The Interaction of Tetraiodofluorescein with Glutamate Dehydrogenase from Bovine Liver

MANFRED KEMPFFLE and ROBERT MÜLLER
Physiologisch-Chemisches Institut der Universität, 53 Bonn, Nussallee 11
West Germany

and HEINZ WINKLER
Max-Planck-Institut für Biophysikalische Chemie, 34 Göttingen-Nikolausberg,
West Germany

Reports from various laboratories have dealt with the binding properties of glutamate dehydrogenase from bovine liver (EC 1.4.1.3) (Pantaloni & Dessen, 1969; Huang & Frieden, 1969; Malcolm, 1972; Brown et al., 1973; Koberstein & Sund, 1973), but unfortunately these reports differ considerably over the number and nature of glutamate dehydrogenase coenzyme-binding sites as well as the magnitude of their dissociation constants.

Fluorescence spectroscopy (Pantaloni & Dessen, 1969; Huang & Frieden, 1969; Brown et al., 1973) and relaxation kinetics (Kempffle et al., 1974) indicated that there are two binding sites for NADH but only one for NADPH. To support these earlier findings difference-spectrophotometric studies were performed with tetraiodofluorescein (Erythrosin B) as indicator to study the binding properties of some nicotinamide nucleotides acting as coenzymes or effectors, since absorption difference spectrophotometric titrations with coenzymes as titrants had failed owing to the high absorbance of these compounds at higher concentrations.

Since it is reported in the literature (Johnson, 1974; Wasserman & Lentz, 1971) that tetraiodofluorescein binds at the active site of nicotinamide nucleotide-dependent dehydrogenases, this dye appears to be an appropriate tool for studying nicotinamide nucleotide interactions with glutamate dehydrogenase. Further, the application of this compound has the great advantage that the perturbation of the dye absorption spectrum, indicating binding to the enzyme, falls in a wavelength range (between 500 and 600 nm) far away from enzyme and nucleotide absorption.

Results

All measurements were carried out in a Cary 118 spectrophotometer, thermostatically controlled at 25°C.

Dye binding to the enzyme. (a) Low dye concentrations (<5 μM). Tetraiodofluorescein at low concentrations, bound to glutamate dehydrogenase, shows a difference spectrum with a maximum at 538 nm and an isosbestic point at 527 nm. Titrations of dye with increasing amounts of enzyme result in saturation of the dye with enzyme. Under conditions of high enzyme/dye ratios it can be assumed that only the high-affinity enzyme binding site will contain tetraiodofluorescein and that all bound dye molecules will have the same spectrum. The presence of an isosbestic point justifies this assumption.

According to the 'classical' procedures of evaluating difference spectra we obtain a difference extinction coefficient

$$\Delta \varepsilon_{538 \text{nm}} = \varepsilon_{\text{bound dye}} - \varepsilon_{\text{free dye}}$$

of $3.21 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ and a $K_d/n$ value of 6 μM (n is number of binding sites).
A Scatchard plot using this calculated value of $\Delta \varepsilon$ yields $n = 1$ and $K_d = 6 \mu M$.

(b) High dye concentrations (>10 $\mu M$). At higher dye concentrations, however, this procedure is no longer justified since more than one site of the enzyme (with different affinity for the ligand) may now be occupied. At enzyme/dye ratios smaller than unity and unity a red shift of the maximum (to 542 nm) as well as the isosbestic points (to 532 nm) is observed. Scatchard plots of the titration curves yield two different straight lines with different slopes, leading to a value of $n = 2.5$ and indicating two binding sites with $K_d$ values of $K_{d1} = 6 \mu M$ and $K_{d2} = 30 \mu M$.

(c) Very high dye concentrations (>60 $\mu M$). High excess of dye leads to non-specific binding to the enzyme, reflected by the appearance of a new shoulder in the difference spectrum at around 515 nm. Moreover, the former maximum is shifted from 538 nm to 552 nm and the isosbestic point to 505 nm.

Displacement of the dye by nicotinamide nucleotides. As the dye apparently binds to the nicotinamide nucleotide-binding sites of the enzyme (Johnson, 1974), displacement of the dye by coenzymes should occur and therefore give information about the properties of these sites. Cuvettes with dye and enzyme were preincubated at 25°C and titrated with coenzyme in one cuvette and buffer in the other one. The appearance of a difference spectrum justifies the assumption that the coenzyme expels the dye from its specific binding site and thus we should be able to predict the nature and number of binding sites for the different nucleotides.

