}$ concentrations on F-actin ATPase activity and on ability of α-actinin to increase F-actin ATPase activity indicated that the α-actinin-induced increase in F-actin ATPase activity was sensitive to the Mg$^{2+}$ to ATP ratio present in the assay medium, two experiments were done to determine the effects of Mg$^{2+}$ to ATP ratio on the ability of α-actinin to increase the specific activity of F-actin ATPase. α-Actinin increases the specific activity of F-actin ATPase over the entire range when the Mg$^{2+}$ to ATP ratio is held constant at one but the level of Mg$^{2+}$ and ATP is varied from 0.5 to 10 mM (Figure 21). F-actin ATPase activity in the absence of α-actinin increases markedly as Mg$^{2+}$ and ATP concentrations are raised from 0.5 to 1 mM, increases slightly as Mg$^{2+}$ and ATP concentrations are raised from 1 to 5 mM, and then remains constant as Mg$^{2+}$ and ATP concentrations are increased to 10 mM (Figure 21). That α-actinin increased F-actin ATPase activity throughout the range of 0.5 to 10 mM Mg$^{2+}$ and ATP while the Mg$^{2+}$ and ATP ratio is maintained at one strengthens the conclusion that neither high ATP nor low Mg$^{2+}$ per se is responsible for loss of the ability of α-actinin to increase the specific activity of F-actin ATPase (Figures 19 and 20). Rather it seems that Mg$^{2+}$ to ATP ratios below 1.0 cause this loss of ability of α-actinin to increase F-actin ATPase activity. Direct evidence confirming the conclusion that ability of α-actinin to
Figure 21. Effect of α-actinin and Mg$^{2+}$ and ATP in equal molar amounts on F-actin ATPase activity. Final concentrations for ATPase assay: 100 mM KCl, 0.05 mM CaCl$_2$, ATP and MgCl$_2$ added in equal molar amounts at the levels indicated, 20 mM Tris-acetate, pH 7.5, 0.5 mM DTT, 1.0 mg F-actin/ml, 0.10 mg α-actinin/ml when added. Temperature = 37°C. Points are averages of 4 determinations on 2 different actin and α-actinin preparations.
The graph shows the relationship between Mg-ATP concentration and ATPase activity in F-actin and F-actin plus α-actinin. The y-axis represents j moles P_i/mg actin/8 hr, while the x-axis represents Mg-ATP concentration in mM. The graph includes two lines: one for F-actin (dashed line) and one for F-actin plus α-actinin (solid line).
accelerate F-actin ATPase activity is lost when the Mg$^{2+}$ to ATP ratio in the incubation medium is less than one is shown in Figure 22. α-Actinin increases F-actin ATPase activity almost equally well anywhere between Mg$^{2+}$ to ATP ratios of 1 to 10, but α-actinin loses the ability of increase F-actin ATPase activity at Mg$^{2+}$ to ATP ratios below 1 (Figure 22). Indeed, α-actinin seems to inhibit F-actin ATPase activity to an increasing extent as Mg$^{2+}$ to ATP ratio decreases from 0.5 to 0.1 (Figure 22). It is also evident from Figure 22 that F-actin ATPase activity in the absence of α-actinin is highest at low Mg$^{2+}$ to ATP ratios (which is to say low Mg$^{2+}$ concentrations in the experiment shown in Figure 22) and decreases as Mg$^{2+}$ to ATP ratio increases from 0.1 to 1.0. It should be noted that all experiments done to assay the effect of Mg$^{2+}$ and ATP ratios and concentrations on F-actin ATPase activity and on ability of α-actinin to increase F-actin ATPase activity were done in the presence of 100 mM KCl and that viscosity experiments described in the preceding section showed that α-actinin cross-links actin extensively under these conditions, even in the presence of ATP and total absence of Mg$^{2+}$ (see Table 3). Consequently, the inability of α-actinin to increase F-actin ATPase at Mg$^{2+}$ to ATP ratios below 1 cannot be due to inability of α-actinin to bind to F-actin filaments under these conditions. Rather, these results suggest that, when
Figure 22. Effect of α-actinin and different molar ratios of Mg$^{2+}$/ATP on F-actin ATPase activity. Final concentrations for ATPase assay: 100 mM KCl, 0.05 mM CaCl$_2$, MgCl$_2$ and ATP in molar ratios as indicated, 20 mM Tris-acetate, pH 7.5, 0.5 mM DTT, 1.0 mg F-actin/ml, 0.10 mg α-actinin/ml when added. Temperature = 37°C. Points are averages of 4 determinations on 2 different actin and α-actinin preparations.
α-actinin is bound to F-actin, the ability of α-actinin to alter the structure of actin monomers in F-actin filaments in a way to increase the ATPase activity of these monomers is either inhibited by ATP uncomplexed with Mg²⁺, or that a small amount of free Mg²⁺ is required for α-actinin to alter the structure of actin monomer.

Because the experiments on the effects of Mg²⁺ and ATP concentrations indicated that either free Mg²⁺ or the Mg·ATP complex was necessary for α-actinin to increase F-actin ATPase activity, a series of experiments were done to determine whether Mg²⁺ was an obligatory requirement for α-actinin to increase F-actin ATPase activity, or whether α-actinin could also alter F-actin ATPase activity in the presence of other cations. Moreover, although heavily loaded SDS-polyacrylamide gels showed that the actin used in these ATPase experiments contained no myosin contamination (Figure 1), the very low Mg²⁺-modified ATPase activity of F-actin compared to the Mg²⁺-modified ATPase activity of reconstituted actomyosin left open the remote possibility that the ATPase activity detected in F-actin preparations actually originates from a minute amount of myosin contaminating these actin preparations, but undetectable in SDS-polyacrylamide gels. Because it was critical for the purpose of the study to establish that α-actinin affects F-actin structure, it was necessary to establish beyond
any doubt that the ATPase activity in F-actin solutions originates from F-actin and not from a minute amount of myosin contaminating the F-actin preparation. Actomyosin ATPase activity varies widely in a very characteristic way in the presence of different cationic activators, and it was hoped that F-actin ATPase activity would also vary widely in the presence of different cation activators but that the relative effects of these different cations on F-actin ATPase activity would differ greatly from their relative effects on actomyosin ATPase activity. This difference in relative effects of different cationic activators could then be used to clearly differentiate F-actin ATPase activity from actomyosin ATPase activity. Consequently, the experiments measuring the effects of different cations on F-actin ATPase activity had two purposes.

Figure 23 shows the effect of different divalent cations on F-actin ATPase activity and on the ability of α-actinin to increase the specific activity of F-actin ATPase. To prevent any complications due to an excess of free cation or free ATP, all experiments shown in Figure 23 were done in 100 mM KCl with 1 mM ATP and 1 mM cationic activator added. F-actin ATPase activity in the absence of α-actinin is similar in the presence of Mg$^{2+}$ plus 0.5 mM Ca$^{2+}$, Mg$^{2+}$ alone, Ca$^{2+}$, or Mn$^{2+}$, as the added cation (Figure 23, Table 6). Addition of Co$^{2+}$ or Ni$^{2+}$ as the cation results in a 27% or
Figure 23. Effect of different divalent cations and \( \alpha \)-actinin on F-actin ATPase activity. Final concentrations for ATPase assay: 100 mM KCl, 1 mM divalent cations (except, 0.05 mM CaCl\(_2\), when added with MgCl\(_2\)), 1.0 mM ATP, 20 mM Tris-acetate, pH 7.5, 0.5 mM DTT, 1.0 mg F-actin/ml, 0.10 mg \( \alpha \)-actinin/ml when added. Temperature = 37°C. Data used in these bar graphs are averages of 4 determinations on 2 different actin and \( \alpha \)-actinin preparations.
- F-ACTIN
- F-ACTIN PLUS
- α-ACTININ

μ MOLES Pi/mg ACTIN/8hr

Mg$^{2+}$ and Ca$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Ba$^{2+}$, Fe$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Co$^{2+}$
Table 5. Effect of divalent cations on F-actin and actomyosin ATPase activity

<table>
<thead>
<tr>
<th>Cations</th>
<th>ATPase activity of F-actin of Mg(^{2+}) and Ca(^{2+})-modified F-actin</th>
<th>ATPase activity of reconstituted actomyosin</th>
<th>% of Mg(^{2+}) and Ca(^{2+})-modified actomyosin ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg(^{2+}) and Ca(^{2+})</td>
<td>0.192 ± 0.005</td>
<td>0.280 ± 0.05</td>
<td>100</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>0.195 ± 0.01</td>
<td>0.320 ± 0.05</td>
<td>102</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>0.214 ± 0.01</td>
<td>0.682 ± 0.06</td>
<td>111</td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>0.181 ± 0.01</td>
<td>0.412 ± 0.032</td>
<td>94</td>
</tr>
<tr>
<td>Ba(^{2+})</td>
<td>0.390 ± 0.01</td>
<td>0.154 ± 0.02</td>
<td>203</td>
</tr>
<tr>
<td>Fe(^{2+})</td>
<td>0.356 ± 0.02</td>
<td>0.301 ± 0.02</td>
<td>185</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>0.009 ± 0.009</td>
<td>0.003 ± 0.002</td>
<td>5</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>0.266 ± 0.002</td>
<td>0.211 ± 0.01</td>
<td>138</td>
</tr>
<tr>
<td>Co(^{2+})</td>
<td>0.245 ± 0.01</td>
<td>0.270 ± 0.01</td>
<td>127</td>
</tr>
</tbody>
</table>

\(^a\)Conditions for assay of F-actin ATPase activity: 100 mM KCl, 1 mM divalent cations (except, 0.05 mM CaCl\(_2\) when added with MgCl\(_2\)), 1 mM ATP, 20 mM Tris-acetate, pH 7.5, 0.5 mM DTT, 1.0 mg F-actin/ml, 37°C. Figures are means plus or minus the standard error of six determinations.

\(^b\)Conditions for assay of reconstituted actomyosin ATPase activity: 100 mM KCl, 1 mM divalent cations (except, 0.05 mM CaCl\(_2\) when added with MgCl\(_2\)), 1 mM ATP, 20 mM Tris-acetate, pH 7.5, 0.5 mM DTT, 0.2 mg actomyosin/ml in the presence of Mg\(^{2+}\) and Ca\(^{2+}\), Mg\(^{2+}\) alone, and Ca\(^{2+}\), and 0.5 mg actomyosin/ml in the presence of Mn\(^{2+}\), Ba\(^{2+}\), Fe\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\) and Co\(^{2+}\), 37°C. Figures are means plus or minus the standard error of six determinations.

\(^c\)F-actin ATPase activity is expressed as μ moles of Pi/mg actin/st h.

\(^d\)Actomyosin and F-actin ATPase activity in the presence of 1 mM MgCl\(_2\) and 0.05 mM CaCl\(_2\) is considered as 100 percent and activities in the presence of other divalent cations are expressed as a percent of this activity.

\(^e\)Actomyosin ATPase activity is expressed as μ mole Pi/mg actomyosin/min.
a 38% increase, respectively, in specific activity of F-actin ATPase in the absence of α-actinin (Figure 23, Table 6). Addition of Cu$^{2+}$ greatly decreases and addition of either Ba$^{2+}$ or Fe$^{2+}$ greatly increases F-actin ATPase activity in the absence of α-actinin (Figure 23, Table 6). α-Actinin increases F-actin ATPase activity by 41 to 84% in the presence of Mg$^{2+}$ plus Ca$^{2+}$, Mg$^{2+}$ alone, Mn$^{2+}$, or Co$^{2+}$ but has only a very small or no significant effect on F-actin ATPase activity in the presence of Ca$^{2+}$, Ni$^{2+}$, or Cu$^{2+}$ (Figure 23). In the presence of Ba$^{2+}$ and Fe$^{2+}$, α-actinin actually seems to inhibit F-actin ATPase activity slightly (Figure 23).

Comparison of the effects of the divalent cations shown in Figure 23 and Table 6 on F-actin ATPase activity with the effects of the same divalent cations on actomyosin ATPase activity clearly shows that the ATPase activity measured in F-actin solutions does not originate from any myosin contamination in these solutions (Table 6). Ca$^{2+}$ and Mn$^{2+}$ activate actomyosin ATPase activity to a far greater extent than they activate F-actin ATPase activity, and Ba$^{2+}$ and Ni$^{2+}$ activate actomyosin ATPase activity to a much lesser extent than they activate F-actin ATPase activity (Table 6). Therefore, these results demonstrate that the effect of α-actinin on the ATPase activity in F-actin solutions originates from an ability of α-actinin to alter the structure of actin
monomers while they are assembled in F-actin filaments.

