BBA 61191

Dual effect of *N*-ethylmaleimide and other maleimides on α-glycerophosphate dehydrogenase activity

From the sigmoidal rate dependence on the substrate concentration, it is known[1-3] that NADH, L-α-glycerophosphate and dihydroxyacetone phosphate produce substrate inhibition when α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate:NAD^+ oxidoreductase, EC 1.1.1.8) is assayed in triethanolamine buffer (pH 7.5). Substrate inhibition is known to be pH dependent[2,4], and kinetic studies suggest that this inhibition is due to the formation of multiple dead-end enzyme–substrate complexes[2].

Incubation of the enzyme with *N*-ethylmaleimide removes the substrate inhibition giving a normal Michaelis–Menten kinetics (Fig. 1). Although this effect is observed in both directions of the enzymatic reaction, the experiments to be described

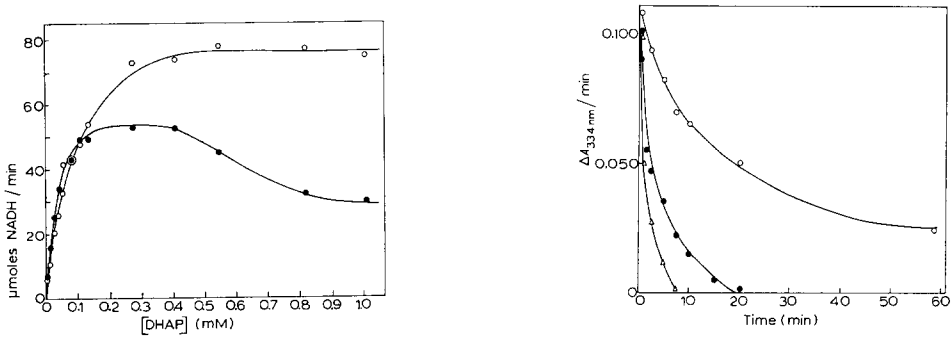

Fig. 1. Effect of *N*-ethylmaleimide on substrate inhibition of glycerol-3-phosphate dehydrogenase by dihydroxyacetone phosphate (DHAP). 1.43 nmoles enzyme were incubated with 14.3 μmoles *N*-ethylmaleimide during 20 sec in 0.1 M triethanolamine buffer (pH 6.9) at 0°. The reaction was quenched by diluting 0.1 ml of the incubation mixture with 0.9 ml of a 10-fold excess cysteine solution in the same buffer. The same results are obtained if, after the addition of cysteine, the incubation mixture is filtered through Sephadex G-25 or dialyzed at 4° against 10 mM phosphate buffer (pH 7.0) containing EDTA and cysteine both at 1 mM. Control experiments were done in the same way with the omission of *N*-ethylmaleimide. Activity was assayed at 25° in 50 mM triethanolamine buffer (pH 7.55). NADH was 47 μM and enzyme was 2.3 nM. ●—●, control; ○—○, enzyme–*N*-ethylmaleimide.

Fig. 2. Time and concentration dependence of the effect of *N*-ethylmaleimide on the glycerol-3-phosphate dehydrogenase. Conditions as in Fig. 1 but the enzyme to *N*-ethylmaleimide ratios were: 1:10 000 (○—○), 1:50 000 (●—●) and 1:100 000 (△—△), respectively. Control enzyme had a $\Delta A_{334\,nm}$/min = 0.060.

here have been done using dihydroxyacetone phosphate as substrate. The Michaelis constant, K_m, for dihydroxyacetone phosphate with both native enzyme and enzyme–*N*-ethylmaleimide complexes measured at noninhibitory substrate concentrations (6.6–130 μM) is equal to 88 μM, which is in agreement with previously obtained values[1-3].

As shown in Fig. 2, depending on the time of incubation and on the molar enzyme to *N*-ethylmaleimide ratio, activation or inhibition of the enzyme activity can be observed.

At each enzyme to *N*-ethylmaleimide concentration ratio investigated, the extent of enzyme activation observed at very short time periods is independent of this ratio. Subsequently, the enzyme rapidly undergoes inactivation with increasing *N*-ethylmaleimide concentrations.

The formation of enzyme–*N*-ethylmaleimide complexes is in both cases pH dependent (Fig. 3). However, the enzyme reaction with *N*-ethylmaleimide leading to activation depends on the basic form of a group with a pK_a of 6.4 (Fig. 3A); that reaction leading to inactivation depends on the basic form of a group with a pK_a of 8.7 (Fig. 3B).

