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Hydrolysis of Trifluoroethyl Phosphate as Evidence that the Serine and Tyrosine Phosphatase Activities of Calcineurin Share the Same Specificity Determinant[1]

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SUMMARY Calcineurin is shown to hydrolyze 2,2,2-trifluoroethyl phosphate. The time course of hydrolysis showed a lag period. The lag was present at all substrate concentrations used and was dependent on substrate concentration. Evaluation of the kinetic constants from the steady state portion of the enzymatic reaction indicated that hydrolysis of this compound fit the Bronsted relationship determined previously for V_{max}/K_m term for the hydrolysis of aromatic phosphate esters. Moreover, a Bronsted relationship for the V_{max} was developed for the substrates containing leaving groups that are more basic than 2,3,4,5-tetrafluorotyrosine. The hydrolysis of 2,3,4,5-tetrafluorotyrosine phosphate did not fit this relationship implying a change in the rate limiting step with this substrate. The significance of these data to the serine and tyrosine phosphatase activities of calcineurin is discussed. A rationale for understanding the determinant of both activities of calcineurin is presented.

The calmodulin-activated protein phosphatase, calcineurin (1,2), has activity with proteins and low-molecular-weight phosphate esters (3-6). Proteins containing each of the different phospho-hydroxy amino acids have been identified as substrates for the enzyme. The importance of the tyrosine protein phosphatase activity is

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Abbreviations Used: EGTA, Ethylene glycol bis(ß-amino ethyl ether)N,N'-tetraacetate; F-Tyr, 3-Fluorotyrosine; F_3Et-P, 2,2,2-Trifluoroethyl phosphate; F_3EtOH, 2,2,2-Trifluoroethanol; F_4-Tyr, 2,3,4,5-Tetrafluorotyrosine; HPLC, High Performance Liquid Chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; P_i, Inorganic phosphate; pNP, para-Nitro phenol; pNPP, para-Nitrophenyl phosphate.

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unclear with respect to the serine protein phosphatase activity of the enzyme (7-9). We have studied (6,10) the low-molecular-weight activity phosphatase of calcineurin aiming to determine features of the chemical mechanism of calcineurin

With the exception of phosphoenolpyruvate (5), no nonaromatic compound has been shown to serve as a substrate for calcineurin. Serine phosphate, threonine phosphate, glucose-6-phosphate, glucose-1-phosphate, and a number of nucleotides have been tested unsuccessfully as substrates (4,5). Phosphoenolpyruvate may be exceptional because it can be considered an "activated" phosphate ester. The pK_a of the enol-hydroxy group has been estimated to be 11.1 (11). Our model of calcineurin catalysis posits that the enzyme is likely to hydrolyze phosphate esters more readily if the resulting product alcohol has a lower pK_a value that may reflect the apparent lability of the ester bond. Serine has a pK_a value of 13.4 (12). The inability of calcineurin to hydrolyze serine phosphate efficiently may be related to the high pK_a value of the serine produced. To examine this possibility, 2,2,2-trifluoroethyl phosphate was synthesized and examined as a substrate for calcineurin. The resulting alcohol, 2,2,2-trifluoroethanol, has a pK_a of 12.4 (13). As such, it may serve as a suitable model for the phosphoseryl protein phosphatase activity of calcineurin.

EXPERIMENTAL PROCEDURES

Materials - pNPP (Sigma 104 substrate), MOPS, EGTA, and phenyl-sepharose were purchased from Sigma. 2,2,2-trifluoroethanol was purchased from Aldrich Chemical. DE-52 cellulose was obtained from Whatman. Other chemicals were obtained from Fisher.

Synthesis of 2,2,2-Trifluoroethyl Phosphate - 2,2,2-trifluoroethyl phosphate was synthesized and purified according to the procedure of Hall and Williams (13). The preparation obtained contained approximately 10% (mol/mol) P_i as a contaminant. The material was purified further by recrystallization from ethanol until the P_i contamination was $\leq 1\%$.

Proteins - Calcineurin was isolated from bovine brain by the method of Sharma et al. (14) and was 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 (15) modified to include chromatography on phenyl-sepharose (16). Protein concentrations were determined by the method of Bradford (17).

