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pH DEPENDENCE AND SOLVENT ISOTOPE EFFECTS IN THE HYDROLYSIS OF PHOSPHOMONOESTERS BY HUMAN PROSTATIC ACID PHOSPHATASE

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Summary

The pH dependence of the human prostatic acid phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate and β-glyceryl phosphate has been studied over a wide range of pH and the values of K_m and V calculated with the aid of the Cleland HYPER program. The pH dependence of K_m shows the effect of substrate ionization: pK values of 5.6 and 6.4 are observed as for the respective values of free substrates. The pH dependence of both K_m and V for each substrate reveals the involvement of an ionizable group in the ES complex which is ascribed to a phosphohistidine-enzyme intermediate. The small deuterium solvent isotope effects which are observed on V are consistent with values observed for solvolysis of phosphoramidates. The measured data for K_m indicates limits on burst-titration experiments of prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2).

Introduction

Several lines of evidence indicate that the acid phosphatase-catalyzed hydrolysis (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) of phosphomonoesters follows the minimal scheme shown in Eqn. 1, where ES is an enzyme-substrate complex, K_s is its dissociation constant, ES′ is a covalent phosphoryl-enzyme intermediate, and P_1 and P_2 are

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} P_1 + ES' \xrightarrow[H_2O]{k_3} P_2 + E \qquad (1)$$

Abbreviation: NO_2PhP, *p*-nitrophenyl phosphate.

the alcohol (or phenol) and phosphate portions of the substrate, respectively. The isolation in essentially quantitative yield of phosphohistidine from an alkaline hydrolyzate of wheat germ acid phosphatase which had been stoichiometrically labeled by incubation with ^{32}P-labeled NO_2PhP or ^{32}P-labeled pyrophosphate ion [1,2] provides convincing evidence for the presence of a covalent intermediate ES′ in at least one acid phosphatase-catalyzed reaction. Less than stoichiometric amounts of phosphohistidine have also been isolated from alkaline hydrolyzates of rat liver [3] and human prostatic [4—6] acid phosphatases, and in unknown stoichiometry from an insoluble microsomal acid phosphatase [7]. Other evidence in support of the reaction scheme outlined in Eqn. 1 includes the observation of the expected ordered uni-bi kinetic scheme [8,9] and the observation of transphosphorylation when phosphate esters are hydrolyzed by prostatic acid phosphatase in the presence of high concentrations of alcohols [10]. The specific chemical nature of the rate-determining step of Eqn. 1 remains open to question. On the basis of a consideration of the structure of the early transition metal oxyanions which act as potent inhibitors representative plant and animal acid phosphatases we have proposed [11] that the transition state of the rate-determining step resembles a trigonal bipyramidal intermediate characteristic of a nucleophilic displacement reaction upon phosphorus. However, such a recognition does not by itself permit a distinction between an initial displacement reaction represented by the k_2 step, and a subsequent displacement reaction involving a molecule of water which is represented by the k_3 step.

In favorable instances studies of the pH dependence of reactions may provide information about changes in rate-determining steps and even provide initial guides to the possible nature of the groups involved. Studies with single isoenzymes of wheat germ acid phosphatase reveal that the rate-determining step changes with pH: a common value of V is seen at high pH even for substrates having widely varying leaving groups, while greatly varying V values are observed at low pH values [12]. In the case of the human prostatic enzyme a common value of V has been observed even at pH 5 with a variety of substrates having alkyl, aryl and even an acyl leaving group [13]. Some previous studies of the pH dependence of prostatic acid phosphatase have been described but they employed a single substrate or covered a limited pH range. Kilsheimer and Axelrod [13] graphically determined K_m and V over the pH range 3—6.7. In a communication unaccompanied by experimental data the effect of pH on K_m for the phosphatase-catalyzed hydrolysis of NO_2PhP at pH 3.2, 6.5 and 6.9 was described [14]. The change of K_m with pH was interpreted as indicating the ionization of some functional groups on the enzyme which had pH values of 3.3 and 6.7 and which were said to be involved in the binding of substrate to enzyme [14]. In the present report we describe the results of a study of the pH dependence of human prostatic acid phosphatase. The parameters K_m and V, calculated by statistically preferable methods [15], have been determined for two substrates over a wide pH range. In connection with this study we have also examined the solvent deuterium isotope effect on the enzymatic reaction. The results are interpreted in terms of a rate-limiting dephosphorylation reaction of an enzyme-bound phosphohistidine intermediate.

