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Leaving Group Dependence in the Phosphorylation of *Escherichia coli* Alkaline Phosphatase by Monophosphate Esters†

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Received January 31, 1986; Revised Manuscript Received April 3, 1986

ABSTRACT: Values of k_{cat} and K_m have been measured for the *Escherichia coli* alkaline phosphatase catalyzed hydrolysis of 18 aryl and 12 alkyl monophosphate esters at pH 8.00 and 25 °C. A Brønsted plot of log (k_{cat}/K_m) (M^{-1} s^{-1}) vs. the pK of the leaving hydroxyl group exhibits two regression lines:

$$\log (k_{cat}/K_m) = -0.19\ (\pm 0.02)\ pK^{ArOH} + 8.14\ (\pm 0.15)$$

$$\log (k_{cat}/K_m) = -0.19\ (\pm 0.01)\ pK^{ROH} + 5.89\ (\pm 0.17)$$

Alkyl phosphates with aryl or large lipophilic side chains are not correlated by the above equations and occupy positions intermediate between the two lines. The observed change in effective charge on the leaving oxygen of the ester (−0.2) is very small, consistent with substantial electrophilic participation of the enzyme with this atom. Cyclohexylammonium ion is a noncompetitive inhibitor against 4-nitrophenyl phosphate substrate at pH 8.00, and neutral phenol is a competitive inhibitor (K_i = 82.6 mM); these data and the 100-fold larger reactivity of aryl over alkyl esters are consistent with the existence of a lipophilic binding site for the leaving group of the substrate. The absence of a major steric effect in k_{cat}/K_m for substituted aryl esters confirms that the leaving group in the enzyme–substrate complex points away from the surface of the enzyme. Arguments are advanced to exclude a dissociative mechanism (involving a metaphosphate ion) for the enzyme-catalyzed substitution at phosphorus.

The presently accepted kinetic scheme for the catalytic action of alkaline phosphatase from *Escherichia coli* possesses four sequential steps passing through a central, phosphoryl-enzyme (eq 1). The phosphoryl group ($-P_i = -PO_3^{2-}$) becomes co-

$$E + XOP_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}XOP_i \underset{k_{-2}[XOH]}{\overset{k_2}{\rightleftharpoons}} E{-}P_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E{\cdot}HOP_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + HOP_i \quad (1)$$

valently attached to serine-102 in the single peptide chain constituting a monomer of the dimeric enzyme (Coleman & Chlebowski, 1979; Bradshaw et al., 1981; Sowadski et al., 1985; Coleman & Gettins, 1983). There is now considerable information concerning the constituents of the active site from X-ray crystallographic, NMR spectroscopic, and chemical work providing ground-state spacial data.

Knowledge of the change in charge on the leaving atom in transfer of the phosphoryl group from the substrate to the enzyme would provide useful data to describe the molecular mechanism of the catalysis. Measurement of the change in charge requires knowledge of the polar effect on the transition state for the phosphorylation step as well as a calibrating polar effect on an equilibrium for phosphoryl group transfer where the charge change may be defined. The most appropriate calibrating equilibrium is the hydrolysis of the monophosphate dianion (eq 2) for which the polar effect has recently been

$$X{-}O{-}P_i \overset{K}{\rightleftharpoons} X{-}O{-}H + H{-}O{-}P_i \quad (2)$$

$$X{-}O{-}H \overset{K_a}{\rightleftharpoons} X{-}O^- + H^+ \quad (3)$$

determined (Bourne & Williams, 1984a). All measurements of charge are referred to the ionization of the hydroxyl species (eq 3) where the charge change is defined as unity (Williams, 1984a).

The rate-limiting step for the enzyme-catalyzed hydrolysis at low substrate concentrations is phosphorylation when the added leaving group concentration is negligible ($k_{-2}[XOH] \ll k_3$). The kinetic equation for k_{cat}/K_m simplifies to eq 4 (Ko & Kézdy, 1967). The free energy difference between ground

$$k_{cat}/K_m = k_2/K_s = k_1k_2/(k_{-1} + k_2) \quad (4)$$

state (E + XOP_i) and transition state of the phosphorylation step is essentially measured by k_{cat}/K_m; the effect of polar substituents on k_{cat}/K_m will therefore directly depend on the charge change (Williams, 1984; Hine, 1959; Jencks, 1971) by virtue of the change in energy of interaction between charge and the substituent. Previous work (Williams et al., 1973) has shown that there is little polar effect of substituents in the

† This work was supported, in part, by the SERC (U.K.) (Grant GRC 75529).

0006-2960/86/0425-4784$01.50/0

phosphorylation of *E. coli* alkaline phosphatase by aryl phosphates. Close examination of the work indicates that alkyl phosphates hydrolyze at a much lower rate than the pK of their leaving conjugate acids would predict. Since reactivity expressed in k_{cat}/K_m may depend on factors other than polar ones, it is essential that the range of leaving groups be extended so that we can examine such possibilities in phosphorylation as changes in rate-limiting step or special binding effects. In particular, we are interested in extending the data into the region where the leaving ability of the alkyloxy group as measured by the pK of the alcohol is similar to that for the aryloxy leaving groups.