As indicated previously in the section discussing viscometric studies of the α-actinin-F-actin interaction, the thin filament in mammalian skeletal muscle contains tropomyosin in addition to F-actin. If the physiological function of α-actinin in vivo involves alteration of actin structure, α-actinin must be capable of causing this alteration in the presence of tropomyosin. Therefore, several experiments were done to determine whether α-actinin can alter F-actin ATPase activity in the presence of tropomyosin. The viscometric studies described in the preceding section showed that α-actinin did not cross-link F-actin filaments in the presence of tropomyosin and suggested that tropomyosin added in vitro did not bind to F-actin filaments in the same way that tropomyosin is bound in vivo. This altered in vitro binding of tropomyosin seemed to completely prevent α-actinin from binding to F-actin filaments in the presence of tropomyosin. Despite this, it was hoped that the inability of viscometric studies to detect binding of α-actinin unless it was accompanied by cross-linking of F-actin filaments did not completely eliminate that possibility that some α-actinin binds to F-actin in the presence of tropomyosin at 37°C and that ATPase assays might simultaneously reveal the existence of this binding and the ability of α-actinin to alter actin structure in the presence of tropo-
myosin. Unfortunately, addition of either 10% or 20% as much α-actinin as actin by weight had no effect on F-actin ATPase activity when tropomyosin was also present (Figure 24), even though addition of the same amounts of α-actinin increases F-actin ATPase activity by 55% and 82% in the absence of tropomyosin (Figure 24). Addition of 25% tropomyosin alone decreases F-actin ATPase activity by 35 to 40%; this indicates that tropomyosin binds to F-actin filaments under the conditions used in this study, even though such binding was undetectable by viscometric measures (see Table 5). Because both the viscometric studies and the ATPase assays suggest that α-actinin cannot bind to F-actin filaments in the presence of tropomyosin added in vitro, it seems likely that failure of α-actinin to alter F-actin ATPase activity in the presence of tropomyosin is due to blocking of the binding of α-actinin to F-actin by improperly positioned tropomyosin strands. In the absence of conclusive evidence showing that α-actinin is not bound to F-actin in the presence of tropomyosin, however, it remains possible that α-actinin is bound under these conditions and that tropomyosin nullifies the ability of α-actinin to alter actin structure. Additional studies are necessary to answer this important question.
Figure 24. Effect of α-actinin and tropomyosin on F-actin ATPase activity. Final concentrations for ATPase assay: 100 mM KCl, 1.0 mM MgCl₂, 0.05 mM CaCl₂, 1.0 mM ATP, 20 mM Tris-acetate, pH 7.5, 0.5 mM DTT, 1.0 mg F-actin/ml, 0.1 or 0.2 mg α-actinin/ml when added as indicated, 0.25 mg tropomyosin/ml when added. Temperature = 37°C. Points are averages of 4 determinations on 2 different actin and α-actinin preparations.
C. Studies on Purified α-Actinin

As described in the Introduction of this thesis, it is possible that the physiological role of α-actinin in skeletal muscle involves structural stabilization of I- and Z-filaments in the Z-disk rather than modification of actin structure in a way that alters the contractile interaction of actin and myosin. Although antibody studies have located α-actinin exclusively in the Z-disk, it is not possible to determine the molecular nature of the interaction of α-actinin with other proteins in the Z-disk by using antibody studies alone. Therefore, the exact content of some important amino acid side-chains and the N- and C-terminal amino acids of purified α-actinin were determined as a necessary prelude to learning whether modification of these amino acid side-chains or the N- and C-terminal amino acids would provide any clues concerning the molecular nature of the interaction of α-actinin with other proteins in the Z-disk.

1. Tryptophan content of purified α-actinin

  The tryptophan content of highly purified α-actinin prepared from several different types of muscles obtained from several different species is shown in Table 7. Tryptophan content of α-actinin from porcine red skeletal (dark semitendinosus) muscle, bovine cardiac muscle, chicken gizzard smooth muscle, chicken breast skeletal muscle, and
Table 7. Assay of tryptophan content in some proteins whose tryptophan content is known and in highly purified α-actinins from different muscles

<table>
<thead>
<tr>
<th>Protein</th>
<th>Moles of tryptophan/mole of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>2.34</td>
</tr>
<tr>
<td>Trypsin</td>
<td>3.57</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>0.0</td>
</tr>
<tr>
<td>Porcine white skeletal α-actinin</td>
<td>26.2 ± 0.2^a</td>
</tr>
<tr>
<td>Porcine red skeletal α-actinin</td>
<td>30.8 ± 0.2^a</td>
</tr>
<tr>
<td>Bovine cardiac α-actinin</td>
<td>30.9 ± 0.2^a</td>
</tr>
<tr>
<td>Chicken breast α-actinin</td>
<td>30.0 ± 0.3^a</td>
</tr>
<tr>
<td>Chicken gizzard α-actinin</td>
<td>30.6 ± 0.1^a</td>
</tr>
<tr>
<td>Rabbit skeletal α-actinin</td>
<td>29.9 ± 1.1^a</td>
</tr>
</tbody>
</table>

^aTryptophan analysis was done according to the procedure of Gaitonde and Dovey (1970). Figures are means plus or minus standard errors of 4 to 6 determinations done on 2 different preparations.

^bMolecular weights of bovine serum albumin, trypsin and α-actinin from all different types of muscles were taken as 68,000 daltons, 23,800 daltons and 200,000 daltons, respectively.
rabbit skeletal muscle are all very similar and are all slightly greater than the tryptophan content of \(\alpha\)-actinin from white skeletal (light semitendinosus) muscle. The reason for the slightly lower tryptophan content of porcine white skeletal muscle \(\alpha\)-actinin is unclear.

2. **Cysteine content of purified \(\alpha\)-actinin**

Experience in protein chemistry has shown that sulfhydryl groups are often intimately involved in the biological functions of proteins. Therefore, a careful study was done to determine the number of sulfhydryl groups in purified \(\alpha\)-actinin. One of the most accurate methods currently available for determining sulfhydryl content of a protein involves measurement of the increase in absorbance at 412 nm caused by reaction of DTNB with ionized sulfhydryl groups at pH 8.1. This method has the advantage that free sulfhydryl groups can be measured independently of those involved in disulfide bonds and that an estimate of the number of sulfhydryl groups on the surface of the protein and available to the solvent can be obtained by measuring the number of sulfhydryl groups that react with DTNB when the protein is dissolved in a nondenaturing solvent. The total number of sulfhydryl groups in the protein can then be determined by measuring the number of sulfhydryl groups that react with DTNB when the protein is completely denatured in a strong denaturing solvent. It is critical when determining
total sulfhydryl content of a protein to insure that conditions used to denature the protein cause complete unfolding of the molecule and exposure of all sulfhydryl groups for reaction with DTNB. For that reason, a careful study was done to ascertain the conditions necessary to denature α-actinin completely.

The results shown in Figure 25 indicate that SDS and DTNB should be added simultaneously to produce maximum reaction of DTNB with sulfhydryl groups. Maximum reaction of DTNB with sulfhydryl groups of denatured α-actinin occurs approximately 18 to 30 min after DTNB and SDS have been added simultaneously (Figure 25). It is also clear from Figure 25 that 0.05 M SDS is as effective in causing complete unfolding of cardiac α-actinin as 0.10 M SDS as long as either concentration of SDS is added simultaneously with DTNB. The reason for the lower availability of sulfhydryl groups when α-actinin is incubated with 0.05 M or .10 M SDS for 1 hr at 22°C before adding DTNB is not entirely clear. The most likely explanation is that reaction with SDS does not cause simple unfolding of a protein into a random coil, but rather than incubation with SDS causes unfolding of proteins followed quickly by conversion into a rod whose dimensions are determined by the size of the protein (Reynolds and Tanford, 1970). This refolding of proteins into rods in SDS may result in some sulfhydryl groups
Figure 25. Reaction of DTNB with bovine cardiac α-actinin in the presence of different concentrations of SDS. Final concentrations: 0.005 μ moles of α-actinin, 0.4 mM DTNB buffered with 10 mM phosphate buffer, pH 8.1, 40 mM Tris-acetate, pH 8.1, in the presence of different SDS concentrations as indicated. Temperature = 25°C. Moles of cysteine/mole of α-actinin were calculated by using the extinction coefficient of 12,000/cm for 3-carboxylacto-4-nitrothiophenolate (Robyt et al., 1971) and a molecular weight of 200,000 daltons for the cardiac α-actinin molecule. Time after addition of DTNB is plotted on the abscissa of each graph. Points are means of 3 determinations made on 2 different bovine cardiac α-actinin preparations.
0.05M SDS and DTNB added at the same time

0.05M SDS added one hour before the addition of DTNB

0.1M SDS and DTNB added at the same time

0.1M SDS added one hour before the addition of DTNB
being buried in the interior of these rods and becoming unavailable for reaction with DTNB. If it occurs before 1 hr of incubation in SDS, this refolding could explain the lowered availability of sulfhydryl groups when α-actinin is incubated in SDS for 1 hr before adding DTNB (Figure 23) because adding SDS and DTNB simultaneously would permit reaction of DTNB with sulfhydryl groups as they became exposed during the initial unfolding of the protein and before refolding into rods. The results obtained when SDS and DTNB are added simultaneously show that bovine cardiac α-actinin contains about 17.5 to 17.8 sulfhydryl groups per mole of 200,000 daltons (Figure 25, Table 8). Measurements done in nondenaturing media show that 6 of these 17.5 to 17.8 sulfhydryl groups in bovine cardiac α-actinin are available for reaction with DTNB in undenatured molecules (Table 8) and are therefore presumably located on the surface of the α-actinin molecule. The results shown in Table 8 also confirm the results shown in Figure 25 and indicate that incubation in 0.05 M SDS results in as many sulfhydryl groups being available for reaction with DTNB as incubation in 0.125 M SDS does.

In a separate study whose results are not shown here, it was found that, after reduction and carboxymethylation (Crestfield et al., 1963), bovine cardiac α-actinin still contains 2 to 3 sulfhydryl groups per α-actinin molecule as
Table 8. Determination of sulfhydryl groups of native cardiac α-actinin and α-actinin denatured with different concentrations of SDS\textsuperscript{a}

<table>
<thead>
<tr>
<th>Amount of SDS used to denature α-actinin</th>
<th>Moles of SH/mole of α-actinin\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 M</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>0.025 M</td>
<td>17.0 ± 0.1</td>
</tr>
<tr>
<td>0.050 M</td>
<td>17.5 ± 0.1</td>
</tr>
<tr>
<td>0.075 M</td>
<td>17.6 ± 0.1</td>
</tr>
<tr>
<td>0.100 M</td>
<td>17.7 ± 0.0</td>
</tr>
<tr>
<td>0.125 M</td>
<td>17.8 ± 0.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Conditions: 0.005 μ moles of α-actinin, 0.4 mM DTNB buffered with 10 mM phosphate, pH 8.1, 40 mM Tris-acetate, pH 8.1, SDS as indicated, 25°C. When added, SDS was added simultaneously with DTNB. Optical density was read at 412 nm 30 min after addition of DTNB and SDS (when added).

\textsuperscript{b}Calculated by using a molecular weight of 200,000 daltons for bovine cardiac α-actinin. Figures are means plus or minus standard errors of three determinations on two different α-actinin preparations.

measured by the DTNB procedure in 0.05 M SDS. These results show that 15 min of treatment with iodoacetate is not sufficient to completely carboxymethylate all sulfhydryl groups in bovine cardiac α-actinin. Previous studies by other investigators have shown that the rate of carboxymethylation of cysteine side-chains is influenced by the type of amino acids adjacent to the cysteine. Because the 2 to 3 sulfhydryl...
groups remaining unreacted after 15 min of treatment with iodoacetate did not seem to form disulfide bonds, carboxymethylation was limited to 15 min in the remaining experiments done in this study to prevent undesirable reaction of iodoacetate with side-chains of amino acids other than cysteine.

3. C-Terminal amino acid analysis of purified α-actinins

The C-terminal amino acids of highly purified α-actinin prepared from several different muscles were determined by using carboxypeptidase A and B on α-actinin molecules that had been completely denatured by SDS. Carboxypeptidase A will specifically release amino acids from the C-terminal end of a peptide chain as long as these amino acids are not proline, arginine or lysine (Ambler, 1967). Carboxypeptidase B will specifically release amino acids from the C-terminal end of a peptide chain as long as these amino acids are arginine or lysine (Ambler, 1967). Consequently, the simultaneous or sequential use of carboxypeptidases A and B followed by determination of amino acids released will identify the C-terminal amino acid of any peptide chain that does not contain proline as its C-terminal amino acid. The time-course of amino acid release from carboxymethylated bovine cardiac α-actinin by CPA is shown in Figure 26. Leucine is the only amino acid released even after 5 hr of incubation
Figure 26. Release of amino acids from bovine cardiac $\alpha$-actinin by carboxypeptidase A. Final concentrations: 0.1 $\mu$ mole of carboxymethylated $\alpha$-actinin was incubated for the times indicated with 10.24 units of carboxypeptidase A at 37°C in 0.05 M SDS, 0.2 M N-ethylmorpholine acetate, pH 8.5. The reaction was stopped by lowering the pH of the supernatant to 2.5 to 3.0 by adding Dowex-50X8 ($H^+$). Amino acids released from $\alpha$-actinin by CPA were analyzed by using a Beckman automatic amino acid analyzer. The molecular weight of bovine cardiac $\alpha$-actinin was assumed to be 200,000 daltons.
with CPA. After 5 hrs, CPA releases 1.7 moles of leucine per mole of bovine cardiac \( \alpha \)-actinin (Figure 26). \( \alpha \)-Actinin has been isolated from six or seven different kinds of muscle thus far, and all these different \( \alpha \)-actinins have been shown to contain two peptide chains of 100,000 daltons each per molecule of \( \alpha \)-actinin. Therefore, the release of 1.7 moles of leucine per mole of bovine cardiac \( \alpha \)-actinin molecule suggests that both of the two peptide chains in \( \alpha \)-actinin contain leucine as their C-terminal amino acid, or that 1.7 moles of leucine is being released from one of the two peptide chains and that the other peptide contains a lysine, arginine, or proline that is not being released by CPA. The latter alternative, of course, requires that \( \alpha \)-actinin preparations contain two different peptide chains having different C-terminal amino acids. That no amino acid other than leucine is released even after 5 hr of incubation with CPA also suggests that the next amino acid after the last leucine released by CPA (the penultimate amino acid if both peptide chains are identical or the third amino acid if two different peptide chains are present in \( \alpha \)-actinin) is arginine, lysine, or proline, and is therefore unavailable for release by CPA.

