DESENSITIZATION OF THE ALLOSTERIC SITES OF GLUTAMATE DEHYDROGENASE BY FLUORODINITROBENZENE

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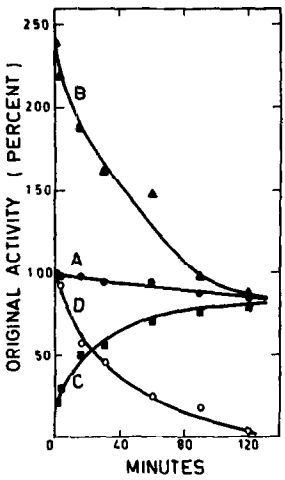
Beef liver glutamate dehydrogenase has been reported to possess several kinds of binding sites: a) substrate or active site for NAD, $NADH_2$, NADP and $NADPH_2$, b) purine nucleotide site, c) non-substrate site which binds only $NADH_2$(Frieden, 1963). The availability of site b) is of considerable interest, in view of the possibility of control of reaction rates by purine nucleotides in vivo. Under certain conditions, some of these nucleotides (e.g. ADP) enhance, and others (e.g. GTP) inhibit, the enzymatically catalyzed reaction. Kinetic studies have shown that purine nucleotides compete for the same site and that relatively high concentrations of oxidized pyridine nucleotides give unexpectedly high reaction rates. This activation has been ascribed to interaction at the purine nucleotide site (Olson and Anfinsen, 1953; Frieden,1959a; di Prisco et al.,1965).

This paper deals with the modifications of the first two kinds of enzymatic sites caused by treating the enzyme with fluorodinitrobenzene, a compound known to react with functional groups present in proteins. Evidence is provided for the existence of separate ADP- and GTP-binding sites; the evidence indicates also that the sites of interaction for "activating" concentrations of NAD are identical to the GTP sites.

Methods - The activity of glutamate dehydrogenase, supplied by Sigma as a sodium phosphate-glycerol solution, was measured at 23-25° by recording the initial reduction of NAD at 340 mμ by means of a Gilford optical density converter and a Beckman model DU monochromator. The unit of activity is defined as described previously (Strecker, 1955). 1-Fluoro-2,4-dinitrobenzene (FDNB) was dissolved in ethanol and added to the enzyme solutions to a final concentration of 1 mM; dinitrophenylation was carried out in the dark at 32°, in 0.05

M phosphate buffer, pH 8.0.

Results and discussion - When glutamate dehydrogenase was treated with FDNB, at pH 8.0 and 32°, almost complete loss of enzymatic activity occurred within 2 hours (Fig. 1, Curve D). The presence of 1 mM NAD during the incubation prevented this inactivation (Fig. 1, Curve A); the substrate L-glutamate, was ineffective. When the enzyme undergoing dinitrophenylation in the presence of NAD was assayed in the presence of ADP (allosteric activator) of GTP (allosteric inhibitor), a time-dependent desensitization was observed with both these modifiers (Fig. 1, Curves B and C). The presence of 1 mM ADP, in addition to NAD, during dinitrophenylation prevented the time-dependent abolition of ADP activation, whereas desensitization with regard to GTP inhibition was unaffected (Fig. 2). Conversely, incubating the enzyme with FDNB in the presence of 0.1 mM GTP (and NAD) completely prevented the desensitization to GTP, but not that to ADP (Fig. 3). Finally, the simultaneous presence of both



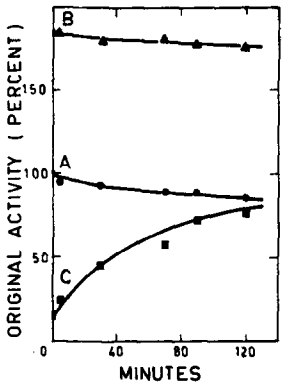


Fig. 2 - Effect of dinitrophenylation in the presence of ADP. Experimental conditions, as in Fig. 1, except that 1 mM ADP was present during incubation with FDNB.

Fig. 1 - Effect of dinitrophenylation on sensitivity to ADP and GTP. 0.3 mμmoles (0.6 mg) of glutamate dehydrogenase were incubated at 32° with 1 mM FDNB in 0.05 M potassium phosphate buffer, pH 8.0, and 1 mM NAD (omitted in the sample of Curve D). The final volume was 0.5 ml. Aliquots (20 μl) were removed at different times and assayed. For Curve A (●) and D (○), the assay mixture consisted of 0.05 M Tris-HCl buffer, pH 9.0, 8.3 mM potassium glutamate and 0.05 mM NAD, in a final volume of 3 ml. Curve B (▲), 0.5 mM ADP, and Curve C (■), 0.02 mM GTP added to the assay mixture.

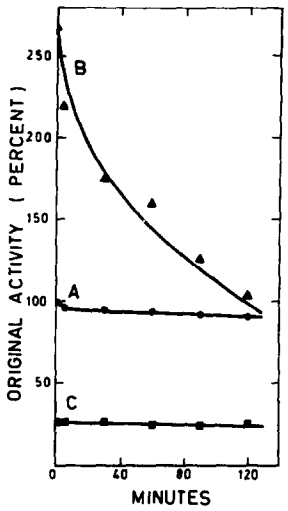


Fig. 3 - Effect of dinitrophenylation in the presence of GTP. Experimental conditions, as in Fig. 1, except that 0.1 mM GTP was present during incubation with FDNB.

