is an impressive example of opportunism in enzyme catalysis.

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Calcineurin-Catalyzed Reaction with Phosphite and Phosphate Esters of Tyrosine†

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ABSTRACT: A convenient synthesis is reported for the preparation of the phosphite ester of tyrosine methyl ester. By use of calcineurin, at 30 °C, a phosphite ester was hydrolyzed with a V_M value [119 nmol/(min·μg of E)] approximately 500 times greater than that obtained with tyrosine phosphate [0.23 nmol/(min·μg of E)] as substrate, but with similar K_M values (12 mM for Tyr-PH ME, 11 mM for Tyr-P). Acid phosphatase, on the other hand, hydrolyzed the phosphite ester with a V_M and K_M value lower than those obtained with tyrosyl phosphate. The temperature dependence of the kinetic parameters (K_M and V_M) was evaluated, and the activation parameters were obtained with both substrates. The entropy of activation associated with the enzymatic hydrolysis of tyrosine phosphate agrees with the entropy change for the hydrolysis of the monoanion of phosphate monoesters. The energy of activation for both substrates was in agreement with the energy change for hydrolysis of the oxygen–phosphorous linkage of phosphate monoester monoanions and phosphite esters. These results are consistent with a scheme of general acid catalysis in the action of calcineurin.

Enzyme-catalyzed protein phosphorylation–dephoshorylation is an important regulatory mechanism (Nimmo & Cohen, 1977; Krebs & Beavo, 1979). In the last several years, much attention has focused upon the tyrosyl phosphorylation of cellular proteins by specific tyrosyl kinases and the dephosphorylation of phosphotyrosyl residues by tyrosyl protein phosphatases. Tyrosyl phosphorylation is associated with the regulation of cellular activities such as proliferation, differentiation, and transformation (Hunter & Sefton, 1982; Heldin & Westermark, 1984; Sefton & Hunter, 1984; Swarup et al., 1984; Sefton, 1985; Coughlin et al., 1988). It has also been suggested that increased cellular phosphorylation of protein tyrosyl residues plays an important role in the regulation of growth processes (Hunter & Cooper, 1983).

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Protein phosphatases specific for phosphorylated tyrosyl residues have been isolated from a number of sources (Chernoff & Li, 1983; Foulkes et al., 1983, 1981; Nelson & Brauton, 1984; Shriner & Brautigan, 1984; Gallis et al., 1981; Hörlein et al., 1982; Swarup et al., 1982; Tonks et al., 1987; Jones et al., 1989), and there have been several reports of phosphotyrosyl protein phosphatase activity associated with a number of broad-specificity acid or alkaline phosphatases (Swarup et al., 1981; Leis & Kaplan, 1982; Li, 1984). Calcineurin, a Ca^{2+}- and calmodulin-dependent type 2B protein phosphatase, has been shown to have an intrinsic phosphotyrosyl protein phosphatase activity toward tyrosyl-phosphorylated human EGF receptor and toward *p*-nitrophenyl phosphate and tyrosine phosphate at neutral pH. Although the enzyme will hydrolyze seryl-phosphorylated proteins, it will not dephosphorylate seryl phosphate (Pallen & Wang, 1983; Chernoff et al., 1984; Pallen et al., 1985; Chan et al., 1986; Kincaid et al., 1986).

A systematic study of the mechanism of action of calcineurin was done by Martin and Graves (Martin et al., 1985; Martin & Graves, 1986; 1987). By exploiting the low-molecular-weight phosphatase activity of calcineurin toward lower molecular weight substrates as a model enzymatic reaction, features of the chemistry of calcineurin catalysis, as well as potential information of the chemistry involved in the dephosphorylation of phosphotyrosyl residues, were examined. From results obtained with fluorinated analogues of tyrosine as substrates for calcineurin, the specificity of the reaction with amino acid derivatives was assumed to be related to the pK_a of the leaving group. A comparison with results obtained from acid hydrolysis suggested a correlation beween an enzymatic and an acid-catalyzed reaction. One interpretation of the Brønsted coefficient (−0.35) obtained in both experiments suggests that hydrolysis of a monoanion form of the substrate may occur by calcineurin. Product inhibition studies indicate that both products—free phosphate and phenolic leaving group—are competitive inhibitors. These inhibition patterns are consistent with a rapid equilibrium random uni-bi kinetic mechanism (Rudolph, 1979), indicating no preference to the order of release of products following hydrolysis. An average pK_a of 6.45 evaluated for the enzyme–substrate complex, which was obtained from pH dependence studies, indicates that a proton transfer is involved in catalysis by calcineurin.

