Leaving Group Dependence and Proton Inventory Studies of the Phosphorylation of a Cytoplasmic Phosphotyrosyl Protein Phosphatase from Bovine Heart†

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ABSTRACT: The k_{cat} and K_m values for the bovine heart low molecular weight phosphotyrosyl protein phosphatase catalyzed hydrolysis of 16 aryl phosphate monoesters and of five alkyl phosphate monoesters having the structure $Ar(CH_2)_nOPO_3H_2$ (n = 1–5) were measured at pH 5.0 and 37 °C. With the exception of α-naphthyl phosphate and 2-chlorophenyl phosphate, which are subject to steric effects, the values of k_{cat} are effectively constant for the aryl phosphate monoesters. This is consistent with the catalysis being nucleophilic in nature, with the existence of a common covalent phosphoenzyme intermediate, and with the breakdown of this intermediate being rate-limiting. In contrast, k_{cat} for the alkyl phosphate monoesters is much smaller and the rate-limiting step for these substrates is interpreted to be the phosphorylation of the enzyme. A single linear correlation is observed for a plot of log (k_{cat}/K_m) vs leaving group pK_a for both classes of substrates at pH 5.0: log (k_{cat}/K_m) = −0.28pK_a + 6.88 (n = 19, r = 0.89), indicating a uniform catalytic mechanism for the phosphorylation event. The small change in effective charge (−0.28) on the departing oxygen of the substrate is similar to that observed in the specific acid catalyzed hydrolysis of monophosphate monoanions (−0.27) and is consistent with a strong electrophilic interaction of the enzyme with this oxygen atom in the transition state. The D_2O solvent isotope effect and proton inventory experiments indicate that only one proton is "in flight" in the transition state of the phosphorylation process and that this proton transfer is responsible for the reduction of effective charge on the leaving oxygen.

The low molecular weight, cytoplasmic bovine heart acid phosphatase, which is also an active phosphotyrosyl protein phosphatase, catalyzes the hydrolysis of aryl phosphate monoesters through a phosphoenzyme mechanism (Zhang & Van Etten, 1990, 1991). The kinetic scheme can be described by an ordered uni-bi sequence with a minimum of a four-step reaction (Scheme I). The existence of a covalent phosphoenzyme intermediate has been implicated by extensive pre-steady-state and steady-state kinetic analyses, and the rate-limiting step in the normal hydrolytic reaction has been identified as the breakdown of this intermediate (Zhang & Van Etten, 1991). The enzyme hydrolyzes a number of phosphotyrosyl peptides and proteins but also shows activity toward aryl phosphate monoesters and toward a selected group of alkyl phosphate monoesters that are related to flavin mononucleotide (Zhang & Van Etten, 1990). The rate-limiting step should be affected by changes in the structure and electronic nature of the substrate. Here, we describe a structure–activity study of leaving group effects on the phosphorylation of the bovine heart low molecular weight phosphotyrosyl protein phosphatase. This study permits an estimation of the change in effective charge on the leaving oxygen atom in this catalytic event. The resulting value of the leaving group effect for the phosphorylation reaction strongly indicates the involvement of electrophilic participation that facilitates the departure of the leaving phenol or alcohol group in the transition state. The nature of this electrophilic interaction might be a positive charge acting as an oxygen anion binding site or a solvent-derived proton that is hydrogen-bonded to the leaving oxygen atom. This was further examined by conducting a proton inventory study using a poor substrate, $Ar(CH_2)_4OPO_3H_2$, whose rate-limiting step becomes the phosphorylation of the enzyme. In this way, we sought to determine if it is a solvent-derived proton, which may be hydrogen-bonded to the leaving oxygen in the transition state, that causes the observed reduction of the effective charge on the oxygen atom.

Scheme I

$$E + pNPP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}pNPP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{–}P + pNP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E{\cdot}P_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P_i$$

Materials and Methods

Enzyme. Homogeneous low molecular weight phosphotyrosyl protein phosphatase was purified from fresh bovine hearts obtained from a local slaughterhouse (Zhang & Van Etten, 1990).

Aryl Phosphate Monoesters. Aryl phosphate monoesters, 2-chlorophenyl, 3-chlorophenyl, 4-chlorophenyl, 4-acetylphenyl, 4-ethylphenyl, 4-fluorophenyl, 4-methylphenyl, 3-nitrophenyl, 4-cyanophenyl, and 4-(trifluoromethyl)phenyl phosphate were synthesized as dicyclohexylammonium salts according to Zhang and Van Etten (1991), while *p*-nitrophenyl phosphate, tyrosine phosphate, phenyl phosphate, and α- and β-naphthyl phosphate were purchased from Sigma.