Calcineurin Assay - Calcineurin was assayed by measuring the release of P_i as previously described (6). Standard assays were performed in 25 mM MOPS, pH 7.0 with 1.0 mM $MnCl_2$. With pNPP as the substrate, calmodulin and calcineurin were added to 35 μg/ml. Both proteins were raised to 0.35 mg/ml with F_3Et-P as substrate. The reaction volume was maintained at 50 μl and the temperature at 30°C. For pNPP hydrolysis, 20% of the reaction mix was assayed. The enzymatic reaction was linear for at least 9 minutes. For reactions with F_3Et-P as substrate, 80% of the reaction mixture was assayed for P_i. The kinetic parameters were evaluated by utilizing either the program of Siano et al. (18) written in OMNITAB language or a BASIC program written for the IBM PC by Dr. D. L. Quinn (Dept. of Chemistry, Univ. of Iowa, Iowa City, Iowa).

RESULTS and DISCUSSION

Hydrolysis of F_3Et-P - Our work with aromatic substrates clearly indicated that the substrate specificity of the low-molecular-weight phosphatase activity of calcineurin was dependent upon, in part, the electronic nature of the substrate, as measured by the pK_a of the phenolic product. Substrates with product alcohols of high pK_a, including Ser-P, Thr-P, AMP, Glc-6-P, and $NADP^+$, were not hydrolyzed very well, if at all, by calcineurin. To determine whether the specificity of calcineurin could be explained by the acidity of the product alcohol and not whether the product is aromatic, 2,2,2-trifluoroethyl phosphate was synthesized (13) and tested as a substrate for calcineurin.

The corresponding trifluoro-alcohol has a pK_a of 12.4 (13) in contrast to the value of 13.6 (12) for serine. Although serine phosphate could not be dephosphorylated by calcineurin, 2,2,2-trifluoroethyl phosphate was hydrolyzed by the enzyme. This compound was not a good substrate and requires high concentrations of enzyme to be hydrolyzed. The time course of the reaction also exhibited a pronounced lag, lasting for up to 15 minutes shown in Figure 1. The lag was present at all substrate concentrations tested and indicated that calcineurin was a hysteretic enzyme with F_3Et-P as substrate. At 12 Mm F_3Et-P, the lag was equivalent for two different enzyme concentrations (not shown) suggesting that the self-association of

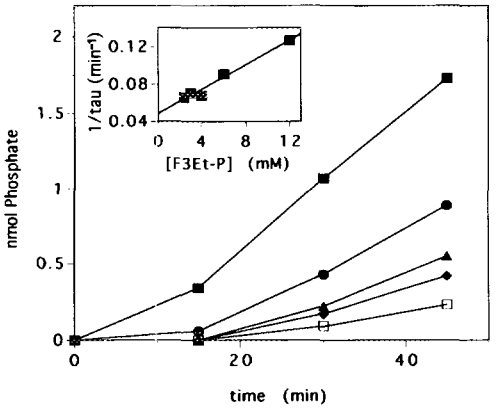

Figure 1 - Time Course of Trifluoroethyl Phosphate Hydrolysis. The hydrolysis of F_3Et-P by calcineurin was monitored by the release of phosphate as described. From top to bottom, the substrate concentration was 12.0, 6.0, 4.0, 3.0, and 2.4 mM trifluoroethyl phosphate. The reaction was initiated by the addition of calcineurin. The linear portion of the time courses were extrapolated to the time at which the level of product equals zero to provide an estimation of the lag time, τ. Inset - The values of $1/\tau$ were plotted vs. the concentration of trifluoroethyl phosphate.

protein molecules was not responsible for the lag period. Moreover, the lag could not be eliminated by incubating the enzyme for 15 minutes under the following conditions: 1) the presence of calmodulin and metal ion and the absence of substrate or 2) the presence of calmodulin and substrate and the absence of metal ion. In the absence of Mn^{2+} from the preincubation mixture, the lag was longer (not shown).

Analysis of the Lag Period - As shown in Figure 1, there was a lag period observed with each of the substrate concentrations used. Extrapolation of the linear portion of the time courses provides an estimation of the lag period, designated τ, for each concentration. For the concentrations used, the lag varied from 7 min with 12.0 mM F_3Et-P to 16 min with 2.4 mM F_3Et-P. The data did demonstrate that the lag was dependent on the substrate concentration. The lag could be fit by the equation $1/\tau = k_{off} + k_{on}[S]$ for the association of substrate with the enzyme (inset, Figure 1), but because the lag lasts minutes, it is difficult to conceive that it merely reflects substrate binding. It is more likely that the lag results from a combination of the association of substrate with the enzyme and the equilibrium between different states of the enzyme (19,20).

