*Biochemica et Biophysica Acta*, 568 (1979) 370—376


BBA 68762

# AN ESSENTIAL CARBOXYLIC ACID GROUP IN HUMAN PROSTATE ACID PHOSPHATASE

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(Received November 15th, 1978)

*Key words: Acid phosphatase; Woodward's reagent K; Tartrate inhibition; Ethylamine analysis; Active site residue; Essential carboxyl group; (Human prostate)*

## Summary

Treatment of homogenous human prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) with low concentrations of Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate) leads to a rapid loss of enzymic activity. The rate of inactivation of the enzyme is reduced in the presence of the competitive inhibitors phosphate and L-(+)-tartrate, but not in the presence of non-inhibitory D-(—)-tartrate. Measurement of the ethylamine produced upon hydrolysis of enzyme modified in the presence of D- and of L-tartrate permitted the quantitative estimation of the number of carboxylic acid residues at the active site. The data indicate that two carboxyl groups per (dimeric) enzyme molecule are essential for catalytic activity. It is proposed that one function of the active site carboxyl group may be to protonate the leaving alcohol or phenol portion of the phosphomonoester substrate during the formation of the covalent phosphoenzyme intermediate.

## Introduction

Recent studies on acid phosphatases (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) have provided an increasingly detailed picture of the mechanism of action of the $50 \cdot 10^3$ molecular weight monomeric isoenzyme from wheat germ [1] and the $90 \cdot 10^3$ molecular weight

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dimeric [2,3] isoenzymes from mammalian sources. The enzymes follow the kinetic scheme shown in Eqn. 1.

$$E + S \overset{K_S}{\rightleftharpoons} E \cdot S \overset{k_2}{\underset{ROH}{\searrow}} E\text{-}P \underset{-H_2O}{\overset{+H_2O}{\underset{k_{-3}}{\rightleftharpoons}}} E \cdot P_i \rightleftharpoons E + P_i \quad (1)$$

In this scheme the Michaelis complex formed from the enzyme and a phosphate ester substrate undergoes a reaction $k_2$ with the loss of the alcohol or phenol portion of the substrate, which results in the formation of a covalent phosphoenzyme intermediate E-P. The stoichiometric formation of a phosphoenzyme intermediate following incubation of substrate with wheat germ acid phosphatase [1,4] and a near-stoichiometric labeling of human prostatic acid phosphatase [5,6] has been established to occur with $\tau$-phosphohistidine as the covalent intermediate. Experiments with transition-state analogs suggest that the breakdown (and by inference the formation) of the phosphoenzyme intermediate occurs by an $S_N2(P)$ mechanism [7] and this reaction is presumably facilitated by coordination of the phosphomonoester with an essential active site arginine residue [8]. The hydrolysis of this phosphoenzyme intermediate is the rate-limiting step in the overall catalytic sequence of reactions catalyzed by human prostatic acid phosphatase [6,9] and (at high pH values) by wheat germ acid phosphatase [10], so that $k_2 > k_3$. Recent mass spectroscopy and $^{31}P$ NMR spectroscopy studies have established that human prostatic acid phosphatase catalyzes a phosphate oxygen-water exchange and have further shown that $k_3 >> k_{-3}$ [11].

Among the outstanding mechanistic problems which remain is the catalytic basis of the rapid initial displacement reaction $k_2$ during which the enzyme becomes phosphorylated and the alcohol or phenol portion of the molecule is released. A comparison of the pH-dependent steady-state rate of hydrolysis of *p*-nitrophenylphosphate and of $\beta$-glycerylphosphate catalyzed by human prostatic acid phosphatase [9] shows the rates to be virtually identical over the pH range 3—7, despite the approximately $10^7$ difference in acidities of the leaving groups. Such an identity in rates (at one pH) had been previously noted with a variety of other substrates [12]. We now know that the identity in rates is due to the fact that the rate-limiting step is the dephosphorylation of the phosphoenzyme, but the fact that $k_2 >> k_3$ even with substrates like alkyl phosphates which possess such poor leaving groups requires an explanation. One possibility could be the involvement of general acid catalysts which would serve to protonate the leaving alcohol group. The pH dependencies of acid phosphatases from potato [13], yeast [14] and human liver [15] imply a possible involvement of carboxyl groups in the overall sequence. In order to test this possibility more directly, we studied the effect on human prostatic acid phosphatase of Woodward's reagent K, since this reagent is known to have a high specificity for the modification of carboxyl groups [16].