Materials and Methods

Materials. Homogeneous human prostatic acid phosphatase was obtained essentially by the method described by Ostrowski [16]. Deuterium oxide (99.5%) was purchased from Columbia Organic Chemicals Co. and ^2HCl (99% deuterium content) was obtained from Stohler Isotope Chemicals. Sigma 104 grade NO_2PhP and Grade II β-glyceryl phosphate (determined before use to have less than 0.04% phosphate ion impurity present) were obtained from Sigma Chem. Co., St. Louis. Other chemicals were of analytical grade.

Enzyme assay. The assay substrate solution was 5 mM NO_2PhP in assay buffer (0.10 M citrate/sodium citrate, pH 5.0). A 10 μl sample of enzyme solution appropriately diluted in assay buffer was added to 1.0 ml of substrate solution at 25°C. After 1 min the reaction was stopped by the addition of 2.0 ml of 0.4 M NaOH. The liberated *p*-nitrophenoxide ion was determined spectrophotometrically at 400 nm. A molar extinction coefficient $\epsilon = 18.3 \cdot 10^3\ l \cdot mol^{-1} \cdot cm^{-1}$ was used in the calculations [17]. Six to eight determinations were performed on each enzyme stock solution. One unit of acid phosphatase activity is defined as the amount required to produce 1 μmol of *p*-nitrophenol/min. Homogeneous enzyme has a specific activity of 280 units/mg. Protein concentrations were determined by the method of Lowry et al. [18] using crystalline bovine serum albumin as a standard.

pH dependence. The buffers used in determining the pH profile were 0.05 M glycine/HCl (pH 2.5—3.6), 0.05 M acetate (pH 3.6—5.6), 0.05 M succinate (pH 5.2—6.9), 0.025 M barbital (pH 6.9—9.0), and 0.05 M glycine/NaOH (pH 8.4—9.4). pH values were measured with a Radiometer model 26 pH meter. Seven substrate concentrations varying from approx. $(1/5) \times K_m$ to $5 \times K_m$ were used in the range of pH < 8. In order to maintain constant ionic strength I at all pH values the highest concentration of NO_2PhP and of β-glycerol phosphate at pH > 8 was for many determinations only approx. $1 \times K_m$; it has however been noted [15] that good kinetic constants can usually be obtained even when the range of substrate concentrations extends only to $\approx K_m$. Identical K_m and V values (within experimental error) were obtained even when the maximal substrate concentration could be extended to $2 \times K_m$ in the pH range 8—9. Triplicate determinations of the initial velocity at each substrate concentration were made at 25.0 ± 0.1°C using a discontinuous assay. The extent of reaction was always < 5%. For the pH profile with NO_2PhP as a substrate I was maintained at 0.15 M by the addition of NaCl. To 1 ml of NO_2PhP solution at the appropriate concentration was added 20 μl of enzyme solution (20 μg/ml; specific activity 240 units/mg). The reaction was stopped after 1 min by the addition of 2 ml of 0.4 M NaOH. The liberated *p*-nitrophenoxide ion was determined spectrophotometrically on a Gilford 2000 spectrophotometer at 400 nm.

For the pH profile with β-glycerol phosphate as a substrate a larger K_m necessitated the use of a higher value of I because the contribution by the substrate became too large to maintain I = 0.15 M. Because chloride and bromide at high (0.7—1.0 M) concentrations inhibited the reaction, citrate was employed to maintain I = 0.7. In addition to 25 or 50 mM buffer all solutions contained 0.1 M NaCl and sodium citrate as necessary to adjust I at the pH to 0.7 M.

