1976

Control of dephosphorylation of bovine heart glycogen synthase D

Ronald Lee Mellgren
Iowa State University

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Control of dephosphorylation of bovine heart glycogen synthase D

by

Ronald Lee Mellgren

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To Jeri, for all the years of encouragement,

patience and love.
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INTRODUCTION

Properties of Mammalian Glycogen Synthases

Synthesis of glycogen in mammalian systems is catalyzed by the enzyme glycogen synthase, first described by Leloir and Cardini (1). The enzyme catalyzes the transfer of a glucosyl residue from UDP-glucose to the nonreducing end of an α-1,4-polyglucan chain referred to as the glucosyl acceptor or primer. The physiologically important primer for the synthase reaction is glycogen, a homopolysaccharide composed of α-1,4-linked chains of glucosyl residues interconnected by α-1,6-bonds. On the average, there are about twelve α-1,4-glucosyl bonds per α-1,6-glucosyl bond (2,3). The nucleotide sugar specificity of skeletal muscle synthase has been investigated (4,5). In decreasing order of reactivity, UDP-glucose, ADP-glucose and TDP-glucose were substrates for the synthase. CDP-glucose and IDP-glucose could not be used as glucosyl donors. The role of primer structure in the skeletal muscle synthase reaction has also been investigated (4-6). Goldemberg (4) reported that glycogens from a variety of sources including shellfish, rabbit liver, rat liver and maize were equivalent as primers. Malto-oligosaccharides were effective only at much higher concentrations, but they gave twice the V_{max} values of the glycogen samples. Brown et al. (5) studied linear and branched oligosaccharides as well as enzymatically modified glycogens

1UDP-glucose:glycogen α-4-glucosyltransferase, EC 2.4.1.11. In the remainder of this thesis, this enzyme will be referred to as "synthase" for the sake of brevity.
as primers. This study confirmed the previous observation that glycogens were better primers than the malto-oligosaccharides. However, these investigators found that the oligosaccharides tested could accept glucosyl residues at about one-fourth the rate of rabbit liver glycogen under conditions where the synthase was saturated with primer. Salsas and Larner (7) presented evidence that the minimum structure required as a primer is glucose. The Km for glucose was very high, 0.9 M in the presence of 0.1 mM UDP-glucose, and no information was given about the Vmax. In the studies of Brown et al. (5), Km's for rabbit liver glycogen, phosphorylase limit dextrin of rabbit liver glycogen and β-amylase limit dextrin of rabbit liver glycogen were very similar, around 2 to 4 µM expressed as end groups. Relative maximum velocities for the three glycogen samples showed a greater variation. The phosphorylase dextrin having one-fourth the Vmax and the β-amylase dextrin having about one-tenth the Vmax of the unmodified glycogen.

As discussed below, synthase can exist in two forms, synthase I and synthase D, and the substrate specificities should be determined for each form. In studies done before the discovery of interconvertible forms of synthase (4,6), it is not possible to tell whether a single form or a mixture of the two forms was present. Little information is available about the primer specificity of the isolated synthase forms. The Km for glycogen for a highly purified skeletal muscle synthase D preparation was 5.7 µg/ml or about 2 µM end groups in the presence of saturating UDP-glucose (8).

Villar-Palasi and Larner (9) first suggested the existence of two
separate forms of glycogen synthase. The two forms could be inter-
converted, and it was found that the interconversion involved phosphory-
lation or dephosphorylation of the synthase (10). This observation sug-
gested a dual function for phosphorylation-dephosphorylation in glycogen
metabolism since the enzyme responsible for glycogen breakdown in
mammalian tissues, glycogen phosphorylase,\(^2\) was already known to exist in
phosphorylated and nonphosphorylated forms (11,12). The nonphosphory-
lated form of synthase, designated synthase I, could be distinguished
from the phosphorylated form, or synthase D, because it is active in the
absence of glucose-6-phosphate whereas the latter form requires this
metabolite for activity.

The two forms of synthase have been studied in a number of tissues
(for a review see reference 13). Both forms from rabbit skeletal muscle
have been purified to homogeneity (14,15). Liver synthase D has been
purified to homogeneity by several groups (16-18). Swine kidney (19) and
swine adipose tissue (20) synthase have also been purified to homogeneity
as judged by polyacrylamide gel electrophoresis. The specific activities
of the apparently pure enzymes vary from 1.9 U/mg for swine adipose
tissue (20) and 9.1 U/mg for swine kidney (19), to 35 U/mg for rabbit
skeletal muscle synthase (21). It is not certain that this difference in
specific activities for synthases isolated from various tissues is the
result of different isozymes of synthase. Another possibility which has
been considered (20) is that denatured synthase co-purifies with active
synthase in some preparation methods. Support for the latter possibility

\(^2\)\(\alpha\)-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1.
came with the finding that an improved method for purification of skeletal muscle synthase I or synthase D results in an enzyme with a specific activity three-fold higher than previously reported for "homogeneous" synthase from the same source (15,21,22).

There is general agreement on the subunit size of synthase; values of 85,000 to 90,000 daltons have been reported for both synthase I and synthase D from several different tissues (15,16,18-20). One group has reported a subunit size of 77,000 to 80,000 daltons for rat liver synthase D (17). This value must be questioned in light of the finding by two other groups that liver synthase D from both rat (16) and rabbit (18) has a subunit molecular weight of 85,000. The possibility of a proteolytic modification cannot be overlooked since it is well known that synthase may be partially hydrolyzed by proteases without destroying its activity (22-24).

Both synthase I and synthase D exist as oligomeric proteins in the enzymatically active forms (14-19). The state of aggregation of skeletal muscle synthase I depends on temperature (25). Both liver and skeletal muscle synthase D may exist in various molecular weight forms. A number of metabolites, including glucose-6-P, UDP-glucose, Mg$^{2+}$ and ATP influence the molecular weight of synthase D from the two tissues (26,27). The first three metabolites listed above cause the aggregation of liver synthase D from an approximately 150,000 dalton species to one of approximately 260,000 to 280,000 daltons, and ATP appears to reverse this effect (27). Synthase D from various tissues may exist as the dimer (17,18,27) trimer (15,16,27), tetramer (17,19) and hexamer (16) of
the subunit.

Studies on Synthase Interconverting Enzymes

Synthase kinases

Friedman and Larner (28) showed that the protein kinase acting on synthase I was not phosphorylase kinase. Since synthase was known to be inactivated in diaphragms treated with epinephrine and in broken cell preparations incubated with cyclic-AMP (29), the effect of cyclic-AMP on the conversion of synthase I to synthase D was studied, and a stimulation of the conversion by cyclic-AMP was reported (30). Soderling et al. (14) demonstrated that the same cyclic-AMP dependent protein kinase could phosphorylate synthase I or the inactive form of phosphorylase kinase. Recently, the synthase kinase reaction has been reinvestigated, and there is good evidence that a cyclic-AMP independent protein kinase is also involved in the phosphorylation of synthase I (31,32).

Another facet of synthase phosphorylation currently being investigated is the number of mole equivalents of phosphate incorporated into the enzyme under different conditions. Smith, Brown and Larner (15) reported six phosphate molecules per 90,000 dalton subunit of the rabbit muscle synthase D, while Soderling et al. (14) found that only one phosphate per subunit was incorporated during phosphorylation of the rabbit muscle enzyme by cyclic-AMP dependent protein kinase. Recent work with skeletal muscle and rabbit liver synthase indicated that only one or two of the serine residues need be phosphorylated to cause conversion of synthase I to synthase D (18,33). The possible significance of the "extra" phosphate
sites on synthase D is currently under investigation.

**Synthase phosphatases**

Friedman and Larner (10) showed that conversion of partially pure $^{32}$P-labeled synthase D to synthase I was accompanied by release of phosphate from protein. Synthase phosphatases have been purified from a variety of tissues (18,34-36). The protein phosphatases active on synthase D are not specific for this phosphoprotein, but have activity on a variety of other substrates including phosphorylase $\alpha$, phosphorylase kinase and phosphohistone (18,34-36). An extensive study of a protein phosphatase from bovine heart showed that phosphatase activity on synthase D, phosphorylase $\alpha$ and phosphohistone co-purified, and that dephosphorylation of any of the three substrates could be inhibited by the addition of another substrate (35). Strong proof for a synthase phosphatase with broad substrate specificity came with the purification to homogeneity of a protein phosphatase from rabbit liver (37). Originally described as a liver phosphorylase phosphatase, this enzyme could also convert highly purified rabbit liver synthase D to synthase I and dephosphorylate phosphohistone (18).

Control of synthase phosphatase may be brought about in two distinct manners. It is possible for a modulator to bind directly to the phosphatase and thus affect its activity, or a modulator may influence the phosphatase reaction by binding to the synthase and altering its properties as a substrate. There is evidence that both of these mechanisms of synthase phosphatase control are important. Fluoride, phosphate and
pyrophosphate appear to have a direct effect on synthase phosphatase, since these anions also inhibit the activity of the phosphatase on other substrates (35). A number of phosphoprotein phosphatases are stimulated by divalent cations, especially \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) (35-41). Since the activation can be observed with different substrates (35,38), at least part of the divalent cation stimulation of synthase phosphatase could be due to interaction of the metal ion with the phosphatase. Control of phosphatase by interaction with regulatory proteins is a possibility which is currently being explored. Multiple forms of synthase phosphatase have been reported from bovine heart (38), rabbit skeletal muscle (42) and rat liver (43). The forms could be separated on DEAE-cellulose chromatography and sucrose density gradient ultracentrifugation. In the case of the heart phosphatases, the larger forms could be dissociated to active small forms by urea or SDS (38). The large molecular weight forms of synthase phosphatase could be complexes between a catalytic subunit and regulator protein or proteins. Brandt et al. (37) have reported the partial purification from rabbit liver of a low molecular weight phosphorylase phosphatase inhibitor protein. Recently, this protein preparation has been shown to inhibit a homogeneous protein phosphatase acting on synthase D (14). Glucose-6-P is an activator of synthase phosphatase (44) which appears to act through binding to synthase D. Not only are the hexose phosphate specificities for synthase D activation and synthase phosphatase activation similar (36,45), but the activation constants for the two effects are nearly the same (45). Furthermore, glucose-6-P activation of phosphatase activity is specific for the substrate synthase D; there is
no effect on phosphohistone phosphatase activity (36). Divalent cations also affect the synthase D reaction and synthase phosphatase reaction in the same way. $\text{Mn}^{2+}$, $\text{Mg}^{2+}$, and $\text{Ca}^{2+}$ activate synthase D and synthase phosphatase. $\text{Mn}^{2+}$ gives the greatest activation in either case (45).

In 1965, Danforth presented evidence for control of the interconversion of the two forms of synthase by glycogen (46). His studies with mouse skeletal muscle revealed an inverse relationship between the amount of glycogen in the muscle and the fraction of total synthase in the synthase I form. Villar-Palasi (47) demonstrated that the relationship could be accounted for by an inhibition of synthase phosphatase by glycogen. Glycogens of high molecular weight were shown to inhibit better (lower concentrations required for half-maximal inhibition) than smaller glycogen molecules. It was also proposed that polysaccharides with a short exterior chain length were better inhibitors than those having relatively long exterior chains. The glycogen inhibition noted in this study was transitory; upon incubation of the crude rat muscle phosphatase at cold room temperatures overnight, about 60% of the phosphatase activity was lost and the remaining activity was not inhibited by glycogen. The glycogen inhibition was postulated to occur as a result of polysaccharide binding to the phosphatase; upon incubation overnight the phosphatase was converted to a form incapable of interacting in the proper fashion with glycogen; hence, the polysaccharide no longer inhibited the phosphatase catalyzed conversion of synthase D to synthase I. A number of more recent studies have indicated that glycogen affects the reaction by binding to the synthase D rather than phosphatase (36,45).
phosphatases used in these studies were purified several hundred-fold and still were sensitive to glycogen inhibition; therefore a simple time dependent transformation of synthase phosphatase to a form insensitive to glycogen inhibition is not likely. Synthase phosphatase in a crude liver system was shown to be less sensitive to glycogen inhibition than the muscle system (49). This observation indicates the importance of glycogen inhibition of synthase phosphatase since liver glycogen is normally present at concentrations three to five-fold higher than muscle glycogen. The lack of response of synthase phosphatase to glycogen inhibition in liver could account for this difference. It remains to be established whether this difference in sensitivity to glycogen inhibition is the consequence of a different synthase or phosphatase being present in liver than in muscle. Another possibility that will be considered in this thesis is that a factor is required to sensitize the phosphatase to glycogen inhibition. The degree of inhibition of the synthase phosphatase by glycogen would be dependent on the amount of the factor in a given tissue.

In the present work, I have studied the dephosphorylation of highly purified bovine heart synthase D by _E. coli_ alkaline phosphatase. The physical properties of the synthase phosphatase activity of alkaline phosphatase were investigated to determine whether the synthase phosphatase activity was a property of the alkaline phosphatase or a contaminating protein phosphatase in the alkaline phosphatase preparation. Control of the reaction by known effectors of synthase phosphatase was
studied to assess the role of substrate in control of the synthase phosphatase reaction. Finally, glycogen inhibition of synthase D de-phosphorylation was studied in detail.
MATERIALS AND METHODS

Materials

UDP-glucose, glucose-6-P, ATP, p-nitrophenyl phosphate, 1,4-diamino-
butane dihydrochloride, rabbit liver glycogen (Type III), calf thymus
histone (Type II-A), crystallized phosphorylase a, DEAE-cellulose,
Sephadex and Sepharose were purchased from Sigma Chemical Company. Oyster
glycogen was from J. T. Baker Chemical Company. Code BAPF E. coli strain
C90 alkaline phosphatase was obtained from Worthington Biochemical Cor-
poration. Crystalline sweet potato β-amylase, a product of Worthington,
and Pseudomonas isoamylase, a product of Hayashibara Company Limited, were
generous gifts from Dr. Dexter French, Iowa State University. Crystalline
bovine serum albumin was purchased from Miles Laboratories. Rabbit
skeletal muscle lactate dehydrogenase was from Boehringer Mannheim Cor-
poration. Rabbit muscle phosphorylase b was a gift from Dr. D. J. Graves,
Iowa State University. A hydrolyzed starch sample with an average degree
of polymerization of 87, referred to hereafter as DP87 dextrin, was a
gift from Dr. J. R. Robyt, Iowa State University. Uniformly labeled $^{14}$C-
glucose and carrier-free $^{32}$P-phosphoric acid were products of New England
Nuclear. All other chemicals used were of reagent quality.

