Biochem. J. (1990) 271, 351–355 (Printed in Great Britain)

A pH-dependent activation–inactivation equilibrium in glutamate dehydrogenase of *Clostridium symbiosum*

Shabih E.-H. SYED* and Paul C. ENGEL†

Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, U.K.

1. On transferring *Clostridium symbiosum* glutamate dehydrogenase from pH 7 to assay mixtures at pH 8.8, reaction time courses showed a marked deceleration that was not attributable to the approach to equilibrium of the catalysed reaction. The rate became approximately constant after declining to 4–5% of the initial value. Enzyme, stored at pH 8.8 and assayed in the same mixture, gave an accelerating time course with the same final linear rate. The enzyme appears to be reversibly converted from a high-activity form at low pH to a low-activity form at high pH. 2. Re-activation at 31 °C upon dilution from pH 8.8 to pH 7 was followed by periodic assay of the diluted enzyme solution. At low ionic strength (5 mM-Tris/HCl), no re-activation occurred, but various salts promoted re-activation to a limiting rate, with full re-activation in 40 min. 3. Re-activation was very temperature-dependent and extremely slow at 4 °C, suggesting a large activation energy. 4. 2-Oxoglutarate, glutarate or succinate (10 mM) accelerated re-activation; L-glutamate and L-aspartate were much less effective. 5. The monocarboxylic amino acids alanine and norvaline appear to stabilize the inactive enzyme: 60 mM-alanine does not promote re-activation, and, as substrates at pH 8.8 for enzyme stored at pH 7, alanine and norvaline give progress curves showing rapid complete inactivation. 6. Mono- and di-nucleotides (AMP, ADP, ATP, NAD^+, NADH, $NADP^+$, CoA, acetyl-CoA) at low concentrations (10^{-4}–10^{-3}M) enhance re-activation at pH 7 and also retard inactivation at pH 8.8. 7. The re-activation rate is independent of enzyme concentration: ultracentrifuge experiments show no changes in molecular mass with or without substrates. 8. The activation–inactivation appears to be due to a slow pH-dependent conformational change that is sensitively responsive to the reactants and their analogues.

INTRODUCTION

Glutamate dehydrogenases (EC 1.4.1.2–4) catalyse the reversible $NADP^+$-dependent oxidative deamination of L-glutamate. They have been much studied because of their complex regulatory behaviour and metabolic importance, but the lack of a detailed three-dimensional framework has limited understanding. Crystallization of NAD^+-dependent glutamate dehydrogenase from the anaerobic bacterium *Clostridium symbiosum* [1] has, however, now led to the solution of the structure at high resolution [2,3; P. J. Baker, K. L. Britton, P. C. Engel, G. W. Farrants, K. S. Lilley, D. W. Rice & T. J. Stillman, unpublished work]. The enzyme is hexameric, with a subunit M_r of 49000 [1], and striking sequence similarity (K. S. Lilley, P. E. Brown & P. C. Engel, unpublished work) to the $NADP^+$-dependent glutamate dehydrogenases of *Escherichia coli*, for which a gene sequence is available [5,6], and *Neurospora crassa*, sequenced via both protein and gene [7,8].

Detailed functional information for the clostridial enzyme is required both in order to understand its structure and to permit comparison with the properties of related glutamate dehydrogenases. The present paper documents the reversible pH-dependent inactivation of the enzyme, including the effects of temperature, ionic strength and various ligands. The interpretation offered in a brief previous report [9] is now revised in the light of new results.

MATERIALS AND METHODS

Glutamate dehydrogenase was purified from cell paste of *Clostridium symbiosum* [10] and stored at 4 °C as a precipitate in 60% (w/v)-satd. $(NH_4)_2SO_4$. Stock solutions of the enzyme were extensively dialysed at 4 °C against Tris/HCl buffer, pH 7 or pH 8.8. Enzyme concentrations were calculated from A_{280} measurements by using an absorption coefficient of 1.05 $cm^2 \cdot mg^{-1}$ determined by the dry-weight method [10]. An independent check of this value, based on amino acid analysis (K. S. Lilley, A. G. Moir & P. C. Engel, unpublished work), gives agreement to within 2%.

