Dependence of the Phosphorylation of Alkaline Phosphatase by Phosphate Monoesters on the pK_a of the Leaving Group†

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ABSTRACT: The hydrolysis and transphosphorylation reactions of a series of phosphate monoesters, $ROPO_3^{2-}$ (R = 2,4-dinitrophenyl, 4-nitrophenyl, phenyl, glucose-1, glycerol-1, methyl, ethyl, and dodecyl), catalyzed by *Escherichia coli* alkaline phosphatase and a mutant enzyme, Ser102Cys, have been studied at alkaline pH using the rates of change in the ^{31}P NMR signals of substrate, the hydrolysis product (inorganic phosphate), and the transphosphorylation product (*O*-Tris phosphate) as the assay. The k_{cat} at pH 8.0 for the wild-type enzyme is ~30 s^{-1} and is independent of the nature of the R group, when the pK_a of the leaving group is <10. Under these conditions the rate of phosphorylation is much faster than dissociation of inorganic phosphate, 15−60 s^{-1}. If the pK_a of the leaving group is between 10 and 15, phosphorylation and dissociation of the product phosphate both contribute to the rate limit. If the pK_a of the leaving group is >15, phosphorylation is rate limiting. A Bronsted plot of log k_{cat} vs pK_a of the leaving group for those substrates for which phosphorylation is rate limiting yields a β_{lg} of ~−0.6. In contrast to the wild-type enzyme, the log k_{cat} values for the S102C mutant enzyme catalyzing the hydrolysis of phosphate esters are linearly dependent on the pK_a's of the leaving group throughout the range of pK_a from 4 to 16. Phosphorylation of C102 is the rate controlling step, and k_{cat} is independent of the Tris concentration as predicted for rate limiting phosphorylation. The Bronsted constant, β_{lg}, is ~−0.3. The catalytic rate for the S102C mutant is at least 50-fold slower than that for the wild-type enzyme. The dependence of both k_{cat} and the β_{lg} value on the nature of the nucleophile suggests that phosphorylation of the enzyme is primarily associative in character.

Escherichia coli alkaline phosphatase is a zinc enzyme which catalyzes the hydrolysis of phosphate monoesters (Reid & Wilson, 1971; Coleman & Chlebowski, 1979; McComb *et al.*, 1979; Coleman, 1992). It is a dimer of identical subunits (MW = 47 000) which bind two Zn ions and one Mg ion at each active center (Plocke *et al.*, 1962; Anderson *et al.*, 1975). The crystal structure of the enzyme shows Zn1 and Zn2 to be 3.9 Å apart and to be bridged by phosphate in the noncovalent enzyme−phosphate complex, E·P,[1] while Zn2 is bridged to Mg3 by the carboxyl group of Asp51 (Kim & Wyckoff, 1991). The catalytic mechanism involves the formation of a phosphoseryl intermediate, E-P, formed by attack of Ser102 on the phosphoryl group of the substrate (Schwartz & Lipmann, 1961; Engstrom, 1961; Engstrom & Agren, 1962; Schwartz *et al.*, 1963; Bradshaw *et al.*, 1981). Either water or another alcohol, $R'O^-$, can be the acceptor for the phosphoryl group from E-P in the second step to give the hydrolysis product, $HOPO_3^{2-}$, or the transphosphorylation product, $R'OPO_3^{2-}$. The reaction pathways for both hydrolysis and transphosphorylation are summarized in Scheme 1.

In Scheme 1, E·ROP, E·P, and E·R′OP are noncovalent Michaelis complexes formed by the enzyme with the substrate and the products, respectively. At acid pH, the rate determining step in the mechanism is the dephosphorylation of the seryl phosphate intermediate, accounting for the bursts observed in rapid-flow kinetics (Halford *et al.*, 1969; Reid & Wilson, 1971; Chlebowski & Coleman, 1974; Coleman, 1992). At alkaline pH the rate limiting step has been concluded to be the dissociation of the product phosphate for all rapidly hydrolyzed phosphate esters, since the phosphate dissociation rate has been shown to be 15−60 s^{-1} by ^{31}P NMR methods (Hull *et al.*, 1976; Gettins *et al.*, 1985), a number similar to k_{cat} values.

Because of the availability of convenient spectrophotometric assays for the release of RO^-, a large number of aryl phosphate monoesters have been tested as substrates for alkaline phosphatase. The pK_a's of the leaving groups have ranged between 4 and 10, and all have been hydrolyzed at approximately the same rate (reviewed in Reid & Wilson, 1971; Hall & Williams, 1986). A series of N-phosphorylated pyridines with leaving group pK_a's from 6 to 10 have been shown to be hydrolyzed by alkaline phosphatase at approximately the same rate (Labow *et al.*, 1993).

There have been fewer data collected on the hydrolysis of alkyl phosphates by alkaline phosphatase, most of which have leaving group pK_a's from 10 to 16. The majority of the data have been collected by detecting the release of phosphate by forming the phosphomolybdate complexes employed in the standard colorimetric detection of inorganic phosphate. Rates reported in the literature for the hydrolysis

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[1] Abbreviations: E-P, the phosphoryl enzyme formed by the phosphorylation of Ser102; E·P, the noncovalent complex of enzyme with inorganic phosphate; P_i, inorganic phosphate. ROP and R′OP refer to phosphate monoesters, while E·ROP refers to the noncovalent Michaelis complex formed between a phosphate monoester and the enzyme.