Because CPB has a very small amount of CPA activity, CPB was used to test whether leucine released from bovine cardiac \( \alpha \)-actinin by CPA originates from both peptide chains
in the α-actinin molecule of whether α-actinin preparations contain two different kinds of peptide chains, and one of these two different peptide chains has a C-terminal amino acid sequence of -leu-leu. Assays of the CPB preparations used in this study on the synthetic substrates, hippuryl-L-arginine and hippuryl-L-phenylalanine, showed that these preparations contained 1.44% as much CPA activity as they did CPB activity. Incubation of bovine cardiac α-actinin with CPB resulted in the slow release of leucine followed by the release of arginine (Figure 27). Because CPB would have released C-terminal arginine or lysine very quickly, the results in Figure 27 indicate that neither peptide chain in bovine cardiac α-actinin contains lysine or arginine as a C-terminal amino acid, and that arginine is probably the amino acid penultimate to C-terminal leucine in the peptide chains of α-actinin. Although the experiments described thus far do not eliminate the possibility that α-actinin preparations contain two kinds of peptide chains with one kind having a C-terminal proline and the other kind having a C-terminal sequence of arg-leu-leu, hydrazinolyzed bovine cardiac α-actinin did not contain free proline. Consequently, the CPA, CPB, and hydrazinolysis results together demonstrate that both peptide chains of bovine cardiac α-actinin molecule contain C-terminal leucine and have arginine as the penultimate C-terminal amino acid.
Figure 27. Release of amino acids from bovine cardiac α-actinin by carboxypeptidase B. Final concentrations: 0.1 μ mole of carboxy-methylated α-actinin was incubated for the times indicated with 21.9 units of carboxypeptidase B at 37°C in 0.05 M SDS, 0.2 M N-ethylmorpholine acetate, pH 8.5. The reaction was stopped as described in the legend for Figure 26. The molecular weight of bovine cardiac α-actinin was taken as 200,000 daltons.
MOLES AMINO ACID RELEASED/MOLE OF α-ACTININ
In another series of experiments, the amino acids released from carboxymethylated bovine cardiac α-actinin by CPA were divided into two aliquots. One aliquot was analyzed for amino acid composition by using an amino acid analyzer without hydrolyzing the sample before analysis. The other aliquot was hydrolyzed with constant boiling 5.7 N HCl at 110°C for 24 hrs, and the hydrolysate then subjected to an amino acid analysis with an amino acid analyzer. Leucine was the only amino acid found in either the hydrolyzed or the unhydrolyzed aliquots; identical amounts of leucine were found in both these two samples. This result proves that the peak ascribed to leucine in the amino acid analyzer elution profile of unhydrolyzed samples was not due to asparagine or glutamine. After showing that bovine cardiac α-actinin contained C-terminal leucine and penultimate C-terminal arginine, CPA and CPB digestion was used to determine the C-terminal amino acids in α-actinins prepared from porcine skeletal muscle, chicken gizzard smooth muscle, and chicken breast muscle. In all these assays, both CPA and CPB were incubated simultaneously with carboxymethylated α-actinin, and release of amino acids was followed during a 5 hr incubation period. The results indicate that α-actinins from these diverse sources all contain leucine as their C-terminal amino acid and that incubation with CPA plus CPB releases between 1.6 and 1.7 moles of
leucine per mole of α-actinin from any of these sources (Figures 28-30). Moreover, approximately 0.35 moles of arginine per mole of α-actinin is released from porcine skeletal α-actinin by incubation with CPA plus CPB (Figure 28). This result indicates that porcine skeletal α-actinin also resembles bovine cardiac α-actinin by having arginine as an amino acid penultimate to its C-terminus. Although no detectable arginine was released from chicken gizzard or chicken breast muscle α-actinin during a 5 hr incubation with CPA plus CPB, the small amount of CPB used in these two incubations (Figures 29, 30) may have failed to release enough arginine in 5 hrs to permit its detection. Consequently, it is possible that chicken gizzard and chicken breast muscle α-actins also possess arginine as an amino acid penultimate to their C-terminal leucine, and that α-actins from all four muscle sources used in this study have identical C-terminal amino acids.

4. N-Terminal amino acid analysis of purified α-actins

The highly fluorescent sulfonamide derivatives of dimethyl-amino-naphthalene-5-sulfonyl chloride (dansyl chloride, DNSCl) are favored for amino terminal analysis of peptides and proteins because they react with amino groups to form bonds that are stable to hydrolysis in 5.7 N HCl for 24 hrs at 110°C, and because the dansylated amino acid derivatives can be detected at 100-fold greater sensitivity
Figure 28. Release of amino acids from porcine skeletal muscle α-actinin by carboxypeptidases A and B. Final concentrations: 0.1 μ mole of carboxymethylated α-actinin was incubated for the times indicated with 11.4 units of carboxypeptidase A and 8.14 units of carboxypeptidase B at 37°C in 0.05 M SDS, 0.2 M N-ethylmorpholine acetate, pH 8.5. The reaction was stopped as described in the legend for Figure 26. The molecular weight of porcine skeletal α-actinin was taken as 200,000 daltons.
Figure 29. Release of amino acids from chicken gizzard α-actinin by carboxypeptidases A and B. Final concentrations: 0.1 μ mole of carboxy-methylated α-actinin was incubated for the times indicated with 10.24 units of carboxypeptidase A and 3.12 units of carboxypeptidase B at 37°C in 0.05 M SDS, 0.2 M N-ethyl-morpholine acetate, pH 8.5. The reaction was stopped as described in the legend for Figure 26. The molecular weight of chicken gizzard α-actinin was taken as 200,000 daltons.
MOLES AMINO ACID RELEASED/MOLE OF α-ACTININ

TIME (hr.)

LEUCINE
Figure 30. Release of amino acids from chicken breast muscle α-actinin by carboxypeptidases A and B. Final concentrations: 0.1 μ mole of carboxymethylated α-actinin was incubated for the times indicated with 10.24 units of carboxypeptidase A and 3.12 units of carboxypeptidase B at 37°C in 0.05 M SDS, 0.2 M N-ethylmorpholine acetate, pH 8.5. The reaction was stopped as described in the legend for Figure 26. The molecular weight of chicken breast α-actinin was taken as 200,000 daltons.
MOLES AMINO ACID RELEASED/MOLE OF α-ACTININ

TIME (hr)

0 1 2 3 4 5

LEUCINE
than fluorodinitro-benzene derivatives of amino acids. The first experiments on the N-terminal amino acids of α-actinin involved some preliminary attempts to insure that complete separation of the dansylated derivatives of all 20 amino acids could be achieved by using thin layer chromatography on polyamide coated plates. Chromatography using the two solvent systems described in the Materials and Methods separates all dansylated derivatives of the amino acids except DNS-histidine, DNS-arginine, and ε-DNS-lysine (Figure 31). An additional chromatographic development in a solvent system containing 1 M ammonia-ethanol (1:1, V/V) as described in the Materials and Methods section separates DNS-histidine, DNS-arginine, and ε-DNS-lysine.

When bovine cardiac α-actinin is treated with dansyl chloride, and the treated protein is hydrolyzed to amino acids by using 5.7 N HCl, the only dansylated amino acid found in the hydrolysate is ε-DNS-lysine. To insure that the dansylation procedure used in this study would actually dansylate the N-terminal α-amino group of a protein, the dansylation procedure was repeated using several different proteins having known N-terminal amino acids. In every instance, the proper N-terminal amino acid was found (Table 9) by using the dansylation procedure described in the Material and Methods section. Two proteins, actin and cytochrome C, whose N-terminal amino acids are known to be
Figure 31. Thin layer chromatography on polyamide coated sheets of standard DNS-amino acids and of amino acids from bovine cardiac α-actinin that had been treated with dansyl chloride before acid hydrolysis. Chromatography was done first in the horizontal direction in H₂O-90% formic acid (100:1.5, V/V) solvent. The plate was then turned and chromatography was done in the vertical direction in a benzene-acetic acid (9:1, V/V) solvent. Circles indicate spots seen when the dried plate was observed under an ultraviolet light. X indicates where the original sample was applied.
Table 9. N-terminal amino acid determination on α-actinins from several different muscles and on proteins containing known N-terminal amino acids by using the dansyl chloride procedure

<table>
<thead>
<tr>
<th>Proteins</th>
<th>N-terminal amino acid found in this study</th>
<th>N-terminal amino acid reported in literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>Phenylalanine and glycine</td>
<td>Phenylalanine and glycine</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>Valine</td>
<td>Valine</td>
</tr>
<tr>
<td>Aldolase</td>
<td>Alanine</td>
<td>Alanine</td>
</tr>
<tr>
<td>Actin</td>
<td>None</td>
<td>Blocked-acetylated</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>None</td>
<td>Blocked-acetylated</td>
</tr>
<tr>
<td>Bovine cardiac α-actinin</td>
<td>None</td>
<td>----</td>
</tr>
<tr>
<td>Porcine skeletal α-actinin</td>
<td>None</td>
<td>----</td>
</tr>
<tr>
<td>Chicken gizzard α-actinin</td>
<td>None</td>
<td>----</td>
</tr>
<tr>
<td>Chicken breast α-actinin</td>
<td>None</td>
<td>----</td>
</tr>
</tbody>
</table>

*Proteins were treated with dansyl chloride and then hydrolyzed with constant boiling HCl. Dansylated amino acids released by acid hydrolysis were separated on polyamide coated plates by the method of Woods and Wang (1967) and Gray (1972).*
acetylated and therefore unavailable for reaction with dansyl chloride, were also included in these tests of the accuracy of the dansylation procedure used in this study. No N-terminal amino acid was found for either actin or cytochrome C (Table 9). Dansylation studies on porcine skeletal α-actinin, chicken gizzard α-actinin, and chicken breast muscle α-actinin also failed to reveal any N-terminal amino acid available for reaction with dansyl chloride (Table 9). These studies, therefore, suggest that the N-terminal amino acids of α-actinin are blocked and unavailable for reaction with dansyl chloride. Because the N-terminal amino acid of other myofibrillar proteins, actin, myosin, and tropomyosin, are acetylated, it was postulated that the N-terminal amino acids of α-actinin were also acetylated, and further studies on the N-terminal amino acids of α-actinin were based on this premise.

The first experiments based on the assumption that the N-terminus of α-actinin is acetylated involved treatment of purified α-actinin with acylase I (amino acylase), an enzyme that hydrolyzes the amide bond of N-acetyl-L-methionine, in the hope that this enzyme would remove any acetate blocking the N-terminus of α-actinin and leave the N-terminal α-amino groups free to react with dansyl chloride. Even after 6 hrs of incubation with acylase I, however, bovine cardiac α-actinin yielded only ε-DNS-lysine after treatment with
dansyl chloride and subsequent hydrolysis to amino acids. Because acylase I treatment was done in a nondenaturing solvent, the failure of acylase I to make the N-terminal \(\alpha\)-amino group of \(\alpha\)-actinin available for reaction with dansyl chloride could be due to either one of two reasons: 1) the N-terminus of \(\alpha\)-actinin is buried in the interior of the tertiary structure of \(\alpha\)-actinin and hence is not available for hydrolysis by acylase I; or 2) the N-terminus of \(\alpha\)-actinin is not acetylated but is blocked by some other group. The simplest way to determine which of these two alternatives is correct is to unfold the \(\alpha\)-actinin molecule completely in a denaturing solvent so that its N-terminus is exposed, and then use acylase I on the denatured molecule. Unfortunately, acylase I was not active on a synthetic substrate in the presence of the denaturing solvent, SDS (Figure 32) so this approach could not be used.

An alternative procedure for obtaining evidence as to whether the N-terminal amino acids of \(\alpha\)-actinin are acetylated is to assay \(\alpha\)-actinin for the presence of acetate. If \(\alpha\)-actinin contains no acetate, then its N-terminal \(\alpha\)-amino groups could not be acetylated. On the other hand, finding that \(\alpha\)-actinin contains approximately 2 moles of acetate per mole of \(\alpha\)-actinin would suggest that the N-terminus of each of the two peptide chains in one \(\alpha\)-actinin molecule was acetylated. Of course, the presence of two moles of acetate
Figure 32. Action of aminoacylase on N-acetyl-L-methionine in the presence and absence of SDS. Final concentrations: 3 ml of 0.015 M N-acetyl-L-methionine in 0.1 M K-phosphate, pH 7.0 was placed in a cuvette and the reaction was initiated by adding 0.1 ml of enzyme in 0.1 M K-phosphate, pH 7.0 (2000 units/ml). Temperature = 25°C. The decrease in extinction at 238 nm was recorded.
per mole of α-actinin would not prove that the N-terminal amino acids of α-actinin were acetylated because the acetate could be bound to places other than the N-terminal α-amino groups, but such results would at least be quite suggestive. To insure the accuracy of the acetate determination, the analysis was first applied to actin, a protein that is known to contain one mole of acetate per mole of protein. This acetate is on the N-terminal α-amino group of actin. The analysis showed that actin contained 0.89 moles of acetate per mole of actin (Table 10), in excellent agreement with what is known about the acetate content of actin. Acetate analysis of bovine cardiac α-actinin, porcine skeletal α-actinin, and chicken breast muscle α-actinin showed that these α-actinins all contain 1.78 to 1.83 moles of acetate per mole protein (Table 10). Although chicken gizzard α-actinin contains only 1.57 moles of acetate per mole of α-actinin, this slight difference is not significantly different because large difference was observed between the two gizzard samples tested than with any other α-actinins. Consequently, quantitative acetate analysis shows that α-actinins from diverse tissue sources all contain approximately two moles of acetate per mole of protein and suggest that both N-terminal α-amino groups in the α-actinin molecule are acetylated.

