Phosphoramidic Acids. A New Class of Nonspecific Substrates for Alkaline Phosphatase from *Escherichia coli*†

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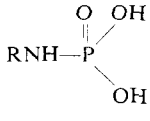
ABSTRACT: Phosphoramidic acid derivatives have been shown to be substrates of alkaline phosphatase from *Escherichia coli*. In addition the broad specificity of the enzyme toward this class of compounds has been established. Thus, it is demonstrated that for monoamidophosphates, $RNHPO_3H_2$, that variations in the structure of the side chain from R = alkyl to aryl to acyl result in only minor differences in their enzymatic hydrolysis rates. The V_{max} for this class of compounds is only about 1.7- to 3.0-fold slower than V_{max} for *p*-nitrophenyl phosphate at pH 8.0, 0.02 M Tris buffer, 0.5 M in KCl. This difference in maximum rates increased in the presence of high Tris concentrations. The K_m's for the amidophosphates are substantially higher than that observed for *p*-nitrophenyl phosphate. Whereas evidence is presented which implicates the same active site as being involved in the hydrolysis of both ester and amide substrates, differences in the effect of Tris and deuterium oxide on the enzymatic hydrolysis rates of the two classes of compounds point to different rate-influencing steps.

Previous kinetic investigations with alkaline phosphatase from *Escherichia coli* have shown that this enzyme hydrolyzes a wide range of monophosphate esters and anhydrides at nearly the same rate (Garen and Levinthal, 1960; Heppel *et al.*, 1962). Using rapid-kinetic techniques evidence favoring a rate-limiting transformation from an "inactive" to an "active" enzyme conformation which preceded any bond making between the enzyme and substrate has been presented (Halford *et al.*, 1969; Reid and Wilson, 1971). Such a scheme adequately accounts for the broad specificity, the effect of acceptors such as Tris on the rate of utilization of substrate, and various other kinetic features of this enzyme (Wilson *et al.*, 1964).

In contrast to the large number of mechanistic investigations employing monophosphate esters as substrates, no definitive studies employing the corresponding phosphoramidic acids have been reported. Indeed there is evidence in the literature to suggest that amidophosphates are not substrates for alkaline phosphatase from *E. coli*, (Lazdunski and Lazdunski, 1969; Dixon and Webb, 1964) even though preparations of intestinal and prostate alkaline phosphatase utilize phosphoramidic acids in both hydrolytic (Winnick, 1946) and transphosphorylation reactions (Meyerhof and Green, 1950; Morton, 1953). There are several notable examples of naturally occurring phosphoramidic acids and a study of the action of *E. coli* alkaline phosphatase on this class of compounds could have biological as well as mechanistic significance. Of particular interest to us is the specificity of alkaline phosphatase toward these substances. In the present study we demonstrate that *E. coli* alkaline phosphatase catalyzes the hydrolysis of P–N bonds in compounds having the following general structure



where R may be an alkyl, aryl, or acyl group.

Experimental Section

Melting points are uncorrected. pH measurements were made on a Radiometer TTTlc automatic titrator equipped with an expanded scale. Ultraviolet spectra were obtained with a Cary 17 recording spectrophotometer, and infrared spectra with a Perkin-Elmer Model 337.

Materials. Water was twice distilled. All reagents, acids, and salts were of reagent grade (Baker, Fisher). Tris was reagent grade Trizma base from Sigma. Deuterium oxide was obtained from Mallinckrodt, minimum isotopic purity 99.8%. Acetic acid-*d*, employed as the acid component of Tris buffers in D_2O was a product of Stohler.

Enzyme Preparation. The enzyme was prepared from *E. coli* by the osmotic shock technique of Neu and Heppel (1964, 1965) and purified by chromatography on DEAE-cellulose using a sodium chloride gradient (Simpson *et al.*, 1968). Enzyme prepared in this manner had a specific activity comparable to the crystalline preparation of Malamy and Horecker (1964). The final preparations were stored in 0.01 M Tris at pH 8.0 at 4°. Enzyme sample stored in this way suffered no detectable loss in activity over a period of 6 months.

Enzyme Assay. The enzyme concentration was determined by assaying the activity with *p*-nitrophenyl phosphate under the conditions described by Malamy and Horecker (1964). Pure enzyme was ascribed a specific activity 3200 μmoles (min^{-1} mg^{-1}).

Commercial Substrates. Disodium *p*-nitrophenyl phosphate hexahydrate (Aldrich) and *N*-(phosphonoamidino)-sarcosine hexahydrated disodium salt (Pfaltz and Bauer) were found to be relatively free of contamination by inorganic orthophosphate and were used without further purification. Commercial samples of *N*-(*p*-chlorophenyl)phosphoramidic acid (*p*-chloroanilidophosphonate) (Pfaltz and Bauer, and K & K Laboratories) were found to contain large amounts of inorganic phosphate (~10 and 30%, respectively) and, therefore, this compound was prepared by us.

Synthetic Substrates. *N*-(α- or β-naphthyl)phosphoramidic

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acids were prepared by hydrolysis of the corresponding dichlorides and isolated as the free acids as described below. *N*-(Phenyl) and *N*-(*p*-chlorophenyl)phosphoramidic acids were also prepared by basic hydrolysis of their respective dichlorides using the same procedures as described for the naphthyl derivatives but were isolated and purified as the anilinium salts. There has been some question in the literature as to the exact composition of phosphoramidic acids derived from different methods of preparation (Chanley and Feageson, 1958) and, therefore, the anilinium salt of phosphoramidic acid was also prepared by hydrogenation of dibenzylanilinophosphonate, mp 90–91 (hexane–cyclohexane). These two compounds had identical uv and ir spectra, and were hydrolyzed at the same rate in the presence of alkaline phosphatase.