Experimental Procedures

Materials. Alkyl phosphates were prepared by two general methods. When the alcohol was not available in large quantity or was not suitable as a solvent, the phosphate was prepared by the following technique exemplified by the preparation of 2,2,2-trifluoroethyl phosphate. A solution of 2,2,2-trifluoroethanol (15 g) in pyridine (150 mL) was added slowly to a stirred, cooled, solution of $POCl_3$ (22.5 mL) in pyridine (150 mL). When the addition was complete, the mixture was stirred for about 45 min; it was then filtered and the pale yellow solution stirred into ice (400 g). The aqueous solution was allowed to warm to room temperature and Na_2CO_3 added until effervescence had ceased. The solvent was removed in a rotary vacuum evaporator and the residue extracted with 90% EtOH/water (3 × 250 mL). The extracts were filtered, combined, and stored at 4 °C until recrystallization had occurred.

The following technique was employed for an alcohol suitable as a solvent (Kirby, 1963). Crystalline phosphorous acid (0.82 g) was dissolved in a mixture of the alcohol (20 mL) and triethylamine (5 mL). Iodine (3.8 g) was added, with stirring, over a 5-min period. The mixture was kept for 5 min before pouring it into acetone (300 mL) containing cyclohexylamine (10 mL). The mixture was kept for 2 h, and the precipitate was recrystallized from ethanol containing a few drops of cyclohexylamine.

Aryl phosphates were prepared by the following technique (Williams et al., 1973) exemplified by the preparation of phenyl phosphate. Phenol (0.2 mol) was dissolved in dry pyridine (100 mL) and added slowly with stirring to a cooled solution of $POCl_3$ (0.2 mol) in dry pyridine in a system protected from moisture. After addition was complete, the stirring was continued for 1/2 h and the mixture then poured onto crushed ice (400 g). The pH was adjusted to pH 9 with cyclohexylamine and the resultant precipitate filtered and recrystallized. Yields of recrystallized phosphate ester salts were between 50 and 80% on the basis of either phenol, alcohol, or phosphorous acid in the three methods. 3-Carboxyphenyl phosphate was prepared according to the method of Chanley and Feageson (1955) and had a melting point of 194–195 °C (lit. mp 193.5 °C). 2-Carboxyphenyl phosphate was prepared according to the method of Chanley et al. (1952) and had a melting point of 160–161 °C (lit. mp 162.5–163 °C). Free phenyl phosphate was prepared from the dicyclohexylammonium salt as previously described (Williams & Naylor, 1971b). 1-Naphthyl phosphate and the 2-isomer were purchased as their disodium salts from Sigma Chemical Co.

The phosphate esters were subjected to ^{31}P NMR, ^{1}H NMR, and infrared spectroscopy. The spectra were consistent with the proposed structures, and the NMR spectra showed no evidence of impurities. Elemental analysis for C, H, and N was carried out by A. J. Fassam with a Carlo Erba instrument; data are given in the supplementary table (see paragraph at end of paper regarding supplementary material). Previous experience (Williams et al., 1973; Cramer, 1960; Cramer et al., 1962; Kugel & Halmann, 1967) has shown that C, H, and N analysis is not satisfactory; the analytical chromatogram often exhibits multiple peaks for nitrogen. Equivalent weights of the substrates were estimated by titration with a Radiometer pH titration assembly comprised of REC Servograph, REA Titratigraph, pH-meter PHM 26, Titrator TTT60, and Autoburette ABU 11. The alkyl phosphates were titrated as their sodium salts formed via passage over Zeo-carb-225 Na^+. Inorganic phosphate analysis of the substrates indicated that the total free inorganic phosphate content was less than would give rise to a maximal concentration of 10^{-6} M at the highest substrate concentration.

Other materials were of analytical reagent grade or were recrystallized or redistilled from bench grade products. Water was double-distilled from an all-glass apparatus.

The enzyme was obtained from the Sigma Chemical Co. (type III, P-4252), and several batches had between 50 and 60 units/mg of protein (quoted value 45 units/mg). The enzyme originated from the C90 strain of *E. coli* employed in our previous studies (Williams et al., 1973; Williams & Naylor, 1971b).

Methods. The enzyme batch was assayed initially at 37 °C by adding a 5-μL aliquot of the sample to 3.0 mL of a buffer at pH 10.4 containing glycine (0.1 M), $ZnCl_2$ (1 mM), $MgCl_2$ (1 mM), and 4-nitrophenyl phosphate (disodium salt) (0.275 mM). The absorption change at 400 nm was measured with a Pye-Unicam SP500 series 2 spectrophotometer coupled directly to a BBC Model B 32K microcomputer. The unit of enzyme activity is that which releases 1 μmol of 4-nitrophenol/min at pH 10.4 and 37 °C. Routine assay of the enzyme was carried out at 25 °C and pH 8.00 with tris(hydroxymethyl)aminomethane buffer at 0.05 M and with 0.25 M NaCl and substrate at 0.275 mM.

Protein concentration was measured by the Warburg–Christian (1941) method from the ratio of the absorbance at 280 and 260 nm and the absorbance at 280 nm.

Kinetics. The velocity of the reaction of aryl phosphate substrates in the presence of enzyme was measured spectrophotometrically by a simple variation of the assay procedure. Extinction coefficients for the optimal wavelengths were measured from spectra of the substrates before and after complete hydrolysis catalyzed by the enzyme (Table I).

