Biochimica et Biophysica Acta, 523 (1978) 422—429

BBA 68396

AN ESSENTIAL ARGININE RESIDUE IN HUMAN PROSTATIC ACID PHOSPHATASE

JOHN J. McTIGUE and ROBERT L. VAN ETTEN *

Chemistry Department, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

(Received September 6th, 1977)

Summary

Treatment of human prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) with either of the arginine-specific modifiers 2,3-butanedione or 1,2-cyclohexanedione in borate buffer at pH 8.1 leads to loss of activity. The inactivation by cyclohexanedione can be partially reversed by 0.2 M hydroxylamine. The rate of inactivation by both modifiers is decreased in the presence of the competitive inhibitors L-(+)-tartrate or inorganic phosphate but not in the presence of the non-inhibitor D-(—)-tartrate. Amino acid analysis of modified acid phosphatase indicates that only arginines are modified and that L-(+)-tartrate protects at least two arginyl residues from modification. A likely role of these arginyl residues is their involvement in binding the negatively charged phosphate group of the substrate.

Introduction

Human prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) is a dimeric enzyme consisting of two identical subunits [1,2] which catalyzes the hydrolysis of a wide range of phosphomonoesters. Chemical modification and radioactive labeling studies have shown an essential role for histidine in the catalytic mechanism [3]. Other studies have eliminated serine [4], tyrosine and tryptophan [5] and cysteine (McTigue, J.J. and van Etten, R.L., unpublished results) from involvement in the mechanism, but have not identified other amino acid residues which may participate in either catalysis or substrate binding. Because the enzyme has a specificity for the monoanionic form of the phosphomonoester substrate [6] we thought it likely that a positively charged recognition site at the active center would be required for binding of the substrate. The involvement of a metal ion in the catalytic or binding function seemed unlikely in view of the inability of various chelating agents to affect enzyme activity (McTigue, J.J. and van

* To whom correspondence should be addressed.

Etten, R.L., unpublished results). Experiments with trinitrobenzenesulfonic acid and pyridoxal 5-phosphate provide no evidence for the presence of lysyl residues at the substrate recognition site [3]. Recent reports have identified arginyl residues of a large number of enzymes acting as positively charged recognition sites for negatively charged substrates and anionic cofactors, particularly those substrates and cofactors containing a phosphate moiety [7—14]. Hence, it seemed possible that arginine could be important for substrate binding in acid phosphatase and we therefore undertook studies with the established reagents for ariginine modification, 1,2-cyclohexanedione and butanedione. It is demonstrated that two arginyl residues per dimeric enzyme molecule are essential for enzyme activity. A preliminary report of these findings has appeared [15].

Materials and Methods

Homogeneous human prostatic acid phosphatase was obtained essentially by the method of Ostrowski [16]. 1,2-Cyclohexanedione, 2,3-butanedione, *p*-nitrophenyl phosphate and L- and D-tartrate were obtained from Sigma Chemical Co. The butanedione was distilled just prior to use. All other reagents were analytical grade. For enzyme and protein assay procedures see ref. 3.

Modification of acid phosphatase by 1,2-cyclohexanedione or 2,3-butanedione. Acid phosphatase (0.2—20 μM) in 1.0 ml of 50 mM borate buffer (pH 8.1) was modified at 30°C by the addition of 50 μl of a solution of cyclohexanedione or butanedione in borate buffer to bring the total concentration of the modifier to 50 mM. Aliquots were taken periodically and assayed for activity. Pseudo first-order rate constants for the initial rate of loss of enzyme activity were obtained from a computer fit of the data to a non-linear least-squares equation. Control experiments were run simultaneously without the modification reagent. Active site protection experiments were performed in the presence of the competitive inhibitors inorganic phosphate or L-(+)-tartrate and control experiments were run simultaneously without the inhibitors or with the non-inhibitory D-(—)-tartrate in place of L-(+)-tartrate (compare ref. 3).

Amino acid analyses. Aliquots of a reaction mixture containing approx. 0.3 mg of the modified acid phosphatase were diluted into ice-cold 50% acetic acid, dialyzed against 10% acetic acid (8 h), 5% acetic acid (12 h), lyophilized and hydrolyzed for 24 h at 110°C in evacuated glass tubes containing 0.5 ml of 6 M HCl/0.5% mercaptoacetic acid to prevent regeneration of arginine [14]. Total amino acid compositions were used to quantitate the amount of arginine present in the sample. Analyses were performed with a Durrum D-500 amino acid analyzer.