To probe further into the catalytic mechanism, a labile monoanion derivative, tyrosyl phosphite ($ROPO_2H^-$), was synthesized and used in enzymatic and acid hydrolysis experiments with tyrosine phosphate. The effect of temperature on calcineurin kinetics was studied, and information about the interactions of the enzyme and substrate at the various kinetically significant steps in the enzymatic reaction was provided.

Experimental Procedures

Materials. Escherichia coli alkaline phosphatase, potato acid phosphatase, calmodulin, L-tyrosine methyl ester, *O*-phospho-L-tyrosine, and all buffers were obtained from Sigma. Phosphonic acid (H_2PHO_3) and disodium phosphonate sodium pentahydrate ($Na_2PHO_3 \cdot 5H_2O$) were obtained from ICN Biomedicals, Inc. Calcineurin was obtained from Dr. Jerry Wang at the University of Calgary. All other chemicals were from Fisher Chemical Co.

Synthesis of Disodium Diphosphonate. Disodium diphosphonate was synthesized according to the method of Yoza et al. (1977).

Synthesis of L-*Tyrosyl O-Phosphite.* An anhydrous mixture of 0.46 g of L-tyrosine methyl ester, 2.2 g of phosphonic acid, and 1.0 g of disodium diphosphonate was heated to 100 °C for 6 h in a hydrolysis tube. After addition of 0.75 g more of disodium diphosphonate, heating was continued for another 66 h. The mixture was then dissolved with crushed ice. L-Tyrosine *O*-phosphite and L-tyrosine methyl ester *O*-phosphite were isolated on a Du Pont ZORBAX ODS reverse-phase HPLC column (C18; 21.2 mm i.d. × 25 cm) employing an elution with 0.1% trifluoroacetic acid applying an acetonitrile gradient (0–4% in 20 min) at a flow rate of 7 mL/min. The retention times were about 10 min for Tyr-PH and 56 min for Tyr-PH ME. Yield 10–20% and 20% for Tyr-PH and Tyr-PH ME, respectively.

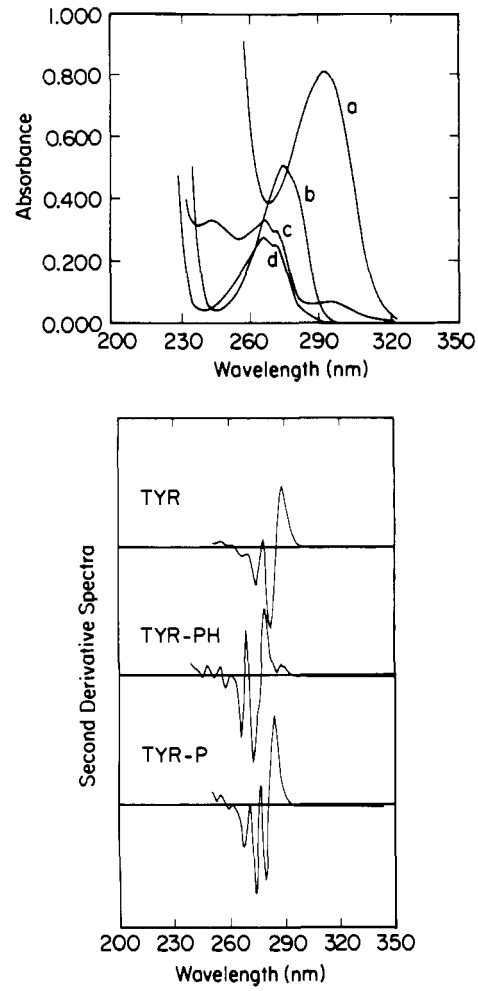

FIGURE 1: Spectra of tyrosyl derivatives. (A) UV spectra of L-tyrosine methyl ester phosphite compared to those of tyrosine: (a) L-tyrosine at pH 11 and (b) at pH 7; (c) L-tyrosine methyl ester phosphite at pH 11 and (d) at pH 7. (B) Second derivative spectra of Tyr, Tyr-PH, and Tyr-P. Conditions are given under Experimental Procedures.

