A Deactivating Conformational Change Induced by Reduced Nicotinamide–Adenine Dinucleotide Phosphate in a *Neurospora* Glutamate Dehydrogenase

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Stopped-flow fluorescence techniques have been used to observe the formation of the binary complex of E•NADPH. At pH 7.5 there is a protein conformational change after the formation of the binary complex. This conformational change can be detected by a decrease in the fluorescence intensity of the complex at 350 nm and by an increase in its fluorescence intensity at 450 nm.

The NADP⁺-specific glutamate dehydrogenase (EC 1.4.1.4) from wild-type *Neurospora crassa* (ST74A) is a hexamer of identical subunits (Gore *et al.*, 1972; Wootton *et al.*, 1972) that can undergo protein conformational changes resulting in a gain or loss of catalytic activity. A conformational change that occurs as the pH of a solution of this enzyme is increased or decreased in the range pH 6.0–7.8 was first reported by West *et al.* (1967). At pH 7.8 nearly all of the enzyme is in a catalytically active conformation, whereas the conformation prevailing at pH 7.0 or below is inactive. The pK of this transition in activity is at pH 7.3, and has been studied by Ashby *et al.* (1974). The conformation of the enzyme at pH 7.3 can be activated by an increase in pH or by the addition of di-, tri- or poly-carboxylic acids such as succinate, citrate or EDTA. Each substrate acid, L-glutamate or 2-oxoglutarate, will activate the enzyme; activation by L-glutamate is enhanced by NADP⁺ and that by 2-oxoglutarate by NADPH (West *et al.*, 1967).

The reduced coenzyme alone has been shown to bind both to the inactive and to the active conformation of the enzyme and to displace the equilibrium in favour of the inactive form (Gore *et al.*, 1972). In the presence of reduced coenzyme at pH values less than pH 8.5 the enzyme becomes catalytically inactive and this form of the enzyme specifically needs the substrate acid 2-oxoglutarate for reactivation.

Methods and materials

Materials. NADPH was obtained from the Sigma Chemical Co., Kingston-upon-Thames, Surrey,

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U.K. All other reagents were of AnalaR grade and were obtained from BDH Chemicals, Poole, Dorset, U.K.

Preparation of *Neurospora* mycelia. The details of the growth of *Neurospora crassa* and the subsequent stages in the purification of the enzyme have been given by Ashby *et al.* (1974).

Solutions and buffers. Solutions of all buffers and nucleotides were made up freshly before use. The concentration of a solution of nucleotide was determined from its E_{340} . The enzyme concentration is expressed as molarity of enzyme subunits (mol.wt. 48000; Wootton *et al.*, 1972), and was determined from its E_{280} (Ashby *et al.*, 1974). The enzyme used in the experiments was shown to have the specific activity of pure *Neurospora* glutamate dehydrogenase; 1 mg of pure enzyme/ml reduces 100 μ mol of NADP⁺ $\cdot$ ml⁻¹ $\cdot$ min⁻¹ in an assay solution containing 2.8 ml of 50 mM-Tris-HCl buffer, pH 8.5, and 0.2 ml of 0.2% (w/v) NADP⁺ in water. The temperature was 35°C (Ashby *et al.*, 1974).

Stopped-flow fluorimetry. The fluorescence changes were observed on a stopped-flow fluorimeter built by M. Iwatsubo. This apparatus was equipped with a xenon lamp as a light-source and had an excitation light-path of 2 mm and an emission light-path of 10 mm. The excitation wavelength was selected by using a Jobin–Yvon HRS 2 monochromator and the fluorescent light observed was selected by using optical filters. For NADPH fluorescence (excitation at 340 nm or 350 nm) a Kodak Wratten filter (no. 98) sufficed to eliminate the excitation light. For the protein fluorescence (excitation at 290 nm) two filters were used simultaneously, a Kodak filter no. 2 to eliminate the excitation light, and an MTO filter (H325a) to eliminate the bound-NADPH fluorescence, the latter allowing the passage of light between 250 and 400 nm with a maximum transmission of 80% at about 340 nm.

In these experiments the reacting solutions were diluted twofold unless otherwise stated, and all concentrations are expressed as final concentrations after mixing. The dead time of the apparatus was 2–3 ms. All experiments were carried out at 20°C unless otherwise stated. The stored oscilloscope traces were photographed on film (36 mm × 24 mm) with a Nikon camera, and the negatives were projected and copied by hand on to graph paper to show the fluorescence changes with time. These copies were used to calculate the reaction rates and the amplitudes of the observed changes.