Because quantitative acetate analysis suggested that
Table 10. Acetate analysis\(^a\) of porcine skeletal actin and of \(\alpha\)-actinins from different muscles

<table>
<thead>
<tr>
<th>Protein</th>
<th>Moles of acetate/mole of protein(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine skeletal actin</td>
<td>0.89 (3)(^c)</td>
</tr>
<tr>
<td>Bovine cardiac (\alpha)-actinin</td>
<td>1.83 (3)</td>
</tr>
<tr>
<td>Porcine skeletal (\alpha)-actinin</td>
<td>1.81 (2)</td>
</tr>
<tr>
<td>Chicken gizzard (\alpha)-actinin</td>
<td>1.57 (2)</td>
</tr>
<tr>
<td>Chicken breast (\alpha)-actinin</td>
<td>1.78 (2)</td>
</tr>
</tbody>
</table>

\(^a\)Acetate analysis was done by the method of Kuo and Younathan (1973).

\(^b\)Molecular weight of 42,000 daltons was used for actin; a molecular weight of 200,000 daltons was used for \(\alpha\)-actinin.

\(^c\)Figures are averages of determinations on the number of different preparations shown in parentheses.

Both N-terminal amino groups in the \(\alpha\)-actinin molecule are acetylated, it seemed that determination of the N-terminal amino acid(s) of \(\alpha\)-actinin would require isolation of an acetylated peptide from an enzymic digest obtained by exhaustive treatment of bovine cardiac \(\alpha\)-actinin with pronase, trypsin, and CPB as described in the Materials and Methods. If the N-terminal amino group of a peptide chain is acetylated, then the peptide containing that acetylated N-terminal amino group should be the only peptide not having
a positive charge in a mixture of peptides released from a peptide chain by extensive enzymic hydrolysis. All other peptides would contain a positively charged \( \alpha \)-amino group at their terminus. Of course, if the acetylated peptide contained a lysine or arginine residue, it would have a positively charged side-chain. The possibility that an acetylated peptide would contain a lysine or arginine residue, however, can be made very small by treating with trypsin to cleave all lysine or arginine bonds and finally treating with CPB to remove the C-terminal lysine or arginine that would result from trypsin treatment. Because the acetylated peptide has no positive charge, it should pass through a Dowex-50 column unretarded. The enzymic digest of \( \alpha \)-actinin was applied to a Dowex-50x8 column, and the void volume was assayed for acetate. The presence of acetate in this fraction indicated that it contained the acetylated amino acids that were present in the intact \( \alpha \)-actinin molecule. This fraction, called the N-acetyl-x fraction, was hydrolyzed with 5.7 N HCl at 110\(^\circ\)C for 24 hrs, and the hydrolysate was subjected to complete amino acid analysis (Table 11). The results showed that the N-acetyl-x peptide fraction contained a number of different amino acids, but only aspartic and glutamic acid were present in molar amounts as large as acetate (Table 11). Because it seemed possible that the N-acetyl-x peptide fraction could be
Table 11. Amino acid composition of an N-acetyl-x fraction isolated from a proteolytic enzyme digest of bovine cardiac α-actinin by elution from Dowex-50X8

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Sample 1 (Moles of amino acids per mole of acetate in the sample)</th>
<th>Sample 2 (Moles of amino acids per mole of acetate in the sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>1.04</td>
<td>0.95</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.34</td>
<td>0.15</td>
</tr>
<tr>
<td>Serine</td>
<td>0.34</td>
<td>0.16</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3.18</td>
<td>1.71</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.24</td>
<td>0.16</td>
</tr>
<tr>
<td>Methionine</td>
<td>--</td>
<td>0.64</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.15</td>
<td>0.18</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.18</td>
<td>0.31</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>--</td>
<td>0.05</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>--</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*a Acetate analysis was done by the method of Kuo and Younathan (1973).*
contaminated with small amounts of large peptides that might not penetrate the cross-linked polystyrene resin completely, and that therefore might not have been bound to column, the N-acetyl-x peptide fraction eluted from the first Dowex-50X8 column was applied to a second Dowex-50X2 column (having less cross-linking), and the void volume eluting from this second Dowex column was collected. The peptide in the void volume from the second column was subjected to acetate analysis and to analysis for complete amino acid composition after hydrolysis in 5.7 N HCl at 110°C for 24 hrs (Table 12). This peptide contained only aspartic and glutamic acid in a molar ratio of 1:2.2:3.8 acetate:aspartic acid: glutamic acid. Consequently bovine cardiac α-actinin ostensibly has the N-terminal amino acid composition of CH₃-C-[asp]₂-(glu)₄.

D. Effect of CAF-Treatment on the C- and N-Terminal Amino Acids of α-Actinin and Actin

CAF is a Ca²⁺-activated factor that has been isolated from skeletal muscle cells. The purified factor has been shown to be a protease that removes Z-disks from intact skeletal myofibrils without causing other ultrastructurally detectable deletions (Busch et al., 1972). Treatment of purified myofibrillar proteins has shown that purified CAF degrades tropomyosin, troponin-T, troponin-I and C-protein,
Table 12. Amino acid analysis of N-acetyl-x fraction of sample 2 isolated from a proteolytic enzyme digest of bovine cardiac α-actinin by elution from Dowex-50X8 and subsequent elution from Dowex-50X2

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Moles of amino acid/mole of acetate in the samplea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>2.23</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3.84</td>
</tr>
</tbody>
</table>

aAcetate analysis was done by the method of Kuo and Younathan (1973).

but, as measured by SDS-polyacrylamide gel electrophoresis, has no effect on the subunit molecular weights of myosin, actin, α-actinin or troponin-C (Dayton et al., 1974). Because α-actinin is located in the Z-disk of skeletal muscle myofibrils and is presumably bound to actin there it is surprising that CAP removes Z-disks and α-actinin from myofibrils (Dayton et al., 1974) without hydrolyzing either actin or α-actinin or both. SDS-Polyacrylamide gel electrophoresis is an extremely sensitive assay for proteolysis if the proteolysis breaks the peptide chain into large pieces. It would, however, be very difficult to detect the proteolytic removal of 5 to 15 amino acids from either the N- or C-terminal end of the peptide chain by using SDS-polyacrylamide gel electrophoresis because such
removal would cause very little change in the subunit mass of the peptide. Also, the 5 to 15 amino acids removed would migrate so fast in SDS-polyacrylamide gel electrophoresis that they too would probably not be detected. Removal of 5 to 15 amino acids from either the N- or C-terminal end of the peptide chains of actin or a-actinin would, of course, alter the N- or C-terminal amino acids of the peptide chains in these proteins. Therefore, N- and C-terminal amino acid analysis was done on control and CAF-treated porcine skeletal actin and a-actinin samples to determine whether CAF was removing a small peptide from the N- or C-terminal end of the peptide chains in these proteins. Removal of such a small peptide might account for the ability of CAF to remove Z-disks and a-actinin from intact myofibrils.

Because SDS-polyacrylamide gel electrophoretic analysis shows that the peptide chains of a-actinin and actin remain intact or almost intact after CAF-treatment, N- and C-terminal analysis was done only on the large, seemingly intact, peptide chains remaining after CAF treatment. This was done because it would be easier to detect a new N- or C-terminal amino acid on these peptide chains than to detect a very small oligopeptide released from these chains by CAF. CPA and CPB digestion releases leucine and arginine from CAF-treated porcine skeletal a-actinin in the same amounts and at the
same rates as it releases these two amino acids from a control sample of porcine skeletal α-actinin (Figure 33). Moreover, phenylalanine was the only amino acid released from either a CAF-treated or a control sample of porcine skeletal actin by digestion with CPA and CPB (Figure 34). The amino acid sequence near the C-terminus of α-actinin peptide chains is not known. It is very unlikely, however, that the arg-leu sequence would appear both at the C-terminus of these peptide chains and again within 5 to 15 amino acids from the C-terminus and that CAF would cleave the peptide chain to expose a new arg-leu C-terminus. Consequently, the results shown in Figure 33 suggest very strongly that CAF does not affect the C-terminus of α-actinin peptide chains. The amino acid sequence of actin is known, and 23 amino acids would have to be removed from the C-terminus of actin before exposing another C-terminal phenylalanine. Removal of such a large piece would be detected by SDS-polyacrylamide gel electrophoresis. Hence, the results in Figure 34 demonstrate that CAF does not affect the C-terminus of actin.

N-Terminal analysis of control and CAF-treated porcine skeletal α-actinin and actin samples done by using the dansyl chloride procedure described earlier showed that the N-terminal amino acids of both α-actinin and actin were still unavailable for reaction with dansyl chloride after CAF-
Figure 33. Release of amino acids from control and CAF-treated porcine skeletal muscle α-actinin by carboxypeptidases A and B. Conditions of CAF-treatment: 5.0 mg porcine skeletal α-actinin/ml; 0.025 mg purified CAF/ml, 100 mM KCl, 5 mM CaCl₂, 10 mM MCE, 100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 hr at 25°C. CAF proteolysis was stopped by addition of 10 mM EDTA. CAF-treated α-actinin was passed through 2.5 x 85 cm Sephadex G-200 column that had been equilibrated with 10 mM Tris-HCl, pH 7.5, and the α-actinin peak was used for carboxypeptidase treatment. Final concentrations for carboxypeptidases assay: 0.1 μ moles of carboxymethylated control and CAF-treated α-actinin was incubated with 11.6 units of carboxypeptidase A and 7.02 units of carboxypeptidase B at 37°C for the times indicated in 0.05 M SDS, 0.2 M N-ethylmorpholine acetate, pH 8.5. The reaction was stopped as described in the figure legend for Figure 26. The molecular weight of both control and CAF-treated porcine skeletal muscle α-actinin was taken as 200,000 daltons.
2.0-

CONTROL α-ACTININ

ARGININE

LEUCINE

TIME (hr)

MOLES AMINO ACID RELEASED/MOLE α-ACTININ

CAF-TREATED α-ACTININ

LEUCINE

ARGININE

TIME (hr)
Figure 34. Release of amino acids from control and CAF-treated porcine skeletal muscle actin by carboxypeptidases A and B. Conditions of CAF-treatment: 4.0 mg procine actin/ml, 0.2 mg purified CAF/ml, 100 mM KCl, 5 mM CaCl₂, 10 mM MCE, 100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 hr at 25°C. CAF proteolysis was stopped by addition of 10 mM EDTA. CAF-treated actin was sedimented by centrifugation, and the actin pellet was dissolved in 1 mM KHCO₃ and used for carboxypeptidase treatment. Final concentrations for carboxypeptidases assay: 0.1 μ moles of carboxymethylated control and CAF-treated actin was incubated for the times indicated with 11.6 units of carboxypeptidase A and 7.02 units of carboxypeptidase B at 37°C in 0.05 M SDS, 0.2 M N-ethylmorpholine acetate, pH 8.5. The reaction was stopped as described in the legend for Figure 25. The molecular weight of both control and CAF-treated porcine skeletal muscle actin was taken as 42,000 daltons.
treatment (Figures 35, 36). Because the N-terminal amino acid of actin is known to be acetylated and because the results described in the preceding section indicated that the N-terminal amino acids of α-actinin are also acetylated, the data shown in Figures 35 and 36 indicate that CAF does not remove amino acids from the N-terminal region of either actin or α-actinin. To test this conclusion, control and CAF-treated samples of actin and α-actinin were subjected to quantitative acetate analysis. CAF-treatment has no effect on the acetate content of either actin or α-actinin (Table 13). Because the acetate of actin is located exclusively on the N-terminal α-amino group of actin, these results demonstrate that CAF does not remove any amino acids from the N-terminal region of actin. Furthermore, because it is highly probable that the acetate in α-actinin is located exclusively on the N-terminal amino acids of α-actinin, it also seems highly likely that CAF removes no amino acids from the N-terminal regions of α-actinin. Consequently, the mechanism whereby CAF removes Z-disks and α-actinin from intact myofibrils remains very unclear. It seems likely, however, that use of CAF as a probe of the molecular nature of the interactions between actin and α-actinin in the Z-disk will produce significant new information on these interaction and on the molecular architecture of the Z-disk.
Figure 35. Thin layer chromatography on polyamide coated sheets of standard DNS-amino acids and amino acids from porcine skeletal $\alpha$-actinin and actin that had been treated with dansyl chloride before acid hydrolysis. Chromatography was done first in a horizontal direction in a water-90% formic acid (100:1.5, V/V) solvent. The plate was then turned and chromatography was done in the vertical direction in a benzene-acetic acid (9:1, V/V) solvent. Circles indicate the spots seen when the dried plate was observed under an ultraviolet light. X indicates where the original sample was applied.
Standard Dansylated Amino Acids

Dansylated Amino Acids of α-Actinin

Dansylated Amino Acids of Actin
Figure 36. Thin layer chromatography on polyamide coated sheets of amino acids from porcine skeletal α-actinin and actin that were treated with dansyl chloride after CAF-treatment and were then hydrolyzed in 5.7 N HCl. Chromatography was done first in a horizontal direction in a H₂O-90% formic acid (100:1.5, V/V) solvent. The plate was then turned and chromatography was done in the vertical direction in a benzene-acetic acid (9:1, V/V) solvent. Circles indicate the spots seen when the dried plate was observed under an ultraviolet light. X indicates where the original sample was applied. CAF-treatment of both α-actinin and actin was done as described in the legends to Figures 33 and 34, respectively.
Dansylated amino acids of CAF-treated α-actinin

Dansylated amino acids of CAF-treated actin
Table 13. Acetate analysis of porcine skeletal α-actinin and porcine skeletal actin before and after CAF-treatment

<table>
<thead>
<tr>
<th>Protein sample</th>
<th>Moles of acetate/mole of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control actin</td>
<td>0.92</td>
</tr>
<tr>
<td>CAF-treated actin</td>
<td>0.89</td>
</tr>
<tr>
<td>Control α-actinin</td>
<td>1.81</td>
</tr>
<tr>
<td>CAF-treated α-actinin</td>
<td>1.77</td>
</tr>
</tbody>
</table>

Acetate analysis was done by the method of Kuo and Younathan (1973).

