Mechanistic aspects of the low-molecular-weight phosphatase activity of calcineurin: a model for phosphotyrosyl-protein phosphatases

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Mechanistic aspects of the low-molecular-weight phosphatase activity of calcineurin:
A model for phosphotyrosyl-protein phosphatases

by

Bruce L. Martin

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of
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1987
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ABBREVIATIONS

pNPP, para-Nitrophenyl phosphate;
Tyr-P, (L)-Tyrosine phosphate;
pNPS, para-Nitrophenyl sulfate;
EGTA, Ethylene glycol bis(β-amino ethyl ether)N,N'-tetraacetate;
HPLC, High Performance Liquid Chromatography;
F-Tyr-P, (DL)-3-Fluorotyrosine phosphate;
F₄-Tyr-P, (DL)-2,3,5,6-Tetrafluorotyrosine;
Pᵣ, Inorganic phosphate;
pNP, para-Nitro phenol;
MOPS, 3-(N-morpholino)propanesulfonic acid;
LG, Phenolic leaving group;
PFP, Pentafluorophenol;
MES, 3-(N-morpholino)ethanesulfonic acid;
PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid);
HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid;
TRICINE, N-[tris-(hydroxy)methyl] glycine;
BICINE, N,N-bis(2-hydroxyethyl)glycine;
tris, tris-(hydroxymethyl)aminomethane;
INTRODUCTION

The regulation of protein function via enzyme catalyzed covalent modification reactions provides a sensitive, reversible mechanism for metabolic control (1,2). A number of covalent modifications have been identified (3,4). Among the most studied is cyclic phosphorylation-dephosphorylation (5-8) of serine or threonine residues in proteins in response to hormonal or neural stimuli. Phosphorylation-dephosphorylation of enzymes on these residues has been implicated in the regulation of glycogen metabolism (9-11), glycolysis/gluconeogenesis (12-15), lipid metabolism (16-19), protein synthesis (20,21), muscle contraction (22,23), and neurotransmitter synthesis (24,25). A number of other systems, involving phosphorylation-dephosphorylation, are known for which the precise role of the modification is not well defined (26-28). A compilation of the enzymes known to be regulated by phosphorylation-dephosphorylation has recently been put together (29,30).

A number of the enzymes, protein kinases, responsible for the phosphorylation events have been isolated and characterized. These kinases can be classified (31,32) upon the basis of the regulatory mechanisms that each is subject to. These enzymes have been extensively characterized with
respect to their biological activators and inhibitors, structural features, and their regulation by phosphorylation as discussed in a number of recent review and research articles (25,33-53). The substrate specificity determinants as identified with peptide substrates have been extensively examined (34,54-71). One of these enzymes, the cAMP-dependent protein kinase, has been additionally characterized with regards to mechanistic features (72-76) of the catalyzed reaction.

Comparatively, much less is known about the enzymes, phosphoprotein phosphatases, responsible for the hydrolysis of the protein phosphate ester. Phosphatase activities involved in the regulation of the various metabolic processes indicated earlier have been described (77-81). Phosphoprotein phosphatases have been isolated by a number of different protocols from a variety of sources. This diversity has provided a great deal of confusion along with a vast amount of factual knowledge concerning these phosphatases (82-84). Ingebritsen and Cohen have tried to systematize the field of phosphatases with the development of a classification scheme (77,85) primarily based upon the relative specificity of each phosphatase toward the α- and β-subunits of phosphorylase kinase and the effect of phosphatase inhibitor proteins. A simple explanation of the
TABLE I

Classification of Phosphoprotein Phosphatases

<table>
<thead>
<tr>
<th>Type</th>
<th>Inhibition by Inhibitor Proteins</th>
<th>Specificity</th>
<th>Phos Kinase Substrate</th>
<th>Phos phosphatase Activity</th>
<th>Other Effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>β-subunit</td>
<td>Broad</td>
<td>High</td>
<td>GSK-3</td>
</tr>
<tr>
<td>2A</td>
<td>No</td>
<td>α-subunit</td>
<td>Broad</td>
<td>High</td>
<td>Polycations ω</td>
</tr>
<tr>
<td>2B</td>
<td>No</td>
<td>α-subunit</td>
<td>Narrow</td>
<td>Low</td>
<td>Ca²⁺/CaM</td>
</tr>
<tr>
<td>2C</td>
<td>No</td>
<td>α-subunit</td>
<td>Broad</td>
<td>Low</td>
<td>Mg²⁺</td>
</tr>
</tbody>
</table>

*The table is adapted from Ingebritsen and Cohen (85).
scheme is depicted in Table I. The phosphatases included in this scheme can account for the majority of phosphatase activity involved in the regulation of metabolic pathways (77-81,85).

Much confusion in the study of the various phosphatases has arisen because of the difficulty in obtaining homogeneous preparations of the enzymes. Extensive investigations have generated disagreement concerning the size and subunit structure of the native, catalytically active form of the best known phosphatase, phosphorylase phosphatase (86-94). Multiple forms (95-100) of this enzyme have been isolated. In general, phosphatases are less well characterized than are kinases. For example, while acceptor specificity determinants of kinases are reasonably well defined, there are only a few reports of the specificity determinants of protein phosphatases as determined with peptide substrates (71,101-104); moreover, these have not been accomplished as systematically as in the situation of the kinase studies.

It is well established that phosphatase 1, or phosphorylase phosphatase, is subject to hormonal regulation (82,105-108). These hormonal effects on phosphatase 1 are modulated through the function of one of the two phosphatase inhibitor proteins specific for this phosphatase.
Inhibitor-2 is postulated to be a subunit in the inactive complex of phosphatase-1 (109). The other phosphatases, phosphatases 2A, 2B, and 2C, described in Table I are not regulated by these inhibitor proteins. Phosphatase 2A is modulated by polycationic compounds (110-113), including histone I, spermine, and putrescine, but little is known of the mechanism or physiological significance of this process. Phosphatase 2C has not been investigated extensively (85). The properties and regulation of phosphatase 2B, or calcineurin, will be discussed in additional detail later.

Recently, the phosphorylation of protein tyrosine residues was observed in proteins of cells transformed by tumor viruses (114-124). Quickly, this modification has been identified in preparations of receptors of growth factors (125-133) after treatment with the appropriate growth factor as well as in cellular proteins following the treatment of a responsive cell with a growth factor. A number of nontreated and nontransformed cells have been shown to possess tyrosine kinase activity (134-141). The field of tyrosine phosphorylation has blossomed in the past 5 years to rival that of the extensively studied serine phosphorylation systems.

A great deal has been determined about the protein kinases responsible for the phosphorylation of tyrosine
residues (142-147). The viral enzymes are found to be identical with the product (114-124,148) of the gene responsible for transformation, the oncogene. General properties of these enzymes include a requirement for Mg$^{2+}$ or Mn$^{2+}$ and a preference for ATP over GTP as a phosphoryl donor (117,119,120,122,130,149-154), the possession of autophosphorylation activity (117,120,154-160), and an apparent requirement for acidic residues amino terminal to the tyrosine residue to be modified (158,161-165), although further examination of specificity determinants utilizing peptide substrates (164,166-171) has not provided confirming information. Comparisons of tryptic peptide maps (172,173) and predicted amino acid sequences (174,175) reveal significant homology amongst these enzymes as well as homology to serine kinases (175).

Initially, the observation that levels of tyrosine phosphate in proteins were elevated in cellular proteins isolated from viral transformed cells was interpreted to indicate a role for tyrosine phosphorylation in the initiation and maintenance of the transformed state (156,176-179). Additional study has not supported this hypothesis (180,181). Current speculation postulates a significant role for tyrosine phosphorylation in growth control (182,183). This speculation is supported by the
observation that cell lines which do not respond well to epidermal growth factor also fail to demonstrate the activation of tyrosine kinase activity in the EGF receptor upon treatment of the cell with EGF (184). An increase in tyrosine kinase activity has also been reported to occur upon fertilization (185,186), during development (187-189) and upon differentiation (190,191) in a number of cell types. These observations suggest that tyrosine phosphorylation may be a signal for cell growth. The increased levels in viral transformed cells may reflect a mechanism for these cells to withstand intercellular signals preventing growth. The transformed cells are then free to undergo uncontrolled growth as is observed in the culture of transformed cells.

To be a physiologically significant regulatory mechanism, the modification reaction must be reversible. The field of phosphotyrosyl-protein phosphatase is as complex as the study of phosphosereryl-protein phosphatases. Phosphatases specific for the hydrolysis of phosphotyrosyl residues in proteins have been isolated from a number of sources including rabbit kidney (192), rat brain (193), liver (194), and spleen (195), Ehrlich Ascites cells (196), A431 (human epidermoid cell line) cells (197), bovine cardiac muscle (198), chick embryo fibroblasts (199), and
chick brain (200). Activity toward phosphotyrosyl-proteins has also been identified in preparations of the nonspecific acid (201-204) and alkaline (204,205) phosphatases and in preparations of a phosphoseryl-protein phosphatase (206) from cardiac muscle. In fact, the nonspecific phosphatases seemingly dephosphorylate phosphotyrosyl phosphoproteins more readily than phosphoseryl phosphoproteins (201,203,205).

As with the phosphorylation of serine/threonine residues, the phosphatases involved in the hydrolysis of phosphotyrosyl residues are less well characterized than the tyrosine protein kinases. The field remains, as yet, at the stage of simply the identification of activities. General features which have emerged, as reviewed (207,208), indicate that these enzymes are sensitive to inhibition by micromolar Zn$^{2+}$ (197), sensitive to inhibition by vanadate (209), less sensitive to inhibition by fluoride than are phosphoseryl-phosphatases, stimulated by EDTA, and possesses maximum activity in the pH range 6.0-7.0.

Of particular interest is the calmodulin-activated phosphatase, calcineurin, due to its ability to dephosphorylate phosphoproteins containing each of the phospho hydroxy-amino acids (210). The phosphoproteins which have been shown to serve as substrates for calcineurin
are listed in Table II (210-218). Also included in this table are the residues from which the phosphoryl moiety is removed. Calcineurin is observed to have a broad specificity toward the phospho hydroxy-amino acid residue of the substrate phosphoprotein while dephosphorylating a limited number of proteins. The mechanistic determinants of this feature of calcineurin remain unknown. It is, as yet, unclear what the relative importance of the phosphotyrosyl and phosphoseryl phosphoprotein phosphatase activities of calcineurin are.

