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ACID PHOSPHATASE FROM RAT LIVER

STUDIES ON THE ACTIVE CENTER

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SUMMARY

Studies on crystalline acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) present further evidence for one active site by spectrophotometric experiments, and the finding of one histidine residue per molecule by amino acid analysis. The technique of dye-sensitized photooxidation with methylene blue shows that the histidine residue is important for catalytic activity. [3-^{32}P]Phosphohistidine was isolated from alkaline hydrolyzates of crystalline acid phosphatase that had been incubated with $^{32}P_i$.

INTRODUCTION

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) forms peptide-bound *O*-phosphorylserine[1-3]. In the case of acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2), however, GREENBERG AND NACHMANSOHN[4] suggested that an amino acid residue other than serine is phosphorylated. BOYER *et al.*[6], DELUCA *et al.*[7] and KREIL AND BOYER[8] discovered a protein-bound phosphohistidine, formed in mitchondria during phosphate participation in oxidative phosphorylation, which implicated succinate thiokinase. More recently phosphohistidine has been found with nucleoside diphosphokinase[9].

Crystalline acid phosphatase obtained from rat liver[5] was previously reported to have a Michaelis–Menten type hyperbolic curve for kinetic studies. This suggested a single active site per molecule.

Spectrophotometric methods[10,11] have been used to show that alkaline phosphatase is reversibly phosphorylated during the hydrolysis cycle, and that under optimal conditions the rate determining step is one common to all substrates, the dephosphorylation of phosphoryl–enzyme. The principle of the method is as follows. If the rate of phosphorylation of the enzyme exceeds the steady state rate, an initial rapid liberation of the alcohol moiety of the substrate would be expected, equal at maximum to the number of active sites. If, on the other hand, phosphorylation is rate-limiting, or if the mechanism does not involve a phosphoryl-enzyme

intemediate, no "burst" of alcohol will be observed. In any event, lack of a burst will preclude a rate-limiting dephosphorylation step, which necessarily implies rapid phosphorylation.

This report describes further evidence for one active site by the spectrophotometric experiments, and the finding of one histidine residue per molecule. The technique of dye-sensitized photooxidation with methylene blue shows that this histidine residue is important for catalytic activity. [3-^{32}P]Phosphohistidine was isolated from alkaline hydrolyzates of crystalline acid phosphatase which had been incubated with $^{32}P_i$.

MATERIALS AND METHODS

Sephadex G-25 was purchased from Pharmacia, Inc. Methylene blue was purchased from Fisher Scientific Co. and crystallized from ethanol. *p*-Nitrophenyl phosphate was obtained from Sigma. $^{32}P_i$ was obtained from New England Corp. and purified by chromatography according to the method of BOYER AND BIEBER[12]. 3-Phosphohistidine was synthesized according to HULTQUIST *et al.*[13].

Radioactivity was measured in solution according to BRAY[14], with a Packard Tri-Carb liquid scintillation spectrometer, Model 3375. Histidine was determined by the diazobenzene sulfonic acid method according to ZETTERQVIST AND ENGSTRÖM[15]. The phosphohistidine content was calculated from the difference between the histidine value obtained after hydrolysis in 1 N HCl, for 5 min at 100°, and the value obtained without hydrolysis. Orthophosphate was determined according to BARTLETT[16]. Acid-labile phosphate was determind as orthophosphate, after treatment of the sample with 0.5 M H_2SO_4 for 30 min at 20°. Crystalline acid phosphatase was made by the method reported previously[5]. Protein concentration was estimated by the biuret method[17], or by measuring the ultraviolet absorbance at 278 mμ, assuming the specific extinction coefficient of crystalline enzyme, $E^{1\%}_{1cm}$, of 6.18. The molecular weight of the enzyme was assumed to be 100 000 as previously published[5].

Enzyme activity was estimated by the method used in the previous paper[5]. Enzyme was incubated for 10 min at 37° with 0.1 M sodium acetate buffer (pH 5.0), and 2.54 mM *p*-nitrophenyl phosphate as substrate, in a final volume of 0.5 ml. The reaction was started by the addition of enzyme and stopped with 1.5 ml of 0.25 M NaOH. The absorbance of the liberated *p*-nitrophenol was read at 410 mμ. One unit of enzyme catalyzed the hydrolysis of 1 μmole of *p*-nitrophenyl phosphate per min. Specific activity is expressed as units per mg of protein.