The molecular weight of control and CAF-treated actin was taken as 42,000 daltons, and the molecular weight of control and CAF-treated α-actinin was taken as 200,000 daltons.

Conditions of CAF-treatment: 4.0 mg porcine skeletal α-actinin or actin/ml, 0.02 mg purified CAF/ml, 100 mM KCl, 5 mM CaCl₂, 10 mM MCE, 100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 hr at 25°C. CAF-proteolysis was stopped by addition of 10 mM EDTA. CAF-treated α-actinin was passed through a Sephadex G-200 column that had been equilibrated with 10 mM Tris-HCl, to remove any small peptides released by CAF-treatments. The α-actinin peak was used for acetate analysis. CAF-treated actin was sedimented by centrifugation, and the actin pellet was dissolved in 1 mM KHCO₃ and dialyzed against 1 mM KHCO₃. The dialyzed protein was used for acetate analysis.
V. DISCUSSION

The goal of this investigation was to determine the physiological role of \( \alpha \)-actinin in biological movement. This objective was approached by studying two general properties of \( \alpha \)-actinin in mammalian skeletal muscle, which is a highly specialized movement system. The existing information indicates that the physiological role of \( \alpha \)-actinin is either to cross-link F-actin filaments structurally or to modify the actin-myosin interaction in a way that strengthens or promotes this interaction at the relatively high ionic strengths that exist in mammalian skeletal muscle in vivo. The two general properties of \( \alpha \)-actinin investigated in this study were selected to provide information on which of these two possible physiological roles of \( \alpha \)-actinin is most consistent with the properties of \( \alpha \)-actinin in vitro. First, the ability of \( \alpha \)-actinin to alter actin structure was investigated by determining whether \( \alpha \)-actinin can alter the ATPase activity of F-actin filaments. If \( \alpha \)-actinin can modify the conformation of actin monomers in the F-actin filament, then \( \alpha \)-actinin may act physiologically to modify the actin-myosin interaction. On the other hand, if \( \alpha \)-actinin cannot modify actin structure, then it seems very unlikely that \( \alpha \)-actinin could function physiologically to alter the actin-myosin interaction. Second, if \( \alpha \)-actinin constitutes the Z-filaments linking F-actin filaments across the Z-disk
in mammalian skeletal muscle, then removal of the Z-disk by a recently discovered Ca\(^{2+}\)-activated protease must be accompanied by proteolytic cleavage of the peptide chains of either α-actinin or actin or both. Because SDS-polyacrylamide gel electrophoresis studies (Dayton et al., 1974) have shown that the Ca\(^{2+}\)-activated protease has very little, if any, effect on the peptide chains of either α-actinin or actin, a careful N- and C-terminal amino acid analysis was done on purified α-actinin to determine whether the Ca\(^{2+}\)-activated protease was removing a very small peptide (10 to 15 amino acids or less) from the N- or C-terminus of the α-actinin molecule. Removal of such a small peptide could break the α-actinin-F-actin complex but, because it would not alter the molecular weight of the peptide chains of α-actinin or actin by a large amount, such removal might not be detected by SDS-polyacrylamide gel electrophoresis.

It is necessary in detailed studies of the biological function of a protein to insure that the protein being studied is as homogeneous as possible so that the biological function ascribed to the protein does not actually originate from a contaminant or the interaction of a contaminant with the major protein. Robson et al. (1970) have shown that DEAE-cellulose chromatography greatly purified the grossly impure preparations of α-actinin that were used in the first studies done with α-actinin (Briskey et al., 1967b;
Even α-actinin preparations purified by DEAE-cellulose chromatography, however, contain up to 10-15% of their protein as a species that sediments with a sedimentation coefficient of 9.1 S in the analytical ultracentrifuge (Robson et al., 1970). A number of different kinds of evidence have suggested that this 9.1 S species is a salt-induced aggregate of the 6.1 S α-actinin species (Goll et al., 1972; Robson et al., 1970), but because of the importance of homogeneity to careful studies of α-actinin function, a preliminary study was done to determine whether two successive column chromatographic purifications using two different kinds of chromatographic material would produce a more homogeneous α-actinin than that produced by the two successive DEAE-cellulose chromatographic purifications as described by Robson et al. (1970).

Use of hydroxyapatite column chromatography after an initial purification by DEAE-cellulose chromatography produced an α-actinin that contained no 9.1 S species in the analytical ultracentrifuge. SDS-Polyacrylamide gel electrophoresis showed that hydroxyapatite chromatography removed a component or components having a subunit molecular weight of 43,000 daltons. This component elutes very close to the α-actinin peak from DEAE-cellulose columns and is frequently included in the α-actinin peak unless only the central part of this peak is collected. Because hydroxyapatite
chromatography removes both the 9.1 S peak observed in sedimentation patterns and the 43,000 dalton material observed in SDS-polyacrylamide gels, it seems likely that the 9.1 S peak is composed, at least in part, of the 43,000 dalton material.

The nature of the 43,000 dalton material is unknown. It may be denatured actin that has been suggested as a major contaminant of crude α-actinin extracts (Goll et al., 1969; Robson et al., 1970). Moreover, the presence of 85% denatured actin has been shown (Robson et al., 1970) to account for the amino acid composition of the first crude α-actinin preparations studied, and it seems likely that some of this contamination could be carried over after a single DEAE-cellulose chromatographic purification. Alternatively, the 43,000 dalton material may contain creatine kinase, which Morimoto and Harrington (1972) and Turner et al. (1973) have shown adheres to myofibrils and is extracted by a high pH, low ionic strength extraction. The possibility that the 43,000 dalton material contains creatine kinase is supported by the findings that antibodies to creatine kinase bind to the M-line region of skeletal muscle myofibrils (Turner et al., 1973) and that antibodies made against DEAE-cellulose purified α-actinin also bind to the M-line as well as to the Z-disk (J. E. Schollmeyer, unpublished paper, 1974).
Because the 43,000 dalton material seems to be the major contaminant in DEAE-cellulose purified \( \alpha \)-actinin, it is reasonable to suggest that the 43,000 dalton component is responsible for eliciting antibodies that bind to the M-line.

Despite the fact that hydroxyapatite chromatography removes the 43,000 dalton material and produces \( \alpha \)-actinin preparations that contain over 95% of their protein as the 100,000 dalton \( \alpha \)-actinin species, hydroxyapatite-purified \( \alpha \)-actinin was not more active in the ATPase and turbidity assays of \( \alpha \)-actinin activity than DEAE-cellulose-purified \( \alpha \)-actinin. This similarity in activity of hydroxyapatite and DEAE-cellulose-purified \( \alpha \)-actinin is probably due to the difficulty in using the ATPase and turbidity assays to precisely quantitate \( \alpha \)-actinin activity (Arakawa et al., 1970b; Goll et al., 1969), particularly when at most only a 10-15% difference in activity would be expected between hydroxyapatite and DEAE-cellulose purified \( \alpha \)-actinin, even if all the 9.1 S species in DEAE-cellulose-purified \( \alpha \)-actinin is inactive in the ATPase and turbidity assays. Consequently DEAE-cellulose chromatography followed by hydroxyapatite chromatography is a very convenient and effective way to produce highly purified \( \alpha \)-actinin from \( P_{0-30} \) crude \( \alpha \)-actinin extracts, and this combination of chromatography is recommended for purification of \( \alpha \)-actinin.
Results of the viscometric studies of α-actinin-F-actin mixtures confirm earlier viscometric results showing that α-actinin greatly increases viscosity of F-actin solutions, regardless of whether the F-actin is polymerized in 1 mM MgCl₂ alone, in 100 mM KCl, or in 100 mM KCl plus 1 mM MgCl₂ (Goll et al., 1969; Holmes et al., 1971). Electron microscope observations of α-actinin-F-actin mixtures (Kawamura et al., 1970) have shown that α-actinin cross-links F-actin filaments laterally to form gel-like networks, and presumably, this cross-linking causes the α-actinin-induced increase in F-actin viscosity. Binding of α-actinin to actin without causing cross-linking, would not produce an increase in viscosity and therefore would not be detected by viscometric measurements. No viscometric studies had been done previously to determine whether α-actinin can cross-link F-actin filaments in the presence of ATP. Because ATP is present in living muscle cells where α-actinin must bind to actin, and because assays of the possible effects of α-actinin on the contractile actin-myosin interaction require the presence of ATP, the effects of ATP on the α-actinin-induced increase in F-actin viscosity were studied. Somewhat surprisingly, ATP decreased the ability of α-actinin to increase F-actin viscosity by 21% when 100 mM KCl or 100 mM KCl plus 1 mM MgCl₂ was used to polymerize the actin, and by 53% when 1 mM MgCl₂ alone was used to
polymerize the actin. It is very unlikely that the added ATP is shortening or depolymerizing F-actin filaments because: 1) increasing the concentration of added ATP by 6-fold caused no additional decrease in viscosity of α-actinin-F-actin mixtures; and 2) ATP at any concentration had no effect on viscosity of F-actin solutions in the absence of α-actinin. Consequently, ATP affects the α-actinin-F-actin interaction. Results of the studies on how Mg$^{2+}$:ATP ratios affect the ability of added ATP to decrease viscosity of α-actinin-F-actin mixtures suggest that ATP prevents the α-actinin-induced cross-linking of F-actin filaments. Because both α-actinin and actin are negatively charged molecules at pH 7.0 (α-actinin contains a net of 159 negative charges per 100,000 daltons assuming no glutamine and arginine and actin contains a net of 31 negative charges per 100,000 daltons at pH 7.0, including the known glutamine and asparagine residues), it is not surprising that the negatively charged ATP anion affects binding of α-actinin to actin if no positively charged cations that are bound by actin or α-actinin are present. The ability of ATP to decrease viscosity of α-actinin-F-actin mixtures is most noticeable when only 1 mM Mg$^{2+}$ is used to polymerize actin and no KCl is present. Under these conditions, addition of ATP to a final concentration of 1 mM or more probably results in chelation of most of the Mg$^{2+}$ to form a Mg$^{2+}$-ATP
complex; hence, most of the positively charged Mg\(^{2+}\) cation is removed. The K\(^+\) cation binds to myosin and probably to the other myofibrillar proteins. Hence, presence of 100 mM KCl results in an excess of positively charged cations capable of binding to actin or \(\alpha\)-actinin in the \(\alpha\)-actinin-F-actin mixtures, and the results obtained in this study show that ATP has much less effect on viscosity of \(\alpha\)-actinin-F-actin mixtures in the presence of 100 mM KCl. Most of the effect of ATP on the \(\alpha\)-actinin-F-actin interaction must be to prevent cross-linking of F-actin filaments. That \(\alpha\)-actinin binds to F-actin in the presence of ATP, even in those instances where \(\alpha\)-actinin causes no increase in F-actin viscosity, is supported by two observations: 1) \(\alpha\)-actinin activates F-actin ATPase in the presence of 1 mM ATP, 1 mM Mg\(^{2+}\) and no KCl even though \(\alpha\)-actinin causes no increase in F-actin viscosity under these conditions; it is very unlikely that \(\alpha\)-actinin alters F-actin ATPase activity without binding to F-actin in some way; and 2) preliminary direct measurements of binding of \(\alpha\)-actinin to F-actin in the presence of 1 mM ATP, 1 mM Mg\(^{2+}\), and no KCl indicate that \(\alpha\)-actinin sediments with F-actin under these conditions (results of these preliminary studies are not shown in this thesis). Although these results suggest that simple binding of \(\alpha\)-actinin to F-actin filaments must differ in some way from interaction of the bound \(\alpha\)-actinin molecule to another
F-actin filament to cause cross-linking, the nature of this difference is not known. Moreover, the viscosity measurements provide no clues about the possible nature of this difference between monovalent and divalent binding of α-actinin to F-actin strands, although these measurements suggest that positively charged cations are essential for divalent binding of α-actinin to actin.

