Reactive groups in mammalian alkaline phosphatase: Loss of enzyme activity after reaction with phenylglyoxal and iodoacetamide

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Recently we obtained evidence for an arginine residue at the active centre of pig kidney alkaline phosphatase (Woodroofs & Butterworth, 1979). Such a residue has been implicated in *Escherichia coli* alkaline phosphatase and in many other enzymes that act on substrates containing a phosphate group (Diemen & Riordan, 1974; Borders & Riordan, 1975). In continuing our study of essential residues in mammalian alkaline phosphatase, we have been working with the calf intestinal enzyme, since it is available commercially in a highly purified form and its amino-acid composition has been reported (Fosset et al., 1974).

Calf intestinal alkaline phosphatase (obtained from the Boehringer Corporation) was dialysed against 0.05 M-Tris/HCl buffer, pH 8.6, before chromatography on DEAE-cellulose by the method described by Fosset et al. (1974). The purified enzyme was stored at -20°C. For reaction with 0-50 mM-phenylglyoxal, 1-2 μg of alkaline phosphatase was preincubated at 20°C in 0.125 M-NaHCO3, pH 8.35. The total reaction volume was 1 cm³. At appropriate times, samples (50 μl) were withdrawn and assayed for residual phosphatase activity at pH 10 (0.1 M-Na2CO3/NaHCO3 buffer) and 30°C with 2.5 mM-p-nitrophenyl phosphate as substrate. The protective action of P, at concentrations up to 200 mM was tested by the inclusion in the preincubation mixture of phosphate that had been adjusted to pH 8.35. For inactivation by iodoacetamide, a very similar procedure was adopted, except that the preincubation was usually carried out at pH 9 (0.1 M-Na2CO3/NaHCO3 buffer). The effect of the H+ concentration on the rate of inactivation was investigated by varying the pH of the preincubation mixture between 8 and 10.5.

Fig. 1 shows that the intestinal enzyme is inactivated by both phenylglyoxal and iodoacetamide. P, protected the enzyme against phenylglyoxal, but has no effect on the rate of inactivation by iodoacetamide. Comparison of the rates of inactivation of alkaline phosphatase suggests that iodoacetamide is more effective than phenylglyoxal on a concentration basis. The rate of inactivation by iodoacetamide is pH-independent between 8 and 9.5, but then decreases markedly above this range. Iodoacetate also inactivates the enzyme, but requires a concentration that is approximately five times higher than those shown for iodoacetamide. N-ethylmaleimide did not inactivate the enzyme.

The inactivation of phenylglyoxal and iodoacetamide could not be reversed by passage through a column of Sephadex G-25, neither was reversal achieved by treatment with β-mercapto
ethanol. Kinetic measurements on treated enzyme that had been separated from the modifying agent by gel filtration showed that $K_m$ values were unaffected and that inactivation was purely a $V_{max}$ phenomenon. Thus chemical modification leads in both of the cases studied to totally inactive enzyme.

The action of phenylglyoxal is presumably on an essential arginine residue(s), which may be at, or close to, the active site, since the competitive inhibitor $P_i$ provides protection. The behaviour of the intestinal enzyme resembles that of pig kidney and E. coli alkaline phosphatases. This similarity is expected for an active-site residue, since it would belong to a class of highly conserved residues in alkaline phosphatase proteins. The non-protection by $P_i$ of attack by iodoacetamide may be indicative of a more remote location for the particular residues involved. Iodoacetamide sensitivity is not shared by the kidney enzyme (M. Wass & P. J. Butterworth, unpublished work), which again may argue for the sensitive residue being remote from the active centre. We cannot yet be sure of the nature of the group attacked by iodoacetamide. The effect of pH on the reaction and the ineffectiveness of $N$-ethylmaleimide point to some other group than thiol. Further investigations are needed to answer this question.