A lag in calcineurin activation by Ni^{2+} has been previously reported (21,22). In that situation, the lag could be abolished by preincubation of the enzyme with Ni^{2+} for 15 min (21,22) although the process was not first order suggestive of a multistage process (21). The lag observed with Ni^{2+} was observed with either pNPP (22) or phosphohistone H1 (21) as the substrate. Additional studies showed that tryptophan fluorescence of calcineurin was quenched and that this effect preceded the activation by Ni^{2+}. The rapid step seemed to be binding of Ni^{2+} followed by a slow change that resulted in activation (21). Heretofore, no lag had been observed in the presence of Mn^{2+}. If the lag was caused by a required conformational change in the protein, it may be dependent on the metal-substrate complex in defining the nature of the conformational change. A conformational change induced by substrate binding by calcineurin may be rapid with aryl substrates, but slow with aliphatic substrates.

Kinetic Analysis of F_3Et-P Hydrolysis - Initial velocities with this substrate were estimated from the linear portion of the time course reached after the lag phase. The initial velocities were used to evaluate the kinetic parameters for the hydrolysis of F_3Et-P. The values were evaluated to be 0.045 ± 0.018 μmol/min/mg CaN and 31.1 ± 12.8 Mm for V_{max} and K_m, respectively; pNPP was used to standardize these results. The resulting value of V_{max}/K_m for F_3Et-P fit the Bronsted relationship developed previously (6) for the tyrosine phosphate derivatives, although the slope was slightly altered to $\beta_{LG} = -0.34$. The relationship including F_3Et-P is shown in Figure 2. The

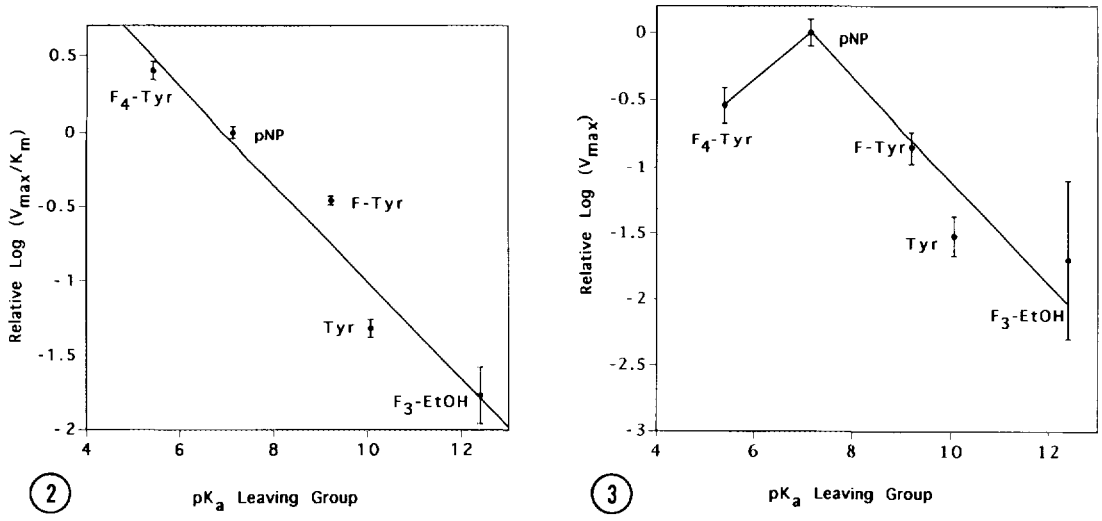

Figure 2 - Bronsted Plot for the V_{max}/K_m Parameter. All values have been standardized to the value for the hydrolysis of pNPP. The line depicted was generated by linear regression; the slope is -0.34. The correlation constant for the relationship is -0.97. The data for hydrolysis of the aromatic substrates were from Reference 6. A similar plot, for the aromatic substrates only, has been published (6).

