Protection of Glutamate Dehydrogenase by Nicotinamide–Adenine Dinucleotide Against Reversible Inactivation by Pyridoxal 5'-Phosphate as a Sensitive Indicator of Conformational Change Induced by Substrates and Substrate Analogues

By SOO-SE CHEN and PAUL C. ENGEL

*Department of Biochemistry, University of Sheffield, Western Bank,
Sheffield S10 2TN, U.K.*

(Received 3 June 1974)

1. The steady residual activity of ox liver glutamate dehydrogenase at equilibrium with the reversible inactivator pyridoxal 5'-phosphate was measured in the presence and absence of various protecting agents. 2. NAD^+ (up to 15 mM) and its 3-acetylpyridine analogue (up to 5 mM) both failed to protect, in contrast with NADH. 3. Partial protection was given by glutarate and by succinate. Adipate and pentanoate were much less effective. 4. Correspondingly, whereas succinate and glutarate were both shown to be strong inhibitors of the catalytic reaction, competitive with glutamate, adipate was only weakly competitive, and the still weaker inhibition by pentanoate was non-competitive. 5. When the enzyme was saturated with glutarate, NAD^+ became a good, although still partial, protecting agent. In the absence of protection, 1.8 mM-pyridoxal 5'-phosphate decreased enzyme activity to 9%, in the presence of 150 mM-glutarate to 29%, and with glutarate and 1 mM- NAD^+ only to 73%. 6. 2-Oxoglutarate also promoted protection by NAD^+ , but neither pentanoate nor succinate did so. The finding with succinate is remarkable in view of findings 3 and 4 above. 7. It seems possible that substrates or analogues possessing the glutarate structure promote a conformational change that alters the mode of NAD^+ binding. This may explain why glutamate is a much better substrate than norvaline or aspartate and why negative interactions in coenzyme binding occur only in the formation of ternary complexes with glutamate or its analogues.

Ox liver glutamate dehydrogenase (EC 1.4.1.3) is reversibly inactivated by pyridoxal 5'-phosphate, which reacts with residue lysine-126, forming a Schiff base (Anderson *et al.*, 1966; Piszkiwicz *et al.*, 1970; Piszkiwicz & Smith, 1971; Moon *et al.*, 1972). It has been suggested that this residue may not be strictly essential (Goldin & Frieden, 1972a) since pyridoxal 5'-phosphate fails to inactivate the enzyme completely, but it is now clear that this failure is merely a consequence of the position of equilibrium between fully inactive enzyme and a non-covalent enzyme-modifier complex (S.-S. Chen & P. C. Engel, unpublished work).

The oxidized substrate, 2-oxoglutarate, and the reduced coenzyme, NADH, both confer on the enzyme limited protection against inactivation by pyridoxal 5'-phosphate (S.-S. Chen & P. C. Engel, unpublished work). Such protection gives a very sensitive, although indirect, indication of enzyme–ligand interaction. The effects of ligands that do not possess strong intrinsic absorbance or fluorescence may be readily detected, since, irrespective of the nature of the protecting agent, the quantity that is measured is the rate of the enzyme-catalysed reaction. Thus, with glutamate dehydrogenase, it is possible to study complexes other than those containing the

reduced coenzyme. The reversibility of the inactivation by pyridoxal 5'-phosphate offers the possibility of measuring a finite residual activity at equilibrium. This steady activity may be determined with greater accuracy and fewer measurements than a rate of inactivation. The present study, based entirely on an equilibrium analysis, illustrates the simplicity of the method.

It has been claimed by Piszkiwicz & Smith (1971) that the coenzymes protect glutamate dehydrogenase only in their reduced forms. In view of the striking differences in steady-state kinetic patterns for the forward and reverse reactions catalysed by glutamate dehydrogenase (Engel & Dalziel, 1969, 1970), it appeared worthwhile to check this claim and explore further the mutual interaction of coenzymes and substrates or their analogues on the surface of the enzyme.

Experimental

Ox liver glutamate dehydrogenase [$(\text{NH}_4)_2\text{SO}_4$ suspension] and coenzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., and were prepared and assayed as described by Engel & Dalziel (1969). Pyridoxal 5'-phosphate, from Koch–Light Laboratories Ltd., Colnbrook, Bucks.,

U.K., was shown by t.l.c. to be free of contamination apart from a minute amount of pyridoxal. 2-Oxoglutarate and the various substrate analogues and inhibitors were all from either BDH Chemicals Ltd., Poole, Dorset, U.K. or Koch-Light.

