Evidence for reactive groups in rat liver acid phosphatase: inactivation by diethyl pyrocarbonate and photo-oxidation in the presence of Rose Bengal

NAVEENAN NAVARATNAM, MALCOLM R. BANNER and PETER J. BUTTERWORTH
Department of Biochemistry, Chelsea College, Manresa Road, London SW3 6LX, U.K.

Diethyl pyrocarbonate and photo-oxidation have been shown to be useful agents for investigating the importance of histidine residues in proteins. The reactions involved occur under mild conditions. We report the effects of these reactions on the activity of rat liver acid phosphatase.

The enzyme was prepared from rat liver by using a modification of the method of Igarashi & Hollander (1968). Two peaks of enzyme activity were obtained by chromatography on DEAE-cellulose. The peaks are designated P1 and P2 according to the order in which they are eluted from the column (Navaratnam et al., 1982). After further purification the specific activities of enzymes P1 and P2 were 57.3 and 167 μmol/min per mg of protein respectively. Both enzymes P1 and P2 were homogeneous on polyacrylamide-gel electrophoresis. They were stable for several weeks when stored at 0–4°C.

From a study of the variation in the rate of inactivation by diethyl pyrocarbonate with pH, it was found that the greatest rate occurred at approx. pH 8 in Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) buffer. To test the effect of diethyl pyrocarbonate on the enzymic activity, portions of enzymes P1 and P2 were each incubated at 20°C with the inhibitor (0–30 mM) in 0.1 M-Hepes buffer, pH 8. The protein concentrations were 15–20 μg in the reaction mixtures. At suitable time intervals, samples were removed and assayed for residual acid phosphatase activity at 30°C and pH 5 (Navaratnam et al., 1981). The protective action afforded by the substrate analogue P1 was tested by including various concentrations of the anion in the inhibitor-containing mixtures.

From the results shown in Fig. 1 it is evident that diethyl pyrocarbonate potently inactivates rat liver acid phosphatase, giving over 90% inhibition after approx. 5 min exposure to the highest concentration of reagent tested. The rate of inactivation was pseudo-first-order and dependent on the concentration of the reagent. The action of the inhibitor is probably on an essential histidine residue at, or close to, the active site, since the competitive inhibitor P1, at concentrations up to 10 mM protected both enzymes P1 and P2 considerably against diethyl pyrocarbonate (results not shown).

Photo-oxidation was performed at 0°C in stirred 0.1 M-Hepes buffer, pH 8, containing Rose Bengal (0–15 μg/ml) and 15–20 μg/ml of enzyme P1. A slide projector was used as the light source and positioned 20 cm from the reaction vessel. Illumination was from the side. At suitable time intervals, samples were withdrawn for assay of residual acid phosphatase activity. A pseudo-first-order loss of activity occurred that amounted to approx. 90% by 4 min of illumination.

The demonstration of the likely importance of histidine residues in two fractions of acid phosphatase from rat liver, together with the observations by other workers studying rat liver (Igarashi et al., 1970), bovine liver (Lawrence & van Etten, 1981), and human liver and prostate (Saint & van Etten, 1978) suggests that histidine is an essential component of the active site of all non-specific acid phosphatases. This may be an important feature that distinguishes them from alkaline phosphatases.

From earlier studies it appears that rat liver has important arginine and cysteine residues in the active site (Navaratnam et al., 1981, 1982). It will be interesting to learn the relative positions of these important residues in the native enzyme structure.

Fig. 1. Inactivation of rat liver acid phosphatase by diethyl pyrocarbonate
(a) Fraction P1 was incubated at pH 8 and 20°C with diethyl pyrocarbonate at the concentrations (mM) shown within the Figure. Samples were withdrawn at the times shown for enzyme assay at pH 5. (b) As in (a) except that enzyme fraction P2 was used in the incubation mixture.


Vol. 11