Methods

Biochemical preparations

Preparation of radiobiochemicals. UDP-$^{14}$C-glucose was made by a
previously described method (50). γ-$^{32}$P-labeled ATP was made by the
method of Glynn and Chappell (51).

**Preparation of polysaccharides** The dextrin III fraction used in primer specificity and phosphatase inhibition studies described later was isolated from an acid hydrolyzate of oyster glycogen. Four grams of oyster glycogen was dissolved in 180 ml of water and brought to 70°C in a water bath. Twenty ml of 1 N HCl was added and the glycogen was hydrolyzed for 8 hours. The hydrolysis was terminated by addition of one equivalent of NaOH and the hydrolyzate was fractionated by ultrafiltration in an Amicon TCF-10 cell. Dextrin III was the fraction which passed through an XM-100A membrane but was retained by a PM-30 membrane. Therefore, the approximate molecular weight range of the dextrin is 30,000 to 100,000. \( \beta \)-amylase dextrins were prepared by the method of Whelan (52) or by incubating an 8% w/v solution of oyster glycogen containing 20 mM sodium acetate, pH 4.7, 0.4% v/v mercaptoethanol, 0.5 mg/ml bovine serum albumin and 0.1 mg/ml \( \beta \)-amylase at room temperature. The reaction mixture was dialyzed during the incubation against the reaction buffer minus bovine serum albumin. The initial dialysis also contained 10 ml of toluene which was layered on top of the buffer to inhibit bacterial growth. The buffer (100 times the volume of sample) was changed daily for four days, and then the sample was dialyzed for one day against water. Protein was denatured by boiling for 5 minutes and removed by centrifugation. The glycogen was precipitated by addition of ethanol to 66% v/v. The entire digestion procedure was repeated except that no albumin was added. This time the ethanol precipitated \( \beta \)-amylase dextrin was washed with absolute ethanol and air dried. Less than 0.5% of the final product
could be hydrolyzed by further treatment with β-amylase. Phosphorylase
limit dextrins of the DP87 dextrin and dextrin III and glycogens were
prepared by the method of Lee (53).

Preparation of aminobutylagarose  Diaminobutane was coupled to
Sepharose 4B by a combination of methods described previously (54,55).
This gel will be referred to as aminobutylagarose. Twenty grams of
powdered CNBr was added with gentle stirring to a slurry of 100 ml
Sepharose 4B and 100 ml of water at 20°C. NaOH was immediately added to
bring the pH to 11 and the pH was maintained at 10.5 to 11.5 by addition
of 8 N NaOH. The temperature was kept at 20-25°C by addition of ice.
After no more CNBr granules were present and the pH was dropping only
slightly, a handful of ice was added to the reaction mixture and the gel
was rapidly washed with a liter of ice water in a Buchner funnel. The
gel was then rapidly transferred to a 0.4 M solution of 1,4-diaminobutane
at pH 9.0 and the suspension was gently stirred for 16-20 hours at 5°C.
A column of the gel was washed with 10 volumes of 50 mM Tris-HCl buffer,
pH 7.8, containing 0.5 M NaCl and then equilibrated in buffer without
NaCl. The gel was stored until needed at 5°C with 0.02% w/v NaN₃ added
to prevent bacterial growth. In early studies with the aminobutylagarose,
it was discovered that the affinity of the gel for synthase was greater
for freshly made preparations of the gel than for aged gel. Since it was
not convenient to make a fresh batch of gel for each synthase preparation,
aminobutylagarose which had been stored at 5°C for at least 2 weeks was
used in the hydrophobic chromatography step of the synthase preparations.
The gel gave an orange stain upon treatment with sodium
2,4,6-trinitrobenzene sulfonate (TNBS) as previously described for agarose gels substituted with primary amines (54).

Enzyme preparations

**Commercial alkaline phosphatase**  This enzyme was obtained as an ammonium sulfate suspension. To make stock solutions of alkaline phosphatase, the suspension was centrifuged and the protein pellet taken up in one to four times the original suspension volume of 0.5 M Tris-HCl buffer, pH 7.8. For kinetic studies with the phosphatase, the stock solution was dialyzed several days at 5°C against the resuspension buffer to remove traces of ammonium sulfate. Stock solutions of the alkaline phosphatase could be stored for one month at 5°C without measurable loss of activity.

**E. coli strain K12 alkaline phosphatase**  Alkaline phosphatase from strain K12 *E. coli* was prepared by a modification of the method described by Simpson *et al.* (56) for the preparation of the strain C90 enzyme. The cells were grown to late log growth phase in a low phosphate medium similar to the one described by Schlesinger (57) except that the buffer was 0.125 M Tris-HCl, pH 7.5, and Bacto-peptone was used rather than Peptiplus. Pre-cycled microgranular DE-52 cellulose was used in the column chromatography step since it can separate the various forms of alkaline phosphatase (56).

**Glycogen synthase**  Synthase D from bovine heart was purified using a combination of previously described methods (8,30,55). Fresh beef hearts were obtained from a local slaughterhouse and immediately immersed
in ice for transporting to the lab. The hearts were then trimmed of fat and cut into approximately 2" by 2" pieces. Tissue could be used either fresh or after quick-freezing in liquid nitrogen and storing at -73°C. The frozen hearts could be kept at -73°C for several weeks with little loss of activity when compared to fresh hearts. Tissue was ground in a chilled commercial meat grinder. The ground tissue was combined with two volumes of 50 mM Tris-HCl buffer, pH 8.2, 5 mM EDTA and 5 mM EGTA, and then blended for 30 to 45 seconds in a Waring blender. The crude homogenate was centrifuged at 10,000 xg and 5°C for 50 minutes and the resulting supernatant was filtered through glass wool. The synthase was precipitated from the first supernatant fraction by addition of ethanol to 30% v/v as previously described (8). The ethanol precipitate was collected by centrifugation and the drained pellets were resuspended in 50 mM Tris-HCl, 5 mM EDTA, 0.4% v/v mercaptoethanol (TEM buffer), pH 7.8. The volume of the preparation at this stage was one-tenth the volume of the crude homogenate. The resuspended ethanol pellet was then centrifuged at 78,000 xg in a refrigerated Beckman ultracentrifuge using the Type 30 rotor for 6 hours and the supernatant was filtered through glass wool in the cold. Oyster glycogen was added to the supernatant to a final concentration of 5 mg/ml and the ultracentrifugation at 78,000 xg was repeated. This time the synthase was recovered in the glycogen pellet and was resuspended in TEM buffer to give one-tenth the volume of the preparation before ultracentrifugation. Glycerol was added to the preparation to a concentration of 25% v/v and 200 μg of salivary α-amylase was added at room temperature. The preparation was dialyzed against 100
volumes of TEM buffer for 2 hours at room temperature and then the sample
was applied to an aminobutylagarose column (40 ml). The column was
washed with TEM buffer plus 25% v/v glycerol (TEMG) until the O.D. \textsubscript{280} of
the column effluent returned to the baseline level. Synthase was
eluted with a 0 to 0.6 M NaCl gradient in TEMG buffer. The total
gradient volume was 12 times the column volume. Rabbit liver glycogen
was then added to the pooled synthase fractions to a final concentration
of 1 mg/ml. Since glycerol interferes with ethanol precipitation of
synthase, glycerol concentration was reduced by dialyzing the enzyme
solution overnight against twenty volumes of TEM buffer at 5°C. The
diazyzed enzyme was chilled to 1°C, and cold 95% ethanol was added to
30% v/v to precipitate the synthase. The resuspended synthase was
dialyzed against 50 mM Tris-HCl, pH 7.5, for 3 hours at 5°C and stored
at -73°C. The synthase at this stage usually assayed as 90 to 95%
synthase I. To prepare synthase D, the thawed synthase was incubated at
30°C in the presence of 1 mM ATP, 10 mM MgCl\textsubscript{2} and 10 \muM cyclic-AMP in
50 mM Tris-HCl buffer, pH 7.5, which also contained 5 mM dithiothreitol.
After two hours of incubation at 30°C, the mixture was allowed to incu-
bate at 5°C for 48 hours. An endogenous kinase converted the enzyme to
the D form in this step. The synthase D, in a total volume of 1 ml was
layered on a 2 ml pad of 50% w/v sucrose in the same buffer as the
synthase, but also containing 100 mM KF. The synthase was then
centrifuged at 145,000 xg and 5°C for 8 hours in the ultracentrifuge using
a Type 40 rotor. This procedure has been shown to remove traces of
synthase phosphatase in the synthase preparation (35). The pellet was
resuspended in 50 mM Tris-HCl, pH 7.5, and dialyzed for three hours against the same buffer at 5°C. The enzyme was then stored at -73°C. Since not all of the synthase was pelleted by the last ultracentrifugation step, the supernatant from the spin was dialyzed against TEM containing 50 mM KF in the cold with 0.5 mg/ml rabbit liver glycogen added. The synthase in this fraction was then collected by precipitation with ethanol as described above except that the final ethanol concentration was 15% v/v. This fraction was combined with the enzyme recovered in the glycogen pellet from the ultracentrifugation step. The final preparation had no contaminating synthase phosphatase activity when assayed under conditions used in subsequent studies.

$^{32}$P-labeled synthase D was prepared by the procedure described above, except that only the synthase that pelleted in the glycogen centrifugation through 50% w/v sucrose was used, and the sucrose pad centrifugation was repeated.

"Glycogen-free" synthase D was prepared in the following manner. One ml of the synthase D preparation described above was incubated with 20 mM dithiothreitol for 30 minutes at 30°C, and then 0.3 ml of glycerol was added. Ten μg of salivary α-amylase was added and the mixture was incubated at 30°C for 45 minutes and then applied to an aminobutylagarose column (0.6 x 4.0 cm) equilibrated at room temperature with 50 mM Tris-HCl, 5 mM dithiothreitol and 25% v/v glycerol at pH 7.5. The column was washed with ten column volumes of the equilibration buffer and the synthase was eluted with buffer containing 0.5 M NaCl. The active fractions were then concentrated and the NaCl removed, by dialysis
against 50 mM Tris-HCl, 1 mM dithiothreitol and 50% v/v glycerol at 50°C for 3 hours and then overnight at -20°C. The final product represented about 50 percent of the original activity of the purified synthase D used as starting material. The glycogen-free synthase D was stored at -20°C, and was stable for a month under these conditions.

In some cases a modification of the method of Thomas and Larner (58) was used to make synthase D. The synthase D prepared by this method was used as a substrate for alkaline phosphatase in some glycogen inhibition studies. The following modifications were made in the synthase preparation. The incubation with sulfate and subsequent treatment with α-amylase were omitted. The synthase was converted to synthase D by incubation with 10 mM MgCl₂, 5 mM ATP and 10 μM cyclic-AMP added before the DEAE step. Ten percent of the synthase in this preparation was in the synthase I form. The specific activity of the preparation was 2 U/mg. There was a contaminating synthase phosphatase activity which accounted for a maximum of 10 percent of the phosphatase activity in subsequent experiments.

**Bovine heart protein phosphatase** This enzyme was prepared as previously described (35). It was necessary to concentrate the phosphatase in order to assay for synthase phosphatase. This was done by dialyzing the phosphatase against 50 mM Tris-HCl, pH 7.5, and 1 mM dithiothreitol made up to 60% in glycerol v/v. The glycerol concentrated phosphatase was stored at -20°C.
Cyclic-AMP dependent protein kinase This enzyme was purified from bovine heart essentially by previously published methods (59,60), or a partially purified preparation was obtained from the aminobutylagarose chromatography step of the synthase preparation. The latter preparation contained neither synthase activity nor phosphohistone phosphatase activity and was used to convert synthase I to synthase D. Kinase prepared by the first method listed was used to phosphorylate histone for use in phosphohistone phosphatase assays.

Human salivary α-amylase Salivary α-amylase was prepared by the method of Bernfeld (61).

Enzyme assays

Glycogen synthase This enzyme was assayed by the method of Thomas et al. (50). A unit of glycogen synthase is defined as the amount of enzyme required to transfer one μmole of glucose from UDP-glucose to glycogen in one minute under conditions of the standard assay. When determining primer $K_m$'s, where very low substrate concentrations were necessary, the reaction was stopped by addition of 1/50 volume of 1 M acetic acid and 1 mg/ml carrier polysaccharide was added to the test mixture before spotting on filter paper. This procedure increased the reproducibility of the assay, presumably because the carrier glycogen prevents the loss of the very low concentration of $^{14}$C-glycogen in the assay during the wash procedures. Since the phosphorylase-treated dextrin III and DP87 samples might be slightly soluble in 66% ethanol, a special assay was devised for these samples. After the reaction was
stopped by addition of 1 M acetic acid and carrier polysaccharide added as described above, the reaction mixture was adjusted to 80% v/v ethanol and the polysaccharide was allowed to precipitate in an ice bath for 30 minutes. The mixture was then centrifuged at 10,000 xg for 5 minutes and the pellet taken up in 0.1 ml of water. The washed pellet was once again adjusted to 80% v/v with absolute ethanol, precipitated in the cold and collected by centrifugation. After precipitating four times with ethanol, control samples showed that all detectable radioactive UDP-glucose had been washed out of the pellet. The pellet was then taken up in 0.1 ml of water and a 70 μl aliquot was transferred to filter paper, dried under a heat lamp and counted in a liquid scintillation counter. Carbohydrate determinations on resuspended pellets from the final ethanol precipitation indicated that less than 10 percent of the carbohydrate was lost during the ethanol precipitation steps. When the β-amylase-treated oyster glycogen sample was assayed by this method, the same Km value was obtained as with the previous method (73 μM end groups for the washed filter paper method and 76 μM end groups for the 80% ethanol precipitation method).

Synthase phosphatase Activity of phosphatases on synthase D was determined by the rate of conversion of synthase D to synthase I. Synthase D was preincubated at 30°C in 50 mM Tris-HCl, 5 mM dithiothreitol, 10 mM MgCl₂ and 100 mM Na₂SO₄ at pH 7.5. In experiments where glycoprotein-free synthase D was used, the preincubation buffer also contained 10% v/v glycerol. The reaction was started by addition of phosphatase, and aliquots were diluted 10 to 20-fold in a stopping buffer
at 4°C to terminate the reaction. The stopping buffer contained 50 mM Tris-HCl, 20 mM dithiothreitol, 1 or 10 mg/ml rabbit liver glycogen, 5 mM EDTA and 50 to 100 mM NaF. Aliquots of the stopped reaction were then assayed for synthase I and total synthase and the rate of conversion of synthase D to synthase I was calculated.