Studies to determine spectrophotometrically the number of active sites were performed by a modification of the method of Fife[10]. The formation of *p*-nitrophenol was followed with a Gilford Model 2000 spectrophotometer at 410 mμ with the cell compartment thermostatically controlled at 25°. Recording of absorbance *vs.* time curves was started within 5 or 6 sec after initiation of the reaction. Extrapolation of the absorbance *vs.* time curves to zero time (Fig. 1) gave absorbance values from which the concentration of *p*-nitrophenol released in an instantaneous burst was calculated. The concentration of *p*-nitrophenol in the burst is assumed to be equal to the concentration of active sites on the enzyme[11]. The reaction appears to be zero order in substrate at pH 8.1.

Amino acid analysis were performed with a Beckman Spinco amino acid

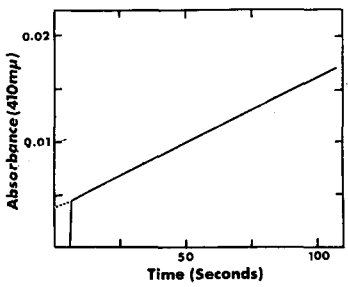


Fig. 1. Acid phosphatase catalyzed hydrolysis of *p*-nitrophenyl phosphate at 25°. The reaction mixture consisted of 22.3 μg of crystalline enzyme and 93 μM of *p*-nitrophenyl phosphate in a total volume of 1 ml in Tris–acetate buffer. I = 0.1, pH 8.1. The reaction was started by the addition of 50 μl of enzyme solution at 25°. The recorder of the Gilford 2000 spectrophotometer was adjusted to the full scale of 0.1 absorbancy and the chart speed of 2 inches/min.

analyzer, Model 120 C. Crystalline enzyme was dialyzed exhaustively against distilled water and hydrolyzed in 6 M HCl at 110° for 21, 48, and 70 h in sealed evacuated tubes[19]. Tryptophan was determined spectrophotometrically by the method of GOODWIN AND MORTON[20].

Photooxidation of the enzyme was done in buffers indicated, with methylene blue, 0.1 mg/ml, at 25°. A 300-W slide projector was used as a light source. The lens of the projector was placed 30 cm from the sample and focused to give the most concentrated light possible.

Isolation of [3-^{32}P]phosphohistidine from an alkaline hydrolyzate of the ^{32}P-labeled crystalline enzyme was done by a modification of the method of WÅLLINDER *et al.*[21]. Crystalline enzyme, 84 μg in 1.1 ml of 0.1 M Tris–acetate buffer (pH 7.4), was incubated with 0.25 ml of 3.1 mM $^{32}P_i$ ($3.3 \cdot 10^7$ counts/min per nmole) for 5 min at 25°. Incubation was interrupted by the addition of 0.15 ml of 1 M NaOH, followed at 1-min intervals by 0.05 ml of 10% sodium lauryl sulfate and 0.3 ml of 1 M Tris–acetate buffer (pH 7.4). 1 mg of crystalline bovine serum albumin was added to the solution. The incubation mixture was chromatographed on a column (2.5 cm × 26 cm) of Sephadex G-25 in 0.025 M Tris–acetate buffer (pH 8.5) containing 0.25% sodium lauryl sulfate (Fig. 4). The protein fraction was precipitated with 4 vol. of acetone, allowed to stand for 2 h at −25° and collected in a glass tube by centrifugation. The precipitate was dried under reduced pressure at 25° and treated with 1 ml of 3 M KOH for 3 h at 100° in the same sealed glass tube[6]. The hydrolyzate was diluted with 9 ml of water and chromatographed, as described in the legend of Fig. 5, on a column of Dowex 1, together with 8.8 μmoles of synthetic 3-phosphohistidine. Fractions were pooled, as can be inferred from the legend of Fig. 5, diluted with 10 vol. of water, and rechromatographed on a separate column of Dowex 1 (Fig. 6). The fractions containing the reference substance were pooled. The pool was concentrated, desalted on Sephadex G-10, and subjected to paper chromatography: (a) 0.1 M aqueous K_2CO_3 –ethanol (3.5:6.5, by vol.), and (b) isopropanol–ethanol–water–triethylamine (30:30:39:1, by vol.). After the chromatograms were dried, phosphohistidine was detected according to the method of ROSENBERG[22]. Radioautography of the papers was carried out with Ilford Gold Seal X-ray film (exposure time 6 days).

