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Slow Conformational Changes of a *Neurospora* Glutamate Dehydrogenase Studied by Protein Fluorescence

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1. The NADP-dependent glutamate dehydrogenase of *Neurospora crassa* undergoes slow reversible structural transitions, with half-times in the order of a few minutes, between active and inactive states. The inactive state of the enzyme, which predominates at pH values below 7.0, has an intrinsic tryptophan fluorescence 25% lower than that of the active state, which predominates at pH values above 7.6. The inactive state can be activated either by an increase in pH or by addition of activators such as succinate. 2. The kinetics of the slow transitions that follow activating and inactivating rapid changes in conditions have been monitored by measurements of protein fluorescence. The results show that the slow reversible conformational change detected by the change in fluorescence is the rate-limiting process for enzyme activation and inactivation. 3. In both directions this conformational change follows apparent first-order kinetics and the rate constant is independent of protein concentration. These kinetics and published measurements of molecular weight are indicative of an isomerization process. 4. In both directions the changes show a large energy of activation and a large positive entropy of activation, consistent with a considerable disturbance of conformation in the transition state. 5. Comparisons of the fluorescence emission spectra of the active and inactive states indicate that the difference in fluorescence is produced by quenching, possibly intramolecular, in the inactive conformation. Iodide ions cause similar quenching. 6. In some mutationally altered forms of the enzyme comparable but modified conformational changes can be followed by protein fluorescence.

The enzyme under study is the NADP-dependent glutamate dehydrogenase [L-glutamate–$NADP^+$ oxidoreductase (deaminating), EC 1.4.1.4] of *Neurospora crassa*. Several mutationally modified forms of this enzyme exist (Fincham, 1962), in some of which single amino acid substitutions have been characterized (Wootton *et al.*, 1973; G. K. Chambers & A. A. Holder, unpublished work). The consequences of these substitutions are currently being studied to investigate the functions of different parts of the enzyme molecule. The enzyme has allosteric properties (West *et al.*, 1967), which are altered in various ways in some of these mutants. In the wild-type enzyme some of these properties involve slow transitions between active and inactive states, which take several minutes to reach equilibrium. Similar slow transitions have been described in other enzyme systems (Frieden, 1970). In one of the mutants, am^{19}, an inactive state is considerably more stable than in the wild-type enzyme and the activation process takes about 1–2h rather than several minutes (Sundaram & Fincham, 1964). The present work, designed to characterize and analyse these slow transitions, demonstrated a 25% difference in protein fluorescence between active and inactive states in the wild-type enzyme, which allows us to monitor activation and inactivation processes directly. The methods described below are being used to examine the comparable but modified processes in both active and inactive mutant forms of the enzyme.

West *et al.* (1967) described an effect of pH on the wild-type enzyme activity in the direction of 2-oxoglutarate amination. Below pH7.2 the enzyme is virtually inactive, above pH8.0 it is fully active and at intermediate pH values it is partially active. If enzyme in the inactive state at low pH is transferred to activating conditions of high pH the activation is slow, taking about 10min to reach equilibrium at 25°C. The inactivation process following a pH change in the opposite direction occurs at a similar low rate. The enzyme in the inactive state at pH7.0 can also be activated by the substrates 2-oxoglutarate or L-glutamate and by the non-substrates succinate, citrate, isocitrate, malate, fumarate, oxaloacetate or EDTA. In the present paper we describe work on pH-dependent transitions of the wild-type enzyme in both directions and activation by dicarboxylic acids, all of which occur at similar low rates. Other slow transitions occur in this enzyme, but are less convenient for the measurement of protein fluorescence because of quenching by the ligands involved. These include inactivation by the substrate NADPH alone at pH values below 7.8 (B. Ashby & J. C. Wootton, unpublished work) and synergistic activation by NADPH and 2-oxoglutarate over a wide range of pH values (West *et al.*, 1967).

Experimental

Materials

Standard laboratory chemicals, of analytical grade, were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Tris (Sigma 7-9), Pipes [piperazine-*NN'*-bis-(2-ethanesulphonic acid)] buffer, 2-oxoglutaric acid and bovine serum albumin (Cohn fraction V) were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. $NADP^+$ and NADPH were purchased either from the Boehringer Corporation (London) Ltd., London W.5, U.K., or from Sigma (London) Chemical Co. Ltd. DEAE-Sephadex and Sephadex G-200 were obtained from Pharmacia Fine Chemicals (G.B.) Ltd., London W.5, U.K.

Growth of Neurospora

Conidia of wild-type *N. crassa* strain STA-4 were grown at 30°C in 250ml conical flasks for 3 days on 100ml of Vogel's minimal medium (Vogel, 1964) containing 1.5% (w/v) of sucrose and 1.5% (w/v) of Davis agar. Growth was allowed to continue for a further 2–3 days in the light at room temperature. Conidia from four to six flasks were suspended in 240ml of sterile water and distributed between 12 2-litre flasks each containing 500ml of N-free Vogel's medium with 4% (w/v) sucrose, 0.02M-NH_4NO_3 and 0.04M-KNO_3. Mycelium from 23h growth at 30°C on a Gallenkamp orbital incubator was harvested by filtration under suction on a Buchner funnel, washed with water and stored at −18°C. The mycelium was freeze-dried before use.

Enzyme assay

Two assay systems were used (Fincham & Coddington, 1963). System C measures the rate of oxidative deamination of glutamate and was used as a routine at all stages of the purification: 2.8ml of 0.16M-sodium glutamate in 0.1M-Tris–HCl buffer, pH8.5 (containing 1mM-EDTA); 0.2ml of 0.2% $NADP^+$ in the same buffer; 1μl or 5μl of enzyme solution; all at 37°C. System A measures the rate of reductive amination of 2-oxoglutarate and was used in activation/inactivation experiments: 2.55ml of 0.1M-Tris–HCl buffer, pH7.4 (containing 1mM-EDTA); 0.10ml of 1M-NH_4Cl; 0.15ml of 0.2M-2-oxoglutaric acid (adjusted to pH7.4 with NaOH); 0.2ml of 0.15% NADPH; 5μl of purified enzyme; all in the same buffer at 25°C.

Changes in absorbance at 340nm were measured on a Unicam SP.800 spectrophotometer attached to a Servoscribe chart recorder (Kelvin Electronics Co., U.K.).

Enzyme activity (initial rate) is expressed in units of μmol of NADPH oxidized/min or μmol of $NADP^+$ reduced/min, though most data are expressed as percentages of the activity of the fully activated state either before inactivation or at the end of activation.

Enzyme concentration is given in mg/ml obtained by applying the formula mg/ml = $E_{280} \times 0.825$, based on the amino acid composition of the protein determined in this laboratory and on the absorbance of a carefully dried protein sample (J. C. Wootton, unpublished work). This value compares with a value of 0.8 cited by Sundaram & Fincham (1964), which was based on an earlier determination of the amino acid composition. The difference arises from a better estimate of the number of tryptophan residues by the more recent work.