Analysis of Synthesized Tyrosyl Phosphite. The purified Tyr-PH[1] and Tyr-PH ME both yielded correct molecular weights on FAB mass spectra. The molecular weights of Tyr-PH and Tyr-PH ME are 245.2 and 259.2, respectively. The UV–vis spectra of Tyr-PH, when compared with spectra of Tyr, showed no pH-dependent shift in the peak position (Figure 1A). The small amount of absorbance seen at 249 nm for tyrosine phosphite is a consequence of the rapid breakdown of this ester under alkaline conditions. Second derivative spectrophotometry indicated a new spectrum for the

[1] Abbreviations: Tyr, L-tyrosine; Tyr-P, L-tyrosine phosphate; Tyr-PH, L-tyrosine phosphite; Tyr ME, L-tyrosine methyl ester; Tyr-PH ME, L-tyrosine phosphite methyl ester; PVA, poly(vinyl alcohol); P_i, inorganic phosphate; PMP, phenyl methylphosphonic acid; *p*-NPMP, *p*-nitrophenyl methylphosphonic acid.

tyrosyl phosphite compared with the spectrum of Tyr and Tyr-P, as shown in Figure 1B. Both phosphite derivatives were shown by ^{31}P NMR to have only one organic phosphite species (shifts in ppm relative to 85% H_3PO_4): Tyr-PH (pH 7.0), δ 4.44 ppm; Tyr-PH ME (pH 7.0), δ 4.33 ppm. ^1H and ^{13}C NMR spectra were determined for tyrosyl phosphite, as compared with spectra of their starting materials.

Tyr-PH (pH 7.0). ^1H NMR: δ 3.03–3.33 (dd, 8) (CH_2); 3.94–3.99 (dt, 5) (CH); 7.11–7.23 (d, 8.4) (Ar); 8.11, 5.93 (PH). ^{13}C NMR: δ 174.00 (C=O); 56.22 (α CH); 35.86 (β CH_2); 130.89–150.42 (aromatic carbons).

Tyr-PH ME (pH 7.0). ^1H NMR: δ 3.84 (CH_3); 3.15–3.37 (dd, 8) (CH_2); 4.36–4.41 (dt, 6) (CH); 7.12–7.29 (d, 8.4), (Ar); 8.11, 5.92 (PH). ^{13}C NMR: δ 53.67 (CH_3); 170.70 (C=O); 54.29 (α CH); 35.34 (β CH_2); 130.15–150.89 (aromatic carbons).

These show the appropriate alterations (in ppm) resulting from the formation of the phosphite ester, and the data are in agreement with the loss of phenol function at δ 6.98–7.22 (d, 8.4), 6.87–7.17 (d, 8.5) in ^1H NMR, δ 156.30, 155.36 in ^{13}C NMR, observed with tyrosine and its methyl ester, respectively, and gain of PH function at δ 8.11, 5.93, 8.11, 5.92 in ^1H NMR observed with tyrosine phosphite and its methyl ester, respectively. NMR spectra were obtained on a Bruker WM-300 NMR spectrometer operating at 300, 75.46, and 121.5 MHz for ^1H, ^{13}C, and ^{31}P, respectively.

The rates of dephosphonylation catalyzed by calcineurin on both tyrosine phosphite and its methyl ester are similar. Studies on the initial rate kinetics show that both substrates share similar K_M and V_M. Because it is difficult to purify large quantities of pure tyrosine phosphite, tyrosine phosphite methyl ester was used instead of tyrosine phosphite in the kinetics study.

Assay for Inorganic Phosphate. P_i was assayed with slight modification of the method developed by Van Veldhoven and Mannaerts (1987). In general, 10–50 μL of sample was added to 200 μL of reagent A [1.75% (w/v) ammonium heptamolybdate·$4H_2O$ in 6.3 N H_2SO_4], and the solution was mixed. Enough H_2O was added to make a final volume of 1200 μL, and the solution was mixed again. After 10 min at room temperature, 200 μL of reagent B [0.035% (w/v) malachite green, 0.35% (w/v) PVA in H_2O] was added, and the solution was mixed again. The absorbance of the solution at 610 nm was determined 30 min later in a DU-7 spectrophotometer. A standard curve was linear over the range 0.5–20 nmol of P_i.