**Alkaline phosphatase**  This enzyme was assayed by hydrolysis of p-nitrophenyl phosphate as previously described (62,63), except that the assay buffer was 50 mM Tris-HCl, pH 7.5, and the assay temperature was 30°C. A unit of alkaline phosphatase is defined as the amount of enzyme necessary to form one μmole of p-nitrophenol from p-nitrophenyl phosphate per minute under the assay conditions specified above.

**Histone phosphatase**  Histone phosphatase activity was assayed by release of radioactivity from 32P-phosphohistone as previously described (35), except that the reaction mixture contained 0.1 M NaCl instead of MgCl₂.

**Protein kinase**  Protein kinase activity was determined as previously described (64).

**Analytical methods**

Protein concentrations were determined by the method of Lowry et al. (65), using crystalline bovine serum albumin as the standard.

Average chain length (CL) of polysaccharides was determined by hydrolysis with Pseudomonas isoamylase, an α-1,6-glucanohydrolase which has been shown to totally debranch glycogen (66). Approximately 6 mg of polysaccharide sample was incubated with 885 U of isoamylase in 0.4 ml of sodium acetate buffer, pH 3.5. After incubation at 30°C for 20 hours,
aliquots were assayed for reducing end groups by the ferricyanide reduction method (67). Exterior chain length (ECL) was determined by incubation of the polysaccharide with β-amylase as described previously (52). In both the isoamylase treatment and the β-amylase treatment, controls were prepared using enzyme which had been boiled for 10 minutes. This procedure corrects for any materials in the reaction mixture which might interfere with the determination of reducing equivalents released by the enzymes. The β-amylase preparation was incubated with maltose to correct for contaminating α-1,4-glucosidase activity which has been shown to be present in some preparations of crystalline β-amylase (68). No maltose-cleaving activity was found in this preparation.

Total carbohydrate concentration of samples was determined by the method of Dubois et al. (69), using glucose as the standard. Glycogen concentration in crude tissue fractions was determined on 66% v/v ethanol-precipitated material from the fractions.
RESULTS

Purification and Properties of Bovine Heart Glycogen Synthase D

Table 1 shows the results of the purification of glycogen synthase using the purification scheme described under "Methods." Three hearts were used and each heart was processed separately to the ethanol precipitation step following the aminobutylagarose column, then the preparations were combined for ultracentrifugation. This preparation was used for most of the alkaline phosphatase studies and as starting material for the preparation of glycogen-free synthase D. The final preparation contained 10 mg/ml glycogen, and 1.5% of the total synthase activity was in the synthase I form.

Homogeneity of the preparation was checked by SDS gel electrophoresis as described previously (70,71). The enzyme was heterogeneous as shown in Figure 1 as there were five minor bands as well as one major band with a molecular weight of 86,000. This band corresponds well with the subunit molecular weight of synthase D from a variety of tissues (15,16,18-20).

When radioactive synthase D was prepared by the modified procedure described in "MATERIALS AND METHODS", the SDS gel pattern showed two protein bands (inset B of Figure 1). One of the bands had a molecular weight of 86,000. The other band was very faint and had a molecular weight of approximately 132,000. The specific activity of the synthase D preparation used in this gel was 32 U/mg protein. The synthase D
Table 1. Purification of bovine heart glycogen synthase

<table>
<thead>
<tr>
<th>Purification Procedure</th>
<th>Volume (ml)</th>
<th>Units/ml</th>
<th>Total Units</th>
<th>Protein mg/ml</th>
<th>Units/mg protein</th>
<th>Yield %</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude supernatant</td>
<td>4023</td>
<td>0.316</td>
<td>1273</td>
<td>24.3</td>
<td>0.013</td>
<td>(100)</td>
<td>1</td>
</tr>
<tr>
<td>First ethanol pellet</td>
<td>584</td>
<td>1.616</td>
<td>943</td>
<td>23.8</td>
<td>0.068</td>
<td>74</td>
<td>5.23</td>
</tr>
<tr>
<td>78,000 x g #1 supernatant</td>
<td>512</td>
<td>1.392</td>
<td>713</td>
<td>17.8</td>
<td>0.078</td>
<td>56</td>
<td>6.00</td>
</tr>
<tr>
<td>78,000 x g #2 pellet</td>
<td>79</td>
<td>5.65</td>
<td>446</td>
<td>12.6</td>
<td>0.45</td>
<td>35</td>
<td>34.6</td>
</tr>
<tr>
<td>Incubation with amylase</td>
<td>79</td>
<td>(0.899)(^a)</td>
<td>(71)(^b)</td>
<td>12.6</td>
<td>(0.071)(^b)</td>
<td>(5.6)(^b)</td>
<td>(5.46)(^b)</td>
</tr>
<tr>
<td>Pooled fractions from aminobutyl-agarose</td>
<td>243</td>
<td>(0.123)(^b)</td>
<td>(30)(^b)</td>
<td>0.065</td>
<td>(1.90)(^b)</td>
<td>(2.4)(^b)</td>
<td>(146)(^b)</td>
</tr>
</tbody>
</table>

\(^a\)Starting material was one fresh beef heart and two frozen hearts. Each heart was processed separately through the second ethanol precipitation and then the preparations were combined for centrifugation through 50% v/v sucrose.

\(^b\)Following amylase treatment there was apparently some reversible inactivation in these fractions. After ethanol precipitation, step 7, there was an increase in the synthase activity.
Table 1. (Continued)

<table>
<thead>
<tr>
<th>Purification Procedure</th>
<th>Volume (ml)</th>
<th>Units/ml</th>
<th>Total Units</th>
<th>Protein mg/ml</th>
<th>Units mg protein</th>
<th>Yield %</th>
<th>Purification</th>
</tr>
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<tr>
<td>Second ethanol pellet</td>
<td>2.76</td>
<td>41.3</td>
<td>114</td>
<td>8.57</td>
<td>4.82</td>
<td>8.9</td>
<td>371</td>
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<tr>
<td>Sucrose Pad pellet&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5</td>
<td>14.2</td>
<td>35.6</td>
<td>1.05</td>
<td>13.6</td>
<td>2.8</td>
<td>1046</td>
</tr>
<tr>
<td>15% ethanol pellet&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>30.1</td>
<td>30.1</td>
<td>2.63</td>
<td>11.4</td>
<td>2.4</td>
<td>876</td>
</tr>
</tbody>
</table>

<sup>c</sup>These two fractions were pooled for the final preparation.
Figure 1. SDS gel electrophoresis of synthase D preparations. Five percent w/v polyacrylamide gels containing 1:29 w/w N,N'-methylenebisacrylamide to acrylamide were prepared in 0.6 by 10 cm gel tubes. Proteins were incubated in 4 M urea, 1% SDS, and 50 mM Tris-HCl buffer, pH 7.5, for 30 minutes at 45°C before applying to gels. Electrophoresis was performed in a Shandon analytical polyacrylamide electrophoresis apparatus at 7 ma per tube. Standard proteins and the synthase D samples were run in separate gel tubes. Ten to twenty µg of each standard protein was applied to the gel in a volume of 40 µl, except in the case of the 32P-labeled synthase D sample which was more dilute. About 3 to 5 µg of this sample was applied. Inset A shows the gel pattern of the synthase D preparation from Table 1. Inset B shows the gel pattern of the 32P-labeled synthase D preparation. Symbols for standard proteins and their molecular weights are: BSA2, bovine serum albumin dimer, 132,000 (70); PHOS a, rabbit muscle phosphorylase a, 94,000 (71); BSA, bovine serum albumin, 66,000 (70); LDH, rabbit muscle lactate dehydrogenase, 36,000 (71). The arrow shows the position of synthase D as determined with either of the two preparations used.
SUBUNIT MOLECULAR WEIGHT \( \times 10^{-3} \)

MOBILITY RELATIVE TO LDH

- \( \text{BSA}_2 \)
- \( \text{PHOS} \)
- \( \text{BSA} \)
- \( \text{LDH} \)

A
B
contained two moles of $^{32}\text{P}$-labeled phosphate per mole of subunit, indicating that conversion of synthase I to synthase D during the preparation of the synthase D resulted in phosphorylation of two sites on the synthase. Since bovine heart synthase I has never been purified, its phosphate content is not known; hence it is not possible to determine the total phosphate content of synthase D prepared from bovine heart synthase I. It has been demonstrated that skeletal muscle synthase I contains no alkali-labile phosphate (15).

When glycogen was removed from synthase D as described under "MATERIALS AND METHODS", the resulting preparation had little activity in the absence of added polysaccharide primer. Figure 2 gives a comparison of the activity of the glycogen-free synthase without added primer and in the presence of 10 mg/ml of β-amylase-treated oyster glycogen. Although the β-amylase dextrin is a very poor primer when compared to native glycogens (giving only 8% of the activity given by glycogen under the experimental conditions used here), the activity of the synthase was greatly increased when this dextrin was included in the reaction. The possibility existed that endogenous primer was soluble in 66% ethanol and would not be detected in the standard assay procedure which involves repeated washing with 66% ethanol to remove unreacted UDP-glucose. To rule out this possibility, a sample of the synthase was incubated under the same conditions as in Figure 2 without added primer. After 10 minutes, 50 mg of Amberlite MB-3 was added to remove UDP-glucose from the reaction mixture. Following a 30 minute incubation with the ion exchange resin, a 40 μl aliquot of the reaction mixture was spotted on a
Figure 2. Activity of glycogen-free synthase D in the absence of added primer and in the presence of β-amylase-treated oyster glycogen. Glycogen-free synthase D was incubated at a concentration of 40 mU/ml in a reaction mixture containing 2.5 mM UDP-\(^{14}\)C-glucose (specific radioactivity 2.5 \(\times\) \(10^6\) cpm/\(\mu\)Mole), 10 mM MgCl\(_2\), 10 mM Na\(_2\)SO\(_4\), 5 mM glucose-6-phosphate, 10 % v/v glycerol and 50 mM Tris-HCl, pH 7.5. The synthase was incubated either in the absence of added primer (○ ○), or in the presence of 10 mg/ml of β-amylase-treated oyster glycogen (● ●) prepared as previously described (52). After 5 minutes at 30°C, an aliquot of the reaction mixture was transferred to a filter paper square for determination of synthase activity as previously described (50).
20 cm x 20 cm sheet of Whatman 3 mm chromatography paper along with a UDP-\(^{14}\)C-glucose standard and a control which was prepared by repeating the above procedure using enzyme which had been boiled for 1 minute. Chromatography was done in an ascending system using 66% v/v ethanol as the solvent. The chromatography was stopped when the solvent front reached the top of the paper. The chromatogram was dried, cut into 1 cm sections and counted in a liquid scintillation system. Chromatography of the reaction mixture gave two peaks of radioactivity, one very small peak at the origin (66 cpm) and a larger peak (205 cpm) which migrated with the UDP-glucose standard. The control also had the larger peak, but the small peak at the origin was missing. Since the small peak was only present in the sample incubated with active synthase, it would appear to be the product of the synthase reaction. This small peak did not migrate with 66% ethanol, indicating that it would probably not be removed in the 66% ethanol wash steps of the synthase assay. Furthermore, the amount of product formed at the origin of the chromatogram was comparable to radioactivity incorporated in the synthase assay without added primer (Figure 2). The larger radioactivity peaks observed with both experimental and control samples were probably traces of UDP-glucose which were not removed completely by the ion exchange resin. The endogenous primer in these experiments may be present in the glycogen-free synthase preparation. Alternatively, there may be contaminating primers in one or more of the reagents used in the synthase assay. Abdullah et al. (72) described a primer contaminating a commercial glucose-1-P preparation in studies on phosphorylase activity in the direction of polysaccharide
synthesis. In any case, the very low level of endogenous primer did not interfere with the primer specificity studies described later. The polysaccharide inhibition studies were all done using the same concentration of synthase D so that any contribution of an endogenous polysaccharide to phosphatase inhibition would be the same for all samples.

The molecular weight of the glycogen-free synthase D was estimated by gel filtration using Sepharose 6B. Figure 3 gives the molecular weight estimation of the synthase compared to rabbit skeletal muscle phosphorylase b, lactate dehydrogenase and urease which were used as calibration standards. The synthase D gave a single symmetrical peak indicating the presence of only one molecular form of the enzyme. By comparison with the standards, the synthase has a molecular weight of approximately 490,000 daltons.

Figure 4 shows the pattern of the glycogen-free synthase D on sucrose density ultracentrifugation. An aliquot of glycogen-free synthase was diluted with four parts of a 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM dithiothreitol and incubated at 30°C for 15 minutes. The enzyme was then placed in an ice bath and 100 μg of phosphorylase b and 25 μg of lactate dehydrogenase were added as internal standards for the ultracentrifugation. The duration of the run was 12 hours. Synthase D migrated as a single symmetrical peak as did the two standards. The molecular weight of the synthase was estimated by comparing the peak position of the synthase with the peak positions of each of the two standards (73). Compared to the lactate dehydrogenase peak the synthase D molecular weight was 233,000, and compared to the phosphorylase b peak
Figure 3. Gel filtration of glycogen-free synthase D on Sepharose 6B. A 0.8 x 12 cm column of Sepharose 6B was equilibrated with 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol and 25% v/v glycerol. Freshly prepared glycogen-free synthase at a concentration of 3 U/ml was applied in the same buffer along with 70 µg phosphorylase b and about 25 µg lactate dehydrogenase as internal standards in a total volume of 0.3 ml. Chromatography was carried out at room temperature. The flow rate was 3 ml/hour. The urease standard was run separately and lactate dehydrogenase was included as an internal standard in this run also. Symbols used for standard proteins and their molecular weights are: LDH, rabbit skeletal muscle lactate dehydrogenase, 140,000 (74); PHOS b, rabbit skeletal muscle phosphorylase b, 200,000 (75); UREASE, jack bean urease, 490,000 (76). Both urease and synthase D were included in the gel matrix since \( \frac{V_o}{V_{LDH}} \) was 0.52 as determined from the elution profile for Blue Dextran 2000.
Figure 4. Sucrose density ultracentrifugation of glycogen-free synthase D. Three-tenths unit of glycogen-free synthase D in 0.2 ml of 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol and 12.5% v/v glycerol was layered on a 5 ml zero to twelve and one-half percent v/v sucrose gradient in the same buffer. The ultracentrifugation was done at 35,000 rpm in an SW-50.1 rotor at 0°C for 12 hours as previously described (27). Protein standards used were rabbit muscle lactate dehydrogenase (LDH) and rabbit muscle phosphorylase b (PHOS b). The arrows indicate the peak positions of the standards.
the molecular weight was 260,000. The molecular weight of lactate dehydrogenase is taken as 140,000 (74), and phosphorylase b dimer has a molecular weight of 200,000 (75).

