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Kinetics Studies of Substituted Aryl Phosphate Hydrolysis by Two Acid Phosphatases[1]

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First-order kinetic constants under subsaturating conditions as well as Michaelis–Menten kinetic parameters have been determined with a wide range of substituted aryl phosphates hydrolyzed in the presence of acid phosphatases of bovine milk and potato. The results obtained suggest that decomposition of an enzyme–phosphate intermediate is rate controlling for both enzymatic reactions. However, while with the potato acid phosphatase no evidence of effects by the substrates on preceding steps in the reaction sequence was found, K_m for the bovine milk enzyme was markedly affected by the nature of the phosphate hydrolyzed.

INTRODUCTION

Acid phosphatases (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2), which are widely distributed in bacteria, plants, and animals, show a low degree of specificity in the hydrolysis of monophosphate esters (*1*). Interest in this group of enzymes has lately been stimulated by their role as indicators of some pathological conditions (*2*).

The mode of action of acid phosphatases from a variety of sources has been examined by a number of authors using kinetics (*3–7*) and other techniques (*8*). The results obtained suggest that a minimal description of the catalysis is given by Eq. [1], where

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' + P' \overset{k_3}{\rightarrow} E + P'' \qquad [1]$$

and E, S, P′, and P″ are, respectively, enzyme, substrate, and first- and second-released products. For the enzyme from potato, Hsu *et al.* (*5*) have shown P′ is the alcoholic moiety, P″ the phosphate. The same authors have suggested (*5*) that isomerization of the ES′ complex also occurs.

Histidine has been implicated in the active site of the phosphatases from measurements of rate versus pH (*4, 6, 7*). The role of that amino acid residue has been clearly demonstrated more recently by van Etten and colleagues (*9–11*), who showed that an intermediate *N′*-phosphoryl histidine species is formed. Evidence that arginine is important in binding the phosphate moiety has been adduced for the prostate acid phosphatase (*12*).

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TABLE 1

FIRST-ORDER RATE CONSTANTS (k_ψ) FROM MEASUREMENTS OF SUBSATURATING SUBSTRATE CONCENTRATIONS AND K_m, V_{max} VALUES, OBTAINED FROM DOUBLE-RECIPROCAL PLOTS, FOR POTATO PHOSPHATASE IN 0.1 *M* ACETATE BUFFER, pH 5.0, 20°C

Substrate	$10^4 \times k_\psi$ (sec^{-1})	$10 \times K_m$ (m*M*)	$10^2 \times V_{max}$ (μmol sec^{-1})
o-NPP (*o*-NO_2)	17.4	0.93	12.5
m-NPP (*m*-NO_2)	13.0	3.32	31.7
p-NPP (*p*-NO_2)[a]	18.7	1.08	18.9
o-MPP (*o*-CH_3)	1.71	8.93	16.9
m-MPP (*m*-CH_3)	13.3	2.50	22.2
p-MPP (*p*-CH_3)	9.0	4.35	21.7
o-CPP (*o*-Cl)	11.5	1.47	26.4
m-CPP (*m*-Cl)	26.5	0.66	12.0
p-CPP (*p*-Cl)	12.6	3.37	37.1
o-APP (*o*-COOH)	—	1.43	41.7
m-APP (*m*-COOH)	—	6.15	47.0
p-APP (*p*-COOH)	—	5.19	56.6
m-BPP (*m*-Br)	11.5	5.10	58.1
p-FPP (*p*-F)	4.71	4.76	38.5
p-PPP (*p*C_6H_5)	10.1	2.33	21.3
p-tBPP (*p*-*tert*.-C_4H_9)	1.81	13.3	23.3
o-tBPP (*o*-*tert*.-C_4H_9)	0	—	—
1-NAP		3.45	22.7
2-NAP		6.25	26.3
6-BNAP		1.89	32.5

[a] All data were standardized against measurements with this substrate.

In the work reported here we examined the effects of modifying the phenolic moiety of phosphate substrates by examining the kinetics of phosphatase reactions with a variety of substituted phenyl and naphthyl phosphates. These studies have enabled us to assess the importance of electronic and of steric factors in the

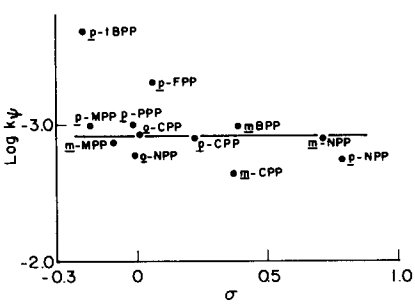

FIG. 1. Plot of log k_ψ, determined for a range of aryl phosphates, hydrolyzed in the presence of potato acid phosphatase, versus the Hammett σ values.

hydrolysis. In a further extension of the study, enzymes from two sources have been examined, namely bovine milk and potatoes. The results obtained may be interpreted on the basis of Eq. [1], and strongly suggest that the k_3 step in that reaction route is rate determining under the conditions employed (compare (*7*)). While the data presented show a common slow step for the two enzymes examined, they also demonstrate that differences are evident in earlier stages of the hydrolyses.

