Evidence for the Importance of Arginine Residues in Pig Kidney Alkaline Phosphatase

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(Received 8 November 1978)

The arginine-specific reagents 2,3-butanedione and phenylglyoxal inactivate pig kidney alkaline phosphatase. As inactivation proceeds there is a progressive fall in $V_{\max}$ of the enzyme, but no demonstrable change in the K_m value for substrate. P_i , a competitive inhibitor, and AMP, a substrate of the enzyme, protect alkaline phosphatase against the arginine-specific reagents. These effects are explicable by the assumption that the enzyme contains an essential arginine residue at the active site. Protection is also afforded by the uncompetitive inhibitor NADH through a partially competitive action against the reagents. Enzyme that has been exposed to the reagents has a decreased sensitivity to NADH inhibition. It is suggested that an arginine residue is important for NADH binding also, although this residue is distinct from that at the catalytic site. The protection given by NADH against loss of activity is indicative of the close proximity of the active and NADH sites.

There is increasing evidence that in a wide variety of enzymes, arginine residues act as positively charged centres for the recognition and binding of anionic substrates (Borders & Riordan, 1975; Borders *et al.*, 1975; Frigeri *et al.*, 1977; Riordan *et al.*, 1977). *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) has been shown to contain arginine residues essential for activity that are located presumably at the active site (Daemen & Riordan, 1974). If interaction of the phosphate group of substrates with an active-site arginine is a common property of phosphatases, then pig kidney alkaline phosphatase would be expected to resemble *E. coli* enzyme in response to chemical modification of arginine residues. Ahlers (1975a) reported rather surprisingly, however, that the activity of pig kidney enzyme is indifferent to treatment with arginine-specific reagents such as butanedione and phenylglyoxal.

It seemed of interest to re-examine the possibility that arginine residues are important in kidney phosphatase, particularly as the enzyme can be inhibited uncompetitively by NADH (Ramasamy & Butterworth, 1973, 1975), and arginine has been implicated in the nucleotide-binding sites of many dehydrogenases (Bleile *et al.*, 1975; Lange *et al.*, 1974; Nagradova & Asryants, 1975). If the NADH site on kidney alkaline phosphatase is structurally related to the coenzyme site on dehydrogenases, arginine residues may be essential for the inhibitory action of

NADH as well as for the catalysis of phosphate ester hydrolysis.

We chose to allow pig kidney alkaline phosphatase to react with 2,3-butanedione and phenylglyoxal to see whether catalytic activity and susceptibility to inhibition were affected. These reagents react most readily with arginine residues in proteins (Yankeelov *et al.*, 1968; Riordan, 1973; Takahashi, 1968) and can serve as useful probes. We were especially interested in obtaining some information about the nature of the inhibitory site and its relationship with the catalytic centre.

Experimental

Alkaline phosphatase

The enzyme was isolated from pig kidney cortex by the method of Wachsmuth & Hiwada (1974) with slight modification. The published procedure suggests concentration of the aqueous portion obtained after butanol extraction by rotary evaporation. This step was replaced by acetone precipitation carried out as follows. An equal volume of ice-cold acetone was added to the aqueous fraction at 0°C and the mixture was stirred for 1–2 h. After centrifugation at 8000g for 30 min, the acetone precipitate was resuspended in 0.01 M-Tris/HCl buffer, pH 8.0, to a volume approx. one-tenth of the original aqueous fraction. The suspension was dialysed for 24 h against 5 litres of the same buffer, with three changes, to remove any remaining traces of acetone and butanol. The specific activity was increased 29-fold by this step and 80%

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of the phosphatase activity was retained. The recommended final step in the published method involving gel filtration on Sephadex G-200 was done twice with some preparations to improve their specific activity. The final activity of different batches varied from 200 to 800 $\mu\text{mol}/\text{min}$ per mg when determined at pH 9.5 and 30°C in 0.05 M-Tris/HCl buffer containing 1 mM-*p*-nitrophenyl phosphate and 10 mM-MgCl₂. Electrophoresis in sodium dodecyl sulphate/polyacrylamide gel (Weber & Osborn, 1969) revealed several minor proteins that comprised up to 30% of the total stained with Coomassie Blue (Fairbanks *et al.*, 1971). A specific activity of 800 $\mu\text{mol}/\text{min}$ per mg of protein is comparable with the value given by Wachsmuth & Hiwada (1974) for enzyme that is assumed to be pure. The enzyme was stored at -15°C in 0.01 M-Tris/HCl buffer, pH 8, containing 0.1% NaN₃.

