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The Equilibrium Position of the Reaction of Bovine Liver Glutamate Dehydrogenase with Pyridoxal 5′-Phosphate

A DEMONSTRATION THAT COVALENT MODIFICATION WITH THIS REAGENT COMPLETELY ABOLISHES CATALYTIC ACTIVITY

By SOO-SE CHEN and PAUL C. ENGEL
Department of Biochemistry, University of Sheffield, Western Bank, Sheffield S10 2TN, U.K.

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1. The activity of bovine liver glutamate dehydrogenase incubated with pyridoxal 5′-phosphate declined to a steady value reached within 30–60min. The residual activity depended on the concentration of modifier up to about 5mM. Above this concentration, however, no further inactivation was produced. The minimum activity obtainable in such incubations was 6–7% of the initial value. 2. K_m values of the modified enzyme were unaltered, whereas $V_{max.}$ was decreased. 3. Activity was fully regained on dialysis against 0.1M-potassium phosphate buffer. 4. Reduction with borohydride rendered the inactivation permanent but did not alter its extent. 5. Enzyme permanently inactivated in this way to the extent of 90% and dialysed was re-treated with pyridoxal 5′-phosphate. In this second cycle activity declined from 10 to 1% of the original activity. 6. This strongly suggests that the failure to achieve complete inactivation in a single cycle reflects a reversible equilibrium between inactive Schiff base, i.e. covalently modified enzyme, and a non-covalent complex. 7. The re-inactivation reaction occurring on dilution was demonstrated directly and a first-order rate constant obtained ($0.048 min^{-1}$). This, in conjunction with an estimate of the forward rate constant for Schiff-base formation, obtained by approximate pseudo-first-order analysis of inactivation at varied modifier concentrations, gives a predicted minimum activity very close to that actually obtained in a single cycle of treatment. 8. The dissociation constant of the non-covalent complex is given by two methods as 0.90 and 1.59mM. 9. The results indicate that covalent modification with pyridoxal 5′-phosphate completely abolishes the activity of glutamate dehydrogenase.

Of the relatively small number of amino acid residues so far implicated in the function of bovine liver glutamate dehydrogenase (EC 1.4.1.3), lysine-126 has received the most attention. This residue is attacked with high specificity by 4-iodoacetamidosalicylic acid (Baker *et al.*, 1962; Malcolm & Radda, 1968, 1970; Holbrook *et al.*, 1973; Wallis & Holbrook, 1973), acetylsulphamidophenylmaleimide (Holbrook & Jeckel, 1969), cyanate (Veronese *et al.*, 1972) and pyridoxal 5′-phosphate (Anderson *et al.*, 1966; Piszkiewicz *et al.*, 1970; Piszkiewicz & Smith, 1971*b*; Wallis & Holbrook, 1973; Brown *et al.*, 1973). If lysine-126 is essential for substrate binding or catalysis, its modification should abolish catalytic activity, but, surprisingly, this has never been directly demonstrated. Diminution of activity has been obtained frequently, but its total abolition has not been achieved even with irreversible modifiers. The assumption that fully modified enzyme is completely inactive has been based on the stoicheiometry of partial inactivation (Holbrook & Jeckel, 1969) and the observed competitive protection by substrates or coenzymes (Baker *et al.*, 1962; Malcolm & Radda, 1968; Wallis & Holbrook, 1973). Mere competition, however, does not necessarily indicate mutually exclusive binding (Reiner, 1959; Dixon & Webb, 1964). Widespread disagreement in the literature as to whether lysine-126 is required for binding of substrate or of coenzyme in any case precludes the unequivocal assignment of a role to this residue.

Goldin & Frieden (1972) have found that, even after prolonged incubation of glutamate dehydrogenase with pyridoxal 5′-phosphate and the apparent incorporation of one molecule of modifier per enzyme subunit, 10% of the initial activity remains. They suggest that lysine-126 may be 'important rather than essential in catalysis'. Partial inactivation could indeed result either from steric hindrance or a conformational effect. More recently Brown *et al.* (1973) have ascribed the residual activity to unmodified enzyme, but have not

explained how unmodified enzyme could persist in the presence of pyridoxal 5'-phosphate at a saturating concentration.

