STUDIES ON THE BINARY AND TERNARY COMPLEXES FORMED BY A NEUROSPORA GLUTAMATE DEHYDROGENASE AND ITS SUBSTRATES

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SUMMARY. The $NADP^+$ specific glutamate dehydrogenase from wild-type Neurospora crassa forms a stable binary complex with NADPH. This can combine with L-glutamate, α-ketoglutarate or the substrate analogue D-glutamate to form ternary complexes which can be distinguished by their different fluorescence properties. The affinity of the enzyme for NADPH diminishes with increases in pH or ionic strength of the solution. Experimental data obtained using modified glutamate dehydrogenases from mutant strains of N. crassa suggest that the reduced-coenzyme binding sites observed fluorimetrically are the same as those observed by enzyme kinetics.

The $NADP^+$ specific glutamate dehydrogenase (E.C.1.4.1.4) from wild-type Neurospora crassa (ST74A) is an oligomeric protein consisting of six identical and equivalent subunits (1, 2) and is capable of undergoing a reversible conformational change induced by a change in pH in the range pH 7.0 to pH 8.0. The enzyme conformation present in solution at pH 8 0 is catallytically active whereas the species prevailing at pH 7.0 is inactive (3). Carboxylic acids such as succinate, citrate or EDTA will activate the enzyme present in solutions of pH 7.0. The only effector molecule shown to inactivate the enzyme (other than H^+) is NADPH (4), and the NADPH-inactivated enzyme is reactivated only by the presence of the substrate α-ketoglutarate (3). It was therefore of interest to observe the spectral properties of the binary complex (enzyme-reduced coenzyme) at pH values where inactivation did (eg pH 7.2) or did not take place (eg pH 9.0)

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Abbreviations: E., free enzyme in solution, E.NADPH, binary complex of enzyme and NADPH.

MATERIALS AND METHODS

NADPH, L-glutamate, D-glutamate and α-ketoglutarate were obtained from the Sigma Chemical Co. Ltd., Kingston-upon-Thames, U. K. All other reagents used were of the Analar grade and were obtained from BDH Chemicals, Poole, Dorset, U. K.

Wild-type Neurospora crassa (ST74A) and the mutant strain R24 were grown according to Ashby et al (5) and the enzyme purified and assayed as in a previous publication (6). Mutant strains of Neurospora-crassa am^1 and am^3 were cultured according to Coddington and Fincham (7) and the enzyme from each strain was purified as the wild-type enzyme. Enzyme concentrations quoted are of the sub-unit of MW 48.000 (8).

Spectrofluorimetry was carried out on a Hitachi-Perkin-Elmer 104 spectrofluorimeter. The operating temperature was $18^{\circ}C$. All buffers were of potassium phosphate adjusted to the required pH by 5M KOH. All spectra are corrected for changes in ionic strength when substrates etc. are added. Control solutions had KCl added to them to maintain an equivalent ionic strength.

RESULTS AND DISCUSSION

Figure 1 shows typical absorption spectra for the excitation of fluorescence for NADPH when in free solution or bound to this enzyme at pH 7.2. There is slight hypochromicity (about 15 - 18%) in the absorbance at 340nm of the nicotinamide moiety of NADPH and the wavelength causing maximum fluorescence emission from the NADPH shifts from 340nm (free) to 349nm when the reduced-coenzyme is in the complex E. NADPH. Cross and Fisher (9) have shown that changes in conformation of NADH molecules give rise to difference spectra resolvable (in the 340nm region) into red or blue shifts with hypo- or hyper chromicity. They suggest that the large red-shift seen with some dehydrogenases could be caused by the nicotinamide ring being in a more hydrophobic environment when in the active site of the enzyme than when in free solution. The addition of L-glutamate to a solution of the binary complex E. NADPH caused the hypochromicity at 340nm to increase to about 20%.

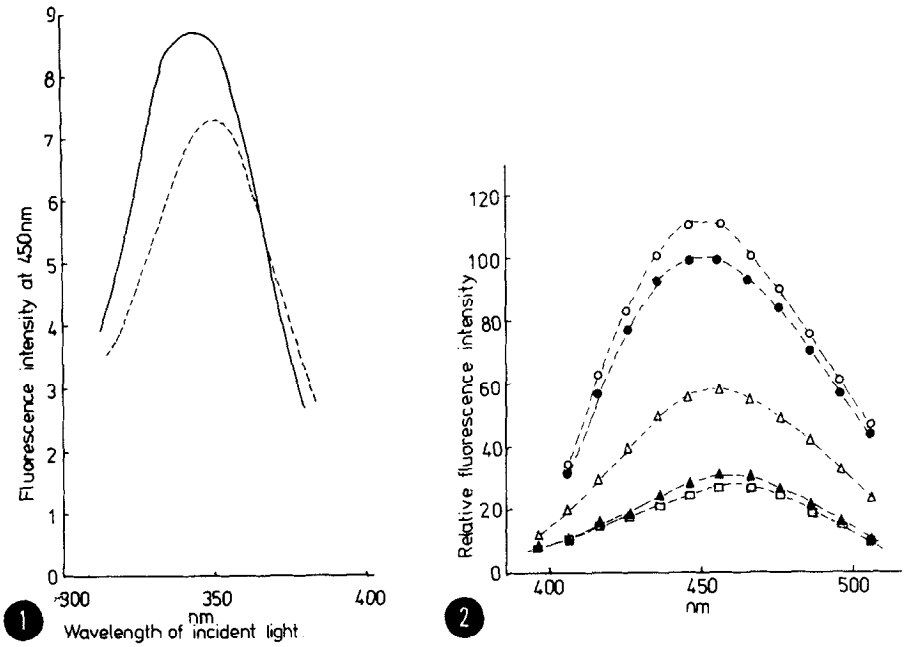

Fig. 1

Spectra showing the wavelengths of light which will excite fluorescence emission (450nm) from a solution of 3μM NADPH in the presence (---) or absence (——) 15μM enzyme in 10mM potassium phosphate buffer pH 7.2