Reaction with 2,3-butanedione

The enzyme (5 μl of a solution containing 1.75 mg/ml) was preincubated in 50 mM-sodium borate, pH 8.3, at 20°C for 20 min in sealed tubes. A portion of a butanedione solution made up in the borate was added to the incubation mixture to give the concentrations indicated in the Figures and bring the reaction volume to 1 ml. Immediately after the addition, a sample (50 μl) was withdrawn from the mixture and assayed for alkaline phosphatase activity. This was regarded as the zero-time sample. Further portions were removed for assay at convenient time intervals. In some experiments the borate was replaced by sodium phosphate (50 mM) or Tris base (50 mM), both adjusted to pH 8.3. Butanedione solutions were prepared fresh each day and the pH was adjusted to 8.3 with NaOH if necessary. Mixtures containing only the enzyme and borate solution were incubated as controls.

Protection studies

The ability of various ligands, e.g. P_i, NADH etc., to protect alkaline phosphatase against 50 mM-butanedione in 50 mM-sodium borate, pH 8.3, was tested by including them in the incubation medium at the concentrations indicated in the Figures. With NADH, inactivation was studied at a number of butanedione concentrations (0–500 mM) and with a range of NADH concentrations (0–10 mM) at each butanedione concentration tested.

Kinetic study of treated enzyme

Incubation mixtures containing enzyme that had been exposed to 100 mM-butanedione for various times were applied, at 4°C, to a column (1 cm $\times$ 15 cm) of Sephadex G-25 equilibrated with 50 mM-sodium borate to remove unchanged butanedione. Zero-time samples, i.e. samples that were separated on the column immediately after the addition of butane-

dione, were used for the determination of control values for K_m and V_{max} . Fractions (1 ml) were collected from the column and the one with the highest enzymic activity was used for K_m and V_{max} measurements. The time from application of the sample to the recovery of the partially inactive enzyme was approx. 5 min. No significant further inactivation took place during this period. Separated enzyme was assayed as described below but with 10–100 μM -*p*-nitrophenyl phosphate and in the absence of Mg²⁺ ions. No re-activation occurred during the assay. Velocities are expressed as μmol of product/min per ml of separated enzyme.

The effects of inhibitors of alkaline phosphatase on the kinetics of treated and native enzyme were investigated by the addition of the particular inhibitor to the assay medium.

Reaction with phenylglyoxal

The experiments were very similar to those performed with butanedione, except that the incubation was carried out in 0.125 M-NaHCO₃, pH 7.5. Phenylglyoxal was prepared fresh daily in the bicarbonate solution and adjusted to pH 7.5 with 0.125 M-Na₂CO₃.

Measurement of activity

The assay mixture (3 ml) contained 0.1 M-Tris/HCl buffer, pH 9, 2 mM-MgCl₂ and 1 mM-*p*-nitrophenyl phosphate. The release of *p*-nitrophenol at 30°C was monitored at 400 nm in a recording spectrophotometer.

Polyacrylamide-gel electrophoresis

Phosphatase (4 mg), treated with 0.5 M-butanedione at 20°C for 48 h and then separated from excess reagent on Sephadex G-25, was subjected to electrophoresis in polyacrylamide gels (Saini & Done, 1970). A current of 8 mA per tube was applied for 6 h. Native enzyme was run in parallel as a control.

Chemicals

General laboratory reagents and solvents were A.R. grade and were used without further purification. 2,3-Butanedione and AMP were obtained from the Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. NADH was from the Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. *p*-Nitrophenyl phosphate was obtained from BDH, Poole, Dorset, U.K., and phenylglyoxal from Aldrich Chemical Co., Wembley, Middx., U.K. Cibacron Blue F3GA was a gift from the Ciba-Geigy Co., Basle, Switzerland.