Alkyl Phosphate Monoesters. Phosphate monoesters having a pendant aromatic group ($Ar(CH_2)_nOPO_3H_2$, n = 1–5) were synthesized following the method of Kirby (Kirby, 1963; Zhang & Van Etten, 1990). The purity of these preparations was established by use of an inorganic phosphate assay, by ^{31}P NMR spectroscopy, and by observation of sharp melting points. Benzyl phosphate and 4-phenylbutyl phosphate (cyclohexylammonium salt) were also crystallized; the X-ray crystal structures of these two substrates have also been solved (unpublished results).

Deuterium oxide (99.9%) was obtained from Aldrich. All the phenols and alcohols were from Aldrich. Other reagents

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were of analytical grade and were used without further purification. Water was double-deionized and distilled in an all-glass apparatus.

Steady-State Kinetics. The initial velocities of the hydrolysis of phosphate monoesters in the presence of phosphotyrosyl protein phosphatase were measured by determining the inorganic phosphate produced during the reaction. The procedure developed by Black and Jones (1983) for measurement of inorganic phosphate was modified for use in this phosphatase enzyme assay. Enzyme-catalyzed hydrolysis reactions were terminated by the addition of 0.2 mL of 10% trichloroacetic acid, followed by the addition of a 0.5-mL mixture (composed of 0.2 mL of 2% ammonium molybdate and 0.3 mL of 14% ascorbic acid in 50% trichloroacetic acid), and then 1 mL of 2% trisodium citrate plus 2% sodium arsenite in 2% acetic acid was added immediately. The blue color was developed for 30 min, and the absorption at 700 nm was measured by use of a Beckman DU-68 spectrophotometer. The amount of inorganic phosphate produced was calculated from the standard curve with KH_2PO_4 as a standard. Steady-state kinetic measurements were performed in a buffer of 100 mM acetate/1 mM EDTA, I = 0.15 M (adjusted by adding NaCl), pH 5.0. The solutions (400 μL) containing various substrate concentrations (seven substrate concentrations ranging from $0.2K_m$ to $5K_m$) were first incubated at 37 °C in a water bath for at least 5 min, and then the reactions were initiated by the addition of a catalytic amount of enzyme. The extent of reaction was in every case less than 1% of the total substrate added. The substrates, which were synthesized as dicyclohexylammonium salts, were used directly. Cyclohexylammonium ion was determined to be a very weak noncompetitive inhibitor of the enzyme, with a K_i of 140 mM at pH 5.0. In order to minimize inhibitory effects on the steady-state behavior of enzyme-catalyzed reactions of these salts, the maximum substrate concentration in kinetic measurements was 20 mM (and was generally much less). The V_{max} and K_m values were obtained by direct fit of the data to the Michaelis–Menten equation with the nonlinear regression program KINFIT (Knack & Röhm, 1981). Replicate measurements as well as standard errors obtained from the fitting programs indicated that k_{cat} and K_m were accurate to better than ±5 and ±9%, respectively.

Proton Inventory Measurement. The proton inventory study of the phosphotyrosyl protein phosphatase catalyzed hydrolysis was conducted according to literature methods (Schowen & Schowen, 1982). Seven mixtures of H_2O and D_2O were prepared with deuterium atomic fractions from 0.0 to 1.0. Stock solutions of 100 mM acetate/1 mM EDTA, I = 0.15 M (adjusted with NaCl), in pure H_2O (pH = 5.0) or pure D_2O (pD = 5.0) were prepared and the mixed H_2O/D_2O buffers were prepared by mixing appropriate volumes of stock solutions. For each H_2O/D_2O mixed buffer system, the pL (L = H or D) value was measured with a Corning 130 pH meter and the meter reading was adjusted with the same deuterium content LCl or NaOL, according to the equation $(pH)_n = 0.076n^2 + 0.3314n$ (Schowen & Schowen, 1982). In each mixture, the pL value was effectively 5.0. Initial velocities at seven substrate concentrations covering $0.2K_m$–$5K_m$ were measured in each H_2O/D_2O mixed buffer system and V_{max} and K_m values were calculated as described above.

Results

Structure–Activity Relationship. The k_{cat} and K_m values were determined for 16 aryl phosphate monoesters differing only in the structure of the phenolic leaving groups and for five alkyl phosphate monoesters having structures of the form $Ar(CH_2)_nOPO_3H_2$ (n = 1–5). The results are summarized in Table I.