Tissue distribution (219) studies indicate that the enzyme is preferentially found within neural tissues. The highest levels occur in the cerebrum, olfactory bulb, and the cerebellum; the cerebrum contains the highest amounts. Within the cerebrum, calcineurin is primarily located in the caudate nucleus and the putamen. Further localization (220) has indicated that calcineurin is associated with the postsynaptic densities and dendritic microtubules within the dendrites. Calmodulin follows a similar distribution pattern (220). In chick retina (221), calcineurin is found localized to both pre- and post-synaptic densities. In agreement with these findings, a developmental study (222) of calmodulin-dependent phosphatase in rat and chick nervous tissues has revealed that levels of the enzyme increase
TABLE II

Phosphoprotein Substrates for Calcineurin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor-1</td>
<td>Thr</td>
</tr>
<tr>
<td>G-Substrate</td>
<td>Thr</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>Thr</td>
</tr>
<tr>
<td>Phosphorylase Kinase</td>
<td>Ser</td>
</tr>
<tr>
<td>R II Subunit Kinase A</td>
<td>Ser</td>
</tr>
<tr>
<td>Myosin Light Chain</td>
<td>Ser</td>
</tr>
<tr>
<td>Protein K.-F.</td>
<td>Ser</td>
</tr>
<tr>
<td>Synapsin-I</td>
<td>Ser</td>
</tr>
<tr>
<td>Casein</td>
<td>Ser, Tyr</td>
</tr>
<tr>
<td>EGF R/K</td>
<td>Tyr</td>
</tr>
<tr>
<td>Histone H1</td>
<td>nd*</td>
</tr>
</tbody>
</table>

*nd, not determined.
during synaptogenesis.

Calcineurin is primarily isolated from bovine brain (223-228). Calmodulin-dependent phosphoprotein phosphatase activity has also been isolated from human platelets (229), skeletal muscle (213, 230), pancreas (231), rat brain (215), cardiac muscle (216, 232), erythrocytes (233), reticulocytes (234), rabbit brain (235), sea urchin (236), Xenopus oocytes (237), and Paramecium tetraurelia (238). The brain is found to have the highest levels of the protein as already discussed. The physical properties of these enzymes that have been determined are found to be similar to the bovine brain form, calcineurin.

Calcineurin was first isolated (223-228) as a calmodulin binding protein of molecular weight 80,000 and designated as CBP80. It was initially assayed by its ability to inhibit the activation of phosphodiesterase (223-228) and adenylate cyclase (226, 227) by calmodulin (then known as the Ca\(^{2+}\) dependent activator protein). It was later shown to possess phosphatase activity toward a number of phosphoproteins (212, 213). As isolated from bovine brain, calcineurin is a heterodimer of 80,000 molecular weight with subunits of 61,000 and 19,000 kilodaltons (225, 227, 228, 239). The larger, A, subunit is the catalytic subunit (239-243) and possesses binding sites for calmodulin
and divalent metal ions \((241,246,247)\). The smaller, \(B\), subunit possesses 4 binding sites for \(Ca^{2+}\) \((225,239,245,248)\) with a \(K_a = 1.0 \times 10^{-6}\) M and is myristylated at its amino terminal residue \((248,249)\). The \(B\)-subunit shares a fairly high degree of homology \((35\%)\) with bovine brain calmodulin \((248,250)\). The function of the \(B\)-subunit is not definite, but appears to serve a regulatory role \((245,251,252)\). Removal of the \(B\)-subunit from the holoenzyme leaves an active \(A\)-subunit \((240,241,251,252)\). The catalytic activity of the isolated \(A\)-subunit is stimulated by both calmodulin and the \(B\)-subunit \((251,252)\). The \(B\)-subunit activates the activity of the \(A\)-subunit greater than does calmodulin \((251)\). Activity is synergistically stimulated in the presence of both calmodulin and the \(B\)-subunit \((251)\).

The enzymatic properties of calcineurin are just beginning to be elucidated. Calmodulin activates the enzyme 5-10 fold \((212,213,240,241,251-255)\). The activation is due to an increase in \(V_M\) with little change in the \(K_M\) value for the substrate \((251,253-255)\). The activation process involves the binding of 2 metal ions \((Ca^{2+})\) to calmodulin which permits the interaction of calmodulin with the phosphatase \((256)\). Additional binding of metal ions \((Ca^{2+})\) produces activation of the enzyme. Calmodulin also induces
the deactivation of the phosphatase activity of calcineurin (257,258) upon prolonged incubation. This deactivation cannot be reversed by the dissociation of calmodulin, but the enzyme can be reactivated by the addition of metal ion in the presence of calmodulin (257).

The phosphatase activity of calcineurin is dependent upon the presence of a divalent metal ion (255,257-263); Mn²⁺ and Ni²⁺ support the highest activity although other metals activate the enzyme to some extent. It might be expected that the B-subunit is responsible for the metal ion activation; however, the A-subunit is known to bind 2 Ni²⁺ ions (246,247) suggesting that the catalytically active metal binds the catalytic subunit. It has been reported that calcineurin contains tightly bound metals, identified as Mn (260) or Fe and Zn (257). The subunit in which these metals are found is not known. The activation by metal ions is thought to be via the inducement of a conformational change in the protein (255,262,263). Support for a metal ion dependent conformational change, particularly in the B-subunit, has been obtained from studies of the interaction of subunit specific antibodies with calcineurin (264). It has recently been shown that activation of calcineurin activity is directly correlated to the binding of metal ion (247).
Calcineurin has been shown to be a substrate for both the Ca^{2+}-activated, phospholipid-dependent protein kinase (265) and a carboxymethyl transferase (266). Phosphorylation has no effect on the in vitro activity of calcineurin whereas methylation appears to have no effect on the basal activity (Mn^{2+} dependent) of the enzyme while reducing the calmodulin stimulated activity. The physiological effects of these modifications remain unknown. Trypsinization (242,243) produces an active fragment of the A-subunit which is not sensitive to calmodulin.

Fallen and Wang (267) determined that calcineurin possessed activity toward the low-molecular-weight phosphate esters, para-nitrophenvl phosphate and free tyrosine phosphate. Subsequently, naphthyl phosphates and phosphoenolpyruvate and other "activated phosphates" have been shown to be hydrolyzed by calcineurin (262,268). A number of other biological esters such as phosphoserine, phosphothreonine, glucose-6-phosphate, and nucleotide phosphates are not substrates for calcineurin (262,268). These low-molecular-weight esters are hydrolyzed with both a higher $V_m$ and a higher $K_m$ (mM) than phosphotyrosyl phosphoproteins (214,217,254,255,262).

The preceding discussion serves to illustrate the enormous amount of information that has been acquired
concerning the enzymes involved in protein phosphorylation-dephosphorylation. However, little is known concerning the features of the chemistry involved in the catalytic reactions. Detailed information about the chemical mechanism involved in protein phosphorylation been determined for only one kinase and not at all for phosphoprotein-specific phosphatases, although the non-specific phosphatases have been well characterized with respect to their chemical mechanisms (269-271).

Calcineurin is clearly the most well defined specific phosphoprotein phosphatase, but still little is known of the mechanism of action of this enzyme. The focus of this research has been to determine features of mechanism of action of calcineurin. We have exploited the low-molecular-weight phosphatase activity of calcineurin as a model enzymatic reaction to examine features of the chemistry of calcineurin catalysis as well as potential information of the chemistry involved in the dephosphorylation of phosphotyrosyl residues. Although it seems clear that the activity of calcineurin toward these esters is of little physiological significance, it enables specific, characterizable changes in the structure of the substrate. The approach we have utilized entails the application of tyrosine phosphate derivatives in which a substituent in the
aryl ring, primarily fluorine atoms, is incorporated. The substrates utilized are shown in Figure 1. Replacement of hydrogen with fluorine is interesting because of the introduction of a highly electronegative atom without the introduction of steric effects common with other substituents. Application of principles from physical organic chemistry and observations from model chemical reactions coupled with investigations of the enzymatic properties of the reaction provide powerful insights into the chemical mechanism of calcineurin and phosphotyrosyl dephosphorylation. The results of these studies may prove useful for the development of potential inhibitors of phosphotyrosyl protein phosphatases. The majority of these results have been published (272,273).
Figure 1. Low-molecular-weight substrates for calcineurin
EXPERIMENTAL PROCEDURES

**Materials** - *Escherichia coli* alkaline phosphatase, potato acid phosphatase, disodium para-nitrophenyl phosphate (Sigma 104 substrate), 3-nitrotyrosine, 3,5-dinitrotyrosine, disodium para-nitrophenyl sulfate, EGTA, SP-sephadex, and phenyl-sepharose were purchased from Sigma. All buffers utilized were also obtained from Sigma. (D,L)-3-fluorotyrosine was obtained from either Sigma or Aldrich. Tetrafluorotyrosine was from Columbia Organic Chemical Co. and pentafluorophenol was from Aldrich. (L)-Tyrosine was purchased from J.T. Baker. DE-52 cellulose was from Whatman and Dowex 50W-X8 was from Bio-Rad. Sterox (0.02%) was from Harleco. All other chemicals (malachite green, metal salts, etc.) were obtained from Fisher.

**Synthesis of Tyrosine Phosphate** - Tyrosine phosphate was synthesized by the method of Rothberg *et al.* (274). The yield was 116.0 mg from 1.4 g of tyrosine (5.75 % yield). The inorganic phosphate contamination (mol/mol) was less than 1%.

**Synthesis of F-Tyr-P and F₄-Tyr-P** - These compounds were synthesized from F-Tyr and F₄-Tyr, respectively, according to Rothberg *et al.* (274). The purification was as follows. The fluorotyrosine phosphate reaction mixture was
solubilized with ice and loaded onto a SP-sephadex column equilibrated with 1.0 M acetic acid and eluted with 1.0 M acetic acid isocratically. F-Tyr-P and P$_A$ eluted in the void while unreacted F-Tyr was retained. F-Tyr-P was separated from P$_A$ on an Ultrasphere-ODS (Altex) reverse phase HPLC column (C$_{18}$; 1.0 X 25.0 cm) employing isocratic elution with 0.1% trifluoracetic acid. The yield was from 10 to 40 mg of F-Tyr-P from 300 mg starting material (2.4-9.6% yield). F$_4$-Tyr-P was purified in a similar fashion by substituting Dowex 50W-X8 for SP-sephadex with H$_2$O as eluant followed by HPLC purification as before. The yield for this material was very low. Inorganic phosphate contamination was less than 1% for F-Tyr-P and less than 4% for F$_4$-Tyr-P.

Analysis of Synthesized Substrates - The purified Tyr-P, F-Tyr-P, and F$_4$-Tyr-P all yielded a single peak on analytical HPLC employing isocratic elution with 0.2 M triethylammonium acetate, pH 6.0, on a 0.46 X 25.0 cm Ultrasphere-ODS reverse phase column. The peak detected was distinct from the respective starting material for each compound. Upon hydrolysis in 6 N HCl at 110°C, Tyr, F-Tyr, and F$_4$-Tyr were formed, respectively, as shown by analytical HPLC just described. F-Tyr and F$_4$-Tyr hydroxylates were shown also to yield their respective starting materials by amino acid analysis. UV-VIS spectrophotometry indicated a
new spectrum for the phosphate ester compared with the starting material and showed no pH-dependent shift in the peak position. All three compounds were shown by $^{31}$P-NMR to have only a single organic phosphate species (shifts in ppm relative to 85% H$_3$PO$_4$); Tyr-P (pH 3.0), $\delta$=-1.85; F-Tyr-P (pH 3.0), $\delta$=-2.85; and F$_4$-Tyr-P (pH 7.0), $\delta$=3.8 ppm. F-Tyr-P and F$_4$-Tyr-P were also analyzed with $^{19}$F-NMR. F-Tyr-P (pH 7.0) resulted in a single peak at $\delta$=-55.6 ppm relative to trifluoroacetic acid (pH 6.8), $\delta$=0.0 ppm. F$_4$-Tyr-P yielded two peaks, each a doublet, at $\delta$=-70 ppm and $\delta$=-80 ppm relative to trifluoroacetic acid. NMR spectra were obtained on a Bruker WM-300 NMR spectrometer operating at 121.5 and 282.4 MHz for $^{31}$P and $^{19}$F, respectively.