Assay for L-*Tyrosine Methyl Ester.* L-Tyrosine methyl ester released from L-tyrosine methyl ester *O*-phosphite was analyzed by second derivative spectrophotometry. A standard curve was linear over the range 0.05–1 mM by plotting the difference in absorbance ($d^2A/d\lambda^2$) between the maximum derivative absorption at 288 nm and the minimum at 283 nm against the concentration of L-tyrosine methyl ester.

Acid Phosphatase Assays. Assays were done in 100 mM NaOAc, pH 4.8, at 30 °C. The amount of enzyme in each assay was 36 μg/mL. Aliquots (40 μL) were removed at various time points and assayed for inorganic phosphate with Tyr-P. With Tyr-PH ME, the assay was performed in a semimicrocuvette, and the second derivative UV spectra were taken at different time intervals for the measurement of Tyr ME.

Calcineurin Assay. Phosphatase activity of calcineurin was assayed in 120 mM MOPS, pH 7.0, 1 mM $MnCl_2$, at 30 °C. Metal ions can influence activation and deactivation (Pallen & Wang, 1983; King & Huang, 1984). Linear progress curves were obtained with phosphite and phosphate esters in the presence of Mn^{2+}, facilitating the kinetic analyses. Calmodulin was included at an equivalent concentration as calcineurin. With Tyr-P, at different time intervals, 50 μL was removed and assayed for P_i with a calcineurin concentration equal to 28 μg/mL. With calcineurin concentration equaling 14 μg/mL with Tyr-PH ME, 20 μL was removed at different time intervals and added to 300 μL of saturated EDTA to stop the reaction. Enough H_2O was added to make a final volume of 500 μL for the measurement of Tyr ME. For the temperature dependence study, assays were performed at 16.5, 20, 25, and 30 °C with calcineurin concentration varying from 14 to 84 μg/mL.

Acid- or Alkaline-Catalyzed Hydrolysis. The acid-catalyzed hydrolysis of the tyrosine phosphate or tyrosine phosphite methyl ester was carried out in sealed tubes at 60 °C in 1 M HOAc–NaOAc buffer, pH 4.8, and at 95 °C in 1 M HOAc–NaOAc buffer, pH 4.0. At pH 10.0 in 250 mM Ches buffer, at 60 °C, the hydrolysis reactions were followed at 294 nm for both substrates. Substrate concentrations were in the range 10^{-4}–10^{-3} M.

Buffers. The pH dependence of calcineurin-catalyzed Tyr-PH ME dephosphonylation was performed with a buffer concentration of 100 mM. The buffers and their pH values were as follows: MES, 5.8, 6.1, 6.4; MOPS, 6.7, 7.0, 7.3, 7.7; Tricine, 7.9, 8.2, 8.5.

Data Analysis. All kinetic data were analyzed by using the Enzfitter program (Leatherbarrow, 1987). The thermodynamic data parameters (ΔG, ΔH, ΔS) were evaluated by fitting the data to the equations:

$$\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$$

$$\log \frac{k}{T} = -\frac{\Delta H^{\neq}}{2.3R}\frac{1}{T} + \log \frac{k_B}{h} + \frac{\Delta S^{\neq}}{2.3R}$$

where k_B is the Boltzmann constant and h is Planck's constant.

Because kinetic studies showed a rapid equilibrium mechanism of calcineurin catalysis (Martin & Graves, 1986), it was assumed that the K_M value was equivalent to K_S. The reciprocal of K_M was utilized to calculate the free energy change for the association of the enzyme with its substrate from the equation

$$\Delta G^{\circ} = -RT \ln (1/K_M)$$

$$-\log (1/K_M) = \frac{\Delta H^{\circ}}{2.3R}\frac{1}{T} - \frac{\Delta S^{\circ}}{2.3R}$$

where K_M is the Michaelis constant.