The method outlined below for the preparation of the *N*-(α- and β-naphthyl)phosphoramidic acids was identical with that used for the preparation of the phenyl analogs. However, the initial precipitate (probably a mixture of the free acid and corresponding monoanilinium salt) after washing with cold dilute hydrochloric acid, followed by a small amount of cold water, was extracted with 200 ml of 95% ethanol. The mixture was then filtered and 3.0 ml of aniline was added to the filtrate to precipitate the anilinium salt. This product was purified by recrystallization from boiling methanol. The yields were generally low 5–10%.

N-(Benzoyl)phosphoramidic acid was prepared by the method of Titherley and Worrall (1909) and was purified by recrystallization from methanol–benzene, mp 146–147° (lit. mp 146–147°) (Zioudrou, 1961).

N-(*n*-Butyl)phosphoramidic acid was prepared from *O,O*-diphenyl *N*-(*n*-butyl)phosphoramidate, isolated as the monopotassium salt, and twice recrystallized from methanol–acetone as described by Benkovic and Sampson (1971).

Preparation of N-(α- and β-Naphthyl)phosphoramidic Acid. In a typical experiment 15.0 g (0.058 mole) of α-naphthylamido dichlorophosphate, prepared by the method outlined by Kropacheva and Parshina (1959), was added in small portions to a rapidly stirred solution of 10% sodium hydroxide at room temperature. Most of the starting material dissolved within a short time, however, in the case of the naphthyl derivatives a small amount of sticky residue deposited along the walls of the flask. After three hours, the stirring was discontinued and the reaction mixture was filtered to remove any insoluble material. The flask containing the filtrate was then placed in a crushed ice–salt water bath and cooled to 0°. Concentrated hydrochloric acid was then added in small portions to the rapidly stirred solution while maintaining the temperature at 0–3°, until precipitation finally ensued. The precipitate was washed twice with cold dilute hydrochloric acid and redissolved in 5% sodium hydroxide. The solution was again filtered, cooled to 0°, and the pH slowly adjusted to approximately 5. The precipitate was collected by suction filtration and washed twice successively with cold dilute hydrochloric acid, water, ethanol, and ether. Finally the product was extracted with boiling acetonitrile. The yield was 4.3 g (33%).

N-(α-Naphthyl)phosphoramidic acid, mp ~230 dec. *Anal.* Calcd for $C_{10}H_{10}NO_3P \cdot H_2O$: C, 49.79; H, 5.03; N, 5.81; P, 12.85. Found: C, 50.46; H, 4.03; N, 5.72; P, 12.99.

N-(β-Naphthylphosphoramidic) acid, mp 249–252 dec. *Anal.* Calcd for $C_{10}H_{10}NO_3P$: C, 53.81; H, 4.53; N, 6.72; P, 13.89. Found: C, 53.92; H, 4.45; N, 6.68; P, 12.84.

Anilinium salt of *N*-(phenyl)phosphoramidic acid, mp 260–267 dec, from anilinophosphodichoridate. *Anal.* Calcd for $C_{12}H_{15}N_2O_3P$: C, 54.10; H, 5.68; N, 10.52; P, 11.64. Found: C, 53.44, H, 5.88; N, 11.08; P, 11.96.

Anilinium salt of *N*-(phenyl)phosphoramidic acid, mp 260–263 dec, from dibenzylanilinophosphonate. *Anal.* Calcd for $C_{12}H_{15}N_2O_3P$: C, 54.10; H, 5.68; N, 10.52; P, 11.64. Found: C, 53.92; H, 6.23; N, 10.41; P, 12.13.

Anilinium salt of *N*-(*p*-chlorophenyl)phosphoramidic acid, mp 282–288 dec. *Anal.* Calcd for $C_{12}H_{14}ClN_2O_3P$: C, 47.90; H, 4.70; N, 9.32; P, 10.31. Found: C, 48.40; H, 4.78; N, 9.45; P, 10.64.

Kinetic Runs. Whenever possible initial rates were measured by following the release of inorganic phosphate (method A) and by monitoring the loss of substrate as reflected by the decrease in optical density at a suitable wavelength (method B). The sensitivity of both of these methods depended on measuring approximately 2×10^{-5} to 4×10^{-5} M product, or roughly 2–5% reaction. Normally, employing data from 2 to 5% hydrolysis would present no difficulty in the calculation of initial rates. But in our case the K_m values of the substrates are much higher than the K_I of phosphate. Therefore, even at these relatively low extents of reaction severe product inhibition by inorganic phosphate occurred which tended to undervalue initial rates by 15–124%, depending on the particular substrate and conditions. Since the Michaelis constants for all the substrates and inorganic phosphate were determined at pH 8.0, it was possible to correct the apparent velocities as described in the Results section. All others are presented as uncorrected values.

METHOD A. The production of inorganic phosphate was measured by a modification of the method described by Chanley and Feageson (1958). The reagents employed herein were prepared exactly as described by these authors. In a typical run 60 μl of stock substrate (6×10^{-2} to 3.0×10^{-1} M) made up in 5% excess of sodium hydroxide was added to 9 ml of buffer. The reaction was initiated by the addition of 15 μl of stock enzyme (0.61 mg/ml). A 3-ml aliquot of the reaction mixture was then quickly pipetted into 9 ml of ice-chilled acetate buffer (pH 4.04), thus quenching the reaction. Timing was begun as the first drop of the substrate solution was introduced into the quenching buffer. Addition of the reaction mixture was followed immediately by 3 ml each of ice-cold ascorbic acid and molybdate reagent. The same procedure was repeated after incubation of the substrate with the enzyme for exactly 5 min. The molybdate solutions containing the substrate, enzyme, and inorganic phosphate were allowed to develop at ice temperature for 20 min. the optical density of the solution contained in a 5-cm cuvet was then read at 660 mμ. Using the ΔOD for 5 min it was possible to calculate an initial rate. Under our conditions the molar extinction coefficient change ($\Delta\epsilon$) was 690 ± 20 M^{-1} cm^{-1}. Good agreement was obtained from this value and that determined by complete hydrolysis of the various substrates employed in this study (Table I). In addition, by dissolving the substrates directly in buffer and measuring the color intensities at $t = 0$ after development times of 20 and 40 min, it was possible to determine the mole percentages of inorganic phosphate contaminating most of the various compounds employed in this study (Table I). Both *N*-(*n*-butyl)phosphoramidic acid and phosphoramidic acid were too labile in the molybdate reagent to determine the amount of phosphate contamination. Phosphoramidic acid itself was too labile to perform rate measurements by the procedure outlined above, however, by carefully standardizing development times and the time required for reading the optical density at 660 it was possible to obtain initial rates

TABLE I: Physical Data for Alkaline Phosphatase Substrates.