The hydrolysis of alkyl phosphates was followed by assaying inorganic phosphate release from the absorbance of a blue phosphomolybdate complex. We employ the Lowry–Lopez (1946) modification of the Fiske–Subba-Row (1925) method. The procedure is as follows: an aliquot (1 mL) of the reaction mixture is added to acetic acid (0.1 mL, 2 M) to bring the pH to approximately 4; ascorbic acid (0.05 mL, 10% solution) and ammonium molybdate (0.05 mL, 10% solution) are added, and the solution is made up to 5 mL with acetate buffer at pH 4. The color is allowed to develop for 10 min and the absorbance measured at 660 nm with a Pye-Unicam SP 500 Model 2 spectrophotometer. The assay was calibrated against standard inorganic phosphate solutions; the molar extinction coefficient estimated ($\Delta\epsilon_{660}$ = 4310 M^{-1} cm^{-1}) for the phosphate present in the assay solution agreed with that from earlier work (Williams & Naylor, 1971b). The standard alkyl phosphate solutions were prepared by filtering a solution of the salts (dicyclohexylammonium or sodium) through a Zeo-carb-225 Na^+ column; this is necessary because at the relatively high substrate concentrations required the cyclohexylammonium counterion concentration would be sufficient

Table I: Michaelis–Menten Data for Aryl Monophosphate Ester Hydrolyses[a]

substrate	pK^{ArOH} [h]	mp (°C)[b]	$K_m \times 10^5$ (M)	k_{cat} (s^{-1})[i]	$k_{cat}/K_m \times 10^{-6}$ [g]	N[e]	λ (nm)	Δε (M^{-1} cm^{-1})
phenyl esters								
4-nitro	7.16	173–176	1.31 ± 0.13	86.2 ± 4.3	6.58 ± 0.46	11	400	9500
2-nitro	7.21	162–165	1.51 ± 0.06	81.0 ± 4.3	5.37 ± 0.07	12	406	1540
3-nitro	8.38	174–175	2.59 ± 0.13	80.2 ± 7.7	3.09 ± 0.15	11	242	2010
3,4,5-trichloro	7.55	163–166	1.28 ± 0.12	87.0 ± 4.3	6.80 ± 0.3	9	308	3130
2-carboxy	9.43[f]	160–161[c]	4.78 ± 0.2	87.1 ± 1.7	1.83 ± 0.04	8	295	2810
2-chloro	8.48	189–191	2.60 ± 0.06	80.2 ± 0.9	3.09 ± 0.03	11	238	1680
3-chloro	9.08	182–184	2.86 ± 0.07	82.8 ± 4.3	2.90 ± 0.08	12	280	810
4-chloro	9.38	197–201	3.68 ± 0.28	78.4 ± 0.9	2.13 ± 0.14	8	288	860
4-phenyl	9.51	186–187	13.7 ± 1.4	77.6 ± 0.9	0.566 ± 0.005	9	288	2980
3-carboxy	9.78	194–195[c]	3.96 ± 0.06	78.4 ± 0.9	1.98 ± 0.03	12	295	950
parent	9.99	213–215	3.72 ± 0.08	77.6 ± 4.3	2.09 ± 0.07	9	268	505
3-methyl	10.09	182–184	4.34 ± 0.09	85.3 ± 5.2	1.97 ± 0.08	12	272	655
3,5-dimethyl	10.15	181–184	4.28 ± 0.17	82.8 ± 4.3	1.93 ± 0.03	10	279	800
4-methyl	10.26	196–198	4.32 ± 0.25	80.2 ± 0.9	1.85 ± 0.9	9	276	470
2-methyl	10.28	189–191	7.36 ± 0.12	93.1 ± 0.9	1.25 ± 0.09	12	271	750
2,6-dimethyl	10.59	198–199	1.15 ± 0.11	81.9 ± 0.9	0.712 ± 0.06	9	274	710
1-naphthyl phosphate[d]	9.24		2.89 ± 0.16	70.7 ± 0.9	2.45 ± 0.16	9	320	860
2-naphthyl phosphate[d]	9.24		3.57 ± 0.1	91.4 ± 2.6	2.56 ± 0.09	10	325	770

[a] Tris(hydroxymethyl)aminomethane buffer (pH 8.00) at 0.05 M, 25 °C; ionic strength maintained at 0.25 M with NaCl. See Experimental Procedures for enzyme concentration and substrate concentration ranges. [b] Melting point refers to the dicyclohexylammonium salt except where stated. [c] Free ester. [d] Disodium salt. [e] Number of data points. [f] pK given for the 4-carboxyphenol; there is an intramolecular interaction in the ionization of the phenol of salicylate monoanion not seen in nucleophilic attack at salicylate ester. [g] Units are M^{-1} s^{-1}. [h] Values of pK^{ArOH} taken from the compilation of Jencks and Regenstein (1970). [i] The value of k_{cat} is determined from V_{max} [M s^{-1} (mg of protein)$^{-1}$] assuming a molecular weight of 94 100 for the enzyme (Bradshaw et al., 1981); per active site the value of k_{cat} is half that quoted.

