Investigations on the Alkaline Phosphatase Catalyzed Hydrolysis of Phosphoramidates. Substituent Effects and Transphosphorylation†

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ABSTRACT: The effect of substituents on the second-order rate constant k_{cat}/K_m for the phosphorylation of *Escherichia coli* alkaline phosphatase by a series of *N*-(X-phenyl)phosphoramidates has been studied. A Hammett plot for this parameter gave a ρ value of $+0.58$. This ρ value and our earlier findings that the enzymatic hydrolysis of phosphoramidates is not subject to a solvent deuterium isotope effect suggests that a proton transfer is not involved in the rate-controlling step. Possible pathways for the catalytic hydrolysis of these substrates by alkaline phosphatase are presented. Four arylphosphoramidates were hydrolyzed in 2 M Tris which acted as an acceptor in competition with water. The product ratio, aniline/PO_4, was found to be constant and equal, within experimental error, to the nitrophenol/PO_4 ratio when *p*-nitrophenyl phosphate was employed as the substrate. These results are consistent with the formation of a common phosphoryl enzyme intermediate in the alkaline phosphatase catalyzed hydrolysis of phosphoramidates and phosphate esters.

It has recently been demonstrated that the hydrolyses of a broad range of phosphoramidates are catalyzed by alkaline phosphatase from *Escherichia coli* (Williams and Naylor, 1971; Yount *et al.*, 1971; Snyder and Wilson, 1972). This enzyme is known to hydrolyze phosphate esters *via* a phosphoryl-transfer mechanism. Both transphosphorylation (Barrett *et al.*, 1969) and labeling studies (Reid and Wilson, 1971; Lazdunski *et al.*, 1971) employing phosphate esters and anhydrides as substrates have provided evidence for a common phosphoryl enzyme in the catalytic pathway. Thus, it was demonstrated that, when a series of phosphate esters were hydrolyzed in media containing a high concentration of Tris, a constant ratio of transphosphorylation to hydrolyses products was obtained (Barrett *et al.*, 1969). In the case of phosphate esters, Tris is known to accelerate the rate of utilization of substrate. This is consistent with the notion that Tris is acting as a kinetic acceptor (nucleophile) toward the phosphorylated enyzme, and schemes have been developed to account for its acceleratory effect on the rate (Reid and Wilson, 1971; Wilson *et al.*, 1964). In a previous communication (Snyder and Wilson, 1972) we reported that high concentrations of Tris actually had a decelerating effect on the rates of hydrolyses of several phosphoramidates. This difference in the effect of Tris on the rates of hydrolyses of phosphate esters and phosphoramidates suggested the possibility that Tris might be playing a different role in phosphoramidate hydrolysis. In view of this finding we felt it necessary to determine whether a common phosphoryl enzyme could be implicated in the catalytic pathway for phosphoramidate hydrolysis. In the present communication we show that arylamidophosphates participate in the transphosphorylation reaction and give evidence that the phosphoryl enzyme is the same as that derived from esters. We also report the results of our findings regarding the effect of substituents on the catalytic parameters k_{cat} and k_{cat}/K_m for a series of arylamidophosphates.

Experimental Section

Enzyme Preparation. The enzyme was prepared and purified as before (Snyder and Wilson, 1972). Its concentration was determined using the assay procedure of Malamy and Horecker (1964) assuming a molecular weight of 89,000 (Simpson *et al.*, 1968) and a specific activity of 3200 μmoles/min per mg.

Substrates. p-Nitrophenyl phosphate was obtained from Aldrich as the hexahydrate. The arylamidophosphates were prepared by hydrolysis of the corresponding dichlorides and isolated as the anilinium salts (Snyder and Wilson, 1972). These substrates all gave satisfactory elementary analyses (phosphate within $\pm 0.5\%$ of theoretical) and were found to be free of contamination by inorganic phosphate.

Kinetic Measurements. Rate measurements were performed both by spectrophotometry at the wavelength indicated (Table I) and by measuring phosphate production. The experimental procedures are the same as outlined before (Snyder and Wilson, 1972).

The spectrophotometric determinations were performed on a Cary 17 recording spectrophotometer ($T = 26° \pm 0.5°$) while the production of phosphate was monitored with the aid of a Zeiss M4 Q III spectrophotometer ($T = 26° \pm 0.2°$). In all kinetic runs the substrate was present in large excess of its K_m (~20- to 100-fold), and thus the measured rates corresponded to V_{max} (uncorrected). Agreement between the two methods was approximately $\pm 15\%$. Because of severe product inhibition by orthophosphate (P_i), even at the relatively low extents of reaction measured (always less than 5%), the observed rate constants tabulated in Tables I and II have been corrected by the method reported earlier (Snyder and Wilson, 1972).