Below pH 4.5 citrate caused some interference with color development and no effort was made to collect low pH data with this substrate. The extent of reaction was measured essentially by the method of Lowry and Lopez [19], taking advantage of the fact that molybdate is a potent competitive inhibitor of acid phosphatases [11,14]. The basis of the determination is the spectrophotometric quantitation of phosphomolybdic acid ($\epsilon = 4.3 \cdot 10^3\ l \cdot mol^{-1} \cdot cm^{-1}$ at 700 nm). To 0.5 ml of substrate solution was added 20 μl of enzyme solution (spec. act. 230 units/mg; protein concentration 18 μg/ml). The enzymatic reaction was stopped after 2 min by the addition of 2 ml of 4.0% (w/v) ammonium molybdate in 0.5 M sodium acetate (pH 4). The phosphomolybdic acid was reduced by the addition of 0.2 ml of 1% ascorbic acid and the color allowed to develop 30 min. The absorption was read using a Gilford 2000 spectrophotometer against a similar sample containing no enzyme. Calculation of the steady-state parameters K_m and V was carried out on a CDC 6500 computer with the program HYPER according to Cleland [15].

The pH vs. rate profile for the enzyme-catalyzed hydrolysis of NO_2PhP in ^2H_2O was determined in the same manner employed for the corresponding reaction in H_2O. Substrate solutions were made up in ^2H_2O and titrated to the appropriate p^2H using ^2HCl. The p^2H was determined with a glass electrode, applying the correction [20] $p^2H = pH_{meter} + 0.40$.

Results

pH dependence of the phosphatase-catalyzed reaction

The Michaelis-Menten parameters and associated error limits calculated by the use of the Cleland HYPER program [15] for the human prostatic acid phosphatase-catalyzed hydrolysis of NO_2PhP are presented in Table I. At the lower pH values V is remarkably independent of pH, and the slight reduction at very low pH is due to irreversible acid denaturation: exposure of the enzyme to pH values between 2.2 and 2.8 followed by readjusting the pH to 5.0 and measurement of activity showed that the enzyme is relatively slowly but irreversibly denatured at low pH and correction of the velocity data at low pH was necessary. Below pH 2 the enzyme is subject to a rapid and highly cooperative acid-induced denaturation process. Above pH 3 the data for V indicates a dependence upon an ionizable group with a dissociation constant K_a in the basic region. From the graphical presentation in Fig. 1 it is seen that the change in slope at the inflection point is from zero to minus one, consistent with the ionization of a single prototropic group. Because of the absence of interfering ionizations [21] the pK_a of this group may be readily estimated by graphical methods [22] to be 7.7—7.8.

The pH dependence of log V/K_m (Fig. 1) shows that the enzymatic hydrolysis of NO_2PhP is dependent on one ionizable group with a pK_a of approx. 5.7. The negative slope indicates [22] that this group is present in either the free enzyme or free substrate. The pH dependence of pK_m indicates the involvement of two groups having pK_a values of $\approx$5.6 and $\approx$7.9. The increase in slope (from -1 to 0) at pH 7.9 indicates that an ionizable group in the ES complex is important in the breakdown of ES to product. The decrease in slope by one unit at pH 5.6 indicates that this inflection in the pK_m versus pH curve is

TABLE I

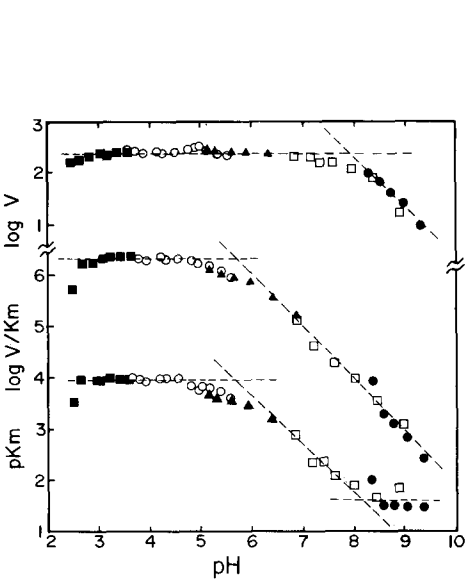
KINETIC CONSTANTS FOR THE PHOSPHATASE-CATALYZED HYDROLYSIS OF *p*-NITROPHENYL PHOSPHATE

Determined at 25.0 ± 0.1°C and I = 0.15 M. Error limits shown are those calculated by the Cleland HYPER program (see Materials and Methods) and should be taken as indications of precision of fit. V expressed in μmoles of phenolic product liberated per mg of protein per min.