EXPERIMENTAL

Reagents. Unless otherwise stated reagents were of analytical grade. Solutions were prepared in distilled/deionized water. Except for *p*-nitrophenyl phosphate (*p*-NPP, Sigma), 1- and 2-napthyl phosphates (1NAP, 2NAP, Calbiochem), and 6-benzoyl-2-naphthyl phosphate (6BNAP; ICN), the aryl phosphates used in this work were synthesized by the methods of Kraft and Katyshkina (*13*), Chanley, *et al.* (*14*), and Friedman *et al.* (*15*). The phosphodichloridates produced by the first named synthetic method were hydrolyzed in wet ether (*16*).

The phosphates were obtained either as the free acids, and crystallized from chloroform, or as the potassium salts, and crystallized from aqueous ethanol. Partial elemental analyses (by Mr. H. Seguin of the Division of Biological Sciences, NRCC) confirmed the identities of the substrates employed, many of which have been synthesized elsewhere (*13–15*).

Isolation and purification of enzymes. Bovine milk acid phosphatase was isolated by the methods described (*16, 17*) followed by gel filtration on Sephadex G-100 (Pharmacia Ltd.) in acetate buffer, 0.1 *M*, pH 4.8. A single protein was so separated: on gel electrophoresis (*18*) a second, minor band of phosphatase activity was detected which was probably an isozyme of the enzyme separated (compare (*6*)).

Potato acid phosphatase was prepared from Canada No. 1 potatoes by the method of Hsu *et al.* (*5*). After ion-exchange chromatography on DEAE-Sephadex (Pharmacia Ltd.) in 0.05 *M* Tris–HCl buffer, pH 7.0, with a linear salt gradient 0–0.25 *M* NaCl, and gel filtration on both Sephadex G-200 and G-50 (Pharmacia Ltd.), gel electrophoresis showed that the product consisted of two separable isozymes.

Kinetics measurement. These were run on Beckman Model 26 or Unicam SP800 spectrophotometers by the method previously described (*19*) for determination of the first-order rate constants obtained at subsaturating conditions. Michaelis–Menten parameters were measured by conventional double-reciprocal plots with a range of substrate concentrations from 8×10^{-6} to 2×10^{-3} *M*. All measurements were made in 0.1 *M* acetate buffer, pH 5.0, at 20°C. Under these conditions spectrophotometric determinations of p*K*'s showed that the substrates employed in this study were all predominantly in their first ionization stage ($ROPO_3H^-$). According to Alvarez (*4*) it is only this form of the substrate which is acted on by the acid phosphatases and evidence confirming this view has been recently reported (*7*). As several batches of each enzyme

were used in this work the kinetic results were standardized against data for *p*-NPP.

RESULTS AND DISCUSSION

The first-order rate constants $k_\psi = V/K_m$ obtained with the acid phosphatase of potato are collected in Table. 1. When these data are plotted against the Hammett σ values, as in Fig. 1, it is immediately apparent that the reactivities of the substrates are unaffected by electronic factors, the results for the *p-tert.*-butyl compound being somewhat anomalous.

This conclusion was confirmed when Michaelis–Menten parameters V_{max} and K_m were estimated (Table 1), as $k_\psi = V_{max}/K_m$. Plots of V_{max} and K_m versus Hammett σ values both gave lines of zero slope; clearly neither Michaelis parameter is affected by the electronic nature of the substituents on

TABLE 2

FIRST-ORDER RATE CONSTANTS (k_ψ) FROM EXPERIMENTS AT SUBSATURATING CONCENTRATIONS AND K_m, V_{max} OBTAINED FROM DOUBLE-RECIPROCAL PLOTS FOR BOVINE MILK ACID PHOSPHATASE IN 0.1 *M* ACETATE, pH 5.0, 20°C

Substrate	$10^4 \times k_\psi$ (sec^{-1})	$10 \times K_m$ (m*M*)	$10^2 \times V_{max}$ (μmol sec^{-1})
o-NPP	3.30	0.88	4.0
m-NPP	1.23	3.20	8.1
p-NPP[a]	2.16	3.60	11.1
o-MPP	0.08	167	5.0
m-MPP	—	115	4.8
p-MPP	—	125	5.0
o-CPP	0.81	12.8	15.4
m-CPP	0.86	11.1	9.5
p-CPP	0.60	11.9	8.6
o-APP	0.66	10.4	7.1
m-APP	0.44	11.4	7.4
p-APP	1.01	7.7	8.3
o-tBPP	0	—	—
m-tBPP	0	—	—
p-tBPP	1.0	9.1	10.9
m-BPP	0.91	5.7	6.4
p-FPP	—	25.6	7.7
6-BNAP	1.00	14.3	9.4
1-NAP	0.26	—	—
2-NAP	0.30	—	—

[a] All data were standardized against measurements with this substrate.

the aryl substrates employed. Further, the comparatively small variations observed are not attributable to other simple substituent or structural effects.