Substrate	Mole % Free P_I	Mole % P_I[a] at Infinity	λ followed	Δε (cal) = ε (sub.) − ε (prod)	Δε (obsd)
N-(Phenyl)phosphoramidic acid[c]	0	99	260	487 ± 37	508
N-(*p*-Chlorophenyl)phosphoramidic acid[c]	2.1	99	305	473 ± 30	494 ± 10
N-(α-Naphthyl)phosphoramidic acid	0	100	335	2107 ± 100	2342 ± 40
N-(β-Naphthyl)phosphoramidic acid	1.0		255	2590 ± 110	2552 ± 30
N-(Phosphonoamidino)sarcosine	0	104[d]	225		774[b]
N-(*n*-Butyl)phosphoramidic acid		94			
N-(Benzoyl)phosphoramidic acid	0	93	260	450	524

[a] Incubated 24 hr in 0.02 M Tris, pH 8.0 buffer with E = 4 μg/ml. [b] Based on the moles of P_I produced. [c] Monoanilinium salt. [d] Based on $10H_2O$.

for *N*-(*n*-butyl)phosphoramidic acid. The background due to hydrolysis of this compound in the molybdate reagent at an initial substrate concentration of 10^{-3} M was 0.81 ± 0.03 or 23% of the optical density at infinity, while the ΔOD due to enzymatic hydrolysis was 0.218 ± 0.012 for three determinations.

The above rate measurements were performed on a Zeiss M 4 Q III spectrophotometer.

METHOD B. Spectrophotometric determinations of initial rates of hydrolysis were obtained with a Cary 17 recording spectrophotometer at 26 ● 0.5°, using the 0.0–0.10 slidewire. In general the reaction was monitored at a wavelength where there was a significant OD change on going from the particular phosphoramidic acid to the corresponding amine. In each case the wavelength was chosen so as to maximize the Δε = ε (substrate) − ε (products), while attempting to minimize the background absorbance of the substrate. Table I summarizes the extinction coefficient data for various substrates. The Δε values for the aromatic derivatives varied slightly between pH 7.0 and 8.0, probably reflecting the second ionization of these compounds, but were essentially invariant above pH 8.0.

In a typical run 20 μl of stock substrate (6×10^{-2} to 3.0×10^{-1} M) made up in a 5% excess of sodium hydroxide was introduced into the reference and sample cuvets, each of which contained 3.0 ml of buffer. Five microliters of stock enzyme (0.61 mg/ml) was introduced into the sample cuvet to initiate the reaction. In general an optical density of 0.01–0.03 was needed to determine the initial slopes. The pH of the reaction mixture was measured at the end of each kinetic run. For solutions in D_2O, the pD was calculated by adding 0.40 of a pH unit to the measured pH.

K_m for Phosphoramidic Derivatives. The K_m values for the phosphoramidic acids were obtained indirectly in this study by using these substrates as inhibitors of *p*-nitrophenyl phosphate hydrolysis. By employing an absorbance scale of 0.0–0.1 at a wavelength of 400 mμ, and by monitoring the release of *p*-nitrophenol to only about 1–3% reaction, hydrolysis of the substrates and thus inhibition resulting from phosphate production was kept at a minimum. At least two different *p*-nitrophenyl phosphate concentrations ranging from 4×10^{-5} to 10^{-4} were used, and five different concentrations of "inhibitor" usually ranging from 2.5×10^{-4} to 1.25×10^{-3}. The enzyme concentration was 0.15–0.30 μg/ml.

At each particular *p*-nitrophenyl phosphate (NO_2PhP) concentration the quotient of the uninhibited and inhibited rate v_0/v when plotted against the "inhibitor" concentration gave a straight line intersecting the ordinate at 1.0. The slope of such a plot was used to calculate K_m according to

$$\frac{1}{K_m} = \left[1 + \frac{NO_2PHP}{K_{m,\,NO_2PHP}}\right] \times \text{slope} \qquad (1)$$

These measurements were performed at 26° on a Zeiss M4 Q III spectrophotometer equipped with a Varian G-4000 recorder.

K_I for Phosphate. K_I for phosphate was determined by the same procedure outlined for the phosphoramidic acids. Plots of v_0/v *vs.* $[P_I]$ were made at six different NO_2PhP concentrations (5×10^{-5} to 10^{-3} M), employing five concentrations of P_I (1.0×10^{-4} to 5.0×10^{-4} M). The enzyme concentration was 0.31 μg/ml. The conditions were pH 8.0, 0.02 M Tris-HCl, KCl at 0.5 M and $T = 26°$.

K_m for p-Nitrophenyl Phosphate. K_m for *p*-nitrophenyl phosphate was determined at 26° in 0.02 M Tris-HCl with KCl at 0.5 M and pH's of 8.0, 9.4, and 10.0. These determinations were made by measuring initial rates of hydrolysis at varying substrate concentrations in the range of K_m. For example, at pH 8.0 rates were determined at five substrate concentrations ranging from 9.3×10^{-7} to 6.6×10^{-6} M *p*-nitrophenyl phosphate. Since such low substrate concentrations result in small absorbance changes, 5.0-cm cells were employed to increase the sensitivity. Reciprocal plots of v^{-1} *vs.* $(s)^{-1}$ resulted in good straight lines intersecting the ordinate at V_{max} from which K_m was calculated according to the Michaelis–Menten equation (1913). The enzyme concentration was 0.15 μg/ml.