Buffer	pH	K_m (mM)	V
Glycine	2.51	0.30 ± 0.01	158 ± 3
	2.66	0.11 ± 0.01	177 ± 5
	2.88	0.11 ± 0.01	199 ± 4
	3.06	0.11 ± 0.02	241 ± 12
	3.24	0.10 ± 0.01	231 ± 8
	3.43	0.11 ± 0.01	250 ± 9
	3.64	0.11 ± 0.01	271 ± 7
Acetate	3.60	0.11 ± 0.01	278 ± 7
	3.80	0.11 ± 0.01	264 ± 7
	3.95	0.12 ± 0.02	238 ± 12
	4.22	0.11 ± 0.02	267 ± 10
	4.34	0.11 ± 0.02	246 ± 11
	4.56	0.11 ± 0.01	254 ± 8
	4.81	0.15 ± 0.01	295 ± 13
	4.95	0.18 ± 0.01	301 ± 6
	5.04	0.16 ± 0.01	256 ± 4
	5.18	0.16 ± 0.01	252 ± 3
	5.39	0.18 ± 0.01	230 ± 9
	5.59	0.24 ± 0.03	219 ± 13
Succinate	5.16	0.20 ± 0.01	261 ± 3
	5.34	0.25 ± 0.02	260 ± 6
	5.62	0.28 ± 0.02	263 ± 5
	5.98	0.34 ± 0.01	259 ± 7
	6.39	0.62 ± 0.01	229 ± 2
	6.87	1.33 ± 0.05	243 ± 4
Barbital	6.89	1.63 ± 0.09	216 ± 4
	7.22	4.67 ± 0.23	198 ± 4
	7.40	4.20 ± 0.30	156 ± 8
	7.63	8.21 ± 0.51	163 ± 3
	8.02	12.3 ± 1.7	116 ± 8
	8.45	22.1 ± 3.0	79 ± 7
	8.98	13.7 ± 3.3	17 ± 2
Glycine	8.37	10.2 ± 1.2	99 ± 5
	8.58	31.8 ± 7.2	60 ± 1
	8.78	31.5 ± 3.6	40 ± 3
	9.03	34.8 ± 11.0	25 ± 1
	9.39	34.0 ± 9.0	9 ± 1

caused by groups situated in either the free enzyme or free substrate [22].

Because the pK_a values of NO_2PhP are known [23,24] to be 1.9 and 5.7 it appeared likely that the inflections at pH 5.6—5.7 in the pK_m and the log (V/K_m) curves were due to the ionization of substrate. To provide further evidence on this point the pH dependence of the enzymatic hydrolysis of β-glycerol phosphate was investigated. It was reasoned that in going from a relatively acidic substrate like NO_2PhP (with pK_2 = 5.7) to a less acidic one



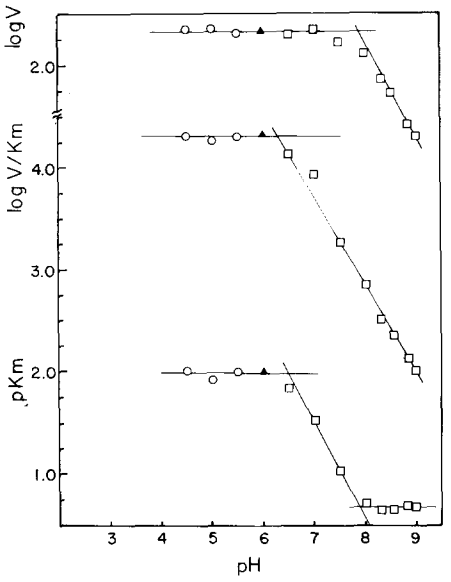


Fig. 1. Effect of pH on log V, log (V/K_m) and pK_m for the prostatic acid phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate. The buffers used were: ■, glycine/HCl; ○, acetate; ▲, succinate; □, barbital, and ●, glycine/NaOH; I = 0.15 M.