Table I: Michaelis–Menten Kinetic Parameters for Hydrolysis of Aryl and Alkyl Monophosphate Ester Hydrolysis

substrate phenyl ester	leaving group pK_a	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\times 10^{-3}$) (M^{-1} s^{-1})
4-nitro	7.14	34.2	0.38	90
3-nitro	8.38	33.3	0.71	47
4-acetyl	8.05	34.2	0.19	180
4-cyano	7.95	31.9	0.97	33
4-fluoro	9.95	29.5	2.6	11
4-chloro	9.38	30.5	1.3	24
3-chloro	9.08	27.5	1.5	18
2-chloro	8.48	3.15	22.	0.14
4-bromo	9.34	26.1	0.92	28
α-naphthyl	9.38	1.2	21.	0.057
β-naphthyl	9.38	29.5	1.2	25
4-(trifluoromethyl)	8.68	34.3	0.98	35
4-methyl	10.26	28.2	3.5	8.1
4-ethyl	10.0	30.4	1.7	18
parent	9.99	27.4	3.6	7.6
tyrosine	10.07	27.1	14	1.9
$ArCH_2OPO_3H_2$	14.84[a]	0.73	38.	0.019
$Ar(CH_2)_2OPO_3H_2$	15.59[a]	18.8	6.7	2.8
$Ar(CH_2)_3OPO_3H_2$	15.79[a]	0.97	2.85	0.34
$Ar(CH_2)_4OPO_3H_2$	15.87[a]	3.54	7.0	0.51
$Ar(CH_2)_5OPO_3H_2$	15.9[a]	2.02	3.8	0.54

[a] The pK_a values of the alkyl alcohols were calculated according to Williams (1984b).

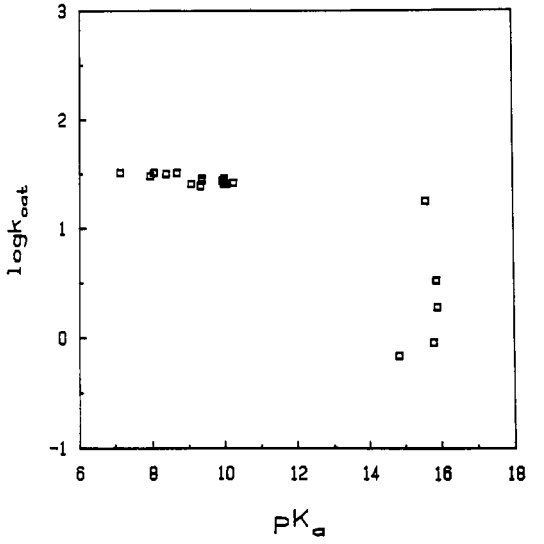


FIGURE 1: Modified Bronsted plot for k_{cat} at pH 5.0, 37 °C. Points are taken from Table I. α-Naphthyl phosphate and 2-chlorophenyl phosphate are not included in the plot (see text).

For the aryl phosphate monoesters, a graph of log k_{cat} vs the pK_a of the leaving group (Figure 1) shows that the k_{cat} values are effectively constant, consistent with the existence of a common, covalent phosphoenzyme intermediate and with its breakdown being rate-limiting. In contrast, for the alkyl phosphate monoesters, the line is sharply curved, indicating a change in the rate-limiting step of the enzyme-catalyzed hydrolysis for this class of compounds. For both classes of substrates, however, there is a relatively good linear correlation between log (k_{cat}/K_m) vs pK_a of the leaving phenols or alcohol, with only benzyl phosphate, and to a lesser extent tyrosine phosphate, showing significant deviations (Figure 2). The relationship can be described by the linear equation

$$\log (k_{cat}/K_m) = -0.28pK_a + 6.88 \quad (n = 19, r = 0.89) \quad (1)$$

Thus, the phosphorylation of the enzyme by substrate has a

Table II: D_2O Solvent Isotope Effect on the Hydrolysis of Some Phosphate Monoesters at pL = 5.0, 37 °C, Catalyzed by the Bovine Heart Low Molecular Weight Phosphotyrosyl Protein Phosphatase

phosphate substrate	k_{cat}^H (s^{-1})	k_{cat}^D (s^{-1})	K_m^H (mM)	K_m^D (mM)	k_{cat}^H/k_{cat}^D	$(k_{cat}/K_m)^H/(k_{cat}/K_m)^D$
p-nitrophenyl	32.2	30.3	0.323	0.265	1.06	0.865
p-ethylphenyl	29.1	25.7	1.48	1.40	1.13	1.07
p-methylphenyl	30.2	23.7	3.59	2.81	1.27	0.997
$Ar(CH_2)_2OPO_3H_2$	17.7	14.2	5.5	2.8	1.25	0.635
$Ar(CH_2)_4OPO_3H_2$	3.33	1.40	7.0	2.9	2.38	0.899

