Ox Liver Glutamate Dehydrogenase

THE ROLE OF LYSINE-126 REAPPRAISED IN THE LIGHT OF STUDIES OF INHIBITION AND INACTIVATION BY PYRIDOXAL 5'-PHOSPHATE

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The time-course of inactivation of bovine liver glutamate dehydrogenase by pyridoxal 5'-phosphate was studied in the presence of varied amounts of 2-oxoglutarate or NADH. Pseudo-first-order analysis reveals that the protection by both these compounds is competitive with respect to the chemical modifier. The competition is only partial, however: saturation with either NADH or 2-oxoglutarate decreases the rate constant for inactivation to a finite minimum and not to zero. Similarly, the plot of activity at equilibrium as a function of the concentration of the protecting substrate or coenzyme reveals that neither NADH nor 2-oxoglutarate protects completely against inactivation. In initial-rate experiments, pyridoxal 5'-phosphate, used as an instantaneous inhibitor rather than a long-term inactivator, displayed non-competitive inhibition with respect to both 2-oxoglutarate and NADH. These results clearly indicate that, although there is mutual hindrance between the binding to the enzyme of pyridoxal 5'-phosphate, on the one hand, and 2-oxoglutarate or NADH on the other, binding is not mutually exclusive. These findings are discussed in terms of the two-step mechanism for inactivation by pyridoxal 5'-phosphate. It is concluded that lysine-126 cannot be solely responsible for binding either the substrate or the coenzyme, but could be essential for the catalytic step.

The intimate chemistry of the catalysis effected by glutamate dehydrogenase [L-glutamate-NAD(P)⁺ oxidoreductase (deaminating), EC 1.4.1.3] remains obscure despite many attempts to obtain information through chemical modification of the enzyme (Goldin & Frieden, 1972a). Of the various amino acid residues implicated in the overall function of the ox liver enzyme, tyrosine-406 and lysines-419 and -422 appear to be involved in allosteric interactions (Price & Radda, 1969; Piszkiwicz *et al.*, 1971; Coffee *et al.*, 1971). Only lysine-27 and lysine-126 (formerly designated 97) have been proposed as functional elements of the active site. Lysine-27 reacts with glyoxal (Deppert *et al.*, 1973), whereas lysine-126 is attacked with a high degree of specificity by several reagents, including pyridoxal 5'-phosphate (Anderson *et al.*, 1966; Piszkiwicz *et al.*, 1970). The latter residue has been shown to be in a highly conserved sequence of amino acids (Piszkiwicz *et al.*, 1970; Moon *et al.*, 1972; Wootton *et al.*, 1973; Blumenthal & Smith, 1973; Holbrook *et al.*, 1973), which shows homology with the sequence in the vicinity of lysine-212 of pig glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (Smith *et al.*, 1970; Forcina *et al.*, 1971), which is also inactivated by pyridoxal 5'-phosphate.

There is no general agreement as to the function of lysine-126. Indeed it has been suggested that this residue is not, after all, essential (Goldin & Frieden, 1972b), since, even in the presence of pyridoxal 5'-

phosphate at high concentrations, a small percentage of catalytic activity persists. In a recent paper, however, we have presented results which explain the residual activity and indicate that lysine-126 is essential (Chen & Engel, 1975a).

There are conflicting claims in the literature as to whether 2-oxoglutarate or NAD(P)H protects the enzyme against inactivation, and, correspondingly, whether lysine-126 is involved in binding the coenzyme or the 2-oxo acid substrate (Baker *et al.*, 1962; Malcolm & Radda, 1968, 1970; Piszkiwicz & Smith, 1971b; Goldin & Frieden, 1972b; Wallis & Holbrook, 1973). Most recently, Brown *et al.* (1973) have claimed on the basis of binding studies that pyridoxal 5'-phosphate abolishes the binding of both NADPH and 2-oxoglutarate at the active site of glutamate dehydrogenase.