Against this background, it appeared important, as a prelude to an examination of the role of lysine-126, to establish clearly whether or not its modification abolishes catalytic activity. A study of the reaction with pyridoxal 5'-phosphate was undertaken with this objective.

Experimental

Materials

Coenzymes and bovine liver glutamate dehydrogenase [$(NH_4)_2SO_4$ suspension] were purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., and prepared and assayed as described by Engel & Dalziel (1969). Pyridoxal 5'-phosphate was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Its purity was tested by paper and cellulose t.l.c. in the dark with solvent systems described by Peterson & Sober (1954). Polygram thin-layer plates were obtained from Camlab, Cambridge, U.K. The chromatography revealed trace contamination with pyridoxal, but this was considered negligible, since the affinity of glutamate dehydrogenase for pyridoxal is much lower than for the phosphorylated compound (Anderson *et al.*, 1966; Piszkiewicz & Smith, 1971*a*). Glutamate and other chemicals were of the highest purity commercially available.

Inactivation experiments

Glutamate dehydrogenase (0.2mg/ml) was incubated at 25°C with various concentrations of pyridoxal 5'-phosphate in 0.1M-potassium phosphate buffer, pH7.0. The incubation mixtures were protected from light with metal foil. At timed intervals, samples (5μl) were withdrawn and assayed for catalytic activity.

Assays were carried out with a recording fluorimeter of the type described by Dalziel (1962). Except where otherwise noted, the reaction mixture (4 or 6ml) contained 10mM-glutamate and 60μM-NAD^+ in 0.111M-sodium phosphate buffer, pH7.0, at 25°C. Re-activation on dilution was negligible in the time (1min) taken for an assay; progress curves showed no sign of acceleration. The sensitive fluorimetric technique allowed sufficient dilution to ensure insignificant interference by excess of pyridoxal 5'-phosphate carried over with the assayed enzyme sample.

Re-activation experiments

Initial inactivation was performed as described above, except that the enzyme concentration was raised to 1mg/ml. When the inactivation process had reached equilibrium, 10μl of the incubation mixture was withdrawn and diluted 100-fold with 0.1M-potassium phosphate buffer, pH7.0. Samples (10–50μl) of the diluted solution were assayed at intervals as described above.

Two-cycle inactivation

Enzyme (2mg/ml) was inactivated in the manner described above. When the residual activity became constant, freshly prepared ice-cold 40mM-$NaBH_4$ was added. The volume added was in slight excess of that required to bleach the solution. After 1h at 4°C in the reduced state such samples were dialysed overnight against 0.1M-potassium phosphate buffer, pH7.0, and centrifuged to remove any particulate matter. The absorbance of the enzyme at 280nm was checked as an indication of protein concentration, and the sample was once again incubated with pyridoxal 5'-phosphate.

Stoicheiometry

Samples of enzyme that had been inactivated, reduced, dialysed and centrifuged as described above were used for determination of the extent of incorporation of pyridoxal 5'-phosphate. Spectra were recorded with a Cary model 14 scanning spectrophotometer. Calculations of the number of molecules of inactivator bound per enzyme subunit were based on an extinction coefficient of 1.07×10^4 litre$\cdot$mol$^{-1}\cdot$cm^{-1} at 327nm for the reduced Schiff-base adduct of pyridoxal 5'-phosphate and the ε-amino group of lysine (Fischer *et al.*, 1963) and 5.2×10^4 litre$\cdot$mol$^{-1}\cdot$cm^{-1} at 280nm for the enzyme (Egan & Dalziel, 1971), with the molecular weight of the enzyme subunit taken to be 56000. Anderson *et al.* (1966) showed that modification of glutamate dehydrogenase by pyridoxal 5'-phosphate, followed by reduction with borohydride, does not significantly alter the maximum absorption at 280nm. This was confirmed in the present work.

Theory and Results

Fig. 1 shows time-courses of inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate at various concentrations. In each incubation the catalytic activity decreased to an equilibrium value within 30–60min, the residual activity being determined by the pyridoxal 5'-phosphate concentration. Fig. 1 (inset) also shows that modifier concentrations above about 5mM produced no further decrease in activity. The lowest values obtained were 6–7% of the initial activity, in reasonable agreement with the results of Goldin & Frieden (1972).

