Short Communications

The Reaction of Diethyl Pyrocarbonate with Pyruvate Kinase

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Diethyl pyrocarbonate inactivates muscle pyruvate kinase with the substitution of 3–4 histidine residues per subunit. Phosphoenolpyruvate, ATP and ADP to a lesser extent, and Mg^{2+} and pyruvate to a small extent, protect against inactivation.

Pyruvate kinase from rabbit muscle (EC 2.7.1.40) catalyses the transfer of a phosphate moiety from phosphoenolpyruvate to ADP, releasing pyruvate. It is immunologically distinct from the L-type liver enzyme (Tanaka *et al.*, 1965) and exhibits different kinetics (Bailey *et al.*, 1968); in particular, it is not activated by fructose 1,6-diphosphate. Hollenberg *et al.* (1971) showed that modification of one lysine residue per subunit causes complete inactivation of the enzyme. Modification studies also indicate a thiol residue at the active site (Kupiecki & Coon, 1959; Flashner, 1971). Mildvan & Cohn (1965) studied the pH-dependence of the dissociation of Mn^{2+} from the enzyme and found evidence for binding by a group with a pK of 6.8. They tentatively suggested this to be a histidine residue. To obtain more direct evidence for involvement of histidine, we have investigated the reaction of pyruvate kinase with diethyl pyrocarbonate. This reagent reacts with several amino acid side chains to form ethoxycarbonyl derivatives at neutral and alkaline pH values (Muhlrad *et al.*, 1967; Ovadi *et al.*, 1967), but at pH 6 it reacts only with the imidazole nitrogen of histidine residues (Muhlrad *et al.*, 1967).

Materials and methods

ADP, ATP, NADH, phosphoenolpyruvate (as sodium salts), lactate dehydrogenase and pyruvate kinase were from Boehringer Corp. (London) Ltd. (London W.5, U.K.). All other chemicals, reagent grade or better, were from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Absorbances were measured with a Pye-Unicam recording spectrophotometer (Pye-Unicam, Cambridge, U.K.). The purity of pyruvate kinase was checked by zone electrophoresis at pH 8.95 on Cellogel (Reeve Angel Scientific Ltd., London E.C.4, U.K.). Virtually all the enzyme ran as a single band, though a trace of material remained at the application line. Before use, the enzyme was diluted to the required concentration with 0.1M-

potassium phosphate buffer, pH 6.0. It was then dialysed against the same buffer overnight to remove $(NH_4)_2SO_4$, since nitrogenous bases catalyse the breakdown of diethyl pyrocarbonate and its derivatives.

Pyruvate kinase activities were measured at pH 7.0 by using a coupled assay with lactate dehydrogenase (Tietz & Ochoa, 1958). The reaction mixture in a cuvette of 10 mm path length at 25°C contained the following in a volume of 3 ml: 0.1 M-potassium phosphate buffer, pH 7.0, 0.2 mM-NADH, 1.2 mM-ADP, 0.6 mM-phosphoenolpyruvate, 25 mM- $MgSO_4$ and 50 μ g of lactate dehydrogenase. The oxidation of NADH was followed by the decrease in absorbance at 340 nm. Concentrations of pyruvate kinase were determined from the absorption at 280 nm by using an extinction coefficient of $0.54 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ (Bücher & Pfeleiderer, 1955). The dialysed enzyme had an activity of $16000 \mu\text{mol/min per mg}$. For the inhibition reaction, the enzyme solution was brought to 25°C and a sample taken for a control reading; diethyl pyrocarbonate (see below), suitably diluted, was then added. Formation of ethoxycarbonyl-histidine residues was followed by observing the increase in absorbance at 240 nm (Ovadi *et al.*, 1967).

A single batch of diethyl pyrocarbonate (BDH) was used in all experiments. It was diluted in 0.1 M-potassium phosphate buffer, pH 6.0, immediately before use. By the method of Holbrook & Ingram (1973) it was 60% pure. A value of $4.25 \times 10^6 \text{ cm}^2 \cdot \text{mol}^{-1}$ for the molar extinction coefficient for ethoxycarbonylhistidine at 240 nm was obtained from the reaction of 5 mM-diethyl pyrocarbonate with 0.3 mM-N-acetylhistidine in 0.1 M-potassium phosphate buffer, pH 6.0 (Holbrook & Ingram, 1973).

Results

The reaction of diethyl pyrocarbonate with pyruvate kinase was studied at inhibitor concentrations in the range 0.59–4.13 mM (a molar excess of 270–1900-fold). There was a rapid loss of activity

Fig. 1. Inhibition of pyruvate kinase by diethyl pyrocarbonate

(a) Reaction of pyruvate kinase ($2.2\mu\text{M}$) in 0.1M -potassium phosphate buffer, pH 6.0, with increasing concentrations of diethyl pyrocarbonate at 25°C . At the times shown, samples were withdrawn and assayed for pyruvate kinase activity as described in the text. The number of modified histidine residues was calculated from the E_{240} and mol.wt. 237 000 (Cottam *et al.*, 1969). Concentrations of diethyl carbonate were as follows: $\circ$, 4.13mM ; $\blacksquare$, 2.95mM ; $\bullet$, 1.48mM ; $\triangle$, 1.18mM ; ∇ , 0.59mM . Dashed lines signify the formation of ethoxycarbonylated histidine residues with respect to time. (b) Relationship between loss of enzyme activity and the production of ethoxycarbonylated residues. The results of two typical experiments are shown: $\blacksquare$, $2.69\mu\text{M}$ enzyme, 1.09mM inhibitor; $\triangle$, $2.32\mu\text{M}$ enzyme, 3.08mM inhibitor.