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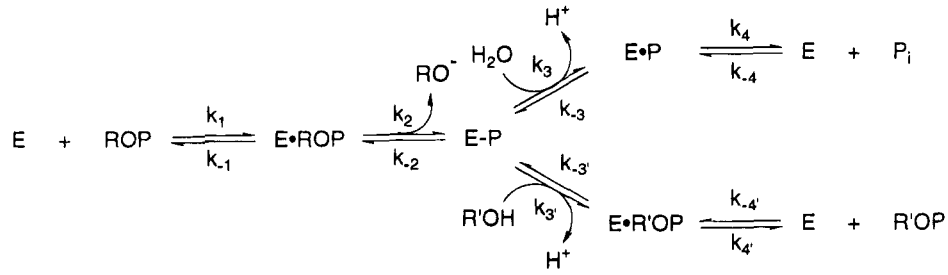
Scheme 1



of the alkyl phosphates show much less agreement. Early data reported ribose 5-phosphate, glucose 1-phosphate, glucose 6-phosphate, and fructose 1,6-bisphosphate to be hydolyzed at 0.7, 0.6−0.9, 0.9, and 0.6 the rate of *p*-nitrophenyl phosphate (Torriani, 1960; Heppel *et al.*, 1962). Williams and Naylor (1971) reported methyl, ethyl, *n*-butyl, and isopropyl phosphates to be hydrolyzed at 0.65, 0.46, 0.5, and 0.03 the rate of 4-nitrophenyl phosphate, while *tert*-butyl phosphate was not detectably hydrolyzed. However, much slower rates for methyl, ethyl, and *n*-butyl phosphates, 0.02 the rate for 4-nitrophenyl phosphate for all three esters, were published by Hall and Williams (1986), while a variety of other alkyl phosphates were hydrolyzed from 0.02 to 0.3 the rate observed for 4-nitrophenyl phosphate. The very slow rates reported for some alkyl phosphates have been attributed to steric interactions of the side chain with the active center (Williams & Naylor, 1971) or to the existence of a lipophilic site on the enzyme that binds aryl groups better (Hall & Williams, 1986).

Much of this variability may relate to the difficulties in the colorimetric assays for phosphate. In order to develop an assay that could be applied to all aryl and alkyl phosphates and measure simultaneously the rates of disappearance of substrate and the appearance of the hydrolysis and transphosphorylation products, we developed an assay employing the ^{31}P NMR signals of the substrates and products. In this paper we report the results of these accurate assays on a variety of aryl and alkyl phosphates with leaving group pK_a's from 4 to 16. We also report the results using a S102C mutant of *E. coli* alkaline phosphatase in which the phosphorylation step has been postulated to be at least partially rate controlling for *p*-nitrophenyl phosphate (Ghosh *et al.*, 1986).

MATERIALS AND METHODS

Sodium salts of 4-nitrophenyl, phenyl, and glycerol-1 phosphates were from Sigma. The sodium salt of glucose 1-phosphate was from Aldrich. Methyl and ethyl phosphates were prepared by the treatment of the dichlorophosphates with sodium hydroxide (Chlebowski & Coleman, 1974). 2,4-Dinitrophenyl phosphate was prepared according to the reported method (Azerad *et al.*, 1963). All the prepared phosphate monoesters were characterized by ^{1}H and ^{31}P NMR spectroscopy. Phosphate monoester samples in which significant phosphate was detected prior to the assay were discarded.

Wild-Type and S102C Mutant Alkaline Phosphatases. The wild-type alkaline phosphatase was isolated from an overproducing strain of *E. coli*, CW3747, and purified as previously described (Applebury *et al.*, 1970). The gene for the S102C mutant of alkaline phosphatase was carried on the plasmid pT4391, a gift of Professor Emil T. Kaiser (deceased). The plasmid was transformed into the host *E. coli* strain K12 SM547, lacking the chromosomal gene for alkaline phosphatase, kindly supplied by Professor Evan R. Kantrowitz. The original *E. coli* strain, AW1043, containing a partially deleted AP gene and used initially to produce the mutant, grew very poorly on the phosphate-poor media required to induce enzyme, and the induction was weak in our hands. In contrast, *E. coli* strain SM547 induced large amounts of mutant enzyme with moderate phosphate limitation, which was easily isolated in homogeneous form. The mutant enzyme was purified by the same method employed for the wild-type enzyme. The concentrations of both the wild-type and mutant alkaline phosphatases were determined spectrophotometrically at 278 nm using $E_{0.1\%} = 0.72$ (Malamy & Horecker, 1964) using a molecular weight for the dimer of 94 000, calculated from the amino acid sequence (Bradshaw *et al.*, 1981).

AP Activities. The hydrolysis of phosphate monoesters catalyzed by both the wild-type and the mutant alkaline phosphatases was carried out in Tris buffer containing 10% D_2O at pH 8, 20 °C. All the reactions were monitored by proton-decoupled ^{31}P NMR spectroscopy. The initial concentrations of phosphate monoesters were determined relative to a standard solution of inorganic phosphate by ^{31}P NMR spectroscopy integrating the ^{31}P NMR resonances. All the initial concentrations of the phosphate monoesters were about 30 mM. The enzyme is saturated at this substrate concentration; thus the measured velocities are k_{cat} values. The concentrations of substrate, $ROPO_3^{2-}$, hydrolysis products, $HOPO_3^{2-}$, and transphosphorylation product, Tris-OPO_3^{2-}, were calculated from the relative areas of their resonances and the total concentrations of substrate and products. As required, the sum of the concentrations of substrate and products, calculated from the NMR signals, remains equal to the initial concentration of phosphate monoester throughout the assays. The concentrations of E·ROP, E·P, E·R′OP, and E-P are much lower than that of substrate, since enzyme concentrations ranged from 8.8×10^{-7} to 7.1×10^{-6} M. The k_{cat} values are expressed per active site, i.e., per monomer.

Assays of alkaline phosphatase, even rapid-flow assays, are subject to product inhibition as the reaction proceeds. The ^{31}P NMR-based assay employed here is no exception, although the use of 30 mM substrate allows collection of signals before the [P_i]:[substrate] ratio becomes high enough to result in significant inhibition. In confirmation of this assumption, the k_{cat} values determined for the aryl phosphates are 30−31 s^{-1}, in agreement with standard spectrophotometric assays of these substrates. One can calculate the rate in the absence of phosphate inhibition by correcting for the

Table 1: Hydrolysis of Phosphate Monoesters Catalyzed by the Wild-Type Alkaline Phosphate

$ROPO_3^{2-}$	pK_a (ROH)[a]	k_{cat} (s^{-1})	$R_{t/h}$
2,4-dinitrophenyl phosphate	4.09	30	1.1
4-nitrophenyl phosphate	7.15	30	0.9
phenyl phosphate	10.00	30	1.0
glucose 1-phosphate	12.46[b]	22	1.0
glycerol 1-phosphate	14.15[c]	16	1.1
methyl phosphate	15.5	5.7	0.8
ethyl phosphate	15.9	7.0×10^{-1}	0.7
dodecyl phosphate	16.2[d]	4.3×10^{-1}	0.2

[a] Streitwieser & Heathcock, 1976. [b] Izatt *et al.*, 1966. [c] Lide, 1990–1991. [d] Estimated from the Taft equation; see Perrin et al., 1981.

exponential component of the decay in the amplitude of the substrate signal (see Figure 1A).