These results strongly suggest the passage of all substrates through a common intermediate, succeeded by a rate-determining step during which the common phosphate moiety is displaced from the enzyme. On the basis of the route of Eq. [1] the data discussed require that k_3, the dephosphorylation step (*5*), be rate limiting. It is probable, on the basis of work with other acid phosphatases, that an *N*-phosphoryl histidine is decomposed in this process (*11, 12, 20–23*). The data discussed here may also be interpreted as suggesting that k_1, k_{-1}, and k_2 are of comparable values as $V/K = k_{-1}k_2/(k_{-1} + k_2)$. However, it seems unlikely that such a balance would prevail for all 17 substrates examined. Evidence of a rate-limiting dephosphorylation in other acid phosphatases, for example from human prostate, wheat germ, and rat livers has been reported (*21–24*) from experiments utilizing a variety of techniques.

As a comparison with these results, and for further examination of the general validity of the mechanistic route discussed here, measurements were made using the same range of aryl substrates with the acid phosphatase isolated from bovine milk. The results from experiments with subsaturating concentrations are collected in Table 2 and confirm those obtained in conventional Michaelis–Menten experiments which are presented in the same table. A Hammett correlation of V_{max} versus σ yielded a straight line of zero slope. When K_m was plotted against the Hammett parameter, Fig. 2 was obtained. It is apparent that V_{max} is not affected by the electronic nature of the substituents on the aryl phosphates employed as substrates. This result again suggests that k_3 is rate limiting.

Figure 2 shows, however, that K_m has a marked response to σ, giving a line of slope (ρ) −1.7. Clearly the electronic characteristics of the substituents on the aryl phosphate substrates employed play a significant role in determining this Michaelis parameter.

It may be shown (*25*) that for reactions of the type described by Eq. [1]:

$$K_m = \frac{k_{-1}k_{-2}\mathrm{P}' + k_{-1}k_3 + k_2k_3}{k_1(k_2 + k_{-2} + k_{-2}\mathrm{P}' + k_3)}, \qquad [2]$$

$$V_{max} = \frac{k_2k_3[\mathrm{E}]}{k_2 + k_{-2}\mathrm{P}' + k_3}. \qquad [3]$$

Because the milk acid phosphatase the plot of log V_{max} vs σ has a slope of zero, we may conclude that values of k_3 must dominate Eq. [3] (that is, that k_3 is significantly lower than k_2, k_{-2}). Alternatively, if the difference between k_3 and the constants of the forward and reverse reactions in the second equilibrium of Eq. [1] is not predominant, that equilibrium must not show a response to the substituents on the phenols examined. This latter assumption implies that it is only in the formation of the enzyme–substrate complex that the electronic nature of the phosphates hydrolyzed is relevant, affecting K_m as is shown in Fig. 2.

While the data of Table 1 suggest that the size of the phosphate substrates may slightly affect their reactivities with the potato acid phosphatase, this phenomenon is not observed in the data from the milk acid phosphatase (Table 2). Only with

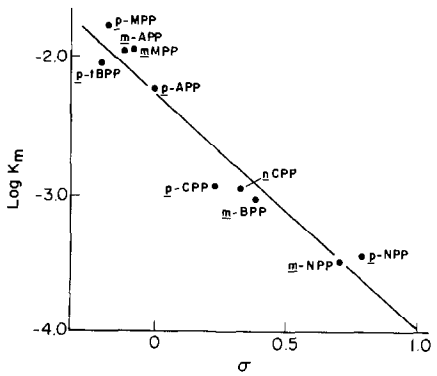

FIG. 2. Log K_m from bovine milk acid phosphatase catalysed hydrolyses of aryl phosphates plotted versus Hammett σ values.

some *tert.*-butyl phenyl phosphates were there no measurable reactions; either these substrates failed to bind to the enzymes, or did so nonproductively. Smaller substituents ($-NO_2$, $-CH_3$, and $-COOH$ for example) at *ortho* positions exerted no significant effects on the reactions examined with either of the acid phosphatases discussed here.

The results reported above may be summarized as showing that for the acid phosphatases of potato and bovine milk the decomposition of the enzyme–phosphate compound is rate controlling. While in work with the former enzyme no evidence of effects by the substrates on preceding steps in the reaction sequence was found, for acid phosphatase from milk K_m was markedly influenced by the electronic nature of the phosphate hydrolyzed.

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