For the inactivation reactions, glutamate dehydrogenase (0.2mg/ml) was incubated at 25°C in test tubes protected from light with metal foil. The buffer, 0.1 M-potassium phosphate, pH 7.0, contained pyridoxal 5'-phosphate and protecting agents, previously adjusted to pH 7.0, at the concentrations indicated in the text. At timed intervals samples of volume 5 μ l were withdrawn for assay of catalytic activity in reaction mixtures containing 40mM-glutamate and 240 μ M-NAD⁺ in 0.111M-sodium phosphate buffer, pH 7.0. The concentrations of reactants were high in order to minimize the effects of protecting agents carried over to the assay mixture with the sample. For measurement of the zero-time activity in each experiment, enzyme from a stock solution was added to an assay mixture already containing the appropriate amount of the protecting agent. Formation of NADH was monitored with a recording fluorimeter of the type described by Dalziel (1962). Reactivation consequent on dilution was negligible in the time (1 min) taken for an assay.

Results and Discussion

The effect of NAD⁺ on inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate was investigated by incubating the enzyme with the inactivator at NAD⁺ concentrations covering the range 0–15mM. These additions of NAD⁺ were without measurable effect on the rate or final extent of inactivation. On the basis of direct studies of binding (Dalziel & Egan, 1972), the enzyme might be expected to be saturated by 15mM-NAD⁺, a concentration also far above the maximum used in kinetic experiments (Engel & Dalziel, 1969).

Although the total failure of NAD⁺ to protect the enzyme is in agreement with the findings of Piszkievicz & Smith (1971), and is consistent with the conclusion (S.-S. Chen & P. C. Engel, unpublished work) that lysine-126 is not essential for coenzyme binding, it is remarkable nonetheless: by contrast, protection by NADH is substantial, though incomplete (Piszkievicz & Smith, 1971; S.-S. Chen & P. C. Engel, unpublished work). This clear-cut difference may indicate that the binding of the oxidized and reduced coenzymes to glutamate dehydrogenase involves different conformations or states of the enzyme, the coenzyme, or both. On the other hand, there is abundant evidence for more than one NADH-binding site per enzyme monomer, one site being catalytically active and at least one regulatory (e.g. Pantaloni & Dessen, 1969; Jallon & Iwatsubo, 1971; Malencik & Anderson, 1972; Shafer *et al.*, 1972;

Koberstein & Sund, 1973; Krause *et al.*, 1974; also references in Goldin & Frieden, 1927b). It is conceivable therefore that, in the absence of glutamate or a suitable analogue, NAD⁺ is bound at only one of these sites.

A difference between the protective capacities of NAD⁺ and NADH was found by di Sabato & Kaplan (1965) in a study of the denaturation of lactate dehydrogenase (EC 1.1.1.27) by urea. They found, however, that, although NAD⁺ gave virtually no protection, the analogue 3-acetylpyridine-adenine dinucleotide protected as effectively as NADH. It seemed that the latter finding might reflect a conformation of the analogue more similar to that of NADH than NAD⁺. For glutamate dehydrogenase, 3-acetylpyridine-adenine dinucleotide is claimed to be a better coenzyme than NAD⁺ (Kaplan *et al.*, 1956), and accordingly the effect of this analogue at various concentrations was tested in our system. Even at 5mM, 3-acetylpyridine-adenine dinucleotide, like NAD⁺, conferred on the enzyme no protection whatsoever against pyridoxal 5'-phosphate (0.9mM). Thus, in its binding to glutamate dehydrogenase, the analogue appears to mimic NAD⁺ more closely than NADH.

It was unfortunately impossible to investigate protection by glutamate, because the ready reaction of pyridoxal 5'-phosphate with the α -amino group of glutamate interferes. The substrate analogue, gluta-

Fig. 1. Effect of 2-oxoglutarate and other di- and mono-carboxylic acids on the time-course of inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate

The control incubation mixture (Δ) contained glutamate dehydrogenase (0.2mg/ml) and pyridoxal 5'-phosphate (1.8mM) in 0.1 M-phosphate buffer, pH 7.0, at 25°C. Other incubations contained in addition 150mM-pentanoate ($\blacksquare$), 150mM-adipate ($\bullet$), 150mM-succinate ($\blacktriangle$), 150mM-glutarate ($\square$), 60mM-2-oxoglutarate ($\circ$). Samples were withdrawn at timed intervals for assay of activity.