Fig. 2. Effect of pH on log V, log (V/K_m) and pK_m for the prostatic acid phosphatase-catalyzed hydrolysis of β-glyceryl phosphate. The buffers used were: ○, acetate; ▲, succinate and □, barbital; I = 0.7 M (see Materials and Methods).

like β-glycerol phosphate [25] (with pK_2 = 6.34) that there should be a corresponding shift in the inflections seen in the pK_m and the log (V/K_m) curves. Data for the β-glycerol phosphate reaction are given in Table II and illustrated in Fig. 2. As can be seen, the inflection in log V remains at pH 7.9 but the other inflections in the pK_m and log (V/K_m) curves are shifted upwards to 6.4 and 6.3, respectively. Again, the negative change in slope associated with these latter inflections is consistent with the ionization of a group on the substrate. With the exception of the predicted shifts, the shapes of the curves for NO_2PhP and β-glycerol phosphate are identical.

Correction of pH dependence for substrate ionization

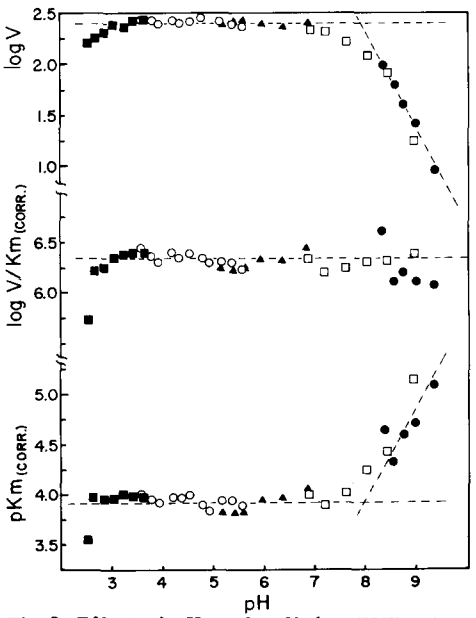
On the basis of the known ionization constants of the isolated substrates [23–25], the direction of the slope change, the shift in apparent pK_a values from substrate to substrate coupled with the corresponding shift in the inflections in the pK_m and the log (V/K_m) curves, as well as experience with a related enzyme [12], the prostatic enzyme can be assigned a specificity for the monoanionic form of the substrate. Such a conclusion has also been reached by Alvarez [26] for the case of potato acid phosphatase. At each pH value the actual proportion of phosphate ester monoanion present may be calculated and this proportion used to correct the apparent K_m values (and in turn the V/K_m ratios). Figs. 3 and 4 present the pH dependence curves for the prostatic acid phosphatese-catalyzed hydrolysis of NO_2PhP and β-glycerol phosphate,

TABLE II

KINETIC CONSTANTS FOR THE PHOSPHATASE-CATALYZED HYDROLYSIS OF β-GLYCEROPHOSPHATE

Determined at 25.0 ± 0.1°C and I = 0.7 M. Error limits shown are those calculated with the Cleland HYPER program and should be regarded only as indications of precision of fit. V is expressed in μmol of phosphate ion liberated per mg of protein per min.

Buffer	pH	K_m (mM)	V
Acetate	4.50	9.8 ± 0.9	223 ± 3
	5.00	12.3 ± 1.5	223 ± 3
	5.50	9.5 ± 0.7	203 ± 4
Succinate	6.00	9.8 ± 0.3	221 ± 8
Barbital	6.55	13.9 ± 1.2	197 ± 5
	7.00	30.0 ± 2.5	240 ± 10
	7.50	92 ± 3.5	164 ± 12
	8.00	192 ± 28	136 ± 20
	8.31	230 ± 15	74 ± 6
	8.55	245 ± 35	56 ± 5
	8.86	197 ± 28	26 ± 3
	9.00	198 ± 27	20 ± 3