Results

To follow the possible reactions of calcineurin and other phosphatases on the phosphite ester of tyrosine, a spectrophotometric assay was developed based on the difference in the UV spectra of the phosphite ester and free tyrosine (Figure 1A). Second derivative spectra of tyrosine phosphite, tyrosine phosphate, and tyrosine are shown in Figure 1B. Because of the sensitivity of derivative spectroscopy for separating spectral information from the background and for discriminating overlapping spectra in the mixtures (Cook et al., 1977; O'Haver, 1979), the small amount of Tyr ME produced could be detected from Tyr-PH ME. On the basis of the relation derived by Hager (1973)

$$A = \ln \frac{I_0}{I} = abc$$

$$\frac{d^2I}{d\lambda^2} \Big/ I = \frac{1}{I_0}\frac{d^2I_0}{d\lambda^2} + \left(bc\frac{da}{d\lambda}\right) - \frac{2}{I_0}\frac{dI_0}{d\lambda}\frac{da}{d\lambda}bc - bc\frac{da^2}{d\lambda^2}$$

Table I: Comparison of the Kinetic Parameters for Enzymatic Hydrolysis of Tyrosine Phosphate and Tyrosine Methyl Ester Phosphite

	acid phosphatase		calcineurin	
substrate	V_M [10^{-10} mol/(min·μg of E)]	K_M (mM)	V_M [10^{-10} mol/(min·μg of E)]	K_M (mM)
Tyr-P	32 ± 4	4.6 ± 0.9	0.23 ± 0.02	11 ± 2
Tyr-PH ME	7.5 ± 0.2	0.44 ± 0.04	119 ± 5	12 ± 1

Table II: Kinetic Parameters for Acid Hydrolysis

	pH 4.0, t = 95 °C		pH 4.8, t = 60 °C		pH 10.0, t = 60 °C	
substrate	k_H (min^{-1})	$t_{1/2}$ (h)	k_H (min^{-1})	$t_{1/2}$ (h)	k_H (min^{-1})	$t_{1/2}$ (h)
Tyr-P	0.017	0.7	0.00056	20.7		
Tyr-PH ME	0.0019	6.0	0.00045	25.6	0.0012	9.8

where A is absorbance, a the absorption coefficient as a function of wavelength for a particular sample, b the path length of radiation through the sample, and c the sample concentration. The derivatives of intensity can be influenced by $a(\lambda)$ and $I_0(\lambda)$. The appearance of product (i.e., the phenolic derivative) may be quantified by plotting the difference in absorbance ($d^2A/d\lambda^2$) of the maximum absorption at 288 nm from the minimum at 283 nm against its concentration. The standard curve is linear over the concentration range of 0.05–1 mM of L-tyrosine methyl ester (results not illustrated). In the presence of the substrate (4 mM), 0.5% of the conversion of substrate to product may be measured by this procedure.

By use of this method, both Tyr-PH and Tyr-PH ME were found to be substrates for acid phosphatase and calcineurin. With enzyme present, Tyr peaks were produced in the second derivative spectrum. The dephosphonylation of tyrosyl phosphite by calcineurin was also shown by ^{31}P NMR to have two peaks—one representing Tyr-PH, δ 4.4 ppm, and the other representing $H_2PO_3^-$, δ 3.3 ppm (results not illustrated).

To quantitate the action of calcineurin on phosphite and phosphate esters and compare these results with acid phosphatase, initial rate kinetics were performed. The kinetic parameters, K_M and V_M values, are summarized in Table I. The values of K_M for calcineurin for both substrates are similar, but the value of V_M is considerably larger for the phosphite derivative. Similar results were obtained with tyrosyl phosphite. With acid phosphatase, both values of K_M and V_M are smaller for the phosphite derivative. Alkaline phosphatase also could hydrolyze the phosphite ester, but, compared with that of tyrosine phosphate, the rate was too slow to be measured. Hence, no kinetic parameters were evaluated.