Preparation of Substrate Solutions - Stock solutions of Tyr-P and F-Tyr-P were prepared from dry solids. Alkaline and acid phosphatase hydrolysis of Tyr-P yielded 100% expected P$_4$ release. Similar treatment of F-Tyr-P yielded 93% and 98%, respectively, expected P$_4$ release. Amino acid analysis of the F-Tyr-P hydrozylate yielded 94% of the expected value. All stock solutions were used as prepared from dry weight with correction for P$_4$ contamination. Stock solutions of F$_4$-Tyr-P were prepared and concentrations determined by amino acid analysis.
Enzymes - Calcineurin, isolated from bovine brain by the method of Sharma et al. (275) was kindly provided by Dr. Jerry H. Wang (Dept. of Medicinal Biochemistry, Univ. of Calgary, Calgary, Alberta, Canada). Calmodulin was purified by the procedure of Sharma and Wang (276) with a slight modification. Following DE-52 cellulose chromatography, the calmodulin was loaded onto phenyl-sepharose and eluted with EGTA (277).

Assay for Inorganic Phosphate - The release of $P_i$ was assayed with a modification of a method employing malachite green as an indicator as developed by Hess and Derr (278). In general, 10-40 µl of sample were added to 30 µl 5 N $H_2SO_4$ and enough $H_2O$ to make a final volume of 200 µl. Malachite green (800 µl) reagent was added; the solution was mixed, allowed to sit for 10 minutes, and then remixed. The absorbance of the solution was then measured at 660 nm. The malachite green was prepared as follows: 3 volumes of 0.045% malachite green in $H_2O$ was mixed with 1 volume of 4.2% ammonium molybdate in 4 N HCl. This mix was stirred for 30 minutes followed by the addition of 1.0 ml 0.02% sterox for each 50 ml of dye solution. The solution was then filtered and ready for use. A standard curve was linear over the range 0.5-8.0 nmol of $P_i$, with a typical extinction coefficient of 0.085 A₆₆₀/nmol $P_i$. Typically,
the reagent was good for 1-2 weeks if stored in the cold. No interference by buffers, metal ions, proteins, or substrates and products of the reactions on the accuracy and reproducibility of the assay was observed.

**Assay for para-Nitrophenol** - The formation of para-nitrophenol was followed at 410 nm. Reaction aliquots were diluted to 1.0 ml with 0.1 M glycine, pH 9.5, mixed, and the absorbance measured at 410 nm. An extinction coefficient of $1.73 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was employed for pNP.

**Assay for Protein** - Acid phosphatase, calmodulin, and calcineurin were all measured by the method of Bradford (279). Alkaline phosphatase was measured by employing an $\varepsilon_{280}^\text{max}=7.2$ (280).

**Alkaline Phosphatase Assay** - Alkaline phosphatase assays were carried out in 10 mM glycine, pH 9.5, 1 mM MgCl$_2$ and 0.1 mM ZnCl$_2$ at 30°C. Initial rate kinetics were followed spectrophotometrically in a reaction volume of 1.25 ml. Dephosphorylation of Tyr-P was followed at 285 nm by measuring the appearance of tyrosine by employing an $\varepsilon_{285}=607 \text{ M}^{-1} \text{ cm}^{-1}$. Hydrolysis of F-Tyr-P was performed at 290 nm by using an $\varepsilon_{290}=1970 \text{ M}^{-1} \text{ cm}^{-1}$ for F-Tyr. The amount of enzyme used in each assay was 0.5 µg. The time course was generated by removing 10 µl aliquots from a 100 µl mixture for the measurement of inorganic phosphate. The
enzyme level was 3.0 μg/ml.

**Acid Phosphatase Assays** - Assays were done in a reaction volume of 100 μl in 100 mM NaOAc, pH 4.8 at 30°C (281). At different intervals, 10 μl were removed and assayed for inorganic phosphate. The protein level was 2.3 μg/ml.

**Calcineurin Assays** - The phosphatase activity of calcineurin was assayed in 25 mM MOPS, pH 7.0, 1 mM MnCl₂ at 30°C. Calmodulin was included at an equivalent concentration (μg/ml) as calcineurin. The reaction volume was maintained at 50 μl when assaying for the release of P₄ whereas assays for pNP release were done in reaction volumes of 100 μl. Generally, activity was measured via the direct measurement of inorganic phosphate, the exceptions being the for the phosphotransferase activity and the inhibition by inorganic phosphate and phosphate analogs. For initial rate studies, 40 μl of the 50 μl were assayed for P₄ with Tyr-P and F-Tyr-P, and 20 μl for pNPP and F₄-Tyr-P. With pNPP, F-Tyr-P, and F₄-Tyr-P, the reaction was linear for 6 minutes, whereas with Tyr-P, the reaction was linear for 15 minutes. Replacement of 1.0 mM MnCl₂ with 20.0 mM MgCl₂ did not affect the linearity of the reaction. Typically, initial rates were taken from a 3 minute reaction point for pNPP, F-Tyr-P, and F₄-Tyr-P and a 9 minute reaction point for Tyr-P.
Calcineurin was used at a concentration of 28 μg/ml with pNPP and Fα-Tyr-P and 35 μg/ml with F-Tyr-P and Tyr-P. The extended time course was generated with a 100 μl reaction mix with 10 μl aliquots assayed for inorganic phosphate. The calcineurin concentration was 17.5 μg/ml.

**Analog Inhibitors** - Inhibition by pNP, tyrosine derivatives, and phosphate analogs was assayed by measuring the amount of P_4 produced. The concentrations of inhibitors used were 19.4-48.5 mM pNP, 10.9-43.6 mM PFP, 5.0-30.0 mM pNPS, and 0.5-4.0 mM for K₂HPO₄ and Na₂SO₄. All tyrosine derivatives were tested at a concentration of 1 mM because of solubility constraints and for comparison with the inhibition by P₄ at 1 mM.

**Inhibition Kinetics** - All inhibition kinetics were performed in 25 mM MOPS, pH 7.0. Inhibition by P₄ was carried out with a pNPP concentration range of 3.3-20.0 mM. The kinetics of the inhibition by pNP were performed in 3.5% ethanol to provide for pNP solubility at the levels required for significant inhibition. At this concentration of ethanol, there was a slight increase in the K_M values for pNPP. Therefore, the range of pNPP concentrations was raised from 8.4 to 50.0 mM. PFP inhibition of calcineurin was studied over a range of 5.0 to 20.0 mM pNPP. Ethanol was not required for these experiments.
Phosphotransferase Assays - Calcineurin was assayed for phosphotransferase activity utilizing a reaction volume of 100 µl with pNPP as the substrate. After a reaction time of 3 minutes, 10 µl were assayed for P₁ and the remaining 90 µl were assayed for pNP.

pH Kinetics - The pH dependence of the kinetic parameters of calcineurin catalysis was performed utilizing substrate concentrations in the range 4.0-60.0 mM for pNPP and 3.3-20.0 mM Tyr-P. The buffer concentration was maintained at 25 mM. The reaction volume was maintained at 50 µl. The effect of replacement of Mn²⁺ with Mg²⁺ on the kinetic parameters was measured utilizing substrate ranges of 8.4-50.0 mM and 3.3-20.0 mM for pNPP and Tyr-P, respectively. The reaction volume was maintained at 50 µl.

pH Stability of Calcineurin Activity - Calcineurin (0.70 mg/ml) was incubated at each pH utilized in the kinetic study at 30°C in a total volume of 100 µl. The buffer concentration during the incubation was maintained at 25 mM. MnCl₂ (1 mM) and calmodulin (0.70 mg/ml) were also present. After incubation for 3 minutes (duration of a typical assay), 4 µl were removed for assaying at pH 7.0. The assay volume was 100 µl, with other conditions as in the standard assay.

Buffers - All assays were performed at 25 mM buffer.
The buffers and the pH values at which they were employed are: MES, 5.8, 6.1, 6.4, and 6.7; PIPES, 6.4, 6.7, 7.0, and 7.3; MOPS, 6.7, 7.0, 7.3, and 7.6; HEPES, 7.0, 7.3, 7.6, and 7.9; TRICINE, 7.6, 7.9, 8.2, and 8.5; and BICINE, 7.9, 8.2, 8.5, and 8.8. Kinetics were performed in MES at pH 5.8, 6.1, and 6.4; in MOPS at pH 7.0 and 7.6; in TRICINE at pH 8.2; and in BICINE at pH 8.8.

**Determination of Kinetic Parameters** - The parameters, $V_M$, $K_M$, and $K_2$ were evaluated utilizing the program of Siano et al. (282) written in OMNITAB language.

**Determination of pK_a Values** - The pK_a values for the free enzyme and enzyme-substrate complex were evaluated by fitting the data to the following equations (283):

\[
(Y)_H = Y - (Y)_H \frac{[H^+]}{K_a} \quad (1)
\]

\[
(Y)_H = Y - K_a \frac{(Y)_H}{[H^-]} \quad (2)
\]

where $(Y)_H$ is the value of $V_M$ or $V_M/K_M$ determined at each pH and $Y$ is the pH independent value for the parameter. The pK_a for the enzyme-substrate complex was evaluated from the $V_M$ vs. pH data set fit to equation 1. The $V_M/K_M$ vs. pH data set was analyzed by fitting the data to equation 2.

**Acid Catalyzed Hydrolysis** - The acid-catalyzed hydrolyses of the tyrosine phosphate analogs were carried out at 110°C in 6 N HCl in hydrolysis tubes. A reaction mixture was set up for each time point taken. Values of $k_H$. 
were determined from plots of log (per cent remaining ester) versus time yielding $t_{1/2}$ from which $k_{H-}$ is calculated from the relationship $k_{H-} = 0.693/t_{1/2}$. Time points taken ranged from 0.5 to 2.5 $t_{1/2}$ minimally. The concentrations employed were 300 μM pNPP, 222 μM Tyr-P, 227 μM F-Tyr-P, and 299 μM F$_4$-Tyr-P.
RESULTS AND DISCUSSION

Both *E. coli* alkaline phosphatase (284) and potato acid phosphatase (281) are known to dephosphorylate phosphate esters of widely different structure with a constant $V_M$ value. In Figure 2, the dephosphorylation of Tyr-P and F-Tyr-P by these two enzymes as well as by calcineurin is demonstrated as a function of time. Each of the two non-specific phosphatases is seen to hydrolyze the two esters with the same progress curve while calcineurin apparently recognizes the two compounds differently. A kinetic investigation of the hydrolysis of these two substrates was performed to determine if the difference observed was merely an effect of $K_M$ or really a difference in $V_M$. As seen in Table III, alkaline phosphatase hydrolyzed both compounds with essentially the same $V_M$, as expected. In contrast to this observation, calcineurin was observed to hydrolyze F-Tyr-P much more readily than Tyr-P.