Enzyme purification

The enzyme was purified by using modifications of the methods (due to M. G. Gore, unpublished work) of Barratt & Strickland (1963) and Fincham & Coddington (1963). Except where indicated, all steps were carried out at 4°C, and EDTA was added as a routine to all buffers to a concentration of 1mM.

Extraction. About 150g of freeze-dried mycelium was blended to a fine powder in a Waring blender run at maximum speed for a total of 30s with intermittent cooling. The powder was suspended in 2½ litres of 0.05M-sodium phosphate buffer, pH7.4, by pouring the buffer into the blender and mixing for a further 30s. After centrifugation (2500rev./min for 45min in an MSE Mistral 6L centrifuge) the supernatant was retained and the precipitate was resuspended in 1½ litres of the same buffer for further extraction. Cell debris was removed by a similar centrifugation and the supernatant pooled with the previous supernatant.

Ammonium sulphate fractionation. Solid $(NH_4)_2SO_4$ was added to the extract to 30% saturation. Precipitated protein was removed by centrifugation at 2500rev./min in a Mistral 6L centrifuge for at least 2h and this was discarded. The supernatant was made 55% saturated with respect to $(NH_4)_2SO_4$ and the precipitate collected by a similar centrifugation for at least 3h. This precipitate was resuspended in a minimum volume (usually 150–200ml) of 0.05M-sodium phosphate buffer, pH6.5, and dialysed overnight against 10 litres of the same buffer.

Protamine sulphate precipitation. About 80ml of a 1% (w/v) solution of protamine sulphate was added to the diffusate and the mixture stirred for 10min. The dense precipitate formed was removed by centrifugation in an MSE High Speed 18 centrifuge (12000rev./min for 20min).

Heat step. A flask containing the supernatant was placed in a water bath at 51°C. After it had reached this temperature the protein solution was left for 10min, cooled and then centrifuged in an MSE High Speed 18 centrifuge at 12000rev./min for 20min. The clear yellow supernatant was made up to 55% satura-

tion with respect to $(NH_4)_2SO_4$, and the precipitate collected by another centrifugation and resuspended in 0.05M-sodium phosphate buffer, pH7.4, to be dialysed overnight against 10 litres of the same buffer.

First DEAE-Sephadex column. The diffusate was loaded on to a column (4.5cm×15cm) of DEAE-Sephadex (A-50) equilibrated with 0.05M-sodium phosphate buffer, pH7.4. This column was eluted with a linear gradient obtained from 300ml each of 0.05M- and 0.15M-sodium phosphate buffer, pH7.4. Active fractions were pooled, then adjusted to 55% saturation with $(NH_4)_2SO_4$ and the centrifuged precipitate was redissolved in a minimum volume (usually 5–10ml) of 0.05M-sodium phosphate buffer, pH7.4.

Sephadex G-200 column. The above sample was applied to a column (3.1cm×70cm) of Sephadex G-200 and eluted with 0.05M-sodium phosphate buffer, pH7.4. Generally two overlapping peaks were obtained, the faster-moving peak containing most of the enzyme activity. The most active fractions were pooled, leaving out those heavily contaminated with the slower-moving protein components.

Second DEAE-Sephadex column. This pool was applied directly to a column (2.5cm×20cm) of DEAE-Sephadex (A-50) equilibrated with 0.05M-sodium phosphate buffer, pH7.4. The column was eluted with a linear gradient obtained from 150ml each of 0.05M- and 0.15M-sodium phosphate buffer, pH7.4. Peak fractions from the single peak of this column were generally about 90–95% pure, as judged by specific-activity measurement by comparison with the highest specific-activity obtained with fully purified protein (90.8μmol of NADPH-produced/min per mg of protein).

Partial ammonium sulphate precipitation. Further purification was achieved by precipitating the concentrated enzyme fractions, pooled from the peak, by 45% saturation with $(NH_4)_2SO_4$, which left minor contaminants in solution together with some of the enzyme. All experiments described in the present paper used enzyme from the 0–45%-saturated-$(NH_4)_2SO_4$ fraction. A summary of the purification is given in Table 1.

Criteria of purity. Polyacrylamide-gel electrophoresis at pH8.7 (Ornstein & Davis, 1962) performed on the 45%-saturated-$(NH_4)_2SO_4$ fraction usually showed a single protein band. A few preparations showed one or more minor bands, estimated at not more than 2% of the major band from heavily loaded gels. The highest specific activity obtained was 90.8μmol of NADPH produced/min per mg of protein, and specific activities greater that 85 units/mg were considered tolerable. Work by Wootton *et al.* (1972) on the subunit structure of the enzyme used protein prepared by substantially the same method. In this case the purified product was homogeneous by equilibrium sedimentation and gave a single band on sodium dodecyl sulphate–polyacrylamide gels.

Storage. Enzyme was stored as a suspension in 45%-saturated $(NH_4)_2SO_4$ in 0.05M-sodium phosphate buffer, pH7.4, at 4°C, and retained full activity for at least 4 weeks. The enzyme did not keep in free solution but lost most of its activity in 3–4 days.

Fluorescence measurements. Protein fluorescence was measured on an Aminco–Bowman SPF 125 spectrophotofluorimeter equipped with a Heath Servo-Recorder, model EU-108 [Heath (Gloucester) Ltd., Gloucester, U.K.].

To measure small changes in fluorescence at protein concentrations down to 0.08mg/ml the sensitivity was increased by scale expansion, and this did not significantly increase the signal-to-noise ratio. An electrical compensator was used to back-off the signal excess from the photomultiplier.

The excitation wavelength was set at 297nm (an emission maximum of the mercury-vapour arc lamp) with a slit width of 0.5mm and the emission was observed at 340nm with a slit width of 2.0mm.

Table 1. *Purification of Neurospora glutamate dehydrogenase from* 120*g of freeze-dried mycelium*

One E_{280} unit of protein is that concentration, contained in 1ml, that has an absorbance of 1 at 280nm in a 1cm light-path. Protein concentration was not determined in this preparation. Crude extracts usually have specific activities of 5 or less.

Step	Volume (ml)	Total activity measured in System C	Total protein (E_{280} units)	Specific activity (enzyme units/E_{280} unit)	Yield (%)
Crude extract after two extractions	4500	26400	—	—	100
50%-satd.-$(NH_4)_2SO_4$ ppt. after dialysis	600	14600	—	—	53
Supernatant after heating	600	12900	—	—	47
First DEAE-Sephadex effluent	160	12600	448	28.1	46
Sephadex G-200 effluent	155	11800	223	53.0	43
Second DEAE-Sephadex effluent	75	7000	96	73.0	25
45%-satd.-$(NH_4)_2SO_4$ ppt. after dialysis	20	6150	74	83.3 (≡90.8 units/mg)	22.5

Under these conditions decay of fluorescence due to photo-oxidation of tryptophan by excessive radiation (Udenfriend, 1969) was negligible. Emission spectra were determined at 5nm intervals from 300 to 400nm and are uncorrected.