Results

Pig kidney alkaline phosphatase is inactivated by butanedione (Fig. 1). The rate of inactivation is greatly enhanced by borate, providing some evidence

Fig. 1. Rate of inactivation of alkaline phosphatase by 2,3-butanedione

The reactions were carried out at pH 8.3 in 50 mM-borate (∇ , ∇), 50 mM-Tris (Δ , $\square$) or 50 mM- P_i ($\blacksquare$, $\triangle$). The concentrations of butanedione employed (mM) are shown in the Figure.

that modification of arginine residues is responsible for the loss of activity (Riordan, 1973). The low rate in the presence of phosphate is no doubt partially attributable to the lack of the stimulatory effect of borate, but another important factor is the probable protection of the active site by phosphate. This protection is reported more fully below.

In Fig. 1 the inactivation appears to follow a pseudo-first-order reaction process. This was not found to be exactly so in every experiment, particularly when incubations with the higher concentrations of butanedione were prolonged. Some deviation from linearity was then seen in the plots of log (activity remaining) versus time, although an initial apparent first-order reaction was always recognizable. Phenylglyoxal also inactivated the phosphatase, and semilogarithmic plots were linear throughout the time course studied.

Treating the initial rate of inactivation as pseudo-first-order and replotting the slopes of semilogarithmic plots obtained at different concentrations of butanedione or phenylglyoxal gave the results shown

Fig. 2. Effect of butanedione and phenylglyoxal concentrations on the rate of inactivation of alkaline phosphatase (a) $k_{app}/2.303$, determined from the slopes of semilogarithmic plots of the type shown in Fig. 1, is plotted against the concentration of butanedione ($\blacksquare$) or phenylglyoxal ($\blacktriangle$). (b) Double-reciprocal plots of data shown in (a). The intercepts on the vertical axis represent $2.303/k$, where k is the rate constant for the reaction of alkaline phosphatase with the particular reagent. The symbols have the same meaning as in (a).

in Fig. 2(a). These replots indicated an approach to a limiting rate of inactivation at high butanedione or phenylglyoxal concentrations. Replotting the data in double-reciprocal form gave linear plots (Fig. 2b) compatible with the assumption of the formation of an intermediate complex between the enzyme and butanedione (or phenylglyoxal) before transformation into the modified inactive enzyme (Jencks, 1969). Values of the rate constants for the transformation of the intermediate complex calculated from the intercepts of Fig. 2(b) are 0.135 min^{-1} and 0.175 min^{-1} for butanedione and phenylglyoxal respectively. The linearity of the double-reciprocal plots is evidence that modification of a sensitive site (presumably the active site) by a single molecule of reagent causes loss of activity.

An investigation of the kinetic properties of enzyme that had been preincubated for various times with 100mM-butanedione produced the following results: untreated control, $K_m = 28.2 \pm 3.0 \mu\text{M}$, $V_{\max} = 0.465 \pm 0.019$; incubated for 10min, $K_m = 23.7 \pm 1.5 \mu\text{M}$, $V_{\max} = 0.325 \pm 0.007$; incubated for 30min, $K_m = 36.8 \pm 4.7 \mu\text{M}$, $V_{\max} = 0.197 \pm 0.001$. The kinetic constants were calculated by weighted regression analysis of data obtained for the variation of velocity with substrate concentration (Cleland, 1967). The units for $V_{\max}$ are μmol of product/min per ml of separated enzyme (see the Experimental section). Treatment with phenylglyoxal gave similar results in that only $V_{\max}$ was affected significantly. Thus it seems that modification completely abolishes enzymic activity and any residual activity of treated preparations

derives from enzyme molecules that have their active site still intact.

AMP, a substrate of alkaline phosphatase, protected the enzyme against 50mM-butanedione (Table 1), as did the competitive inhibitor P_i . P_i was more effective against phenylglyoxal than against butanedione. At 50mM, P_i decreased the rate of inactivation with 10mM-phenylglyoxal by approx. 80%, whereas to overcome 50mM-butanedione in the presence of borate, 100mM- P_i was needed. As already mentioned (Fig. 1), butanedione in 50mM- P_i is very unreactive towards alkaline phosphatase.