Results

Demonstration of Phosphamidase Activity by Alkaline Phosphatase. In most instances we could detect little or no spontaneous hydrolysis of the various phosphoramidic acids even after 1 hr when these compounds were dissolved in pH 8.0, 0.02 M Tris buffer. The exception to this was *N*-(α-naphthyl)-phosphoramidic acid whose rate of spontaneous hydrolysis was approximately 7.5% that of the enzyme-catalyzed reactions. In *all* cases examined, when alkaline phosphatase was added to buffer containing the phosphoramidic acid derivative, there was an appreciable amount of hydrolysis within

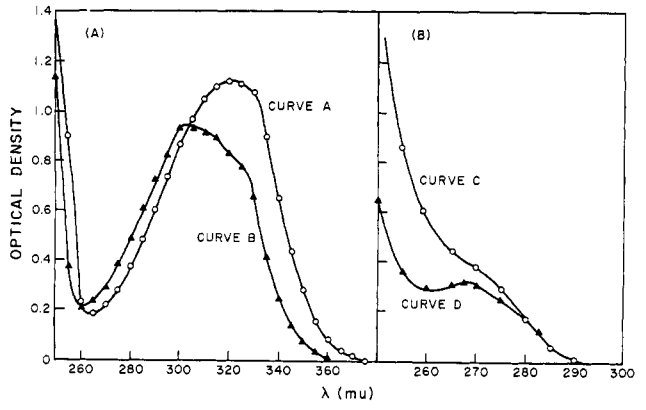

FIGURE 1: Substrate and product spectra before and after incubation with the enzyme. (A) Substrate is 2×10^{-4} M *N*-(α-naphthyl) phosphoramidic acid (curve A), and product spectrum is identical with that of α-naphthylamine. (B) Substrate is 5×10^{-4} M *N*-(benzoyl)phosphoramidic acid (curve C), and product spectrum (curve D) is identical with that of benzamide. Conditions: pH 8.0, 0.02 M Tris-HCl, KCl = 0.5 M. Incubation for 24 hr with 4 μg/ml of enzyme.

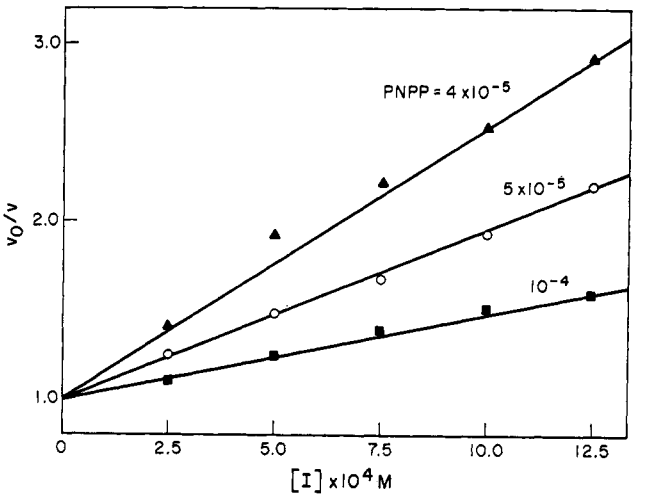

FIGURE 2: Competitive inhibition of the alkaline phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate (PNPP) by *N*-(*p*-chlorophenyl)phosphoramidic acid (I). Conditions: pH 8.0, 0.02 M Tris-HCl, KCl = 0.5 M; E = 0.15 μg/ml.

5 min. This could be detected either by measuring the production of orthophosphate (method A) or in most cases by monitoring the decrease in optical density (method B).

The spectra for *N*-(α-naphthyl)phosphoramidic acid and for *N*-(benzoyl)phosphoramidic acid before and after incubation with alkaline phosphatase for 24 hr are shown in Figure 1A,B. The product spectrum for the α-napthyl derivative is identical with that of α-naphthylamine, clearly establishing this as a product of the hydrolysis. In addition, the final OD readings of the two other aryl derivatives were in good agreement with those calculated on the basis of the experimentally determined extinction coefficients of their respective anilines. The calculated and observed $\Delta\epsilon$'s are summarized in Table I.

When *N*-(benzoyl)phosphoramidic acid was employed as a substrate the remote possibility of C–N rather than P–N bond cleavage had to be considered. The product spectrum of Figure 1B which is identical with that of benzamide clearly establishes the P–N bond as the point of cleavage.

Orthophosphate is demonstrated to be the other product of the hydrolysis by the data collected in Table I. Incubation of substrates for 24 hr with the enzyme produced quantitative amounts of orthophosphate, within experimental limits.

K_m and K_I Determinations. Typical plots for the determination of K_m of a phosphoramidic acid derivative are illustrated by Figure 2. Similar plots for the determination of K_I for orthophosphate employing *p*-nitrophenyl phosphate as the substrate is shown in Figure 3A. The value of K_I for phosphate as determined from the slopes of these lines was $1.3 \pm 0.1 \times 10^{-6}$ M. Values of K_m for the various substrates are collected in Tables II and III. Due to experimental difficulties inherent in the measurement of K_m for *p*-nitrophenyl phosphate, these values are only numerically approximate but are accurate in their relationship to each other. The value of K_m for *p*-nitrophenyl phosphate at pH 8.0 was found to be $1.3 \pm 0.2 \times 10^{-6}$ M. This value is in reasonable agreement with an earlier value of 1.0×10^{-6} M determined in these laboratories under slightly different conditions (Wilson and Dayan, 1965). The value of K_m for *N*-(*p*-chlorophenyl)phosphoramidic acid of $2.2 \pm 0.3 \times 10^{-5}$ is substantially higher than a previously published value of 4.3×10^{-6} (Reid and