A further investigation of the kinetic parameters of calcineurin with the other substrates given in Figure 1 reveal that calcineurin, indeed, does not seem to hydrolyze these phosphate esters with a constant maximum velocity. The kinetic parameters of calcineurin are compiled in Table IV. This initial observation suggests that, unlike the non-
Figure 2. Progress curve for the hydrolysis of Tyr-P and F-Tyr-P. The substrates were hydrolyzed by E. coli alkaline phosphatase ( , ), potato acid phosphatase ( , ), and calcineurin ( , ) at pH 7.0. In all cases, the substrate concentration is 10mM. Open symbols represent the hydrolysis of Tyr-P and closed symbols represent the hydrolysis of F-Tyr-P. Other details are given in the "Experimental Procedures" section.
30

A, Acid Phosphatase [pH 4.8]
□, Alkaline Phosphatase [pH 9.5]
○, Calcineurin [pH 6.8]

TYR-P
F-TYR-P

CONCENTRATION P_i [mM]

TIME [Min]

120 240 360
**TABLE III**

Comparison of Hydrolysis of Tyr-P and F-Tyr-P by Alkaline Phosphatase and Calcineurin*

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Alkaline Phosphatase</th>
<th>Calcineurin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_m^b$</td>
<td>$K_m^c$</td>
</tr>
<tr>
<td>Tyr-P</td>
<td>1.16±0.06</td>
<td>47.9±7.5</td>
</tr>
<tr>
<td>F-Tyr-P</td>
<td>1.52±0.13</td>
<td>93.6±20.9</td>
</tr>
</tbody>
</table>

*Amounts of enzyme used were: 1.75 μg calcineurin and 0.5 μg alkaline phosphatase.

$V_m^b$ is given units of $10^{-8}$ M/min.

$K_m^c$ is in units mM for calcineurin catalyzed hydrolysis and μM for alkaline phosphatase.
### TABLE IV

**Kinetic Parameters for Calcineurin-catalyzed Hydrolysis**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pKₐ LG</th>
<th>Relative $v$</th>
<th>$K_m$ (mM)</th>
<th>Relative $V_m/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-P</td>
<td>10.07</td>
<td>0.030±0.003</td>
<td>6.20±0.7</td>
<td>0.048±0.002</td>
</tr>
<tr>
<td>F-Tyr-P</td>
<td>9.21</td>
<td>0.14±0.02</td>
<td>4.20±0.7</td>
<td>0.35±0.02</td>
</tr>
<tr>
<td>pNPP</td>
<td>7.14</td>
<td>1.00±0.1</td>
<td>10.1±1.4</td>
<td>1.00±0.1</td>
</tr>
<tr>
<td>F₄-Tyr-P</td>
<td>5.40</td>
<td>0.29±0.07</td>
<td>1.7±0.6</td>
<td>2.50±0.4</td>
</tr>
</tbody>
</table>

*a Corrected for the amount of enzyme (1.75 µg/assay for F-Tyr-P and Tyr-P, 1.4 µg/assay for pNPP and F₄-Tyr-P) added in assay. For comparison, the $V_m$ with pNPP is 3.7 µmol/min/mg enzyme.

*Reference 285.

*Reference 286.

*Reference 287.

*The value of $K_m$ (pNPP) = 14.4 mM in kinetic experiments utilizing F₄-Tyr-P. The value $V_m/K_m$ (F₄-Tyr-P) is corrected for this difference.

*Reference 288.
specific acid (289) and alkaline (290) phosphatases, calcineurin does not generate a phosphoryl-enzyme intermediate during the hydrolysis of phosphate esters. Figure 3 demonstrates that the maximal velocity of calcineurin is not independent of the leaving group in contrast to the situation found for the nonspecific phosphatases (281,284).

The failure to observe hydrolysis of these phosphate esters with a constant $V_m$ suggests that the calcineurin-catalyzed hydrolysis does not proceed with rate limiting decomposition of a common intermediate; that is, the rate limiting step in calcineurin catalysis does not involve the decomposition of a covalent or noncovalent phosphoryl-enzyme complex. Further investigation of this point was performed by examining whether calcineurin possessed phosphotransferase activity. The possession of phosphotransferase activity implies the existence of a phosphoryl-enzyme as illustrated in the reaction scheme given in Figure 4. The inclusion of an alcohol in the reaction mixture provides a second phosphoryl acceptor in addition to water, the normal acceptor. Phosphotransferase activity of calcineurin was assayed for utilizing tris or ethylene glycol as possible phosphoryl acceptors in competition with water. Tris has been shown to serve as an
Figure 3. Dependence of log ($V_m$) for calcineurin catalysis upon the $pK_a$ of the leaving group. The line on the high side of the $pK_a$ range was generated from a linear regression fit of the data from Table IV with a correlation coefficient of $-0.99$. 
Figure 4. Reaction scheme depicting the transphosphorylation of a competing alcohol. The reaction scheme presumes the formation of a phosphoryl-enzyme as an intermediate.
acceptor in alkaline phosphatase-catalyzed trans-phosphorylation reactions (291), whereas ethylene glycol has been shown to be an acceptor in an acid phosphatase system (292). In the presence of either acceptor, the initial velocity of the calcineurin-catalyzed hydrolysis of pNPP was the same, independent of which product was measured as shown in Figure 5. That is, there was no transfer of the phosphoryl group to either tris or ethylene glycol. This supports the contention that calcineurin does not generate a phosphoryl-enzyme intermediate in its mechanism of action.

The mechanistic interpretation of enzymatic reactions is greatly simplified by the application of physical organic chemistry, specifically with the aid of Brønsted or Hammett plots (293). A modified Brønsted plot is a linear free-energy relationship in which the logarithm of the rate constant is related to the energy term, expressed as a $pK_a$ value. The varied species may either be the incoming nucleophile or the leaving group. The slope term in the linear relationship, designated $\beta_{\text{nu}}$ or $\beta_{\text{lo}}$, reflects the amount of bond cleavage/formation in the transition state.

The relationship is more directly applied to the $V_M/K_M$ term than the $V_M$ term (294). The $V_M$ term is dependent upon a number of features, including accumulation of intermediates and nonproductive binding, which may
Figure 5. Phosphotransferase activity of calcineurin. Calcineurin was assayed in the presence of either tris or ethylene glycol by measuring the release of $P_i$ (○) or pNP (●). A) The reaction was performed in the presence of tris. B) The reaction was performed in the presence of ethylene glycol. In both situations, the activity of calcineurin was measured as described in the "Experimental Procedures" section.
The figure shows two graphs. The upper graph plots the relationship between the concentration of TRIS (M) and the rate of reaction (V in nmol/min). The data points are represented by circles, with solid circles indicating PNP and open circles indicating P_i.

The lower graph plots the relationship between the concentration of ethylene glycol (M) and the rate of reaction (V in nmol/min). The data points are represented similarly, with solid circles indicating PNP and open circles indicating P_i.
complicate the analysis. These features are more likely to cancel out in the $V_m/K_m$ term. The application of Brønsted plots to enzyme-catalyzed reactions is complicated, however, because of multiple interactions occurring within and about the active site of an enzyme. This effect is lessened by comparison of the enzymatic reaction to model chemical reactions of the respective substrates. Comparison of the $\beta_{LO}$ values for enzymatic reactions with the $\beta_{LO}$ for well defined chemical reactions, then, can reveal mechanistic information of the enzymatic reaction. This value, in conjunction with values of $\beta_{LO}$ obtained in model studies, yields valuable information about the mechanism of calcineurin catalysis.

As a model reaction for the enzymatic catalyzed hydrolyses of the tyrosine phosphate analogs utilized, the acid catalyzed hydrolyses of these compounds was also studied. As described in the Experimental Procedures, the kinetics of the hydrolysis of these compounds in 6 N HCl at 110°C was investigated. Table V collects the data for this reaction. Figure 6 shows that the rate constant of this reaction is subject to a dependence upon the electronic properties of the compounds.

The results of this analysis indicate that the specificity of calcineurin is dependent upon the nature of
TABLE V

Kinetic Parameters for Acid-catalyzed Hydrolysis

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pKₐ LG</th>
<th>t₁/₂ (min)</th>
<th>kₚ⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-P</td>
<td>10.07</td>
<td>56.8</td>
<td>0.012</td>
</tr>
<tr>
<td>F-Tyr-P</td>
<td>9.21</td>
<td>21.1</td>
<td>0.033</td>
</tr>
<tr>
<td>pNPP</td>
<td>7.14</td>
<td>5.1</td>
<td>0.136</td>
</tr>
<tr>
<td>F₄-Tyr-P</td>
<td>5.40</td>
<td>1.2</td>
<td>0.563</td>
</tr>
</tbody>
</table>

* pKₐ values from Table IV.

** Determined from plots of log (% remaining reactant) versus time.
Figure 6. The dependence of $\log (k_{H^+})$ for acid-catalyzed hydrolysis upon the $pK_a$ of the leaving group for the tyrosine phosphate derivatives. The line shown was generated with linear regression. The slope of the line is $-0.35$. The correlation coefficient is $-0.99$. The data are from Table V.
the substrate, particularly the phenolic leaving group. Figure 7 shows that, as the $pK_a$ of the resulting phenol is lowered, the activity of calcineurin (measured by $V_{M}/K_M$) is increased. The $pK_a$ of the phenol is also taken to be a measure of the lability of the phosphate ester bond. A similar treatment has also been done for a number of hydrolytic enzymes (295-300) as well as the nonenzymatic hydrolyses (301,302) of phosphate esters. The $\beta_{Lo}$ value for the calcineurin-catalyzed hydrolysis of the phosphate esters employed here was observed to be -0.35. This value is the same as the value of $\beta_{Lo} = -0.35$ observed for the acid-catalyzed hydrolysis of the same phosphate esters (Figure 6). The equality of the $\beta_{Lo}$ values for the two processes suggests that a relationship between the enzymatic- and acid-catalyzed hydrolyses exists. Figure 8 shows that there is a 1:1 correlation between the rate constants for the enzymatic and chemical hydrolysis. A 1:1 correlation between enzymatic and chemical hydrolysis was also observed by Benkovic et al. (295) for sulfate ester hydrolysis by acid and an aryl sulfatase from Aspergillus oryzae. They interpreted this finding as indicating that the effect of substrate structure on the enzymatic activity is manifested in the velocity term, not the binding term. Our results suggest that the electronic nature of the substrate may also
Figure 7. The dependence of log \( \frac{V_{\text{cat}}}{K_M} \) for calcineurin catalysis upon the \( pK_a \) of the leaving group. The line shown was generated with linear regression. The slope of the line is -0.35. The correlation coefficient is -0.94. The data are from Table IV.
Figure 8. Relationship of enzymatic hydrolysis to chemical hydrolysis. The line shown was generated with linear regression; the correlation coefficient is 0.97. The slope of the line is 0.96. The data are from Tables IV and V.
Figure 9. Dependence of the $K_m$ for calcineurin-catalyzed hydrolysis of each tyrosine phosphate derivative upon the $pK_a$ of the leaving group. The line depicted was generated for the data from the amino acid substrates only. The line shown was generated by linear regression; the correlation coefficient is 0.99. The data are from Table III.
affect the binding of substrate. As shown in Figure 9, when only the amino acid substrates are considered, there is a relationship between the $K_M$ for the amino acid substrate and the $pK_a$ of the leaving group. Not unexpectedly, the $K_M$ value for pNPP deviates greatly from this relationship. It is unclear that calcineurin is subject to electronic effects on substrate binding and catalytic specificity.