Monosodium L-glutamate was of the purest grade obtainable from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Nucleotides were of the highest grades from Boehringer Corp., Lewes, East Sussex, U.K. or Sigma Chemical Co., Poole, Dorset, U.K. Other chemicals (analytical grade) were from BDH Chemicals Ltd., Atherstone, Warwickshire, U.K., or Fisons Scientific Ltd., Loughborough, Leics., U.K. Initial-rate measurements were made at 25 °C with a recording fluorimeter [11], calibrated as described by Engel & Hornby [12]. Unless otherwise stated, the buffer was 20 mM-Tris/HCl at pH 8.8, with 1 mM-NAD^+ and glutamate at 10 mM (System I) or 40 mM (System II).

In re-activation experiments, typically enzyme solutions at pH 8.8 in Tris/HCl were diluted into the same buffer at pH 7 pre-equilibrated at 31 °C in a water bath. Conventional sedimentation-velocity experiments were carried out at 25 °C with a Spinco model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). Protein solutions were preincubated at 25 °C for 90 min before ultracentrifugation either at pH 7 in a standard 12 mm single-sector Dural cell (60000 rev./min) or at pH 8.8 in a standard single-sector Kel-F cell (50000 rev./min).

Density-gradient centrifugation [13] at pH 7 and 8.8 was carried out with 5–20% (w/v) sucrose gradients in the SW39 rotor of a Spinco model L preparative ultracentrifuge. The temperature was set at 25 °C and the rotor was taken up to

* Present address: Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, U.K.
† To whom correspondence should be sent.

41000 rev./min. Total run time was 4 h. Samples were collected by gently puncturing the bottoms of the tubes and counting equal numbers of drops for each fraction. The protein boundary was located by assaying 10–100 μl samples for enzyme activity (System II) and measuring protein concentration [14].

RESULTS

Non-linearity of progress curves

When crude extracts of *C. symbiosum* at pH 7 (0.1 M-potassium phosphate) were assayed at pH 8.8 (System I) for glutamate dehydrogenase, they showed a markedly decelerating reaction. In the fluorimeter, full-scale deflection corresponded at most to 1 μM-NADH produced, so that non-linearity could not be explained by approach to equilibrium. In assays at pH 7, the traces were linear, despite a less favourable equilibrium. Identical behaviour was seen with the purified enzyme used for all the detailed studies described here.

In an assay at pH 8.8, after 5.3 min the rate had declined from 13.2 to 0.56 $\mu M \cdot min^{-1} \cdot \mu g^{-1} \cdot ml$; a second identical enzyme addition increased the rate to 13.7 $\mu M \cdot min^{-1} \cdot \mu g^{-1} \cdot ml$, and a new decline ensued. The additive rates suggested that the decline was due to a change in the enzyme rather than in reactant concentrations. Enzyme instability can be excluded, since the rate decreased (23-fold) to an essentially constant non-zero value. By contrast, when stock enzyme at pH 8.8 in 20 mM-Tris/HCl was assayed in System I, and thus kept at a constant pH, an accelerating reaction was observed. The final linear rate was 23-fold less than the initial rate given by the stock solution of enzyme at pH 7 assayed in the same buffer.

Incubations of stock enzyme solutions at altered pH

These observations suggested reversible conversion from a low-activity form at pH 8.8 into a high-activity form at pH 7.0. This was further tested by diluting enzyme stock (4.5 mg/ml) in 20 mM-Tris/HCl, pH 8.8, 20–40-fold into 20 mM-Tris/HCl, pH 7.0 at 25 °C, and then assaying samples (10 μl) in System I (4 ml). Activity increased over several hours from 0.68 $\mu M \cdot min^{-1} \cdot \mu g^{-1} \cdot ml$ to a constant value of 14 $\mu M \cdot min^{-1} \cdot \mu g^{-1} \cdot ml$. In the converse experiment, upon dilution into buffer at pH 8.8, enzyme previously stored at pH 7 was completely converted into the low-activity form in less than 1 min. Changing the chemical nature of the buffer did not alter the pattern: dilution from pH 8.8 into 0.1 M-potassium phosphate, pH 7, gave the same result as dilution into Tris buffers at pH 7.