Measurement of K_m's. The K_m values for the various phosphoramidates were obtained by using these substrates as inhibitors of *p*-nitrophenyl phosphate hydrolysis. The methods employed for these determinations are the same as outlined previously (Snyder and Wilson, 1972). Laidler (1958) has

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TABLE I: Kinetic Data for Alkaline Phosphatase Substrates as Determined by Spectrophotometry.[a]

Amidophosphate[b]	λ followed, nm	$\Delta\epsilon_{calcd} = \epsilon_{sub} - \epsilon_{prod}$	$\Delta\epsilon_{obsd}$	k_0(uncorrected)[c]	k_0(corrected)[c]
p-Methoxyphenyl	260	1062		0.52	0.66
p-Methylphenyl	260	1320	1332	0.29	0.32
Phenyl	260	487	508	0.23	0.28
p-Chlorophenyl	305	473	494	0.44	0.55
p-Bromophenyl	270	1556	1483	0.42	0.48
m-Chlorophenyl	262	1177	1186	0.68	0.80

[a] Conditions: pH 8.00, 0.02 M Tris-HCl, 0.5 M in KCl, $T = 26°$. [b] Substrate concentration ranges from 7.5×10^{-4} to 2.0×10^{-3} M. [c] Relative to *p*-nitrophenyl phosphate $k_0 = 1.0$. V_{max} for *p*-nitrophenyl phosphate under these conditions is 25.1 μm $\times$ mg^{-1} min^{-1} or $k_{cat} = 37.1$ sec^{-1} (Snyder and Wilson, 1972).

TABLE II: Kinetic Data for Alkaline Phosphatase Substrates as Determined from Orthophosphate Production.[a]

Amidophosphate[b]	k_0(uncorrected)[c]	k_0(corrected)[c]	K_m ($\times 10^5$ M)	k_{cat}/K_m ($\times 10^{-5}$ M^{-1} sec)
p-Methoxyphenyl	0.44	0.76	4.5 ± 0.3	6.2
p-Methylphenyl	0.23	0.30	2.6 ± 0.5	4.3
Phenyl	0.29	0.37	1.9 ± 0.1	7.2
p-Chlorophenyl	0.36	0.52	2.2 ± 0.3	8.8
p-Bromophenyl	0.39	0.52	2.2 ± 0.1	8.8
m-Chlorophenyl	0.56	0.83	2.4 ± 0.8	12.8

[a] Conditions: same as in Table I. [b] Substrate concentrations were 10^{-3} M. [c] Based on $k_0 = 1.0$ for *p*-nitrophenyl phosphate.

TABLE III: Substituent Effects on the Kinetic Parameters k_{cat} and k_{cat}/K_m for the Alkaline Phosphatase Catalyzed Hydrolysis of Arylamidophosphates.

Amidophosphate	log k_{cat}[a]	log k_{cat}[b]	log (k_{cat}/K_m^{-5})[a]	log (k_{cat}/K_m^{-5})[b]	σ
p-Methoxyphenyl	1.45	1.39	0.79	0.73	−0.268
p-Methylphenyl	1.05	1.08	0.63	0.66	−0.170
Phenyl	1.13	1.02	0.86	0.74	0.000
p-Chlorophenyl	1.28	1.31	0.94	0.97	+0.227
p-Bromophenyl	1.28	1.23	0.94	0.91	+0.232
m-Chlorophenyl	1.49	1.47	1.10	1.09	+0.373

[a] From phosphate production. [b] From spectrophotometry.

demonstrated that the kinetic equation for the velocity of one substrate in the presence of a second competing substrate has the same form as that for competitive inhibition. Further, it was shown that the inhibition constant for the second substrate "K_I" is kinetically equivalent to K_m, the true Michaelis constant. Therefore, even though we have used the various phosphoramidates as inhibitors of the *p*-nitrophenyl phosphate reaction, we refer to the inhibition constants obtained in this study as K_m values. Each K_m value reported in Table II is the average of four individual K_m determinations carried out at four different *p*-nitrophenyl concentrations in the range of 4×10^{-5} to 10^{-4} M. Five different inhibitor concentrations ranging from 2.5×10^{-4} to 1.2×10^{-3} M were employed for the plot of the quotient of the uninhibited to inhibited rate (v_0/v) *vs.* inhibitor at each particular *p*-nitrophenyl phosphate concentration. The enzyme concentration was 0.15–0.30 μg/ml. K_m was determined from the slope of such a plot and, as before (Snyder and Wilson, 1972), the extent of hydrolysis was kept low to minimize the effect of product inhibition by orthophosphate.