The presence of an ATPase activity in F-actin solutions has made it possible to study the effect of α-actinin on actin structure by using ATPase activity as a measure of actin conformation. When adopting this approach, it is critical to establish that the ATPase activity in F-actin solutions is not an actomyosin ATPase activity. If a small amount of myosin contaminates F-actin solutions and α-actinin modified the actomyosin ATPase activity resulting from the presence of this myosin, then any effect of α-actinin on this ATPase activity could be ascribed to cross-linking or repositioning of F-actin filaments so as to enhance the opportunity for myosin to interact with these filaments rather than to a direct effect of α-actinin on actin structure. SDS-Polyacrylamide gel electrophoresis of purified actin at heavy loads showed no myosin contamination in the actin preparations used in this study. The specific activity of Mg²⁺-modified F-actin ATPase, however, is only 0.2% to 0.8% of the specific activity of Mg²⁺-modified
actomyosin ATPase, and it might be very difficult to detect 0.2% myosin contamination, even by using heavy loads on SDS-polyacrylamide gels. Comparison of the properties of F-actin ATPase activity with actomyosin ATPase activity, however, shows that ATPase activity in the F-actin preparations used in this study clearly differs from actomyosin ATPase activity in at least three ways: 1) different divalent cations have widely different effects on the specific activities of F-actin and actomyosin ATPase; for example, if specific activity in the presence of 1 mM Mg$^{2+}$ plus 0.05 mM Ca$^{2+}$ is considered 100%, Ba$^{2+}$ activates F-actin ATPase by 203% but inhibits actomyosin ATPase, Ca$^{2+}$ activates F-actin ATPase by 111% but activates actomyosin by 244%, and Mn$^{2+}$ activates F-actin ATPase by 94% but activates actomyosin ATPase by 147%; 2) the specific activity of the Mg$^{2+}$-modified F-actin ATPase is highest at pH 5.0, decreases rapidly between pH 5.0 and 7.0, and decreases slowly between pH 7.0 and 8.5; the specific activity of Mg$^{2+}$-modified actomyosin ATPase, however, is low at pH 5.0, increases between pH 5.0 and 6.5, remains relatively constant between pH 6.5 and 8.0 and then increases between pH 8.0 and 8.5 (Seraydarian et al., 1967); and 3) α-actinin has no effect on the specific activity of F-actin ATPase between pH 5.0 and 6.0, but increases F-actin ATPase activity between pH 7.5 and 8.5 with the greatest increase occurring at pH 8.5; when tested with actomyosin,
however, α-actinin increases the specific activity of actomyosin ATPase between pH 6.0 and 7.5, with the greatest increase occurring at pH 6.5, but α-actinin has no effect on actomyosin ATPase activity above pH 8.0 (Seraydarian et al., 1967). These three lines of evidence demonstrate that ATPase activity in F-actin preparations used in this study originates from F-actin itself and not from a minute amount of actomyosin contamination.

The specific activity of F-actin preparations used in this investigation ranges from 0.020 to 0.030 μ moles of Pi released per mg of protein per hr at pH 7.5 and 37°C with 1 mM Mg²⁺ as an activator. This specific activity increased to 0.030 to 0.040 μ moles of Pi released per mg of protein per hr in the presence of α-actinin in 1 mM Mg²⁺ at pH 7.5 and 37°C. These activities may be compared with a specific activity of 16.8 μ moles of Pi released per mg of protein per hr for reconstituted actomyosin at pH 7.5 and 37°C with 1 mM Mg²⁺ as an activator, a specific activity of 0.020 μ moles of Pi released per mg of protein per hr for F-actin at pH 5.0 and 25°C, a specific activity of 0.090 to 0.100 μ moles of Pi released per mg of protein per hr for F-actin at pH 5.0 and 37°C, a specific activity of 0.27 μ moles of Pi per mg of protein per hr for F-actin at pH 5.0 and 45°C (Kuroda and Maruyama, 1972), a specific activity of 0.20 μ moles of Pi released per mg of protein per hr for F-actin at
pH 8.1 and 53°C (Asai and Tawada, 1966), and a specific activity of 0.015 to 0.017 μ moles of Pi released per mg of protein per hr for F-actin in the presence of tropomyosin at pH 7.5 and 37°C. Hence, it is clear that even under conditions that activate F-actin ATPase, the specific activity of F-actin ATPase is much lower than the specific activity of actomyosin ATPase.

The extensive studies described in this thesis clearly show that α-actinin alters the conformation of actin monomers while they are incorporated into a F-actin filament. The activation of F-actin ATPase by α-actinin is not a non-specific effect caused simply by binding of a protein to F-actin filaments because: 1) the ability of α-actinin to accelerate F-actin ATPase activity requires Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$; α-actinin does not activate F-actin ATPase activity in the absence of added divalent cation (i.e., in 100 mM KCl alone) or if the added divalent cation is Ba$^{2+}$, Fe$^{2+}$, Cu$^{2+}$, or Ca$^{2+}$; 2) not all proteins that bind to F-actin filaments increase F-actin ATPase activity; indeed, as shown in this study, addition of tropomyosin decreases F-actin ATPase activity. α-Actinin increases F-actin ATPase activity by 80 to 90% when the α-actinin:F-actin ratio is 1:10 by weight. The best estimates currently available indicate that the α-actinin content of skeletal muscle myofibrils is about 10% of the actin content of these myofibrils by weight.
Consequently, α-actinin will increase F-actin ATPase activity when α-actinin is added at approximately the same levels that exist *in vivo* in skeletal muscle myofibrils.

By using a molecular weight of 200,000 daltons for the α-actinin molecule and 42,000 daltons for actin monomers, it can be calculated that a weight ratio of 1:10 α-actinin to actin corresponds to 1 α-actinin molecule added for every 48 G-actin monomers present. α-Actinin is located only in the Z-disk in skeletal muscle myofibrils *in vivo*, and if α-actinin has a physiological role in modifying the actin-myosin interaction, α-actinin located at only one end of the actin filament must be able to modify actin monomers all along the actin filament. Therefore, it is important to determine whether α-actinin bound to only one out of 48 actin monomers changes conformation of all actin monomers in the filament or changes conformation only of those monomers to which it is bound. The results of the present study show that increasing the amount of α-actinin added from 10% of the actin present by weight to 20% of the actin present increases the percent α-actinin activation of F-actin ATPase activity from 65% to 94%. If α-actinin activated the ATPase activity of only those actin monomers to which it is bound, doubling the amount of α-actinin added should double the α-actinin-induced increase in F-actin ATPase activity rather than increasing this activity only 1.45 times.
Furthermore, addition of 10% as much α-actinin as actin increases the rate of release of nucleotide from 80% of actin monomers in F-actin filaments (Schmidt, unpublished results). These two findings demonstrate that α-actinin bound to only 1 out of 48 actin monomer changes the conformation of most of the actin monomers in the F-actin filament and that α-actinin can, therefore, exert the long range effects on actin structure that are necessary if α-actinin is to modify the actin-myosin interaction in vivo. Unfortunately, it was not possible in this study to show that α-actinin could also exert a long range effect on actin structure in the presence of tropomyosin, which is also a component of the thin filament in vivo. The viscosity studies showed that α-actinin does not cross-link F-actin filaments in the presence of tropomyosin but it was still possible that α-actinin was binding to F-actin filaments without cross-linking them when tropomyosin was present. α-Actinin, however, also had no effect on F-actin ATPase activity in the presence of tropomyosin. Moreover, direct binding studies (Goll et al., 1972) have shown that no detectable α-actinin binds to tropomyosin-F-actin complexes at 37°C, although the precision of the direct binding experiments do not eliminate the possibility that 0.03 parts of α-actinin to 1.0 part of F-actin bind to tropomyosin-F-actin complexes at 37°C. Consequently, it seems that tropomyosin added in vitro does not
bind to F-actin filaments in the same way that tropomyosin is thought to bind to F-actin filaments in vivo. Rather, tropomyosin added in vitro seems to block the ends of F-actin filaments and prevents α-actinin from binding to these ends. Unfortunately, the available evidence does not conclusively eliminate the possibility that a small amount of α-actinin (0.03 parts or less to 1 part of F-actin) is binding to tropomyosin-F-actin complexes at 37°C but that this α-actinin is unable to modify F-actin structure in the presence of tropomyosin. Additional studies, probably using different methods to add tropomyosin to F-actin filaments in vitro, are necessary to answer the important question of whether α-actinin can alter actin structure in the presence of tropomyosin.

The F-actin ATPase results, together with the studies on viscosity of α-actinin-F-actin mixtures, also provide some useful information on the mechanism of F-actin ATPase activity. Asakura et al. (1963b) have suggested that F-actin ATPase activity originates from transient perturbations during which two of the four noncovalent bonds that a given actin monomer forms with its neighbors in the F-actin filament are broken. Asakura et al. (1963b) suggest that an actin monomer having two of its four bonds to neighboring actin monomers broken undergoes a conformational change to a "f-actin" state in which the nucleotide in this monomer
momentarily becomes available for exchange with medium nucleotide. Incorporation of a molecule of medium ATP is then followed quickly by dephosphorylation of the ATP to ADP and healing of the two broken bonds to reform the F-actin filament. In this scheme, addition of \( \alpha \)-actinin would increase the incidence (or equivalently, accelerate the rate) of "f-actin" production and thereby increase F-actin ATPase activity. On the other hand, because ultrasonic treatment ruptures long F-actin filaments to short filaments that coexist with actin monomers (Nakaoka and Kasai, 1969), and also increases F-actin ATPase activity, it has been suggested that F-actin ATPase activity originates from rapid cycles of depolymerization-repolymerization occurring exclusively at the ends of the short polymers, rather than by formation of a "f-actin" state. Addition of \( \alpha \)-actinin causes an increase in F-actin viscosity rather than the decrease in viscosity that would be expected if \( \alpha \)-actinin ruptured long F-actin filaments into short pieces; hence, \( \alpha \)-actinin does not increase F-actin ATPase activity by breaking F-actin filaments into short pieces to increase the number of ends available for depolymerization-repolymerization cycles. Moreover, binding of \( \alpha \)-actinin to F-actin causes cross-linking of F-actin filaments rather than promoting depolymerization of actin monomers on the ends of F-actin filaments. Hence, \( \alpha \)-actinin does not accelerate F-actin ATPase activity by
binding to the ends of existing F-actin filaments and greatly increasing the rate at which actin monomers at the ends of these filaments depolymerize. Finally, α-actinin does not bind to G-actin monomers (Holmes et al., 1971) so α-actinin does not increase F-actin ATPase by accelerating the rate at which G-actin monomers repolymerize onto the ends of existing F-actin filaments. In summary, the effect of α-actinin on F-actin ATPase activity confirms earlier data indicating that F-actin ATPase activity does not originate from cycles of depolymerization-repolymerization at the ends of the filament, but rather, that F-actin ATPase activity must originate from formation of a "f-actin" or some similar species.

Although the effects of α-actinin on F-actin viscosity and F-actin ATPase activity indicate that splitting of ATP by F-actin must involve formation of a "f-actin" species rather than originating from depolymerization-repolymerization cycle at ends of F-actin filaments, the F-actin ATPase data obtained in this study provide little evidence concerning the mechanism of ATP hydrolysis by actin monomers. For example, α-actinin activates F-actin ATPase activity only when the Mg$^{2+}$ concentration is as large or larger than the ATP concentration. α-Actinin inhibits F-actin ATPase activity at Mg$^{2+}$ concentrations lower than the ATP concentration. This behavior may indicate that Mg·ATP is required as a substrate if α-actinin
is to accelerate F-actin ATPase activity, and that if ATP is the substrate, α-actinin inhibits F-actin ATPase activity. On the other hand, free Mg$^{2+}$ or Mg·ATP may be necessary for stabilization of actin monomers at the ends of F-actin filaments, and F-actin ATPase activity in the absence of free Mg$^{2+}$ or Mg·ATP may originate from depolymerization-repolymerization cycles rather than from formation of a "f-actin" species. As indicated in the preceding paragraph, α-actinin would probably show the rate of depolymerization-repolymerization cycles occurring at the ends of F-actin filaments, and would therefore inhibit any ATPase activity originating from such cycles. In the absence of additional information, it is difficult to be more exact concerning the effect of Mg$^{2+}$/ATP ratios on the ability of α-actinin to increase F-actin ATPase activity.

Careful studies on the sulfhydryl content of purified α-actinin by using DTNB in denaturing solvents show that α-actinin contains 18 moles of sulfhydryl groups per mole of α-actinin. Assay of the number of sulfhydryl groups available to DTNB in nondenaturing solvents indicates that 6 of these 18 sulfhydryl groups are located on the surface of the α-actinin molecule, or at least in areas of the tertiary structure of α-actinin that are exposed to the solvent. The DTNB assay of sulfhydryl groups does not measure those groups involved in disulfide linkages unless pH of the assay is
raised to 10.5 and then lowered to 8.1, or unless the disulfide bonds are reduced before addition of DTNB. Performic acid oxidized disulfide bonds as well as sulfhydryl groups, however, and the number of sulfhydryl groups as determined by the number of cysteic acid residues obtained from amino acid analysis of performic acid oxidized α-actinin (Robson and Zeece, 1973) is the same as the number of sulfhydryl groups as determined by DTNB analysis. This result indicates that the α-actinin molecule has 18 sulfhydryl groups and no disulfide bonds.

N- and C-terminal amino acid analysis shows that α-actinin contains C-terminal leucine and probably has arginine as the amino acid penultimate to the C-terminus. The N-terminal amino group of α-actinin is acetylated, and the results described in this study suggest that the amino acid composition of N-terminal peptides of bovine cardiac α-actinin is $\text{CH}_3\text{C}^\text{O}[\text{asp}_2\text{glu}_4]$. The N- and C-terminal amino acid analysis of α-actinin provides additional evidence that the two peptide chains that constitute the α-actinin molecule are identical because one mole of α-actinin contains two moles of leucine and two moles of acetate. Previous electrophoretic studies have shown that the two subunit peptide chains of α-actinin cannot be separated by SDS-polyacrylamide gel electrophoresis; hence, these two chains have very similar molecular mass. Moreover, the two
peptide chains of α-actinin from bovine cardiac muscle cannot be separated by polyacrylamide gel electrophoresis in urea so these two chains also have very similar net charge at alkaline pH. Consequently, the two subunit peptide chains in α-actinin molecules must be very similar, if not identical.