Results

The reaction of butanedione with human prostatic acid phosphatase results in a loss of enzymic activity. Studies involving variation of the pH, temperature, borate and modifier concentration were used to determine the optimal conditions for modification of acid phosphatase by butanedione. The inactiva-

tion reaction exhibits pseudo first-order kinetics over the first 50% of reaction, but at longer reaction times the kinetics of the inactivation reaction are not consistent with any simple scheme. The initial pseudo first-order rate constant is independent of enzyme concentration (0.2—20 μM), but is dependent on the modifier concentration giving rate constants of 0.005, 0.011 and 0.018 min^{-1} for inactivation with 20, 50 and 100 mM butanedione, respectively. The presence of the competitive inhibitors inorganic phosphate or L-(+)-tartrate protects acid phosphatase against inactivation and reduces the rate of inactivation by factors of 3 and 6, respectively.

The reaction of cyclohexanedione in borate buffer with acid phosphatase also causes rapid inhibition of enzyme activity (Fig. 1). Blumenthal and Smith [14] have recommended pH 9.0 and 30°C for optimal inactivation with cyclohexanedione, but under these conditions acid phosphatase loses activity relatively rapidly even in the absence of modification reagents. The modification of acid phosphatase with cyclohexanedione was therefore run in borate buffer at pH 8.1 and 30°C and under these conditions the enzyme is stable for the duration of the modification experiment. The initial pseudo first-order rate constant for inactivation of acid phosphatase by 50 mM cyclohexanedione was 0.037 min^{-1} and complete inactivation of the enzyme was achieved after 150 min. To determine if the modified residues were present at or near the active site competitive inhibitors were added to the modification mixture; the rate of inactivation by cyclohexanedione was reduced by a factor of 3 upon addition of inorganic phosphate and by a factor of 5 by L-(+)-tartrate. The control experiments in which D-(—)-tartrate was used rather than L-(+)-tartrate exhibited the same rate of inactivation as experiments carried out in the absence of competitive inhibitors. Thus, the protective effect of L-(+)-tartrate is not due to the reaction of the modifier or borate with tartrate. Fig. 2 shows the loss of activity of acid phosphatase due to cyclohexanedione modification and the partial regeneration of acid phosphatase activity upon addition of

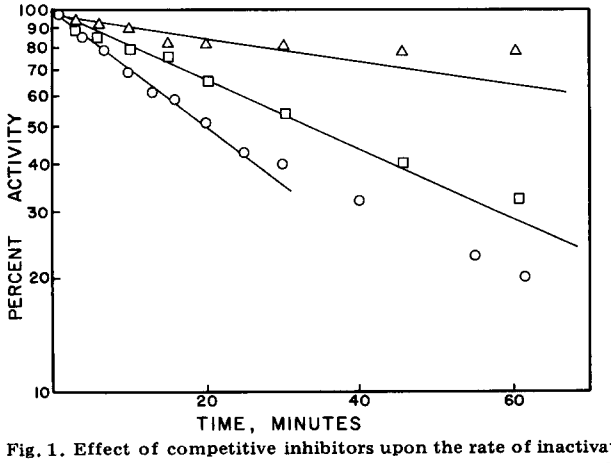

Fig. 1. Effect of competitive inhibitors upon the rate of inactivation of acid phosphatase by cyclohexanedione. Symbols: ○———○, enzyme alone; □———□, enzyme plus 0.1 M inorganic phosphate; △———△, enzyme plus 0.1 M L-(+)-tartrate. Enzyme plus 0.1 M D-(—)-tartrate had the same rate of inactivation as enzyme alone.