Studies on Dephosphorylation of Synthase D
by *E. coli* Alkaline Phosphatase

In early studies it was discovered that incubation of glycogen synthase D with commercial *E. coli* alkaline phosphatase resulted in a time dependent conversion of the synthase D to a form which did not require glucose-6-P for activity. Subsequently, it was found that incubation of this alkaline phosphatase-treated synthase with cyclic-AMP dependent protein kinase and ATP resulted in reconversion of the synthase I to synthase D. The time course for conversion of synthase D to synthase I by alkaline phosphatase and the reversal of this conversion by cyclic-AMP dependent protein kinase and ATP is shown in Figure 5. In order to reverse the alkaline phosphatase-catalyzed conversion of synthase D to synthase I, it was necessary to first remove the alkaline phosphatase from the reaction mixture. If this was not done there was no noticeable reconversion by kinase even if 50 mM KF, a known inhibitor of protein phosphatases (77,78), was included in the incubation. KF does not inhibit *E. coli* alkaline phosphatase, and it is likely that the phosphatase hydrolyzed the ATP present in the reaction mixture before the kinase could phosphorylate the synthase. Alkaline phosphatase was removed from the reaction mixture by gel filtration on a small Sepharose 6B column. Since the synthase is bound to glycogen, it is largely excluded from
Figure 5. Alkaline phosphatase catalyzed conversion of synthase D to synthase I and reconversion to synthase D by cyclic-AMP-dependent protein kinase. Synthase D was incubated at a concentration of 1.5 U/ml in the standard synthase phosphatase reaction mixture (0.5 x 5 cm). Alkaline phosphatase was added to a final concentration of 5 U/ml. After 2 hours at 30°C the sample was chromatographed on a small column of Sepharose 6B as described in "MATERIALS AND METHODS" to remove alkaline phosphatase. Only that portion of the synthase recovered which was free of alkaline phosphatase was used in the kinase reaction, and it represented 40% of the original synthase activity. Cyclic-AMP-dependent protein kinase was added to the synthase along with ATP to 10 mM, cyclic-AMP to 10 μM and MgCl₂ to 5 mM.
Sepharose 6B and could be readily separated from the relatively small alkaline phosphatase. The synthase from the column was then incubated with 10 mM ATP, 5 mM MgCl$_2$, 10 $\mu$M cyclic-AMP and partially purified cyclic-AMP dependent protein kinase (the aminobutylagarose fraction described in "MATERIALS AND METHODS"). Figure 5 shows that the alkaline phosphatase-catalyzed conversion could be completely reversed by protein kinase. When $^{32}$P-labeled synthase D was incubated in the presence of alkaline phosphatase, the conversion reaction catalyzed by alkaline phosphatase was paralleled by release of $^{32}$P from synthase D (Figure 6). The complete conversion of synthase D to synthase I was accompanied by complete removal of synthase-bound $^{32}$P-labeled phosphate. In an experiment using $\gamma$-$^{32}$P-labeled ATP, it was found that the reconversion of the synthase I to synthase D by cyclic-AMP dependent protein kinase was paralleled by incorporation of $^{32}$P into protein.

Figure 7 shows that the time course of synthase D dephosphorylation by alkaline phosphatase was linear for at least 15 minutes. The time course was linear over a nine-fold range of alkaline phosphatase concentrations, and at least 15% of the synthase D could be converted to synthase I without affecting the linearity of the reaction. Figure 8 shows that the rate of conversion of synthase D to synthase I is linearly dependent on alkaline phosphatase concentration.

Characterization of the Synthase Phosphatase Activity Associated with Alkaline Phosphatase

While the reversal of alkaline phosphatase catalyzed dephosphorylation of synthase D by cyclic-AMP dependent protein kinase indicated the
Figure 6. Correlation of conversion of synthase D to synthase I with release of protein-bound phosphate by alkaline phosphatase. The incubation conditions used in this experiment were the same as in Figure 5, except that $^{32}$P-labeled synthase D was used as substrate. Aliquots of the reaction mixture were removed and assayed for synthase D to I conversion (•—•) or $^{32}$P released from trichloroacetic acid insoluble materials (○—○) as described in "MATERIALS AND METHODS." A control reaction mixture was incubated under the same conditions except that no alkaline phosphatase was included. After 65 minutes there was no detectable $^{32}$P released nor conversion to synthase I in the control.
Figure 7. Time course of conversion of synthase D to synthase I by *E. coli* alkaline phosphatase. Synthase D was incubated in the standard synthase phosphatase reaction buffer described in "MATERIALS AND METHODS" at a concentration of 1.5 U/ml. Alkaline phosphatase was present at varying concentrations: (◊ — ◊), 1.8 U/ml; (▲ — ▲), 1.4 U/ml; (Δ — Δ), 0.9 U/ml; (● — ●), 0.45 U/ml; (○ — ○), 0.22 U/ml. Aliquots were taken at 3 minute intervals of incubation and percent synthase I determined.
Figure 8. Dependency of the conversion rate of synthase D to synthase I on the concentration of alkaline phosphatase. The conversion rates determined from the data in Figure 7 were plotted against alkaline phosphatase concentration.
phosphatase nature of the catalyst, it was necessary to demonstrate rigorously that the activity on synthase D was associated with the alkaline phosphatase itself and not a contaminating protein phosphatase in the alkaline phosphatase preparation.

Figure 9 shows the effect of prolonged heating at 89°C on alkaline phosphatase activity and the synthase D activity associated with the alkaline phosphatase preparation. The experimental protocol was designed so that only irreversible denaturation of alkaline phosphatase would be observed. This was necessary since the synthase phosphatase assay requires a longer incubation time than the alkaline phosphatase assay. The alkaline phosphatase was incubated in 30 µl aliquots in covered 250 µl plastic tubes, and tubes were removed to a 30°C water bath after a given length of time at 89°C. Alkaline phosphatase and synthase D phosphatase activities of the samples were measured approximately one hour after the last sample was transferred to the 30°C bath. Under these conditions, only irreversible heat denaturation of the alkaline phosphatase is measured (79). Heat treatment of the alkaline phosphatase resulted in a simultaneous loss of activity on synthase D and p-nitrophenyl phosphate. The half-life for both activities was about 50 minutes. Stability at very high temperatures is a well-documented characteristic of E. coli alkaline phosphatase (62,79), while protein phosphatases are usually very temperature sensitive (36,47,64). The observation that the synthase phosphatase activity is relatively insensitive to heat treatment lends additional evidence that it is an activity of the alkaline phosphatase itself.
Figure 9. Heat inactivation of alkaline phosphatase activities on synthase D and p-nitrophenyl phosphate. Alkaline phosphatase was incubated at 89°C at a concentration of 4 U/ml in 50 mM Tris-HCl buffer, pH 7.5. Aliquots were removed and incubated at 30°C prior to determination of synthase phosphatase and p-nitrophenyl phosphate phosphatase activity as described in "MATERIALS AND METHODS." Open circles indicate activity on synthase D, and closed circles indicate activity on p-nitrophenyl phosphate.
Since *E. coli* alkaline phosphatase is denatured by reducing agents (80), inactivation of synthase phosphatase and alkaline phosphatase activities by dithiothreitol was examined. As Figure 10 shows, both activities were sensitive to dithiothreitol treatment and the rate of activity loss was the same whether synthase D or p-nitrophenyl phosphate was used as substrate. Under the conditions of the experiment, which were similar to the conditions used in the synthase phosphatase assay, the half-life of both activities was about 150 minutes extrapolated from the region of first order decay. After 150 minutes in the presence of dithiothreitol, little or no activity was lost using either synthase D or p-nitrophenyl phosphate as substrate. This deviation from first-order denaturation kinetics for both activities further supports the suggestion that the activities are both properties of the same enzyme, i.e., not only are the rates of denaturation the same, but the shapes of the denaturation curves for the different activities are identical. Dithiothreitol does not interfere in the synthase phosphatase assay when using alkaline phosphatase because the duration of the assay is much shorter (10 to 20 minutes) than the half-life for dithiothreitol denaturation.

Attempts were made to separate the synthase phosphatase activity from alkaline phosphatase by gel filtration using Sephadex G-200. The elution pattern from such an experiment is given in Figure 11. The two activities appeared as a single symmetrical peak which was included in the gel matrix, indicating that the synthase phosphatase and alkaline phosphatase activities are very similar in size.
Figure 10. Inactivation of alkaline phosphatase activities on synthase D and p-nitrophenyl phosphate by dithiothreitol. Alkaline phosphatase was incubated as in Figure 9 except that the temperature was 30°C, and dithiothreitol was present at a concentration of 20 mM. Aliquots were removed for determination of synthase phosphatase and p-nitrophenyl phosphate phosphatase activities at various times. Open circles indicate activity on synthase D, and closed circles indicate activity on p-nitrophenyl phosphate.
Figure 11. Sephadex G-200 gel filtration of alkaline phosphatase; co-elution of activities on synthase D and p-nitrophenyl phosphate. Ten units of alkaline phosphatase in 0.3 ml of 50 mM Tris-HCl, pH 7.5, was applied to a 0.8 by 14 cm column of Sephadex G-200 equilibrated in the same buffer. The fractions from the column were assayed for synthase phosphatase activity and activity on p-nitrophenyl phosphate. The arrows indicate the exclusion volume of the column, determined by the elution volume of Blue Dextran 2000 (BD2000), and the elution volume of cytochrome c (CYTO c). Open circles represent activity on p-nitrophenyl phosphate, and closed circles give synthase phosphatase activity.
The synthase phosphatase and alkaline phosphatase activities could not be separated by ion exchange chromatography. A sample of the preparation was applied to a column of DE-52 cellulose, and the phosphatase activities were eluted with a NaCl gradient. The results are presented in Figure 12. The synthase phosphatase and alkaline phosphatase activities were recovered in the same fractions and the activity peaks coincided exactly. The peak tubes from this experiment were pooled, concentrated by ultrafiltration in an Amicon 8-MC ultrafiltration device with a PM-10 membrane, and then dialyzed against 10 mM Tris-HCl, pH 8.5. The DE-52 chromatography was then repeated with a much shallower gradient of NaCl (0 to 0.1 M NaCl in a 50 ml gradient). The results were the same as in the first chromatography. The peak tubes of synthase phosphatase and alkaline phosphatase activity coincided, and the elution profiles for both activities were very similar.

If *E. coli* are grown under the proper set of conditions, multiple forms of alkaline phosphatase can be purified from the organism (79). These forms differ in content of tightly bound phosphate (81) and can be separated by DE-52 anion exchange chromatography. The commercial alkaline phosphatase preparations used in the present study contained significant amounts of only one form. To further investigate the nature of the synthase phosphatase activity associated with alkaline phosphatase, strain K12 *E. coli* were grown under conditions known to produce multiple forms of alkaline phosphatase. Alkaline phosphatase was purified from the bacteria as described in "MATERIALS AND METHODS." Figure 13 shows the results of DE-52 cellulose chromatography of the alkaline phosphatase.
Figure 12. DEAE-cellulose chromatography of commercial alkaline phosphatase. Ten units of alkaline phosphatase in 10 mM Tris-HCl buffer, pH 8.5, was applied to a 4.5 ml column of DE-52 microgranular DEAE-cellulose in the same buffer. The phosphatase was eluted with a 0 to 0.125 M NaCl gradient in 20 ml of buffer. The effluent was assayed for synthase phosphatase activity (o—o) and activity on p-nitrophenyl phosphate (●—●). The NaCl concentration was determined by conductivity measurement against standard NaCl solutions (---······)
ALKALINE PHOSPHATASE (O.D. 410)

SYNTHASE PHOSPHATASE (cpm)

FRACTION NUMBER

[NaCl], M
Figure 13. DEAE-cellulose chromatography of *E. coli* strain K12 alkaline phosphatase. Approximately 10 units of this enzyme from the osmotic shock extraction step were applied to a 20 ml column of DE-52 cellulose. The phosphatase was eluted with a salt gradient as described in Figure 12 except that the total gradient volume was 500 ml. Fractions were assayed for synthase phosphatase activity (○ — ○), phosphohistone phosphatase activity (● — ●) and activity on p-nitrophenyl phosphate (▲ — — ▲). The NaCl concentration (●●●●) was also measured.
preparation. Phosphatase activities on p-nitrophenyl phosphate, synthase D and phosphohistone were measured. Synthase phosphatase and phosphohistone phosphatase activities were associated with two of the alkaline phosphatase peaks, but the alkaline phosphatase peak eluting at low salt concentrations had little activity on synthase D and virtually no activity on phosphohistone. The possibility of a contaminating inhibitor of alkaline phosphatase activity on synthase D and phosphohistone being present in the low salt alkaline phosphatase fraction was explored. It was discovered that Sephadex G-200 gel filtration of the low salt peak increased the activity of the phosphatase on synthase D and phosphohistone. After gel filtration, the low salt alkaline phosphatase had activity on the protein substrates equivalent to the other two peaks. The results indicate that there was an inhibitor of synthase phosphatase and phosphohistone phosphatase activities of alkaline phosphatase in the low salt fraction, and gel filtration removed the inhibitor. The observation that the synthase phosphatase and histone phosphatase activities of alkaline phosphatase exist in multiple forms which also have activity on p-nitrophenyl phosphate is evidence that these protein phosphatase activities are properties of all forms of alkaline phosphatase irrespective of the amount of phosphate bound to the enzyme. It is clear that the synthase phosphatase activity of commercial alkaline phosphatase is not due to a contaminant activity of one of the enzyme forms with a different phosphate content.
Alkaline Phosphatase Activity on Other Proteins

As Figure 13 shows, all forms of alkaline phosphatase could dephosphorylate phosphohistone as well as glycogen synthase. Since other investigators have described the activity of alkaline phosphatases on phosphohistone (82, 83) activity on this substrate was examined more closely. Figure 14 shows a time course of phosphohistone dephosphorylation by alkaline phosphatase. Alkaline phosphatase was not able to completely dephosphorylate the phosphohistone and addition of another 1 U/ml alkaline phosphatase after 25 hours of incubation did not release more phosphate from the phosphohistone. In similar experiments, it was demonstrated that removal of phosphate released from the phosphohistone during incubation with alkaline phosphatase by dialysis did not increase the amount of $^{32}$P-labeled phosphate released from the phosphohistone. Addition of more alkaline phosphatase to the dialyzed reaction mixture also did not result in release of more phosphate. Therefore, the incomplete dephosphorylation of phosphohistone was not the result of product inhibition by phosphate.