## Materials and Methods

Human prostatic acid phosphatase was purified by affinity chromatography on Sepharose-bound *N*-(6-aminohexyl)-L-tartramic acid as previously described

[17]. Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate) was purchased from Sigma Chemical Co.; ethylamine hydrochloride from Eastman Chemical Co.; 3′-pyridinesulfonic acid from Aldrich Chemical Co. All other chemicals were of analytical reagent grade.

*Enzyme activity assay.* A solution of the modified or the native enzyme was added to 2 ml 5 mM *p*-nitrophenylphosphate in 50 mM sodium acetate/100 mM NaCl (pH 5) at 25°C, and the reaction was stopped after 1—5 min by addition of 0.4 ml 1.25 M NaOH. The absorbance due to *p*-nitrophenolate ion was measured at 400 nm ($\epsilon = 1.81 \cdot 10^4\ M^{-1} \cdot cm^{-1}$). Protein concentration was determined using crystalline serum albumin as the standard [18].

*Reaction of Woodward's reagent K with human prostatic acid phosphatase.* The enzyme was stored at 4°C in 20 mM sodium cacodylate (pH 5). The stock enzyme solution or any other component of the incubation mixture was made completely free of acetate. A solution of Woodward's reagent K in 1 mM HCl [19] was prepared fresh each day. The reaction was carried out in 20 mM pyridinesulfonic acid pH 3.6 at 25°C. Portions of the reaction mixture were removed at different time intervals and diluted into 5—8 vols. ice-cold 2 M sodium acetate (pH 5.). This ensures rapid termination of the modification reaction by lowering the temperature, diluting the reagent, and causing a reaction of the excess Woodward's reagent K with the high concentration of acetate. The enzyme activity of the modified protein was then assayed as already described.

*Determination of the ethylamine released upon hydrolysis of the modified protein.* The enzyme solution modified by Woodward's reagent K was equilibrated against 1 mM HCl by concentrating and diluting using a PM-10 membrane [20] and then dialysed against 1 mM HCl for 24 h [21]. The sample was lyophilised and transferred to a hydrolysis tube. Hydrolysis was carried out at 110°C for 22 h. Amino acids and ethylamine were determined on a Durrum D-500 analyser. The separation of the ethylamine peak from that of arginine was increased by changes in the standard Durrum program. Instead of changing from 50 to 65°C at 50 min, the change was from 51 to 59°C at 39 min. The $Na^+$ concentration was 1.0 rather than 1.1 M, and the pH was 6.7 rather than 7.9. Ethylamine and amino acid standards were run with each determination of a set of experimental points. Previous workers [19,22] reported that the color yield of ethylamine was about one-third of that obtained from arginine or histidine; the amino acid analyses in these earlier studies were carried out using Beckman 120C analysers. However, we find the color yield of ethylamine to be comparable with those of histidine and arginine when the amino acid analysis is done with a Durrum analyser. This made it possible to perform reliable analyses of even relatively low ethylamine values along with the other amino acids by using 0.5 mg modified protein. In order to calculate the total number of ethylamine residues released upon hydrolysis of the modified enzyme, well-resolved peaks of a stable amino acid such as arginine or lysine were used. Calculations were then based on the known [23] number of arginine-residues present/dimeric molecule of the enzyme.

## Results

$9.0 \cdot 10^{-8}$ M human prostatic acid phosphatase is rapidly inactivated by $4.5 \cdot 10^{-4}$ M Woodward's reagent K as shown in Fig. 1. The enzyme is protected against inactivation by reagent K in the presence of various competitive inhibitors. L-(+)-Tartrate is a strong competitive inhibitor of this and homologous acid phosphatases, and its action may be due to its action as a transition-state analog [7]. Although 0.25 mM L-(+)-tartrate or $9.0 \cdot 10^{-2}$ M phosphate almost completely protects the enzyme against inactivation (Fig. 1), D-(—)-tartrate at a similar concentration affords little or no protection towards inactivation by reagent K. The relative degree of protection of the enzyme by L-(+)-tartrate versus that by D-(—)-tartrate with respect to inactivation by Woodward's reagent K is consistent with their respective $K_i$ values.