^{31}P NMR. All ^{31}P NMR spectra were recorded on a GE Omega 300 spectrometer at 121.4 MHz. The gated decoupling mode was used in order to remove the nuclear Overhauser effect. A 45° pulse and a repetition time of 5 s were used. The chemical shifts are reported relative to 1% phosphoric acid.

RESULTS

Hydrolysis and Transphosphorylation Reactions of Phosphate Monoesters Catalyzed by the Wild-Type Alkaline Phosphatase. The hydrolysis and transphosphorylation reactions of 2,4-dinitrophenyl, 4-nitrophenyl, phenyl, glucose-1, glycerol-1, methyl, ethyl, and dodecyl phosphates catalyzed by the wild-type alkaline phosphatase were measured in 1 M Tris at pH 8 and 30 mM substrate, 20 °C, standard conditions for AP assays. The k_{cat} values of the phosphate esters were measured from the initial rate of disappearance of the ^{31}P NMR signal for the esters. The k_{cat} values and the pK_a's of the leaving groups are listed in Table 1 in the order of increasing pK_a values for the leaving groups. Under these conditions, the k_{cat} is the sum of the rates for the hydrolysis and transphosphorylation reactions which do not proceed at the same rate (see Discussion). The ratios of the rates for the two reactions, $R_{t/h} = k_{transphosphorylation}/k_{hydrolysis}$, are given for each substrate in Table 1. The individual rate constants were determined by the initial buildup of the ^{31}P NMR signals for *O*-Tris phosphate and inorganic phosphate.

The $^{31}P\{^1H\}$ NMR spectra during the hydrolysis and transphosphorylation reactions of 4-nitrophenyl phosphate are shown in Figure 1A for wild-type *E. coli* alkaline phosphatase. Each sampling of the reaction mixture consisted of 32 transients requiring 3.5 min of signal averaging. Therefore, each peak in Figure 1 represents the signal amplitude at the midpoint of each 3.5 min interval. The substrate, 4-nitrophenyl phosphate, signal is at −0.6 ppm, that of *O*-Tris phosphate at 4.1 ppm, and that of inorganic phosphate at 2.6 ppm under the assay conditions in 1 M Tris at pH 8. While the ^{31}P NMR assay described here has the great advantage of being applicable to all phosphate esters hydrolyzed by alkaline phosphatase, ^{31}P NMR is an inherently insensitive detection method and both signal collection times and signal:noise become unsatisfactory at the lower end of the concentration ranges needed for satisfactory determination of K_m. Hence, the substrate concentrations were set well above the range where a dependence of velocity on substrate concentration was observed, i.e., 30 mM.

Table 2: Hydrolysis of Phosphate Monoesters Catalyzed by the Wild-Type Alkaline Phosphatase at Different Concentrations of Tris

$ROPO_3^{2-}$	Tris (M)	k_{cat} (s^{-1})	$R_{t/h}$
4-nitrophenyl phosphate	0.1	14	0.2
	0.5	26	0.6
	1.0	30	0.9
	1.4	36	1.0
glycerol 1-phosphate	0.1	9.2	0.2
	0.5	15	0.7
	1.0	16	1.1
	1.4	17	1.4
methyl phosphate	0.1	5.3	0.04
	0.5	5.8	0.2
	1.0	5.7	0.8
	1.4	5.6	0.8

If the assay shown in Figure 1A is continued long enough, the hydrolysis of the transphosphorylation product by the enzyme becomes significant, e.g., the last four spectra in Figure 1A. An equilibrium between the hydrolysis product, phosphate, and the *O*-Tris phosphate is ultimately achieved, yielding a [phosphate]:[*O*-Tris phosphate] ratio of ~10. If wild-type alkaline phosphatase is added to a solution of inorganic phosphate in 1 M Tris at pH 8, 20 °C, the phosphorylation of Tris via the formation of E-P by inorganic phosphate can be followed by ^{31}P NMR spectroscopy. Equilibrium is reached after several days with a final [inorganic phosphate]:[*O*-Tris phosphate] ratio of ~10 in 1 M Tris at pH 8, the same as observed when starting with an ester substrate only. This ratio is a function of the concentration of Tris. The log k_{cat} values for the hydrolysis plus transphosphorylation reactions of 2,4-dinitrophenyl, 4-nitrophenyl, phenyl, glucose-1, glycerol-1, methyl, ethyl, and dodecyl phosphates, catalyzed by the wild-type alkaline phosphatase, are plotted vs the pK_a's of the leaving groups in Figure 2. For substrates with leaving groups having a pK_a <10, the log k_{cat} does not vary with pK_a, but k_{cat} begins to fall when the pK_a of the leaving group is above 12. The nonlinear part of the curve represents the region where the enzyme reaction is changing from rate limiting dissociation of product to rate limiting phosphorylation.

In the case of rapidly hydrolyzed phosphate esters, the addition of Tris to the reaction mixture enhances the activity, since the rate of formation of the transphosphorylation product, *O*-Tris phosphate, is additive to that for hydrolysis as illustrated in Table 2 by data for 4-nitrophenyl phosphate. This can only occur if the rate limiting step occurs after the phosphorylation step (Scheme 1). Thus, a loss of the ability of Tris to increase k_{cat} for a given phosphate ester must accompany a shift to rate limiting phosphorylation. The observed increase in k_{cat} accompanying transphosphorylation not only requires that the rate limiting step occur after phosphorylation of the enzyme but also requires that the dissociation of the transphosphorylation product, *O*-Tris phosphate, is much more rapid than the dissociation of the hydrolysis product, inorganic phosphate.[2]

[2] Why the dissociation of $ROPO_3^{2-}$ is more rapid than that of inorganic phosphate if the latter is bound as the dianion is unclear. One possibility is that coordination of phosphate to both Zn ions as well as hydrogen bonding of the other two oxygens to the guanidino group of Arg166 induces the formation of the trianion, not possible with the phosphate ester. The trianion would be expected to dissociate very slowly, perhaps requiring protonation first.