The temperature control for the Arrhenius plots was achieved as follows. All solutions were carefully equilibrated in a water bath at the temperature of the experiment. Circulating water from the same bath was used to control the temperature of the holder of the 1cm-square silica cuvette. The temperature of each solution was measured before every determination, as was the temperature of the mixture in the cuvette afterwards. No change greater than ±0.25°C was observed in experiments of up to 20min duration. Variations in protein fluorescence intensity with temperature were slight over the range studied (approx. 1% fall in intensity/°C rise in temperature was noted) and such variations were negligible in a given experiment at one particular temperature.

The fluorimeter was calibrated by using a series of bovine serum albumin standard solutions. Fluorescence intensity is expressed in arbitrary units relative to the bovine serum albumin standard. Fluorescence changes are expressed as a percentage of the total fluorescence change taking place during each process investigated.

Preparation of samples for fluorescence experiments

To obtain a fluorescence titration curve samples of purified enzyme in 45%-saturated-$(NH_4)_2SO_4$ suspension at pH7.4 were dialysed against 0.1M-sodium phosphate buffer at a series of pH values. These solutions, as well as all protein solutions used in other experiments, were filtered through Millipore filters (HAWP 0.45μm; 25mm) immediately after dialysis to remove insoluble material. Each solution was adjusted to the required concentration, as judged by absorbance at 280nm, by dilution with the appropriate buffer. The fluorescence intensity of each sample was determined. To calibrate the system and to check for linearity of fluorescence with protein concentration, a series of dilutions of the enzyme was made at the three pH values 8.0, 7.2 and 6.5, and the fluorescence of each was determined by comparison with a bovine serum albumin standard curve. The absorbance of fluorescence solutions at the excitation wavelength of 297nm ranged from 0.04 to 0.4 depending on the protein concentration.

Rapid changes in the pH of enzyme solutions were achieved as follows. For a pH change from pH6.8 to 7.8 the sample of purified enzyme was dialysed against 0.1M-Tris–HCl buffer, pH6.8, and the pH change was effected by addition of 0.1M-Tris base (pH10.4) to 1.5ml of this enzyme solution contained in the fluorescence cell. After the contents of the cell had been mixed, the fluorescence change was monitored on the chart recorder.

A change in pH from pH7.8 to 6.8 was achieved by the addition of 0.5ml of 0.1M-NaH_2PO_4 (pH about 4.5) to 1.5ml of enzyme in 0.1M-sodium phosphate buffer, pH7.8.

Use of the allosteric effector succinate as an activator was studied by addition of 0.2ml of disodium succinate (from 0 to 500mM) in 0.1M-sodium phosphate buffer, pH7.0, to 2.8ml of enzyme in the same buffer, and again the fluorescence change was recorded.

Results

Relationship between protein concentration and fluorescence

In any investigation of fluorescence phenomena it is necessary to establish the relationship between fluorphor concentration and fluorescence intensity. This is particularly important in protein fluorescence studies since association or dissociation of protein molecules could lead to non-linearity even at low concentrations. Over the entire range of protein concentrations and pH values examined in the present work there was a linear relationship between protein concentration and fluorescence (Fig. 1). At higher

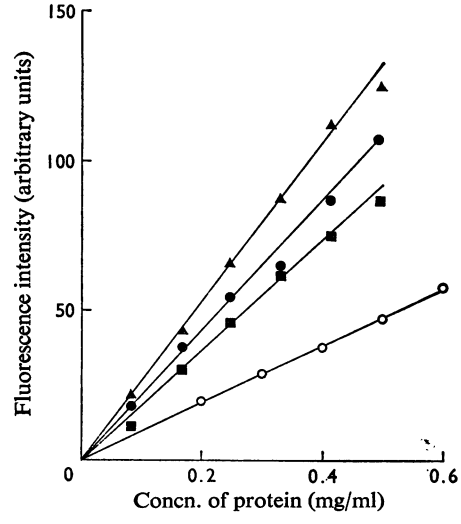

Fig. 1. *Relationship between protein concentration and fluorescence intensity at 25°C*

▲, Glutamate dehydrogenase at pH8.0; ●, glutamate dehydrogenase at pH7.2; ■, glutamate dehydrogenase at pH6.5; ○, bovine serum albumin standard at pH7.2. The excitation wavelength was 297nm and the emission was observed at 340nm. The buffer was 0.1M-sodium phosphate in all cases.

concentrations (not shown) fluorescence intensity fell off rapidly owing to a combination of self-absorption and light-scattering.

Relationship between enzyme activity, protein fluorescence and pH

Enzyme activity (initial rate) measured in System A for enzyme preincubated at a number of pH values in 0.1M-sodium phosphate buffer shows the profile given in Fig. 2(*a*). The p*K* of the titration curve is about 7.1. Disappearance of enzyme activity at low pH is not a simple matter of protonation of residues involved in the activity since the change is slow, taking minutes to go to completion (West *et al.*, 1967). The fluorescence titration curve of native enzyme in 0.1M-sodium phosphate buffer (Fig. 2*b*) also has a p*K* at about 7.1, which is almost the p*K* of the buffer. To eliminate the possibility that this titration curve could simply represent quenching of protein fluorescence by protonated buffer ions the experiment was repeated in 0.1M-Tris–HCl and 0.1M-sodium Pipes buffers of p*K* values 7.8 and 6.8 respectively. In each case the titration curve (Fig. 2*b*) is similar to that obtained in sodium phosphate buffer. The total fluorescence change between high and low pH values is about 25%. It seems likely that the pH inactivation/activation process involves a conformational change and that the protein fluorescence change is directly related to it.

A comparable titration curve (Fig. 2*c*) was obtained with the use of the fluorescent dye 2-*p*-toluidinylnaphthalene-6-sulphonate, first used by McClure & Edelman (1966). 2-*p*-Toluidinylnaphthalene-6-sulphonate is characterized by a low quantum yield in water and a high quantum yield in non-polar solvents. Apparently there are more non-polar regions available to the dye in the low pH state of the enzyme since the fluorescence intensity is greater. The p*K* of the curve is again about 7.1. Use of the dye as a conformational probe is obviated in the present case since protein fluorescence can be used as an intrinsic probe with the advantage that the system is not complicated by possible consequences of the binding of 2-*p*-toluidinylnaphthalene-6-sulphonate to the enzyme.

Kinetics of the fluorescence change produced by pH change

West *et al.* (1967) showed that enzyme at pH7.0 could be activated by preincubation at a higher pH, and that the activation took some minutes to go to completion and could be followed by measurement of the activity of enzyme samples taken at various times after the start of preincubation. Since protein fluorescence is apparently directly related to enzyme activity it should be possible to follow the activation process by monitoring changes in fluorescence.