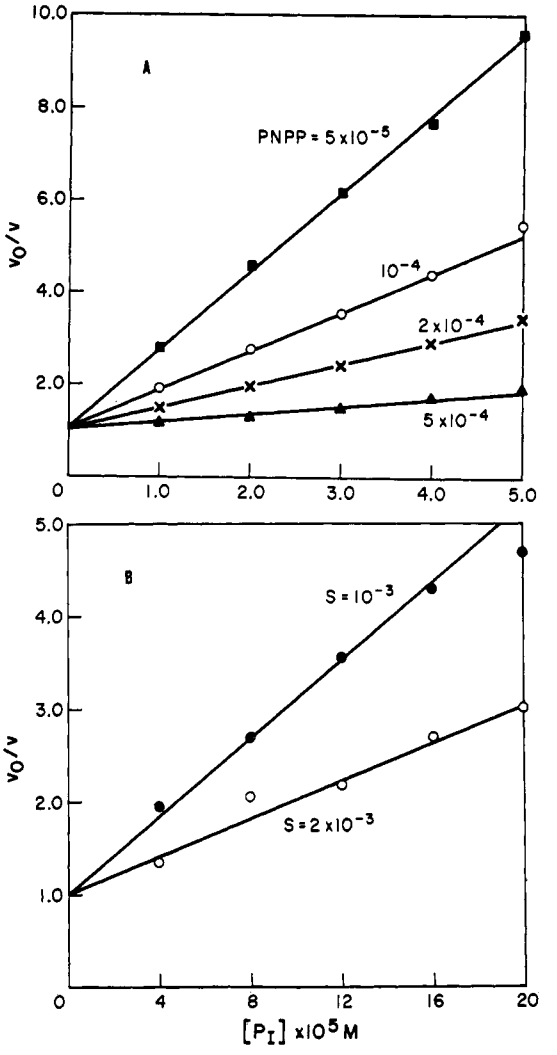

FIGURE 3: Competitive inhibition of the alkaline phosphatase-catalyzed hydrolysis. (A) Of *p*-nitrophenyl phosphate (PNPP) by inorganic phosphate (P_i). Conditions: pH 8.0, 0.02 M Tris-HCl, KCl = 0.5 M; E = 0.30 μg/ml. (B) Of *N*-(*p*-chlorophenyl)phosphoramidic (S) by inorganic phosphate (P_i). Conditions: pH 8.0, 0.02 M Tris-HCl, KCl = 0.5 M; E = 1.0 μg/ml.

TABLE II: Catalytic Constants for the Alkaline Phosphatase Catalyzed Hydrolyses of Several Phosphoramidic Acids.[a]

Substrate	Concn (10^{-4} M)	v[b] (min^{-1} mg^{-1})	v[c] (min^{-1} mg^{-1})	V_{max} (μmoles min^{-1} mg^{-1})	K_m (10^{-5} M)
N-(Phenyl)phosphoramidic acid[d]	10	5.7	7.3	9.3	1.9 ± 0.1
	20		8.4	9.7	
N-(*p*-Chlorophenyl)phosphoramidic acid[d]	10		9.1	13	2.2 ± 0.3
	20	11	11	13	
N-(α-Naphthyl)phosphoramidic acid	4.0	6.8	7.0	11	1.6 ± 0.1
N-(β-Naphthyl)phosphoramidic acid	4.0	7.5	6.3	8.5	1.2 ± 0.1
N-(Benzoyl)phosphoramidic acid	200		10	14	32 ± 10
N-(Phosphonoamidino)sarcosine	4.1		10	15	1.1 ± 0.2
(creatine phosphate)	6.2	12			
N-(*n*-Butyl)phosphoramidic acid	10		6.3	14	4.7 ± 0.4
p-Nitrophenyl phosphate	2	25.1			0.13 ± 0.02

[a] Conditions: pH 8.0, 0.02 M Tris, KCl at 0.5 M, T = 26°. [b] Determined spectrophotometrically. [c] Determined by phosphate production. [d] Monoanilinium salt.

Wilson, 1971). This latter value is certainly wrong because a commercial sample containing large amounts of orthophosphate was used.

It was also of interest to see if orthophosphate would compete with *N*-(*p*-chlorophenyl)phosphoramidic acid for the active site. Figure 3B demonstrates that phosphate is a competitive inhibitor of *N*-(*p*-chlorophenyl)phosphoramidic acid hydrolysis, just as it is of phosphate ester hydrolysis. Furthermore, the K_I of 1.0 ± 0.1 × 10^{-6} M calculated on the basis of this data was in good agreement with that calculated on the basis of competition studies with *p*-nitrophenyl phosphate.

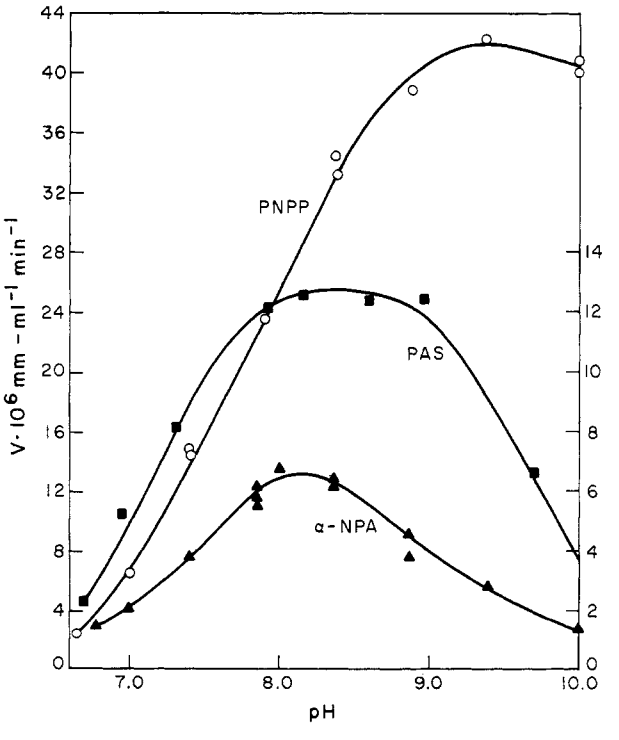

FIGURE 4: pH–rate profiles for the alkaline phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate (PNPP), *N*-(α-naphthyl)-phosphoramidic acid (α-NPA), and *N*-(phosphonoamidino)sarcosine (PAS). Left-hand scale refers to PNPP, right-hand scale refers to α-NPA and PAS. Conditions: pH 6.5–7.0 Tris-HAc, pH 7.4–9.4 Tris-HCl, pH 9.6–10.0 ethanolamine-HCl, all 0.02 M in basic component with KCl = 0.5 M. For PNPP rates determined at E = 0.30 μg/ml but calculated for 1.02 μg/ml. For α-NPA and PAS E = 1.02 μg/ml. PNPP = 10^{-3} M; α-NPA = 4 × 10^{-4} M; PAS = 6.2 × 10^{-4} M.