To monitor re-activation in long-term incubations, a choice had to be made between assay at pH 7 (accelerating rates) and at pH 8.8 or some other pH. Assays at pH 7 gave poor reproducibility, and, despite the declining rates, assay at pH 8.8 (System I and II) was more satisfactory.

Influence of ionic strength on re-activation

When a stock enzyme solution (4.5 mg/ml), prepared by dialysis against 5 mM-Tris/HCl, pH 8.8, was diluted 45-fold into 5 mM-Tris/HCl, pH 7, at 31 °C, almost no re-activation was observed (Fig. 1). Higher concentrations of Tris/HCl or Tris/acetate gave much greater rates and extents of re-activation. Similar enhancement was given by NaCl (10–200 mM; results not shown). With 50 mM-NaCl or more, re-activation was maximal after 60 min. At 100 mM, the effects of NaCl, LiCl, KCl, RbCl, CsCl and also NaI, NaBr, $NaNO_3$ and NaF were indistinguishable. Substitution with bivalent ions (Mg^{2+}, Ca^{2+}, SO_4^{2-}) to give the same ionic strength (I 0.1) produced the same effect. Thus, under conditions of maximal re-activation, no ion-selectivity was seen. However, when 30 mM-NaCl, which gives slower re-activation, was compared with other salts, some selectivity

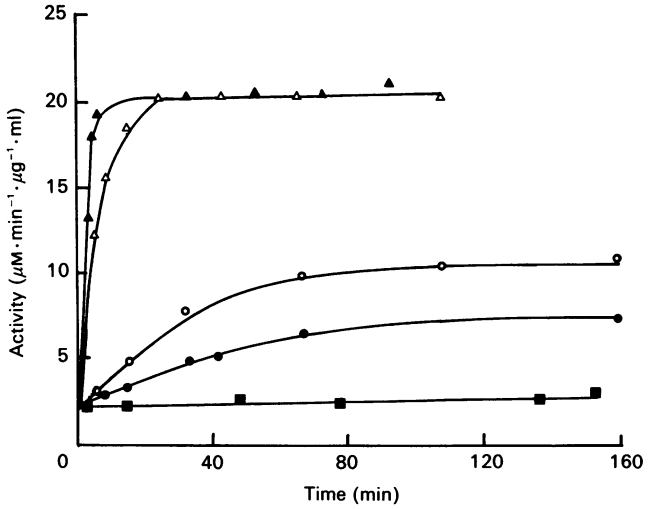

Fig. 1. Time courses of re-activation of clostridial glutamate dehydrogenase in Tris/HCl buffers of various concentrations at pH 7 and 31 °C

The stock solution of inactivated enzyme was diluted 45-fold from 5 mM-Tris/HCl, pH 8.8, into Tris/HCl, pH 7, at 5 mM (■), 10 mM (●), 20 mM (○), 50 mM (△) or 200 mM (▲) to give an enzyme concentration of 0.1 $mg \cdot ml^{-1}$. Samples (10 ml) were withdrawn periodically for assay in System II. The same enzyme concentration and temperature were used for experiments shown in Figs. 2–4.

emerged. Thus Na^+ gave faster re-activation than K^+, Cl^- was more effective than F^-, and bivalent ions (SO_4^{2-} as 10 mM-Na_2SO_4 and Mn^{2+} as 10 mM-$MnCl_2$) were more effective than univalent ions at the same ionic strength. Despite these differing rates, most salts gave the same final activity after 24 h.