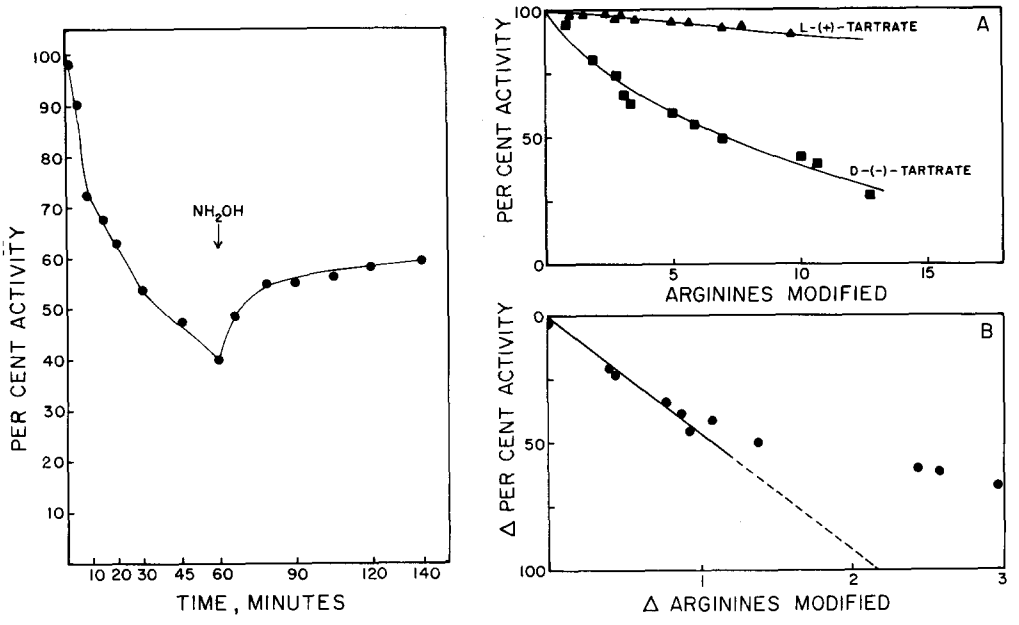

Fig. 2. Effect of reaction with cyclohexanedione on the activity of acid phosphatase. At the time indicated by the arrow, neutral hydroxylamine solution was added to a final concentration of 0.2 M.

Fig. 3. Relation between acid phosphatase activity and arginine modification upon cyclohexanedione treatment in the presence of L- or D-tartrate. Enzyme was modified by cyclohexanedione as described under Materials and Methods. Aliquots were taken from both tartrate solutions at the same time and assayed for enzyme activity. Others were freed of excess reagent by dialysis against acetic acid and subjected to amino acid analysis after acid hydrolysis. The number of arginines modified is given per mol of enzyme. (A) Correlation of the enzyme activity and the number of arginines modified by cyclohexanedione in the presence of 0.1 M L-(+)-tartrate (▲) or 0.1 M D-(—)-tartrate (■). (B) Correlation of the differences between enzyme activity and the number of arginines modified in the L- and D-tartrate-containing enzyme solutions.

neutral hydroxylamine. Smith and coworkers [12—13] have shown that hydroxylamine treatment of cyclohexanedione-modified enzymes results in the regeneration of arginine residues from the arginine modification derivative. The reversibility of the inactivation reaction was also shown using an enzyme sample treated with cyclohexanedione in borate buffer until it had approx. 20% residual activity; upon dialysis against 0.025 M sodium barbital (pH 8.1) at 4°C for 12 h to remove borate its activity increased to 45% of the unmodified control, thus indicating a necessity for borate to stabilize the modified arginine. Therefore, the reversal of cyclohexanedione inactivation of acid phosphatase by NH_2OH or by dialysis is consistent with arginyl modification.

The specificity of cyclohexanedione for arginine modification in prostatic acid phosphatase is consistent with the results of amino acid analysis. Because of the difference in inactivation of the enzyme by cyclohexanedione in the presence of the inhibitory and non-inhibitory L- and D-tartrate isomers, respectively, differential modification [17] can be used to distinguish essential active site arginyl residues from all others. To establish the possibility of differential modification, an enzyme sample which was modified by cyclohexanedione in the presence of L-(+)-tartrate for 50 min and had approx. 80% residual activity,

was dialyzed against 50 mM borate buffer (pH 8.1) to remove the inhibitor and unreacted modifier. Treatment of this enzyme solution with cyclohexanedione in the absence of the competitive inhibitor now led to a loss of enzymatic activity at a rate similar to that observed when the native enzyme was exposed directly to the modifying reagent in the absence of any competitive inhibitor. Therefore, the modification reaction was performed in the presence of the same amount of L- or D-tartrate and aliquots were taken from both tartrate solutions at the same time and analyzed for enzyme activity and amino acid content as described in Materials and Methods. Amino acid analyses (Table I) show a decrease of up to 13 arginyl residues but no loss of lysyl or any other amino acid residues. It is noteworthy that in the presence of a competitive inhibitor (L-(+)-tartrate) up to 10 arginyl residues are modifed by cyclohexanedione without significant loss of enzyme activity while in the presence of the non-inhibitory form of tartrate there is only a small additional increase in the total number of arginines modified accompanied by a large decrease in enzymic activity (Fig. 3A).