Because tyrosyl phosphite has a pK_a value of about 0.8, it is a monoanion in the enzyme assays, whereas tyrosyl phosphate ($pK_{a,2}$ = 5.8) may exist both as the monoanion and as the dianion. Therefore, a difference in velocities may appear between tyrosyl phosphate and the phosphite if the enzyme only bound or acted on the monoanion. If it did, the pH activity curves could differ for the two substrates. But calcineurin possesses the same pH optimum of approximately 7.0 with Tyr-PH ME in the presence of Mn^{2+} (results not illustrated), as with Tyr-P (Martin & Graves, 1986). Thus the pH activity curves reflect changes in ionization of critial groups in the enzyme and not the substrate.

To determine whether the increased V_M value obtained with the specific phosphatase, calcineurin, is related to the difference in acid lability of the phosphite ester, the first-order rate constants for acid hydrolysis of tyrosyl phosphite ethyl ester and tyrosyl phosphate were measured at pH 4.8 at 60 °C, pH 4.0 at 95 °C, and pH 10.0 at 60 °C. The kinetic parameters are summarized in Table II. The k_H values are smaller and the half-lives are longer for tyrosyl phosphite in acid conditions. Thus, tyrosyl phosphite is more stable than tyrosyl phosphate is under acid conditions. But in an alkaline condition, tyrosyl phosphite is more labile than tyrosine phosphate is, as indicated by the k_H and $t_{1/2}$ values shown in Table II. The hydrolysis of the tyrosyl phosphate reaction at pH 10.0 is too slow to be detected; and, hence, no kinetic parameters were evaluated.

To better understand the action of calcineurin toward its substrates and the reason that the V_M value for tyrosyl phosphite was so much higher than that for tyrosyl phosphate, the temperature dependence of the kinetic parameters was evaluated. A linear relationship was obtained between the −log $(1/K_M)$ and $1/T$ for both substrates along the temperature range of 16.5–25 °C. A sharp break in both plots of −log $(1/K_M)$ vs $1/T$ and of log (V_M/T) vs $1/T$ for both substrates at temperatures above 25 °C was observed. These results and the calculated thermodynamic parameters are shown in Figure 2 and Table III.

Table III: Temperature Dependence of the Kinetic Parameters and the Activation Parameters for Enzymatic Hydrolysis of Tyrosyl Phosphate and Tyrosyl Phosphite

	Tyr-P		Tyr-PH ME	
temp (°C)	K_M[a] (mM)	V_M[a] [mol/(min·μg of E)]	K_M[a] (mM)	V_M[a] [mol/(min·μg of E)]
16.5	37	1.1×10^{-10}	118	1.9×10^{-7}
20.0	34	2.7×10^{-10}	114	2.7×10^{-7}
25.0	33	4.2×10^{-10}	90	4.7×10^{-7}
30.0	11	3.5×10^{-10}	12	1.2×10^{-7}
$\Delta G°$ (kcal/mol)[b]	−2		−1	
$\Delta G^{\neq}$ (kcal/mol)[b]		30		26
$\Delta G^{\ddagger} = \Delta G° + \Delta G^{\neq}$ (kcal/mol)[b]		28		27
$\Delta H°$ (kcal/mol)[b]	2		6	
$\Delta H^{\neq}$ (kcal/mol)[b]		26		17
$\Delta H^{\ddagger} = \Delta H° + \Delta H^{\neq}$ (kcal/mol)[b]		28		23
$\Delta S°$ (eu)[b]	14		24	
$\Delta S^{\neq}$ (eu)[b]		−16		−29
$\Delta S^{\ddagger} = \Delta S° + \Delta S^{\neq}$ (eu)[b]		−2		−5

[a] The standard deviation is less than 20%. [b] $E + S \rightarrow ES^{\neq} \rightarrow ES$, $\Delta G°$, $\Delta H°$, $\Delta S°$; $ES \rightarrow ES^{\neq\neq}$, $\Delta G^{\neq}$, $\Delta H^{\neq}$, $\Delta S^{\neq}$; $E + S \rightarrow ES^{\neq} \rightarrow ES \rightarrow ES^{\neq\neq} \rightarrow E + P$, $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$. The standard deviation is about 20% calculated by using the linear regression method.