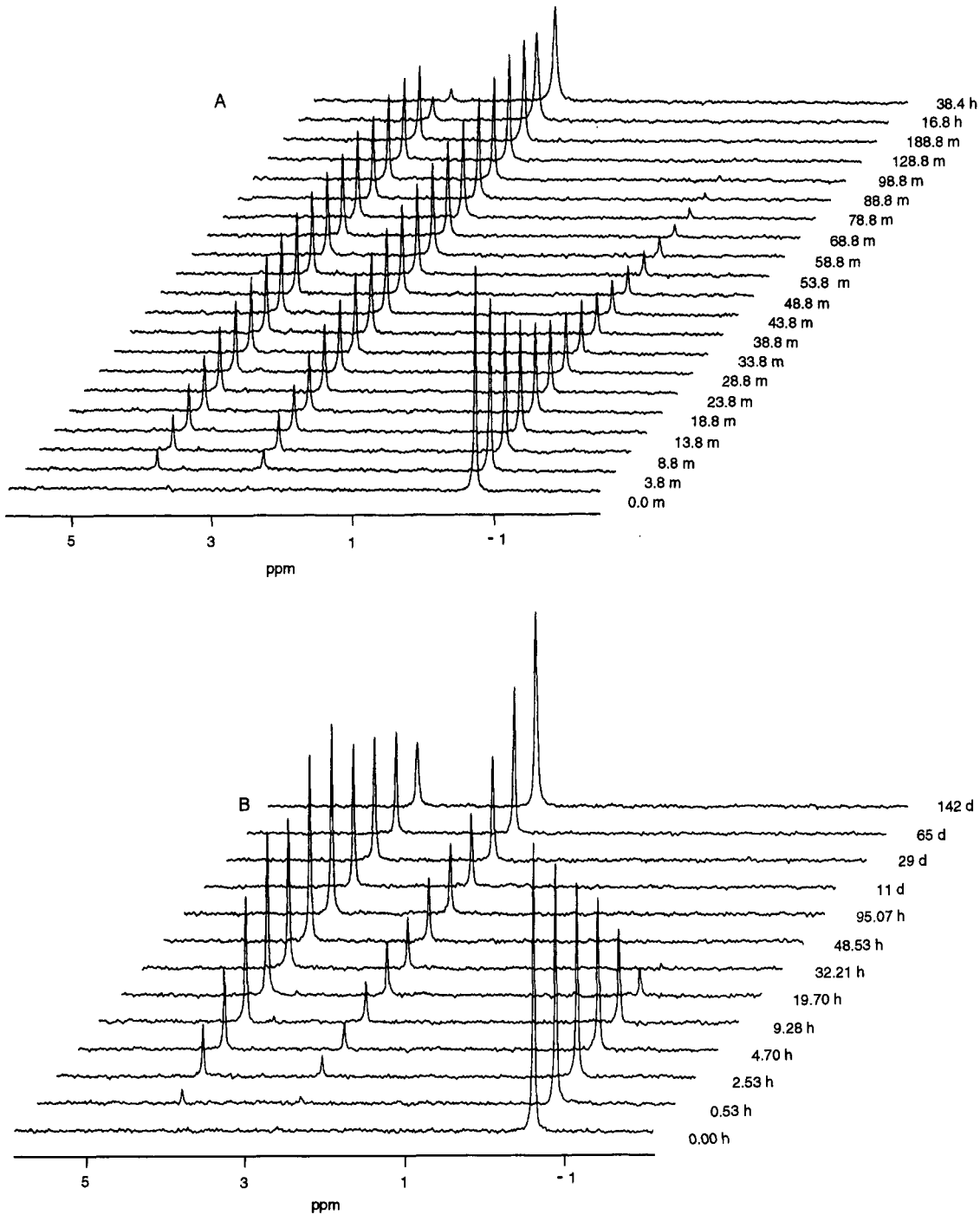


FIGURE 1: $^{31}P\{^{1}H\}$ NMR spectra following the hydrolysis and transphosphorylation reactions of 4-nitrophenyl phosphate catalyzed by the wild-type *E. coli* alkaline phosphatase in 1 M Tris, pH 8, 20 °C (A), and a S102C mutant enzyme in 0.9 M Tris, pH 8, 20 °C (B). The resonances of 4-nitrophenyl phosphate, inorganic phosphate, and *O*-Tris phosphate are at −0.6, 2.6, and 4.1 ppm, respectively. In the figure, m, h, and d represent minutes, hours and days.

The ratio, $R_{t/h}$, shown in Table 1, expresses the *O*-Tris phosphate:P_i ratio observed at the time of the initial assay point. Since *O*-Tris phosphate is a substrate for the enyzme, these numbers are not the maximum initial transphosphorylation:hydrolysis ratios. They are close to such numbers for the rapidly hydrolyzed aryl phosphates, since the Tris hydroxyl groups have $pK_a > 10$ and the poor leaving group renders *O*-Tris phosphate a relatively poor substrate. However, in the case of poor substrates like methyl, ethyl, and dodecyl phosphates, *O*-Tris phosphate is a good substrate on a relative scale, and significant hydrolysis of the transphosphorylation product takes place by the time the data for the initial assay point have been collected. The above conclusion is confirmed by the progressive fall in $R_{t/h}$ as the substrates become poorer (Table 1). The competitive hydrolysis of *O*-Tris phosphate in the presence of the slowly hydrolyzed substrates potentially introduces small errors in the k_{cat} values, but this error is small (see k_{cat} vs [Tris], Tables 2 and 4).