Maximal Rate V_{max} for the Various Substrates. The velocity and corrected maximal velocity for the various substrates investigated are collected in Table II. The reproducibility of values determined by both methods was approximately ±10%. Rate determinations were performed in triplicate. As Table II shows, agreement between the two methods was generally good.

Due to the high values of K_m relative to K_I for phosphate, it was necessary to correct the experimentally determined rates even though the percentage of reaction followed was low (usually less than 5%). These corrections were based on a knowledge of the percentage of the reaction (and thus a knowledge of the mean phosphate concentration ($\bar{P}_I$) during a given run), and the standard equation for competitive inhibition. Depending on the particular substrate (S) and the

$$V_{max} = v + v\frac{K_m}{(S)} + v\frac{K_m(P_I)}{K_I(S)} \qquad (2)$$

conditions chosen these corrections (right-hand portion of eq 2) usually amounted from 15 to 35% of the experimentally determined rate (v) but in one case was 124%. The value of V_{max} for *p*-nitrophenyl phosphate listed in Table II is in good agreement with that determined by others under similar conditions (Lazdunski and Lazdunski, 1969).

pH–Rate Profiles. The rate of utilization of three substrates as a function of pH is shown in Figure 4. Rate data presented for *N*-(α-naphthyl)phosphoramidic acid and *N*-(phosphoroamidine)sarcosine were determined spectrophotometrically and are uncorrected. Bell-shaped profiles were observed for all phosphoramidic acids for which pH–rate profiles were performed. These included the *N*-phenyl, *N*-*p*-chlorophenyl, and *N*-β-naphthyl derivatives as well as those shown in Figure 4. From the midpoints of the curves the left-hand leg was associated with a group having an apparent pK_a of 7.0–7.3 for phosphoramidate hydrolysis. However, these values are only

TABLE III: K_m As a Function of pH for *N*-(α-Naphthyl)phosphoramidic Acid (α-NPA) and *p*-Nitrophenyl Phosphate (NO_2PhP).

pH	K_m(α-NPA), 10^{-5} M	$K_m(NO_2PhP)$, 10^{-5} M
8.00	1.6	0.13
9.36	17	1.1
10.00	109	5.2

apparent and may actually be somewhat higher as there is a marked dependence of the observed rates on substrate concentration at higher pH values. Experiments where the concentration of substrate was doubled in the region 7.0–8.0 gave no higher values of v indicating that this portion of the curve was related to the maximal velocity rather than K_m. On the other hand, the right hand leg of the curve is associated with a change in K_m with pH since dilutions of sample in the region 9.0–10.0 resulted in significant changes in the rate. This is consistent with the results presented in Table III which demonstrate that the K_m of both *N*-(α-naphthyl)phosphoramidic acid and *p*-nitrophenyl phosphate increase markedly as the pH increases in the region of 8.0–10.0.

The sigmoidal nature of the *p*-nitrophenyl phosphate profile as opposed to that for the phosphoramidic acids is a consequence of its much lower K_m, ensuring saturation at all pH's at a concentration of 10^{-3} M. Thus the differences in the shapes of the profiles for the two types of substrates is more apparent than real. Had a lower concentration of *p*-nitrophenyl phosphate been employed, one would also observe a bell-shaped pH–rate profile for this substrate. Under our conditions we can assign a pK_a of 7.8 to the group controlling the left hand side of the *p*-nitrophenyl phosphate curve (*i.e.*, V_{max}). This is in agreement with the pH–rate studies of Neumann *et al.* (1967) but is higher than the most recently reported value of 7.43 by Krishnaswamy and Kenkare (1970).

pD–Rate Profiles. Figure 5 shows the effect of changing pD on the rates of hydrolysis of *p*-nitrophenyl phosphate *N*-(α-naphthylphosphoramidic acid. From the midpoints of the left hand portions of these curves an apparent pK_a of 7.8 can be assigned to the group controlling V_{max}^0 for both of these substrates.

Examination of the maximal rates for both substrates at the pH (pD) optimum in deuterium oxide and water reveals that there is a significant solvent isotope effect for the hydrolysis of the phosphate ester $k_{H_2O}/k_{D_2O} = 1.9$ in agreement with earlier findings (Lazdunski and Lazdunski, 1969), whereas the hydrolysis of the amidophosphate exhibits no such effect $k_{H_2O}/k_{D_2O} = 1.0$.

Effect of Tris. Whereas Tris accelerates the hydrolysis of phosphate esters, a decrease in the rates of hydrolyses of amidophosphates is observed at high Tris concentrations (Table IV).

Effect of KCl. Potassium chloride enhances the rates of hydrolyses of both types of substrates (Figure 6).

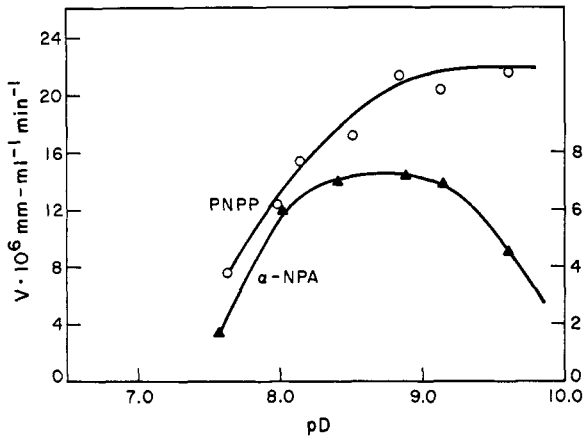

FIGURE 5: pD–rate profiles for the alkaline phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate (PNPP) and *N*-(α-naphthyl)-phosphoramidic acid (α-NPA). Left-hand scale refers to PNPP, right-hand scale refers to (α-NPA). Conditions: 0.02 M Tris-HAc buffer, 0.5 M in KCl; PNPP = 10^{-3} M; α-NPA = 4×10^{-4} M; PNPP rates determined at E = 0.30 μg/ml but calculated for 1.02 μg/ml; for α-NPA, E = 1.02 μg/ml.