Transphosphorylation Experiments. The ratio of transphosphorylation/hydrolysis was determined by incubating the substrate and enzyme for a fixed period of time in pH 8.2, 2 M Tris (or 0.1 M Tris), T 26°. The enzyme concentration ranged from 1.0 to 2.0 μg/ml and incubation periods from 15 to 30 min. The substrate concentrations were 2×10^{-3} M, and the extent of hydrolysis never exceeded 20%. The number of moles of a particular aniline produced in a given period of

TABLE IV: Product Ratios for the Hydrolysis of Alkaline Phosphatase Substrates in Tris Buffer.[a]

Compound	Aniline (Nitrophenol)/PO_4 Ratio 2 M Tris	0.1 M Tris
p-Nitrophenyl phosphate	2.2 ± 0.1	1.0 ± 0.1
N-(*m*-Chlorophenyl)-phosphoramidate	2.3 ± 0.3	1.0 ± 0.1
N-(Phenyl)phosphoramidate	2.0 ± 0.3	1.1 ± 0.1
N-(*p*-Methylphenyl)-phosphoramidate	2.0 ± 0.2	*b*
N-(*p*-Methoxyphenyl)-phosphoramidate	2.2 ± 0.4	*b*

[a] pH 8.2; T = 26°; μ = 1.0 adjusted with KCl. [b] Not determined.

time was calculated from the change in optical density. The production of phosphate was measured by a slight modification of the procedure outlined for the rate measurements (Snyder and Wilson, 1972), except that for transphosphorylation studies an equivalent of 1 M acetic acid was added to the incubation mixture to neutralize the Tris and the development times were extended to 30 min. By measuring the amount of the particular aniline and phosphate produced in a fixed period of time, the amount of transphosphorylation/hydrolysis was easily determined. The ratios tabulated in Table IV are an average of at least three individual measurements of phosphate and aniline production.

Results and Discussion

Substituent Effects. The rate constants for the various substrates employed in this study are reported in Tables I and II. The corrected values of k_0 determined by the two methods are in substantial agreement. The corrections for phosphate production during the course of the kinetic runs can be seen to be quite important, particularly when rates were determined by measuring phosphate production where the percentage hydrolysis was 2–5%. The spectrophotometric method required smaller corrections because it was only necessary

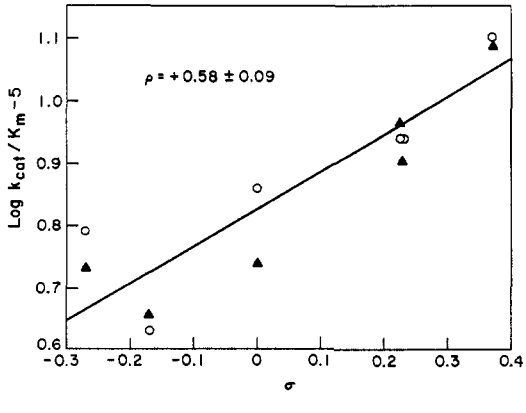
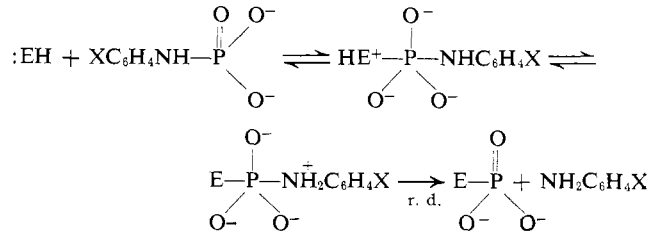


FIGURE 1: Hammett plot for k_{cat}/K_m for the alkaline phosphatase catalyzed hydrolysis of substituted arylamidophosphates. Circles represent values determined by measuring phosphate production, triangles represent values obtained from spectrophotometry.

SCHEME I



to follow the reaction for 1–2% of completion in order to obtain initial rates.