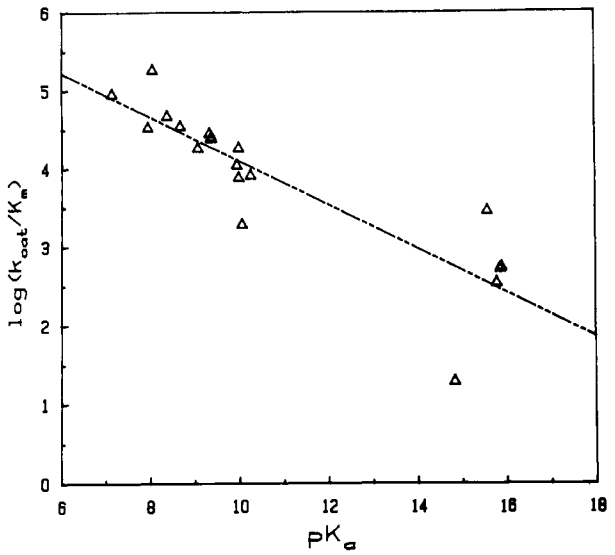


FIGURE 2: Modified Bronsted plot for k_{cat}/K_m at pH 5.0, 37 °C. Points are taken from Table I. α-Naphthyl phosphate and 2-chlorophenyl phosphate are not included in the plot (see text). The line is generated by a linear regression method; the slope is −0.28 with a correlation coefficient of 0.89.

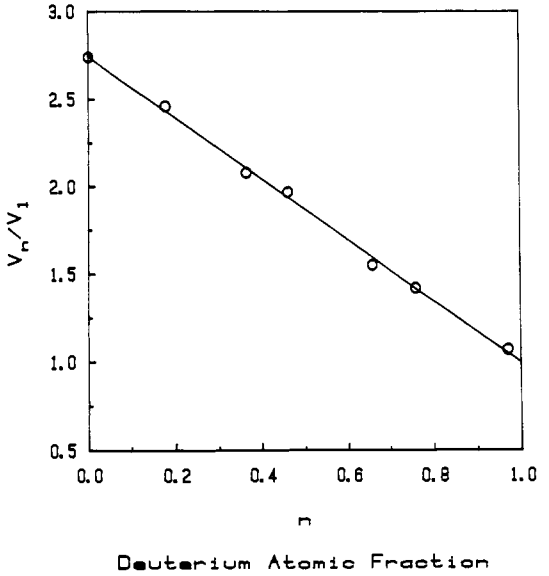


FIGURE 3: Proton inventory graph of V_{max} for the hydrolysis of 4-phenylbutyl phosphate at pL = 5.0, 37 °C. The line is generated by a linear regression method; the correlation coefficient is 0.999.

Bronsted β_{lg} value of −0.28. The single linear relationship for log (k_{cat}/K_m) vs pK_a with both aryl and alkyl phosphate monoesters (the latter having structures of the form $Ar(CH_2)_nOPO_3H_2$) suggests that the transition-state structures are similar for all of these substrates and that a uniform mechanism is utilized over the wide range of pK_a values that was studied for the phosphorylation of the low molecular weight phosphotyrosyl protein phosphatase.

Solvent Isotope Effect and Proton Inventory. Values of V_{max} and K_m for 4-nitrophenyl phosphate, 4-methylphenyl phosphate, 4-ethylphenyl phosphate, 2-phenylethyl phosphate, and 4-phenylbutyl phosphate in H_2O and D_2O acetate buffer at pL = 5.0 are summarized in Table II.

A proton inventory experiment was performed with use of 4-phenylbutyl phosphate as a substrate with seven concentrations of D_2O in a buffer of 100 mM acetate/1 mM EDTA, I = 0.15 M, pL 5.0. The V_n/V_1 vs n plot (where n is the deuterium atomic fraction) gives a straight line (eq 2) with

$$V_n/V_1 = -1.75n + 2.74 \quad (2)$$

a least-squares correlation coefficient of 0.999 (Figure 3). We therefore conclude that the solvent isotope effect on the phosphorylation step is due to the transfer of a single-solvent derived proton in the transition state.