Alkaline phosphatase activity on phosphorylase a was also examined. Under a variety of reaction conditions no activity was found. Phosphorylase phosphatase was assayed in the following manner. Phosphorylase a was dialyzed for two days at 5°C against 50 mM Tris-HCl, 5 mM dithiothreitol, pH 7.5, and then preincubated with the same buffer plus any additions at 30°C for 5 minutes. Reaction was started by the addition of alkaline phosphatase. Aliquots were removed at various times during the incubation and diluted ten to one hundred-fold in a stopping buffer
Figure 14. Dephosphorylation of $^{32}$P-phosphohistone by alkaline phosphatase. $^{32}$P-phosphohistone was incubated in 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol and 0.1 M NaCl at 30°C. Phosphohistone concentration was 1.2 mg/ml. Alkaline phosphatase was added at a concentration of 1 U/ml, and 50 μl aliquots were removed for phosphohistone phosphatase assay as described in "MATERIALS AND METHODS." After 25 hours at 30°C, an additional 1 U/ml alkaline phosphatase was added.
containing 40 mM β-glycerophosphate, pH 6.0, and 1 mg/ml bovine serum albumin. Alkaline phosphatase has no activity at pH 6.0. Phosphorylase activity was then measured colorimetrically (84) in the absence and presence of AMP to determine the extent of conversion of the phosphorylase a to phosphorylase b. When 0.2 mg/ml phosphorylase a was incubated with 8 U/ml alkaline phosphatase, no phosphorylase phosphatase activity could be detected after 150 minutes of incubation at 30°C. Addition of glucose at 5 mg/ml or theophylline at 2.5 mM had no significant effect.

Although both synthase D and phosphohistone are substrates for E. coli alkaline phosphatase, they are extremely poor substrates compared to small phosphoesters and phosphoanhydrides. A comparison of the activity of alkaline phosphatase on synthase D, phosphohistone and γ-32P-ATP, each substrate being present at a concentration of 1 μM 32P-phosphoester bond or 32P-phosphoanhydride bond, illustrates the magnitude of difference in reaction rate between protein substrates and small substrates. Taking the relative reaction rate on synthase D as unity, the rate of phosphohistone dephosphorylation is 3.2 and the rate of hydrolysis of the γ-phosphate of ATP is 826, almost three orders of magnitude greater than the rate of dephosphorylation of synthase D.

Effect of Cations and Anions on Synthase Phosphatase Activity of Alkaline Phosphatase

Since a number of protein phosphatases are stimulated by divalent cations (35,36,38-41), it was of interest to see if the alkaline phosphatase-catalyzed conversion of synthase D to synthase I was specifically
stimulated by divalent cations. These experiments were of particular interest because it is now known that $\text{Mg}^{2+}$ has a direct effect on bovine heart synthase D activity (85). Divalent cations could affect synthase phosphatase by binding to the phosphatase, the synthase D, or both phosphatase and synthase D. Cation specificity experiments with alkaline phosphatase were designed to assess the role of cation binding to synthase D in the synthase phosphatase reaction. It is well known that the activity of \textit{E. coli} alkaline phosphatase on p-nitrophenyl phosphate is not greatly stimulated by a variety of cations even when assayed at low ionic strength (79). Moreover, the stimulation is not cation specific and appears to be related to the ionic strength increment produced by addition of the cation (79). Table 2 gives the results of a survey of cations as effectors of alkaline phosphatase activity on p-nitrophenyl phosphate and synthase D. The data show that the synthase phosphatase activity of alkaline phosphatase is specifically stimulated by divalent cations, especially $\text{Mn}^{2+}$, but the activity on p-nitrophenyl phosphate was more related to the buffer ionic strength. Of the cations tested, $\text{Mn}^{2+}$ gave the greatest activation of the synthase phosphatase activity. This is in agreement with results others have reported using mammalian synthase phosphatases (35,36). Addition of both $\text{Mg}^{2+}$ and $\text{Mn}^{2+}$ at 10 mM resulted in the same activity as $\text{Mn}^{2+}$ alone, suggesting that these cations may interact at the same site. Addition of 150 mM NaCl and 10 mM $\text{Mg}^{2+}$ together resulted in the same activity as $\text{Mg}^{2+}$ alone, indicating that ionic strength does not greatly influence the activity of the alkaline phosphatase on synthase D either in the presence or absence of
Table 2. Cation effects on alkaline phosphatase activity with glycogen synthase D and p-nitrophenyl phosphate as substrates

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Relative Activity on p-Nitrophenyl phosphate&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Buffer Ionic Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>~40</td>
<td>79</td>
<td>0.04</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;, 10 mM</td>
<td>118</td>
<td>103</td>
<td>0.09</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;, 10 mM</td>
<td>(100)</td>
<td>(100)</td>
<td>0.09</td>
</tr>
<tr>
<td>MnCl&lt;sub&gt;2&lt;/sub&gt;, 10 mM</td>
<td>280</td>
<td>93</td>
<td>0.09</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;, 10 mM + MnCl&lt;sub&gt;2&lt;/sub&gt;, 10 mM</td>
<td>270</td>
<td>103</td>
<td>0.14</td>
</tr>
<tr>
<td>NaCl, 150 mM</td>
<td>75</td>
<td>137</td>
<td>0.19</td>
</tr>
<tr>
<td>NaCl, 150 mM, + MgCl&lt;sub&gt;2&lt;/sub&gt;, 10 mM</td>
<td>100</td>
<td>134</td>
<td>0.24</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard assay conditions were 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol.

<sup>b</sup> Synthase D was present at a concentration of 2 U/ml in the assay mixture described above. Synthase phosphatase was assayed as described in "MATERIALS AND METHODS." Alkaline phosphatase concentration was 1.5 U/ml.

<sup>c</sup> The concentration of p-nitrophenyl phosphate was 2 mM NaOH in the standard assay mixture. Alkaline phosphatase was present at a concentration of 3 mU/ml. The samples were incubated at 30° C in the presence of the alkaline phosphatase, and the reaction terminated by adding one-half volume of 0.2 M. The absorbance at 410 nm was then determined.
divalent cations. A number of other cations were tested, including Zn\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\) and Cr\(^{3+}\), but these metal ions inhibited synthase activity at the concentrations used. In similar experiments it was found that K\(^+\), like Na\(^+\), gave little stimulation of the activity on synthase D even at high concentrations (140 mM). The specific divalent cation effects on the synthase phosphatase activity of alkaline phosphatase suggest that the activation is a result of cations binding to the synthase D. In a separate experiment the concentration dependence of the Mg\(^{2+}\) activation was determined under the conditions used in Table 2. Half-maximal activation was observed at 6 mM Mg\(^{2+}\). This value is similar to the \(A_2\) determined previously for activation of synthase D in the standard synthase D reaction conditions (85).

The effect of various anions on the synthase D phosphatase and alkaline phosphatase activities is shown in Table 3. The conditions in this experiment were the same as in Table 2 except that 10 mM MgCl\(_2\) was present under all conditions tested. The activity on p-nitrophenyl phosphate was increased only slightly by addition of the anions, and the activation was related to ionic strength as in the cation experiments. Phosphate was the only exception, since it caused significant inhibition at a concentration of 10 mM. Sulfate specifically activated the synthase phosphatase activity of alkaline phosphatase. The activation occurred at relatively low concentrations of sulfate, and this anion caused greater than two-fold stimulation of synthase phosphatase activity at a concentration of 50 mM. A kinetic plot of the sulfate activation data from this experiment gave half-maximal activation at 4 mM sulfate.
Table 3. Anion effects on alkaline phosphatase activity with glycogen synthase D and p-Nitrophenyl phosphate as substrates

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative Activity&lt;sup&gt;b&lt;/sup&gt; on Synthase D</th>
<th>Relative Activity&lt;sup&gt;c&lt;/sup&gt; on p-Nitrophenyl phosphate</th>
<th>Buffer Ionic Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(100)</td>
<td>(100)</td>
<td>0.09</td>
</tr>
<tr>
<td>NaCl, 10 mM</td>
<td>106</td>
<td>108</td>
<td>0.10</td>
</tr>
<tr>
<td>150 mM</td>
<td>100</td>
<td>134</td>
<td>0.24</td>
</tr>
<tr>
<td>NaNO&lt;sub&gt;3&lt;/sub&gt;, 10 mM</td>
<td>119</td>
<td>111</td>
<td>0.10</td>
</tr>
<tr>
<td>150 mM</td>
<td>160</td>
<td>130</td>
<td>0.24</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;, 5 mM</td>
<td>171</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>10 mM</td>
<td>194</td>
<td>111</td>
<td>0.12</td>
</tr>
<tr>
<td>20 mM</td>
<td>206</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>50 mM</td>
<td>229</td>
<td>124</td>
<td>0.24</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;S&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;, 50 mM</td>
<td>183</td>
<td>122</td>
<td>0.24</td>
</tr>
<tr>
<td>NaH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;, 10 mM</td>
<td>0</td>
<td>24</td>
<td>0.12</td>
</tr>
</tbody>
</table>

<sup>a</sup>The standard incubation mixture as in Table 2, except that 10 mM MgCl<sub>2</sub> was present in all samples.

<sup>b</sup>Synthase D phosphatase activity was determined as in Table 2.

<sup>c</sup>Activity on p-nitrophenyl phosphate determined as in Table 2.
Sulfate has previously been shown to stimulate the activity of bovine heart synthase phosphatase in the absence of glucose-6-P (45). In contrast, chloride did not activate the synthase phosphatase activity of alkaline phosphatase even when present at 150 mM. Nitrate activated at a concentration of 150 mM, but did not activate at 10 mM. Phosphate inhibited the reaction.

Glycogen Inhibition of Alkaline Phosphatase Activity on Synthase D

Since glycogen is a well-known inhibitor of synthase phosphatase in several tissues including skeletal muscle (36,47), cardiac muscle (45), liver (49,86) and polymorphonuclear leukocytes (87), the effect of glycogen on the synthase phosphatase activity of E. coli alkaline phosphatase was studied. Figure 15 compares the glycogen inhibition patterns of alkaline phosphatase and bovine heart protein phosphatase action on synthase D. In this experiment glycogen-free synthase D was used as substrate, and varying amounts of rabbit liver glycogen were added to the reaction mixture. When either protein phosphatase or alkaline phosphatase was used to dephosphorylate synthase D, inhibition by glycogen occurred at very low glycogen concentrations, and there was very little further inhibition by glycogen at concentrations from 0.5 to 25 mg/ml (0.19 mM to 9.6 mM glycogen end groups). Since studies were to be undertaken comparing the effectiveness of polysaccharides as primers for synthase D and synthase phosphatase inhibitors, it was of interest to know whether glucose-6-P and UDP-glucose, both present in the
Figure 15. Inhibition of alkaline phosphatase and protein phosphatase activities on glycogen-free synthase D by rabbit liver glycogen. Synthase phosphatase was determined under the standard conditions defined in "MATERIALS AND METHODS" using either 2 U/ml alkaline phosphatase (•—•) or 5 U/ml bovine heart protein phosphatase (o—o). In some cases, 5 mM glucose-6-P and 25 mM UDP-N-acetylglucosamine were added to the protein phosphatase reaction mixture (Δ—Δ)
synthase assay, would have any effect on the glycogen inhibition pattern. It was not possible to use UDP-glucose in the phosphatase assay since under these conditions the synthase D would actively synthesize glycogen and the structural characteristics of the polysaccharide inhibitor would be changing during the course of the incubation. UDP-N-acetylglucosamine was used instead. This nucleotide sugar does not serve as a glycosyl donor for synthase (88), but it does give competitive inhibition of the synthase reaction with respect to UDP-glucose. The addition of glucose-6-P and UDP-N-acetylglucosamine did not affect the glycogen inhibition curve of the protein phosphatase. It was not possible to do the same experiment with the alkaline phosphatase since glucose-6-P is hydrolyzed by this phosphatase.

Figure 16 shows a Dixon plot of the data from Figure 15. Half-maximal inhibition for either protein phosphatase or alkaline phosphatase occurred at about 100 μg/ml (40 μM end groups) glycogen. The similarity in half-maximal inhibition for glycogen inhibition of the two phosphatases indicates that the glycogen binds to the synthase D to cause inhibition.

While the concentration dependence of glycogen inhibition appears to be independent of the phosphatase used to dephosphorylate synthase, the extent of inhibition at "saturating" concentrations of glycogen is different for alkaline phosphatase than for protein phosphatase. This observation suggests that the nature of the phosphatase acting on synthase D may determine the extent of inhibition by glycogen.

The sensitivity of phosphatase activity to glycogen inhibition
Figure 16. Kinetics of rabbit liver glycogen inhibition of synthase phosphatase activities. Data from Figure 15 were replotted. Open circles show glycogen inhibition of protein phosphatase, and closed circles show glycogen inhibition of alkaline phosphatase activity on synthase D.
\[ \frac{1}{V} \times 10^2 \]

(\% SYNTHASE I)\(^{-1}\)

GLYCOGEN (mg/ml)
at very low glycogen concentrations observed in this experiment is in contrast to previous studies which show glycogen inhibition at much higher concentrations of polysaccharide (36,45,47,49).