Protection by these agents is expected if butanedione and phenylglyoxal inactivate by reaction at an active-site arginine residue. More difficult to explain, however, was the finding that 37.5mM- Mg^{2+} decreased by 50% the rate of inactivation of alkaline phosphatase by 10mM-phenylglyoxal. Mg^{2+} has been postulated to cause a conformational change at the active site of kidney alkaline phosphatase (Ahlers, 1975b) and so protection by the cation against phenylglyoxal may be more evidence for the postulate. Mg^{2+} ions did not restore the activity of enzyme

Table 1. Effect of various ligands on the rate of inactivation of alkaline phosphatase by 2,3-butanedione and phenylglyoxal

Values for k_{app} were obtained by least-squares fitting of plots of $2.303 \log$ (percentage of residual activity) against time of incubation with reagent.

Reagent	Ligand	$10^3 \times k_{\text{app}}$ (min^{-1})
Butanedione (50mM)	—	65.3 ± 1.83
	AMP (2.5mM)	32.7 ± 1.9
	AMP (10mM)	17.96 ± 0.23
	AMP (20mM)	12.9 ± 0.48
	NADH (5mM)	20.3 ± 2.07
	Nicotinamide (40mM)	51.3 ± 2.0
	Nicotinamide (80mM)	28.9 ± 9.05
Phenylglyoxal (10mM)	—	49.9 ± 3.49
	NADH (1mM)	36.4 ± 3.3
	NADH (10mM)	16.35 ± 1.04
	P_i (25mM)	13.15 ± 0.73
	P_i (50mM)	10.89 ± 0.73

Fig. 3. Kinetic analysis of protection by NADH. Values for $k_{\text{app}}/2.303$ were obtained from a series of experiments in which the concentration of butanedione was varied at several fixed concentrations of NADH. The results are plotted in double-reciprocal form. The concentrations of NADH in the reaction mixtures were: zero (Δ); 0.25mM ($\blacksquare$); 0.75mM ($\square$); 5mM ($\blacktriangle$). The lines were fitted to the experimental points by eye.

that had reacted with butanedione or phenylglyoxal. In kinetic experiments, 5mM-MgCl₂ increased $V_{\max}$ of native enzyme 2-fold. The same increase was found with enzyme that had been treated with 10mM-phenylglyoxal for 30min, suggesting that only intact phosphatase molecules remaining in the preparation could be stimulated by Mg²⁺ ions.

The uncompetitive inhibitors of pig kidney alkaline phosphatase NADH and nicotinamide were also found to protect the enzyme. In fact, of the several ligands tested, NADH appeared to be the most effective in protecting alkaline phosphatase against butanedione and phenylglyoxal (Table 1). The kinetics of the protection were studied by determining the dependence of k_{app} on butanedione concentration at a series of NADH concentrations ranging from 0 to 5mM. The slopes of the initial linear portion of plots of log (activity remaining) versus time were used as an estimate of $k_{app}/2.303$ in each case. Plots of $2.303/k_{app}$ against the reciprocal of the butanedione concentration are shown in Fig. 3. The plots representing several NADH concentrations intersect on the vertical axis, suggesting that NADH can compete with butanedione in forming the complex with alkaline phosphatase (Chen & Engel, 1975). A secondary plot of the slopes of the lines in Fig. 3 against NADH concentration (Fig. 4) is hyperbolic and so it would appear that the competition is only partial.

It has already been described how enzyme that is still active after exposure to either of the reagents behaves towards the substrate as though it is unchanged at the active site. When such a preparation

Fig. 4. Secondary plot of the kinetic data for the protection by NADH

Weighted regression analysis was used to calculate the slopes of lines providing the best fit to the experimental points shown in Fig. 3. The slopes are plotted against the corresponding concentrations of NADH. Where the standard error for the slope value extends beyond the region occupied by the symbol, the error is represented by a vertical bar.