Discussion

The present study was undertaken first to establish whether *E. coli* alkaline phosphatase has phosphamidase activity; second, to determine the specificity of the enzyme toward these substrates; and third, to ascertain differences and/or similarities with respect to the catalytic mechanisms for the hydrolysis of the nitrogen and oxygen analogs.

Having demonstrated that phosphoramidic acids are alkaline phosphatase substrates it was reasonable to assume that catalysis occurred at the same active site for these substrates as for phosphate esters. Nonetheless, it was important to establish this point. The competition studies showing the *N*-(*p*-chlorophenyl)phosphoramidic acid competitively inhibits *p*-nitrophenyl phosphate hydrolysis, and further that inorganic phosphate competitively inhibits hydrolysis of both types of substrates with the same K_I, suggests the identity of the catalytic site.

The present study not only shows phosphamidase activity by alkaline phosphatase but demonstrates the broad specificity of the enzyme toward phosphoramidic acids. Thus, although the basicity of the leaving groups for the various sub-

TABLE IV: Effect of Tris on the Rate of Hydrolysis of Alkaline Phosphatase Substrates.[a,b]

Concn of Tris (M)	α-NPA	PPA	p-Cl-PPA	NO_2PhP
0.02	7.4	7.6	13	29.6
0.10	7.8	6.7	14	33.8
0.20	8.3		15	38.3
0.80	7.6	4.8	12	47.7
1.20	6.3	4.7	13	49.6
2.00	5.2	5.1		47.4
$V_{2.0\ M\ Tris}/V_{0.02\ M\ Tris}$	0.65	0.67		1.60

[a] Conditions: determined spectrophotometrically, pH 8.2, ionic strength adjusted to 1.0 using KCl, T = 26°, E = 1.02 μg/ml. [b] Units = mm ml^{-1} min^{-1}. Abbreviations: α-NPA = *N*-(α-naphthyl)phosphoramidic acid; PPA = *N*-(phenyl)-phosphoramidic acid; *p*-Cl-PPA = *N*-(*p*-chlorophenyl)-phosphoramidic acid; NO_2PhP = *p*-nitrophenyl phosphate.

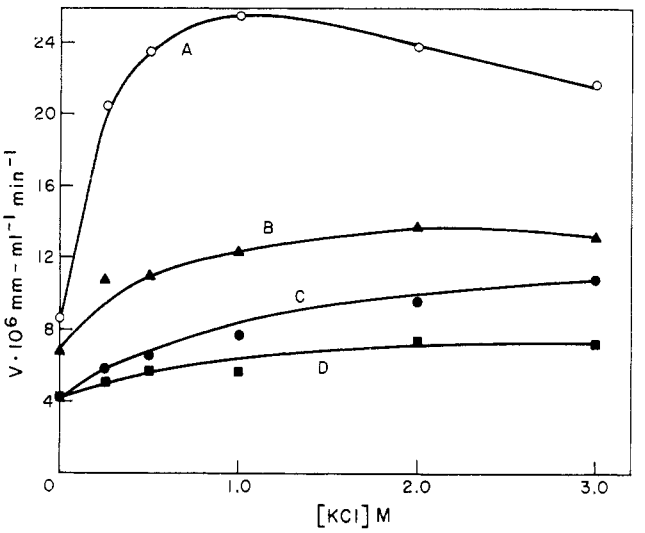

FIGURE 6: Effect of potassium chloride on the alkaline phosphatase-catalyzed hydrolysis of several substrates. (A) 10^{-3} M *p*-nitrophenyl phosphate; (B) 2×10^{-3} M *N*-(*p*-chlorophenyl)phosphoramidic acid; (C) 4×10^{-4} M *N*-(α-naphthyl)phosphoramidic acid; (D) 10^{-3} M *N*-(phenyl)phosphoramidic acid. Conditions: 0.02 M Tris-HCl (pH 8.00). Runs using PNPP were performed at E = 0.30 μg/ml but calculated for 1.02 μg/ml. All others performed at E = 1.02 μg/ml.

strates employed varied by more than nine powers of ten, there was less than a twofold difference in the rates of enzymatic hydrolysis at pH 8.0. In addition, it is shown that phosphoramidates have reasonably high maximum velocities (V_{max}), the hydrolysis of the enzyme–substrate complex occurring 30–60% as fast as for phosphate esters. However, the data in Table II indicate that the K_m values are distinctly higher than for *p*-nitrophenyl phosphate.

Having demonstrated that catalysis very probably occurs at the same site, it is pertinent to examine whether the enzyme hydrolyzes both classes of substrates in the same manner. It appears that the hydrolysis of phosphate esters is determined to a large extent by a conformational change of the enzyme. The slower rate of hydrolysis of the phosphoramidates suggests that some other step is rate influencing with these substrates although it is possible to suppose that the rate of the conformational change can be influenced by the substrate. This is so if we assume that the actual entity undergoing the conformational change is the enzyme–substrate complex (E·S) (Halford *et al.*, 1969). We should then have to say that the conformational change has a maximum rate that is achieved with phosphate esters but not with phosphoramidates.