TABLE I

EFFECT OF SUBSTRATE CONCENTRATION ON THE NUMBER OF ACTIVE SITES

The concentration of *p*-nitrophenol in a burst was determined as described in the text. The reaction mixtures consisted of various concentrations of *p*-nitrophenyl phosphate, and 22.3 μg of crystalline enzyme, in a total volume of 1.0 ml Tris–acetate buffer (pH 8.1), $I = 0.1$. Five determinations were made under each experimental condition. Standard deviations of the concentrations of *p*-nitrophenol in bursts were within 6% of the means.

Substrate concentration (mM)	*Number of active sites*
0.0435	0.801
0.0927	1.04
0.142	1.02
0.201	1.06

RESULTS

Spectrophotometric determination of the number of active sites

Although the measurements were done at an alkaline pH, the results (Table I) suggest that the number of active sites is one, under the assumption that all the enzyme molecules are active. These results can be interpreted on the basis of the usual enzyme–substrate saturation phenomena.

TABLE II

AMINO ACID COMPOSITION OF CRYSTALLINE ACID PHOSPHATASE

Amino acid	*Residues/mole*	
	Experimental	*Nearest integer*
Lysine	64.6	65
Histidine	0.98	1
Arginine	31.3	31
Aspartic acid	94.3	94
Threonine*	65.7	66
Serine*	79.8	80
Glutamic acid	100.9	101
Proline	39.6	40
Glycine	95.9	96
Alanine	143.2	143
Half-cystine***	1.67	2
Valine	58.3	58
Methionine	7.64	8
Isoleucine**	53.6	54
Leucine	66.9	67
Tyrosine*	32.1	32
Phenylalanine	33.7	34
Tryptophan§	3.91	4
Total		976

* Corrected for decomposition by extrapolation of the values obtained at 21, 48 and 70 h back to zero time.

** The value obtained after 70 h hydrolysis was used.

*** Estimated as cysteic acid by performic acid oxidation.

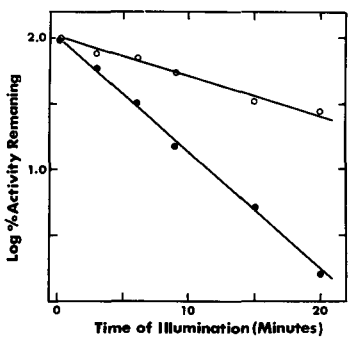
§ Determined spectophotometrically[13].

Amino acid analysis of crystalline acid phosphatase

The amino acid composition of the enzyme is shown in Table II. Values for those amino acids that were stable to acid hydrolysis were derived by taking the average of the three values obtained at the times shown in Table II. The corresponding values for serine, threonine and tyrosine were corrected for decomposition by extrapolation to zero time, and the values obtained in the 70-h analysis were used for isoleucine. Half-cystine was estimated as cysteic acid by performic acid oxidation. Tryptophan was determined spectrophotometrically[20]. The number of amino acid residues was calculated based upon the molecular weight of 100 000. The minimal molecular weight of acid phosphatase, obtained by summation of the residue weight times the number of residues for each amino acid, is 101 760. It is noted in Table II that the number of residue of histidine per molecule is one.

Evidence of histidine oxidation as the cause of inactivation

When a solution of acid phosphatase was photooxidized in the presence of methylene blue, the rate of loss of activity was first order with respect to active



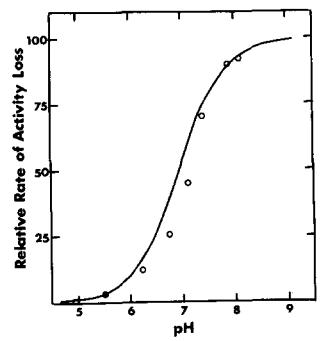


Fig. 2. First-order plot of the loss of activity as a function of length of time of illumination. Conditions were: crystalline enzyme 12 μg/ml, and methylene blue 0.1 mg/ml in Tris–acetate buffer, I = 0.1, pH 7.4 (●—●); or sodium phosphate buffer, I = 0.1, pH 7.4 (○—○).