and rapid formation of ethoxycarbonylhistidine residues (Fig. 1). After about 4min the rate of inactivation began to slow down and inactivation ceased after about 20min. Total loss of activity was accompanied by the production of 3–4mol of ethoxycarbonylhistidine/mol of enzyme subunit (assuming mol.wt. 237 000 and four subunits/molecule; Cottam *et al.*, 1969). Increasing the concentration of inhibitor increased the initial rate of inactivation of enzyme approximately in proportion to the concentration of inhibitor. At the lower end of the range complete loss of activity was not attained. This was probably because of the hydrolysis of diethyl pyrocarbonate, since a second addition of inhibitor at 30min (not illustrated) caused a further loss of activity.

Fig. 2. Inhibition of pyruvate kinase by 1.18mM -diethyl pyrocarbonate in the presence of substrates

$\blacktriangle$, $2.38\mu\text{M}$ enzyme; $\triangle$, $1.98\mu\text{M}$ enzyme, 39.7mM-Mg^{2+} ; $\bullet$, $2.36\mu\text{M}$ enzyme, 8.20mM-ADP ; $\circ$, $1.98\mu\text{M}$ enzyme, 39.7mM-Mg^{2+} , 8.12mM-ADP ; $\blacksquare$, $1.98\mu\text{M}$ enzyme, $7.82\text{mM-phosphoenolpyruvate}$; $\square$, $2.36\mu\text{M}$ enzyme, 39.7mM-Mg^{2+} , $7.46\text{mM-phosphoenolpyruvate}$; ∇ , $2.05\mu\text{M}$ enzyme, 39.7mM-Mg^{2+} , 7.86mM-ATP ; ∇ , $2.05\mu\text{M}$ enzyme, 39.7mM-Mg^{2+} , 9.92mM-pyruvate .

To test for protection against inactivation various substrates and Mg^{2+} were added before addition of diethyl pyrocarbonate (Fig. 2). Phosphoenolpyruvate and Mg^{2+} together gave the greatest protection, with a 75% retention of activity. Mg^{2+} alone offered relatively little protection in relation to its high concentration. Surprisingly, both ADP and ATP, which protected to some extent, caused a slight initial activation; after about 1 min inactivation began and then followed the usual pattern. Similar results were obtained with 3 mM-diethyl pyrocarbonate. Under these conditions the initial activation with ADP and ATP was not seen, but there was no loss of activity in the first sample taken at 30 s; thereafter there was a loss of activity.

To check that no reaction occurred at pH 6 with amino or thiol groups, 2.7 mM-lysine and 2.7 mM-*N*-acetylcysteine were incubated with 5.5 mM-diethyl pyrocarbonate in 0.1 M-potassium phosphate buffer for 25 min. The amino groups of the lysine did not react and less than 1% of the thiol groups of *N*-acetylcysteine were ethoxycarbonylated, as judged by the u.v. difference spectra (220–280 nm). At pH 8.0 both groups were substituted, and the u.v. difference spectra which were recorded did not change when the pH was lowered to 6. It was concluded that under the conditions of the pyruvate kinase experiments very little substitution of these groups occurred.

Discussion

The lack of reaction of diethyl pyrocarbonate with *N*-acetylcysteine and lysine provides further evidence that at pH 6 this reagent is virtually specific for histidine groups (Muhlrad *et al.*, 1967). The inactivation of pyruvate kinase by this reagent with concomitant substitution of histidine groups therefore provides evidence for the involvement of histidine groups in the active site. Extrapolation (Fig. 1) suggests that 3–4 histidine residues must be substituted per subunit for complete inactivation. However, not all of these may be located at the active site. The relative ineffectiveness of Mg^{2+} to protect against inactivation, compared with the protection afforded by the metal ion against reagents modifying the lysine residue (Hollenberg *et al.*, 1971), suggests that the histidine residues may not be liganded to Mg^{2+} , as was originally proposed by Mildvan & Cohn (1965) on the basis of pH-profile studies of Mn^{2+} binding. The pattern of protection offered by substrates also differs from the pattern found for lysine residues. Thus phosphoenolpyruvate gave the greatest protection against histidine substitution, whereas it was without effect on lysine substitution (Hollenberg

et al., 1971). ATP and ADP, however, protect against substitution of both groups. These findings suggest that a histidine residue or residues is co-ordinated with the phosphate group of phosphoenolpyruvate and is involved in phosphate transfer to ADP. In contrast, the lysine residues appear to be liganded to the metal ion and so involved in the binding of nucleotides. Similar studies with diethyl pyrocarbonate have indicated the presence of histidine at the active sites of adenylate kinase and creatine kinase (Pradel & Kassab, 1968). It is possible that histidine may function in these reactions in a way comparable with the role of histidine in the ribonuclease reaction (Findlay *et al.*, 1962; Deavin *et al.*, 1966). An unusual feature of the experiments involving protection with ATP and ADP was the small but consistent initial activation. This suggests that the enzyme normally exhibits less than its maximum activity and raises the possibility that there may be, as yet unidentified, activators of the muscle enzyme. In this connexion the inhibition by creatine phosphate under physiological conditions is of interest (Kemp, 1973).

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