The sizeable deuterium isotope effect for *p*-nitrophenyl phosphate ($k_{H_2O}/k_{D_2O} = 1.9$) and the lack of such an effect for *N*-(α-naphthyl)phosphoramidic acid ($k_{H_2O}/k_{D_2O} = 1.0$) also points to a different rate-influencing step. Similarly the effect of Tris (an acceptor) which increases the rate of hydrolysis of *p*-nitrophenyl phosphate but at very high concentration (2 M) decreases the rates of hydrolyses of phosphoramidates again suggests a different rate-influencing step. Tris may have a small accelerating effect at lower concentrations.

In the case of phosphate esters it is known from rapid sampling studies of dephosphorylation and of steady-state levels of phosphorylation that dephosphorylation is rapid (Aldridge *et al.*, 1964; Reid and Wilson, 1971). It is all the more evident in the case of phosphoramidates that dephosphorylation cannot be rate limiting. It would appear that the conformational change has become less rate limiting and that the phosphorylation step is the principal rate-influencing process. The small effect of Tris (at lower concentrations) supports this suggestion.

The enzyme-catalyzed hydrolytic rates bear little resemblance to nonenzymatic rates of hydrolysis. The observation that amidophosphates are hydrolyzed more slowly by the enzyme than the corresponding esters is in sharp contrast to the known reactivities of these substances in nonenzymatic hydrolysis. Here, phosphoramidic acids have been shown to be far more reactive than the corresponding esters. The monoanion of *N*-(phenyl)phosphoramidic acid hydrolyzes about 10^3 times as fast as the monoanion of phenyl phosphate at 20° (Cox and Ramsay, 1964). Phosphoramidic acid has, in fact, been successfully employed as a synthetic phosphorylating agent (Chambers and Khorana, 1958). Moreover, failure to observe any substantial changes in rate with changes in the R group is not consistent with nonenzymatic hydrolysis rates of phosphoramidates. Large variations in the nonenzymatic hydrolysis rates have been observed among the classes of compounds employed in this study (Benkovic and Sampson, 1971; Chanley and Feageson, 1958, 1962; Halmann *et al.*, 1960).

Added in Proof

While this manuscript was in press, the hydrolysis of several arylamidophosphates by alkaline phosphatase from *Escherichia coli* was reported (Williams and Naylor, 1971).

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Ionization Behavior of the Catalytic Carboxyls of Lysozyme. Effects of Ionic Strength†

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ABSTRACT: The pH difference titration of the β-ethyl ester derivative of the Asp-52 residue of lysozyme relative to native lysozyme has been obtained. The difference curve reflects the ionization behavior of both Asp-52 and Glu-35. Four microconstants describe the ionizations of the two interacting catalytic groups. The macroscopic ionization constant, including all forms of the enzyme, for Asp-52 is 4.5 while that for Glu-35 is 5.9 in 0.15 M KCl at 25°. The ionization of each group is strongly dependent on ionic strength in a manner opposite to that found for simple carboxylic acids. A Debye–Hückel treatment of the electrostatic potential from the rest of the enzyme adequately accounts for the ionic strength dependence. The results indicate that lysozyme in KCl solution has a net positive charge on the protein surface of about 6 to 7 over the pH range 4–7. If lysozyme were not charged and the two groups did not interact, Asp-52 and Glu-35 would have pK^0's of 5.3 and 5.8, respectively.

Recently we prepared, isolated, and identified a derivative of hen egg-white lysozyme which consists of the β-ethyl ester of the catalytic residue aspartic acid 52 (Parsons *et al.*, 1969; Parsons and Raftery, 1969). By subjecting this well-characterized derivative and native lysozyme to a differential measurement we could hope to gain detailed knowledge about the active site region in which effects from the rest of the protein were cancelled out. An obvious comparison to make when dealing with a singly esterified enzyme is a proton difference titration. It is worth noting that this is a preferred comparison even if another method such as pH-dependent perturbed protein ultraviolet (uv) absorbance is available. This is so because the hydrogen ion titration has a predictable stoichiometric span. Accordingly, more complex difference curves can be fitted with confidence.

Little information has been available about aspartic acid residue 52 (Asp-52). Kinetic data suggest that its pK is between 3.5 and 4.5 in the catalytic complex (Rupley, 1967; Parsons *et al.*, 1969; Rand-Meir *et al.*, 1969). A group with an apparent pK of about 3.2 affects the tryptophanyl chromophores (Donovan *et al.*, 1961; Lehrer and Fasman, 1967). A group of about pK 3.6 is the only acidic group to affect the electrophoretic mobility (Beychok and Warner, 1959), and two groups of pK 3.2 become normalized in guanidine hydrochloride solution (Donovan *et al.*, 1960). None of these effects, which may be due to several different groups, have been assigned to Asp-52. It will be apparent from the results presented in this paper that these effects do not arise from Asp-52. However, other unassigned observations which will be discussed below probably originate from this amino acid residue.

In contrast to Asp-52, information about the other catalytic residue, glutamic acid 35 (Glu-35), has been obtained previously. A single carboxyl of pK 6.0–6.5 appears in pH titrations (Sakakibara and Hamaguchi, 1968), becomes normalized in guanidine hydrochloride solution (Donovan *et al.*, 1960), perturbs tryptophanyl chromophores (Ogasahara and Hamaguchi, 1967; Donovan *et al.*, 1961; Lehrer and Fasman, 1967), affects the binding of inhibitors and substrates (Dahlquist *et al.*, 1966; Lehrer and Fasman, 1966; Rupley, 1967; Rand-Meir *et al.*, 1969), and affects the proton magnetic resonance chemical shift values of bound inhibitors (Dahlquist and Raftery, 1968). The X-ray crystallographic results (Blake *et al.*, 1967, 1965) place Glu-35 in a hydrophobic region of the enzyme consistent with all of these effects.

This paper reports on some aspects of the pH difference titration of the Asp-52 ester derivative. Four microconstants for the ionization of Asp-52 and Glu-35 in the native enzyme have been accurately determined in solutions containing 0.02–0.50 M KCl at 25°. A previous communication briefly described the results in 0.15 M KCl (Parsons and Raftery, 1970). Subsequent papers (Parsons and Raftery, 1972a,b) report on the effects of temperature and of inhibitors and substrates

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