Fig. 2

The relative intensity of fluorescence emitted from a solution of 3μM NADPH in 10mM potassium phosphate buffer pH 7.2 also containing (-▲-▲-) 15mM α-ketoglutarate and 15μM enxyme, (-Δ-Δ-) 30mM D-glutamate and 15μM enxyme, (-●-●-) 15μM enzyme, (-○-○-) 30mM L-glutamate and 15μM enzyme, (-□-□-) control solution with no additions.

A more marked change occurred in the fluorescence emission spectrum of NADPH when it formed the binary complex with enzyme (Figure 2). At pH 7.2 the position of the peak of NADPH fluorescence shifts towards the blue end of the spectrum (from 465 to 450nm) when the NADPH forms the binary complex and this blue shift is accompanied by a four-fold increase in the intensity of the fluorescence at 450nm. This is much larger than for the comparable complex formed between NADPH and the bovine glutamate dehydrogenase (10) and suggests that

the NADPH is more immobilised in complex with the Neurospora enzyme (11).

When the pH of a solution of binary complex was increased from pH 7.4 to pH 9.0 there was an almost total disappearance of the enhancement of fluorescence at 450nm and the position of peak fluorescence emission returned to 465 nm. At pH 9.0 the enzyme is only weakly inactivated by NADPH at concentrations which completely inactivates enzyme at pH 7.5 (4). It is therefore possible that the weak inactivation of enzyme at pH 9.0 is due to the enzymes low affinity for NADPH. Similar changes in the fluorescence emission spectrum of a solution of E. NADPH occurred if the ionic strength of the solution was increased by the addition of KCl, NaCl or by using potassium phosphate buffer of a higher molarity.

A 10% increase in the fluorescence of enzyme-bound NADPH was observed when 30mM L-glutamate was added to the solution at pH 7.4. This strongly suggests that there is a formation of a ternary complex E. NADPH L-glutamate. The substrate analogue D-glutamate had a much stronger effect on the fluorescence of the binary complex than L-glutamate. The addition of 30mM D-glutamate to a solution of binary complex caused the fluorescence at 450nm to decrease by about 50%. This large difference in fluorescence was used to monitor the saturation of the enzyme (enzyme-reduced coenzyme complex) by D-glutamate. The decrease in fluorescence of enzyme-bound NADPH on addition of D-glutamate, (presumably due to formation of an abortive ternary complex E. NADPH. D-glutamate) was half completed when 6-8mM D-glutamate had been added to the solution. It is possible, that the D-glutamate decreases the fluorescence of the solution by displacing NADPH from the binary complex, however it has been shown to be a potent competitive inhibitor for L-glutamate and α-ketoglutarate (4) which suggests that it is able to bind to the enzyme in the presence of $NADP^+$ or NADPH.

Although great care was taken to exclude NH_4^+ ions it is almost certain that the spectra obtained are of solutions which contain a small contaminating amount of NH_4^+. However, the addition of NH_4Cl to the solutions (1 - 100mM) had no effect on the spectra other than that of increasing ionic strength.

The oxidised substrate α-ketoglutarate decreased the fluorescence of NADPH almost to that of NADPH in free solution. This is certainly due to the formation of a ternary complex, E. NADPH. α-ketoglutarate. The fluorescence intensity at 450nm from a solution of binary complex was decreased by half when 3-5 mM α-ketoglutarate was added.

This experiment was carried out differently from the others since any contaminating NH_4^+ ions would complete a reaction mixture and lead to oxidation of NADPH. However, at pH 7.2 the binary complex is catalytically inactive and even in the presence of 20mM α-ketoglutarate it does not regain full activity for several minutes at 10°C (4). A solution of 10mM α-ketoglutarate with binary complex (see legend to Figure 2) was shown to be stable for at least 30 seconds. Therefore the spectrum of the complex E. NADPH. α-ketoglutarate was obtained by adding an aliquot of 0.5M α-ketoglutarate solution (adjusted to pH 7.2 by 5M KOH) to a sample of binary complex, rapidly mixing and reading the fluorescence of the solution at one wavelength. This procedure never took more than 10 seconds and using separate enzyme samples and gradually increasing the wavelength of fluorescence observed it was possible to obtain the spectrum given in Figure 2.

Similar studies were carried out using purified enzyme prepared from strains of Neurospora crassa containing a modified $NADP^+$-linked glutamate dehydrogenase. These enzymes were extracted and purified from the mutant strains am^1 and am^3 (7) and R24 (12). The latter two enzymes am^3 and R24 were both shown to bind NADPH with spectra of excitation of fluorescence and those of fluorescence emission similar to those of the wild-type enzyme. These enzymes are both able to catalyse the conversion of L-glutamate to α-ketoglutarate although their modified structures tend to favour the stability of the inactive enzyme conformation. However, the enzyme from the mutant am^1 is catalytically inactive under any conditions (13). This enzyme showed no tendency to bind NADPH, i.e. there was no enhancement of NADPH fluorescence at 450nm when the reduced coenzyme was added to a solution of glutamate dehydrogenase. This clearly suggests that this mutant is inactive through an inability to bind reduced coenzyme and that the NADPH binding sites observed fluorimetrically in the above experiments are in the active site of the enzyme.

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