Influence of temperature on re-activation

Glutamate dehydrogenase was re-activated with 0.1 M-NaCl in 5 mM-Tris/HCl adjusted to pH 7 at different temperatures (Fig. 2). Re-activation was complete after 40 min at 25 °C and 31 °C, but negligible even after 2 h at 4 °C. This implies a large activation energy, but this was difficult to estimate, since semi-logarithmic plots were biphasic.

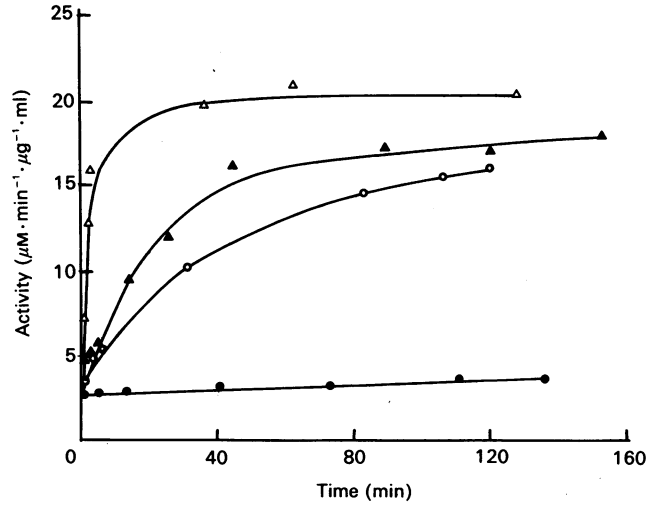

Fig. 2. Effect of temperature on the re-activation of glutamate dehydrogenase in 5 mM-Tris/HCl, pH 7, in the presence of 0.1 M-NaCl

●, 4 °C; ○, 10 °C; ▲, 15 °C; △, 25 °C.

Effects of substrates and substrate analogues on re-activation

The $NADP^+$-linked glutamate dehydrogenases of *Neurospora crassa* and *Candida utilis* display a pH-dependent activation–inactivation equilibrium in the reverse sense from that seen here, that is, the enzyme is activated at high pH. With these glutamate dehydrogenases the equilibrium is markedly affected by di- and tri-carboxylic acids [15–17]. The effects of substrates, substrate analogues and dicarboxylic acids on the re-activation of *Clostridium symbiosum* glutamate dehydrogenase were therefore investigated (Fig. 3). Concentrations were calculated to match the ionic strength of 30 mM-NaCl, used as a control, according to eqn. (1):

$$I = 0.5 \Sigma c_i z_i^2 \quad (1)$$

where I is the ionic strength, c_i the concentration of species i and z_i its charge. For multiply charged species, especially zwitterions, z_i is a somewhat ambiguous entity. Here a net point charge has been assumed so that, for example, together with the univalent counterions 10 mM-glutarate/succinate, contributes the same ionic strength as 30 mM-NaCl. For glutamate there is the added complication of the extent to which opposite charges cancel out: the fully charged ion could be regarded as a point charge of anything from 1 to 3. It is thus surprising that 20 mM-glutamate gave considerably slower re-activation than 30 mM-NaCl (Fig. 3). With aspartate, even 60 mM gave re-activation not much faster than the control. The monocarboxylic amino acid norvaline gave much less re-activation at 60 mM than the NaCl control. Most notably, 60 mM-alanine produced no re-activation whatsoever, despite its ionic-strength contribution. This suggests that alanine stabilizes the less-active form of the enzyme. These effects of monocarboxylic amino acids correlate well with the observation that norvaline and alanine, as substrates at pH 8.8 for enzyme previously stored at pH 7, gave markedly curved reaction traces, showing complete inactivation over the time course of an assay (a few minutes). By contrast, dicarboxylates lacking the amino group strikingly enhanced re-activation. Thus 10 mM-glutarate, 2-oxoglutarate or succinate gave full re-activation within 10–20 min (e.g. glutarate; Fig 3).