Table II: Michaelis–Menten Data for the Hydrolysis of Alkyl Monophosphate Esters[a,b]

alkyl phosphate	pK^{ROH}	mp (°C)[e]	$K_m \times 10^3$ (M)	k_{cat} (s^{-1})	$k_{cat}/K_m \times 10^{-2}$ (M^{-1} s^{-1})	N[c]
1,1,1,3,3,3-hexafluoroisopropyl[d]	9.3[f]		0.211 ± 0.002	2.87 ± 0.09	136 ± 40	4
2,2,2-trifluoroethyl[d]	12.43[f]		0.698 ± 0.004	1.46 ± 0.04	20.9 ± 0.6	5
3-phenylpropyl[d]	15.99[h]		3.63 ± 0.13	11.6 ± 1.1	32.1 ± 2.5	5
2-phenylethyl[d]	15.52[h]		3.12 ± 0.08	21.0 ± 0.8	67.5 ± 0.8	5
benzyl	14.84[h]	208–210	2.38 ± 0.01	11.0 ± 0.2	46.4 ± 0.5	4
methyl	15.54[f]	169–172	2.56 ± 0.01	1.84 ± 0.06	7.21 ± 0.21	5
ethyl	15.90[f]	180–181	2.34 ± 0.01	1.64 ± 0.05	6.98 ± 0.19	6
n-propyl	16.04[g]	189–191	2.62 ± 0.16	1.59 ± 0.04	6.07 ± 0.21	7
n-butyl	16.07[i]	184–185	2.84 ± 0.11	1.50 ± 0.03	5.28 ± 0.11	6
propargyl[d]	13.55[f]		2.49 ± 0.01	1.35 ± 0.01	19.7 ± 8.6	4
2-chloroethyl[d]	14.31[f]		1.05 ± 0.03	1.28 ± 0.01	12.2 ± 0.3	5
cyclohexylmethyl[d]	16.16[h]		3.06 ± 0.12	3.24 ± 0.01	10.6 ± 0.3	4

[a] Tris(hydroxymethyl)aminomethane buffer (pH 8.00) at 0.05 M, 25 °C; ionic strength maintained at 0.25 M with NaCl. [b] See Experimental Procedures for range of enzyme and substrate concentrations. [c] Number of data points. [d] Isolated as the disodium salt. [e] Melting points quoted for the dicyclohexylammonium salts. [f] Ballinger & Long (1960). [g] Takahashi et al. (1971). [h] Calculated from the Taft equation for substituted methanols (Williams, 1984b). [i] Sauers et al. (1975).

to cause significant inhibition. The concentrations of the alkyl and aryl phosphate in the stock solutions were measured by titration with the Radiometer pH-titration set. Initial rates were measured in both alkyl and aryl phosphate ester hydrolyses over periods of up to 8 min; little curvature was observed.

The values of K_m and V_{max} and the associated mean standard deviations were calculated with a fourth power weighted analysis as suggested by Cornish-Bowden (1979). Substrate concentrations covered a minimum span between $0.2K_m$ and $10K_m$; total enzyme concentration per assay was ca. 7×10^{-10} M for the aryl phosphates and ca. 2×10^{-9} M for the alkyl phosphates.

Results

The hydrolysis of both alkyl and aryl phosphates in the presence of alkaline phosphatase obeyed good Michaelis–Menten kinetics within the ranges of substrate concentration (see Experimental Procedures) employed. The inorganic phosphate vs. time plots for the hydrolysis of alkyl phosphates exhibited curvature, and initial rates were employed in the computations; the curvature is presumably due to the inorganic phosphate released during hydrolysis acting as competitive inhibitor. The kinetic results, where comparable, are in agreement with those of previous work (Williams et al., 1973; Williams & Naylor, 1971b). The data are collected in Tables I and II.

Cyclohexylammonium ion acts as a noncompetitive inhibitor against both 2-carboxyphenyl phosphate (K_i = 4.53 ± 1.15 mM) and phenyl phosphate (K_i = 5.95 ± 0.71 mM) and the results are depicted in Figure 1. The substrates employed were free of any cyclohexylammonium counterions. The presence of the cyclohexylammonium counterion in the aryl phosphate esters should have very little effect at the concentrations of substrate employed. Other ammonium ions have been shown to inhibit the *E. coli* enzyme (van Belle, 1972).

The effect of ionic strength at various pHs (Wilson et al., 1964) was confirmed (supplementary Figure 1). Supplementary Table II collects data for k_{cat} and K_m for the alkaline phosphatase catalyzed hydrolysis of 4-nitrophenyl phosphate in buffers with varying Na/K ratios; there is essentially no effect of replacing K for Na at pH 8.00, so that it is possible to compare results from different laboratories where either K^+ or Na^+ are used as counterions.

The effect of pH on k_{cat} and K_m (supplementary Figure 2) for 2-carboxyphenyl phosphate is similar to that observed for 4-nitrophenyl phosphate (Lazdunski & Lazdunski, 1966; Krishnaswamy & Kenkare, 1970). The values of k_{cat} and K_m

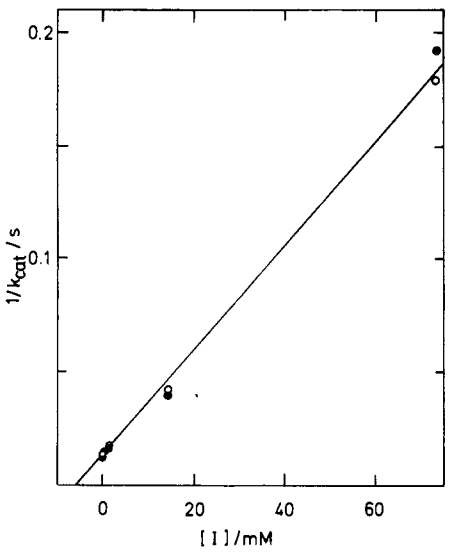


FIGURE 1: Plot illustrating noncompetitive inhibition of alkaline phosphatase from *E. coli* by cyclohexylammonium chloride at 25 °C and pH 8.00, and 0.05 M tris(hydroxymethyl)aminomethane buffer and ionic strength made up to 0.25 M with NaCl. Points are (●) 2-carboxyphenyl phosphate and (○) phenyl phosphate as substrates, and the line is calculated from the parameters given in the text.

are consistent with sigmoidal pH dependencies while k_{cat}/K_m has a bell-shaped pH profile.