It seemed to us that the issue might be resolved by taking full advantage of the sensitivity of an analysis based on measurements of catalytic activity. The nature of the interference by a chemical modification at the active site of an enzyme may be investigated by studying the antagonism between the modifying agent and substrates. One may determine both the effect of a single added substrate on the time-course and final extent of inactivation (substrate protection), and the pattern of inhibition by the chemical modifier in steady-state initial-rate experiments. In experiments of the latter type, covalent modification of the

enzyme is not involved; one is concerned only with a non-covalent enzyme-inhibitor complex. Experiments of both types are reported in the present paper.

It is evident in the present context that a nomenclature based solely on reversible and irreversible inhibition (Dixon & Webb, 1964) is inadequate. The ambiguity lies primarily in the failure to distinguish between covalent and non-covalent enzyme-ligand interactions. We have attempted to avoid this confusion in the present case by reserving the term 'inhibition' for non-covalent interactions, by definition reversible, though not always readily so. Diminution of activity through covalent modification we term 'inactivation'. Thus a ligand may be an inactivator, an inhibitor, or both. Examples of the three categories for glutamate dehydrogenase are pyridoxal, glutarate and pyridoxal 5'-phosphate. Inactivation may be irreversible (e.g. alkylation by iodoacetamidosalicylic acid) or reversible, as in the present case.

Experimental

Materials

The sources and handling of ox liver glutamate dehydrogenase and pyridoxal 5'-phosphate are detailed elsewhere (Chen & Engel, 1975a). Coenzymes were purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Other substrates and chemicals were from normal commercial sources and were of the highest purity available.

Inactivation experiments

These were performed as described previously (Chen & Engel, 1975a), except that the incubation mixtures were supplemented with varied amounts of 2-oxoglutarate or NADH. In some experiments these compounds were used at such high concentrations that the small amounts transferred to the assay mixture were sufficient to cause inhibition. In determining the corresponding 'zero-time' values for 100% activity, therefore, enzyme without pyridoxal 5'-phosphate was added to assay mixtures already containing the appropriate amount of the protecting agent. Since the modification at lysine-126 produces a Schiff base that is totally inactive (Chen & Engel, 1975a), it is possible to vary the composition of the assay mixture in this way without invalidating the estimates of the extent of enzyme modification. In the

studies of protection by NADH, the problem of product inhibition by NADH in the catalytic assay was further counteracted by increasing the concentration of NAD⁺ in the assay mixture from 60 μ M to 600 μ M. Likewise, when 2-oxoglutarate was used as the protecting agent, the concentration of glutamate in the assay solution was increased from 10 mM to 40 mM.

Inhibition experiments

In studies of its behaviour as an instantaneous inhibitor rather than a long-term inactivator, pyridoxal 5'-phosphate was added in various amounts to the catalytic reaction mixture. Substrate concentrations were varied in the normal way to determine the pattern of inhibition. To start the reaction, 10 μ l of a stock solution (0.37–1.48 mg/ml) of the enzyme was added to 3 ml of reaction mixture. Under these conditions the light-sensitivity and strong fluorescence of pyridoxal 5'-phosphate proved a major obstacle to fluorimetric assay. Reactions were therefore monitored spectrophotometrically at 25°C with Unicam SP.500 optics and a Gilford 240 photometer linked to a chart recorder. Rates were calculated from changes in E_{340} on the basis of an extinction coefficient of 6.22×10^6 cm²/mol (Horecker & Kornberg, 1948) for NADH.

Theory and Results

The reaction of glutamate dehydrogenase with pyridoxal 5'-phosphate has been shown to proceed in two stages (Piszkiwicz & Smith, 1971b; Chen & Engel, 1975a), involving initial reversible formation of a non-covalent complex, which can then react further to form a Schiff base. With the concentrations of pyridoxal 5'-phosphate normally used, the initial second-order process is very rapid, whereas the subsequent first-order covalent reaction occurs only slowly. This difference in rates allows separate study of inhibition, resulting from formation of the non-covalent complex, and inactivation, resulting from formation of the Schiff base.