Fig. 3. Relative rate of activity loss by photooxidation as a function of pH at 25° (○). Buffers were 0.1 ionic strength: sodium acetate pH 4.5–6.2; Tris–acetate, pH 6.75–9.0. Illumination time was 5 min. Otherwise, conditions were as described in the legend to Fig. 2. The curve is calculated per cent ionization of imidazole.

enzyme. Fig. 2 shows a plot of the logarithm of per cent residual activity as a function of time. The figure shows that photooxidation is higher in Tris–acetate buffer than in phosphate buffer which might be expected to protect the active site. Measurements of the first-order rate of loss of activity in a series of buffered solutions from pH 4.5 to 9.0 gave the results shown in Fig. 3. The rate of loss of activity was markedly dependent upon the H^+ concentration. Open circles indicate the measurements and the curve is calculated per cent ionization of imidazole. Amino acid analysis of photooxidized acid phosphatase indicated that histidine was the only amino acid that was destroyed to any appreciable extent. Cysteine, tyrosine and methionine remained unaltered compared with control hydrolyzates. The destruction of histidine by photooxidation seemed to follow an all-or-none pathway; that is, a 50% inactivated enzyme preparation lost about 50% of the total histidine.

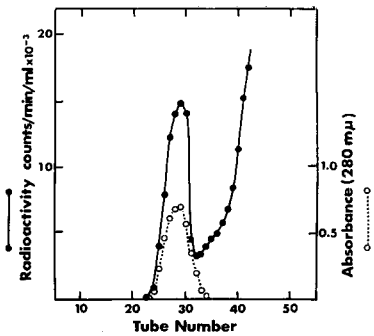


Fig. 4. Separation of ^{32}P-labeled acid phosphatase on Sephadex G-25. Crystalline enzyme (8.4 μg) was labeled by $^{32}P_i$ as described in the text. The sample with 1 mg bovine serum albumin was placed on a column, 2.5 cm × 26 cm, of Sephadex G-25 equilibrated with 25 mM Tris–acetate (pH 8.5), containing 0.25% sodium lauryl sulfate. 2-ml fractions were collected.

Isolation of [3-^{32}P]phosphohistidine

Crystalline enzyme was labeled by $^{32}P_i$ as described previously. The sample with 1 mg bovine serum albumin was placed on a column of Sephadex G-25. The chromatography on the Sephadex G-25 column of the incubation mixture showed that the protein peak was coincident with the peak of radioactivity (Fig. 4). The ^{32}P-labeled enzyme was stable to alkali but labile to acid: exposure to pH 1.5, adjusted by 0.3 M trichloroacetic acid, for 1 min at 100° liberated all radioactivity as $^{32}P_i$; and exposure to pH 10.0 adjusted by 0.1 M KOH for 60 min at 60° liberated no radioactivity. A first-order rate constant for the acid-catalyzed hydrolysis of the ^{32}P-labeled enzyme at pH 3.8 and 80° of 0.02–0.03 per min was obtained. This is in

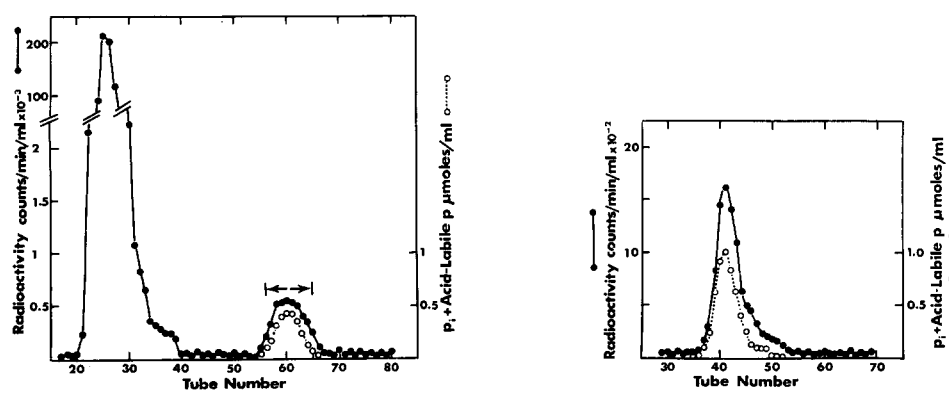


Fig. 5. Chromatography on Dowex 1-X8 (bicarbonate form) of an alkaline hydrolyzate of 84 μg of ^{32}P-labeled enzyme. Column, 1.6 cm × 26 cm. Elution was performed with a linear gradient of 0.2–0.8 M $KHCO_3$. Total elution volume was 400 ml, collected in fractions of 4 ml. Synthetic 3-phosphohistidine (8.8 μmoles) was added to the alkaline hydrolyzate as a reference substance. Fractions containing 3-phosphohistidine were pooled as indicated in the figure. This sample was rechromatographed as described in Fig. 6.