Phenol acts as a weak competitive inhibitor to the hydrolysis of 4-nitrophenyl phosphate. The derived value for K_i is (82.6 ± 15.1 mM) for a substrate concentration range of 5–100 μM and phenol concentration from zero through 8.3 to 50 mM at pH 8.00, 0.25 M NaCl, and 0.05 M tris(hydroxymethyl)-aminomethane buffer.

Discussion

E. coli alkaline phosphatase is a mixture of isozymes; Lazdunski and Lazdunski (1967) have shown that the isozymes have identical kinetic properties within experimental error. The isozymes differ by the existence of an N-terminal arginine in one of the otherwise identical single-chain monomers making up the dimeric enzyme. Perusal of the data in the recent disclosure of the X-ray crystallographic structure (Sowadski et al., 1985) indicates that the N-terminal of each of the monomers is not close to the active center. We therefore consider that kinetic results are independent of the composition of the isoenzyme mixture.

The value of k_{cat} for alkyl phosphates is variant and well below the substantially constant value observed for aryl phosphates; under conditions of enzyme saturation the rate-limiting step for the aryl phosphate reaction is dephosphorylation [$k_{cat} = k_3k_4/(k_3 + k_4 + k_{-3})$]. The alkyl phosphate k_{cat} is lower than that for the aryl phosphates and is not a measure of dephosphorylation. The value of k_{cat}/K_m seems to correlate with the pK of the leaving hydroxyl function depending on whether the hydroxyl is a phenol (eq 5) or an alcohol (eq 6) [the Brønsted plot is exhibited in the preliminary communication by Hall and Williams (1985)]. 4-Phenylphenyl

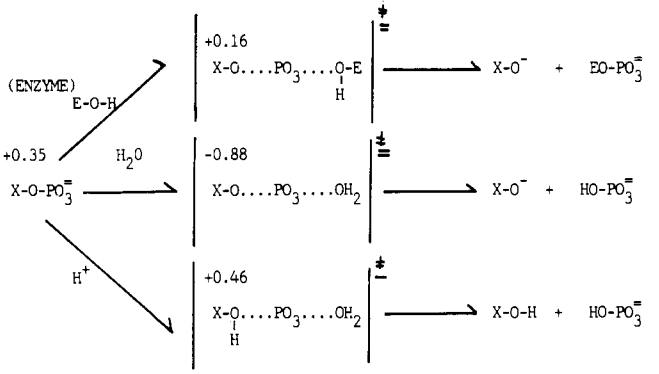
$$\log (k_{cat}/K_m) = -0.188\ (\pm 0.02)\ pK^{ArOH} + 8.14\ (\pm 0.15) \quad (5)$$

$$r = 0.950,\ n = 16$$

$$\log (k_{cat}/K_m) = -0.194\ (\pm 0.01)\ pK^{ROH} + 5.89\ (\pm 0.17) \quad (6)$$

$$r = 0.989,\ n = 8$$

Scheme I: Effective Charge on Oxygen through Some Phosphorylation Reactions



phosphate and 2,6-dimethylphenyl phosphate are omitted from the aryl correlation, and the alkyl phosphates possessing strong lipophilic groups are omitted from the alkyl correlation. The existence of two correlation lines appears to be significant considering the large number of substrates studied. The effect of polar substituents is similar for alkyl as for aryl phosphate esters, and this results from a change in effective charge (Williams, 1984a) of approximately −0.2 compared with a change of −1.35 on the same atom in the calibrating equilibrium (eq 2) from ground to product state. All effective charges are referred to the defined charge change of −1 in the standard ionization equilibrium (eq 3). Spontaneous hydrolysis of the dianion (Scheme I) involves an increase in negative effective charge on the leaving oxygen of about −1.23 (Kirby & Varvoglis, 1967). The hydrolysis of the monoanionic monophosphate occurs through the species protonated on the leaving oxygen (Scheme I), and the change in effective charge from dianionic ground state to the transition state is +0.11 (Kirby & Varvoglis, 1967). It is considered that P–O bond fission is well-advanced in both spontaneous hydrolysis of the dianion and proton-catalyzed hydrolysis. The charge change on the leaving oxygen in the transition state for phosphorylation of the enzyme is close to that for proton-catalyzed hydrolysis of the dianion, and this may be due to electrophilic participation by positively charged groups such as an arginine, hydrogen-bonding of a donor to the oxygen, or metal ion interaction either directly at the ether oxygen or indirectly through the $-PO_3^{2-}$ group. The effect through participation at the $-PO_3^{2-}$ group must be transmitted through the phosphorus atom to the ether oxygen.

Esters with partial aromatic character (benzyl, phenylethyl, and phenylpropyl phosphates) or with large lipophilic groups (cyclohexylmethyl phosphate) occupy an intermediate position on the Brønsted plot (Hall & Williams, 1985) between alkyl and aryl esters. The difference between alkyl and aryl esters in their k_{cat}/K_m values could be due to some sort of lipophilic binding site for the leaving group (the binding interaction would constitute some 2.5 kcal/mol of binding energy). Nonsubstrate moieties such as phenol or cyclohexylammonium ion possess inhibitory effects similar to that constituting the energy difference between the reactivity of alkyl and aryl phosphates; such species do not possess the phosphoryl ($-PO_3^{2-}$) function and therefore do not bind simply at the "phosphoryl" binding site. Positional and steric variation of the substituents on the aryl function indicate that only the most bulky groups reduce the reactivity of the phosphorylation reaction significantly; this suggests considerable freedom of the leaving group consistent with the data obtained for the ground-state structure (Sowadski et al., 1985). Such freedom cannot include the *ether* function which must be in close proximity to the enzyme surface in order to attain the observed electrophilic participation.