The behavior of both maleimide and *N*-phenylmaleimide is similar to that of *N*-ethylmaleimide, while maleic acid, succinimide and 2-methylimidazol do not activate the enzyme. The rate constant for the formation of the inhibited enzyme –

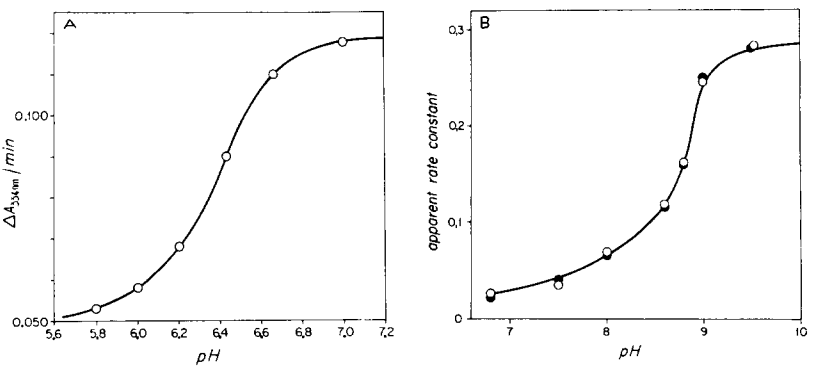

Fig. 3. pH dependence of the formation rate of the enzyme–*N*-ethylmaleimide complexes. Activity was assayed as described in Fig. 1. (A) "Active" enzyme–*N*-ethylmaleimide complex. 50 mM triethanolamine buffer was used throughout. The enzyme to *N*-ethylmaleimide ratio was 1:10 000. (B) "Inactive" enzyme–*N*-ethylmaleimide complex. For pH 9 and 9.5, 100 mM Tris buffer was used, and for these two experimental values the enzyme to *N*-ethylmaleimide ratio was 1:5000. ●—●, the theoretical curve obtained taking 8.78 as the pK_a.

N-ethylmaleimide complex depends on the N-substituted maleimide used; inductive electron withdrawal markedly increases the rate constant k (l/mole·min): 0.450, 0.716 and 5.03 for *N*-ethylmaleimide, maleimide and *N*-phenylmaleimide, respectively.

It has been demonstrated[5,6] that *N*-ethylmaleimide reacts with the side chains of cysteine and histidine and with the α-amino group of some amino acids.

The pK_a of 6.4 for the formation of the active enzyme – *N*-ethylmaleimide complex suggests that a histidyl residue is involved; for the inhibited complex, a pK_a of 8.7 suggests the participation of –SH groups.

Furthermore, photo-oxidation of the enzyme with Rose Bengal abolishes the formation of the active enzyme–*N*-ethylmaleimide complex. These preliminary results suggest that if histidine is involved, perhaps it reacts *via* the formation of an acyl imidazol intermediate[5] which subsequently acylates another amino acid residue in the enzyme molecule. In our laboratory an additional study is being made to clarify this reaction and to establish which residue finally binds the *N*-ethylamaleimide molecule in the active enzyme–*N*-ethylmaleimide complex.

The present findings suggest that *N*-ethylmaleimide acts as an "activator" by blocking the binding site for the formation of the dead-end enzyme–dihydroxyacetone phosphate complex[2].

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Enzymatically active membranes: Some properties of cellophane membranes supporting cross-linked enzymes

Some recent studies[1–3] have emphasized the biological interest of enzymes linked to insoluble macromolecules.

In a previous work, we have shown that bifunctional agents can be used to bind enzymes to insoluble sheets and membranes[4]. In this paper, we report the preparation of catalytically active cellophane–glucose oxidase (β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) membranes, using glutaraldehyde as the cross-linking agent[5,6], and we describe some properties of the bound enzyme as compared with the soluble one. Similar techniques have been employed successfully using other enzymes such as trypsin, chymotrypsin, urease, carbonic anhydrase, urate oxidase (uricase) and different supporting membranes.

Cellophane sheets (0.05 mm thick) are impregnated with an enzyme solution containing 6 mg/ml of protein in water. The water is evaporated by perventilation at 4°. The coupling between enzyme molecules is obtained by dropwise addition of a solution of 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 6.8)[5] on both surfaces of the membrane. The preparation is then left overnight at 4°. The membrane is rinsed with a physiological saline solution until the excess of uncoupled protein and glutaraldehyde is completely eluted. This is controlled by the decay of the absorption at 280 mμ of the rinsing solution. Nitrogen determination shows that 0.1 mg of protein is bound to 1 cm^2 of cellophane.

In order to measure the activity of the bound enzyme, the membrane is dipped in a solution of glucose through which pure oxygen is bubbled. At intervals samples