It has been shown (Piszkiwicz & Smith, 1971a,b; Chen & Engel, 1975a) that the inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate may be subjected to pseudo-first-order analysis. Since the Schiff base is completely inactive (Chen & Engel, 1975a), this may be done simply by plotting $\ln(A_0/A_t)$ against time, where A_0 is the initial specific activity,

Scheme 1. Two-step mechanism for reaction of bovine glutamate dehydrogenase with pyridoxal 5'-phosphate

Fig. 1. Inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate: protection by 2-oxoglutarate

Time-courses of inactivation of glutamate dehydrogenase (0.2mg/ml) by 0.6mM-pyridoxal 5'-phosphate in 0.1M-potassium phosphate buffer, pH7, 25°C, in the presence of 2-oxoglutarate at the concentrations (mM) indicated.

and A_t that at time t . Such plots are linear for the first few minutes of reaction, deviating only as the rate of breakdown of the Schiff base begins to be significant. The slope of the linear portion provides an estimate of $k_{app.}$, the apparent pseudo-first-order rate constant, and a plot of $1/k_{app.}$ against the reciprocal of the pyridoxal 5'-phosphate concentration yields estimates of $1/k_{+2}$ and k_{-1}/k_{+1} (Scheme 1) (Piszkiewicz & Smith, 1971b; Chen & Engel, 1975a). As discussed previously this analysis is approximate when applied to a reversible reaction (Chen & Engel, 1975a). Rigorous analysis of the rate of approach to equilibrium gives an apparent rate constant which is the sum of the forward and reverse rate constants (Frost & Pearson, 1961). In the context of protection studies, however, such analysis would require unwarranted assumptions as to the properties of the covalently modified enzyme. Since the equilibrium constant for conversion of the non-covalent complex into the Schiff base lies well over towards the Schiff base, the approximate treatment seems justifiable.

Fig. 1 shows the effect of 2-oxoglutarate at various concentrations in incubations of glutamate dehydrogenase with 0.6mM-pyridoxal 5'-phosphate. The substrate decreases both the rate of inactivation and its final extent. Similar experiments were carried out for a series of pyridoxal 5'-phosphate concentrations,

Fig. 2. Kinetic analysis of protection by 2-oxoglutarate: pseudo-first-order plots

Conditions were as in Fig. 1, with concentrations of 2-oxoglutarate (mM) as indicated.

Fig. 3. Kinetic analysis of protection by 2-oxoglutarate: double-reciprocal plots

The values of $k_{app.}$ (see the text) corresponding to each concentration of 2-oxoglutarate were obtained from the slopes of the appropriate lines in Fig. 2 and similar plots of results obtained with different concentrations of pyridoxal 5'-phosphate.

Fig. 4. Kinetic analysis of protection by 2-oxoglutarate: secondary plot

For each concentration of 2-oxoglutarate a slope may be obtained from the corresponding line in Fig. 3. These slopes are replotted against the concentration of 2-oxoglutarate. The different symbols correspond to those used in Fig. 3.

yielding a value of k_{app} for each combination of substrate and pyridoxal 5'-phosphate concentrations. The set of pseudo-first-order plots for one pyridoxal 5'-phosphate concentration (0.6 mM) is shown in Fig. 2. Values of $1/k_{app}$ were then plotted against $1/[pyridoxal\ 5'-phosphate]$ in order to determine the pattern of protection by substrate (Fig. 3). Although the ordinate intercepts are small, the protection appears, within the error, to be competitive. As pointed out by Dixon & Webb (1964), however, mere competition does not establish that the binding of two compounds by an enzyme is mutually exclusive. It is essential to establish whether or not the slopes of the lines plotted in Fig. 3 approach a limiting upper value as the concentration of 2-oxoglutarate is increased. The plateau in the replot (Fig. 4) indicates clearly that protection by 2-oxoglutarate is partial only. This means that enzyme saturated with the substrate is still open to attack by pyridoxal 5'-phosphate. Competitive behaviour is still to be expected (Dixon & Webb, 1964), provided that 2-oxoglutarate does not substantially alter the rate constant for conversion of the non-covalent enzyme-pyridoxal 5'-phosphate complex into the Schiff base.