Fig. 6. Rechromatography on Dowex 1-X8 (bicarbonate form) of 3-phosphohistidine-containing fractions from the chromatography in Fig. 5. Pooled fractions were diluted with 10 vol. of water and chromatographed on a column, 0.8 cm × 14.5 cm, of Dowex 1-X8. Elution was performed with a linear gradient of 40 ml of 0.2 M to 40 ml of 0.8 M $KHCO_3$. 1-ml fractions were collected.

good agreement with that of synthetic 3-phosphohistidine[13]. When an alkaline hydrolyzate of ^{32}P-labeled enzyme was applied to a column of Dowex 1, radioactive material was eluted at the second peak in Fig. 5 with the reference substance (8.8 μmoles 3-phophohistidine) added. On rechromatography, radioactive material was eluted parallel with the reference 3-phosphohistidine added (Fig. 6). The first peak in Fig. 5 was composed of $^{32}P_i$. The identity of the ^{32}P-labeled component with [3-^{32}P]phosphohistidine was finally established by paper chromatography. The stoichiometric relation of [3-^{32}P]phosphohistidine isolated to the enzyme is calculated with the following assumption. If it is assumed that the incorporation of $^{32}P_i$ to the enzyme did not depend on an exchange reaction but on binding of $^{32}P_i$ to a previously unphosphorylated histidine molecule, the specific activity of [3-^{32}P]phosphohistidine isolated was the same as that of the $^{32}P_i$ added. The molecular weight of the enzyme is 100 000. 84 μg of the enzyme corresponds to 0.84 nmole. The total amount of [3-^{32}P]phosphohistidine isolated after rechromatography (Fig. 6) was 0.24 nmole which was calculated from the total amount of radioactivity isolated as 3-phosphohistidine (4425 counts/min) and the specific radioactivity of P_i ($1.8 \cdot 10^4$ counts/min per nmole). From these results it appears that [3-^{32}P]phosphohistidine acounts for 29% of the amount of the enzyme used.

DISCUSSION

The prior kinetic study[5] showed a Michaelis–Menten type hyperbolic curve for crystalline acid phosphatase with *p*-nitrophenyl phosphate as substrate. This is consistent with either a single active site or multiple sites without interaction. Spectrophotometric phosphorylation experiments in this report have brought additional evidence for a single active site. The photooxidation of histidine is pH dependent[23,24], the curve being sigmoidal and having an inflection near pH 7.0. The amino acids tryptophan, tyrosine and methionine, which are all susceptible to photooxidation, do not show this pH dependence[23,24]. Thus, it is significant that acid phosphatase, when photooxidized with methylene blue, gave a sigmoidal profile which was almost identical with that for the photooxidation of histidine. The essentiality of histidine residue for the enzyme activity was further strengthened by studies of the stability towards alkali and the acid-lability of the ^{32}P-labeled enzyme. However, this evidence would not exclude any change in the physical properties of the enzyme after photooxidation, were histidine situated in another site than the active center. Concerning the isolation of [3-^{32}P]phosphohistidine, the recovery of [3-^{32}P]phosphohistidine from the initial amount of the enzyme was 29%. The recovery in each chromatography was about 80%, so that the loss of the recovery of [3-^{32}P]phosphohistidine amounted to approx. 50% through the three chromatographic steps. The remaining 20% of the loss of the recovery was accounted for by the alkaline hydrolysis and by the condition of having stopped the reaction of the enzyme with $^{32}P_i$. The rather low yield of phosphohistidine was explained in some part by the course of experimental procedures. However, the yield of phosphohistidine and the lack of evidence of catalytically competent phosphorylated enzyme do not rule out the possibility that these observations are a result of a non-essential side-reaction. Further experiments are now being undertaken to solve this problem.

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