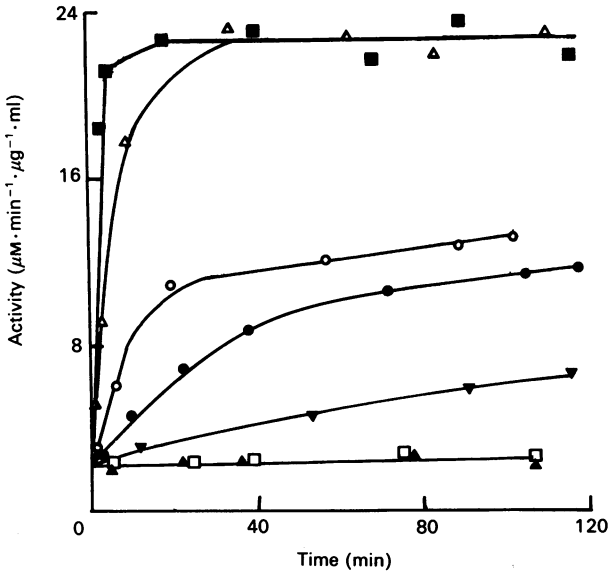

Fig. 3. Re-activation of glutamate dehydrogenase in 5 mM-Tris/HCl, pH 7, in the presence of dicarboxylic acids and amino acids

▲, 15 mM-alanine; □, 60 mM-alanine; ▼, 60 mM-norvaline; ○, 60 mM-aspartate; ■, 10 mM-succinate; △, 10 mM-glutarate. The curve for 30 mM-NaCl (●) is included for reference.

Effects of NAD(H) and other adenine nucleotides on re-activation

Various adenine nucleotides were tested for their effects on re-activation of glutamate dehydrogenase in 5 mM-Tris/HCl at 31 °C. These included the natural coenzymes NAD^+ and NADH, $NADP^+$, which is not an effective coenzyme for this glutamate dehydrogenase, AMP, ADP and ATP, which are indicators of energy status and, in the case of ADP and AMP, also fragments of the coenzyme structure, and CoA and acetyl-CoA, which are involved in the further metabolism of glutamate in *C. symbiosum*. All these nucleotides are inhibitors of the enzyme competitive with NAD^+ [10]. Whereas adenosine at 0.2 mM had no effect whatsoever on re-activation, all the nucleotides triggered regain of activity. ATP was particularly effective, promoting complete re-activation within 30 min at 0.1 mM, but all three mononucleotides were significantly more effective than the dinucleotides. Typical results are shown in Fig. 4. When nucleotides were added to enzyme undergoing the transition from pH 7 to pH 8.8 at 31 °C, inactivation, though not prevented, was nevertheless retarded. In this case NADH was considerably more effective than ATP (both at 1 mM).