Figure 3 - Bronsted Plot for the V_{max} Parameter. All values have been standardized to the value for the hydrolysis of pNPP. The line depicted was generated by linear regression for the substrates with a leaving group more basic than F_4-Tyr. The equation of the line is log (V_{max}) = -0.33 pK_a + 2.20. The correlation constant for the relationship is -0.94. The kinetic parameters for the hydrolysis of F_3Et-P were determined herein; values for the other substrates were from Ref. 6.

inclusion of F_3Et-P demonstrated that the Bronsted relationship is not limited to aromatic substrates. Moreover, the fit of this result demonstrated that the Bronsted relationship is important for product alcohols for a range of approximately 7 pK_a units.

More significantly, the V_{max} value could be compared with values for other substrates. We previously reported (6) that the dependence of V_{max} upon the properties of the substrate did not fit a linear relationship throughout the range of leaving group pK_a values investigated, although no plot was shown. Inclusion of the value for F_3Et-P illustrates that a relationship between the log (V_{max}) and the pK_a of the leaving group does exist for the substrates with leaving groups more basic than F_4-Tyr (Figure 3). The relationship was nonlinear, with a change in the slope of the line in the acidic region below the pK_a value, 7.14, for para-nitrophenol. The instance of a change in the slope occurs in the region between the pK_a values for pNP and F_4-Tyr. This deviation may be because of a change in the mechanism of calcineurin for the F_4-Tyr leaving group. We have argued that the transfer of a proton to the leaving group is a necessary part of the mechanism of calcineurin. With a pK_a value of 5.40,

F_4-Tyr may not require this protonation to be an effective leaving group at pH 7.0, the condition used to evaluate the kinetic parameters (6).

Other Substrates - The hydrolysis of serine phosphate (15 mM) with levels of enzyme (0.35 mg/ml) required for the hydrolysis of 2,2,2-trifluoroethyl phosphate was also reexamined. Calcineurin failed to catalyze hydrolysis through 5 hours of incubation. Another model compound, phosphorylethanolamine, also did not serve as a substrate (enzyme = 0.35 mg/ml; substrate = 15 mM) for calcineurin. The results obtained in this work also demonstrated that the amino and carboxyl groups of the amino acid substrates did not affect the hydrolysis of the phosphate esters.

Serine/threonine vs. Tyrosine Phosphatase Activity - The relative significance of the phosphotyrosyl and phosphoseryl protein phosphatase activities of calcineurin has not been well established. Chan et al. (9) have examined the dephosphorylation by calcineurin of defined peptide substrates containing phosphotyrosyl or phosphoseryl residues. Their results suggested that the specificity of calcineurin toward phosphotyrosyl residues was on the same order of magnitude as toward phosphoseryl residues. Specifically, the values for V_{max}/K_m in the presence of Ni^{2+} were the same order of magnitude for both phosphotyrosine or phosphoserine substrates. For a peptide corresponding to the sequence of a phosphorylation site in the type II regulatory subunit of the cAMP dependent protein kinase, there was only a 2-fold difference between the phosphotyrosine and phosphoserine substrates. In the presence of Mn^{2+}, the phosphoserine substrates were 1 to 2 orders of magnitude better than the phosphotyrosine substrates. These studies do not exclude phosphotyrosine substrates as important, but may implicate an influential role of the metal ion on the specificity of the enzyme. In subsequent reports (23,24), the same authors suggested that calcineurin specificity with phosphoserine substrates was related to higher order structures of the substrate, but specificity with phosphotyrosine substrates was dependent on the phosphotyrosine residue. The latter conclusion is consistent with our studies of low molecular weight substrates.

The apparent dependence on higher order structures with phosphoserine substrates may reflect the effect of structure on the properties of the phosphoserine residue. The RII peptide used as a substrate (9,23) has a number of charged residues. With these charged residues, it would not be unexpected that these residues would affect the properties of a phosphoserine residue and make it more labile. The hydrolysis of F_3Et-P by calcineurin may serve as a model for the phosphoseryl phosphatase activity of calcineurin. The inclusion of the kinetic parameters for this substrate with the parameters for the aromatic substrates suggested that the

specificity of both the phosphoseryl and phosphotyrosyl phosphatase activities of calcineurin can be rationalized by a single model that has calcineurin specificity defined by the environment of the target residue.

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