The incompleteness of protection by 2-oxoglutarate is also shown by an analysis of the activity at equilibrium. This is plotted against 2-oxoglutarate concentration in Fig. 5. Each plot shows a separate plateau. If 2-oxoglutarate could protect completely, all these curves would be asymptotic to 100% activity. The equilibrium analysis is, of course, entirely independent of the kinetic analysis and its attendant approximations.

Fig. 5. Equilibrium analysis of protection by 2-oxoglutarate. Each point represents the minimum activity attained in a single incubation of glutamate dehydrogenase with pyridoxal 5'-phosphate. The conditions were as for Fig. 1 except for the concentration of pyridoxal 5'-phosphate, which was as indicated (mM).

Fig. 6. Inhibition of glutamate dehydrogenase by pyridoxal 5'-phosphate in steady-state initial-rate experiments: 2-oxoglutarate as varied substrate

Initial rates in 0.111 M-sodium phosphate buffer, pH7, at 25°C were measured spectrophotometrically at 340 nm. Reactions were initiated by addition of enzyme to a solution already containing 0.12 mM-NADH, 50 mM-NH₄Cl and pyridoxal 5'-phosphate at the concentrations (mM) indicated. The Figure shows the Lineweaver-Burk plots. The units of activity (v) were μmol of NADH/min per mg. The inset shows secondary plots of the slopes (Δ) and intercepts ($\circ$) against pyridoxal 5'-phosphate concentration.

Fig. 7. Kinetic analysis of protection by NADH

Values of k_{app} for the inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate in the presence of NADH were obtained from pseudo-first-order plots similar to those shown in Fig. 2. Conditions were as for Fig. 1. Double-reciprocal plots are shown, similar to those in Fig. 3. In this plot the separate lines correspond to different concentrations of NADH present in the incubation. The concentrations (mM) are indicated. The inset shows a secondary plot, similar to Fig. 4, of the slopes and intercepts of the double-reciprocal plots against pyridoxal 5'-phosphate concentration.

The conclusion that pyridoxal 5'-phosphate and 2-oxoglutarate may be simultaneously bound to glutamate dehydrogenase was further tested by determining the pattern of inhibition by pyridoxal 5'-phosphate in steady-state initial-rate experiments. The Lineweaver-Burk plots (Fig. 6) show non-competitive behaviour, in contrast with the strict competition expected if 2-oxoglutarate can completely displace the chemical modifier.

The interaction of NADH and pyridoxal 5'-phosphate on the surface of the enzyme was also investigated. Fig. 7 shows plots of $1/k_{app}$ against $1/[\text{pyridoxal } 5'\text{-phosphate}]$ derived from pseudo-first-order analysis of inactivation experiments with various combinations of pyridoxal 5'-phosphate and NADH concentrations. Within experimental error, protection by NADH, as by 2-oxoglutarate, appears to be competitive. The replot of slopes against NADH concentration (Fig. 7, inset) shows that this competition is again only partial. As with 2-oxoglutarate (Fig. 5), it was possible to confirm this conclusion by plotting equilibrium activity against the NADH

Fig. 8. Equilibrium analysis of protection by NADH

The plot is analogous to Fig. 5, except that the protecting agent was NADH rather than 2-oxoglutarate. The concentration of NADH is indicated along the abscissa, and the concentrations of pyridoxal 5'-phosphate (mM) are shown beside each curve.