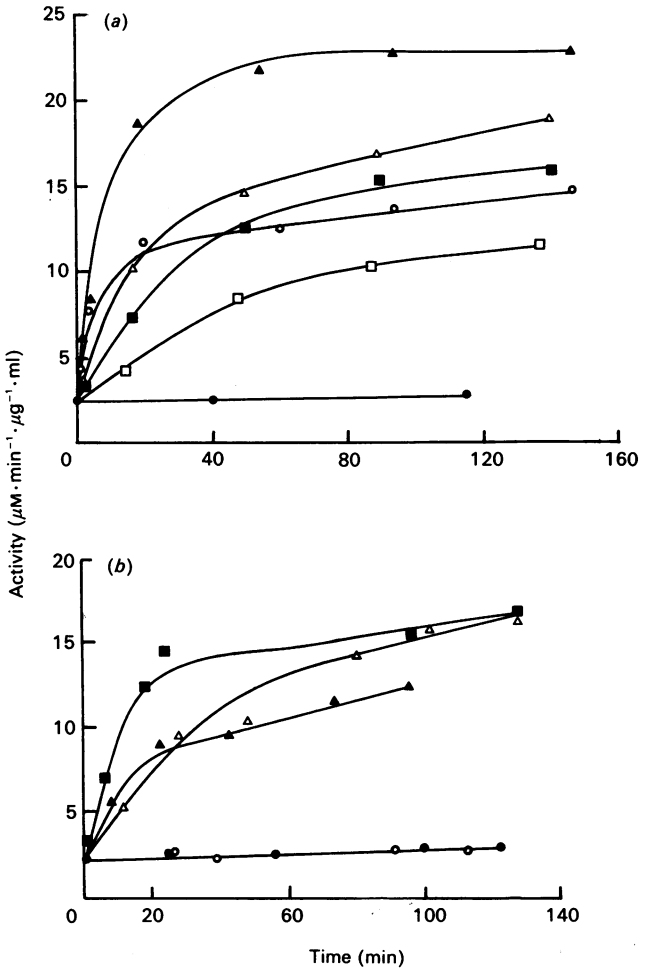

Fig. 4. Effects of coenzyme and coenzyme analogues on the re-activation rates of glutamate dehydrogenase in 5 mM-Tris/HCl, pH 7

All nucleotides were present at a concentration of 0.2 mM. (*a*): ●, no added nucleotide; □, CoA; ○, NADH; ■, acetyl-CoA; △, ADP; ▲, ATP; (*b*): ●, no added nucleotide; ○, adenosine; ▲, $NADP^+$; △, AMP; ■, NAD^+.

Effects of pH and protein concentration on re-activation and on the sedimentation properties of glutamate dehydrogenase

In view of the much-studied linear aggregation of ox liver glutamate dehydrogenase [18–20], it seemed initially plausible that the activity changes of clostridial glutamate dehydrogenase might be due to changes in quaternary structure. Re-activation experiments were carried out at 31 °C with 5 mM-Tris/HCl and 0.1 M-NaCl for four different final concentrations of enzyme over a 10-fold range (0.06–0.6 mg·ml^{-1}). Time courses for regain of activity were superimposable. There is thus no evidence for involvement of aggregation or dissociation in the activation–inactivation equilibrium. This conflicts with the conclusion in our earlier report [9], and it is now clear that the variation of glutamate dehydrogenase concentration in the first study was accompanied by a significant variation in pH. Analytical ultracentrifugation and sucrose-density centrifugation [13] allowed us to explore a wide range of glutamate dehydrogenase concentrations at high and low pH in Tris/HCl buffer. Symmetrical schlieren peaks were seen at pH 7 and pH 8.8 (Fig. 5). The $s^0_{20,w}$ values from plots of $1/s_{20,w(observed)}$ against 1/[glutamate dehydrogenase] were 11.01 S and 11.69 S at pH 7 and 8.8 respectively. These small differences may reflect slight alterations in axial ratio, but are not consistent with a dissociation equilibrium. Traces of slower-sedimenting material (Fig. 5) are not a function of protein concentration and appear to be due to impurities, in line with evidence from gel electrophoresis.

The decrease in sedimentation coefficient with concentration argues against association–dissociation under the conditions studied here. A similar concentration-dependence of $s_{20,w}$ was observed by sucrose-density-gradient centrifugation at pH 7 and pH 8.8 for low glutamate dehydrogenase concentrations (12–1000 μg·ml^{-1}). It remained possible that changes in quaternary structure might occur only in the presence of the assay substrates. This was tested directly by preincubating the enzyme (1 mg·ml^{-1}) with an equilibrium mixture at pH 8.8 containing 0.38 mM-NAD$^+$, 39.4 mM-glutamate, and NADH, 2-oxoglutarate and NH_4Cl all at 0.62 mM; sucrose-density-gradient centrifugation gave no indication of dissociation.