Figure 17 shows the glycogen inhibition of alkaline phosphatase activity on a synthase D preparation made as described by Thomas and Larner (58) compared to the inhibition of a heart protein phosphatase on a similar substrate (45). The concentration dependence of glycogen inhibition in this experiment is similar for both phosphatases acting on synthase. A comparison of Figure 15 and Figure 17 indicates that the glycogen inhibition of synthase D dephosphorylation by glycogen depends on the method used to prepare the synthase D since glycogen was much less inhibitory with the more impure substrate. Figure 17 also shows that glycogen did not affect the activity of alkaline phosphatase on p-nitrophenyl phosphate. That glycogen inhibits the activity of alkaline phosphatase on synthase D but not the activity on p-nitrophenyl phosphate clearly indicates that glycogen interacts with synthase D to inhibit synthase phosphatase activities.

The sensitivity of glycogen inhibition of heart synthase phosphatase observed with highly purified synthase D as compared to a synthase D preparation of lower purity could be the result of removing a factor from the highly purified preparation which decreased the binding affinity of the synthase D for glycogen or competed with synthase D for glycogen binding. Alternatively, the purification scheme for synthase D used in the present work may have resulted in the production of an altered synthase D which bound glycogen very tightly. To test the possibility
Figure 17. Glycogen inhibition of alkaline phosphatase activity on synthase D of lower purity. A partially purified bovine heart synthase D described in the text was used in this experiment. Synthase D phosphatase was determined as described in "MATERIALS AND METHODS" except that rabbit liver glycogen was added at increasing concentrations up to 27 mg/ml. The effect of glycogen on the p-nitrophenyl phosphate phosphatase activity of alkaline phosphatase was also determined. The symbols used are (o—o) synthase phosphatase activity, (●—●) p-nitrophenyl phosphate phosphatase activity and (-----) a glycogen inhibition curve previously published from this laboratory for a bovine heart protein phosphatase acting on a similar synthase D preparation (35).
that some factor which affects glycogen binding by synthase D is removed during preparation, a partially purified fraction from beef heart was prepared by ethanol precipitation, and its effect on glycogen inhibition of synthase D dephosphorylation was studied. Figure 18 shows the effect of 30% v/v ethanol precipitated heart fraction on the glycogen inhibition curve for protein phosphatase activity on the highly purified glycogen-free synthase D. The 30% ethanol fraction was prepared essentially as in the early steps of the synthase preparation using 50 g of beef heart as starting material and contained 70 µg/ml glycogen, 1 U/ml glycogen synthase, 70% of which was in the synthase I form, and no detectable synthase phosphatase activity under the conditions of the experiments shown in Figure 18. There was contamination of the synthase D substrate in these experiments by the synthase present in the 30% ethanol fraction. In all experiments this amounted to 10 to 15% of the total synthase present in the phosphatase incubation or about 5% of the synthase D, since the synthase in the ethanol fraction was about 30% synthase D. When diluted in the phosphatase incubations, the glycogen content of the 30% ethanol fraction (14 µg/ml) was lower than the concentration of rabbit liver glycogen required to inhibit the phosphatase to a significant extent. As Figure 18 shows, the 30% ethanol fraction decreased the sensitivity of the synthase phosphatase activity to glycogen inhibition. When assayed in the presence of the 30% ethanol fraction, the synthase phosphatase was sensitive to glycogen inhibition at the levels of glycogen normally found in heart (89). Assuming 100% recovery of the factor or factors responsible for the desensitization of synthase
Figure 1B. Partial reversal of glycogen inhibition by a factor or factors present in a partially purified heart fraction. Synthase phosphatase was determined by conversion of synthase D to I as described in "MATERIALS AND METHODS." The 30% ethanol fraction was prepared by blending 50 g of beef heart in 100 ml of 50 mM Tris-HCl, 5 mM EDTA and 5 mM EGTA, pH 8.2. The 10,000 xg supernatant obtained from this homogenate was made up to 30% v/v ethanol at 0°C, and the pellet was collected by centrifugation at 10,000 xg. The pellet was resuspended in one-tenth of the original extract volume of 50 mM Tris-HCl, pH 7.5, and the preparation was dialyzed against 100 volumes of the same buffer at 5°C for 5 hours. The dialysis buffer was changed every 1.5 hours. Insoluble materials were removed by centrifugation. Glycogen-free synthase D was used as substrate, and bovine heart protein phosphatase was present at 4 U/ml. Since the samples containing the 30% ethanol fraction contained a low but measurable amount of synthase I, it was necessary to do zero time assays of % synthase I as well as 10 minute time points. The rate of conversion was then determined by subtracting the zero time % synthase I from the % synthase I after 10 minutes of incubation with phosphatase. The total phosphatase reaction volume was 25 µl. Additions were either 5 µl of the 30% ethanol fraction (● — ●) or 5 µl of the buffer used in the 30% ethanol fraction (○ — ○). The arrow shows the approximate glycogen concentration in heart tissue (84).
INHIBITION BY GLYCOGEN

% INHIBITION

GLYCOGEN (mg/ml)

10

15

0

10

20

30

40

50
phosphatase to glycogen inhibition during the preparation of the 30% ethanol fraction, the concentration of the factor in this experiment would correspond to the concentration in the heart tissue itself.

The 30% ethanol fraction also contained a phosphatase inhibitor which decreased the activity of the synthase phosphatase in the presence or absence of glycogen. This inhibitor completely abolished alkaline phosphatase activity on synthase D; thus it was not possible to use this phosphatase for studies on the 30% ethanol fraction.

The phosphatase inhibitor and glycogen inhibition desensitizing factor (GDF) could be differentiated by heating the 30% ethanol fraction for five minutes in a boiling water bath. As shown in Table 4, the GDF was sensitive to heating when a simple assay comparing synthase phosphatase activity in the presence or absence of 0.2 mg/ml rabbit liver glycogen was employed. At this concentration of glycogen, synthase phosphatase was inhibited about 50% when incubated in the absence of GDF (Figure 15). However, if GDF was present the synthase phosphatase activity was not significantly inhibited by this concentration of glycogen (Figure 18). As the data in Table 4 show, heat treatment of the 30% ethanol fraction resulted in loss of its ability to reverse glycogen inhibition of synthase phosphatase, indicating that GDF was heat labile. The phosphatase inhibitor was not destroyed by heat treatment. Before heat treatment, addition of 5 μl of the 30% ethanol fraction inhibited the phosphatase activity 54%, and after heat treatment 5 μl inhibited 45%. Thus, the phosphatase inhibitor was stable. From the difference in heat stability, it appears that the phosphatase inhibitor and GDF
Table 4. Heat denaturation of a glycogen desensitizing factor (GDF) in the 30% ethanol fraction

<table>
<thead>
<tr>
<th>Addition</th>
<th>Phosphatase Activity b (Δ% synthase I/min.)</th>
<th>% Inhibition by Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μl GDF</td>
<td>1.67</td>
<td>--</td>
</tr>
<tr>
<td>5 μl GDF, and 0.2 mg/ml glycogen</td>
<td>1.54</td>
<td>9</td>
</tr>
<tr>
<td>5 μl boiled GDF</td>
<td>2.20</td>
<td>--</td>
</tr>
<tr>
<td>5 μl boiled GDF, and 0.2 mg/ml glycogen</td>
<td>0.87</td>
<td>60</td>
</tr>
</tbody>
</table>

a The standard synthase phosphatase assay as described in "MATERIALS AND METHODS" was used, except that the total reaction volume was 25 μl. Synthase D concentration in the assay was 1.5 U/ml.

b The partially purified bovine heart protein phosphatase described in "MATERIALS AND METHODS" was used.
are not the same factor. The presence of the phosphatase inhibitor in
the 30% ethanol fraction made it impossible to study the kinetics of GDF
effects on glycogen inhibition, but extensive dialysis of the 30% ethanol
fraction (72 hours) against several changes of 50 mM Tris-HCl, 5 mM
dithiothreitol, pH 7.5, resulted in loss of the phosphatase inhibitor
with little loss in the GDF activity. In the future studies it should be
possible to design an assay for GDF and characterize it by using these
dialyzed preparation of the factor.

The studies presented here indicate that highly purified synthase D
binds glycogen very tightly, and as a result phosphatase activity on the
synthase D is very sensitive to glycogen inhibition. If a less highly
purified synthase D preparation is used as substrate, or if the reaction
mixture is supplemented with a crude ethanol precipitated fraction from
bovine heart, glycogen is not as effective at inhibiting the phosphatase
reaction.

Role of Polysaccharide Structure in the Synthase Reaction
and in Inhibition of Synthase Phosphatase

While a number of investigators have described the effect of glyco-
gen structure on the synthase reaction (4,5,90), and others have studied
the role of glycogen structure on synthase phosphatase inhibition (47,91),
in no case has highly purified glycogen-free synthase D been utilized
to study these two effects of glycogen in the same study. To compare the
primer binding site of synthase D for glycogen with the site involved in
polysaccharide inhibition of synthase phosphatase (a process that can now
be said to occur through substrate effects) a series of polysaccharides were prepared that differed in size and branching characteristics. The properties of these polysaccharides as primers and synthase phosphatase inhibitors were studied as a basis for comparing the two phenomena. Table 5 gives the structural characteristics of the polysaccharides used in these studies. The phosphorylase and β-amylase-treated polysaccharides were prepared as described in "MATERIALS AND METHODS." The branching characteristics of the rabbit liver glycogen (CL = 16, ECL = 10, ICL = 5) and oyster glycogen (CL = 12, ECL = 7.5, ICL = 3.6) used in these studies are within the range normally observed for various glycogen samples (2,3,92). Since the Pseudomonas isoamylase used to determine average chain length cannot cleave side chains less than 3 glycosyl residues long, the chain length of the β-amylase-treated oyster glycogen was estimated from the chain length of the parent oyster glycogen and the percent hydrolysis by β-amylase (38%). Further treatment with β-amylase under conditions used to estimate exterior chain length (52) resulted in hydrolysis of 4% of the dextrin; thus giving a calculated exterior chain length of 2.8 glucosyl units. The phosphorylase-treated glycogen samples appeared to be degraded nearly to the phosphorylase limit dextrin (ECL = 4.0). The phosphorylase-treated dextrin III sample had a calculated exterior chain length of 3.5, somewhat lower than the theoretical phosphorylase limit dextrin. It may be that the acid hydrolysis used in the preparation of dextrin III produced a significant number of exterior chains less than 4 glucosyl units long. Interior chain lengths of the glycogens and dextrins were calculated from the equation
Table 5. Effects of polysaccharide structure on catalytic activity of synthase D and on polysaccharide inhibition of synthase phosphatase activity of alkaline phosphatase

<table>
<thead>
<tr>
<th>Polysaccharide Sample</th>
<th>I. Structural Characteristics</th>
<th>Approximate $M_w$ $^a$</th>
<th>$\text{CL}$</th>
<th>$\text{ECL}$</th>
<th>$\text{ICL}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit liver glycogen</td>
<td></td>
<td>$10^6 - 10^7$</td>
<td>16.0</td>
<td>10.1</td>
<td>4.9</td>
</tr>
<tr>
<td>Oyster glycogen</td>
<td></td>
<td>$10^6 - 10^7$</td>
<td>12.0</td>
<td>7.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Phosphorylase-treated rabbit liver glycogen</td>
<td></td>
<td>$10^6 - 10^7$</td>
<td>9.6</td>
<td>4.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Phosphorylase-treated oyster glycogen</td>
<td></td>
<td>$10^6 - 10^7$</td>
<td>8.8</td>
<td>4.2</td>
<td>3.6</td>
</tr>
<tr>
<td>$\beta$-amylase-treated oyster glycogen</td>
<td></td>
<td>$10^6 - 10^7$</td>
<td>(7.4)$^b$</td>
<td>2.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Phosphorylase-treated Dextrin III</td>
<td></td>
<td>$10^5$</td>
<td>7.5</td>
<td>3.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Phosphorylase-treated DP87 Dextrin</td>
<td></td>
<td>$10^4$</td>
<td>14.1</td>
<td>4.3</td>
<td>8.8</td>
</tr>
</tbody>
</table>

$^a$Glycogen and glycogen dextrin approximated molecular weights were based on values from literature sources (93). Phosphorylase-treated Dextrin III molecular weight was estimated from the behavior of the parent polysaccharide on ultrafiltration (see "MATERIALS AND METHODS"). Phosphorylase-treated DP87 Dextrin molecular weight was estimated from the degree of polymerization of the parent polysaccharide.

$^b$CL for the $\beta$-amylase-treated oyster glycogen was estimated from the known CL of the parent glycogen and the percent of the sample hydrolyzed by incubation with $\beta$-amylase.

$^c$Relative Vmax was determined at polysaccharide concentrations greater than 100 times the $K_m$ concentration.

$^d$Glycogen samples gave a biphasic inhibition pattern. Half-maximal inhibition was determined at low concentrations of glycogen (less than 0.2 mg/ml). At higher concentrations of glycogen, there was no additional glycogen inhibition (Figure 15).
<table>
<thead>
<tr>
<th>Km (μM end groups)</th>
<th>Relative $V_{\text{max}}$</th>
<th>$D_2$ (μM end groups)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>(100)</td>
<td>38$^d$</td>
</tr>
<tr>
<td>1.2</td>
<td>105</td>
<td>41$^d$</td>
</tr>
<tr>
<td>1.0</td>
<td>26</td>
<td>--</td>
</tr>
<tr>
<td>1.4</td>
<td>28</td>
<td>--</td>
</tr>
<tr>
<td>73.3</td>
<td>8.3</td>
<td>2,320</td>
</tr>
<tr>
<td>8.7</td>
<td>9.2</td>
<td>--</td>
</tr>
<tr>
<td>11.0</td>
<td>8.7</td>
<td>970</td>
</tr>
</tbody>
</table>
ICL = CL - ECL - 1 (2). Interior chain lengths of the glycogens and dextrins derived from glycogens fall within the normal range of values reported for glycogen (2). The phosphorylase-treated DF87 dextrin was derived from a starch sample; therefore the interior chain length of this sample is similar to values found for amylopectin samples (2).

**Primer specificity of glycogen-free synthase D**

Prior to specificity studies with the various polysaccharides just described, preliminary experiments were done to find the proper conditions for determining primer kinetic parameters. The reaction rate is linear for at least eight minutes using either β-amylase-treated oyster glycogen (Figure 2), a poor primer, or rabbit liver glycogen, a good primer. The very low synthase activity in the absence of added primer shown in Figure 2 was only measurable at the highest concentrations of synthase used in the primer specificity studies. Furthermore, Figure 19 reveals that the Kₘ for glycogen (1.2 μM end groups) or β-amylase-treated glycogen (73 μM end groups) does not change with increasing synthase D concentration. This indicates that endogenous primer in the incubation mixture does not interfere with the determination of primer Kₘ values for the characterized polysaccharides under the conditions used in these experiments.