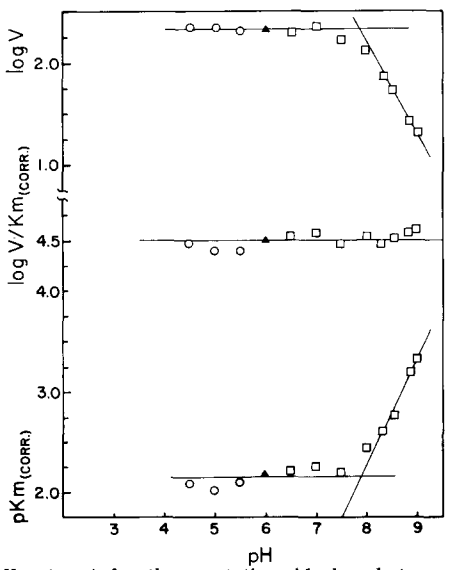


Fig. 3. Effect of pH on log V, log (V/K_m (corr)) and pK_m (corr) for the prostatic acid phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate. The substrate monoanion concentration at each pH was calculated using pK_2 = 5.7 [23,24]; conditions and buffers as for Fig. 1.

Fig. 4. Effect of pH on log V, log (V/K_m) (corr)), and pK_m (corr) for the prostatic acid phosphatase-catalyzed hydrolysis of β-glyceryl phosphate. The substrate monoanion concentration at each pH was calculated using pK_2 = 6.34 [25]; conditions and buffers as for Fig. 2.

TABLE III

HUMAN PROSTATIC ACID PHOSPHATESE-CATALYZED HYDROLYSIS OF *p*-NITROPHENYL PHOSPHATE IN WATER AND DEUTERIUM OXIDE

Determined at 25.0 ± 0.1°C and I = 0.15 M. The error limits shown are obtained from Cleland HYPER program. The values of p^2H were determined using the glass electrode correction $p^2H = pH_{meter} + 0.40$. Data for the reaction in H_2O taken from Table I.

Buffer	pH	K_m (mM)	V	p^2H	K_m (mM)	V	$V(H_2O)$ / $V(^2H_2O)$	$K_m(H_2O)$ / $K_m(^2H_2O)$
Glycine	2.88	0.11 ± 0.01	199 ± 4	2.85	0.39 ± 0.05	144 ± 6	1.3	0.3
Acetate	3.95	0.12 ± 0.02	238 ± 14	3.94	0.14 ± 0.01	216 ± 3	1.1	0.9
	5.04	0.16 ± 0.01	256 ± 4	504	0.13 ± 0.01	215 ± 4	1.2	1.3
Succinate	5.98	0.34 ± 0.01	259 ± 7	5.98	0.21 ± 0.01	244 ± 2	1.1	1.6
Barbital	7.40	4.2 ± 0.30	156 ± 8	7.41	3.6 ± 0.3	154 ± 3	1.0	1.2
	8.02	12.3 ± 1.7	116 ± 8	8.02	14.4 ± 1.0	128 ± 4	0.9	0.9
	8.45	22.1 ± 3.0	79 ± 7	8.40	43.1 ± 13.2	111 ± 22	0.7	0.5

respectively, after correction for the ionization of the substrate. The log V versus pH curves are of course unchanged. They exhibit an identical dependence upon an ionizable group of $pK_a \approx 7.8$ and an almost complete independence of pH in the range 3—7 (particularly after taking into account the slow acid denaturation at very low pH). The graphs of log (V/K_m (corr)) and of pK_m (corr) for NO_2PhP and β-glycerol phosphate (Figs. 3 and 4, respectively) more clearly illustrate the significant ionization behavior in the enzymatic reaction. The positive one-unit change in slope of the pK_m (corr) curve for both substrates at approx. pH 7.8 represents the ionization of the reaction intermediate reflected in the log V plot. The log (V/K_m (corr)) versus pH plot, representing the sum of the log V and the pK_m (corr) plots, is virtually constant for both substrates over the entire range of pH. A detailed analysis of the pH dependence of enzymatic reactions proceeding via two intermediates has been described [27].

Effects of ^2H_2O

The parameters V and K_m for the prostatic acid phosphatase-catalyzed hydrolysis of NO_2PhP in 99% ^2H_2O are given in Table III. Noteworthy is the absence of any significant solvent isotope effect upon V over a wide range of acidities.