Results and discussion

When NADPH saturates the reduced-coenzyme-binding sites of *Neurospora* glutamate dehydrogenase (E) there is an enhancement of the nucleotide fluorescence at 450 nm and a decrease in the protein fluorescence (by about 45–50%) at 350 nm (Gore *et al.*, 1972; Holbrook *et al.*, 1972). These fluorescence changes, which occur on formation of the binary complex, were used in preliminary studies involving the use of a stopped-flow device to deter-

Fig. 1. Observation of the rate of formation of the binary complex formed between *Neurospora* glutamate dehydrogenase and NADPH

The two syringes contained respectively 40 μ M-NADPH in 20 mM-potassium phosphate buffer, pH 6.5, and 4.0 μ M-enzyme in 50 mM-phosphate buffer, pH 6.5, at 10°C. The scales were 20 mV and 20 ms per division. Total fluorescence intensity due to the enzyme was 280 mV before reaction. The arrows indicate the origin of the trace. The inset shows $\log[100 - \text{percentage of the total change in fluorescence}]$ plotted against time (ms).

mine the rate of binding of NADPH to this enzyme. It became clear that in some conditions the binding of NADPH to the enzyme is at least partially limited by a factor other than the concentration of the NADPH or the enzyme. Fig. 1 shows a typical curve describing the formation of the binary complex E·NADPH with time (ms) obtained when enzyme (2 μ M in 50 mM-potassium phosphate buffer, pH 6.5) is mixed with 20 μ M-NADPH in the same buffer. The stopped-flow device was arranged to allow observation of the protein fluorescence, which is quenched partially by non-radiative energy transfer from the aromatic amino acids of the protein to the bound NADPH on formation of the binary complex. The residual protein fluorescence has already been shown to be proportional to the degree of saturation of the reduced-coenzyme-binding sites by NADPH at pH 6.3 (Holbrook *et al.*, 1972) and pH 7.5 (M. G. Gore, unpublished work), although no information is yet available for the system at pH 8.5. Therefore the enhancement of nucleotide fluorescence seen on formation of the binary complex E·NADPH was used to study the rate of binding of NADPH to the enzyme when the pH was above pH 7.5. The inset of Fig. 1 is a graph showing a semi-logarithmic plot of the change in fluorescence of the solution (350 nm) with time (ms), from which was calculated the first-order rate constant $k_{app.}$ (s^{-1}) for the formation of the binary complex. A series of concentrations of NADPH was used and the resultant values of $k_{app.}$ were plotted against the concentration of NADPH, the slope of this graph describing the 'on-rate' of NADPH to the binary complex. Typical values of the 'on-rate', i.e. k_+ , were $4.5 \times 10^6 M^{-1} \cdot s^{-1}$ at pH 6.5, $7.7 \times 10^6 M^{-1} \cdot s^{-1}$ at pH 7.5 and $10 \times 10^6 M^{-1} \cdot s^{-1}$ at pH 8.5; all reactants were dissolved in 50 mM-potassium phosphate buffer adjusted to the required pH by 5 M-KOH at 10°C. It was noted, especially at pH 7.5, that the rate of binding of NADPH to the enzyme was biphasic with time. A normal decrease in protein fluorescence takes place over the first 50–100 ms of the reaction, presumably owing to the formation of the complex E·NADPH. Values of $k_{app.}$ for this part of the curve are of the order of 10 – $100 s^{-1}$, depending on the molarity of the NADPH in the reacting solution. However, by using a slower oscilloscope time-scan it is possible to observe a second phase of binding of NADPH to the enzyme that lasts for about 20 s after mixing of the reactants at 10°C. The use of a 1.5–20-fold molar excess of NADPH over enzyme subunits did not affect the rate of this slow process, although the rate of the initial fast phase of reaction was affected. Temperature, however, had a great effect on this second phase of binding; e.g. at 10°C the process had a rate constant of $18.2 min^{-1}$ whereas at 25°C the rate constant became $98.4 min^{-1}$. Recordings at six temperatures suggested an activation energy

of about $80\text{ kJ}\cdot\text{mol}^{-1}$, which is of the order to be expected for protein conformational changes.