Table 5 gives the kinetic constants determined for the various polysaccharides. When unmodified glycogen samples were studied, the synthase D concentration in the assays was 2 mU/ml. For the phosphorylase dextrins of oyster and rabbit liver glycogen, a synthase D concentration of
Figure 19a. Effect of synthase D concentration on primer Km. Synthase activity was determined as in Figure 3, except that in Panel A, where rabbit liver glycogen was used as primer, the synthase D concentration was 2 U/ml (o — o) or 0.7 U/ml (● —●).

Figure 19b. In panel B, where β-amylase-treated oyster glycogen was used as primer, 40 U/ml (o — o) or 20 U/ml (● —●) synthase D was present.
A. 

\[
\frac{RLG}{V} \times 10^2 \\
\left( \frac{\mu M \text{ end groups}}{cpm} \right)
\]

B. 

\[
\frac{\beta-DEXTRIN}{V} \times 10^2 \\
\left( \frac{\mu M \text{ end groups}}{cpm} \right)
\]
2 mU/ml or 4 mU/ml was used, and for the β-amylase-treated oyster glycogen the synthase D concentration was 20 mU/ml. The duration of the assay was five minutes and the reaction was stopped by addition of 1 M acetic acid and processed as described in the "DISCUSSION."

Eadie-Hofstee plots for determination of kinetic constants gave straight lines over a wide range of substrate concentrations, one-half to five thousand times the apparent $K_m$ in the case of rabbit liver glycogen. All of the kinetic constants were determined under conditions in which the average chain length of the primer was not increased by more than one glucosyl residue during incubation. Ideally, kinetic parameters should be determined under conditions where a minimum number of chains have been lengthened, since increasing the chain length of the polysaccharide in effect produces a new substrate which may have significantly altered binding affinity or relative velocity compared to the unmodified primer. In practice, however, it was only possible to meet the criterion given above. The average chain length of the rabbit liver glycogen and oyster glycogen samples was extended by nearly one glucosyl residue during incubation, a value that represents the greatest degree of chain elongation observed in the polysaccharides studied. These samples, which have relatively long exterior chain lengths (Table 5), should be less sensitive to an extension of the average chain length by one glucosyl unit than a polysaccharide with a very short exterior chain length such as the β-amylase-treated oyster glycogen.

Table 5 shows that exterior chain length of the primer affected the $K_m$ for the polysaccharides. The glycogen samples and their phosphorylase
limit dextrins all had a Km of about 1 μM nonreducing end groups. The phosphorylase limit dextrins of the smaller polysaccharides had a somewhat higher Km indicating that the synthase D showed a preference for high molecular weight primers. Such a preference has been documented for skeletal muscle synthase (4,5). The most dramatic change in Km occurred when the exterior chain length was decreased from about 4, (the theoretical value for a phosphorylase limit dextrin), to between 2 and 3. The Km for the β-amylase-treated oyster glycogen was fifty-fold higher than for the other glycogen samples and also much higher than the Km values for the small polysaccharides. It was possible to determine the Km and relative Vmax for the β-amylase-treated oyster glycogen under conditions where the average chain length was extended by less than one-tenth of a glucosyl residue because the apparent Km of the β-amylase-treated oyster glycogen is much higher than the Km for the parent glycogen, while the relative Vmax is much lower. Since the β-amylase-treated oyster glycogen sample used in these studies was not taken to the limit of β-amylolysis, the possibility that the true β-amylase limit dextrin would not serve as a primer was explored. A β-amylase dextrin of the same parent oyster glycogen used in the specificity studies was prepared by extensive incubation with β-amylase as described in "MATERIALS AND METHODS." Less than 0.5% of the dextrin was hydrolyzed when incubated with β-amylase under conditions used for determining exterior chain length of the glycogen samples; thus indicating that the new β-amylase-treated glycogen was essentially taken to the limit dextrin. When both samples were incubated at a concentration of 10 mg/ml with the glycogen-
free synthase D, it was found that the new dextrin gave about one-third the activity of the old dextrin. It is therefore likely that a true \( \beta \)-amylase limit dextrin of glycogen can serve as a primer for synthase D. Brown et al. (5) found that a \( \beta \)-amylase limit dextrin of glycogen could act as a primer for rabbit skeletal muscle synthase, but these investigators reported a \( K_m \) for the dextrin that was only about two-fold greater than the \( K_m \) for glycogen. The discrepancy between their data and that reported here might be explained by either a difference in the primer specificity of synthase I and synthase D since the studies by Brown et al. were done with skeletal muscle synthase I, or by a difference in the primer specificity of heart synthase (used in the present study) and the skeletal muscle enzyme used by Brown. Vardanis (91) has presented evidence from ultracentrifugation studies that mouse liver synthase binds glycogen better than \( \beta \)-amylase limit dextrin of glycogen. If the \( K_m \) of synthase for primers reflects the binding affinity for the polysaccharides, then Vardanis' data supports the findings of the present work; that is, \( \beta \)-amylase-treated glycogen should be a poorer primer in terms of \( K_m \) than native glycogen.

The relative \( V_{max} \) values for the different polysaccharides (Table 5) varied more with exterior chain length than did the \( K_m \)'s. [This observation is in agreement with results obtained using skeletal muscle synthase (5).] Although phosphorylase treatment of oyster glycogen and rabbit liver glycogen did not affect the \( K_m \) for these substrates, the \( V_{max} \) was decreased four-fold below the \( V_{max} \) for the parent glycogens. A further decrease was noted for the phosphorylase-treated dextrins of the smaller
polysaccharides and the β-amylase-treated oyster glycogen which all gave $V_{\text{max}}$ values about 10% of the values for the unmodified glycogen samples. Rabbit liver glycogen and oyster glycogen behaved identically as primers despite the fact that the rabbit liver glycogen had a longer average exterior chain length. This observation correlates well with the earlier finding that skeletal muscle synthase can utilize glycogens from a variety of sources equally well as substrates (4).

**Effect of polysaccharide structure on phosphatase inhibition**

Several of the polysaccharides used in the primer specificity studies were tested for inhibition of alkaline phosphatase activity on synthase D. Synthase phosphatase activity was determined by conversion of synthase D to synthase I as described in "MATERIALS AND METHODS" except in the case of the oyster glycogen sample where radioactive synthase D was used and the synthase phosphatase activity was determined by release of protein-bound $^{32}$P. Synthase D concentration in the phosphatase incubation was always 1.5 U/ml, and alkaline phosphatase was present at 2 U/ml. Both the β-amylase-treated oyster glycogen sample and the phosphorylase-treated DP87 sample gave linear plots of $1/v$ vs. polysaccharide concentration. The plot for the β-amylase-treated oyster glycogen is given in Figure 20. The rabbit liver glycogen sample gave a more complex inhibition pattern as shown in Figure 16. The reason for the complex pattern is evident upon inspection of Figure 15. It is clear that for either the alkaline phosphatase or the protein phosphatase glycogen cannot completely inhibit synthase D dephosphorylation; therefore
Figure 20. Inhibition of alkaline phosphatase activity on synthase D by β-amylase treated oyster glycogen. Synthase D phosphatase activity was measured as described in "MATERIALS AND METHODS." Synthase D was present at 1.5 U/ml and alkaline phosphatase at 2 U/ml.
$\frac{1}{V} \times 10^2$

$(\% \text{SYNTHASE I})^{-1}$

$\beta$-DEXTRIN (mg/ml)
the Dixon plots plateau at high glycogen concentrations (Figure 16). At present, the reason for the incomplete inhibition of synthase phosphatase activity by glycogen remains unknown. The oyster glycogen sample gave the same complex pattern of synthase phosphatase inhibition observed for the rabbit liver glycogen sample.

The concentration of various polysaccharides necessary to give half-maximum inhibition of alkaline phosphatase activity is presented in Table 5. A comparison of the polysaccharide Km data and the concentrations of the polysaccharides giving 50% inhibition of alkaline phosphatase activity on synthase D reveals a direct relationship between the two properties of the polysaccharides. When compared as either synthase primer or phosphatase inhibitor, the relative efficiency of the polysaccharides is rabbit liver glycogen = oyster glycogen > phosphorylase-treated DP87 dextrin > α-amylase-treated oyster glycogen.
DISCUSSION

This work reports the highest specific activity yet obtained for heart glycogen synthase. Previous purification procedures based on a method for preparation of the enzyme from skeletal muscle resulted in specific activities of 1.0 to 4.7 U/mg (58). By centrifuging the preparation through 50% sucrose or precipitating with 15% v/v ethanol, the specific activity of the synthase could be increased to greater than 7 U/mg (35). In the present work, the synthase D routinely used for synthase phosphatase assays had a specific activity of 13 U/mg. The procedure used in making $^{32}$P-labeled synthase D resulted in an enzyme preparation that was nearly homogeneous on SDS gel electrophoresis and had a specific activity of 32 U/mg. A key step in the purification method now in use involves two ultracentrifugation steps following the first ethanol precipitation. The purification procedure through the first ultracentrifugation step is similar to the procedure used by Brown and Larner (8) for the purification of rabbit skeletal muscle synthase D; however, in their procedure the synthase was recovered in the pellet of the first ultracentrifugation step. In the purification scheme for the bovine heart preparation, most of the synthase remains in the supernatant in this step. A number of factors could account for the differences in behavior of the two preparations, but most likely there was a difference in the glycogen content of the two preparations at this step. Since synthase binds very tightly to glycogen, a higher concentration of glycogen in the rabbit skeletal muscle preparation could account for a
greater proportion of the synthase appearing in the glycogen-containing pellet. Glycogen analysis of frozen hearts has shown that the glycogen level can be very low (see the preparation of GDF in the "RESULTS" section). Furthermore, preparations from bovine heart were largely in the synthase I form as extracted (70% synthase I or greater in three different preparations), and it is known that percent synthase I is high when glycogen levels are low (94).

The synthase could be nearly quantitatively removed from the supernatant of the first ultracentrifugation step by addition of oyster glycogen and recentrifugation under the same conditions as before. This step resulted in a three to five-fold purification of synthase with little or no loss of activity. The two ultracentrifugation steps also removed a great amount of insoluble material which would have interfered with the chromatography step used later.

Hydrophobic chromatography (95,96) is a term coined by Shaltiel to describe the separation of proteins on hydrocarbon-substituted agarose derivatives, where at least part of the resolution is due to hydrophobic interactions of the protein and agarose-bound ligand. This technique has been previously used to purify synthase (21,55,96). In early experiments using aminobutylagarose it was discovered that columns of the hydrophobic resin could be reused after washing with 1 M NaCl as described by Shaltiel (97). The affinity of the synthase for the resin was altered, however, and the synthase appeared in progressively earlier fractions of the salt gradient used for elution. Subsequently, it was discovered that staining aminobutylagarose and aminoethylagarose prepared by the
same method with 2,4,6-trinitrobenzenesulfonate (98,99) resulted in a red-orange stain for the aminobutylagarose and a yellow stain for the aminethylagarose. Washing the aminobutylagarose with 1% v/v SDS before or after staining resulted in loss of coloration so that the SDS treated aminobutylagarose and the aminethylagarose gave the same yellow stain. Treatment of aminethylagarose with SDS did not affect the intensity of staining. The TNBS staining intensity of aminobutylagarose also decreased with aging at 5°C, as did the affinity of the gel for synthase.

The observations on staining characteristics of aminethylagarose and aminobutylagarose suggest that in the case of the aminobutylagarose there is aggregation of noncovalently bound diaminobutane with the agarose-bound ligand. Treatment with SDS or aging results in loss of noncovalently bound diaminobutane.

Table 6 makes a comparison of the physical properties of bovine heart synthase D and of synthase D from other tissues. The specific activity of homogeneous bovine heart synthase D is probably around 30 to 35 U/mg since a preparation which had one major protein band on SDS polyacrylamide gel electrophoresis and one minor band had a specific activity of 32 U/mg. This value is similar to the highest specific activities reported for synthase D from skeletal muscle (21,22) and liver (18). The subunit molecular weight of 86,000 agrees with values previously reported for synthase from a variety of tissues (15,16,18-20). Two different procedures were used to estimate the molecular weight of glycogen-free synthase D. Sucrose density gradient ultracentrifugation gave an estimated molecular weight of 247,000, indicating that the active
Table 6. Properties of glycogen synthase D from various tissues

<table>
<thead>
<tr>
<th>Source of Synthase D</th>
<th>Specific Activity&lt;sup&gt;a&lt;/sup&gt; (U/mg protein)</th>
<th>Molecular Weight</th>
<th>Subunit Molecular Weight</th>
<th>Mole P&lt;sub&gt;Subunit&lt;/sub&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit liver</td>
<td>25</td>
<td>170,000</td>
<td>85,000</td>
<td>1</td>
<td>(18)</td>
</tr>
<tr>
<td>Rat liver</td>
<td>(37)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>260,000</td>
<td>85,000</td>
<td>12</td>
<td>(16)</td>
</tr>
<tr>
<td>Rabbit skeletal muscle</td>
<td>35</td>
<td>250,000</td>
<td>85,000</td>
<td>3</td>
<td>(22,100)</td>
</tr>
<tr>
<td>Porcine kidney</td>
<td>9.1</td>
<td>370,000</td>
<td>92,000</td>
<td>-</td>
<td>(19)</td>
</tr>
<tr>
<td>Porcine adipose tissue</td>
<td>1.9</td>
<td>-</td>
<td>90,000</td>
<td>-</td>
<td>(20)</td>
</tr>
<tr>
<td>Bovine heart</td>
<td>32</td>
<td>247,000&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86,000</td>
<td>2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>this thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>490,000&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>A unit of glycogen synthase catalyzes the transfer of one micromole of glucose from UDP-glucose to glycogen in one minute at 30°C.

<sup>b</sup>Synthase D activity determined at 37°C and pH 7.4.

<sup>c</sup>Molecular weight determined by sucrose density ultracentrifugation.

<sup>d</sup>Molecular weight determined by Sepharose 6B gel filtration.