A similar fall in the initial $R_{t/h}$ ratios is observed for the S102C mutant enzyme (see Table 3 below). Limited concentration dependence data suggest that the S102C mutation results in changes in K_m. A change in relative K_m values could account for the observation that hydrolysis of the *O*-Tris phosphate product takes place earlier in the course of the reaction for all substrates hydrolyzed by the mutant

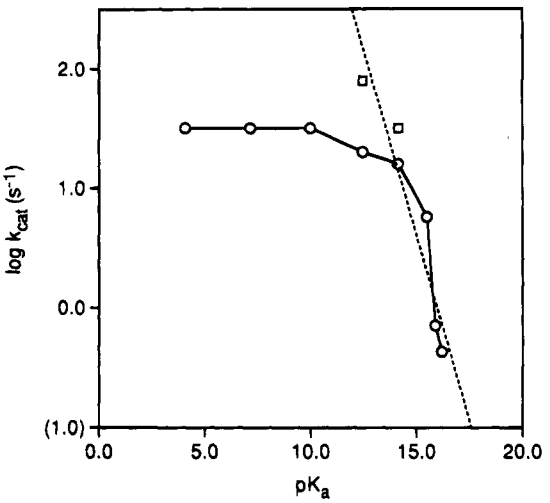


FIGURE 2: Plot of log k_{cat} (s^{-1}) (○) for the hydrolysis of phosphate monoesters catalyzed by *E. coli* alkaline phosphatase vs the pK_a of the leaving group. The open squares (□) are the calculated phosphorylation rates for glucose 1-phosphate and glycerol 1-phosphate. The slope of the dashed line is −0.6.

enzyme. If the $R_{t/h}$ ratios for both the wild-type and mutant enzymes are corrected for the hydrolysis of the *O*-Tris phosphate product, the $R_{t/h}$ ratio should be similar for all substrates as the mechanism predicts (Scheme 1). The maximum ratio, $R_{t/h}$, however, may be different for the wild-type and mutant enyzmes.

As the pK_a of the leaving group is increased, the k_{cat} (wild-type enzyme) for methyl phosphate is the first one which shows no increase on the addition of Tris, even though transphosphorylation continues to take place (Table 2). Thus, k_{cat} for methyl phosphate reflects the rate of phosphorylation of the enzyme. On the other hand, glycerol 1-phosphate, which demonstrates an intermediate k_{cat}, 16 s^{-1}, shows an attenuated enhancement by Tris (Table 2), compatible with the postulate that both phosphorylation and dissociation of product contribute to the rate limit. The rates of phosphorylation of the enzyme by glucose 1-phosphate and glycerol 1-phosphate required to lower k_{cat} from 30 s^{-1} to 22 s^{-1} and 16 s^{-1}, respectively, are 82 s^{-1} and 34 s^{-1}, based on the reasonable assumption that the rates for steps after the formation of E-P are the same for all substrates. These calculated phosphorylation rates are represented by the squares in Figure 2 which are incorporated into the dashed line drawn through the log k_{cat} values for the three substrates for which the phosphorylation step is completely rate limiting. These logarithms of the phosphorylation rates vs the pK_a's of the leaving groups (12.5−16.2) can be considered to represent a Bronsted plot for the first step of the mechanism for the wild-type enzyme, namely, the phosphorylation of Ser102. The β_{lg} value is ∼−0.6.

Hydrolysis and Transphosphorylation Reactions of Phosphate Monoesters Catalyzed by the S102C Mutant of Alkaline Phosphatase. The hydrolysis and transphosphorylation reactions of 2,4-dinitrophenyl, 4-nitrophenyl, phenyl, glycerol-1, methyl, and ethyl phosphates catalyzed by the S102C mutant alkaline phosphatase were studied under similar conditions to those for the wild-type enzyme, 0.9 M Tris at pH 8, 20 °C, and 30 mM substrate. The ^{31}P NMR assay of the S102C mutant enzyme using 4-nitrophenyl phosphate is shown in Figure 1B. The k_{cat} values and the ratios, $R_{t/h}$, of $k_{transphosphorylation}/k_{hydrolysis}$ are given in Table 3. The plot of

Table 3: Hydrolysis of Phosphate Monoesters Catalyzed by the Mutant Alkaline Phosphatase (Ser102Cys)

$ROPO_3^{2-}$	pK_a (ROH)	k_{cat} (s^{-1})	$R_{t/h}$
2,4-dinitrophenyl phosphate	4.09	5.0×10^{-1}	2.1
4-nitrophenyl phosphate	7.15	1.6×10^{-1}	1.3
phenyl phosphate	10.00	6.9×10^{-3}	1.2
glycerol 1-phosphate	14.15	3.9×10^{-3}	0.8
methyl phosphate	15.5	6.8×10^{-4}	0.6
ethyl phosphate	15.9	2.0×10^{-4}	0.5

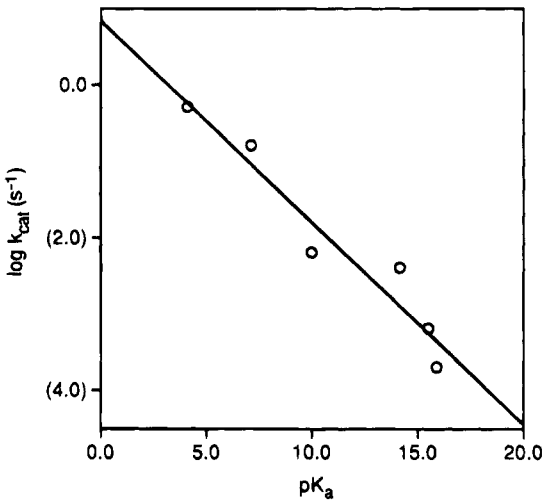


FIGURE 3: Plot of log k_{cat} (s^{-1}) (○) for the hydrolysis of phosphate monoesters catalyzed by the S102C mutant *E. coli* alkaline phosphatase vs the pK_a of the leaving group. The slope of the line is −0.3.