A typical time-course for the increase in protein fluorescence on raising the pH of a protein sample from pH6.8 to pH7.8 is shown in Fig. 3(*a*), which includes a tracing obtained from a chart recording of the output of the fluorimeter. A parallel experiment, in which samples of enzyme were taken for assay in System A at various times after the pH change,

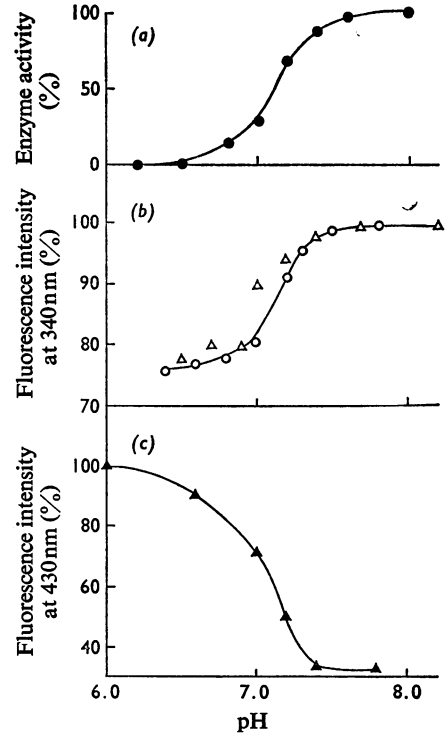

Fig. 2. *pH-dependence of enzyme activity, protein fluorescence and 2-p-toluidinylnaphthalene-6-sulphonate enhancement at 25°C*

(*a*) ●, Enzyme activity measured in System A at pH7.4 and 25°C; the enzyme was preincubated at each of the pH values shown for 1h at 25°C before assay. Activity data are expressed as percentages of the maximum attained; 5μg of enzyme preincubated at pH8.0 gave a rate of 0.13μM-NADPH oxidized/min under the conditions described. (*b*) Fluorescence intensity expressed as percentages of the maximum attained: ○, 0.41mg of enzyme/ml in 0.1M sodium phosphate buffer; △, 0.41mg of enzyme/ml in 0.1M-sodium Pipes buffer. The excitation wavelength was 297nm and the emission wavelength was 340nm. (*c*) 2-*p*-Toluidinylnaphthalene-6-sulphonate fluorescence enhancement: ▲, 0.82mg of enzyme/ml in the presence of 0.45M-2-*p*-toluidinylnaphthalene-6-sulphonate. The excitation wavelength was 366nm and the emission was observed at 430nm. The data are expressed as percentages of the maximum fluorescence intensity attained.

demonstrates a close correlation between fluorescence change and change in the enzymic activity of the protein (Fig. 3*a*, continuous curve and closed circles).

A semilogarithmic plot of the fluorescence time-course is linear (Fig. 3*a*), which indicates that the change is first-order with respect to protein concentration; the rate constant is $0.67 min^{-1}$ at 26°C.

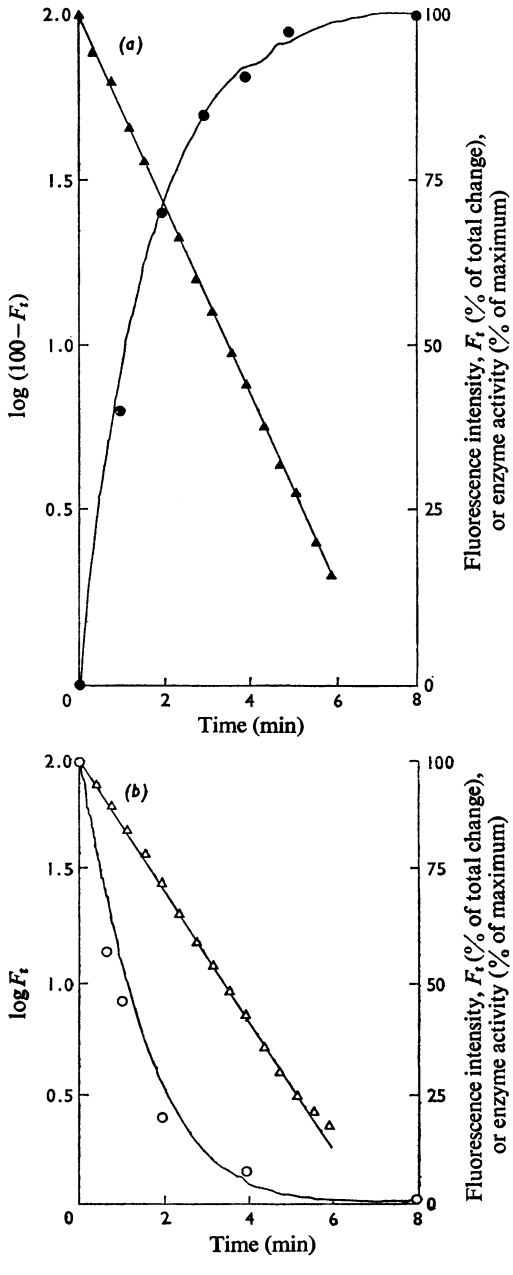

Fig. 3. *Kinetics of the pH-dependent activation and inactivation processes: correlation between protein fluorescence and enzyme activity*

(*a*) Activation of enzyme preincubated for 1h at pH6.8 by a shift in pH to 7.8 performed as described in the text. The continuous trace is a tracing of the chart-recorder output of the fluorimeter and shows the change in protein fluorescence with time. The excitation wavelength was 297nm and the emission wavelength was 340nm. ●, Enzyme activity data obtained in a parallel experiment, measured in System A at pH7.4 and 25°C. The maximum rate attained with 4μg of enzyme was 0.15μM-NADPH oxidized/min. ▲, First-order plot of the fluorescence data. The experiment was performed at 26°C; the final protein concentration was 0.41mg/ml. (*b*) Inactivation of enzyme preincubated for 1h at pH7.8 by a shift in pH to 6.8 performed as described in the text. The continuous trace is a tracing of the chart-recorder output of the fluorimeter: ○, Enzyme activity data obtained in a parallel experiment, measured in System A at pH7.4 and 25°C. The activity at time zero of a 3μg sample was 0.12μM-NADPH oxidized/min. △, First-order plot of the fluorescence data. The fluorescence measurements were performed at 27°C; the final protein concentration was 0.33mg/ml.

Decrease in protein fluorescence on a pH change from 7.8 to 6.8 again showed a first-order time-dependence and was closely correlated with a disappearance of enzyme activity (Fig. 3*b*, continuous curve and open circles). The rate constant was $0.69 min^{-1}$ at 27°C.

Protein fluorescence change was measured after both a decrease in pH (from 7.8 to 6.8) and a rise in pH (from 6.8 to 7.8) for a series of protein concentrations between 0.08 and 0.70mg/ml. In each direction differences in rate constant at different protein concentrations were negligible (Fig. 4).