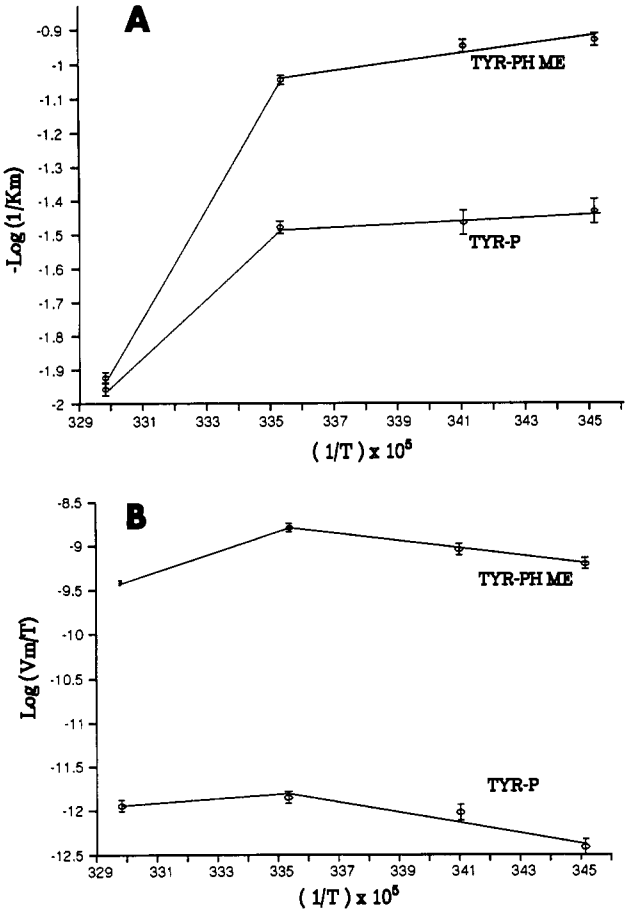

FIGURE 2: Arrhenius plot of (A) $-\log (1/K_M)$ and (B) $\log (V_M/T)$ against reciprocal absolute temperature. Conditions are given under Experimental Procedures.

Discussion

A systematic study of calcineurin-catalyzed dephosphorylation of fluorinated analogues of tyrosine (Martin et al., 1985) suggests that, in contrast to acid and alkaline phosphatase but similar to the acid-catalyzed hydrolysis of these substrates, the effectiveness of the reaction depends upon the electronic nature of the substrate. Presumably, the fluoro group increases the electron-accepting tendency of the leaving phenyl group, causing displacement of electrons from the PO_4 tetrahedron. Reduction of π-bonding may weaken the tetrahedron to facilitate the bonding of an oxygen atom to the d-orbital of phosphorous in the transition state (Van Wazer, 1958). The replacement of an OH group by H may facilitate similar changes in the properties of the phosphorous tetrahedron. The phosphite ester used in this work, like the fluoro analogues, was more effectively utilized by calcineurin, but with this compound, no enhancement in reaction rate was found with acid phosphatase.

A mechanism suggesting that the hydrolysis of phosphate ester monoanions proceeds with protonation of the ester linkage in a rapid preequilibrium step followed by the elimination of the products has been considered as a model for the calcineurin-catalyzed dephosphorylation reaction. This assumption is based on the observations that acid hydrolysis of monoanionic phosphate esters proceeds with a Brønsted coefficient of −0.35 and that the same value has been seen in the calcineurin reactions (Martin et al., 1985). The application of this model to calcineurin catalysis would require monoanion formation on the enzyme. The tyrosyl phosphite was synthesized because it could only be monoanionic at all pH's used in the enzymatic study. Interestingly, an increase in effectiveness

Table IV: Arrhenius Parameters for Hydrolysis of Monoaryl Phosphates[a]

reaction	substrate	ΔH (kcal/mol)	ΔS (eu)
monoanion	*o*-nitro	27	5
monoanion	*m*-nitro	30	4
monoanion	*p*-nitro	26	−1
monoanion	*o*-chloro	26	−4.5
monoanion	2,6-dimethyl	29	2.5
acid	*o*-nitro	19	−23
acid	*m*-nitro	19	−25

[a] Calculated from data from Table VII in Kirby and Varvgolis (1967).