The N- and C-terminal amino acid analyses also contribute to the concept that α-actinin is a highly conserved protein whose molecular properties remain nearly constant regardless of the type of muscle from which it is isolated. α-Actinin isolated from bovine cardiac muscle, porcine skeletal muscle, chicken breast muscle, and chicken gizzard muscle (a vertebrate smooth muscle) all contain two moles of C-terminal leucine per mole of α-actinin, all have N-terminal amino groups completely blocked from reaction with dansyl chloride, all contain two moles of acetate per mole of α-actinin, all contain two subunits of approximately 100,000 daltons each per intact α-actinin molecule, and all are extracted and purified by using the same general procedures. Moreover, antibodies made against α-actinin from one species or muscle source cross-reacts with antibodies made against α-actinin from other species or muscles. Indeed, Schollmeyer has recently shown that antibodies against

\[\text{Schollmeyer, J. E., Ames, Iowa. Localization of } \alpha-\text{actinin in myofibrils. Private communication. 1974.}\]
chicken gizzard $\alpha$-actinin cross-react with $\alpha$-actinin in the acrosomal process of Limulus sperm. Taken together, these results strengthen the hypothesis that $\alpha$-actinin is a widely distributed and highly conserved myofibrillar protein.

Although approximately four moles of glutamic acid are found per mole of acetate in a peptide fraction that presumably originates from the N-terminal region of the peptide chains in bovine cardiac $\alpha$-actinin, several studies (Gordon and Noltmann, 1973; Narita and Ishii, 1962; Winstead and Wold, 1964) have shown that the presence of glutamic acid in an acetylated N-terminal peptide fraction that has been isolated on the basis that such a fraction would have no positive charges may be an artifact. Any free glutamic acid released during the extensive proteolytic treatment required to break peptide chains into very small peptides may cyclize during the proteolytic treatment to produce pyrrolidine carboxylic acid. Because it has no positive charge, pyrrolidine carboxylic acid will pass through a Dowex cation exchange column unretarded along with the acetylated N-terminal peptide. Although pyrrolidine carboxylic acid has no free amino group and is therefore ninhydrin negative, pyrrolidine carboxylic acid is converted back to glutamic acid during acid hydrolysis of the peptide fraction. Hence, glutamic acid can be erroneously found in a fraction thought to contain only an acetylated N-terminal peptide. If the
glutamic acid found associated with the acetylated N-terminal peptide from bovine cardiac α-actinin originates in this way, then the N-terminal sequence of bovine cardiac α-actinin is $\text{CH}_3\text{C}-\text{asp-asp}$.

Additional evidence is needed, however, to show conclusively whether the glutamic acid in the acetylated peptide fraction that presumably originates from the N-terminal region of the peptide chains in α-actinin is an artifact, or is actually part of the N-terminal sequence of the peptide chains in α-actinin.

The discovery of a $\text{Ca}^{2+}$-activated proteolytic enzyme that very quickly removes Z-disks from skeletal muscle myofibrils without causing detectable proteolysis of either purified actin or purified α-actinin suggested that this enzyme might be useful as a probe of the molecular architecture of the Z-disk. α-Actinin binds to F-actin but not to myosin, tropomyosin, the tropomyosin–troponin complex, or G-actin (Holmes et al., 1971), and antibody binding results indicate that α-actinin is located exclusively in Z-disks of skeletal muscle myofibrils (Schollmeyer et al., 1973). Because α-actinin binds only to F-actin among the known myofibrillar proteins, α-actinin must form a complex with F-actin somewhere in the Z-disk. If α-actinin constituted the Z-filaments that pass through the Z-disk to connect F-actin filaments from opposite sarcomers, proteolytic removal of the Z-disk would be expected to be accompanied by extensive
degradation of α-actinin. SDS-Polyacrylamide gels of purified α-actinin and purified actin indicated that neither of these proteins are degraded by the Ca\(^{2+}\)-activated protease. Removal of a small peptide (10 to 15 amino acid residues) from either the N- or C-terminal end of the peptide chains of α-actinin or actin, however, might be undetectable in SDS-polyacrylamide gel electrophoresis, because removal of such a small peptide would not change the mass of α-actinin or actin peptide chains enough for this change to be detected by SDS-polyacrylamide gel electrophoresis. Removal of such a small peptide from the C-terminal end of the peptide chains of α-actinin or actin might be expected to change the C-terminal amino acid of these peptide chains, and cleavage of a small peptide from the N-terminal end would remove the acetate and unblock the N-terminal amino group of either α-actinin or actin.

Careful analyses, however, show that neither the C- nor the N-terminal ends of either α-actinin or actin are altered by 60 min of incubation with the purified Ca\(^{2+}\)-activated protease under conditions where the protease completely removes Z-disks in five min. Because the N-terminal amino groups of both α-actinin and actin are blocked, and because removal of a small peptide would liberate an unblocked N-terminal amino group, these results demonstrate conclusively that the Ca\(^{2+}\)-activated protease does not remove a small
peptide from the N-terminal region of either α-actinin or actin. The amino acid sequence of the peptide chains of α-actinin is not known, and it is possible that the Ca$^{2+}$-activated protease removes a small peptide from the C-terminal region of α-actinin to produce a new C-terminal sequence of arg-leu, the same C-terminal sequence found in the native α-actinin molecule. The likelihood that the C-terminal region of the peptide chains of α-actinin has two arg-leu sequences and that the Ca$^{2+}$-activated protease specifically cleaves the second of these two sequences is very small, however. Because the amino acid sequence of actin is known and phenylalanine, which is the C-terminal amino acid of native actin, does not appear again until 23 amino acids from the C-terminus, it can be concluded that the Ca$^{2+}$-activated protease has no effect on the C-terminal region of purified actin. These results show that, under conditions where the Z-disk is removed rapidly, the Ca$^{2+}$-activated protease has no effect on purified actin and very probably also has no effect on purified α-actinin.

It is very difficult to envision how the Ca$^{2+}$-activated protease removes Z-disks without degrading either α-actinin or actin or both if α-actinin constitutes all or part of the Z-filaments that connect the ends of actin filaments across the Z-disk. Consequently, the specificity of the Ca$^{2+}$-activated protease suggests that α-actinin must constitute
part or all of the amorphous or matrix component (Kelly and Cahill, 1972) of the Z-disk rather than constituting the Z-filaments. This indirect evidence supports the direct antibody binding results indicating that α-actinin is located in the amorphous or matrix phase of the Z-disk (J. E. Schollmeyer, unpublished results, 1974).

The effects of the Ca\(^{2+}\)-activated protease on Z-disks and on purified actin and α-actinin are difficult to explain even if α-actinin is located in the amorphous part of Z-disks. Very brief incubation of myofibrils with the Ca\(^{2+}\)-activated protease releases α-actinin that seems unaltered on the basis of its migration in SDS-polyacrylamide gels, and yet the in vitro binding studies (Holmes et al., 1971) show that α-actinin binds only to F-actin among the known myofibrillar proteins. Thus, it is somewhat difficult to imagine how a proteolytic enzyme can release α-actinin from an α-actinin-F-actin complex without breaking peptide bonds in either α-actinin or actin or both. This ostensible dichotomy could be due to any one of three different possibilities. 1) Interaction between α-actinin and F-actin causes a conformational change in either α-actinin or actin or both, and this conformational change leaves the previously resistant protein vulnerable to proteolysis by the Ca\(^{2+}\)-activated protease. Very recent preliminary results, however, indicate that neither actin or α-actinin are degraded by the
Ca\(^{2+}\)-activated protease even when \(\alpha\)-actinin is bound to actin in vitro (Dayton and Zeece, unpublished results). 2) And as yet, undiscovered myofibrillar protein that binds \(\alpha\)-actinin to F-actin and that is very labile to proteolysis by the Ca\(^{2+}\)-activated protease and by numerous other proteolytic enzymes exists in the Z-disk. This suggestion does not explain why \(\alpha\)-actinin exhibits such a pronounced binding affinity for F-actin in vitro. 3) Actin in the Z-disk exists in the form of a single-strand instead of the double-stranded helix found in the remainder of the thin filament, and single-stranded F-actin is vulnerable to proteolysis by the Ca\(^{2+}\)-activated protease. This last possibility is consistent with what is known about the molecular architecture of the Z-disk, but no experimental evidence exists to show either that single-stranded F-actin actually exists in the Z-disk or that such actin is degraded by the Ca\(^{2+}\)-activated protease. Additional studies will be required to learn how the Ca\(^{2+}\)-activated protease can liberate \(\alpha\)-actinin from myofibrils without causing any evident degradation of either actin or \(\alpha\)-actinin.

In summary, this goal of this study was to learn whether \(\alpha\)-actinin had a structural role to connect thin filaments across the Z-disk in skeletal muscle myofibrils, or whether \(\alpha\)-actinin had a role in modifying the actin-myosin interaction in vivo. The results show that \(\alpha\)-actinin can modify
the structure of actin monomers in a F-actin filament, but that the Ca$^{2+}$-activated protease has no effect on either purified actin or purified α-actinin. These results favor the view that α-actinin can act physiologically to alter the actin-myosin interaction and that α-actinin does not constitute the Z-filaments that cross-link F-actin filaments through the Z-disk in mammalian skeletal muscle. On the basis of the results obtained thus far, however, the possibility that α-actinin in the amorphous phase strengthens Z-disks in a lateral direction or that α-actinin may act to keep actin filaments polymerized during contraction in vivo cannot be eliminated. Additional information is needed, therefore, to demonstrate that α-actinin functions physiologically to alter the actin-myosin interaction.
VI. SUMMARY

This study attempted to learn the physiological role of α-actinin 1) by determining whether α-actinin could alter the structure of actin monomers while they are aggregated in a F-actin filament, and 2) by using a Ca\(^{2+}\)-activated protease that removes Z-disks to determine whether α-actinin could constitute the Z-filaments that connect actin filaments across the Z-disk. Based on its biological and molecular properties, α-actinin may have either one or both of two possible physiological roles in skeletal muscle: 1) α-actinin may strengthen or promote the actin-myosin interaction and thereby be directly involved in the contractile process; if α-actinin alters the actin-myosin interaction, it must do so by altering actin conformation because α-actinin binds only to F-actin among the known myofibrillar proteins; 2) α-actinin may link individual sarcomeres in series to form myofibrils by connecting actin filaments across the Z-disk; this role indicates that α-actinin constitutes part or all of the Z-filaments that are observed connecting thin filaments across the Z-disk in electron micrographs of skeletal muscle. If α-actinin cannot alter the structure of actin monomers, it would be impossible for α-actinin to be involved in the actin-myosin interaction. If α-actinin is not a component of the Z-filaments, it is unlikely that α-actinin has
the sole function of connecting actin filaments across the Z-disk.

To insure that the properties of α-actinin preparations being studied originated from α-actinin itself and not from contaminants in the α-actinin preparations, several studies were done to determine the best method to purify α-actinin. A new procedure was developed for purification of α-actinin; P0-30 crude α-actinin extracts were purified first by chromatography on a DEAE-cellulose column and then by chromatography on hydroxyapatite columns. Hydroxyapatite chromatography removes a contaminant having a subunit molecular weight of 43,000 daltons as measured by SDS-polyacrylamide gel electrophoresis and also removes a component having a sedimentation coefficient of 9.1 S. The 43,000 dalton material observed in SDS-polyacrylamide gel electrophoresis may constitute part or all of the 9.1 S material observed in the analytical ultracentrifuge. Neither the 43,000 dalton material nor the 9.1 S species is completely removed from α-actinin that has been purified by two successive passages through DEAE-cellulose columns unless the α-actinin peaks are selected very carefully. Hydroxyapatite purified α-actinin contains over 95% of its protein as the 100,000 dalton α-actinin species as judged by SDS-polyacrylamide gel electrophoresis, and sediments as a 6 S boundary in the analytical ultracentrifuge with no trace of
more slowly or more rapidly sedimenting species. Although SDS-polyacrylamide gel electrophoresis and analytical ultracentrifugation indicate that hydroxyapatite-purified α-actinin is more homogeneous than α-actinin purified by two passages through DEAE-cellulose columns, hydroxyapatite-purified and DEAE-cellulose-purified α-actinin have similar activities in the ATPase and turbidity assays of α-actinin activity.