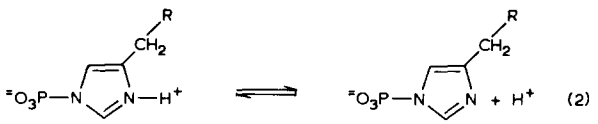
Discussion

It is common in published studies on acid phosphatases to describe the pH dependence of the enzyme with a pH profile measured using a single fixed substrate concentration, often NO_2PhP (compare for example Fig. 3 in ref. 28 and Fig. 2 in ref. 29). Consideration of the present data as illustrated in Figs. 1—4 makes it clear that the sharp "pH optima" commonly observed in the region of pH 4—5 are artifacts resulting from the combined pH dependence of the enzyme and the ionization of the substrate (which in turn affects the apparent K_m value). Because V is in fact almost completely independent of pH over the range 3—7 it is clear that the shape of the optima observed for this and similar acid phosphatases will be markedly affected by the concentration and pK_a of the substrate employed. The use of high fixed substrate concentrations should result in relatively broad plateaus in the pH profile (cf. ref. 28), particularly if less acidic (and thereby more typical) phosphomonoester substrates are employed.

Many theoretical treatments of the pH dependence of enzymatic reactions include no mention of the effects of substrate ionization or explicity assume that substrate ionizations are not involved in the systems considered. There are few examples in the literature where explicit consideration has been given to the effect of substrate ionization upon the apparent pH dependence of enzymatic reactions; there are seemingly more examples where a failure to consider a substrate ionization step resulted in a misleading interpretation. Thus, studies with NO_2PhP substrate of the pH dependence of an acid phosphatase from *Neurospora* led one author [30] to conclude that an inflection in the log (V/K_m) curve at pH 5.5 suggested the presence "of a histidine residue in or near the active site of the enzyme", when in fact the inflection was due to the

ionization of substrate. Similarly, it was concluded that substrate is bound to prostatic acid phosphatase by two groups present in the active site of the enzyme and having pK_a values of 3.3 and 6.7 [14]; the present results show that those estimates in fact represented acid-catalyzed denaturation and substrate ionization, respectively.

In view of the identification of phosphohistidine as a stoichiometric intermediate in reactions catalyzed by wheat germ acid phosphatase [1,2] as well as its implication by results in related system [3,6,10], it seemed likely that the rate-limiting step of Eqn. 1 was the dephosphorylation of a covalent phosphoenzyme intermediate. If so, then the same phosphoenzyme intermediate should be formed by reaction of either NO_2PhP or β-glycerol phosphate, consistent with the identity of the V values and the pK values which are seen in the log V versus pH curves. In other words, for both substrates the value of V is the same, and the pK_a corresponding to the ionization of the postulated reaction intermediate is also independent of the leaving group (*p*-nitrophenol or glycerol). (Similar behavior has been observed in the high pH region with homogeneous isoenzymes of wheat germ acid phosphatase [12].) Further data support this interpretation. It is known from photooxidation studies [31] and from studies of the reaction with diazonium reagents [32] that at least one histidine residue is essential for the activity of prostatic acid phosphatase. It is also known that the pK_a values for ionization of τ- and π-phosphohistine * are 6.4 and 7.3, respectively [33,34]. The unprotonated form of phosphohistidine is much less reactive than the zwitterionic or fully protonated forms [32] and this is consistent with the fall-off in the log V plot seen above pH 7 (Figs. 1—4). Thus, using the example of τ-phosphohistidine, the ionization process evidenced in the decreasing V values would be represented by Eqn. 2, where R represents the remainder of the protein.



(2)

The protonated phosphohistidine species shown in Eqn. 2 can undergo subsequent decomposition either via a metaphosphate species (PO_3^-) or by nucleophilic attack of a molecule of water [11]. In contrast to a mechanism involving a general base-catalyzed transfer of a solvent-derived proton, it would be expected that there should be no significant deuterium solvent isotope effect on the proposed rate-limiting step V (k_3 of Eqn. 1). This is in fact the case (Table III), with V showing effects between 1.3 and 0.7 even at the extremes of acidity studied. It is of interest in this regard that the solvent deuterium isotope effects observed for the solvolysis of phosphoramidic acid and of *N*-(*p*-chlorophenyl)amidophosphoric acid are 1.20 and 0.76, respectively [35].