compared to tyrosyl phosphate is not observed in the binding parameter, K_M, but in the V_M value. A study of temperature's effect on the catalytic reaction supports the view that hydrolysis of a bound monoanion occurs. Activation parameters for the hydrolysis of a number of phosphate monoesters are collected in Table IV. The entropies of activation for the hydrolysis of monoanions are all close to zero. The small negative value for the entropy of activation, $\Delta S^{\ddagger} = -2$ eu, associated with the enzymatic hydrolysis of tyrosine phosphate is in contrast to the larger negative entropy values (~25 eu) for the hydrolysis of phosphate monoester acids (Table IV), in which the transition state is heavily hydrated (Bunton et al., 1967), but is in agreement with the entropy change for the hydrolysis of the monoanions. Similarly, $\Delta S^{\ddagger}$ for the phosphite ester had a small megative value (−5 eu).

A linear relation was obtained between the $-\log (1/K_M)$ and $1/T$, for both substrates, along the temperature range of 16.5–25 °C. Valus for $\Delta H^{\neq}$ of 26 and 17 kcal/mol for tyrosine phosphate and tyrosine phosphite methyl ester, respectively, were obtained from the plot of $\log (V_M/T)$ vs $1/T$. These results are in agreement with the $\Delta H^{\neq}$, 24–30 kcal/mol, for the hydrolysis of the oxygen–phosphorous linkage of phosphate monoester monoanion (Table IV) and with the 17–26 kcal/mol for the hydrolysis of the oxygen–phosphorous linkage of phosphite esters (Behrman et al., 1970), thereby strongly supporting the suggestion that cleavage of the ester bond is the rate-limiting step (Martin & Graves, 1986). Studies on the hydrolysis of some monophenyl phosphates (Bunton et al., 1967) suggest, however, that breaking the phosphorous–oxygen bond is not the only requirement for hydrolysis. That tyrosyl phosphite is more stable than tyrosyl phosphate in an acid but more labile in an alkaline condition indicates that a nucleophilic attack by water may also be part of the rate-limiting step for the hydrolysis of tyrosyl phosphite. A phosphite tetrahedron, which may be more readily available for bonding of an oxygen atom in the activated complex, gave a lower activation energy, $\Delta H^{\neq} = 17$ kcal/mol, consistent with the fact the phosphite ester is a better substrate than tyrosine phosphate for calcineurin. The sharp break in both plots of $-\log (1/K_M)$ vs $1/T$ and $\log V_M$ vs $1/T$ for both substrates at temperatures above 25 °C is not caused by enzyme inactivation because K_M values would not differ from enzyme concentration but may reflect the possibility that calcineurin can exist in two or more stable conformational forms over different kinetic properties.

A change in entropy during enzyme–substrate binding ($\Delta S°$) may be partly explained by solvent effects (Laidler & Peterman, 1979). First, the chemistry of phosphate esters, which has been extensively studied, shows that the singly charged form, $ROP(O_2^-)(OH)$, is the most labile, whereas the fully ionized species, $ROPO_3^{2-}$, is the most stable, and the un-ionized form, $ROP(O)(OH)_2$, is relatively stable toward hydrolysis (Van Wazer, 1958). During the course of reaction,

the negative charge of the phosphate ester may be neutralized by electrostatic interactions with a positively charged group of protein or partly neutralized by hydrogen bonding with some group of protein. A release of water molecules may contribute to some gain of entropy. Second, an intramolecular hydrogen bonding between the ester oxygen and a protein residue is also favored by the entropy gain of releasing bound water molecules. A study of the mechanism of hydrolysis of salicyl phosphate suggests that the reaction proceeds with a preequilibrium internal proton transfer by the carboxylic acid group to the ester oxygen followed by the rate-limiting step releasing the products (Bender & Lawlor, 1963). According to the results of our study, the entropy of activation, $\Delta S^{\ddagger} = -2$ eu, associated with the calcineurin-catalyzed tyrosine dephosphorylation is in agreement with the entropy change (−1.2 eu) reorted by Chanley et al. (1952) for salicyl phosphate hydrolysis. This result implies that calcineurin catalysis may also involve a neighboring carboxyl group in acid catalysis. The results of the Martin and Graves (1986, 1987) study have suggested an acid catalysis as part of the catalytic mechanism of calcineurin, but no functional group has been identified yet.

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