Discussion

The aqueous hydrolysis reactions of phosphate monoesters have characteristic pH optima at around 4. This is interpreted to indicate that the monoanion is the most reactive species (Cox & Ramsay, 1964). The mechanism of the monoanion hydrolysis in aqueous solution at moderately acidic pH is generally considered to be a fast, preequilibrium proton transfer to the leaving oxygen, followed by an unimolecular decomposition. The proton transfer facilitates the process of hydrolysis because ROH rather than the higher energy RO^- species is formed in the reaction. The reaction is also favored by the negative charge on the phosphate group. This mechanism is consistent with an entropy of activation that is nearly zero (Jencks, 1962; Kirby & Varvoglis, 1967). Also, the monoester monoanion hydrolysis reactions display a striking insensitivity to the nature of the leaving groups. As demonstrated by Kirby and Varvoglis (1967), the hydrolysis of phosphate monoesters at pH 3–5 exhibits a β_{lg} of −0.27, while a β_{lg} of −0.32 was observed for a similar reaction (Bunton et al., 1967). This is typical behavior for reactions involving protonation of the leaving group, and the small magnitude of β_{lg} is readily rationalized on the basis that substituents that favor protonation (i.e., electron donating) also hinder the expulsion of the leaving group and vice versa.

Leaving group dependence studies are known in many instances to yield useful and significant information about reaction mechanisms (Hall & Williams, 1986; Davis et al., 1988; Donarski et al., 1989). Such linear free energy relationship investigations have been successfully applied to a number of biological systems, especially hydrolytic enzymes (Williams, 1970; Williams & Bender, 1971). Recently, a related and important concept called "effective charge" has been formulated (Williams, 1984a). It has been demonstrated to be very useful in mechanistic studies, not only of enzyme-catalyzed reactions but also of solution reactions in general. By definition, the effective charge is the hypothetical charge that would be required to give the same observed substituent effect as that observed in a standard equilibrium where the charge is defined. If we define a standard equilibrium (also called calibrating equilibrium) that resembles the reaction being studied, then we can determine the effective charge of an atom in the reactant, in the product, and in the transition state by measuring the effects of substituents on the equilibrium con-

stant (β_{EQ}) and on the rate constant (β_{1g}). The value β_{EQ} is a measure of the change in the effective charge from reactant to product, whereas the value β_{1g} is a measure of the change in the effective charge from reactant to transition state.

In the present case, we define the ionization of phenols as the standard equilibrium (eq 3), since it commonly correlates

$$\underset{(0)}{\text{Ar-O-H}} \rightleftarrows \text{H}^+ + \underset{(-1)}{\text{Ar-O}^-} \quad (3)$$

reactions of esters with varied leaving groups. The effective charge on the oxygen of the ionized and unionized phenol are defined to be −1 and 0, respectively. The effective charges on the oxygen adjacent to phosphono, phosphono monoanion, and phosphoryl groups have been calculated from literature equilibrium constants for the hydrolysis of corresponding esters (Bourne & Williams, 1984) and are given in square brackets in eq 4. The effective charges are relative to the charges

$$\underset{[+0.82]}{\text{X-O-PO}_3\text{H}_2} \overset{\beta_{EQ} = -0.09}{\rightleftharpoons} \underset{[+0.73]}{\text{X-O-PO}_3\text{H}^-} \overset{\beta_{EQ} = -0.38}{\rightleftharpoons} \underset{[+0.35]}{\text{X-O-PO}_3^{2-}} \overset{\beta_{EQ} = -1.35}{\rightleftharpoons} \underset{[-1.0]}{\text{X-O}^-} + \text{HOPO}_3^{2-} \quad (4)$$

defined for the phenolic and phenolate oxygen in the standardizing equilibrium.

Earlier work showed that the bovine liver enzyme catalyzes a stereospecific phospho-group transfer from phenyl (*R*)-[^{16}O,^{17}O,^{18}O]phosphate to (*S*)-propane-1,2-diol with an overall retention of configuration at phosphorus (Saini et al., 1981). This is consistent with a double-displacement mechanism, provided that each attack on phosphorus proceeds by an in-line $S_N2(P)$ mechanism. Extensive steady-state kinetic analyses, ^{18}O-exchange experiments and pre-steady-state burst-titration experiments indicate that the low molecular weight phosphotyrosyl protein phosphatase catalyzes the hydrolysis of aryl phosphate monoesters through a covalent phosphoenzyme intermediate and that the rate-limiting step is the breakdown of this intermediate. A measurement of the effective charge on the leaving oxygen atom during the formation of this phosphoenzyme intermediate was sought in order to better understand the transition-state structure and the catalytic mechanism for phospho-group transfer from the substrate to the enzyme.