The chemistry of phosphate esters has been extensively studied with a similar analysis. Table VI provides the $\beta_{me}$ values for a number of chemical reactions (301-303) as well as the reactions of tyrosine phosphate analogs as studied in the present work. The data suggest three plausible chemical mechanisms: 1) nucleophilic attack on the substrate by the enzyme with electrophilic assistance by a group on the enzyme, possibly bound metal ion; 2) hydrolysis of the substrate by H$_2$O coordinated to Mn$^{2+}$ with electrophilic assistance by a group on the enzyme; and 3) unimolecular breakdown of metal coordinated substrate following proton transfer from the protein.

The first mechanism involves nucleophilic attack on the substrate by the enzyme generating a phosphoryl-enzyme intermediate. A mechanism of this type is known to occur in the mechanism of action of alkaline and acid phosphatases (281,304). Nucleophilic attack on phosphate esters by a
## TABLE VI

Values of the Brønsted Coefficient for Phosphate Reactions

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$\beta$</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Catalyzed Hydrolysis</td>
<td>-0.33</td>
<td>Present Study</td>
</tr>
<tr>
<td>Calcineurin Catalysis</td>
<td>-0.32</td>
<td>Present Study</td>
</tr>
<tr>
<td>Monoanion Hydrolysis</td>
<td>-0.32</td>
<td>301,302</td>
</tr>
<tr>
<td>Dianion Hydrolysis</td>
<td>-1.23</td>
<td>301,302</td>
</tr>
<tr>
<td>Monoanion + Nucleophile</td>
<td>-1.00</td>
<td>303</td>
</tr>
<tr>
<td>Dianion + Nucleophile</td>
<td>-1.00</td>
<td>303</td>
</tr>
</tbody>
</table>
number of different nucleophiles has been shown to have a $\beta_{le} = -1.00$ (303). In a study of *E. coli* alkaline phosphatase, Williams et al. (305) have shown that, for a series of substituted phenyl phosphates, $k_o/K_M (V_M/K_M)$ does show a dependency upon the electronic characteristics of the substrate, whereas $k_o (V_M)$ is constant. Hammett analysis of these data (305) yields a sensitivity lower than expected (corresponding to a value of $\beta_{le}$ less negative than expected) for a nucleophilic attack. This is interpreted as indicating electrophilic assistance in either binding of substrate or phosphorylation of free enzyme. Similar results were obtained for a number of proteolytic enzymes (296-299) which are known to proceed via a covalent acyl-enzyme intermediate generated by nucleophilic attack on the substrate. Electrophilic assistance has also been suggested to have a role in aryl sulfatase action (306). The electrophilic assistance is presumed to come from some electrophilic protein group or divalent metal ion coordinated to the ester. The coordination results in increased polarization of the ester linkage. The phosphorous center will become electropositive, making it more susceptible to nucleophilic attack. It is of interest that calcineurin has been reported to require tightly bound metal for full activity (259,260,263).
The second model proposes a different role for metal ion: activation of the nucleophilicity of H₂O for direct hydrolysis by manganese coordinated H₂O on the phosphate ester. Coordination of H₂O to Mn²⁺ lowers the pKₐ of H₂O from 15.74 to 10.6 (307) which serves to enhance the nucleophilicity of the water molecule. A similar role has been proposed for Zn²⁺ in the mechanism of action of alkaline phosphatase (269, 270). Hydrolysis of phosphate ester dianions has been observed to proceed with a β_L = -1.23 (301). The value of β_L = -0.35 for calcineurin catalysis may be indicative of electrophilic assistance by the protein to the attack by water.

Finally, Kirby and Vargolis (301) and Bunton et al. (302) have observed values of β_L = -0.27 (301) and -0.32 (302) for the hydrolysis of phosphate ester monoanions. This is the same value of β_L obtained for both the calcineurin- and acid-catalyzed hydrolysis observed in this report. Kirby and Vargolis (301) and Bunton et al. (302) have argued that monoanion hydrolysis proceeds via specific acid catalysis with protonation of the ester-linkage occurring in a rapid preequilibrium step followed by the elimination of the phenol. A similar mechanism may be employed in the acid-catalyzed hydrolysis of phosphate esters. Application of this model to calcineurin catalysis
would require monoanion formation on the enzyme, possibly via coordination with protein or metal ion.

One feature which distinguishes these models is the presence or absence of a phosphoryl-enzyme intermediate. The failure to detect phosphotransferase activity is consistent with the absence of phosphoryl-enzyme formation. These results suggest that direct nucleophilic attack on the substrate by the enzyme is not the chemical mechanism. These data are insufficient, however, to distinguish between the remaining possibilities.

The lack of phosphotransferase activity provides insight into the kinetic steps in the mechanism of calcineurin. There are preparations of alkaline (308,309) and acid (296) phosphatases which do not show phosphotransferase activity. It has been suggested that this is because of a change in the rate-limiting step to phosphorylation of free enzyme (309). Application of these observations to calcineurin suggests that the rate-limiting step occurs prior to the release of products. Candidates for the rate-limiting step are binding of substrate, a conformational change, or cleavage of the phosphate ester bond. The observation that log \( \frac{V_m}{K_m} \) is a function of the pK\(_a\) of the leaving group is consistent with a rate-limiting cleavage step provided it is accepted that the pK\(_a\) of the
leaving group is a measure of the lability of the phosphate ester bond. It seems unlikely that binding of substrate could be rate-limiting. However, there is a relationship between the ability of a compound to serve as a substrate (reflected as $V_m/K_m$) and its $K_m$ value, again only for the amino acid substrates. This relationship is shown in Figure 10. This indicates that electronic effects on substrate binding may explain the substrate specificity of calcineurin. Finally, it is possible that a conformational change in the enzyme might be the rate-limiting step. Our data are insufficient to determine the rate-limiting step. However, the evidence is not conclusive. Protonation and departure of the leaving group might still be the rate-limiting step.

Further information regarding the chemical mechanism of calcineurin can be obtained from an examination of the kinetic mechanism of the enzymatic reaction. Product inhibition studies were performed to determine the kinetic mechanism. Both $P_i$ and pNP show concentration dependent inhibition of the calcineurin catalyzed hydrolysis of pNPP as shown in Figures 11A and 11B. As seen, inhibition by $P_i$ occurs at much lower concentrations than for pNP. A kinetic study indicates that both products are competitive inhibitors as indicated by the Lineweaver-Burk plots shown
Figure 10. Relationship of $K_M$ to $V_m/K_m$. The line shown was generated with linear regression for the amino acid substrates only. The correlation coefficient is -0.98. The data are from Table IV.
Figure 11. Inhibition of Calcineurin by $P_i$ and pNP.

Calcineurin was assayed in the presence of varied concentrations of $P_i$ or pNP at a pNPP concentration of 10 mM at 30°C. Activity was measured by determining the amount of pNP or $P_i$, respectively, released after 3 minutes. Other conditions are given in the "Experimental Procedures" section. A) Inhibition by $P_i$. B) Inhibition by pNP
Figure 12. Kinetics of the Inhibition of Calcineurin by P1.
Calcineurin was assayed over a range of pNPP concentrations from 3.3 mM to 20.0 mM in the presence of 0.0 (○), 1.0 (●), 2.0 (▲), and 3.0 (▲) mM P1. Other conditions are given in the "Experimental Procedures" section.
Figure 13. Kinetics of the Inhibition of Calcineurin by pNP. Assays were performed in the presence of 0.0 (○), 19.4 (●), 34.0 (▲), 39.0 (▲), and 48.5 (□) mM pNP over the range of pNPP concentrations of 8.4 mM to 50.0 mM. Other conditions are as given in the "Experimental Procedures" section.
in Figures 12 and 13. Replots of the slope vs. [I] for both products are given in Figures 14A and 14B. These figures indicate that P is a linear competitive inhibitor with a $K_i = 1.1 \pm 0.2$ mM while PNP is observed to be a nonlinear competitive inhibitor (the dashed line represents an estimation of the lower limit of the $K_i$ value for PNP). The nonlinearity prevents conclusive identification of the kinetic mechanism. Therefore, an analog of PNP, pentafluorophenol, was tested as an inhibitor. Pentafluorophenol resembles tetrafluorotyrosine, the product of the hydrolysis of tetrafluorotyrosine phosphate, which is a substrate for calcineurin. The results of the inhibition study with PFP indicate that PFP is a competitive inhibitor, with $K_i = 26.4 \pm 3.1$ mM. This is shown in Figure 15 in the form of Lineweaver-Burk. A replot of the slope values from Figure 15 vs. the PFP concentration indicates that PFP is a linear competitive inhibitor (not shown). The rapid equilibrium random uni-bi kinetic mechanism (310) is illustrated in Figure 16A. An ordered hydrolysis, this indicates that there is no preference to the order of release of products following hydrolysis. In the hydrolytic direction, this indicates that there is no inhibition study with PPP indicate that PPP is a competitive inhibitor, with $K_i = 26.4 \pm 3.1$ mM. The results of the inhibition study with PPP indicate that PPP is a competitive inhibitor, with $K_i = 26.4 \pm 3.1$ mM. This is shown in Figure 15 in the form of Lineweaver-Burk. A replot of the slope values from Figure 15 vs. the PPP concentration indicates that PPP is a linear competitive inhibitor (not shown). The rapid equilibrium random uni-bi kinetic mechanism (310) is illustrated in Figure 16A. An ordered hydrolysis, this indicates that there is no preference to the order of release of products following hydrolysis. In the hydrolytic direction, this indicates that there is no inhibition study with PPP indicate that PPP is a competitive inhibitor, with $K_i = 26.4 \pm 3.1$ mM. The results of the inhibition study with PPP indicate that PPP is a competitive inhibitor, with $K_i = 26.4 \pm 3.1$ mM. This is shown in Figure 15 in the form of Lineweaver-Burk. A replot of the slope values from Figure 15 vs. the PPP concentration indicates that PPP is a linear competitive inhibitor (not shown). The rapid equilibrium random uni-bi kinetic mechanism (310) is illustrated in Figure 16A. An ordered hydrolysis, this indicates that there is no preference to the order of release of products following hydrolysis. In the hydrolytic direction, this indicates that there is no
Figure 14. Replots of Slope vs. [Inhibitor] from the Kinetics of Inhibition of Calcineurin by $P^i$ and pNP. A) Replot from the inhibition by $P^i$. The line shown was generated by linear regression analysis. The correlation coefficient is 0.99. B) Replot from the inhibition by pNP. The dashed line represents an estimation of the $K_i$ for pNP as given in Table VIII.
Figure 15. Kinetics of the Inhibition of Calcineurin by PFP. Assays were performed in the presence of 0.0 (○), 10.9 (●), 21.8 (△), 43.6 (▲) mM PFP over the range of pNPP concentrations of 5.0 mM to 20.0 mM. Other conditions are as given in the "Experimental Procedures" section.
Figure 16. Sequential kinetic schemes for the hydrolysis of phosphate esters. In these diagrams, E represents enzyme, ROP the substrate phosphate, ROH the product alcohol, and Pi inorganic phosphate. A) A random kinetic scheme. B) An ordered kinetic scheme in which Pi is the final product released.
generated in the mechanism of action of calcineurin. Coupled with the dependence of substrate hydrolysis upon the pK$_a$ of the phenolic leaving group and the absence of phosphotransferase activity at pH 7.0, this provides convincing evidence that calcineurin does not form a covalent phosphoryl-enzyme. This would be in stark contrast to the situation observed during the catalysis by alkaline (290) and acid phosphatases (289).