Fig. 9. Inhibition of glutamate dehydrogenase by pyridoxal 5'-phosphate in steady-state initial-rate experiments: NADH as varied substrate

The conditions and units of v were as in Fig. 6, except that the concentration of 2-oxoglutarate was fixed (0.5 mM) and the concentration of NADH was varied. The slopes (Δ) and intercepts (○) of the Lineweaver-Burk plots are replotted against inhibitor concentration in the inset.

concentration: Fig. 8 shows that finite inactivation occurs even in the presence of saturating amounts of NADH. Thus it appears that NADH also may be

bound to the enzyme in the presence of the modifier.

A study of inhibition by pyridoxal 5'-phosphate with respect to NADH present at varied concentrations was also undertaken. Although non-competitive inhibition was observed (Fig. 9), in support of the conclusion that NADH and the modifier may be simultaneously bound to the enzyme, undue weight must not be attached to this evidence; progress plots showed considerable curvature, hindering the accurate estimation of initial rates. The possibility of distortion of the kinetic pattern through systematic error cannot therefore be ruled out.

The insets in Figs. 6 and 9 show the slopes and intercepts of the Lineweaver-Burk plots replotted against inhibitor concentration. The replots yield four different apparent K_i values (2.4, 4.1, 2.7 and 1.7 mM). Such values are often equated (Baker *et al.*, 1962; Malcolm & Radda, 1970; Wallis & Holbrook, 1973) with the dissociation constant governing the combination of the inhibitor with the free enzyme. This is not justified. There are thought to be eight kinetically significant enzyme-containing complexes involved in the three-substrate reaction catalysed by glutamate dehydrogenase, each involving a different combination of bound substrates (Engel & Dalziel, 1970). The results presented here establish that pyridoxal 5'-phosphate binds not only to free enzyme but also to enzyme-substrate and enzyme-coenzyme complexes. With the high fixed substrate concentrations used in the inhibition experiments, it is quite possible that combination of free enzyme with pyridoxal 5'-phosphate makes only a minor contribution to the observed inhibition. True dissociation constants could be determined from such kinetic measurements by evaluating the separate K_i affecting each of the eight kinetic parameters of the reciprocal initial-rate equation (Engel, 1968; Engel & Dalziel, 1970), but this does not seem worthwhile at present. The dissociation constant governing combination of free enzyme with pyridoxal 5'-phosphate has been determined from inactivation experiments (Chen & Engel, 1975a) as 1.59 mM, lower than any of the apparent values given above.

Discussion

In this study we have sought to discover how blockage of lysine-126 abolishes the activity of glutamate dehydrogenase. It is not possible to exclude an allosteric effect: the modification of lysine-126 has been shown previously to interfere with interactions among the nucleotide-binding sites of this enzyme (Malcolm & Radda, 1970; Piskiewicz & Smith, 1971a; Goldin & Frieden, 1972b; Wallis & Holbrook, 1973). It is nevertheless perhaps more likely that lysine-126 is in the vicinity of the active site, especially since total modification leads to total inactivation (Chen & Engel, 1975a). The modification must in any case

directly or indirectly prevent either the correct binding of reactants or successful subsequent catalysis.

Churchich & Anderson (1968) reported that in glutamate dehydrogenase partially inactivated with pyridoxal 5'-phosphate, the dissociation constant for NADH was increased from $4.6 \mu\text{M}$ to $50 \mu\text{M}$. Piskiewicz & Smith (1971b) observed competitive protection of glutamate dehydrogenase against pyridoxal 5'-phosphate by NADH and NADPH but not by 2-oxoglutarate, NAD^+ or NADP^+ . Malcolm & Radda (1970) and Wallis & Holbrook (1973), however, did find protection of lysine-126 4-iodoacetamidosalicylic acid by 2-oxoglutarate at higher concentrations. Wallis & Holbrook (1973) also found that the blockage at lysine-126 largely prevented the binding of 2-oxoglutarate but had relatively little effect on nucleotide binding. In binding studies, Brown *et al.* (1973) were unable to detect binding of either NADPH or 2-oxoglutarate at the active site of enzyme partially inactivated by treatment with pyridoxal 5'-phosphate and NaBH_4 . When, however, these authors blocked lysine-126 with pyridoxal rather than pyridoxal 5'-phosphate, they were able to show binding of NADPH, though still not of 2-oxoglutarate.