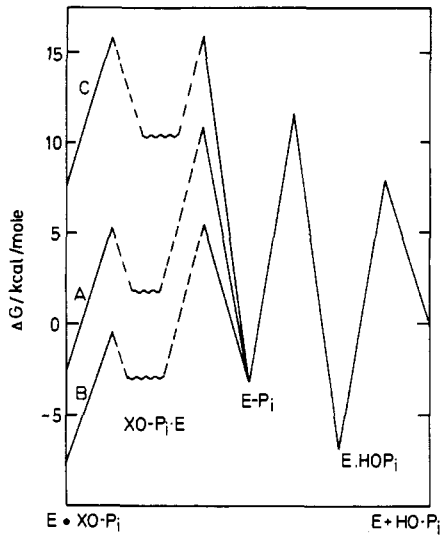


FIGURE 2: Free energy profile for *E. coli* alkaline phosphatase catalyzed hydrolysis of phosphate esters according to the reaction scheme of eq 1. Details of calculations are given in the text. Three profiles are given [formation of EtOH (A), EtO^- (B), and 4-nitrophenoxide ion (C)] for the hydrolysis of ethyl phosphate and 4-nitrophenyl phosphate. The energy of the enzyme–substrate complex is not known and is denoted by a waved line. The energy of E–P_i and the conjugate leaving group (XOH or XO^-) is scaled to the same level.

Equilibrium constants for hydrolysis of phosphate monoesters obey eq 7 (Bourne & Williams, 1984a) where the

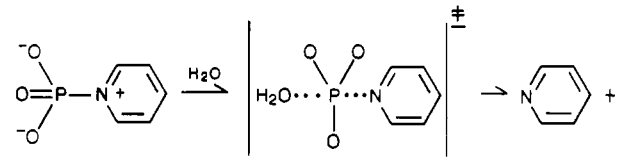
$$\log K = \log ([H^+][HO\text{–}PO_3^{2-}][XO^-]/[XO\text{–}PO_3^{2-}]) = -1.35pK^{XOH} + 7.5 \quad (7)$$

concentration of water is defined as unity. The free energy diagrams are depicted (Figure 2) for hydrolysis of 4-nitrophenyl phosphate and ethyl phosphate for pH 8.00 to give respectively the 4-nitrophenoxide and ethoxide ions as standard-state products and also, in the case of the ethyl ester, the neutral alcohol. Standard states of all the species (except the proton) are taken as 1 M. The free energy profiles have as a common portion the hydrolysis of the phosphoryl-enzyme intermediate, where the energy levels are calculated from published data (Chlebowski et al., 1977; Chlebowski & Coleman, 1972; Fernley & Walker, 1969; Halford et al., 1969). The second-order rate constant for phosphorylation of the enzyme by the free substrate (k_{cat}/K_m) and the overall equilibrium constant enable us to calculate energy levels for the rest of the diagram except that for the enzyme–substrate complex. We assume that the rate constant for association of enzyme with substrate is 10^7 M^{-1} s^{-1} analogous to that for the association of the enzyme with inorganic phosphate (Chlebowski et al., 1977) which is similar to that for other rate constants for complexation between enzymes and substrates (Hammes, 1968). The small value of the polar effect for the aryl phosphates could be due to rate-limiting diffusion of substrate to enzyme; the rate constants (see Table I) are high enough to accommodate this possibility. The observation that alkyl phosphates have a similar low sensitivity to polar substituents but react at about 2 orders of magnitude slower than aryl esters indicates that diffusion is not limiting the rate; the diffusion rate constant chosen above is almost certainly a lower limit.

Catalysis of phosphoryl group transfer could occur through the dissociative pathway where the PO_3 group of atoms becomes almost free (as a putative metaphosphate ion species, PO_3^-) in the transition state. This pathway requires strong activation of the leaving group by electrophilic participation; electrophilic participation at the $-PO_3^{2-}$ group would quench

Scheme II: Dissociative Mechanism Retarded by Electrophilic (E^+) Interaction at the Phosphoryl Group

the internal nucleophilicity of the oxygens which serve to provide electrons to expel the leaving group. In cases where catalysis at the leaving group is already high such as in the hydrolysis of *N*-phosphorylpyridines (Bourne & Williams, 1984b; Skoog & Jencks, 1984), the reactivity is not very high nor is the internal nucleophilicity of the oxygens sufficiently powerful to expel the leaving group without some assistance from the entering nucleophile (eq 8).