As noted, pNP is a nonlinear competitive inhibitor. Nonlinear inhibition by pNP has been observed previously in the noncompetitive inhibition of an aryl sulfatase (311). The nonlinearity may be due to the existence of multiple binding sites for pNP coupled with a conformational change resulting in inhibition. Initial binding at a single site may be insufficient to generate the inhibited conformation. Segel (312) has described a kinetic analysis (equation 3)

\[
\log \left( \frac{V_1}{V_o - V_1} \right) = -n \log [I] + \log K_i \quad (3)
\]

for multiple binding sites. Application to the inhibition of calcineurin by pNP indicates that 3 binding sites for pNP exist. Another explanation for the nonlinearity may be stacking interactions of pNP molecules generating a more potent inhibitor than a single molecule alone. With respect
to this point, it is interesting to note that calcineurin is seemingly subject to substrate inhibition in the presence of high pNP concentrations (see Figure 13, data for 39.0 and 48.5 mM pNP).

As noted, P$_4$ inhibited calcineurin at much lower concentrations than does pNP. This suggests that the phosphoryl moiety of the substrate is of more importance in the binding of substrate than the phenolic function. However, the $K_m$ values for tyrosine phosphate derivatives seem to show a relationship to the $pK_a$ of the tyrosyl leaving group (Figure 9). It was, therefore, of interest to examine the potential role of the aryl function in substrate binding. This was performed by testing a number of tyrosine, serine, and threonine derivatives as inhibitors of calcineurin. None of the compounds tested produced significant inhibition. This was independent of substitution in the aryl ring ($X=\text{F}, \text{NO}_2, \text{or F}_4$), blockage of the phenolic hydroxyl (phosphate or sulfate), or blockage of the amino acid functional groups (N-acetyl or ester derivatives) as shown in Table VII. These results and the weak inhibition by pNP and PFP, evidenced by the values of $K_i$ shown in Table VIII, are consistent with poor binding of the phenolic portion of the substrate. It is unclear why tyrosine phosphate derivatives do not inhibit very well
### TABLE VII

Inhibition of Calcineurin by Tyrosine Derivatives

<table>
<thead>
<tr>
<th>Addition</th>
<th>Percent Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>(L)-Tyr</td>
<td>96</td>
</tr>
<tr>
<td>(D)-Tyr</td>
<td>90</td>
</tr>
<tr>
<td>(D,L)-3-F-Tyr</td>
<td>94</td>
</tr>
<tr>
<td>(D,L)-2,3,4,5-F₄-Tyr</td>
<td>98</td>
</tr>
<tr>
<td>3-NO₂-Tyr</td>
<td>90</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>91</td>
</tr>
<tr>
<td>Serine</td>
<td>95</td>
</tr>
<tr>
<td>Threonine</td>
<td>82</td>
</tr>
<tr>
<td>Tyr Ethyl Ester</td>
<td>95</td>
</tr>
<tr>
<td>N-Acetyl-Tyr Ethyl Ester</td>
<td>92</td>
</tr>
<tr>
<td>Serine Methyl Ester</td>
<td>83</td>
</tr>
<tr>
<td>Serine Phosphate</td>
<td>91</td>
</tr>
<tr>
<td>Threonine Phosphate</td>
<td>98</td>
</tr>
<tr>
<td>Tyrosine sulfate</td>
<td>91</td>
</tr>
</tbody>
</table>

*Activity was determined at a pNPP concentration of 10 mM in the absence or presence of 1 mM of each tyrosine analog tested as an inhibitor. The assay was performed at 30°C for 3 minutes. Activity was determined by measuring the amount of P₄ released.*
## TABLE VIII

Inhibition Parameters for Product and Product Analogs

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>([I]_{0.5} (\text{mM})^b)</th>
<th>(K_i (\text{mM})^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNP</td>
<td>31.1</td>
<td>&gt;19.1(^a)</td>
</tr>
<tr>
<td>PFP</td>
<td>40.9</td>
<td>21.9</td>
</tr>
<tr>
<td>pNPS</td>
<td>49.4</td>
<td>nd(^e)</td>
</tr>
<tr>
<td>HPO(_3^-)</td>
<td>1.2</td>
<td>0.82</td>
</tr>
<tr>
<td>SO(_3^-)</td>
<td>13.9</td>
<td>nd(^e)</td>
</tr>
</tbody>
</table>

\(^a\)Calcineurin activity was measured at 10 mM pNPP under the conditions described in the "Procedures" section.

\(^b\)Values of \([I]_{0.5}\) were obtained by fitting the concentration dependent inhibition of calcineurin to a first order exponential equation, except for pNP for which the data were directly regressed.

\(^c\)The values of \(K_i\) were obtained as described in the "Experimental Procedures" section.

\(^d\)As discussed in the text, pNP is a nonlinear inhibitor. The \(K_i\) value given is obtained the three lowest pNP concentrations.

\(^e\)nd = not determined.
because the $K_m$ values for these compounds are on the order of 2-6 mM (Table IV), lower than the value for pNPP of 10-15 mM. The $V_m$ values are also much lower. These compounds would be expected to bind better than pNPP but not be hydrolyzed as quickly.

To gain additional information concerning substrate-directed requirements for binding, sulfate analogs of the substrate and product were examined in further detail as inhibitors. The concentration dependence of these inhibitors was examined, and $[I]_{0.5}$ ($[I]_{0.5}$ represents the inhibitor concentration required to achieve 50% inhibition) values were evaluated for these as well as the inhibitors utilized in the kinetic studies. These values, collected in Table VIII, reveal that simply an anionic center is not sufficient for significant inhibition. In particular, pNPS (monoanionic) is not a very potent inhibitor with $[I]_{0.5}$ approximately 50 mM. It should be noted that pNPS is not hydrolyzed by calcineurin. Sulfate ion (dianionic) is not a very potent inhibitor relative to phosphate ion. Sulfate ion does, however, more closely approach the inhibitory strength (compare $[I]_{0.5}$ values) of phosphate than do the other compounds tested. Neither methyl nor ethyl phosphonate inhibited calcineurin to any significant degree over the same concentration range (not shown). This may be
an indication that calcineurin requires a dianion for tight binding.

The inhibition studies provide strong evidence that calcineurin does not generate a phosphoryl-enzyme. These studies also provide confirmation that the rate-limiting step does not involve decomposition of a common intermediate as suggested from specificity studies (Table IV, Figure 6). Three alternatives readily present themselves as possibilities for the rate-limiting step: 1) a conformational change in the protein; 2) cleavage of the oxygen-phosphorous bond; and 3) protonation and departure of the phenolic leaving group. Although a relationship between the $K_M$ value for a tyrosine phosphate derivative and the $pK_a$ of the tyrosyl hydroxyl group has been observed (Figure 9), substrate binding can not be rate-limiting since the kinetic mechanism reveals the reaction to be in rapid equilibrium. The observed dependency of calcineurin action upon the $pK_a$ of the leaving group is consistent with either of the last two possibilities inasmuch as the $pK_a$ may reflect the importance of protonation of the leaving group or simply the lability of the ester bond. As discussed earlier, the lack of phosphotransferase activity suggests that the rate-limiting step comes prior to the release of products but is not conclusive. Examination of the pH dependence of the
kinetic parameters of calcineurin with aromatic substrates containing leaving groups with differing pK\(_a\) values should provide additional insight concerning the role of protonation of the leaving group.

Investigation of the pH dependence indicates that the maximal activity of calcineurin increases with increasing pH until reaching a maximum at pH 7.0. Past pH 7.0, the maximal activity remains constant. This occurs for both substrates tested, pNPP and Tyr-P, as seen in Figures 17A and 18A. Data analysis as described in the "Experimental Procedures" yields similar values for the pK\(_a\) with the two substrates; an average pK\(_a\) of 6.45 \(\pm\) 0.07 is evaluated for the enzyme-substrate complex. The analysis was performed assuming that the acidic limb of the profile is the result of the ionization of a single residue. The pK\(_a\) evaluated is not due to ionization of either substrate as Tyr-P has been found to have a pK\(_a\) value of 5.8 (313) while that of pNPP is 5.5 (271). This indicates that a proton transfer is involved in catalysis by calcineurin. We suggest that this proton is transferred to the phenolic leaving group to facilitate its departure. The residue involved was not identified, but may be a histidine based upon the magnitude of the pK\(_a\) value. This is not conclusive evidence because many functional groups have been identified in proteins with
Figure 17. The Effect of pH on the Kinetic Parameters of Calcineurin with pNPP as the Substrate. Kinetic parameters for the hydrolysis of pNPP by calcineurin were evaluated as described in the "Experimental Procedures" section. The concentration of pNPP was varied from 3.0 to 60.0 mM. All other conditions and the buffers employed are described in the "Experimental Procedures" section. A) Panel A depicts the dependence of log $V_M$ on pH. B) Panel B shows the effect of pH on the log ($V_M/K_M$) term. The lines drawn have slopes of zero and negative one. For both parameters, the value at pH 7.0 has been set equal to one.
Figure 18. The Effect of pH on the Kinetic Parameters of Calcineurin with Tyr-P as the Substrate. The Tyr-P concentration was varied from 3.3 to 20.0 mM. Kinetic parameters were evaluated as described in the "Experimental Procedures" section. The buffers used are listed in the "Experimental Procedures" section. A) Panel A depicts the dependence of log $V_M$ on pH. B) Panel B shows the effect of pH on the log ($V_M/K_M$) term. The lines drawn have slopes of zero and negative one. For both parameters, the value obtained at pH 7.0 has been set equal to one.
a pKₐ's of 6-7. Lacking additional evidence precludes assignment of the responsible group. However, it is noteworthy that histidine residues have been implicated in the binding of substrates (314) and catalysis (315) by alkaline phosphatase as well as in catalysis by acid phosphatase (316).