Table 4: Hydrolysis of Phosphate Monoesters Catalyzed by the Mutant Alkaline Phosphatase (Ser102Cys) at Different Concentrations of Tris

$ROPO_3^{2-}$	Tris (M)	k_{cat} (s^{-1})	$R_{t/h}$
2,4-dinitrophenyl phosphate	0.04	5.1×10^{-1}	0.0
	0.2	5.7×10^{-1}	1.1
	0.4	4.7×10^{-1}	1.9
	0.9	5.0×10^{-1}	2.1
4-nitrophenyl phosphate	0.1	1.6×10^{-1}	0.3
	0.4	1.7×10^{-1}	1.3
	0.9	1.6×10^{-1}	1.3
	1.3	1.5×10^{-1}	1.8
methyl phosphate	0.1	8.1×10^{-4}	0.1
	0.4	6.8×10^{-4}	0.4
	0.9	6.8×10^{-4}	0.6
	1.3	5.9×10^{-4}	1.5

log k_{cat} vs the pK_a of the leaving group is shown in Figure 3. The k_{cat} values for the mutant enzyme show no dependence on Tris concentration for any of the substrates, although the transphosphorylation reaction takes place efficiently, as indicated by the $R_{t/h}$ ratios (Table 4). Thus, as the linear relationship between log k_{cat} and the pK_a of the leaving group implies, phosphorylation must be rate limiting for the hydrolysis of all the above substrates by the S102C mutant. The slope of the line in Figure 3, ∼−0.3, can be interpreted as the Bronsted β_{lg} value for the phosphorylation of Cys102.

DISCUSSION

The mechanism of hydrolysis of phosphate monoesters by alkaline phosphatase has been shown to be a nucleophilic one involving the formation of a covalent phosphoseryl

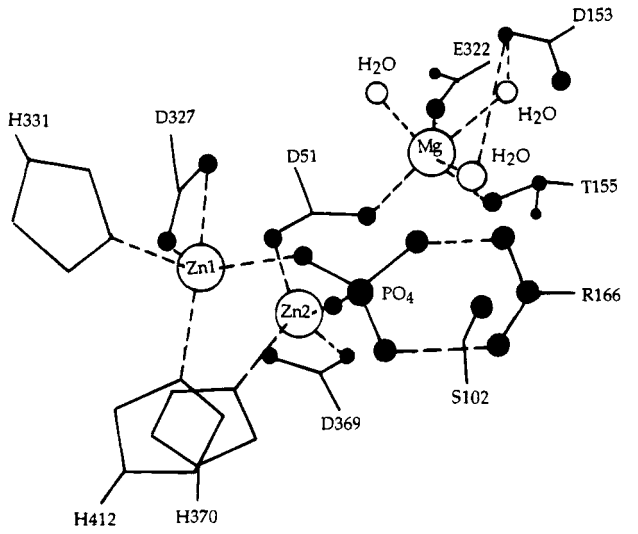


FIGURE 4: Structure around the active center of the complex of *E. coli* alkaline phosphatase with inorganic phosphate, E·P. Drawn from the coordinates of the crystal structure of the phosphate complex of the zinc−magnesium enzyme (Kim & Wyckoff, 1991).

intermediate with Ser102 (Engstrom, 1961; Schwartz et al., 1963; Reid & Wilson, 1971). The central role of the phosphoseryl intermediate has been supported by studies with $^{16}O,^{17}O,^{18}O$-labeled substrates which show that the alkaline phosphatase reaction proceeds with retention of configuration around phosphorus as expected from two sequential in line nucleophilic reactions, the first by Ser102 on the phosphate ester, the second by solvent water on the phosphoseryl intermediate (Jones *et al.*, 1978). The crystal structure of the native Zn enzyme with P_i bound shows the phosphate to form a bridging ligand between the two Zn ions, Zn1 and Zn2, located 3.9 Å apart in each active center of the dimer (Kim & Wyckoff, 1991). The other two phosphate oxygens are hydrogen bonded to the guanidino group of Arg166. The configuration of the active center of the phosphate complex of the native zinc−magnesium enzyme is illustrated in Figure 4. Inorganic phosphate in the presence of the Cd derivative of alkaline phosphatase will slowly phosphorylate the enzyme (Gettins & Coleman, 1983). The high resolution crystal structure of the phosphorylated Cd enzyme shows the phosphoseryl(102) to be in the position within the active center expected if seryl(102) were the initial nucleophile (Kim & Wyckoff, 1991).

From the above crystal structures, it has been inferred that, in the noncovalent Michaelis complex with a phosphate monoester, the same ligand bridge is formed with Zn1 coordinating the ester oxygen of the substrate, the interactions with Zn2 and Arg166 being similar to those observed for the complex with inorganic phosphate (Coleman, 1992). This model would place the phosphorus of the substrate in an ideal position to be attacked by the OH of Ser102. Zn2 is close enough to the Ser102 OH to activate the seryl hydroxyl and to coordinate the ester oxygen of the phosphoseryl intermediate (Figure 4). These structural features would significantly aid both phosphorylation and dephosphorylation of the enzyme.

The k_{cat} values for the hydrolysis of aryl phosphates by *E. coli* alkaline phosphatase reported in the literature range from 13 s^{-1} to 45 s^{-1} (per active site) in 1 M Tris, the latter acting as an acceptor of the phosphoryl group in addition to solvent water. Transfer of the phosphoryl group from a common covalent intermediate, E-P, would explain the small dependence of k_{cat} for alkaline phosphatase on the pK_a of the leaving group if dephosphorylation of E-P were rate limiting at alkaline pH. However, when ^{31}P NMR relaxation transfer and inversion transfer methods were used to show that the dissociation rates of inorganic phosphate from the enzyme−product complex, E·P, were 15−60 s^{-1} (Hull *et al.*, 1976; Gettins *et al.*, 1985), i.e., similar to k_{cat} values, the inference was made that dissociation of phosphate was likely the rate limiting step at alkaline pH. When it was realized that most preparations of *E. coli* alkaline phosphatase contain bound phosphate, phosphate-free enzyme was prepared, and the bursts of RO^- at alkaline pH, expected if dissociation of phosphate were rate limiting, were observed (Bloch & Schlessinger, 1973). These bursts occur within the dead time of stopped-flow instruments, ~3 ms; thus, the rate of both the phosphorylation and dephosphorylation of Ser102 must be at least 300 s^{-1} at alkaline pH.

