INTERACTION OF GLUTAMATE DEHYDROGENASE WITH FLUORESCENT DYES

G.H. Dodd and G.K. Radda

Department of Biochemistry, University of Oxford, England.

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The binding of the dye bromothymol blue has been advocated as a simple test for the capacity of a ligand to induce a transition of state in a protein and as an independent method of studying the binding of effectors to allosteric proteins. (Ullman, Vagelos and Monod, 1964).

We now report our studies on the binding of several fluorescent dyes to beef liver glutamate dehydrogenase (L-glutamate-NADP oxidoreductase (deaminating), EC 1.4.1.3) (GDH), one of which (1-anilino-8-naphthalene sulphonate, ANS) can be used as a probe for detecting some transitions of state in the enzyme.

The binding of carefully purified eosin (which is known to be an inhibitor of GDH, Wolff and Wolff, 1957) is typical of fluorescein dyes. It occurs at relatively few sites, some of which appear competitive with ADP binding sites (Table I). These results were obtained by measuring the fluorescence polarisation of eosin on binding and analysing the titration curves by Scatchard's method (Scatchard, Coleman and Shen, 1957).

Of the effectors only ADP seems to have a significant effect on eosin binding. This may be accounted for by the suggestion that the ADP and GTP binding sites are not identical (Coleman and Frieden, 1966), particularly since GTP binding is

TABLE I

Binding of Eosin to GDH

Added substance	Number of binding sites/400,000 subunit	Average K association (M^{-1})
None	2.5	3.5×10^5
1 mM L-Glutamate	2.4	3.0×10^5
1 mM GTP	2.1	2.6×10^5
1 mM ADP	1.1	1.6×10^5
1 mM NAD	2.8	1.1×10^5
0.2 mM diethyl stilbestrol	2.6	3.6×10^5

Experimental conditions: GDH 2 mg/ml titrated with eosin in 0.1 M phosphate buffer, pH 7.7. Limiting value obtained by titrating eosin (2×10^{-7} M) with GDH.

stronger than that of ADP in the presence of NADPH (Frieden, 1963). It is also likely that the binding of diethyl stilbestrol also occurs at a different site from ADP. The inhibition by eosin is reversed by ADP, and eosin binding can also be reversed by dilution or gel filtration. Because of the ready photo-reducibility of fluorescein dyes by NADH the effect of this coenzyme on eosin binding could not be investigated.

ANS which has been shown to be a suitable probe for hydrophobic binding sites (Stryer, 1965; Daniel and Weber, 1966) is an inhibitor of both glutamate and alanine dehydrogenase activities of GDH. At high concentration this dye also promotes the polymer-monomer dissociation of GDH. (It should be noted that the term monomer is used for the 400,000 subunit of GDH which is made up of several peptide units). This dissociation is facilitated by NADH. ANS at low concentrations has relatively little effect on the enzyme although it is bound to the protein

as shown by a large increase in its fluorescence quantum yield. (A 6-fold increase in fluorescence at 2 μM ANS and 2 mg/ml GDH). At an ANS concentration of 2 μM less than 1 mole of ANS is bound per mole of enzyme at enzyme concentrations shown in Table II. Protein transitions of state then can be detected by titrating the enzyme with effector in the presence of 2 μM ANS. The fluorescence intensity of the ANS-enzyme complex is unaffected by either NADH or GTP alone but it increases (indicating increased ANS binding) when titrated with NADH in the presence of GTP or with GTP in the presence of NADH (Fig. 1 and 2). These changes take place over the same NADH concentration range as the changes observed in the ORD properties of GDH (Bayley and Radda, 1966) The sigmoidicity of the curves is dependent on the relative concentration of the effector to coenzyme. This is consistent with heterotropic interactions between NADH and GTP. (Monod,