Our experiments establish the protective effect of both 2-oxoglutarate and NADH. Nevertheless, although there is clearly mutual hindrance between the binding of these compounds and the binding of the modifier, equally clearly the antagonism is not total. This distinction between abolition and impairment of a property is important in the discussion that follows.

If we consider first the experiments in which pyridoxal 5'-phosphate was used as an inhibitor rather than an inactivator, the non-competitive behaviour with respect to both 2-oxoglutarate (Fig. 6) and NADH (Fig. 9) indicates that the non-covalent enzyme-pyridoxal 5'-phosphate complex can bind both these compounds. The linear secondary plots suggest that inhibition is probably complete rather than partial (insets in Figs. 6 and 9), since they show no plateaux. It is possible in either case, however, that a plateau might be obtained with higher inhibitor concentrations.

The inhibition experiments give no information as to the properties of the covalently modified enzyme, and it might still therefore be argued that once lysine-126 is chemically modified, the enzyme loses the ability to bind coenzyme or substrate or both. The protection experiments show conclusively that this is not the case. If the covalently modified enzyme is unable to bind a given compound that can form a complex with the native unmodified enzyme, that compound should be able completely to displace the equilibrium in favour of active enzyme. This occurs with neither 2-oxoglutarate nor NADH. Since these compounds give only partial protection against

reversible inactivation (Figs. 4 and 7, inset), it follows that the modified enzyme can bind them. It should be emphasized that such conclusions may only be drawn because the covalent inactivation is reversible.

In view of the high concentrations of 2-oxoglutarate used in these experiments, it is necessary to consider the possibility that the partial protection is a non-specific salt effect. This explanation, however, is not compatible with the very small degree of protection given by valerate and adipate (150mM) (Chen & Engel, 1974).

Our conclusion that the integrity of lysine-126 is not essential for the binding of 2-oxoglutarate or NADH has to be reconciled with, for instance, the evidence of Brown *et al.* (1973) suggesting that the inactivated enzyme cannot bind substrate. The apparent discrepancy probably arises from the fact that binding measurements give a much less sensitive indication of events at the active site than do measurements of catalytic activity. Direct observation of a binary enzyme-2-oxoglutarate complex by binding measurements (Cross *et al.*, 1972) only followed the inference of its existence from kinetic experiments, earlier binding measurements having failed to detect it (Fisher, 1960). Since the binding of substrate to the modified enzyme is weaker still, it is not surprising that it was missed by even the careful measurements of Brown *et al.* (1973).

If the binding of coenzyme and substrate does not depend on the integrity of lysine-126, another explanation must be found for the inactivation. Binding, though possible, may nevertheless be defective, so that the reactants are not properly orientated on the enzyme surface. Alternatively the malfunction may be in catalysis rather than binding.

Establishing the nature of the malfunction in the modified enzyme would not necessarily define the role of lysine-126 in glutamate dehydrogenase. Indeed the residue might not be directly involved in the normal activity of the enzyme; its substitution may, for example, block access to some other, essential residue. This seems less likely, however, in view of the inactivation caused by reaction of lysine-126 with cyanate, a much less bulky modifying agent than pyridoxal 5'-phosphate (Veronese *et al.*, 1972). It is thus at least possible that lysine-126 plays a direct role, either in the orientation of the reactants or in the catalytic process. This view receives indirect support from the finding that several other nicotinamide nucleotide-linked dehydrogenases are also inactivated by the reaction of a lysine residue with pyridoxal 5'-phosphate, suggesting a possible common role for lysine in these enzymes (Chen & Engel, 1975b). For some of these dehydrogenases the three-dimensional structures as well as the primary sequences are known. Identification of the essential lysine residues in these enzymes may yield clues to the function of lysine-126 in glutamate dehydrogenase.

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