Fig. 2. Concentration-dependence of the protection of glutamate dehydrogenase by 2-oxoglutarate, substrate analogues and NAD^+

Each point represents the final activity obtained in an incubation under the conditions of Fig. 1. Fig. 2(a) shows results obtained with glutarate ($\square$), succinate (∇), and pentanoate ($\circ$) used as protection agents at the concentrations indicated. Fig. 2(b) shows protection by 2-oxoglutarate at various concentrations. In both figures the filled symbols and dashed lines indicate supplementation with NAD^+ at concentrations shown on the upper scale.

rate, was used as a substitute. Although it lacks the amino group, it is a strong inhibitor, competitive with glutamate by virtue of its two correctly spaced carboxylate groups (Caughey *et al.*, 1957). Fig. 1 shows that, whereas, in the absence of protecting agents 1.8mm-pyridoxal 5'-phosphate decreased the activity of glutamate dehydrogenase to 9% of the initial value, in the presence of 150mm-glutarate the final activity was considerably higher (28%). Protection was limited, however: in the presence of 150 and 300mm-glutarate the activity at equilibrium was essentially the same (Fig. 2a). Thus saturation with glutarate makes it less easy, but not impossible, for pyridoxal 5'-phosphate to inactivate the enzyme. This pattern of partial protection is similar to that found with 2-oxoglutarate and with NADH (S.-S. Chen & P. C. Engel, unpublished work). For comparison, Fig. 1 shows the somewhat greater protection given by a saturating concentration (60mm) of 2-oxoglutarate.

In view of the high concentrations of glutarate

required to achieve a modest degree of protection, the specificity of the effect might be questioned. Similar experiments were performed therefore with adipate and succinate, the next higher and lower homologues in the dicarboxylic acid series. The results, shown in Fig. 1, indicate that 150mm-adipate was much less effective as a protecting agent than the same concentration of glutarate. Succinate, on the other hand, was as effective as glutarate. Pentanoate, which has the same carbon-chain length as glutarate, but only one carboxyl group, was also tested. Even at 150mm this compound gave very little protection.

The contrast between the effects of glutarate and adipate indicates that the protection by glutarate is not merely an ionic-strength effect. On the other hand the effectiveness of succinate was surprising, since Caughey *et al.* (1957) had found both succinate (K_i 110mm) and adipate (K_i 160mm) to be much feebler inhibitors of glutamate dehydrogenase than glutarate (K_i = 0.58mm). This discrepancy was investigated, and the results of steady-state inhibition

Fig. 3. Lineweaver-Burk plots showing inhibition of glutamate oxidation by various mono- and di-carboxylic acids

The initial rates were measured at 25°C in 0.111M-phosphate buffer, pH 7.0, with a fixed concentration of NAD^+ (64 μM). The concentration of glutamate was varied between 4 and 40mM. All inhibitors were present at a concentration of 150mM. Note the different scales used in (a) and (b). Control (○), pentanoate (Δ), adipate (□), succinate (▽), glutarate (▽).

experiments are shown in Fig. 3. In the presence of 64 μM - NAD^+ , in phosphate buffer at pH 7.0 and 25°C, 150mM-adipate gave weak inhibition ($K_i = 67\text{mM}$) competitive with glutamate. In contrast with the earlier findings, however, under our experimental conditions succinate was a strong competitive inhibitor ($K_i = 0.47\text{mM}$) comparable with glutarate ($K_i = 0.27\text{mM}$) in this respect. These results accord well with the relative protective capacities of the three dicarboxylic acids, as shown in Fig. 2. It should be noted that Caughey *et al.* (1957) used a higher pH (8.4) and NAD^+ concentration (640 μM) for their experiments. The inhibitory effect of 150mM-penta-

noate was also tested: Fig. 3 shows that this compound was a rather feeble inhibitor, and that the inhibition was not strictly competitive with glutamate. The observed inhibition may be due in part to non-specific hydrophobic interactions with the hydrocarbon chain of pentanoate, or to electrostatic interactions. Markau & Steinhübel (1972) report inhibition by acetate and by 2-methylpentanoate, in neither case strictly competitive with glutamate, and attribute the effect to the lack of internal charge compensation in these compounds.

