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Titration of malate dehydrogenase with 2-hydroxy-5-nitrobenzyl bromide

The reagent, 2-hydroxy-5-nitrobenzyl (HNB) bromide devised by KOSHLAND *et al.*[1-3], represents a new means of modification and identification of tryptophanyl residues in proteins. In view of our interest in the role of tryptophan in dehydrogenase enzymes[4,5], we have studied the action of HNB bromide on malate dehydrogenase (EC 1.1.1.37) from pig heart mitochondria.

A modified procedure of BARMAN AND KOSHLAND[3] was adopted in which reagent in acetone was added to enzyme in aqueous solution. Other methods and reagents were the same as described[5,6]. Preliminary experiments indicated that native malate dehydrogenase was inert toward HNB bromide. Thus, malate dehydrogenase in acetone–water (1:4, v/v) at pH 6–8 neither bound HNB bromide nor was it specifically inhibited by the reagent. Following denaturation of the enzyme in 8 M urea, however, it reacted with HNB bromide. Discontinuous titration of malate dehydrogenase was performed by adding HNB bromide in acetone at various concentrations to the enzyme in 8 M urea, 0.5 M sodium citrate, pH 3.0. After incubation, protein was separated from excess reagent by gel filtration. The enzyme was then partially reactivated by dilution in the presence of mercaptan[4,7,8]. Recovery of activity after this treatment in the absence of HNB bromide was about 10%, similar to previous studies in which mercaptan was not included in the denaturing medium[8]. The activity loss may have been due to sulfhydryl oxidation or other undetermined irreversible reactions. A plot

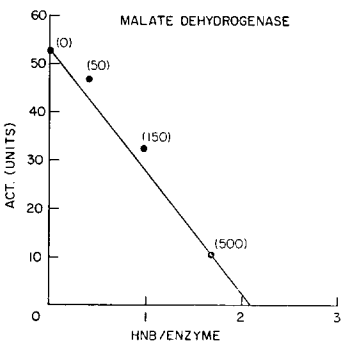

Fig. 1. Titration of malate dehydrogenase with HNB bromide. All work was done at 25°. Malate dehydrogenase, 28.5 nmoles, was incubated 1 h in 0.8 ml 8 M urea, 0.5 M sodium citrate, pH 3.0. HNB bromide, 0–500 mM in acetone, 0.2 ml, was then added and allowed to react 10 min. The protein was separated from excess reagent by gel filtration on a Sephadex G-25 column (0.8 cm × 35 cm). The column was preequilibrated and eluted with 8 M urea, 0.5 M citrate, pH 3.0. The fractions containing the protein were assayed spectrally for HNB and protein, and for enzyme activity after dilution. Ultraviolet spectra were taken on aliquots diluted 2:3 with 2 M NH_4OH. HNB and protein were calculated from $A_{280\ nm}$ and $A_{414\ nm}$ with the values malate dehydrogenase $\varepsilon_{(280\ nm)}$, 19 600, HNB $\varepsilon_{(414\ nm)}$, 18 500. For reactivation by dilution, aliquots were diluted 1:50 with 0.1 M mercaptoethanol, 0.1 M pyrophosphate, pH 8.6, and incubated 3 h. Enzyme activity was then determined from the initial $\Delta A_{340\ nm}$ with final concentrations: diluted enzyme, 1:60; NAD, 1 mM; L-malate, 10 mM; and glycine, 0.1 M, pH 9.8. The numbers in parentheses denote concentration of HNB bromide (mM) in the acetone.

Abbreviation: HNB, 2-hydroxy-5-nitrobenzylgroup.

of residual enzyme activity *vs.* protein-bound HNB is shown in Fig. 1. The titration curve of malate dehydrogenase indicates complete inhibition accompanied by binding of 2 moles reagent. The inactivation is almost certainly due to reaction with tryptophanyl residues, and the stoichiometry suggests one reactive tryptophanyl residue per subunit of enzyme. Other amino acids, such as cysteine, methionine or tyrosine, react with HNB bromide in alkaline media, but at the low pH used here, the reaction should be relatively specific for tryptophan[1-3]. The end point is in accordance with previous studies of tryptophan content[5,6], and it implies a 1:1 adduct with attachment of the methylene group of HNB to C-3 of tryptophan[5,6,9].

Since the enzyme did not react in neutral aqueous solution, but only in 8 M urea, the residues that were modified may normally exist in a sterically inacessible environment. The loss of enzyme activity on modification of tryptophanyl residues may indicate that the reactive tryptophanyl residues are essential for activity, or the activity loss may have been due to the prevention of refolding of the molecule to an active conformation.

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Isozymes of lactate dehydrogenase in the outer segments of the retina

The question concerning the presence of enzymes in the outer segments of the retina is of considerable interest because of the possibility of their participation in the first stage of the photoreception process.

We have shown earlier the presence of lactate dehydrogenase (EC 1.1.1.27, L-lactate–NAD oxidoreductase, LDH) activity in a fraction of bovine retina segments[1]. The activity of lactate dehydrogenase observed in this fraction was not due to the