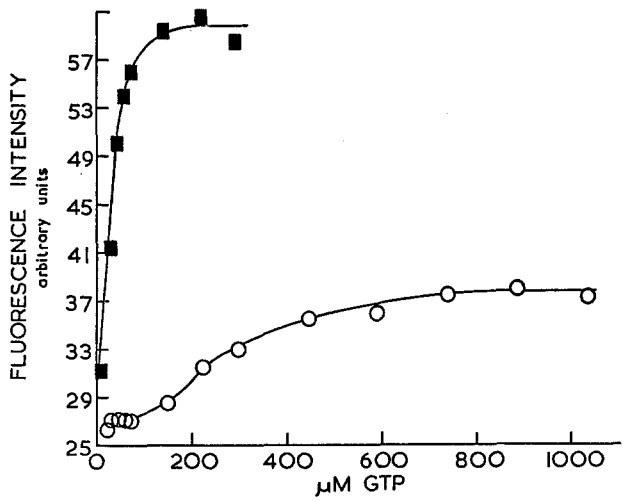

Fig. 1. Titration of GDH with GTP in the presence of 2μM ANS and NADH.

squares: NADH 6.1 x 10^{-6}M
circles: NADH 6.1 x 10^{-7}M
GDH concentration 1 mg./ml. λ excitation 410 mμ λ emission 550 mμ (NADH contribution to fluorescence under these conditions is small).

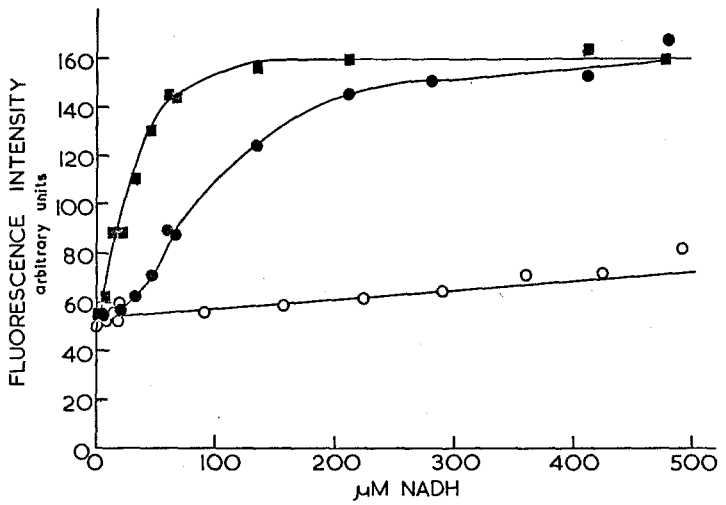

Fig. 2. Titration of GDH with NADH in the presence of 2μM ANS and GTP

squares: GTP 10^{-3}M
solid circles: GTP 10^{-4}M
open circles: no GTP

Wyman and Changeux, 1965). Similar transitions are caused by GTP in the presence of NADPH and by diethyl stilbestrol or phenanthridine in the presence of NADH. ADP reverses the GTP effect while the NAD-GTP, NAD-NADH or ATP-NADH couples have no effect on ANS binding. (Table II).

The detailed interpretation of our results will be presented elsewhere. We believe that the observed increases in ANS binding are not a result of the dissociation of the enzyme into subunits, as these changes are observable at enzyme concentration where GDH is largely dissociated. We therefore attribute the changes in ANS binding to a conformational transition in the protein. Apart from the ease of observation of the transitions, our method is sensitive enough to study the changes at lower enzyme concentrations than is practicable with ORD or spectroscopy (Bayley and Radda, 1966) and thus may provide a link between kinetic and physical studies.

Table II.

The effect of inhibitors on ANS binding to GDH

enzyme concentration (mg/ml)	Added substance	Ligand used in titration	Ligand concentration at half point (μM)	total fluorescence enhancement
0.1	10^{-4} M NADH	GTP	30	1.7
1.0	6.1×10^{-5} M NADH	GTP	12	1.3
1.0	6.1×10^{-6} M NADH	GTP	20	1.9
1.0	6.0×10^{-7} M NADH	GTP	200	1.5
1.0	6.1×10^{-5} M NADH + 5×10^{-3} M NAD	GTP	30	1.3
0.1	10^{-4} M NADH + 10^{-3} M GTP	ADP	480	0.4
1.0	10^{-3} M NADPH	GTP	13	1.4
0.1	10^{-4} M NADH	diethyl stilbestrol	6	2.0
0.1	10^{-4} M NADH	phenanthridine	60	1.8
2.0	9.3×10^{-4} M GTP	NADH	20	3.0
2.0	9.3×10^{-5} M GTP	NADH	80	3.0
2.0	none	NADH (up to 8×10^{-4} M)	-	none

Conditions: 0.1 M phosphate buffer, pH 7.7 in the presence of 2×10^{-6} M ANS.

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