Figure 3, as already discussed, indicates that the phosphate esters utilized in this study are not hydrolyzed with a constant maximal velocity. In fact, the plot is nonlinear with a break occurring at a pKₐ of the leaving group of approximately 7.0. This may be suggestive of a change in the mechanism (rate limiting) for the hydrolysis of F₄-Tyr-P in comparison to Tyr-P, F-Tyr-P, and pNPP. This is supportive of an important proton transfer from the enzymic group of pKₐ = 6.5 to the leaving group. Tyrosine, 3-fluorotyrosine, and para-nitrophenyl phosphate all have phenolic groups with pKₐ values higher than 6.5, while the pKₐ of tetrafluorotyrosine is 5.4. Tetrafluorotyrosine does not require the proton transfer to be a good leaving group. The enzyme does not require the full use of its catalytic apparatus to achieve the hydrolysis of this substrate because of the good leaving group.

Plots of log Vₘ/Kₘ vs. pH indicate a steady decline in the Vₘ/Kₘ value as the pH increases past pH 8.0 for both
substrates. This is shown in Figures 17B and 18B. The subtle differences in the plots in the high pH range are probably due to experimental error. Data analysis (equation 2) indicates that the free enzyme has a pKₐ of 8.0 ± 0.2 consistent with the plots in Figures 17B and 18B. Correction for ionization of the substrate yields a similar pKₐ value regardless of whether the substrate is a monoanion or dianion. The pKₐ of 8.3 (317) for cysteine most closely matches the value of 8.0 evaluated for calcineurin. Recent work by King (318) has shown that modification of calcineurin by sulfhydryl reagents inactivates the enzyme. This suggests an important role for cysteine in calcineurin activity. Possibly, the group identified from the log 
(Vₐ/Kₐ) vs. pH profiles may be water coordinated to metal ion. Water bound to Mn²⁺ is known to ionize with a pKₐ of 10.6 (306). It is possible that the pKₐ of Mn²⁺ coordinated water is reduced when bound to the protein. In fact, Dougherty and Cleland (319) have speculated that the pKₐ of 7.8 (320) evaluated from studies of rabbit muscle pyruvate kinase is due to water coordinated to Mn²⁺.

The apparent shift in the pH optimum when calcineurin is assayed in the presence of Mg²⁺ (compare Figs. 19A and 19B) is consistent with this assignment since the pKₐ of water bound to Mg²⁺ in solution is 11.4 (321) compared to
Figure 19. The Effect of pH on the Activity of Calcineurin in the Presence of Mn\(^{2+}\) or Mg\(^{2+}\). Calcineurin activity, measured at the indicated pH at 30°C, was assayed by measuring the amount of P\(_i\) released. The pNPP concentration was maintained at 10 mM. The buffer concentration was 25 mM in all cases. The buffers utilized are given in the "Experimental Procedures" section. A) The effect of pH in the presence of 1 mM Mn\(^{2+}\). B) The effect of pH in the presence of 20 mM Mg\(^{2+}\).
the value of 10.6 for the manganese complex. The shift is not seemingly due to a change in the mechanism of calcineurin since the enzyme retains its substrate specificity upon the replacement of Mn$^{2+}$ by Mg$^{2+}$ as shown in Table IX and also fails to demonstrate phosphotransferase activity at pH 8.2 in the presence of Mg$^{2+}$, shown in Table X. These results suggest that the mechanism of the enzyme is the same regardless of the metal ion utilized. The effect of substituting other metal ions for Mn$^{2+}$ in the reaction mixture does not appear to be dependent upon the substrate. The effect of different metal ions are demonstrated in Table XI. The table indicates that regardless of the substrate utilized, each metal ion will support a certain level of calcineurin activity. This effect is also seemingly independent of calmodulin.

The similarity of the pH dependent profiles for both pNPP and Tyr-P provides evidence that hydrolysis of the two substrates proceeds via the same mechanism and that protonation of the phenolic leaving group is not the rate-limiting step. The dependency of $V_{\text{m}}$ and $V_{\text{m}}/K_{\text{m}}$ upon the phenolic pK$_{\text{a}}$ must reflect the lability of the phosphate ester bond. Therefore, it seems likely that cleavage of the ester bond is the rate-limiting step.

Kinetic analysis also reveals that the $K_{\text{m}}$ values for
### TABLE IX

**Effect of Metal Ion on the Substrate Specificity of Calcineurin**

<table>
<thead>
<tr>
<th>M$^{2+}$</th>
<th>pH</th>
<th>$(V_m)<em>{TYR-P}/(V_m)</em>{PNPP}$</th>
<th>$(V_m/K_m)<em>{TYR-P}/(V_m/K_m)</em>{PNPP}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn</td>
<td>7.0</td>
<td>0.039</td>
<td>0.026</td>
</tr>
<tr>
<td>Mg</td>
<td>8.2</td>
<td>0.054</td>
<td>0.140</td>
</tr>
</tbody>
</table>

*The ratio of values for pNPP to Tyr-P were obtained from double-reciprocal plots of initial velocity data. Initial velocities were determined at 30°C. Mn$^{2+}$ was maintained at 1 mM while a concentration of 20 mM Mg$^{2+}$ was employed. The higher concentration is required to obtain sufficient velocities at all substrate concentrations utilized. Kinetics at pH 7.0 were performed in 25 mM MOPS buffer; those at pH 8.2 were done in 25 mM BICINE buffer.*
### TABLE X

Phosphotransferase Activity of Calcineurin

<table>
<thead>
<tr>
<th>[Acceptor] (Molar)</th>
<th>Tris 1 mM Mn(^{2+}) pH 7.0</th>
<th>Tris 20 mM Mg(^{2+}) pH 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>0.10</td>
<td>1.03</td>
<td>1.05</td>
</tr>
<tr>
<td>0.20</td>
<td>1.09</td>
<td>1.03</td>
</tr>
<tr>
<td>0.30</td>
<td>1.18</td>
<td>1.03</td>
</tr>
</tbody>
</table>

*The results are presented as the ratio of the activity as measured by pNP release to activity as measured by P\(_i\) release under the conditions indicated. The pNPP concentration was maintained at 10.0 mM. The reactions were done at 30°C with a reaction time of 3 minutes.*
### TABLE XI
Metal Ion Dependence of Calcineurin

<table>
<thead>
<tr>
<th>Me²⁻</th>
<th>Tyr-P</th>
<th>F-Tyr-P</th>
<th>+CaM pNPP</th>
<th>-CaM pNPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Mg</td>
<td>12.3</td>
<td>33.6</td>
<td>13.2</td>
<td>10.0</td>
</tr>
<tr>
<td>Ca</td>
<td>17.1</td>
<td>12.2</td>
<td>10.7</td>
<td>16.7</td>
</tr>
<tr>
<td>Ni</td>
<td>1135</td>
<td>122.9</td>
<td>93.1</td>
<td>190.5</td>
</tr>
<tr>
<td>Zn</td>
<td>5.5</td>
<td>20.6</td>
<td>3.9</td>
<td>11.9</td>
</tr>
<tr>
<td>Cu</td>
<td>40.7</td>
<td>19.4</td>
<td>35.8</td>
<td>29.2</td>
</tr>
<tr>
<td>Co</td>
<td>40.3</td>
<td>51.5</td>
<td>20.8</td>
<td>37.5</td>
</tr>
<tr>
<td>Fe</td>
<td>41.3</td>
<td>69.7</td>
<td>16.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Ba</td>
<td>51.5</td>
<td>47.4</td>
<td>15.8</td>
<td>17.9</td>
</tr>
<tr>
<td>None</td>
<td>14.6</td>
<td>51.2</td>
<td>3.4</td>
<td>25.1</td>
</tr>
</tbody>
</table>

"Activity was measured at 30°C utilizing conditions described under "Experimental Procedures". All metal ions are present at a concentration of 1 mM and were introduced as the chloride salt except for Fe²⁺ which was the sulfate salt.

"Activity is expressed as the percent activity obtained in the presence of 1 mM Mn²⁺ for a given substrate. Only the hydrolysis of pNPP was studied in the absence of calmodulin."
both substrates increase with increasing pH from approximately 3 mM to approximately 110 mM over the entire range of pH studied. As mentioned, calcineurin is more potently inhibited by compounds containing two anion charges, although differences between phosphate and sulfate (both dianions at pH 7.0) are apparent. However, the increase of $K_M$ with pH suggests that the monoanion is more strongly bound than is the dianion. The differences between the binding of substrates and inhibitors may not be because of differences in the number of negative charges, but may be a result of differences in the affinity of the ligand for the metal ion required in calcineurin catalysis. Tighter binding to the metal ion may be a necessary condition for binding to calcineurin.

The finding that the $K_M$ values for both substrates and the activity ($V_M$) of calcineurin are increased at alkaline pH suggests that, while the monoanion is bound more tightly, the dianion form of the substrate is hydrolyzed more quickly. This signifies that calcineurin may be transferring a proton to the substrate during the binding process to allow substrate binding at conditions where less of the monoanion exists. The effect of pH on the $V_M/K_M$ term is consistent with this conclusion. As the group involved in substrate binding ($pK_a=8.0$) becomes deprotonated, the
\( \frac{V_m}{K_m} \) term decreases, indicating that upon deprotonation, this group is no longer able to interact with substrate; it is no longer able to transfer its proton to the substrate.
Calcineurin has been reported to dephosphorylate a limited number of phosphoproteins (210-218). Analysis of the residue dephosphorylated indicates that the enzyme is active against each of the phospho-hydroxyamino acids. The rationale for this behavior is unknown.

Examination of the sequences about the phosphorylated residue from a number of protein substrates reveals little conclusive evidence about specificity determinants. Table XII provides a schematic of the phosphorylation sites for a number of proteins which have been tested as substrates for calcineurin. The proteins have been grouped according to their ability to serve as a substrate for calcineurin. In all but one case, there is the presence of basic residues N-terminal to the modified residue. Basic residues near the residue to be phosphorylated are, seemingly, very important for the majority of serine/threonine kinases. As a first estimation, the presence, number, or distance from the modified residue of these basic residues seems insufficient to determine the fate of the protein as a substrate for calcineurin.