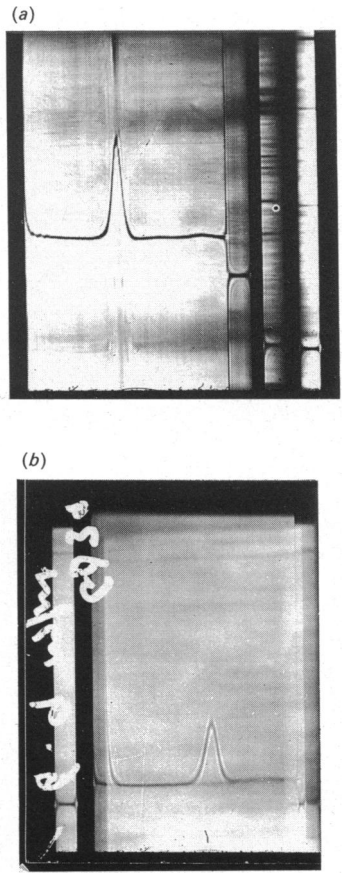

Fig. 5. Analytical ultracentrifugation of glutamate dehydrogenase at 25 °C

(*a*) Glutamate dehydrogenase at a concentration of 8 mg/ml in 20 mM-Tris/HCl, pH 7. The photograph was taken with schlieren optics at 48 min. (*b*) Glutamate dehydrogenase at a concentration of 6 mg/ml in 20 mM-Tris/HCl, pH 8.8. The photograph taken with schlieren optics at 72 min. In both cases sedimentation was from right to left as illustrated. Further details are given in the Materials and methods section.

DISCUSSION

In overall conclusion, it appears that the glutamate dehydrogenase of *Clostridium symbiosum* undergoes a slow pH-dependent transition between a high-activity and a low-activity state. The two pH values employed in this study, namely 7.0 and 8.8, were arbitrarily chosen. Thus, although the enzyme equilibrated at pH 8.8 retains 4–5% of the activity of enzyme stored at pH 7.0, neither activity level necessarily reflects a single molecular state of the protein. More probably there is a poised equilibrium between the active and inactive states. There is a considerable kinetic barrier to the interconversion so that, at low temperature or low ionic strength, the enzyme may remain metastable in its low-activity state. For the corresponding transition in *Candida utilis* glutamate dehydrogenase [17], the major effect of temperature is on the position of the activation–inactivation equilibrium. This is not the case here.

The inability of dicarboxylates and nucleotides to activate the high-pH form of the enzyme without a change to low pH further suggests a large activation energy. Similarly alanine and norvaline were unable to promote inactivation at pH 7, even though they clearly stabilize the low-activity state once it is formed. In an apparently analogous way, NADPH inactivates *N. crassa* glutamate dehydrogenase only at pH values below 7.8 [15].

The effects of alanine and norvaline are in striking contrast with the activation of ox liver glutamate dehydrogenase by monocarboxylic amino acids [21–24].

Although large macroscopic pH changes are perhaps unlikely to occur in the cell, the pH of the enzyme's microenvironment may differ sufficiently from the macroscopic pH to affect the conformational equilibrium, which could also respond to changes in metabolite concentrations. Frieden [25] has discussed the possible metabolic significance of several 'hysteretic' enzymes that respond slowly to rapid changes in metabolite concentrations. Since there is no evidence of changes in quaternary structure, inactivation–activation is presumably due to a slow conformational change in the enzyme hexamer. Though opposite in sense, this seems analogous to the pH-dependent activity changes in *N. crassa* glutamate dehydrogenase, which are similarly modulated by di- and tri-carboxylic acid salts. Direct evidence for conformational change in *C. symbiosum* glutamate dehydrogenase with altered pH has come from measurements of c.d. and is reported elsewhere [26].

At first sight, the accelerating reaction time courses seen at pH 8.8 are surprising, since these assays involve no change in pH. They do, however, involve transfer from a medium without substrates to a complete reaction mixture. Since individual additives influence activation markedly, it is probable that the reaction mixture displaces the equilibrium towards the active state of the enzyme. Correspondingly, assays (System II) of

fractions from sucrose density gradients containing an equilibrium mixture of the reactants at pH 8.8 showed much greater linearity.