Thermodynamic parameters of the fluorescence changes produced by pH changes

The rate of the pH-induced fluorescence change showed a high temperature-dependence both in the direction of enzyme activation (pH change 6.8–7.8) and in the direction of enzyme inactivation (pH change 7.8–6.8). Arrhenius plots prepared by using rate constants determined at a number of carefully controlled temperatures are shown in Fig. 5, and the thermodynamic parameters calculated from the data are in Table 2.

The change in both directions shows a large energy of activation and a large positive entropy of activation, both of the magnitude often found for protein denaturation. In both directions the entropic component, $T\Delta S^*$, of the energy of activation is almost as

large as the heat of activation, ΔH^* (Table 2). The free energy of activation ($\Delta H^* - T\Delta S^*$) is therefore small [approx. 17kJ·mol^{-1} (4kcal·mol^{-1})] in both directions. Experimental errors result in 95% confidence limits of approx. ±12.5kJ·mol^{-1} (±3kcal·mol^{-1}) for the values of E_a, ΔH^* and $T\Delta S^*$. It is not possible to use the present data for interpretations based on equilibrium thermodynamics because the changes in the two directions occur at different pH values.

Dependence of activating fluorescence change on NaCl concentration

Rate constants were determined for a change in pH from 6.8 to 7.8 at 26°C for a series of samples containing from 0 to 0.25M-NaCl. Results plotted in Fig. 6 show a linear decrease in rate constant as the NaCl concentration increases.

Activation by succinate and corresponding change in protein fluorescence

Of the large number of dicarboxylic acids known to reactivate the enzyme at pH7.0 (West *et al.*, 1967), succinate was chosen as an example of an allosteric effector since it has been used to activate the mutant enzyme forms *am*^{19} and *am*^{19}-*R*24 (Sundaram & Fincham, 1964). Succinate was used in preference to the substrate, 2-oxoglutarate, since the latter compound absorbs at 340nm and interferes with fluorescence emission at this wavelength.

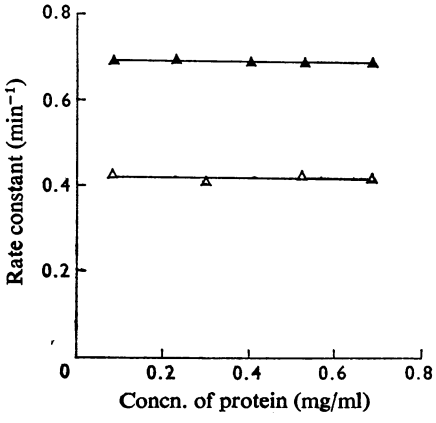

Fig. 4. *Effect of protein concentration on the rate constant for activation and inactivation*

▲, First-order rate constants for the fluorescence change that follows a pH jump from 6.8 to 7.8. at 26°C; △, first-order rate constants for the fluorescence change that follows a pH jump from 7.8 to 6.8 at 24°C. The pH changes were effected as described in the text.

Addition of disodium succinate at pH7.0 to samples of enzyme in 0.1M-sodium phosphate buffer, pH7.0, gave rise to an increase in protein fluorescence.

Both the extent and rate of the fluorescence increase were dependent on succinate concentration. Results

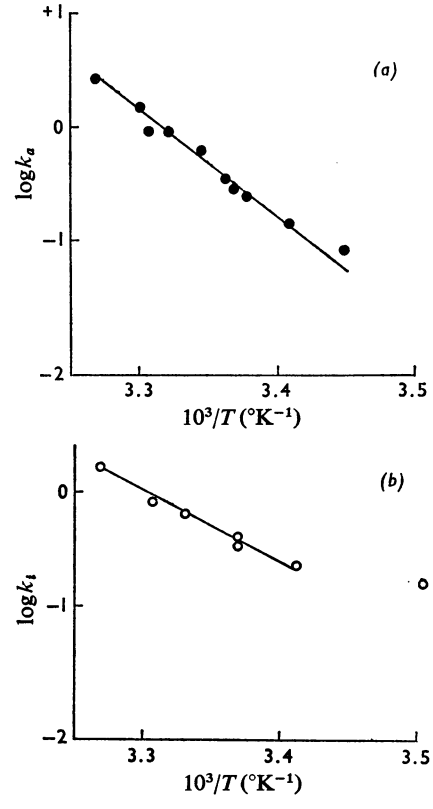

Fig. 5. *Temperature-dependence of the rate of the pH-dependent activation and inactivation processes*

(*a*) Arrhenius plot of rate constants (●) for the direction of enzyme activation determined by fluorescence measurements after a change in pH from 6.8 to 7.8. The pH changes and temperature control were performed as described in the text. The final concentration of protein was 0.41 mg/ml. The line through the points represents a linear regression line. (*b*) Arrhenius plot of rate constants (○) for the direction of enzyme inactivation determined by fluorescence measurements after a change in pH from 7.8 to 6.8 as described in the text. The experiment was performed in Tris–HCl buffer, which has a high temperature coefficient for pH. Consequently the pH change at 33°C was from pH7.7 to 6.7, but this adequately covers the pH range of the fluorescence change. The final protein concentration was again 0.41 mg/ml. The line through the points represents a linear regression line. The point at 12°C has been neglected because of preliminary evidence for a temperature transition in the activity of the enzyme at about 15°C.

Table 2. *Thermodynamic parameters of the changes in protein fluorescence after pH changes*

The values of the energy of activation, E_a, are calculated from the regression lines of the Arrhenius plots in Fig. 5 ($E_a = 2.303 \times R \times$slope). The values of the entropies of activation (ΔS^*) are calculated, by using the same measurements of the rate constants, k, from the regression lines of plots of $T \cdot \ln(k/T)$ against T ($\Delta S^* = R \times$slope). The heat of activation ($\Delta H^* = E_a - RT$) and the entropic component of the energy of activation ($T\Delta S^*$) are obtained by further calculations.