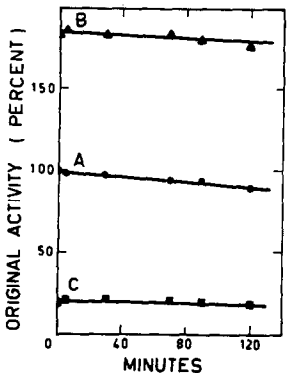


Fig. 4 - Effect of dinitrophenylation in the presence of both modifiers. Experimental conditions, as in Fig. 1, except that 1 mM ADP and 0.1 mM GTP were present during incubation with FDNB.

modifiers (and NAD) yielded curves such as those of Fig. 4, thus proving that under these conditions desensitization to both modifiers could be prevented. The loss of catalytic activity during dinitrophenylation in the absence of NAD could not be prevented by substituting ADP, GTP, or both, for the pyridine nucleotide.

The data summarized in Figures 1-4 indicate that the FDNB-induced desensitization to an activator (ADP) or an inhibitor (GTP) can be selectively prevented by protecting with ADP or GTP, respectively, during dinitrophenylation. It seems reasonable to conclude, therefore, that the site for ADP and that for GTP are not identical, and can be differentiated from one another as a result of a chemical modification of the enzyme, such as dinitrophenylation. A recent report (Colman and Frieden, 1966) has shown that acetylation of glutamate dehydrogenase induces considerable changes in its kinetic properties; in view of the marked difference exhibited by ADP and GTP in protecting against such changes, overlapping but not identical sites have been suggested for these nucleotides, in agreement with the conclusion drawn from the data reported above.

An interesting question remained open, namely the determination of the site

at which activating concentrations of NAD(P) interact (Frieden 1959a; di Prisco et al., 1965). Experiments were conducted in which aliquots of glutamate dehydrogenase were incubated with FDNB together with NAD, ADP or GTP. After 2 hours the reaction rate was measured as a function of NAD concentration. Fig. 5 shows double reciprocal plots obtained from these measurements. The control curve (A) was biphasic, as a consequence of abnormal activation at NAD concentration higher than 0.1 mM; similar biphasic curves had been reported previously (Olson and Anfinsen, 1953; Frieden, 1959a). Dinitrophenylation, carried out in the presence of NAD in order to protect against inactivation, abolished this abnormal activation: the plot (Curve B) was linear over the whole range of NAD concentrations, thus indicating desensitization to NAD when this cofactor acts as a modifier, and also inability of the protecting NAD present during the incubation to prevent this effect. Curve C, obtained when ADP was present during FDNB treatment, was also linear, proving that desensitization to NAD occurred in spite of the presence of the adenine nucleotide. On the other hand, the presence of GTP during dinitrophenylation yielded plots of the type of Curve

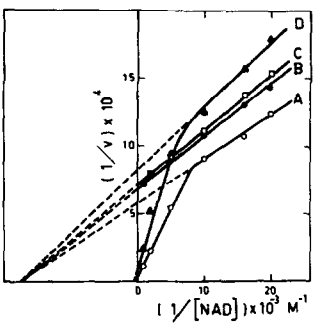


Fig. 5 - Effect of NAD concentration on the reaction rate after dinitrophenylation. The samples were treated with FDNB for 2 hours under the conditions of Fig. 1, then chilled and assayed at pH 9.0 in a system containing 0.025 M potassium glutamate and varying concentrations of NAD (final volume, 3 ml). 1 mM NAD was present in all samples during incubation. For Curve A (○), FDNB was not present during the incubation. Curve B (●), dinitrophenylation in the absence of purine nucleotides. Curve C (□), 1 mM ADP, and Curve D (▲), 0.1 mM GTP present during dinitrophenylation. Rates are expressed as units min^{-1} ml^{-1}.

D, indicating NAD interaction at a site other than catalytic. This site and the GTP site, therefore, must be identical. It has been suggested that the activation by NAD may not occur through the binding of this cofactor at the same site

responsible for the binding of ADP (Anderson and Reynolds, 1966). These findings provide experimental support in favor of this hypothesis.

The data reported in Fig. 5 show also that no change in either the Michaelis or the activation constants of glutamate dehydrogenase occurred as a result of dinitrophenylation in the presence of NAD. The values of these constants have been calculated to be 63 μM and 2 mM respectively, in good agreement with the values of the literature (Frieden, 1959b; Colman and Frieden, 1966).

Work to be reported elsewhere is now in progress to determine the uptake of FDNB, as well as to identify the amino acid residues that are dinitrophenylated in parallel with the loss of catalytic activity, ADP activation and GTP inhibition.

REFERENCES

Anderson, B.M., and Reynolds, M.L., J. Biol. Chem., 241, 1688 (1966).
Colman, R.F., and Frieden, C., J. Biol. Chem., 241, 3652 (1966).
di Prisco, G., Arfin, S.M., and Strecker, H.J., J. Biol. Chem., 240, 1611 (1965).
Frieden, C., J. Biol. Chem., 234, 809 (1959a).
Frieden, C., J. Biol. Chem., 234, 815 (1959b).
Frieden, C., J. Biol. Chem., 238, 3286(1963).
Olson, J.A., and Anfinsen, C.B., J. Biol. Chem., 202, 841 (1953).
Strecker, H.J., in Methods in Enzymology, Vol. II, ed. by Colowick, S.P., and Kaplan, N.O., Academic Press, p. 220 (1955).