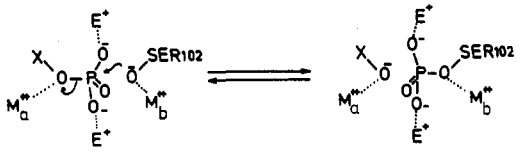


$$HOPO_3^{2-} + H^+ \quad (8)$$

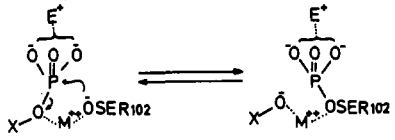
In the mechanism of phosphoryl group transfer (eq 8) the bonding of entering and leaving group atoms with the central phosphorus is very weak in the transition state (Williams & Bourne, 1984b; Skoog & Jencks, 1984), and the process is almost dissociative (that is, a mechanism where leaving group departure precedes entering group attack). An associative mode of catalysis involves converting the dianionic phosphoryl group into a neutral phosphate ester by protonation or some other form of electrophilic interaction with the negatively charged oxygens. This process activates the phosphorus to nucleophilic attack; 4-nitrophenyl dimethyl phosphate is some 10^4-fold more reactive toward imidazole attack than is 4-nitrophenyl phosphate dianion (Williams & Naylor, 1971a; Kirby & Jencks, 1965). Electrophilic interaction with the leaving oxygen will also contribute to catalysis; this catalytic pathway will involve strong bonding between leaving and entering atoms and the central phosphorus in the rate-limiting transition state. Binding of the substrate with the enzyme seems to be mostly at the phosphoryl group, and an electrophilic interaction appears to be the only reasonable mode of attachment; this would favor the associative form of catalysis and retard the dissociative path (Scheme II) by opposing the flow of electrons to the oxygen in the P–O bond undergoing fission.

The enzyme must provide an electrophile to accept the leaving oxygen anion or an acid to donate a proton; the enzyme must also provide a base to accept a proton from serine-102 or an electrophile to stabilize its oxyanion. In an analogous acyl group transfer enzyme, chymotrypsin, the prototropic group (an imidazolyl function), donates a proton to the leaving group and, at a different time, in its basic form accepts a proton from serine-195. Such a conservative use of a single group in two catalytic operations is only possible in the phosphorylation of alkaline phosphatase if displacement at phosphorus occurs with an "adjacent" stereochemistry resulting in a retention of configuration in a single step (Cleland, 1977) at a chiral phosphorus atom. The work of Jones et al. (1978) indicates that phosphoryl group transfer catalyzed by *E. coli* alkaline phosphatase involves retention of configuration at the phosphorus atom. The simplest explanations of this result are (a) phosphoryl-enzyme formation and decomposition *each* with inversion involving "in-line" attack and expulsion and (b) two *retention* displacements with "adjacent" attack and expulsion. *Both* mechanistic types have good precedent (Buchwald et al.,

Scheme III: Inversion Mechanism of Enzyme Phosphorylation with Metal Ion Stabilization of the Hydroxyl Anion



Scheme IV: Retention Mechanism of Enzyme Phosphorylation with Metal Ion Stabilization of the Hydroxyl Ion



1984a,b). In-line displacement requires two separate "acid–base" pairs to provide nucleophile and electrophile (Cleland, 1977). Scheme III illustrates the simplest path for the double-inversion mechanism where the oxyanions are stabilized by metal ions M_a^{2+} and M_b^{2+}. The double-inversion scheme requires the electrophile donating to the leaving group to be different from that involved with serine-102; the double-retention mechanism (Scheme IV) could involve the same metal ion with the leaving oxygen atom and with the serine.

We have depicted metal ions acting as electrophiles effectively reducing the pK's of alcohol leaving groups, serine-102 hydroxyl or water. The action of the metal to activate an hydroxyl function as a nucleophile has considerable precedent (Wagner et al., 1974; Jones et al., 1984), and its function is not to increase the reactivity of the oxyanion (indeed this must be reduced from that of the free anion) but to enable the oxyanion to exist at a pH far below that corresponding to the normal pK of the hydroxyl function.

The results of this paper indicating strong electrophilic activation of the leaving oxygen are consistent with either of the proposed general mechanisms. Proton removal from complexed hydroxyl functions will be at a diffusion-controlled rate because the pK's of these species are generally low; catalysis of proton transfer is not necessary (Eigen, 1964), and no special general base is required.

Any more detailed analysis of the mechanistic function of the various groups other than serine-102 will have to await the results of more detailed study of the topology of the active site although it seems clear that arginine-166 is acting as an electrophilic species either at the ether link or to help bind the $-PO_3^{2-}$ group. The acidic and basic limbs in the pH dependencies appear to involve a histidine residue and another group with p$K \sim 8$ (Krishnaswamy & Kenkare, 1970) in the phosphorylation step (k_{cat}/K_m). Inhibition constants for phosphonate species have bell-shaped pH dependencies (Lazdunski & Lazdunski, 1967) similar to those for k_{cat}/K_m; this indicates a possible involvement of the prototropic groups in K_s rather than in k_2, and further studies will have to be undertaken to confirm this.

Supplementary Material Available

Two tables giving analytical and physical data of aryl and alkyl monophosphate esters and effect of alkali metal cation on Michaelis–Menten parameters and two figures giving the dependence on ionic strength of k_{cat}/K_m for phosphorylation and the dependence on pH of the Michaelis–Menten parameters for alkaline phosphatase (6 pages). Ordering information is given on any current masthead page.