The failure to achieve complete inactivation even after prolonged incubation with pyridoxal 5'-phosphate at saturating concentrations can be explained in two ways. Either the covalently modified enzyme is still active, albeit to a lesser extent than the native enzyme, as suggested by Goldin & Frieden (1972), or the enzyme is not all covalently modified. The second alternative is strongly supported by kinetic measurements made on the partially inactivated enzyme. Initial rates of reaction in 0.111 M-sodium phosphate, pH 7 (Engel & Dalziel, 1969), were measured with various glutamate concentrations (4–40 mM with 60 μM-NAD^+), and also with various NAD^+ concentrations (24–120 μM with 40 mM-glutamate). Under these conditions the apparent K_m values for glutamate (9.2 mM) and NAD^+ (38 μM) were identical for the native and the modified enzyme. The decreased activity is thus entirely attributable to a decrease in $V_{max.}$. This suggests a decrease in the amount of active enzyme present rather than a partial inactivation due to alteration of the properties of active enzyme. These findings and conclusions are entirely in accord with those of Brown *et al.* (1973), but it is now necessary to explain how active enzyme persists in the presence of high concentrations of pyridoxal 5'-phosphate.

A possible explanation emerges as a result of the findings of Piszkiewicz & Smith (1971*b*), who showed that formation of a kinetically significant non-covalent enzyme–pyridoxal 5'-phosphate complex precedes the covalent reaction (Scheme 1). In their analysis, however, the second step was treated as being irreversible ($k_2 \gg k_{-2}$). The existence of a measurable reverse action was established by Anderson *et al.* (1966), who demonstrated full recovery of activity after dialysis. A significant rate of breakdown of the Schiff base is also implicit in the mere fact (Fig. 1) that different amounts of residual activity are obtained with different modifier concentrations; if k_{-2} were not significant by comparison with k_2 all the inactivation curves should lead to the same residual activity.

If the rate constant k_{-2} is included in the analysis (Scheme 1), it is apparent that saturation with pyridoxal 5'-phosphate must distribute the enzyme between the non-covalent complex and the Schiff base according to the ratio k_{-2}/k_{+2}. If it is assumed that the non-covalent complex dissociates immediately on dilution and is therefore measured as active enzyme, whereas the Schiff base dissociates so slowly that it behaves as inactive enzyme in an assay, it then follows that the assayable activity cannot be decreased below

$$\frac{k_{-2}}{k_{+2}+k_{-2}} \times \text{initial activity}$$

even with very high concentrations of the inactivator. Anderson *et al.* (1966) showed that $NaBH_4$, by reducing the Schiff base, rendered inactivation by pyridoxal 5'-phosphate irreversible. This enabled us to test the two alternative hypotheses outlined above by exposing the enzyme to two cycles of treatment with the inactivator. In such an

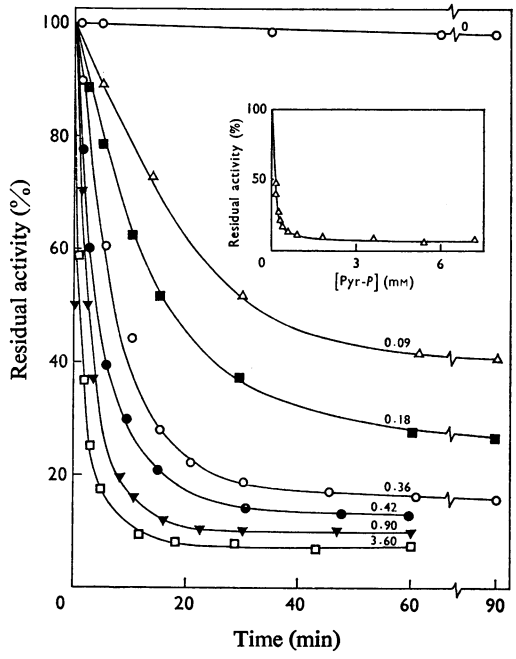

Fig. 1. *Time-courses of inactivation of glutamate dehydrogenase* (0.18–0.20 *mg/ml*) *with various pyridoxal 5'-phosphate concentrations*

Reaction was performed at 25°C in 0.1 M-potassium phosphate buffer, pH 7.0. Values on curves are [Pyr-*P*] (mM).