The difference in the number of arginines modified in the presence of the inhibitory and non-inhibitory forms of tartrate should indicate the number of arginines at or near the active site which were protected from cyclohexanedione modification by the competitive inhibitor. Fig. 3B shows the correlation of this difference in the number of arginines modified with the difference in activity of the enzyme in the two tartrate solutions. When there is a difference of 50% in the enzyme activity between the two solutions, the amino acid analysis shows that the enzyme in the L-tartrate solution contains one more arginyl residue than that in the D-tartrate solution. Extrapolating the initial modification data to zero activity (Fig. 3B) demonstrates that the modification of only two arginyl residues (of those which may be protected by competitive inhibitors) leads to a complete loss of activity. At relatively high percentages of inactivation L-tartrate protects more than two arginines. This could be due to the presence of more than one arginine at the active site or, more likely, to the increased availability of arginines to modification once prostatic acid phosphatase has been substantially inactivated and presumably subjected to some degree of structural alteration.

TABLE I

STOICHIOMETRY OF ARGININE MODIFICATION BY 1,2-CYCLOHEXANEDIONE IN THE PRESENCE OF L- OR D-TARTRATE

Results are expressed as residues per acid phosphatase molecule assuming 764 residues per molecule [23]. Conditions of the reaction are described in Fig. 3.

Reaction time:	Control	10 min		18 min		25 min		80 min	
Tartrate:		L	D	L	D	L	D	L	D
Activity (%):	100	99	80	98	65	97	53	92	24
Arginine	34.4	32.8	32.3	32.0	31.2	29.6	28.6	24.6	21.5
Lysine	43.0	42.4	42.3	42.9	42.9	42.7	43.1	42.8	42.3
Glutamate	106.1	106.3	106.0	107.1	107.0	103.2	105.5	104.0	105.6
Glycine	41.9	43.8	42.3	42.5	42.9	42.5	42.9	42.0	41.6
Phenylalanine	31.7	31.4	31.8	31.7	31.9	31.3	31.9	31.3	32.0
Histidine	26.9	27.0	27.5	26.5	27.2	27.0	26.7	26.5	27.0

Discussion

Previous studies have implicated histidyl residues in the mechanism of action of human prostatic acid phosphatase (see ref. 3 and references cited there). The present study also implicates arginyl residues in the mechanism of acid phosphatase. By using the established methods of arginine modification with 2,3-butanedione [11] or 1,2-cyclohexanedione [12,13] it is found that human prostatic acid phosphatase is readily inactivated by either reagent. Amino acid analysis of the cyclohexanedione-inactivated enzyme shows that the decrease in enzymic activity can be correlated with the loss of arginyl residues and that a competitive inhibitor reduces the rate of inactivation by preventing two arginines per dimeric enzyme molecule from being modified. Also, the partial regeneration of activity of the cyclohexanedione-inactivated enzyme either by the addition of NH_2OH or by the removal of borate ion is in accord with the chemical details of arignine modification by cyclohexanedione [12,13].

L-(+)-Tartrate protects prostatic acid phosphatase against inactivation by both butanedione and cyclohexanedione. These modification experiments were performed in borate buffer because of the reported enhancement of the reaction rate of butenedione and cyclohexanedione with arginine residues in the presence of borate [11—13]. However, borate also reacts with vicinal hydroxyl groups [18] and because L-(+)-tartrate contains such hydroxyl groups it might be argued that the apparent protection towards inactivation by cyclohexanedione or butanedione afforded by L-(+)-tartrate was due to the complexing of borate by L-(+)-tartrate which would decrease the borate concentration available for stabilization of the modified arginyl residues of acid phosphatase. We therefore examined inactivation of acid phosphatase by cyclohexanedione and butanedione in the presence and absence of the non-inhibitor D-(—)-tartrate and found no significant difference in the rate of inactivation of the enzyme in the presence of D-(—)-tartrate. Therefore, the presence of vicinal diols in tartrate does not appear to affect the borate stabilization of the argininines modified by butanedione or cylohexanedione. This is probably due to the requirement that borate only complexes with vicinal diols in a constrained cis configuration such as that found in the modified arginines [11—13] or in the ribosyl moieties of NAD and ADP [19], but not present in L- or D-tartrate. This result demonstrates that the protection of acid phosphatase by the competitive inhibitors L-(+)-tartrate is due to interaction of the inhibitors at the active site and not due to reaction of the inhibitor with the borate buffer. In this study relatively high concentrations of the active site-protecting reagent were required and the use of a steroisomer of a competitive inhibitor proved very advantageous in investigating modification at the active site because it provided a control for not only the potential chemical reactivity between the active site protector and the modifier or enzyme but also for any non-specific interactions (ionic strength effects, etc.) which could influence the rate of inactivation.