The apparent second-order rate constant k_{cat}/K_m relates reaction rate to the concentration of free substrate and free enzyme (Fersht, 1985). Here, k_{cat}/K_m monitors the phosphorylation of free enzyme by free substrate. By studying the effect of polar substituents on k_{cat}/K_m, we may obtain information about the nature of the transition-state structure in the phosphorylation step. Accordingly, k_{cat} and K_m values for 21 substrates (16 aryl phosphate monoesters and five alkyl phosphate monoesters with a structure of $Ar(CH_2)_nOPO_3H_2$, n = 1–5) were measured at 37 °C, pH 5.0 (Table I). The log k_{cat} versus pK_a of the leaving group (Figure 1) shows a discontinuity, indicating a change of the rate-determining step among these two classes of substrates. In contrast, log (k_{cat}/K_m) vs pK_a shows a relatively good linear correlation with a β_{1g} = −0.28 for both classes of substrates (Figure 2).

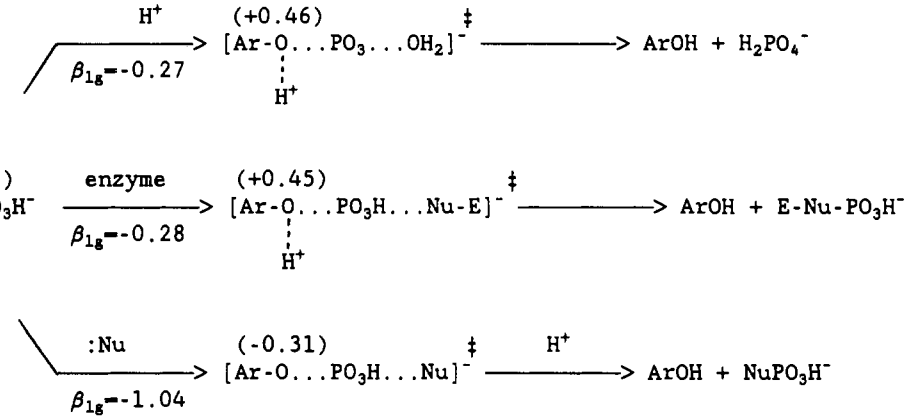
The enzyme shows low activity toward α-naphthyl phosphate or 2-chlorophenyl phosphate. The lack of reactivity of these two substrates may be attributed to the fact that the adjacent carbon or chlorine substitutions on the substrate provide steric hindrance that renders the phosphorous less accessible to an attacking group at the enzyme active site, consistent with the nature of the catalysis being nucleophilic (Zhang & Van Etten, 1990, 1991). These two substrates are therefore not included in the Bronsted-type plot. The point for benzyl phosphate deviates strongly from the line, perhaps also as a result of steric effects. The benzylic CH_2 is disordered in the crystal structure of benzyl phosphate (unpublished results), but it is unclear if there is any relationship between this fact and the lack of reactivity of the ester. Although tyrosine phosphate and 4-acetylphenyl phosphate show k_{cat} values that are similar to those of the other aryl substrates, both of them deviate from the log (k_{cat}/K_m) vs pK_a plot. Tyrosine phosphate has a substantially higher K_m value, which may be due to the presence in the substrate of a protonated amino group that hinders the binding process (Zhang & Van Etten, 1990). 4-Acetylphenyl phosphate has a rather low K_m value, which may indicate that the presence of a carbonyl group results in some additional binding interaction. 2-Phenylethyl phosphate has a k_{cat} that places it near the borderline of the two substrate groups.

A β_{1g} of −1.04 has been observed for reactions of the monoanion of 4-substituted 2-nitrophenyl esters with nicotinamide, while a Bronsted β value of +0.56 has been observed for reactions of substituted pyridines with the monoanion of 2,4-dinitrophenyl phosphate (Kirby & Varvoglis, 1968). The strong dependence on both the nucleophile and the leaving group is consistent with a classical $S_N2(P)$ mechanism, in which bond breaking and bond making are both well underway in the transition state. Thus, a purely nucleophilic displacement reaction on the phosphorus atom of 2,4-dinitrophenyl phosphate monoanion is quite different from an acid-catalyzed hydrolysis of the monoanion, which is effectively a unimolecular elimination (Kirby & Varvoglis, 1968). Furthermore, the entropy of activation for the nucleophilic displacement reaction was −23 eu, consistent with a bimolecular mechanism.

The effective charges on the leaving oxygen atom for the acid-catalyzed hydrolysis of an aryl phosphate monoanion, for the phosphorylation of the bovine heart phosphotyrosyl protein phosphatase by a phosphate monoester, and for the nucleophilic reaction between phosphate monoester monoanion and a nucleophile were each calculated according to Williams (Williams, 1984a) and are summarized in Scheme II. As one can see, the effective charge on oxygen for the enzyme-catalyzed phosphorylation reaction is substantially more positive (+0.45) than the value that would be expected for a typical nucleophilic substitution reaction (−0.31). In fact, it is almost identical with the value observed for the acid-catalyzed hydrolysis of an aryl phosphate monoanion (+0.46). Since the enzyme-catalyzed phosphorylation reaction is nucleophilic in nature, the enzyme must provide an electrophile to accept the leaving oxygen anion (or an acid to donate a proton) in order to reduce the effective charge (or neutralize the negative charge) on the phenolate (or alcoholate) oxygen atom.