The pattern of hydrolysis rates for alkyl phosphates with leaving group pK_a's from 10 to 16 by alkaline phosphatase is much less clear than for aryl phosphates. In the absence of convenient spectrophotometric assays of RO^- release, most of the hydrolysis rates have been measured by the colorimetric analysis of the inorganic phosphate released. Reported rates for the hydrolysis of individual alkyl phosphates have shown large variation; e.g., rates for the hydrolysis of methyl and ethyl phosphates have varied from 0.5 (Williams & Naylor, 1971) to 0.02 the rate of 4-nitrophenyl phosphate (Hall & Williams, 1986). A group of 10 different alkyl phosphates with leaving group pK_a's from 12 to 16 have been reported to be hydrolyzed at about the same rate, 1.5−2.0 s^{-1} (per dimer), compared to 86 s^{-1} for 4-nitrophenyl phosphate (Hall & Williams, 1986). Despite the slow rates, little dependence on pK_a of the leaving group was apparent from these data.

There are several advantages to the use of the ^{31}P NMR signals of the substrate and products for alkaline phosphatase assays. (1) The assay can be applied equally well to aryl and alkyl phosphates, allowing comparisons with the same assay. (2) The substrate concentration and those of the transphosphorylation and hydrolysis products can be followed sequentially on the same reaction mixture. (3) High accuracy and appropriate blank reactions can be obtained even for very slowly hydrolyzed substrates. These features are illustrated by the two assays pictured in Figure 1. The data obtained for phosphate monesters with leaving group pK_a's from 4 to 16 show that as the pK_a becomes >10, k_{cat} for alkaline phosphatase slows in a systematic way with increasing pK_a of the leaving group (Table 1). If the postulate is made that this slowing is due to the slowing of the phosphorylation step to the point where it contributes to the rate limit, determination of the ability of the transphosphorylation reaction to enhance k_{cat} will be a measure of how much $k_{phosphorylation}$ contributes to the rate limit. Only when the pK_a of the leaving group reaches 15.5 (methyl phosphate) has the Tris enhancement of k_{cat} competely disappeared, suggesting that phosphorylation has become exclusively rate limiting (Table 2).

The transphosphorylation reaction still accounts for a similar percentage of the reaction products as in the case of rapidly hydrolyzed substrates, but the sum of the rates of hydrolysis and transphosphorylation remains constant (Table 2). For substrates like glucose 1-phosphate and glycerol

l-phosphate, whose leaving groups have pK_a's between 12 and 14, it is apparent that both the phosphorylation step and the dissociation of the phosphate product must jointly contribute to the rate limit. The k_{cat}'s have slowed, but some enhancement of k_{cat} by the presence of the transphosphorylation reaction is still present (Table 2).

The plot of log k_{cat} vs the pK_a of the substrate leaving group for the wild-type enzyme shown in Figure 2 can be interpreted as a Bronsted plot only for the values of k_{cat} below 5 s^{-1}, i.e., where $k_{phosphorylation}$ is rate limiting. This limits the slope to a relatively small range of pK_a of the leaving group, but we chose substrates to cover as many short intervals of pK_a as possible from 14 to 16. The rates determined by the NMR assay readily distinguish these substrates such that the log k_{cat}'s are inversely proportional to the pK_a's of the leaving groups (Figure 2). The hydrolysis rates of methyl and ethyl phosphates are intermediate in this group, as the pK_a's of the methanol and ethanol, 15.5 and 15.9, predict; however, the NMR assay clearly detects the difference between the rates of hydrolysis of these two esters. The k_{cat} for ethyl phosphate, 0.7 s^{-1} (per active site), agrees with the rate reported in Hall and Williams (1986), 1.64 s^{-1} (per dimer), but not with an earlier much higher figure of 0.46 the rate of hydrolysis observed for 4-nitrophenyl phosphate (Williams & Naylor, 1977). The rate for methyl phosphate of 5.7 s^{-1} (per active site) is considerably faster than that reported by Hall and Williams (1986), 1.84 s^{-1} (per dimer), but the faster rate is in line with the lower pK_a of its leaving group (Figure 1), a relative rate difference also shown by the mutant enzyme hydrolyzing these two alkyl phosphates (Figure 3).

One can extend the Bronsted plot for the native enzyme by calculating the phosphorylation rates for glucose 1-phosphate and glycerol 1-phosphate, 82 and 34 s^{-1}, respectively, as illustrated in the Results. Thus, the rates for phosphorylation of the enzyme by five substrates whose leaving groups span a pK_a range from 12.5 to 16.2 can be used to construct a Bronsted plot with a β_{lg} value of -0.6 (dashed line, Figure 2). This β_{lg} value is somewhat smaller than the β_{lg} value, ~ -1.0, observed for uncatalyzed nucleophilic hydrolysis of the dianions of phosphate monesters and also observed for the nonenzymatic hydrolysis of phosphate triesters, the latter with no possibility of the dissociative metaphosphate mechanism (Khan & Kirby, 1970). The smaller dependence of the log k_{cat} on the pK_a of the leaving group for the enzyme-catalyzed reaction could be due to the coordination of the oxygen of the leaving group to Zn1.

The S102C mutant of alkaline phosphatase was first described by Ghosh *et al.* (1986) who reported k_{cat} for the hydrolysis of 2,4-dinitrophenyl phosphate by the mutant enzyme as 15 s^{-1} compared to a value of 18 s^{-1} for the wild-type enzyme. While a k_{cat} of 15 s^{-1} implies that dissociation of product is still rate limiting, a k_{cat} of 4.7 s^{-1} was reported for 4-nitrophenyl phosphate and k_{cat} was unaffected by the addition of Tris. Thus the rate of hydrolysis of 4-nitrophenyl phosphate by the S102C mutant was suggested by Ghosh *et al.* (1986) to be controlled by the phosphorylation step. We find the homogeneous S102C mutant, isolated from cells which overproduce the enzyme in large quantities, to have a much lower activity than reported in the earlier studies. We cannot account for this difference. The DNA sequence of the mutant gene is the same as reported earlier, and the transfer into the new AP^- host which enabled the isolation of large quantities of the mutant enzyme should not have affected activity. In contrast, we find that the Cys SH in the context of the alkaline phosphatase active center is such a poor nucleophile compared to the Ser OH that phosphorylation is rate limiting for all substrates with leaving group pK_a's between 4 and 16, as suggested by the linear relationship between log k_{cat} and pK_a throughout this range (Figure 3). That phosphorylation is the rate limiting step even for the most rapidly hydrolyzed substrate, 2,4-dinitrophenyl phosphate, is shown by the lack of effect of Tris on k_{cat} for this substrate as well as the others (Table 4). The Bronsted β_{lg} from the plot of log k_{cat} vs pK_a of the leaving group for the mutant enzyme is -0.3, substantially less than for the wild-type enzyme, -0.6.