This second rate of binding was not present if enzyme at pH 6.5 was mixed with NADPH in buffer at pH 6.5 or above. There was a very small change in fluorescence at pH 8.5 when enzyme and NADPH were mixed. However, the maximum change in amplitude of the slow fluorescence change occurred when enzyme in 10mM-Tris-HCl buffer, pH 8.5, was mixed 1:6 (v/v) with NADPH in 50mM-potassium phosphate buffer, pH 6.3, so that the resultant pH of the solution was pH 6.5 (Fig. 2). This type of experiment was repeated with $20\text{ }\mu\text{M}$ -NADPH in solution in buffers of different pH so that the final pH produced in the stopped-flow apparatus could be varied from pH 6.0 to 8.8. In each experiment the buffer used was 10mM-Tris-HCl, pH 8.5, for the solution of the enzyme. This was sufficient to ensure that the enzyme was always in the catalytically active conformation before being mixed with the reduced coenzyme. The solution of NADPH was made up in either 50mM-potassium phosphate buffer, pH 6.0–7.8, or in 50mM-Tris-HCl buffer, pH 7.9–8.8, all adjusted at the temperature of the experiment, which was 10°C . It was clearly demonstrated that only if the final pH was below pH 8.5 was there any significant amount of the slow decrease in protein fluorescence. The inset to Fig. 2 shows the amplitude of the slow fluorescence change (mV) that occurs when enzyme originally at pH 8.5 is mixed 1:6 (v/v) with a solution of NADPH so that the final pH is between 6.5 and 8.8 (for conditions of reaction see the legend of Fig. 2).

A pK at pH 7.75 was obtained from this experiment, and this agrees closely with the pK obtained from steady-state kinetic data for the inactivation of the enzyme by NADPH (M. G. Gore, unpublished work). It is therefore suggested that the slow change in fluorescence seen in Fig. 2 is due to the inactivating conformational change occurring in the protein which limits the rate of binding of some molecules of NADPH to the enzyme. A suitable model for the system would be one where the affinity for the NADPH is higher in the inactive conformation of the enzyme than in the active conformation of the enzyme. Therefore as the enzyme assumes the inactive conformation more high-affinity binding sites for the reduced coenzyme become available. This would result in a loss of protein fluorescence at 350nm due to more non-radiative energy transfer from the enzyme's aromatic amino acids to the nicotinamide moiety of NADPH. If this is occurring then one might expect there to be a slow increase in the nucleotide fluorescence at 450nm (excited by light at 340nm) at the same rate as the slow decrease in protein fluorescence. The stopped-flow fluorimeter was arranged to allow observation of the nucleotide fluorescence and the experiments of Fig. 2 were repeated. A slow increase in fluorescence at 450nm was observed after the rapid enhancement of NADPH fluorescence emission of 450nm which occurred after the initial formation of the binary complex. This slow change in NADPH fluorescence not only had the same rate constant as the slow decrease in protein fluorescence but was also

Fig. 2. Decrease in protein fluorescence observed when a solution of $14\text{ }\mu\text{M}$ -enzyme in 10mM-Tris-HCl buffer, pH 8.5, is mixed 1:6 (v/v) with a solution containing $12\text{ }\mu\text{M}$ -NADPH in 50mM-phosphate buffer, pH 6.5

The final pH of the solution was 6.5. Trace A: 20mV and 20ms per division. Trace B: 20mV and 2000ms per division. Both syringes were thermostatically controlled at 10°C . The total fluorescence intensity due to the enzyme was 517mV before reaction with the NADPH. The arrows indicate a point 54mV immediately below the origin of the traces. The inset shows the amplitude in millivolts of the slow change in protein fluorescence that occurs after the formation of the binary complex E·NADPH plotted against the final pH of the reaction mixture (see the text).

the same percentage of the total change in fluorescence at 450 nm as the slow decrease in protein fluorescence was of the total change in fluorescence at 350 nm. This, of course, would be expected from the data of Holbrook *et al.* (1972), if the slow process observed is in fact due to more molecules of NADPH binding to enzyme. Ashby *et al.* (1974) have determined rate constants (k_{+1} , k_{-1}) for the conformational changes that occur when the pH of a solution of enzyme is perturbed:

E_a and E_i are the uncomplexed active and inactive conformations (respectively) of the enzyme E. They found k_{+1} and k_{-1} were 0.67 min^{-1} and 0.69 min^{-1} respectively at 26°C . It is clear that k_{+1} is very different from the rate constant of the conformational change observed in the present work (k_{+2} in the scheme):

where $E_a\text{NADPH}$ and $E_i\text{NADPH}$ are the catalytically active and inactive conformations respectively in the form of a binary complex with NADPH. Therefore it appears that the binding of NADPH to the active conformation of the enzyme 'energizes' the structure so that it more readily goes to the inactive conformation than in the absence of NADPH. It is also possible that the 'back-reaction' of rate k_{-1} is severely decreased in rate by the presence of the reduced coenzyme.

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