Except for the *p*-methoxyphenyl derivative both k_0 (corrected) and k_{cat}/K_m increase with increasing electron-withdrawing substituents. The quantity k_{cat}/K_m is the second-order rate constant for the reaction between enzyme and substrate to produce the phosphoryl enzyme intermediate, regardless of the rate-determining step. We have constructed a Hammett plot for this quantity (Figure 1). Our results differ from those of Williams and Naylor (1971) in that we find that those substrates with better leaving groups are better substrates. A least-squares treatment of the log k_{cat}/K_m values determined by the two kinetic methods employed in this study gives a ρ value of 0.58 ± 0.09. The positive sign of ρ indicates an increase in electron density on the nitrogen in the transition state. Electron-withdrawing groups would effect the dispersion of charge and thereby stabilize the transition state. If the slow step in the formation of the phosphoryl enzyme were a proton transfer to the nitrogen atom, then ρ would be expected to be negative. The substituent effects observed in this study coupled with our previous finding that there was no solvent deuterium isotope effect in the catalytic hydrolysis of phosphoramidates by alkaline phosphatase (Snyder and Wilson, 1972) indicate that a proton transfer is not the rate-controlling step in the phosphorylation reaction.

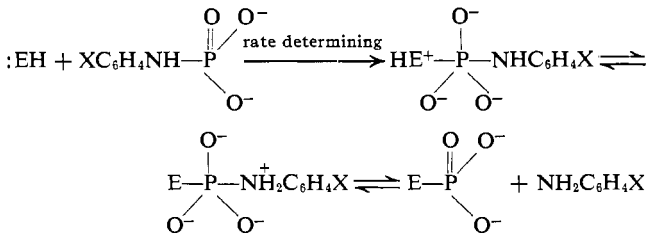
Protonation of the nitrogen function would appear to be a necessary step in the hydrolysis of these substrates because the alternative, expulsion of the aniline as the anion, would be highly unfavorable. The phosphoryl enzyme could arise *via* a direct displacement process or it could be formed through a two-step mechanism involving the formation of a pentacovalent addition intermediate. Each of these possibilities might or might not involve a preequilibrium protonation giving rise to four possible schemes. Preequilibrium protonation would tend to make ρ negative, which is opposite to what we observe. However, this does not necessarily rule out a "preequilibrium protonation" step because the ensuing expulsion of aniline which would have a positive ρ might outweigh the negative protonation effect. Therefore, our results could be interpreted in terms of a pathway illustrated by Scheme I.

We have arbitrarily selected the addition–elimination sequence; however one could also easily imagine a concerted displacement on the zwitterionic form of the monoanion.

An alternative pathway consistent with our findings would be a rate-determining nucleophilic attack on the unprotonated form of the substrate followed by a rapid proton transfer (from some donor at the active site) which is concerted with the expulsion of aniline. This is illustrated by Scheme II. In Scheme II nucleophilic attack by the enzyme is rate determining. Protonation and expulsion of the aniline are written as distinct steps only for clarity of exposition.

Transphosphorylation Experiments. Table IV shows that

SCHEME II



high concentrations of Tris (2 M) lead to a considerable amount of transphosphorylation, while at lower concentrations (0.1 M) we were able to detect little or no transphosphorylation. The relatively large errors reported in this study as compared to those reported for the corresponding phosphate esters (Barrett *et al.*, 1969) are a consequence of the great lability of amidophosphates to acid, thus necessitating a less sensitive method of phosphate analysis.

The observation that a series of substrates having different leaving groups gives a constant ratio of products when hydrolyzed in the presence of two nucleophiles is evidence for a common covalent phosphoryl enzyme intermediate. More detailed arguments for the use of this criterion have been presented elsewhere (Barrett *et al.*, 1969; Jencks, 1969). The ratio of substituted aniline/phosphate produced for the four phosphoramidates studied herein is 2.1 ± 0.1 and is the same within experimental error as that determined for *p*-nitrophenyl phosphate. This constancy in the ratio of products for the various substrates employed in this study is evidence for the formation of a common phosphoryl enzyme in phosphoramidate and phosphate ester hydrolysis.

A rate-determining conformational change of the enzyme has been proposed to account for some observations of the alkaline phosphatase catalyzed hydrolysis of phosphate esters (Trentham and Gutfreund, 1968; Halford *et al.*, 1969; Reid and Wilson, 1971). These proposals can account for the observation that the maximum velocity is about the same for almost all esters and that Tris accelerates ester hydrolysis even though dephosphorylation is not the rate-determining step. Dephosphorylation is, in fact, about ten times faster with ester substrates (Wilson and Reid, 1971) than the phosphorylation sequence which includes the conformational change.

Since these phosphoramidates are hydrolyzed more slowly than esters, and since the same phosphoryl enzyme is formed as the intermediate, it is evident that the phosphorylation sequence must be rate determining.

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