<sup>e</sup>Determined from the radioactivity of <sup>32</sup>P-synthase D prepared as in "MATERIALS AND METHODS."
synthase under the conditions of ultracentrifugation exists as a trimer. Active trimers of synthase D have been reported for skeletal muscle (15) and liver (16,27). A molecular weight of 490,000 was estimated from gel filtration on Sepharose 6B. The different molecular weights estimated by sucrose gradient ultracentrifugation and gel filtration may reflect actual differences in the aggregation states of the synthase D, or the synthase may be a nonglobular protein which would have a larger apparent molecular weight on gel filtration than on ultracentrifugation. There is evidence for multiple molecular weight forms of synthase D (25-27). Liver synthase D has been reported to aggregate on aging to a form with a molecular weight of approximately 500,000 determined by ultracentrifugation (16).

The purified $^{32}$P-labeled synthase D contained two radioactive phosphates per 86,000 dalton subunits. Assuming that the synthase I used as starting material contained no phosphate the synthase D would have two phosphates per subunit. Although it is known that rabbit skeletal muscle synthase I contains virtually no alkali-labile phosphate per subunit (15,000), it was not possible to prepare the quantities of bovine heart synthase I necessary for the colorimetric determination of phosphate (101). Dephosphorylation of radioactive synthase D with alkaline phosphatase resulted in loss of both phosphates and a parallel conversion to the synthase I form (Figure 6). These results indicate that a maximum of two phosphorylation sites on the synthase molecule are necessary to bring about interconversion between the two enzyme forms. The results do not rule out the possibility that only one of the two phosphorylation
sites is sufficient to cause interconversion. The parallel rates of dephosphorylation and conversion to synthase I brought about by alkaline phosphatase may simply reflect a lack of specificity for the two phosphorylated sites on synthase D. Studies with skeletal muscle (14) and liver (18) synthase D indicate that one site on the substrate is phosphorylated during conversion of synthase I to synthase D. Nimmo and Cohen reported the incorporation of two phosphates per subunit, only one of which resulted in actual conversion of synthase I to synthase D (31). Not enough is known about the protein kinases involved in phosphorylation of synthase, and it may be that the stoichiometry of phosphate incorporation depends on the kinase or kinases present during phosphorylation and the conditions of phosphorylation (33).

The evidence accumulated so far indicates that bovine heart synthase D is very similar to highly purified synthase D from other mammalian tissues. The highest specific activities reported using assay conditions employed in the present work are 25 U/mg for rabbit liver synthase D (18), 35 U/mg for rabbit skeletal muscle synthase D or I (21,22) and 32 U/mg for bovine heart synthase D. The above values are similar enough to suggest that the turnover numbers of the different synthases are the same. On the other hand, an apparently homogeneous preparation of swine adipose tissue synthase had a specific activity of 1.86 U/mg (20). The possibility exists that inactive synthase copurifies with the active enzyme in the purification scheme for the adipose tissue synthase (20). It is also possible that the adipose tissue synthase is an isozyme having a lower turnover number than the synthases from skeletal muscle, heart and
liver. The $K_m$ for glycogen reported in the present work is similar to
the glycogen $K_m$ reported for skeletal muscle synthase D under the same
assay conditions (8). The similarity of muscle, liver and heart synthase
D in respect to $K_m$ for UDP-glucose and $K_a$ for glucose-6-P has been docu-
mented previously (13).

The studies on dephosphorylation of synthase D by $E. coli$ alkaline
phosphatase were undertaken to determine the effect of various factors
known to affect mammalian synthase phosphatase activity on a nonspecific
phosphatase. The bacterial phosphatase was a logical choice for a model
nonspecific phosphatase. The lack of specificity of $E. coli$ alkaline
phosphatase activity on small phosphomonoester substrates is well docu-
mented (62,78). Furthermore, observations concerning the control of
glycogen metabolism in $E. coli$ and the cellular location of enzymatically
active alkaline phosphatase in this organism argue against a physiologi-
cal role of alkaline phosphatase in dephosphorylation of synthase D.
Glycogen deposition in $E. coli$ is controlled at the UDP-glucose pyro-
phosphorylase reaction (102), and the glycogen synthase from this
organism has not been reported to exist in interconvertible forms. The
alkaline phosphatase itself does not exist in its active form inside the
cell (103). The active dimer is formed in the periplasmic space after
the monomer is transported to the outside of the cell membrane. One
would not expect such a phosphatase to be sensitive to metabolites
affecting synthase phosphatase activity.

After the initial discovery that alkaline phosphatase could convert
synthase D to synthase I, it was necessary to investigate two
possibilities concerning the synthase converting activity present in the alkaline phosphatase preparation. First of all, there was the possibility that the "conversion" seen in the early experiments was brought about by a contaminating protease in the alkaline phosphatase preparation. Yeast synthase D can be converted to a "pseudo-synthase I" by proteolytic attack (24). The second possibility considered was the presence in the alkaline phosphatase preparation of a contaminating protein specific phosphatase from _E. coli_. The synthase I produced by alkaline phosphatase does not appear to be a proteolytically modified synthase. It can be readily reconverted to synthase D in the presence of a cyclic-AMP-dependent protein kinase (Figure 5) while a synthase I produced by proteolytic cleavage of the phosphoseryl residue or residues responsible for synthase D characteristics should not be reconverted to synthase D by a kinase. A contaminating protein phosphatase is much more difficult to rule out; however, the many similarities in the physical properties of the synthase phosphatase and alkaline phosphatase activities indicate that they are the same enzyme.

While _E. coli_ alkaline phosphatase has activity on bovine heart synthase D and phosphohistone, it does not completely dephosphorylate phosphohistone and it has no detectable activity on phosphorylase a.

These results suggest that alkaline phosphatase may be used to differentiate between phosphorylated sites on proteins. This approach might yield information about the nature of the kinases and phosphatases involved in phosphorylation and dephosphorylation of the various sites. That alkaline phosphatase can totally convert synthase D to the I form and not
convert phosphorylase a to b indicates a potential for specificity in the phosphatase action on synthase D and phosphorylase a. The inability of nonspecific phosphomonoesterases to dephosphorylate phosphorylase a has been demonstrated previously (104). The observation that some protein phosphatases have activity on both synthase D and phosphorylase a (18, 35, 36) argues against the need for specific synthase phosphatase and phosphorylase phosphatase activities in the cell; however, there is always the possibility that modification of a general protein phosphatase under a given set of conditions in vivo might produce a phosphatase which is specific for one of the above substrates. The specificity of alkaline phosphatase as a protein phosphatase suggests that such a transformation is feasible.

The study of the cation specificity for activation of alkaline phosphatase activity on synthase D confirms previous data on the relative effectiveness of various cations in activating bovine heart protein phosphatase (35). Generalizations cannot be made at this time concerning the implications of this observation on the physiological control of synthase phosphatase. The binding of divalent cations directly to the synthase phosphatase activity or activities found in the cell is certainly not ruled out by the data presented here, but it is only possible to conclude that one must also consider the effect of metal ion binding by the macromolecular substrate. It is intriguing to speculate that an increase in concentration of a specific physiologically important cation, such as Mg$^{2+}$, might affect the synthase phosphatase activity by binding to both the phosphatase and synthase. There is some evidence that bovine
heart protein phosphatases may be directly stimulated by divalent cations (35,38). Binding of divalent cations by both phosphatase and synthase D might provide a mechanism for a significant amplification of synthase phosphatase activity by simultaneously increasing the levels of activated phosphatase and "activated" substrate. This possibility can only be investigated when there is available a highly purified protein phosphatase which is shown to be stimulated by directly binding divalent cations. The \( \text{Mg}^{2+} \) binding site on synthase D for activation of synthase phosphatase may be the same as the \( \text{Mg}^{2+} \) site for activation of the synthase D itself. Half-maximal activation of alkaline phosphatase activity on synthase D occurs at 6 mM \( \text{Mg}^{2+} \), while about 4 mM \( \text{Mg}^{2+} \) gives half-maximal activation of synthase D (85). Total \( \text{Mg}^{2+} \) concentration in rat heart is about 10 mM (105), but the \( \text{Mg}^{2+} \) appears to exist mainly in complexes with metabolites (106). Computer simulations based on known dissociation constants for \( \text{Mg}^{2+} \) complexes with various anions predict that at 12.5 mM total \( \text{Mg}^{2+} \) concentration in rat heart only about 0.22 mM free \( \text{Mg}^{2+} \) will be present (107). This level of \( \text{Mg}^{2+} \) is too low to affect synthase D dephosphorylation by binding to synthase D, at least under the conditions employed in the \textit{in vitro} experiments. It is known that the free \( \text{Mg}^{2+} \) concentration varies with different physiological conditions (107); hence it is possible that direct effects of \( \text{Mg}^{2+} \) on synthase D could influence the rate of synthase D dephosphorylation \textit{in vivo} under some conditions.

Several anions were shown to activate alkaline phosphatase-catalyzed dephosphorylation of synthase D. The anion specificity studies were all
done in the presence of Mg$^{2+}$ because in the absence of this cation there was very little activity under the assay conditions and it was difficult to see changes in activity. Of the anions tested, nitrate, sulfate and thiosulfate activated the synthase phosphatase activity of alkaline phosphatase at high concentrations. The effect was not merely related to ionic strength changes as in the case of activation of alkaline phosphatase activity on p-nitrophenyl phosphate. High concentrations of chloride had no noticeable effect on the synthase phosphatase activity. The effects of nitrate and sulfate could be differentiated by the concentration dependence of their activation. Nitrate did not activate much at low concentrations while sulfate activated to a significant extent at the lowest concentrations tested. As was expected, phosphate was inhibitory when using either synthase D or p-nitrophenyl phosphate as substrate.

The studies on glycogen inhibition of the synthase phosphatase activity of alkaline phosphatase firmly support the concept that polysaccharides inhibit synthase phosphatase by binding to the substrate. Concentration dependence of glycogen inhibition was the same when alkaline phosphatase was used to dephosphorylate synthase D or when a bovine heart protein phosphatase was used (Figures 15 and 16). Furthermore, glycogen does not inhibit the activity of alkaline phosphatase on the small substrate p-nitrophenyl phosphate, indicating no direct interaction of glycogen with alkaline phosphatase. Finally, the structural characteristics that make polysaccharides good primers for synthase D also make them better inhibitors of alkaline phosphatase activity on
synthase D (Table 5). A comparison of the polysaccharide structural requirements for primer and phosphatase inhibitor functions indicates a similarity in the two binding sites. This observation suggests that the synthase phosphatase glycogen inhibition site may be the polysaccharide substrate binding site.

The specificity of the glycogen-free bovine heart synthase D for polysaccharide primers does not vary a great deal from the previously reported specificity of skeletal muscle synthase (5) except in the case of the β-amylase treated glycogen sample. Brown et al. (5) reported a Km of 1.7 μM end groups for KOH-extracted rabbit liver glycogen, and a Km of 2.1 μM end groups for the phosphorylase-limit dextrin of this glycogen. The relative Vmax of the phosphorylase dextrins were also around one-fourth of the glycogen Vmax. While the relative velocity with the β-amylase dextrins used in the present study and that by Brown et al. are nearly the same, the Km reported in the latter study was similar to the Km's for the glycogen and phosphorylase limit dextrin of glycogen. In the present study it was discovered that the Km for the β-amylase dextrin was much higher than for glycogen or phosphorylase-limit dextrin samples. Possible explanations for this difference in activity on β-amylase dextrins were discussed under "RESULTS."

The smaller phosphorylase-treated polysaccharides were better primers than the β-amylase treated glycogen in terms of Km but not Vmax. The Km values of the smaller polysaccharides were significantly higher than the Km's for the phosphorylase-treated glycogen samples which had similar average outer chain lengths (Table 5). Previous studies have
indicated that oligosaccharides have higher Km's than glycogen samples (4, 5), but there is disagreement over whether these smaller primers have lower or higher relative Vmax's compared to glycogen (4, 5). The present study indicates that even fairly large polysaccharides (10^4 to 10^5 daltons) with exterior chain lengths similar to the phosphorylase dextrins of glycogen are not comparable to the larger polysaccharides in terms of primer Km and Vmax.

If highly purified glycogen-free synthase D is used as substrate for bovine heart protein phosphatase or alkaline phosphatase, the synthase phosphatase reaction is inhibited by very low levels of glycogen (Figure 14). Addition of an ethanol-precipitated fraction from bovine heart shifted the glycogen inhibition to a more physiological range (using bovine heart protein phosphatase). The nature of the factor or factors responsible for this "desensitization" of the phosphatase to glycogen inhibition is not yet known. However, the factor is non-dialyzable and heat sensitive, suggesting the involvement of a macromolecule with an ordered structure. The presence of a GDF in the less highly purified synthase D preparations used in most of the glycogen inhibition studies reported previously (4, 47, 49) could account for the relative insensitivity to glycogen inhibition observed with these preparations. Villar-Palasi reported that partially purified rat muscle synthase phosphatase was not inhibited by glycogen, but synthase phosphatase in crude extracts was inhibited (47). Ethanol-precipitated phosphatase from crude extracts was inhibited by glycogen, but incubation of crude phosphatase preparations in the cold overnight resulted in
sixty percent loss of phosphatase activity and loss of glycogen inhibition. This loss of glycogen inhibition was interpreted as proof that glycogen inhibition results from binding of glycogen by synthase phosphatase. As discussed in the introduction to this work, current evidence strongly suggests that glycogen interacts with synthase to inhibit the synthase phosphatase reaction. The data presented in this dissertation confirms this hypothesis. An alternative interpretation of Villar-Palasi's data allows for synthase-directed glycogen inhibition of synthase phosphatase. If it is assumed that a glycogen inhibition desensitizing factor is denatured by incubation in the cold overnight, the 60% loss of synthase phosphatase activity described by Villar-Palasi could be the result of glycogen inhibition by endogenous glycogen in his assay system (0.17 to 0.20 mg/ml). As shown in Figure 15, this concentration of glycogen inhibits bovine heart synthase phosphatase about 40% in the absence of GDF. Furthermore, once this initial inhibition by glycogen is attained, there is little more inhibition observed at glycogen concentrations up to 25 mg/ml. Therefore, the synthase phosphatase would appear to be insensitive to glycogen inhibition. The control of synthase phosphatase by glycogen must be studied in more detail in the future. If there is a physiologically important GDF in tissues, the level of this factor could indirectly control the dephosphorylation of synthase D by making the synthase phosphatase more or less susceptible to glycogen inhibition.
REFERENCES


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