	In the direction of enzyme activation at pH7.8	In the direction of enzyme inactivation at pH6.8
Energy of activation (E_a)		
(kJ·mol^{-1})	169.5	110.0
(kcal·mol^{-1})	40.5	26.3
Entropy of activation (ΔS^*)		
(J·mol^{-1}·deg^{-1})	427.8	306.7
(cal·mol^{-1}·deg^{-1})	102.2	73.3
Heat of activation (ΔH^*) at 26°C		
(kJ·mol^{-1})	166.9	107.5
(kcal·mol^{-1})	39.9	25.7
$T\Delta S^*$ at 26°C		
(kJ·mol^{-1})	150.6	91.6
(kcal·mol^{-1})	36.0	21.9

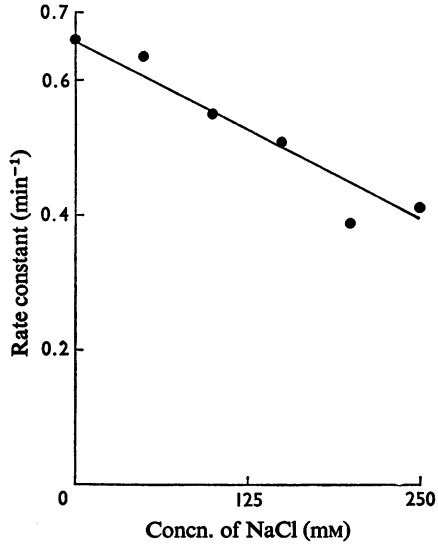

Fig. 6. *Effect of NaCl concentration on the rate constant for activation*

●, Rate constants for the direction of enzyme activation determined by fluorescence measurement at 26°C after a pH change from 6.8 to 7.8 as described in the text.

obtained at two succinate concentrations are given in Fig. 7, which also shows enzyme activity data gained in parallel experiments under identical conditions. There is excellent correlation between the fluorescence curve and the time-course of enzyme activation of each succinate concentration.

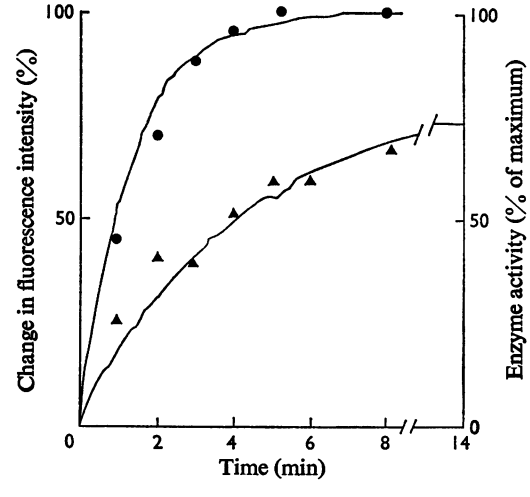

Fig. 7. *Activation by succinate and corresponding change in protein fluorescence*

●, Enzyme activity measured at various times after addition of disodium succinate to a final concentration of 33.3 mM to enzyme preincubated for 1 h at pH7.0 at 25°C. Activity was measured in System A at pH7.4 at 25°C. The maximum activity obtained for 3 μg of protein was 0.125 μM-NADPH oxidized/min, which is the maximum activity attained at any succinate concentration. The continuous trace through these points is a tracing of the fluorescence change obtained under identical conditions. ▲, Enzyme activity assayed as above at various times after addition of disodium succinate to a final concentration of 6.7 mM to enzyme preincubated for 1 h at pH7.0 at 25°C. The continuous trace through these points is a tracing of the corresponding change in protein fluorescence under the same conditions. The final concentration of protein in each experiment was 0.33 mg/ml.

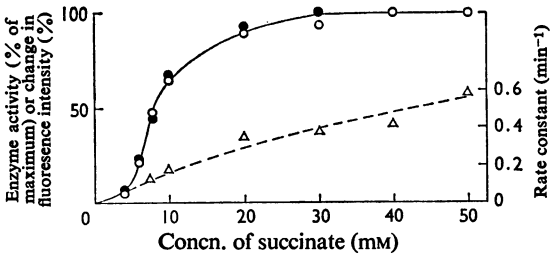

Fig. 8. *Activation by succinate: concentration-dependence of the extent of activation and the rate of activation*

●, Enzyme activity measured in System A at pH7.4 at 25°C after addition of disodium succinate to enzyme pre-incubated for 1 h at pH6.9 at 25°C and after the activation had been followed to completion. The maximum activity attained with 3μg of enzyme was 0.125μM-NADPH oxidized/min. ○, Fluorescence intensity measured after completion of activation under the same conditions as above. △, First-order rate constant determined over the whole time-course of activation by fluorescence measurement. The final protein concentration was 0.33 mg/ml in each case.

When increasing amounts of succinate are added to a solution of the protein in sodium phosphate buffer at pH6.9, the fluorescence increases by about 25% at saturation, and the variation with the concentration of added succinate is clearly sigmoidal (Fig. 8); there is a close correlation between equilibrium fluorescence values and enzyme activity.

Nature of the fluorescence change

A qualitative investigation of the nature of the fluorescence change was attempted by examination of fluorescence emission spectra of the enzyme under a variety of conditions. The spectra are not corrected for poor photomultiplier response at lower wavelengths. Excitation at 297nm excludes any contribution from tyrosine residues. The spectra can be interpreted in terms of contributions from tryptophan residues in hydrophilic and hydrophobic environments although the true situation may be rather more complex. An increase in the polarity of the solvent causes a shift of the tryptophan spectrum to longer wavelengths and an increase in the quantum yield, as measured by the increase in area under the fluorescence band (Lehrer & Fasman, 1967). The wavelength of maximum fluorescence of tryptophan varies over the range 300–350nm in going from less polar to more polar solvents (Cowgill, 1967). Emission spectra of the enzyme are given in Figs. 9 and 10 and are analysed below in terms of the two tryptophan contributions.

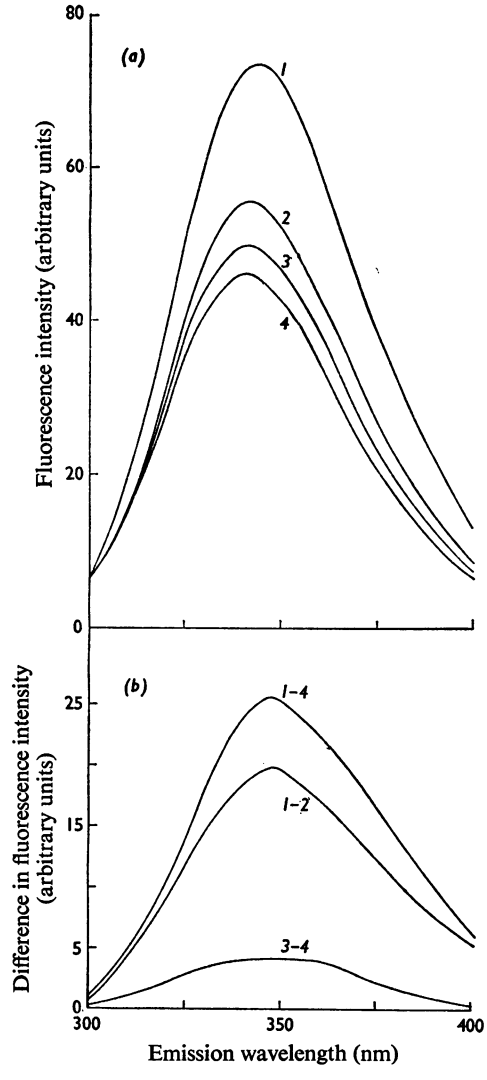

Fig. 9. *Nature of the fluorescence change: effect of pH and KI on the emission spectra at 25°C*

(*a*) Emission spectra obtained from 0.28mg of protein/ml by excitation at 297nm under the following conditions: spectrum *1*, pH8.0 in the absence of KI; spectrum *2*, pH8.0 in the presence of 30mM-KI; spectrum *3*, pH6.5 in the absence of KI; spectrum *4*, pH6.5 in the presence of 30mM-KI. 30mM-KCl was added to the mixtures used for the determination of spectra *1* and *3* to maintain the ionic strength. Spectra *1* and *2* were identical in the presence and in the absence of 150mM-disodium glutamate. (*b*) Difference spectra obtained from the above emission spectra.