The striking similarity between β_{1g} obtained for the phosphorylation of the low molecular weight phosphotyrosyl protein phosphatase and that for the acid-catalyzed hydrolysis of phosphomonoesters strongly suggests the involvement of electrophilic participation in the transition state, perhaps analogous to the oxyanion hole of serine proteases (Venkatasubban & Schowen, 1984; Robertus et al., 1972). Alkaline phosphatase also catalyzes the hydrolysis of phosphate monoesters via a double-displacement mechanism, with the formation and breakdown of a phosphoenzyme intermediate on the catalytic pathway (Kim & Wyckoff, 1991). The change in effective charge on the leaving oxygen of the ester for the phosphorylation of alkaline phosphatase is only −0.2 (Hall & Williams, 1986). A β_{1g} of −0.35 was revealed for k_{cat}/K_m of calcineurin, a calmodulin-activated protein tyrosine phosphatase, during the hydrolysis of substituted tyrosine phos-

Scheme II



phates (Martin, et al., 1985). However, k_{cat} for these substrates was not constant, thus excluding the possibility of a rate-limiting dephosphorylation of a covalent phosphoenzyme intermediate. In any event, irrespective of the detailed mechanism that is operating, it is clear that phosphomonoesterases, including acid, alkaline, and protein tyrosine phosphatases, all employ a substantial electrophilic interaction during the hydrolysis of phosphate monoesters.

The nature and identity of the electrophile is of considerable mechanistic interest. In the case of alkaline phosphatase, three possible candidates have been advanced, i.e., an active site arginine, hydrogen bonding of a donor to the oxygen, or metal ion interaction either directly at the phenolic oxygen or indirectly through the phosphate group (Hall & Williams, 1986). Through the use of site-directed mutagenesis, it was concluded that the presence of the arginine was not required to explain the observed small change in effective charge on the leaving oxygen of the phosphate monoester during the course of catalysis and that this role was most likely fulfilled by one of the active site zinc ions (Butler-Ransohoff et al., 1989). In contrast, the low molecular weight phosphotyrosyl protein phosphatase is a monomeric, non-metalloenzyme. A chemical modification study established the presence of at least one essential arginine residue in the active site (Zhang, 1990). Thus, the possible identity of the electrophile in the low molecular weight phosphatase is likely to be either an active site arginine or hydrogen bonding from a donor to the oxygen. We sought to resolve this issue by conducting a proton inventory experiment on this catalytic step. The proton inventory technique is based on measurements of the rate constants of reaction in a series of mixtures of H_2O and D_2O. Under favorable conditions, proton inventory experiments enable one to determine the number of protons responsible for the observed solvent isotope effect (Venkatasubban & Schowen, 1984).

Initial measurements of the solvent isotope effect were conducted on a number of substrates at 37 °C, pL = 5.0, in a pH-independent region of k_{cat}. The results are summarized in Table II. For good substrates like *p*-nitrophenyl phosphate, 4-ethylphenyl phosphate, and 4-methylphenyl phosphate, there is no significant D_2O solvent isotope effect on either k_{cat} or k_{cat}/K_m. We know that for aryl phosphate monoesters, the rate-limiting step for the enzyme-catalyzed hydrolysis is the breakdown of the covalent phosphoenzyme intermediate, so that k_{cat} in this case reduces to k_3 (see Scheme I), a rate constant for a single chemical event (Zhang & Van Etten, 1991). The absence of a D_2O solvent isotope effect therefore indicates that there is no proton transfer in this catalytic step. In the case of k_{cat}/K_m, which monitors the phosphorylation of the enzyme by the substrate, the absence of a significant D_2O solvent isotope effect appears to suggest the same conclusion. However, solvent isotope effects can be caused by direct proton transfer in the transition state of the rate-limiting step of an enzymic reaction, but they can also be due to the perturbation of some equilibrium constants of ionizable groups on enzyme or substrate. Although we conducted our experiment at the plateau region of the pL rate profile, the fact that our substrate phosphate monoesters ionize in this region complicated the explanation. In addition, since $k_{cat}/K_m = k_2/K_s$ (Ko & Kezdy, 1967), the observed solvent isotope effect on k_{cat}/K_m is a combination of effects on both the rate and equilibrium constants. So, even if there is a real solvent isotope effect on the phosphorylation step (k_2), one might not detect it because a similar effect on the substrate–enzyme dissociation constant (K_s) could lead to a cancellation.