At present it is not known whether the inactive form of the enzyme can bind substrates. Interestingly, however, the crystallographic studies have not yet detected binding of the dicarboxylate substrate, even in the presence of coenzyme (P. J. Baker, K. L. Britton, P. C. Engel, G. W. Farrants, K. S. Lilley, D. W. Rice & T. J. Stillman, unpublished work). Conceivably crystallization stabilizes the low-activity state, and the disability in that state may be a malfunction of the glutamate/2-oxoglutarate binding site. Our evidence [27] suggests that a triad of lysine side chains binds the two carboxylate groups. Their ionization state would govern the competence of the binding site. It is relevant, therefore, that amino acid substrates lacking the second carboxylate group stabilize the low-activity state. Such ligands would suppress positive charge at the binding site for the missing γ-carboxylate, and subunit co-operativity could account for the inactivation.

Clearly an explanation of the nature of the pH-dependent transition in activity will be essential to an overall understanding of the catalytic action and allosteric kinetics [10] of this enzyme. The present results point to a number of new lines of experimentation.

We are grateful to the Science and Engineering Research Council for supporting this work, both through a CASE studentship to S.E.-H.S. in conjunction with Imperial Biotechnology Ltd., and subsequently through a project grant to P.C.E.

REFERENCES

1. Rice, D. W., Hornby, D. P. & Engel, P. C. (1985) J. Mol. Biol. **181**, 147–149
2. Rice, D. W., Baker, P. J., Farrants, G. W. & Hornby, D. P. (1987) Biochem. J. **242**, 789–795
3. Baker, P. J., Farrants, G. W., Rice, D. W., Stillman, T. J. (1987) Biochem. Soc. Trans. **15**, 748–751
4. Reference deleted
5. McPherson, M. J. & Wootton, J. C. (1984) Nucleic Acids Res. **11**, 5257–5266
6. Valle, F., Becerril, B., Chen, E., Seeburg, P., Heyneker, H. & Bolivar, F. (1984) Gene **27**, 193–199
7. Wootton, J. C., Chambers, G. K., Holder, A. A., Baron, A. J., Taylor, J. G., Fincham, J. R. S., Blumenthal, K. M., Moon, K. & Smith, E. L. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 4361–4365
8. Kinnaird, J. H. & Fincham, J. R. S. (1983) Gene **26**, 253–266
9. Syed, S. E. H. & Engel, P. C. (1986) Biochem. Soc. Trans. **14**, 157
10. Syed, S. E. H. (1987) Ph.D Thesis, University of Sheffield
11. Dalziel, K. (1962) Biochem. J. **84**, 224–254
12. Engel, P. C. & Hornby, D. P. (1987) Biochem. Soc. Trans. **15**, 386–387
13. Martin, R. G. & Ames, B. N. (1961) J. Biol. Chem. **236**, 1372–1379
14. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
15. West, D. J., Tuveson, R. W., Barratt, R. W. & Fincham, J. R. S. (1967) J. Biol. Chem. **242**, 2132–2138
16. Ashby, B., Wootton, J. C. & Fincham, J. R. S. (1974) Biochem. J. **143**, 317–329
17. Gore, M. G. & Greenwood, C. (1975) Biochem. Biophys. Res. Commun. **52**, 997–1002
18. Olson, J. A. & Anfinsen, C. B. (1952) J. Biol. Chem. **197**, 67–79
19. Frieden, C. (1959) J. Biol. Chem. **234**, 809–814
20. Josephs, R. (1971) J. Mol. Biol. **55**, 147–153
21. Neumann, P., Markau, K. & Sund, H. (1976) Eur. J. Biochem. **65**, 465–472
22. Yielding, K. L. & Tomkins, G. M. (1961) Proc. Natl. Acad. Sci. U.S.A. **47**, 983–989
23. Hershko, A. & Kindler, S. H. (1966) Biochem. J. **101**, 661–664
24. Syed, S. E. H. & Engel, P. C. (1984) Biochem. J. **222**, 621–626
25. Frieden, C. (1970) J. Biol. Chem. **245**, 5788–5799
26. Syed, S. E. H., Engel, P. C. & Martin, S. R. (1990) FEBS Lett. **262**, 176–178
27. Lilley, K. S. & Engel, P. C. (1988) Biochem. Soc. Trans. **16**, 875–876

Received 16 May 1990; accepted 5 June 1990