The formation of both reversible and irreversible complexes prior to covalent bond formation with Woodward's reagent K or other similar modifying reagents has been observed with various enzymes [19,20,24]. In order to explore the nature of such a complex in the present case, the inactivation of human prostatic acid phosphatase with various concentrations of reagent K was studied as a function of time (Fig. 2). The pseudo first-order rate constant $k_{app}$ corresponding to each concentration of the reagent K was calculated from the equation $k_{app} = (0.693/t_{1/2})$ where $t_{1/2}$ denotes the time required for 50% inactivation of the enzyme at a particular concentration of reagent K. When the reciprocals of $k_{app}$ were plotted against reagent K concentration (diagram omitted) the resulting line passed through the origin, indicating that the interaction between reagent K and the enzyme does not involve the reversible formation of a stable complex. The reaction of reagent K with the enzyme may lead directly to covalent bond formation without the intermediate formation

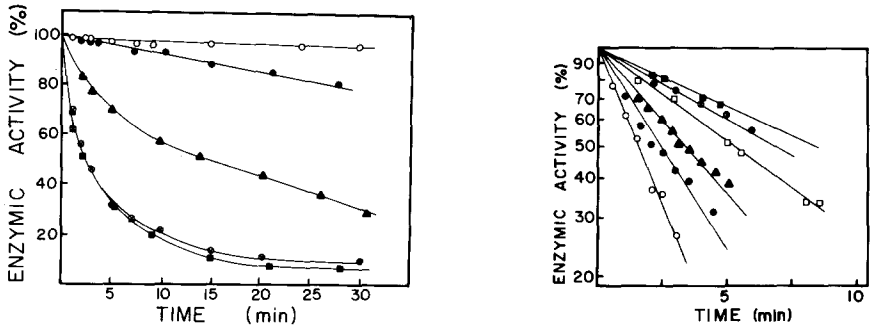


Fig. 1. Rate of inactivation of human prostatic acid phosphatase ($9.0 \cdot 10^{-8}$ M) by Woodward's reagent K ($4.56 \cdot 10^{-4}$ M) in the present of various competitive inhibitors. Activity was measured as a function of time in the presence of: ○———○, 90 mM phosphate; ●———●, $2.25 \cdot 10^{-4}$ M L-(+)-tartrate; ▲———▲, $2.25 \cdot 10^{-5}$ M L-(+)-tartrate; ⊗———⊗, $2.25 \cdot 10^{-4}$ M D-(—)-tartrate, and ■———■, in the absence of any competitive inhibitors.

Fig. 2. Rate of inactivation of human prostatic acid phosphate ($8.33 \cdot 10^{-8}$ M) as a function of the concentration of Woodward's reagent K in 15 mM pyridinesulfonic acid, pH 3.5. Concentrations of Woodward's reagent K were: ○———○, $9.0 \cdot 10^{-4}$ M; ●———●, $7.5 \cdot 10^{-4}$ M; ▲———▲, $6.25 \cdot 10^{-4}$ M; □———□, $5.0 \cdot 10^{-4}$ M; ⊗———⊗, $4.0 \cdot 10^{-4}$ M; ■———■, $3.8 \cdot 10^{-4}$ M.

of a non-covalent enzyme-inactivator complex of significant stability.

There are potentially as many as 154 side chain carboxyl groups in human prostatic acid phosphatase [23], and it is likely that carboxyl groups in addition to those present at the active site also have a significant reactivity towards reagent K. In order to quantitate the extent of modification of the active site carboxyl groups, the inactivation of the enzyme with reagent K was carried out in the presence of L-(+)- and of D-(—)-tartrate; the difference in the number of equivalents of $C_2H_5NH_2$ released upon hydrolysis of the protein modified in the presence of D-(—)-tartrate and in the presence of L-(+)-tartrate provided a measure of the number of carboxyl groups modified at the active site. L-(+)-tartrate is $10^3$ times stronger as a competitive inhibitor [6] than is D-(—)-tartrate. The concentration of both the L-(+)- and D-(—)-tartrate was near the $K_i$ of the weaker inhibitor.

If data for the residual enzymatic activity are graphed versus the difference in the number of ethylamine molecules released upon hydrolysis of the derivative modified in the presence of D-tartrate less that resulting upon modification in the presence of L-tartrate (Fig. 3), it is possible to estimate the number of carboxylic acid groups located at the active site. The number of carboxyl groups modified are 1.9 if all the points up to 68% remaining activity are taken into account (Fig. 3), while a value of 2.2 for the carboxyl groups modified is obtained if the points up to 60% residual activity are included. These calculations were done by a linear least-squares extrapolation with the $y$-intercept passing through zero percent inactivation.