$^1$H/$^2$H isotope exchange studies in intact erythrocytes


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When $\text{H}$-labelled lactate is added to suspensions of intact erythrocytes, three types of $^1\text{H}/^2\text{H}$ exchange process may be discerned: (1) equilibration of the methyl $\text{H}$ label with pyruvate methyl $\text{H}$; (2) net loss of label from the methyl $\text{H}$ pool to solvent; and (3) loss of $\text{H}$ from the C-2 position to solvent. The first two processes may be separated by analysis of exchange kinetics. The individual exchange processes may be used to obtain kinetic information about specific enzyme systems in intact erythrocytes.

Protein $\alpha$-NH$_2$ groups catalyse the exchange of pyruvate $\text{H}$ with solvent water, probably by the formation of transient Schiff's-base links. Haemoglobin, present in high concentrations in erythrocytes (6 mm), causes rapid loss of labelled $\text{H}$ from added pyruvate. If lactate is also present, changes in $\text{H}$ labelling at the methyl position of this metabolite occur, since the enzyme lactate dehydrogenase communicates the pyruvate methyl $\text{H}$ exchange to lactate:

$$\text{Lactate dehydrogenase} \quad \begin{array}{c} \text{CH}_3 \text{CHOH} - \text{COO}^- \quad \text{CH}_3 \text{CHOH} + \text{NADH} \quad \text{COO}^- \quad \text{NAD}^+ \end{array}$$

The kinetics of such a two-step isotope exchange are described by sets of double exponential solutions to the set of differential equations. One such set of solutions for the initial conditions of adding 10 mm protonated lactate and 10 mm protonated pyruvate to cells in $\text{H}_2\text{O}$ buffer is:

$$[\text{[}^1\text{H}]\text{lactate}] = \left( \alpha e^{-\beta t} - \beta e^{-\beta t} \right)$$

$$[\text{[}^2\text{H}]\text{lactate}] = \frac{\alpha - \beta}{\alpha}$$

$$[\text{[}^1\text{H}]\text{pyruvate}] = \left( \beta (k_1 - \alpha) e^{-\beta t} - \alpha (k_2 - \beta) e^{-\beta t} \right)$$

$$[\text{[}^2\text{H}]\text{pyruvate}] = \frac{k_1 (k_2 - \alpha)}{k_2}$$

where $\alpha = \frac{k_1}{k_2}$, $k_1 = k_2 + k_3$, $k_2 = k_4$, $k_3 = k_5$, $k_4 = k_6$, $k_5 = k_7$, $k_6 = k_8$, and the $k_i$ are defined in eqn. (1) above. Some data illustrating this model are shown in Fig. 1.

Exchange at the C-2 position of lactate is manifested as an inversion of the methyl resonance in the spin-echo spectrum (at $\tau = 68$ ms) as shown in Fig. 2. The exchange is dependent on the activities of four glycolytic enzymes: aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase and lactate

$$\text{Pyruvate} \quad \begin{array}{c} \text{Lactate} \quad \text{Pyruvate} \quad \text{Lactate} \quad \text{Pyruvate} \end{array}$$

Fig. 1. Exchange of pyruvate and lactate methyl $^1\text{H}$ with solvent $^2\text{H}$ catalysed by human erythrocytes in $^2\text{H}_2\text{O}$-containing Krebs–Ringer buffer

(a) Peak height of methyl resonance of appropriate metabolite in $\text{H}$ n.m.r. spectra ($\tau = 10$ ms) versus time. In (b) lactate dehydrogenase has been inhibited by approx. 90% by the addition of 4 mm-oxalate. The lines were obtained from the model described in the text by using $k_1 = k_2, k_3 = 0.06$; in (a) $k_1 = 0.06$, in (b) $k_2 = 0.006$.

Fig. 2. Effect of exchange at the C-2 position on the lactate methyl peak intensity

The n.m.r. spectra were obtained by using spin-echo methods (Brown et al., 1977) at 470 MHz with $\tau = 68$ ms. (a) Incubation of 12 mm-lactate, $^2\text{H}$-labelled at the C-2 position, with packed cells in $^2\text{H}_2\text{O}$ at 37°C; (b) incubation of 12 mm-lactate, $^1\text{H}$-labelled at the C-2 position, with packed cells in $^2\text{H}_2\text{O}$ at 37°C.