Consequently, the solvent isotope effect was measured by use of the poor substrate 4-phenylbutyl phosphate, in the expectation that the altered substrate structure should cause the rate-limiting step of the enzyme-catalyzed reaction to change. (Recall that the plot of log k_{cat} versus pK_a showed that the data for 4-phenylbutyl phosphate deviated strongly from the line defined by aryl phosphate monoesters, consistent with a change in the rate-limiting step.) Interestingly, we did in fact observe a D_2O solvent isotope effect of 2.4 on k_{cat} for 4-phenylbutyl phosphate. 2-Phenylethyl phosphate is on the borderline between the two structurally different classes of substrates, and its k_{cat} value may be the result of substantial contributions from both steps.

The turnover number k_{cat} and K_m values for 4-phenylbutyl phosphate at pH 5.0, 37 °C, are 3.33 s^{-1} and 7.0 mM, respectively. The rate constant k_2 for the phosphorylation of the low molecular weight phosphotyrosyl protein phosphatase by 4-phenylbutyl phosphate can be calculated according to $k_{cat}/K_m = k_2/K_s$. The true equilibrium constant K_s for the dissociation of 4-phenylbutyl phosphate from the enzyme was determined to be 6.6 mM at pH 5.0, 37 °C, by steady-state kinetic measurements in which 4-phenylbutyl phosphate was treated as an inhibitor of nitrophenyl phosphate hydrolysis. 4-Phenylbutyl phosphate was found to be a pure competitive inhibitor against *p*-nitrophenyl phosphate. Therefore, $k_2 = k_{cat}/K_mK_s = 3.14\ s^{-1}$, and $k_{cat} \simeq k_2$. We conclude that for 4-phenylbutyl phosphate, the rate-limiting step for the enzyme-catalyzed hydrolysis is the phosphorylation of the enzyme, i.e., the formation of the covalent phosphoenzyme intermediate. The observed D_2O solvent isotope effect indicates the involvement of a proton(s) in the transition state of the phosphorylation step. Note that although in this case k_{cat} and k_{cat}/K_m monitor the same chemical event, k_{cat}/K_m does not show a significant D_2O solvent isotope effect, consistent with the preceding analysis.

A proton inventory experiment was then performed with use of 4-phenylbutyl phosphate as a substrate at pL = 5.0, 37 °C in seven H_2O/D_2O solvent mixtures with different deuterium contents. The V_n/V_1 vs n plot is effectively linear with a correlation coefficient $r = 0.999$ (Figure 3). Together, the results from the solvent isotope effect and the proton inventory experiment are consistent with the conclusion that a single proton is in flight in the transition state for the phosphorylation of the low molecular weight phosphotyrosyl protein phosphatase. The reduction of the effective charge on the leaving oxygen is probably due to the transfer of a solvent-derived proton to the oxygen atom in the transition state. The active site arginine is most likely to be involved in the substrate binding of the phosphate moiety via an ionic interaction. This would favor an associative mechanism of catalysis and retard the dissociative path by opposing the flow of electrons to the oxygen in the P–O bond undergoing fission (Hall & Williams, 1986). The identity of the enzymic nucleophile remains to be established.[1]

In conclusion, from the leaving group dependence study, we found that for aryl phosphates, k_{cat} is independent of the nature of the leaving group, consistent with the existence of a common covalent phosphoenzyme intermediate and with the breakdown of this intermediate being rate-limiting. For alkyl phosphates having the structure $Ar(CH_2)_nOPO_3H_2$, the rate-limiting step changes to the phosphorylation of the enzyme, that is, the formation of the phosphoenzyme intermediate. The phosphorylation of the low molecular weight phosphotyrosyl protein phosphatase shows a rather small dependence on the pK_a of the leaving group, with a $\beta_{lg} = -0.28$ for both classes of substrates. This reflects a small change in effective charge on the leaving oxygen and a more positive effective charge on the oxygen atom in the transition state than would be expected for a pure nucleophilic substitution reaction. This strongly suggests an electrophilic participation that acts to neutralize the negative charge on the oxygen atom in the transition state. The catalytic mechanism for the phosphorylation is conserved for the two classes of substrates. The results from solvent isotope effect and proton inventory experiments are consistent with a concerted proton transfer coupled with the nucleophilic bond-making and bond-breaking processes in the transition state.

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[1] Added in proof: Phosphoenzyme trapping experiments and ^{31}P NMR spectroscopy have led to the identification of the nucleophilic residue as a cysteine (Zhang, Z.-Y., Zhou, M.-M., and Van Etten, R. L., submitted for publication).

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