The structural relationships between bound phosphate and Zn1 at the active center (Figure 4) suggest that Zn1 is in a position to coordinate the ester oxygen of the bound ROP substrate, thus activating the leaving group and perhaps imparting some dissociative character to the phosphorylation of alkaline phosphatase. However, one would predict that a change in the character of the nucleophile would not have dramatic effects on either β_{lg} or k_{cat} if the mechanism were dissociative. Since the nature of the nucleophile appears to be a major factor in determining the value of β_{lg} as well as k_{cat} (Tables 1 and 3), an associative mechanism is more likely.

It has been suggested that the mechanism of alkaline phosphatase is associative because *O*-phosphorothioate monoesters are hydrolyzed by alkaline phosphatase at least 10^2-fold slower than phosphate monoesters (Breslow & Katz, 1968; Mushak & Coleman, 1972). The ratio of k_{cat}'s is similar to the k_S/k_O observed for the nonenzymatic hydrolysis of phosphate triesters and the inverse of that observed for the hydrolysis of the dianions of phosphate monoesters, the latter hydrolyzed by a dissociative metaphosphate mechanism. However, the hydrolysis of *O*-phosphorothioate monoesters by the wild-type enzyme, despite the slow rate, is enhanced at least 5-fold by 1 M Tris (Mushak & Coleman, 1972); hence, phosphorylation of the enzyme cannot be rate limiting. While k_{cat} does not identify which enzymatic step is most slowed by the phosphorothioate, rapid-flow kinetic data for phosphorothioate hydrolysis show that phosphorylation of Ser102 and the dephosphorylation of seryl *O*-phosphorothioate are both very slow (Chlebowski & Coleman, 1974). The dephosphorylation is so slow that this step is in fact rate limiting at alkaline pH, a finding which would explain the Tris effect. Thus, associative mechanisms for both the phosphorylation and dephosphorylation steps catalyzed by alkaline phosphatase are supported by the data on hydrolysis of *O*-phosphorothioate monoesters.

The possibility cannot be excluded that the S102C change could have altered other features of this complex active center some distance from residue 102, which might in turn alter the mechanism in some other manner. A crystal structure of the S102C mutant will be required to determine the limits of any change in structure. In the absence of phosphate, the crystal structure of the zinc enzyme shows the oxygen of Ser102 to be ligated to Zn2. In contrast, the structure of the E•P complex of the zinc enzyme shows a phosphate oxygen to be a ligand to Zn2, while the oxygen of Ser102 is disordered (Kim & Wyckoff, 1991). Based on the surrounding electron density, the Ser OH must occupy a position approximately as indicated in Figure 4 and may retain some transient interaction with Zn2 during some stages of phos-

phate ester hydrolysis. Zn2 could form a 5-coordinate complex with the nucleophile, but the crowding of the large sulfur atom in such a coordination sphere might actually weaken the interaction with $-S^-$ compared to $-O^-$. While differential interactions with the metal ion may alter the character of a sulfur vs an oxygen nucleophile at the active center of alkaline phosphatase, such changes would be more likely to be reflected in k_{cat} or β_{lg} if the mechanism were associative rather than dissociative, the latter involving the creation of a strong electrophile on departure of RO^-.

One of the predictions of the data in Figure 2 is that phosphorylation of S102 in the wild-type enzyme by rapidly hydrolyzed oxyphosphate esters must be very fast. Rapid-flow kinetic studies with the 4-nitrophenyl phosphate at pH <6 can be used to estimate the rate of phosphorylation, k_2, since k_2 is equal to the pre-steady-state burst rate in the absence of phosphate. However, if all phosphate is removed from the enzyme, the burst occurs in the dead time of the instrument, 3 ms. The presence of P_i slows the burst rate because P_i competes with ROP for the formation of E·ROP; thus, estimates for the true value of k_2 can be derived from determinations of burst rates at several [P_i] followed by extrapolations to zero [P_i]. In the presence of a rapidly hydrolyzed phosphate ester, inorganic phosphate does not phosphorylate the enzyme significantly because the phosphorylation rate constant for P_i is so slow, $\sim 0.2\ s^{-1}$ (Applebury *et al.*, 1968). The value of k_2 determined in this manner for 4-nitrophenyl phosphate at pH <6 is at least $10^3\ s^{-1}$ (Chlebowski and Coleman, unpublished), tending to confirm the projection of a very rapid phosphorylation reaction made from the data in Figure 2.

These studies show that only when the pK_a of the leaving group is >15 does phosphorylation of the alkaline phosphatase become rate limiting and k_{cat} show a dependence on the pK_a of the leaving group. Despite the fact that Zn1 is in a position to stabilize the leaving group, i.e., to activate the dissociation of the leaving alcoxide or phenoxide, phosphorylation of the wild-type enzyme appears to be largely associative in character, since the nature of the activated nucleophile is a major factor affecting the phosphorylation rate. The activation of the nucleophile and the leaving group by two different Zn ions greatly accelerates the rates of phosphorylation and dephosphorylation of alkaline phosphatase. The rate of phosphorylation is $>10^3\ s^{-1}$ for substrates with leaving group pK_a's ~4, compared to uncatalyzed rates of $\sim 10^{-5}\ s^{-1}$ for similar model nucleophilic reactions. The zinc-containing active center environment is much more effective in activating a seryl nucleophile than a cysteinyl nucleophile, and phosphorylation of Cys102 is rate limiting for the hydrolysis of all phosphate esters by the S102C mutant enzyme.

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