It has been shown previously that the binding of NAD^+ by glutamate dehydrogenase is greatly affected by the presence of substrate or substrate analogues: with high fixed glutamate concentrations, Lineweaver-Burk plots of e_0/v against $1/[\text{NAD}^+]$, where e_0 is the total enzyme concentration and v is the initial steady-state rate, show a characteristic pattern with abrupt transitions indicative of mixed negative and positive co-operativity (Dalziel & Engel, 1968; Engel & Dalziel, 1969; Engel & Ferdinand, 1973). The abrupt transitions are not found with low concentrations of glutamate. Correspondingly, the weak binding of NAD^+ to free enzyme, directly studied by Dalziel & Egan (1972), shows no sign of site interaction, whereas in the presence of glutarate, NAD^+ binding is initially much tighter, but shows negative interactions. It was of interest therefore to discover whether inhibitory substrate analogues affected the capacity of NAD^+ to protect the enzyme against pyridoxal 5'-phosphate.

Fig. 2(a) shows the striking results of experiments in which glutarate, at a saturating concentration in the enzyme incubation mixture, was supplemented with various amounts of NAD^+ . In the presence of 1mM- NAD^+ , the final activity was 73% of the initial value, as compared with 28% without NAD^+ . Moreover, the protective effect of NAD^+ was half-maximal with 50 μM -coenzyme. Thus in the presence of glutarate, the previously ineffective NAD^+ is a potent protecting agent. Fig. 2(b) shows that 2-oxoglutarate brings about a similar change in the protective capacity of NAD^+ . Saturation with 2-oxoglutarate alone raised the final activity from 9 to 40% of the starting value, but in the presence of 1mM- NAD^+ the activity was raised to 80%. The conclusion appears inescapable that the presence of a substrate or analogue alters not only the tightness, but also the mode of NAD^+ binding. It seemed just possible, however, that a small contamination of the protecting agents with glutamate might have given rise to sufficient NADH during the incubation to account for the apparent effect of NAD^+ . To test this the enzyme (0.2mg/ml) was incubated with NAD^+ (5mM) and 2-oxoglutarate or glutarate (150mM), but without pyridoxal 5'-phosphate. No NADH production could be detected fluorimetrically over the course of incubation for 1 h at 25°C with instru-

mental settings which would have given a 2cm recorder-pen deflection for 0.1 μ M-NADH produced.

Steady-state initial-rate studies of glutamate dehydrogenase (Engel & Dalziel, 1969) revealed a marked contrast between the kinetic patterns with glutamate and norvaline as substrates. With the latter amino acid, in which the γ -carboxyl group of glutamate is replaced by a methyl group, there is no sign of the deviations from Michaelis-Menten kinetics that characterize the reaction with glutamate. It has been suggested (Engel & Dalziel, 1969) that this difference may reflect a conformational change in the ternary complex initiated by glutamate but not norvaline. It was of interest therefore to establish whether pentanoate, which bears the same relation to norvaline as glutarate does to glutamate, is capable of promoting protection by NAD⁺. When 5mM-NAD⁺ was present in inactivation incubations in addition to 150mM-pentanoate, it gave no additional protection (Fig. 2a). Unfortunately this negative result cannot be regarded as conclusive evidence that the glutarate structure is required to effect the postulated conformational change. The weak protection and the non-competitive nature of the feeble steady-state inhibition by pentanoate itself suggest that this compound may be largely bound outside the active site. It is possible therefore that pentanoate fails to promote the conformational change simply because too little of it binds at the substrate site.

An unequivocal result, however, was obtained with succinate. As shown above, this compound at a concentration of 150mM is a strong competitive inhibitor and a good protecting agent. Nevertheless, when 5mM-NAD⁺ was present in addition to 150mM-succinate, it gave no further protection (Fig. 2a).

In their detailed study of the structural requirements for competitive inhibitors of glutamate dehydrogenase, Caughey *et al.* (1957) established the importance of the presence of two correctly spaced, negatively charged groups. Thus glutarate, isophthalate and 5-bromofuroate, all showing the correct spacing (0.75 nm), were found to be strong inhibitors, whereas very similar compounds without this spacing were poorer inhibitors. This established criteria for complementarity to the substrate-binding site.