There are two possible effects of a conformational change on the protein fluorescence spectrum. One effect would be to alter the polarity of the environ-

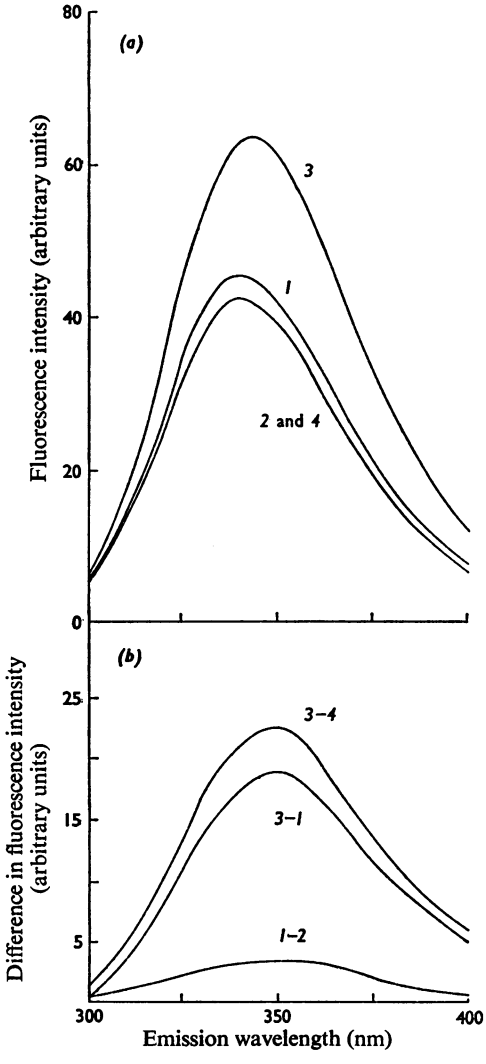

Fig. 10. *Nature of the fluorescence change: effect of succinate and KI on the emission spectrum at 25°C and pH7.0*

(*a*) Emission spectra obtained from 0.22mg of protein/ml by excitation at 297nm under the following conditions: spectrum *1*, pH7.0 in the absence of KI; spectrum *2*, pH7.0 in the presence of 30mM-KI; spectrum *3*, pH7.0 in the presence of 40mM-disodium succinate but in the absence of KI; spectrum *4*, pH7.0 in the presence of 40mM-disodium succinate and in the presence of 30mM-KI. 30mM-KCl was added to the mixtures used for the determination of spectra *1* and *3* to maintain the ionic strength. (*b*) Difference spectra obtained from the above emission spectra.

ment of some of the tryptophan residues, which would lead to a shift in the emission maximum. The way to observe such a shift most clearly is to draw the difference spectrum of the spectra obtained before and after a conformational change. For instance, the difference spectrum obtained from a change that leads to the appearance of more residues in a hydrophilic environment would have a peak at 350nm and a large trough at lower wavelengths, due to a red shift and an increase in quantum yield.

The second possible effect would be a change in the quenching brought about by certain groups on other parts of the protein, candidates for which are protonated carboxyl groups, thiol groups and histidine residues (Cowgill, 1967, 1970), or by protons (Weber, 1961). Since such groups are likely to be found in polar environments, only tryptophan residues in hydrophilic regions of the protein would be quenched, and the difference spectrum obtained by subtracting the quenched from the unquenched spectrum would have a maximum at 350nm. The quantum yield would be decreased to an extent dependent on the efficiency of the quenching group or agent and the number of tryptophan residues involved.

Both mechanisms may of course operate together, so that tryptophan residues move into a hydrophilic environment to be quenched by one of the groups mentioned above.

Emission spectra obtained at pH8.0 and 6.5 are shown in Fig. 9(*a*). There is clearly a shift in the emission maximum to lower wavelengths in going from pH8.0 to 6.5 and the quantum yield, measured as the relative area under each curve, is decreased by some 30%. The difference spectrum (Fig. 9*b*) shows a peak at 350nm and there is no trough at lower wavelengths that would correspond to a shift of tryptophan residues to a non-polar environment. Further evidence for a quenching-type mechanism is provided by experiments in which iodide ions were used as quenching agents, along the lines indicated by Lehrer (1967). At pH8.0 addition of KI produces a fluorescence change similar to that produced by a change to lower pH (Fig. 9*a*). The difference spectrum, obtained by subtracting quenched from unquenched spectra, has a peak at about 350nm and the fall in quantum yield at high concentrations of KI approaches that produced by pH change. There is little further quenching of fluorescence by iodide on protein at pH6.5 (Fig. 9*b*). Addition of glutamic acid to 150mM (well above its K_m) had no effect on iodide quenching at pH8.0. There was thus no protection of any tryptophan residue by glutamate.

Similar results were obtained with enzyme that had been reactivated by succinate at pH7.0 (Fig. 10). Quantum yield is increased in the presence of succinate and the difference spectrum has a maximum at 350nm. Iodide quenches out all of the fluorescence increase caused by addition of succinate and returns the fluorescence yield to that of the enzyme at pH7.0 in the absence of succinate.

Discussion

It seems certain from the evidence given in the present paper that the observed changes in protein fluorescence reflect a reversible slow conformational change that is rate-limiting for activation and inactivation. The rate of change of fluorescence closely parallels the rate of change of enzyme activity for both activating and inactivating pH changes (Fig. 3) and for succinate activation (Fig. 7). The equilibrium protein fluorescence and the enzyme activity have identical dependence on pH (Fig. 2) and on succinate concentration (Fig. 8). The change follows first-order kinetics in both directions and the rate constants are independent of protein concentration in the range 0.08–0.8mg/ml. This suggests that the process is an isomerization not involving a change in the state of aggregation of the molecules.