Fig. 5. Effect of reaction with butanedione on the inhibition of alkaline phosphatase by NADH and Cibacron Blue. Enzyme that had been pretreated for 30min as described in the text was assayed for alkaline phosphatase activity with 50 μ M-*p*-nitrophenyl phosphate in the presence of NADH ($\blacktriangle$) or Cibacron Blue ($\blacksquare$). The response of untreated enzyme to the inhibitors is shown by ($\triangle$) for NADH and ($\square$) for Cibacron Blue.

was tested for its sensitivity to reversible inhibition by NADH, however, it was found to be considerably less sensitive to the inhibitor than was native enzyme (Fig. 5). A similar result was obtained with the chromophore Cibacron Blue F39A. This dye is believed to bind to alkaline phosphatase at the NADH site (M. N. Woodroffe & P. J. Butterworth, unpublished work). Electrophoresis on polyacrylamide gel of enzyme that had received prolonged treatment with butanedione revealed an increase in mobility towards the anode of approx. 85% (mean of four determinations). The reaction of butanedione with arginine residues results in neutralization of positive charges and insertion of negative ones on stabilization of the complex by borate (Riordan, 1973). Thus an increase in migration rate at pH 8.0 is expected. The magnitude of the change in mobility suggests that several arginine residues in addition to any at the active site react per enzyme molecule. A decrease in sensitivity to NADH could mean that arginine is important for binding the coenzyme and that such a residue (or residues) is separate from an arginine at the active site, since NADH inhibition can be impaired, yet catalytic activity remains intact.

Discussion

The specificity for arginine residues of butanedione and phenylglyoxal, the catalytic effect of borate on the rate of inactivation, and the protection by P_i and AMP all point to the existence of an essential arginine

residue at the active site of pig kidney alkaline phosphatase. The residue could be a constituent of the binding site for the phosphate portion of substrate molecules, as has been proposed for several other enzymes (Lange *et al.*, 1974; Daemen & Riordan, 1974; Borders & Wilson, 1976). The failure of Ahlers (1975a) to find inactivation of pig kidney enzyme by butanedione and phenylglyoxal probably resulted from the particular buffer systems that he used in his studies. In particular, he did not use borate when treating with butanedione.

The kinetics of inactivation are indicative of the formation of a dissociable enzyme-inhibitor complex before covalent modification and loss of activity. The loss seems to follow the reaction of one arginine residue per active centre, i.e. two residues presumably, per molecule of the dimeric kidney enzyme (Ramasamy & Butterworth, 1974; Cathala *et al.*, 1975).

There are approx. 65 arginine residues per molecule (Wachsmuth & Hiwada, 1974) and the greatly increased electrophoretic mobility after treatment with butanedione suggests that more than the two active-site ones can become modified. We did not attempt amino acid analysis to identify the number of modified arginines, because of the difficulty of obtaining enzyme that was absolutely pure.

The decreased sensitivity to NADH and Cibacron Blue F3GA of active enzyme that had been exposed to butanedione and phenylglyoxal is more evidence that arginine residues besides those at the active site are attacked, and that separate and distinct arginine residues are involved in the binding of NADH. It is very surprising therefore that NADH protects the active site so effectively against butanedione through a partially competitive mechanism. The coenzyme could be binding to an active-site arginine residue through the pyrophosphate backbone, but the partial-competitive mechanism would not strongly support this idea. In addition, kinetic experiments appear to eliminate the likelihood of binding at the substrate-free active site of uncompetitive inhibitors, such as NADH (Ramasamy & Butterworth, 1973, 1975; Byers *et al.*, 1972). The NADH may induce a conformation change at the active site or bind sufficiently closely to this region to interfere with the approach of attacking reagents. Evidence for hindrance at the active site is provided by the finding that Cibacron Blue F3GA seems to bind to a hydrophobic region on alkaline phosphatase adjacent to the active site and displace NADH (M. N. Woodroffe & P. J. Butterworth, unpublished work). Since it is believed that the dye binds to relatively hydrophobic regions in proteins that recognize NADH (Beissner & Rudolph, 1978), it is reasonable to believe that

NADH becomes bound through an arginine residue close to the active site. This implies the existence of two reactive arginine residues that are not widely separated per subunit, one for the binding of substrate, the other for NADH.

M.N.W. thanks the M.R.C. for a research studentship.

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