The results of this study clearly indicate that the substrate specificity of the low-molecular-weight
TABLE XII

Phosphorylation Sites of Proteins tested as Substrates for Calcineurin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequencea–b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Substrates</strong></td>
<td></td>
</tr>
<tr>
<td>Phosphorylase Kinase α-subunit</td>
<td>F R R L S I S T E S</td>
</tr>
<tr>
<td>Myosin Light Chains</td>
<td>A A A E G G S S N V F S M</td>
</tr>
<tr>
<td>R II Subunit cAMP dep Kinase</td>
<td>P G R F D R R V S V A A E</td>
</tr>
<tr>
<td>Phosphatase Inhibitor Protein 1</td>
<td>R R R R P T P A T</td>
</tr>
<tr>
<td>G-Substrate</td>
<td>P R R K D T P A L H T S</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>R R R R P T P A M L F R</td>
</tr>
<tr>
<td><strong>B) Not Substrates</strong></td>
<td></td>
</tr>
<tr>
<td>Phosphorylase Kinase β-subunit</td>
<td>A R T K R S G S V Y E P L</td>
</tr>
<tr>
<td>Phosphorylase a</td>
<td>E K R K Q I S V R G L A</td>
</tr>
<tr>
<td>Glycogen Synthase Site 1</td>
<td>P Q W P R R A S C T S S S</td>
</tr>
<tr>
<td>Glycogen Synthase Site 2a</td>
<td>P L S R T L S V S S L P</td>
</tr>
<tr>
<td>Pyruvate Kinase</td>
<td>A G Y L R R A S L A Q L T</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>H I S R K R S G E A T V</td>
</tr>
</tbody>
</table>

*aSequences of various phosphorylation sites taken from Ingebritsen and Cohen (85).

*bThe underlined residue is the site of modification.*
phosphatase activity of calcineurin is dependent upon, in part, the electronic nature of the substrate, as measured by the pKₐ of the phenolic product. Substrates with product alcohols of high pKₐ including Ser-P, Thr-P, AMP, Glc-6-P, and NADP⁺ are not dephosphorylated very well, if at all, by calcineurin (267). However, activated phosphate esters such as phosphoenolpyruvate and naphthyl phosphate can be dephosphorylated (262, 268) quite readily by calcineurin even though the pKₐ of the resulting alcohol may be rather high (322). Extension of these points to protein substrates suggests that phosphorylated hydroxy residues (Tyr, Ser, and Thr) in proteins will be more readily dephosphorylated if the phosphorylated residue is in a microenvironment that will serve to increase the lability of the phosphate ester bond. This may explain why calcineurin is able to dephosphorylate proteins with phosphoseryl, phosphothreonyl, or phosphotyrosyl residues.

Kinetic investigation of the enzymatic reaction reveals that calcineurin catalysis proceeds via a random kinetic mechanism. No evidence for the generation of a phosphoryl-enzyme intermediate has been observed. Both alkaline (323) and acid (304) phosphatases are known to utilize an ordered kinetic mechanism in which a phosphoryl-enzyme is formed as an intermediate (289, 290). The simplest model, consistent
with this kinetic scheme, the specificity of the enzyme, and the failure to observe phosphotransferase activity, suggests that calcineurin-catalyzed hydrolysis of phosphate esters proceeds via the direct transfer of the phosphoryl moiety to the acceptor (water) through a concerted reaction involving proton transfers during the binding and hydrolytic steps. Metal ion is suggested to serve as the means of positioning the substrate in the proper orientation for nucleophilic attack possibly by water (hydroxide) coordinated to the metal ion. The close spatial arrangement obtained would be necessary for a concerted reaction to occur. The departure of the leaving group is facilitated by transfer of a proton from some group \((pK_a = 6.5)\) on the enzyme to the leaving group. This occurs concomitant with the attack of the water molecule at the phosphorous center. This picture of the reaction explains the lack of a phosphoryl-enzyme intermediate, as evidenced by the lack of phosphotransferase activity and the random kinetic mechanism, and the importance of the leaving group \(pK_a\). This model is presented in Figure 20.

As discussed, the \(V_m\) observed for the hydrolysis of tetrafluorotyrosine phosphate does not fit the relationship found for the values of \(V_m\) observed with the other substrates. This is indicative of a change in mechanism for this substrate. The change in mechanism may be at the step
Figure 20. Proposed chemical mechanism for the hydrolysis of low-molecular-weight phosphate esters by calcineurin. The model involves the attack on the phosphate group by a water coordinated to the metal ion involved in catalysis. Concomitant with this nucleophilic attack, a proton is transferred from calcineurin to the substrate leaving group. Refer to the text for further discussion.
involving the proton transfer to the leaving group since tetrafluorotyrosine does require a proton transfer to be a good leaving group.

Direct transfer to water is known to occur in the ATPase reaction catalyzed by yeast inorganic pyrophosphatase (324). A proton transfer in the hydrolysis reaction may provide the electrophilic assistance postulated previously. Electrophilic assistance has also been suggested to occur in the mechanism of action of E. coli alkaline phosphatase (305) and an aryl sulfatase (306). It will be of interest to determine if the dephosphorylation of phosphoproteins catalyzed by calcineurin proceeds in a similar fashion as the hydrolysis of nonprotein substrates.

An analogous study (325) with the application of a fluorotyrosyl containing peptide as a potential substrate for the insulin receptor/tyrosine kinase along with a model acylation reaction has demonstrated that the phosphorylation of tyrosine residues is also affected by the chemistry about the phenolic hydroxyl group. The acylation of the tyrosyl phenolic hydroxyl group by N-acetyl imidazole (326) was only observed for tyrosine and fluorotyrosine. Analysis of the relative reactivity of these two derivatives as a function of pH reveals that, under conditions where the concentrations of reactive species are equal (completely deprotonated
hydroxyl), then tyrosine is 17-fold more reactive than is 3-fluorotyrosine. This is consistent with the expected Brønsted relationship. Examination of the phosphorylation of peptides incorporating fluorotyrosine derivatives, suggests that the protonation state of the phenolic hydroxyl group is an important feature involved in the regulation of tyrosine phosphorylation. The fluorotyrosine-containing peptide is phosphorylated with only a slightly lower $K_M$ value, but with a 2.5-fold lower $V_M$ value than is the tyrosine-containing peptide. The simplest model to explain these observations, in comparison with the chemical acylation, provides that the substrate hydroxyl group is deprotonated on the enzyme prior to the phosphorylation event.

The importance of the hydroxyl protonation state is further supported by studies of the phosphorylation of peptides in which the tyrosine has been replaced with a serine or threonine residues (327). The substituted peptides cannot be phosphorylated by the insulin receptor/kinase, but do serve as competitive inhibitors suggesting that the change does not affect the binding process. The simplest explanation would suggest that the high $pK_a$ of the serine or threonine hydroxyl prevents the phosphorylation reaction. However, it is possible that some
other feature of the tyrosine side chain may be the determining factor.

In parallel with the results obtained for the hydrolysis of tyrosine phosphate derivatives by calcineurin, it seems clear that the modification of tyrosine residues is highly dependent upon the chemistry at the specific site of modification. It is obvious that this, in turn, will be affected by the environment in which the residue is found.

This study represents the first detailed examination of the chemical mechanism of action of any phosphoprotein specific phosphatase. These studies also demonstrate the utility of fluorinated derivatives in the examination of bioorganic reaction mechanisms as well as the application of physical organic chemistry to an enzymatic reaction. Moreover, these studies provide an initial examination of the chemical features involved in the phosphorylation-dephosphorylation of tyrosine residues in proteins.

A number of additional experiments suggest themselves as tests of the putative mechanism. Specifically, determination of the rate-limiting step and the stereochemistry of the reaction would provide important information for the development of any mechanism for calcineurin. Conclusive evidence is required relative to the formation of a covalent intermediate. Finally, a more complete explanation for the
activity of calcineurin toward phosphoseryl-, phosphothreonyl-, and phosphotyrosyl-containing phosphoproteins remains to be advanced.

The earlier discussion of the possible rate-limiting steps resulted in the discarding of either binding of substrate or the protonation and departure of the leaving group as the rate-limiting step for the hydrolysis of low-molecular-weight substrates. Cleavage of the phosphate ester bond or some conformational change in the enzyme were left as choices. Examination of the solvent isotope effect would provide information concerning the role of water in the reaction, possibly in the cleavage step.

Although calcineurin is active toward a number of phosphoseryl- and phosphothreonyl-containing phosphoproteins, the enzyme is inactive toward the free amino acids, phosphoserine or phosphothreonine. We have suggested (272) that the lack of activity is a result of the high $pK_a$ in the resulting product alcohol ($pK_a=13.6$ for serine (328)). 2,2,2-trifluoroethanol has a $pK_a$ of 12.4, between the values for tyrosine and serine. Synthesis of 2,2,2-trifluoroethyl phosphate may provide a useful substrate, if hydrolyzed, to obtain additional information of the effect of the $pK_a$ of an aliphatic alcohol. This compound may also serve as a model for the dephosphorylation of phosphoseryl...
residues in substrate proteins.

The question of whether calcineurin catalysis involves the formation of a phosphoryl-enzyme remains inconclusive. All kinetic evidence obtained argues against the formation of a phosphoryl-enzyme. However, it is possible that a phosphoryl-enzyme may be produced upon cleavage of the phosphate ester bond without the release of the phenolic leaving group. This group might remain noncovalently associated with the protein until hydrolysis of the phosphoryl-enzyme occurs with the concomitant release of both products. This mechanism would also demonstrate random order kinetics, but actually be an ordered mechanism. The differentiation between these two situations could be approached in two ways. Incubation of the enzyme with either $^{32}\text{P}_1$ or $^{32}\text{P}_4$-labelled substrate might achieve the radiolabelling of the enzyme. Both of these methods have demonstrated labelling of alkaline phosphatase (329,330). Simply the isolation of a $^{32}\text{P}_4$-labelled protein would provide strong evidence for a phosphoryl-enzyme intermediate. However, if this intermediate does form, no evidence concerning its chemical properties, in particular its lifetime presently exists. A second approach to this feature of the mechanism would be the examination of the stereochemistry (331) of the hydrolysis reaction employing
the chiral compound, \([\text{\textsuperscript{17}O}, \text{\textsuperscript{18}O}]\)-\textit{para}-nitrophenyl phosphorothioate. Hydrolysis of this compound with retention of its stereochemistry would reflect an equal number of substitution reactions consistent with the hydrolysis of a phosphoryl-enzyme. Inversion of the stereochemistry during hydrolysis would be indicative of an odd number of transfer steps consistent with the direct hydrolysis of the ester. An odd number of transfers is also consistent with the formation of two different phosphoryl-enzyme forms prior to the hydrolysis of the intermediate by water. This chiral compound is known to be a substrate for the alkaline phosphatase (332). All alkaline and acid phosphatases, for which the stereochemistry has been determined (333), show an inversion of stereochemistry and have been shown to proceed via a phosphoryl-enzyme intermediate. No information has been acquired for any phosphoprotein-specific phosphatase.
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