By means of the experiments described above we can discriminate between the different dye-binding sites exploiting their different absorption spectra and thus we can correlate spectral perturbations with distinct binding sites. GTP alone does not change the difference spectrum and thus is apparently unable to expel the dye from its binding sites. ADP displaces the dye from the binding site with higher affinity, reflected by a spectral change at 538 nm. NADP on the other hand shows a spectral perturbation at 542 nm indicating a binding process to the lower affinity site. The results with NAD are more complex. As claimed in the literature two sites with different affinity should be detectable. Depending on the enzyme/dye ratios we obtain spectral changes at 538 nm or 542 nm and even inversion of the sign of these changes occurs. This confirms that the enzyme exhibits two different binding sites with NAD.

All experimental evidence supports the idea that the ADP-binding site ($\lambda_{\text{max}} = 538$ nm) is identical with the stronger binding site for NAD ("adenine-binding site"), which we would like to identify with a 'storage site' for NAD. On the other hand the NADP-binding site ($\lambda_{\text{max}} = 542$ nm) seems to be identical with the weaker binding site for NAD ("nicotinamide-binding site") and obviously represents the catalytic site of the enzyme.

A quantitative description of these displacement experiments can be obtained by considering the following reaction scheme:

$$E + D \overset{K_1}{\longrightarrow} ED, \quad E + L \overset{K_2}{\longrightarrow} EL, \quad ED + L \overset{K}{\longrightarrow} EL + D$$

with $E =$ enzyme, $D =$ dye and $L =$ ligand and

$$K_1 = \frac{[ED]}{[E][D]}, \quad K_2 = \frac{[EL]}{[E][L]}, \quad K = \frac{[EL][D]}{[ED][L]}$$

Consequently

$$K_2 = K'K_1$$

$K_1$ is determined from a Scatchard plot of the dye-titration experiments, whereas $K$ is determined by displacement experiments. The great advantage of these displacement experiments is that they offer a method for determining dissociation constants which would otherwise be experimentally inaccessible.

Studies on the Mechanism of the 2-Oxoglutarate Dehydrogenase System

C. L. McMinn and J. H. Ottaway

Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

As an integral part of the tricarboxylic acid cycle, the 2-oxoglutarate dehydrogenase system has been intensively investigated. However, only an elementary kinetic study has been reported for the mammalian enzyme (Massey, 1960). Most work has been devoted to structural studies, and the proposed mechanism of action of the system has been based on knowledge of the reactions catalysed by the subunits and cofactors within the complex (Sanadi, 1963), as shown below:

\[
\text{E1-TPP} + \text{OG} \rightarrow \text{E1-TPP-Succ} + \text{CO}_2
\]

\[
\begin{align*}
\text{E2-lip} + \text{E1-TPP-Succ} & \rightarrow \text{E2-lip-S-H} + \text{E1-TPP} \\
\text{E2-lip} + \text{CoA} & \rightarrow \text{E2-lip-S-H} + \text{Succ-CoA} \\
\text{E2-lip} + \text{E3-FAD} & \rightarrow \text{E2-lip-S} + \text{E3-FADH}_2
\end{align*}
\]

\[
\text{E3-FADH}_2 + \text{NAD}^+ \rightarrow \text{E3-FAD} + \text{NADH} + \text{H}^+
\]

Where TPP represents thiamin pyrophosphate, OG is 2-oxoglutarate, Succ is succinate, lip is lipoate and Succ-CoA is succinyl-CoA (3-carboxypropionyl-CoA). For purposes of a computer simulation of this enzyme system, a more detailed study of kinetics and mechanism was necessary.

To this end, a series of initial-velocity studies was carried out by the Fromm (1967) method. This is a steady-state kinetic approach to the study of the mechanism of action of three substrate enzymic reactions, by which Ping Pong mechanisms can be distinguished from sequential mechanisms. The procedure involves measuring the initial velocity at varying concentrations of one substrate at a series of fixed concentrations of the other two substrates, which are maintained at a constant ratio to each other. The process is repeated for each substrate as the variable one. Lineweaver–Burk plots of the resulting data yield converging lines if a quaternary complex is involved and one or more sets of parallel lines if the mechanism is Ping Pong.

2-Oxoglutarate dehydrogenase was prepared from fresh pig heart by the method of Sanadi (1969). Concentration ranges used in this study were 0.015 mM to 0.75 mM for 2-oxoglutarate, 0.0025 mM to 0.1 mM for CoA and 0.01 mM to 0.333 mM for NAD+. The system was assayed for NADH production by using an SP 1800 recording spectrophotometer.

The mechanism proposed by Sanadi (1963), in the notation of Cleland (1963), is a Hexa Uni Ping Pong reaction. According to Fromm’s (1967) hypothesis this should yield three sets of parallel lines. This was not found to be the case. Only with varying [2-oxoglutarate] was there a parallel set of plots. The remaining two sets of assays yielded...