Registry No. *p*-$O_2NC_6H_4OPO_3H_2$, 330-13-2; *o*-$O_2NC_6H_4OPO_3H_2$, 6064-84-2; *m*-$O_2NC_6H_4OPO_3H_2$, 13388-91-5; 3,4,5-$Cl_3C_6H_2OPO_3H_2$, 88766-68-1; *o*-$HO_2CC_6H_4OPO_3H_2$, 6064-83-1; *o*-$ClC_6H_4OPO_3H_2$, 13428-19-8; *m*-$ClC_6H_4OPO_3H_2$, 77368-40-2; *p*-$ClC_6H_4OPO_3H_2$, 13388-88-0; *p*-$PhC_6H_4OPO_3H_2$, 46817-52-1; *m*-$HO_2CC_6H_4OPO_3H_2$, 77368-39-9; $PhOPO_3H_2$, 701-64-4; *m*-$MeC_6H_4OPO_3H_2$, 22987-28-6; 3,5-$Me_2C_6H_3OPO_3H_2$, 65696-02-8; *p*-$MeC_6H_4OPO_3H_2$, 6729-45-9; *o*-$MeC_6H_4OPO_3H_2$, 18351-85-4; 2,6-$Me_2C_6H_4OPO_3H_2$, 13388-86-8; $(CF_3)_2CHOPO_3H_2$, 101686-67-3; $CF_3CH_2OPO_3H_2$, 2805-15-4; $Ph(CH_2)_3OPO_3H_2$, 86977-01-7; $Ph(CH_2)_2OPO_3H_2$, 18110-43-5; $PhCH_2OPO_3H_2$, 1623-07-0; $MeOPO_3H_2$, 512-56-1; $EtOPO_3H_2$, 1623-14-9; $PrOPO_3H_2$, 1623-06-9; $BuOPO_3H_2$, 1623-15-0; $HC{\equiv}C\text{-}CH_2OPO_3H_2$, 55343-62-9; $Cl(CH_2)_2OPO_3H_2$, 4167-12-8; cyclohexylmethyl phosphate, 33026-79-8; 1-naphthyl phosphate, 1136-89-6; 2-naphthyl phosphate, 13095-41-5; cyclohexylammonium ion, 29384-28-9; phenol, 108-95-2; alkaline phosphatase, 9001-78-9.

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Mechanism of β-Aryl Ether Dimeric Lignin Model Compound Oxidation by Lignin Peroxidase of *Phanerochaete chrysosporium*†

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Received March 3, 1986; Revised Manuscript Received April 10, 1986

ABSTRACT: Homogeneous lignin peroxidase (LiP) oxidized 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxy-4-propylphenoxy)-1,3-dihydroxypropane, I, and 1-(4-ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)-2-methoxyphenoxy]-1,3-dihydroxypropane, III, to yield four products from each substrate. The isolated products indicate that both the α,β and β-ether bonds were cleaved in the dimeric substrates or oxidized derivatives. LiP oxidized 1-(4-ethoxy-3-methoxyphenyl)-2-[4-(hydroxymethyl)phenoxy]-1,3-dihydroxypropane, II, to yield only two products, suggesting that the α,β bond of the substrate was cleaved. LiP oxidized 1-phenyl-2-(2-methoxy-4-propylphenoxy)-1,3-dihydroxypropane, IV, to yield products that indicated that the β-ether bond was cleaved but the α,β bond remained intact. Cleavage of I in $H_2{}^{18}O$ resulted in 42% incorporation of ^{18}O at the 1-position of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane, X. Oxidation of the 1-[^{18}O]hydroxy-labeled dimer IIIa resulted in only 50% retention of the ^{18}O at the 1-position of the phenylglycerol X. Oxidation of the β-^{18}O-ether dimer resulted in 99% retention of the ^{18}O in the phenylglycerol product but only 85% of the ^{18}O in the phenolic counterpart product. Finally, when the 3-[^{18}O]hydroxy-labeled β-ether dimer IIIb was used as a substrate, the phenolic product XV retained 26% of the ^{18}O, suggesting that a rearrangement had taken place. These results are explained by mechanisms involving oxidation of either the phenyl (A) and/or the phenoxy (B) ring of the dimer to an aryl cation radical by the H_2O_2-oxidized enzyme. Oxidation of the A ring of I, II, or III to an aryl cation radical is followed by α,β cleavage, giving rise to a benzaldehyde and a C_6C_2 benzylic radical. The latter would be scavenged by O_2 to yield a glycolaldehyde and a phenol. The phenol would retain the ^{18}O label orginally in the β-ether oxygen of the substrate. Oxidation of the B ring in dimers I, III, or IV apparently is followed by intramolecular attack by the 1-hydroxyl on the methoxylated aryl cation radical carbon, releasing methanol and forming a benzodioxane radical transition state. This intermediate is hydrolyzed to yield a phenylglycerol and a catechol. Alternatively, oxidation of the B ring is followed by intramolecular attack of the 3-hydroxyl on the ether-linked B ring carbon, leading to a γ-aryl ether dimer. Radical cleavages of this intermediate yield a phenol and a benzaldehyde.

Lignin is a complex, optically inactive, and random phenylpropanoid polymer that comprises 20–30% of woody plants (Sarkanen, 1971; Crawford, 1981). Under nitrogen limitation, the white rot basidiomycete *Phanerochaete chrysosporium* efficiently degrades lignin during the secondary metabolic phase of growth (Kirk et al., 1978; Weinstein et al., 1980; Gold et al., 1982). Under ligninolytic conditions, *P. chrysosporium* produces at least two heme peroxidases (Gold et al., 1984; Kuwahara et al., 1984; Tien & Kirk, 1984; Glenn & Gold, 1985), one of which, lignin peroxidase (ligninase, diarylpropane oxygenase), has been purified to homogeneity (Gold et al.,

† This work was supported by Grant DMB 8311441 from the National Science Foundation and by the Crown Zellerbach Co.

0006-2960/86/0425-4790$01.50/0