On the basis of the present studies it seems possible to go beyond the conclusions of Caughey *et al.* (1957). Not only do compounds with the glutarate structure bind tightly at the active site; they also apparently bring about a conformational change in the protein which markedly alters the binding of NAD⁺. This change evidently accounts for the acquired susceptibility to protection by NAD⁺, for the possibilities of site interaction mentioned above, and, to some extent, presumably for the much higher catalytic rates achieved with glutamate as substrate than, for example, with aspartate (Olson & Anfinsen, 1953;

Struck & Sizer, 1960) or norvaline (Struck & Sizer, 1960; Engel & Dalziel, 1969). The contrast between the ability of glutarate, a good inhibitor, to 'switch on' the protection by NAD⁺, and the complete failure of succinate, although also a good inhibitor, to do so, emphasizes the importance of the correct spacing of the carboxylate groups.

In the past, much of the discussion of the regulatory behaviour of glutamate dehydrogenase has centred around homotropic and heterotropic effects in the binding of the coenzymes and the effector nucleotides ADP and GTP (for review see Goldin & Frieden, 1972b). The results reported here focus attention on the likely importance of a highly specific substrate-induced change from an enzyme conformation able to bind coenzyme only weakly to one that binds coenzyme tightly. This may well be similar to the conformational transition detected by measurements of fluorescence and circular dichroism at half-saturation of the enzyme with NAD(P)⁺ in the presence of glutarate (Bell & Dalziel, 1973). Further study of the interplay of substrate and coenzyme in modulating the conformation of glutamate dehydrogenase may clarify the basis of the kinetic complexity of glutamate oxidation (Engel & Dalziel, 1969; Engel & Ferdinand, 1973).

This work was supported by a research grant (to P. C. E.) from the Enzyme Chemistry and Technology Committee of the Science Research Council and by financial assistance (to S.-S. C.) from the British Council and the Lee Foundation, Singapore. The help from these sources is gratefully acknowledged.

References

- Anderson, B. M., Anderson, C. D. & Churchich, J. E. (1966) *Biochemistry* **5**, 2893-2900
- Bell, J. E. & Dalziel, K. (1973) *Biochim. Biophys. Acta* **309**, 237-242
- Caughey, W. S., Smiley, J. D. & Hellerman, L. (1957) *J. Biol. Chem.* **224**, 591-607
- Dalziel, K. (1962) *Biochem. J.* **84**, 244-254
- Dalziel, K. & Egan, R. R. (1972) *Biochem. J.* **126**, 975-984
- Dalziel, K. & Engel, P. C. (1968) *FEBS Lett.* **1**, 349-352
- di Sabato, G. & Kaplan, N. O. (1965) *J. Biol. Chem.* **240**, 1072-1076
- Engel, P. C. & Dalziel, K. (1969) *Biochem. J.* **115**, 621-631
- Engel, P. C. & Dalziel, K. (1970) *Biochem. J.* **118**, 409-419
- Engel, P. C. & Ferdinand, W. (1973) *Biochem. J.* **131**, 97-105
- Goldin, B. R. & Frieden, C. (1972a) *J. Biol. Chem.* **247**, 2139-2144
- Goldin, B. R. & Frieden, C. (1972b) *Curr. Top. Cell. Regul.* **4**, 77-117
- Jallon, J.-M. & Iwatsubo, M. (1971) *Biochem. Biophys. Res. Commun.* **45**, 964-971
- Kaplan, N. O., Ciotti, M. M. & Stolzenbach, F. E. (1956) *J. Biol. Chem.* **221**, 833-844
- Koberstein, R. & Sund, H. (1973) *Eur. J. Biochem.* **36**, 545-552

- Krause, J., Bühner, M. & Sund, H. (1974) *Eur. J. Biochem.* **41**, 593–602
- Malencik, D. A. & Anderson, S. R. (1972) *Biochemistry* **11**, 2766–2771
- Markau, K. & Steinhübel, I. (1972) *FEBS Lett.* **28**, 115–120
- Moon, K., Piskiewicz, D. & Smith, E. L. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 1380–1383
- Olson, J. A. & Anfinsen, C. B. (1953) *J. Biol. Chem.* **202**, 841–856
- Pantaloni, D. & Dessen, P. (1969) *Eur. J. Biochem.* **11**, 510–519
- Piskiewicz, D. & Smith, E. L. (1971) *Biochemistry* **10**, 4544–4552
- Piskiewicz, D., Landon, M. & Smith, E. L. (1970) *J. Biol. Chem.* **245**, 2622–2626
- Shafer, J., Chiancone, E., Vittorelli, L. M., Spagnuolo, C., Mackler, B. & Antonini, A. (1972) *Eur. J. Biochem.* **31**, 166–181
- Struck, J., Jr. & Sizer, I. W. (1960) *Arch. Biochem. Biophys.* **86**, 260–266