It is likely that under the conditions used here the enzyme exists entirely in the hexameric state both above and below the pH transition described. This is supported by the following sedimentation-equilibrium studies, which show similar and homogeneous molecular weights at both pH7.4, where the enzyme is almost entirely in the active state, and pH7.0, where the enzyme is almost entirely in the inactive state. Wootton *et al.* (1972), using pH7.4 and a protein concentration of 0.5mg/ml, obtained a mean molecular weight of 275000. Blumenthal & Smith (1973), using pH7.0 and protein concentrations of 0.4, 0.6 and 0.8mg/ml, quote a molecular weight of 288400±7900, identical at the three protein concentrations. Neither study gave any indication of size inhomogeneity. The two estimates agree within experimental error, but in both cases the actual value of the molecular weight is subject to a range of uncertainty wider than the experimental error because the true partial specific volume of the protein may differ from the values (0.731 and 0.726ml/g respectively from the two laboratories) calculated from the amino acid composition. If the maximum plausible range of values of partial specific volume is taken to be 0.71–0.75ml/g the corresponding maximum plausible range of values of molecular weight (Wootton *et al.*, 1972) is 250000–305000. In both studies evidence was obtained from physical measurements and partial amino acid sequencing that the enzyme consists of six identical subunits of molecular weight about 48000.

Sundaram & Fincham (1964) described the slow activation of a mutant form of the enzyme isolated from the revertant *am*^{19}-*R*24 (a prototrophic strain derived by a single mutational step from the auxotrophic mutant *am*^{19}). This enzyme is inactivated by brief heating at 60°C and activated by relatively high concentrations of succinate or EDTA. The activation, monitored by measurement of enzyme activity, was interpreted in terms of a slow conformational change, with the use of similar arguments to those applied here. The process follows first-order kinetics and its energy of activation [110kJ·mol^{-1} (26kcal·mol^{-1})] is consistent with a considerable change of protein structure. The active and inactive forms of the *am*^{19}-*R*24 enzyme are of different mobilities in polyacrylamide-gel electrophoresis, but their very similar sedimentation characteristics suggest that they are both hexamers. The difference in electrophoretic mobility probably reflects a difference in conformation that affects the shape or the surface charge of the molecule. The fastest rates of activation obtained with this enzyme were about five times slower than the conformational changes described here for the wild-type enzyme after succinate activation. With the enzyme of the original mutant *am*^{19} the corresponding activation is about 50 times slower than in the wild-type, and this increased stability of an inactive conformation probably explains the fact that the *am*^{19} enzyme is inactive *in vivo*.

There are advantages in using protein fluorescence to monitor these conformational changes, rather than the less direct property of enzyme activity. Fluorescence can be recorded continuously and can be used to follow relatively fast changes such as those occurring in the range 30–40°C. In appropriate cases it can be used to monitor comparable but altered conformational changes in mutant forms of the enzyme, including those that are inactive under any conditions. For example, recent work (B. Ashby, unpublished work) has shown that the completely inactive enzyme from the mutant *am*^{1} closely resembles the wild-type enzyme in the fluorescence states and the slow conformational changes described here, except that the p*K* of the fluorescence titration curve is shifted from 7.1 to 6.7. This conformational normality of the *am*^{1} enzyme had been postulated previously (Coddington *et al.*, 1966) to explain the fact that some hybrid hetero-oligomers between the *am*^{1} and *am*^{19} proteins show nearly normal enzyme activity. Presumably the *am*^{1} subunits in the hybrid molecules correct through quaternary interactions the conformation of the potentially active *am*^{19} subunits, which would otherwise be stable in an inactive state. The *am*^{1} subunits themselves are presumably inactive because of an amino acid substitution that affects the active site locally but does not greatly affect the conformation otherwise. The methods described in the present paper will facilitate investigations of conformational characteristics of potentially active mutant enzymes and of hybrid molecules between the enzymes from two mutants or from a mutant and wild-type.

A simple speculative model to explain the observed fluorescence spectra and difference spectra (Figs. 9 and 10) is that the inactive low-fluorescence structure has a quenching group in the proximity of one of the tryptophan residues. This group could be a side chain

on another part of the protein, or a component of the solvent such as a proton. In the active high-fluorescence structure this group is absent or moved away and is replaced by a similarly hydrophilic but non-quenching moiety. It is possible that in the unquenched active conformation the mobile quenching group itself might form part of the active site, and that its movement to another part of the molecule during inactivation might result simultaneously in quenching and loss of activity. Iodide ions, in mimicking the quenching effect, could be acting at the same tryptophan residue as the mobile group or at others. The enzyme contains 6–7 tryptophan residues per subunit (Wootton *et al.*, 1972, and unpublished work; Blumenthal & Smith, 1973).

In contrast with their considerable difference in tryptophan fluorescence, the active and inactive states of the enzyme show identical circular-dichroism spectra over the wavelengths of absorption by aromatic chromophores (M. G. Gore and C. Greenwood, personal communications). This identity implies that there is no major difference between the two states in the orientation or in the degree of exposure to solvent of the aromatic chromophores. This is consistent with the quenching model proposed in the present paper, since a quenching group would not necessarily affect the absorption properties of the quenched chromophores.

Consideration of these spectroscopic results and the thermodynamic parameters (Table 2) suggests that the active and inactive states may not differ greatly from each other in conformation, except for the postulated quenching group, but that considerable disturbance of the conformation occurs during the transition between the states. The large positive entropies of activation are consistent with either of (or a combination of) the following possibilities. (1) The transition state is more open and unfolded than the two stable states and has fewer intramolecular bonds. (2) The transition state makes fewer ordered interactions with water and with other solutes than do the two stable states. One attractive model that uses the former possibility visualizes a large part of the enzyme structure 'breathing out' during the transition. This might be necessary if in the two stable states the group responsible for the proposed intramolecular quenching has its possible movement considerably restricted because of steric blocking by other parts of the molecule or by intramolecular bonding. Other models are possible, however, and a detailed structural description of the process is obviously very remote.

The slow transitions discussed in the present paper can be readily demonstrated in fresh crude extracts by activity measurements and they do not change in kinetics during purification of the enzyme. It is very likely that they are a property of the enzyme *in vivo*. Frieden (1970) has discussed the possible metabolic significance of several enzymes (which he termed 'hysteretic') that respond slowly to a rapid change in concentration of a substrate or modifier. The mechanism of the slow response can be a slow isomerization as in the present case, a slow aggregation or disaggregation, or a slow ligand-binding step. Such enzymes occur at control points in multiply branched pathways or in pathways that utilize common intermediates. In these situations their slow response might result in a 'buffering' of the rate of concentration change of intermediates. Clearly the NADP-dependent glutamate dehydrogenase of *Neurospora* is well placed, immediately before a multiple-branch point, to exert this type of regulation. The enzyme is the major pathway of primary amination when the organism is growing on separate sources of carbon and nitrogen. The physiological pH, measured on undiluted homogenates, is usually 7.2–7.4, close to the p*K* of the pH-dependent transition. In this range the enzyme is poised to respond in the direction of either activation or inactivation to sufficiently sustained changes in concentration of 2-oxoglutarate, other acids of the tricarboxylic acid cycle, or NADPH. It is possible that the function of the slow response time in this enzyme is to isolate amino acid biosynthesis from the effects of sudden fluctuations in carbon and NADPH metabolism and to damp out short-term oscillations.

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