α-Actinin, at a ratio of 0.1 part of α-actinin to 1.0 part of actin by weight, increases the specific viscosity of F-actin two-to-three fold, regardless of whether the actin has been polymerized in 100 mM KCl, in 100 mM KCl plus 1 mM Mg^{2+}, or in 1 mM Mg^{2+} alone. Addition of 1 mM ATP decreases the viscosity of α-actinin-F-actin mixtures by 21% if the actin was polymerized in 100 mM KCl or in 100 mM KCl plus 1 mM Mg^{2+} and by 53% if the actin was polymerized in 1 mM Mg^{2+} alone. Assays done with increasing ATP concentrations showed that ATP was not acting directly to rupture F-actin filaments and thereby decrease the viscosity of α-actinin-F-actin mixtures. Studies at different Mg^{2+}/ATP ratios showed that ATP was most effective in decreasing viscosity of α-actinin-F-actin mixtures if Mg^{2+}/ATP ratios were one or less than one. It was suggested that Mg^{2+} was involved in cross-linking negatively charged F-actin filaments by negatively charged α-actinin molecules and that
complexing Mg\(^{2+}\) with ATP removed the ability of Mg\(^{2+}\) to function with \(\alpha\)-actinin to cross-link F-actin filaments. At relatively high concentrations, positively charged K\(^+\) cations evidently can replace Mg\(^{2+}\) in causing cross-linking of negatively charged \(\alpha\)-actinin molecules and F-actin filaments because ATP caused little decrease in viscosity of \(\alpha\)-actinin-F-actin mixtures when 100 mM KCl was present. Direct binding experiments and the finding that \(\alpha\)-actinin increases F-actin ATPase activity in the presence of 1 mM ATP and 1 mM Mg\(^{2+}\) but in the absence of KCl showed that, although complexing of Mg\(^{2+}\) by ATP prevents cross-linking of F-actin filaments by \(\alpha\)-actinin, it does not prevent simple, monovalent binding of \(\alpha\)-actinin to F-actin filaments.

\(\alpha\)-Actinin, at ratios of 0.1 part of \(\alpha\)-actinin to 1.0 part of actin by weight, increases the specific activity of F-actin ATPase by 70 to 90\% at pH 7.5 and Mg\(^{2+}\)/ATP ratios of one or greater than one. The presence of a sulfhydryl reagent such as DTT decreases F-actin ATPase activity by 15 to 25\%, but DTT does not diminish the ability of \(\alpha\)-actinin to increase F-actin ATPase. The effects of \(\alpha\)-actinin on the specific activity of F-actin ATPase depends on pH, the Mg\(^{2+}\)/ATP ratio, and the type of divalent cation present. Although F-actin ATPase activity was highest at pH 5.0 and decreased as the pH increased from 5.0 to 8.5 \(\alpha\)-actinin increased F-actin ATPase only at pH values above 6.0. The
ability of α-actinin to activate F-actin ATPase increased as the pH increased from 6.0 to 8.5. At Mg$^{2+}$/ATP ratios of 1 or greater and pH values of 7.5, α-actinin accelerated F-actin ATPase activity. Increasing the Mg$^{2+}$/ATP above 1 had little effect on the ability of α-actinin to activate F-actin ATPase. Decreasing Mg$^{2+}$/ATP ratios below 1, however, resulted in increasing inhibition of F-actin ATPase by α-actinin, hence, α-actinin activation of F-actin ATPase activity seems to require Mg$^{2+}$ATP as the substrate instead of free ATP. F-Actin ATPase activity in the absence of α-actinin increases as the Mg$^{2+}$/ATP ratio decreases below 1, so F-actin alone can use free ATP as a substrate. α-Actinin increases F-actin ATPase activity when Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$ are the divalent cations present, has little or no effect on F-actin ATPase activity when Ca$^{2+}$, Ni$^{2+}$, or Cu$^{2+}$ are the divalent cations present, and slightly inhibits F-actin ATPase when Ba$^{2+}$ or Fe$^{2+}$ are the divalent cations present. Although the specific activity of Mg$^{2+}$-modified F-actin ATPase is only 0.2 to 0.8% of the specific activity of Mg$^{2+}$-modified actomyosin ATPase, the ATPase activity in F-actin preparations is not due to minute contamination by myosin because: 1) SDS-polyacrylamide gel electrophoresis of actin at heavy loads show no myosin contamination in these actin preparations; 2) the pH dependence of F-actin ATPase activity differs completely from the pH dependence of actomyosin ATPase
activity; 3) the relative effects of different divalent cations on F-actin ATPase activity differs completely from the relative effects of these same divalent cations on actomyosin ATPase activity; and 4) α-actinin increases F-actin ATPase activity between pH 6.5 and 8.5 but not between pH 5.0 and 6.0, whereas α-actinin increases actomyosin ATPase activity between pH 6.0 and 8.0 but not between pH 8.0 and 8.5. An α-actinin to actin ratio of 0.1 to 1.0 by weight corresponds to a molecular ratio of one α-actinin molecule to every 48 actin monomers in the F-actin filament. This one α-actinin molecule seems able to affect ATPase activity of most of the 48 actin monomers associated with it on the average because: 1) doubling the amount of α-actinin added increases F-actin ATPase by only 1.45-fold instead of twofold; and 2) recent studies by other investigators have shown that 0.1 part of α-actinin to 1.0 part of F-actin by weight increases the rate of exchange of 80% of the total bound nucleotide in F-actin. Consequently, a single α-actinin molecule can alter the structure of actin monomers over a very long distance in a F-actin filament.

Viscosity measurements show that addition of tropomyosin prevents cross-linking of F-actin filaments by α-actinin, regardless of whether Mg2+ or ATP is present. Also, α-actinin does not increase F-actin ATPase activity in the presence of tropomyosin. Direct binding experiments have
shown that tropomyosin added \textit{in vitro} prevents binding of α-actinin to F-actin filaments, although the experimental error associated with direct binding measurements cannot eliminate the possibility that up to 0.03 parts of α-actinin to 1 part of F-actin by weight is bound in the presence of tropomyosin. Consequently, it seems that tropomyosin added \textit{in vitro} does not bind to F-actin filaments in the same way that tropomyosin \textit{in vivo} is bound, and that \textit{in vitro} binding of tropomyosin blocks the ends of F-actin filaments and prevents α-actinin from binding to these ends.

Tryptophan content of α-actinins from porcine white skeletal, porcine red skeletal, bovine cardiac, chicken gizzard, chicken breast, and rabbit skeletal muscle is $25.2 \pm 0.2$, $30.8 \pm 0.2$, $30.9 \pm 0.2$, $30.0 \pm 0.3$, $30.6 \pm 0.1$ and $29.9 \pm 1.1$ moles per mole of α-actinin, respectively. Bovine cardiac α-actinin has 18 moles of cysteine per mole of α-actinin but contains no cystine. Six of these 18 cysteine residues in an α-actinin molecule are located on the surface of the molecule or in an area of the molecule that is readily accessible to solvent, and the remaining 12 cysteine residues are buried inside the tertiary structure of bovine cardiac α-actinin where they are inaccessible to reaction with DTNB. Carboxymethylation of bovine cardiac α-actinin in 8 M urea for 15 min with iodoacetate results in carboxymethylation of 15-16 of the total 18 moles of
cysteine/mole of $\alpha$-actinin.

As determined by assay with CPA and CPB, leucine is the C-terminal amino acid and arginine is the amino acid penultimate to the C-terminal amino acid in both peptide chains of bovine cardiac and porcine skeletal $\alpha$-actinins. Leucine is also the C-terminal amino acid of chicken gizzard and chicken breast $\alpha$-actinin. The N-terminal $\alpha$-amino groups of the two peptide chains of bovine cardiac, porcine skeletal, chicken gizzard, and chicken breast $\alpha$-actinins were unavailable for reaction with dansyl chloride, even after the $\alpha$-actinin molecules were denatured in 0.05 M SDS, pH 8.5 to 9.0. Acetate analysis showed that porcine skeletal, bovine cardiac, chicken gizzard, and chicken breast muscle $\alpha$-actinins all contained approximately 1.8 moles of acetate per mole of $\alpha$-actinin. This finding suggests that the two N-terminal amino groups in the $\alpha$-actinin molecule are both acetylated. A N-acetylated peptide was isolated from a proteolytic enzyme digest of bovine cardiac $\alpha$-actinin by passing the proteolytic digest through two Dowex-50 cation exchange columns in succession. Amino acid analysis showed that this peptide had 2 moles of aspartic acid and 4 moles of glutamic acid per mole of acetate. Hence, the amino acid composition of the N-terminal region of $\alpha$-actinin evidently is $\text{N-acetyl}[(\text{asp})_2-(\text{glu})_4]$. 
A Ca\(^{2+}\)-activated protease that removes Z-disks and α-actinin from myofibrils without causing other ultrastructurally detectable deletions has no effect on the N- and C-terminal amino acids of either α-actinin or actin. α-Actinin is located exclusively in the Z-disk of skeletal muscle and is presumably bound to actin there because in vitro studies have shown that α-actinin binds only to F-actin among the known myofibrillar proteins. That the Ca\(^{2+}\)-activated protease removes Z-disks without degrading purified α-actinin or purified actin suggests that α-actinin does not constitute the Z-filaments that connect actin filaments across the Z-disk and that are quickly removed by the Ca\(^{2+}\)-activated protease. Consequently, α-actinin must constitute part or all of the amorphous or matrix component of Z-disks. The nature of the binding of α-actinin in the amorphous component to the Z-disk remains unclear.
VII. CONCLUSIONS

As a result of this study, the following conclusions are justified.

1. Homogeneity of α-actinin purified by two passages through a DEAE-cellulose column can be improved by using chromatography on a hydroxyapatite column. Hydroxyapatite chromatography removes a component having a subunit molecular weight of 43,000 daltons as determined by SDS-polyacrylamide gel electrophoresis and also removes the 9.1 S species that is observed in analytical ultracentrifuge diagrams of α-actinin preparations purified by DEAE-cellulose chromatography. α-Actinin purified by hydroxyapatite chromatography is as active in the ATPase and turbidity assays of α-actinin activity as α-actinin that has been purified by two passages through DEAE-cellulose columns.

2. Highly purified α-actinin increases F-actin viscosity to an equal extent whether actin is polymerized in 100 mM KCl, in 100 mM KCl plus 1 mM Mg$^{2+}$ or in 1 mM Mg$^{2+}$ alone. Addition of ATP decreases the α-actinin-induced increase in F-actin viscosity slightly when actin is polymerized by 100 mM KCl or by 100 mM KCl plus 1 mM Mg$^{2+}$, and greatly when actin is polymerized by 1 mM Mg$^{2+}$ alone. Because α-actinin increases F-actin viscosity by cross-linking
F-actin filaments, ATP probably decreases viscosity of α-actinin-F-actin mixtures by decreasing the α-actinin-induced cross-linking of F-actin filaments. ATP does not prevent binding of α-actinin to F-actin filaments even when ATP completely eliminates the α-actinin-induced increase in F-actin viscosity.

3. Binding of α-actinin to F-actin alters the ATPase activity of F-actin preparations. Because F-actin ATPase depends on the structure of actin monomers in the F-actin filament, binding of α-actinin alters the structure of these monomers. The α-actinin-induced alteration in conformation of actin monomers occurs at physiological levels of α-actinin and under physiological ionic conditions. The physiological level of α-actinin corresponds to only 1 α-actinin molecule for 48 actin monomers, and binding of this much α-actinin alters the conformation of most of the actin monomers. Therefore, binding of α-actinin has a long range conformational effect on F-actin filaments. In vitro addition of tropomyosin prevents both the α-actinin-induced increase in F-actin viscosity and the α-actinin-induced increase in F-actin ATPase activity. Binding of tropomyosin added in vitro to F-actin filaments probably covers the ends of these filaments and thereby prevents binding of α-actinin added in vitro.
4. Tryptophan content of α-actinins from porcine red skeletal muscle, porcine white skeletal muscle, bovine cardiac muscle, chicken gizzard smooth muscle and chicken breast muscle varies from 26.2 ± 0.2 to 30.9 ± 0.2 moles per mole of α-actinin.

5. Bovine cardiac α-actinin has 18 moles of cysteine per mole and contains no cystine. Six of these 18 cysteine residues in one α-actinin molecule are located on the surface of the molecule and the remaining 12 residues are buried in the tertiary structure of the molecule. Carboxymethylation of bovine cardiac α-actinin with iodoacetate in 8 M urea for 15 min results in modification of 15-16 of the 18 total moles of cysteine/mole of α-actinin.

6. Leucine is the C-terminal amino acid of both peptide chains in α-actinins from bovine cardiac muscle, porcine skeletal muscle, chicken gizzard muscle and chicken breast muscle. Arginine is the amino acid penultimate to the C-terminal amino acid in α-actinin from bovine cardiac muscle and porcine skeletal muscle.

7. The N-terminal amino groups of α-actinins from bovine cardiac α-actinin, porcine skeletal muscle, chicken gizzard muscle, and chicken breast muscle are unavailable for reaction with dansyl chloride. Approximately 1.8 moles of acetate are found per mole of α-actinin for α-actinins from each of these four different muscle sources. Therefore,
N-terminal amino groups in the peptide chains of these α-actinins are probably acetylated. An N-acetylated peptide purified from a proteolytic digest of bovine cardiac α-actinin by two passes through a Dowex-50 column contained 2 moles of aspartate and 4 moles of glutamate per mole of acetate in the peptide. Therefore, the amino acid composition of the N-terminal region in the peptide chains of bovine cardiac α-actinin is CH$_3$-C[(asp)$_2$-(glu)$_4$].

8. Ca$^{2+}$-Activated proteolytic enzyme that very quickly removes Z-disks and releases α-actinin from skeletal muscle myofibrils causes no degradation of purified porcine skeletal α-actinin or actin. Because α-actinin binds only to F-actin among the known myofibrillar proteins, removal of Z-disks without degradation of α-actinin or actin suggests that α-actinin cannot constitute the Z-filaments that connect actin filaments across the Z-disk. The nature of the molecular interactions of α-actinin in the Z-disk remains unclear.
VIII. BIBLIOGRAPHY


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