Under the same conditions cyclohexanedione inactivates acid phosphatase approximately three times faster than butanedione. Other reports have appeared of differences in inactivation rates using arginine-specific α-dicarbonyl compounds to modify an enzyme [7,20,21]. The differences in reactivity may

be due to differences in the hydrophobic character of the dicarbonyl compounds which lead to variations in the interactions between the modifiers and the reactive arginyl residues. The higher rate of inactivation by cyclohexanedione than by butanedione represents a minimal difference which would be increased if more nearly optimal conditions were sought. Powers and Riordan [22] have suggested that a hydrophobic environment plays a role in the selective modification of only a small number of the total arginyl residues present in certain proteins. Such a hydrophobic environment at the active site of acid phosphatase has been proposed by Rybarska and Ostrowski [5].

It is possible that the loss of activity might be due to the modification of an essential amino acid other than arginine or due to a structural change associated with arginine modification. Amino acid analysis of the enzyme modified by cyclohexanedione shows the decrease of 13 arginyl residues when the enzyme loses 76% of its activity. No other change is observed in the amino acid composition and this result is consistent with the reported high degree of specificity of cyclohexanedione for arginine modification [12,13,20]. Thus, under the experimental conditions employed it is reasonable to conclude that only arginyl residues are modified by cyclohexanedione. The inactivation is probably not due to a structural change affecting the active site region because in the presence of a competitive inhibitor at least 10 arginyl residues can be modified without a significant loss of enzyme activity. This conclusion is strongly supported by the results of differential modification experiments (Fig. 3) in which the modification of one active site arginyl residue per subunit is correlated with total inactivation of the enzyme and tends to eliminate conformational changes as being responsible for inactivation of acid phosphatase by cyclohexanedione. By the differential modification study, the arginines modified in the presence of a competitive inhibitor can be excluded from an essential role in enzyme activity. In the presence of L-(+)-tartrate up to 10 arginyl residues are modified without significantly affecting the enzymatic activity, while in the presence of the non-inhibitory D-(—)-tartrate the loss in activity is associated with the modification of approximately two essential arginyl residues per dimeric molecule. In this study the precise number of arginyl residues protected from modification by L-(+)-tartrate is subject to experimental error since there are 34 arginines present and the difference in the number of arginines modified in the presence of the two tartrate solutions represents a small difference between two relatively large numbers. Nevertheless, there results support an essential role for an active site arginyl residue in the enzyme mechanism.

The function of the essential arginyl residue of acid phosphatase which has been identified in this study is unknown because it is not possible to assign functional roles to amino acids in enzymes based solely on protection experiments. However, our results are similiar to those reported by Daemen and Riordan [7] for butanedione inactivation of *Escherichia coli* alkaline phosphatase and in that study the modification of the enzyme markedly decreased phosphate binding. The data suggested a role for the essential arginyl residues of alkaline phosphatase as binding sites for the negatively charged phosphate group of the substrate. Furthermore, the participation of an arginyl residue in the binding of anionic substrates or cofactors, particularly those containing a

phosphate moiety, has been proposed in several other examples of enzyme modification studies and it was suggested that arginyl residues serve as positively charged recognition sites for negatively charged ligands involved in an enzyme mechanism [9,10]. Such an electrostatic interaction would, of course, be enhanced in an environment of low dielectric constant. Therefore, it seems likely that electrostatic interaction between the positively charged arginine group at the active site and the negative charged phosphate ester substrate is important in the binding step and probably subsequent catalytic steps in the mechanism of acid phosphate.

Acknowledgements

This research was supported by 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 Professor Dr. Ulrich Hachmeister of Universitäts Klinikum Giessen, German Federal Republic and in part from material supplied by Dr. T.I. Malinin through the support of U.S. National Prostatic Cancer Project grant CA 15480. The authors thank Mr. William Kohr for the amino acid analyses.

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