The kinetic equations relating the usual Michaelis-Menten parameters to the

* These have also been termed 3- and 1-phosphohistidine in the biochemical literature and also (incorrectly) N'-phosphoryl histidine. The τ and π nomenclature is that recommended by the I.U.B.

individual rate constants of Eqn. 1 are well known [36], and may be summarized by Eqns. 3—5, where K_s is the true substrate

$$V = k_{cat}[E]_0 \tag{3}$$

$$k_{cat} = (k_2/k_3)/(k_2 + k_3) \tag{4}$$

$$K_m = K_s[k_3/(k_2 + k_3)] \tag{5}$$

dissociation constant and $[E]_0$ is the enzyme concentration. For substrates where $k_2 >> k_3$ we have $V = k_3[E]_0$, so that (at fixed enzyme concentrations) the pH dependence of V is a consequence of the pH dependence of k_3, here postulated to be the hydrolysis of the phosphoenzyme intermediate. Since the molecular weight of the homogeneous enzyme is known [37] to be $1.02 \cdot 10^5$ we may estimate k_3 with the aid of Table I and Eqns. 3 and 4 at the pH values 4.5, 8.5 and 9 to be 425, 134 and 42 s^{-1}, respectively.

The pH dependence of K_m may also be readily interpreted in terms of the postulated reaction mechanism. A bend in the pK_m versus pH curve which is convex to the pH axis (the slope increasing with pH) corresponds to the ionization of an intermediate which is in fact the reactant in the slow step of the overall transformation [27]. This is evidenced in an identical way in the case of both substrates as a change from —1 to 0 slope in the region of pH 7.8 (Figs. 1 and 2). From Eqn. 5 and the condition that $k_2 >> k_3$ we see that although K_s increases (substrate binding becomes poorer with increasing pH), the ratio k_3/k_2 decreases due to the ionization of the phosphohistidine intermediate to its unreactive fully ionized form (Eqn. 2), thus resulting in a change in slope at pH 8. By correcting for the monoanionic specificity of the enzyme we eliminate the apparent pH dependence of K_s term and as a result the change in slope due to the ionization of the intermediate of the slow step is revealed even more clearly (Figs. 3 and 4). As required by such a reduction in rate of the k_3 step under conditions where $k_2 >> k_3$, the K_m (corr) value becomes smaller (Figs. 3 and 4 *.

Finally, the present results have important implications with respect to attempts to conduct burst-titration [36] experiments of prostatic acid phosphatase [3,4,10]. The amplitude B in such burst-titration experiments is given by Eqn. 6 where $[E]_0$ is the stoichiometric enzyme concentration

$$B = [E]_0 \times \frac{[k_2/(k_2 + k_3)]^2}{[1 + (K_m/[S]_0)]^2} \tag{6}$$

and $[S]_0$ is the substrate concentration. Under conditions where $k_2 >> k_3$ and $[S]_0 >> K_m$ the amplitude of the burst is equal to the stoichiometric enzyme concentration. Choosing as an example [10] pH 8.1 where K_m = 15 mM (Table I), and $[NO_2PhP] = 3.1 \cdot 10^{-4}$ M (calculated using a molecular weight of 371 g/mol for the hexahydrate), the amplitude B of the theoretical burst would be expected to be 0.04% of the stoichiometric enzyme concentration and thus experimentally unobservable. Even with 2.1 mM substrate [10] at pH 8.5

* Data obtained for substrate analogs suggests K_s may be approx. 0.1—0.01 M; if so this suggests that k_2/k_3 may be in the range of 10^2—10^3. Further studies on this point are underway.

(where $K_m \cong 25$ mM) a theoretical burst of only 6% × $[E]_0$ would be expected to result. Research is being continued on this interesting problem to resolve this seeming contradiction regarding the apparent observation of a burst (Röhm, K.-H., Van Etten, R.L., unpublished results).

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