At greater percentages of inactivation, large numbers of ethylamine residues were obtained upon hydrolysis. This suggests that L-(+)-tartrate protects carboxyl groups besides those present at the active site from modification. It is reasonable to expect that the presence of the competitive inhibitor stabilizes the protein with respect to denaturation, and that many additional carboxyl residual will be subject to modification in the denatured state. The reactivity of non-functional carboxyl groups towards Woodward's reagent K has similarly

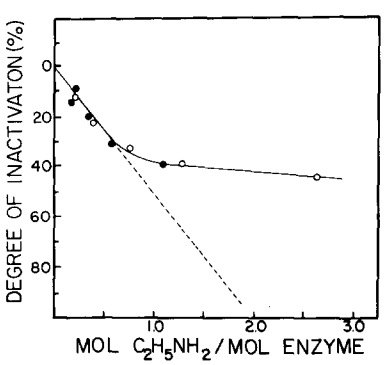


Fig. 3. Modification of human prostatic acid phosphatase by Woodward's reagent K. Ethylamine incorporated at the active site has been calculated per mol protein from the difference of mol ethylamine bound in the presence of D-(—)- and of L-(+)-tartrate. Concentrations of Woodward's reagent K, enzyme and D- or L-tartrate were: ○———○, $2.92 \cdot 10^{-2}$, $3.9 \cdot 10^{-6}$ and $6.53 \cdot 10^{-4}$ M, respectively, and ●———●, $2.05 \cdot 10^{-2}$, $5.2 \cdot 10^{-6}$ and $2.80 \cdot 10^{-4}$ M, respectively.

been observed in the modification of other enzymes [19,20], particularly at high degrees of covalent modification.

## Discussion

Two active site carboxyl groups are modified in native human prostatic acid phosphatase. This implies that a single carboxyl residue/active site is modified by the Woodward's reagent K, since it is known that the enzyme consists of identical subunits [2,25]. The presence of such active site carboxyl groups is evidenced from the fact that L-(+)-tartrate completely protects the enzyme against inactivation by reagent K whereas no protection is offered by the weak inhibitor D-(−)-tartrate (Fig. 1). We have also observed that human liver acid phosphatase is similarly inactivated by Woodward's reagent K and protected by competitive inhibitors [15], but quantitative studies such as those described here were not carried out with the human liver enzyme because of the small amount of enzyme available.

A kinetically detectable, stable complex is not formed during the reaction between Woodward's reagent K and human prostatic acid phosphatase, but the inactivation reaction (in the absence of competitive inhibitors) is very rapid (Fig. 2). Upon binding of reagent K at the active site of the enzyme, a carboxylate ion might facilitate the removal of a proton from position 3 of the isoxazolium ion, thus creating a highly reactive ketoketenimine which then could react rapidly with carboxylic groups [19,22].

Carboxyl groups evidently play an important role in the enzymic reaction mechanism of the acid phosphatases. They may participate in a general acid-catalyzed reaction in the ES complex, donating a proton to the departing alcohol or phenol. At low pH, $V$ varies with the kind of substrate used in the cases of human liver acid phosphatase [15] and wheat germ acid phosphatase [10], so that the rate-determining step in these reactions may be the release of alcohol or phenol. A common $V$ value for the substrates $\beta$-glycerylphosphate and $p$-nitrophenylphosphate was observed with human prostatic acid phosphatase [9] under similar conditions. Thus, the step involving the release of the alcohol or phenol is not rate limiting in the case of human prostatic acid phosphatase, where dephosphorylation of the phosphoenzyme [6] is apparently the slow step in the overall enzymic reaction. Although the pH dependence of steady-state reactions of animal and plant acid phosphatases do not necessarily reveal the kinetic involvement of a carboxylic acid group [9,10,13,14], the present work demonstrates the presence of one carboxyl group at or near the active site of human prostatic acid phosphatase. Since L-(+)-tartrate protects the enzyme from inactivation by reagent K, the involvement of an active site carboxyl group is indicated in some stage of the overall turnover reaction. A possible role for such a group may be to protonate the alcohol or other leaving group in the phosphorylation step ($k_2$) preceding the rate-limiting dephosphorylation of the phosphoenzyme.

## Acknowledgements

This research was supported by research grant CA 10585 from the National Cancer Institute of the U.S. Public Health Service. The enzyme was obtained

in part from material supplied by Prof. Dr. Ulrich Hachmeister of the Universitäts Klinikum Giessen, F.R.G., and by Dr. T.I. Malinin through the support of the U.S. National Prostatic Cancer Project grant CA 15480. The authors thank Mr. William Kohr for his invaluable help in obtaining amino acid analyses.

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