$$\text{E + pyridoxal 5'-phosphate} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{E–pyridoxal 5'-phosphate} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{E'–pyridoxal 5'-phosphate}$$

Free enzyme — Non-covalent complex — Schiff base (covalently modified enzyme)

Scheme 1. *Two-stage mechanism for reversible inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate*

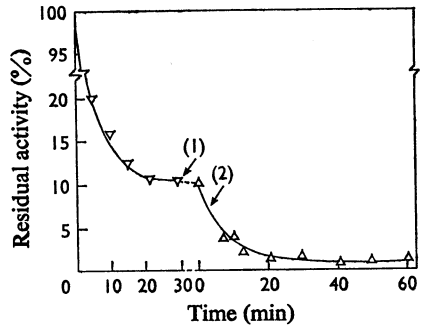

Fig. 2. *Repeated treatment of glutamate dehydrogenase with pyridoxal 5'-phosphate*

Glutamate dehydrogenase (2mg/ml) was incubated with 1.8mM-pyridoxal 5'-phosphate for 30min and then reduced by $NaBH_4$ and dialysed overnight against 0.1M-potassium phosphate buffer, pH7.0. The reduced sample was then treated again with pyridoxal 5'-phosphate. Arrow (1), $NaBH_4$ reduction and dialysis; arrow (2), re-inactivation curve.

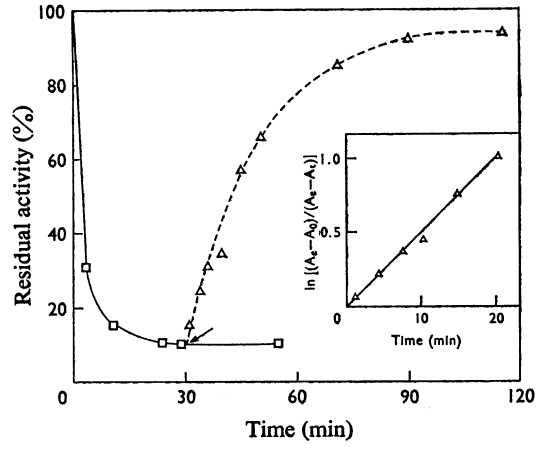

Fig. 3. *Re-activation of glutamate dehydrogenase by dilution after inactivation by pyridoxal 5'-phosphate*

Glutamate dehydrogenase (1mg/ml) was incubated with 0.9mM-pyridoxal 5'-phosphate (□). After 30min 10μl of incubation mixture was withdrawn and diluted 100-fold into 0.1M-phosphate buffer, pH7.0. Activity was assayed at time-intervals (△) from the time indicated by the arrow. The inset shows a first-order plot of the re-activation process. The slope gives an estimate of k_{-2}. For definition of A_e, A_0, A_t see the text.

experiment (Fig. 2), the first cycle of treatment with 1.8mM-pyridoxal 5'-phosphate left 10% residual activity at equilibrium. Borohydride immediately bleached the solution by reducing the excess of pyridoxal 5'-phosphate. The activity of the enzyme after dialysis was still 10% of its original value. A second cycle of treatment under identical conditions decreased the activity from 10% to approx. 1%, i.e. 10% of 10%. Treatment with borohydride in the absence of pyridoxal 5'-phosphate caused no appreciable loss of enzyme activity.

This experiment clearly demonstrates that the residual activity after the first cycle reflects the existence of an equilibrium between the inactive form of the enzyme, i.e. the Schiff base, and other forms that appear active in the catalytic assay. The second cycle decreases activity to a value well below the minimum of 6–7% obtained at the highest pyridoxal 5'-phosphate concentrations in the first cycle. A third cycle carried out as above would presumably leave 0.1% residual activity, if that were measurable.

From absorption measurements at 327nm it was determined that enzyme samples with 10% residual activity after one cycle of inactivation contained 0.85–1.10 molecules of pyridoxal 5'-phosphate, bound as a reduced Schiff base, per enzyme subunit. Two separate experiments indicated incorporation of 1.21 and 1.22 molecules per subunit after the second cycle. This indicates that non-specific modification of residues other than lysine-126 occurs to a small extent. In similar experiments, but at pH8, Goldin & Frieden (1972) found somewhat higher incorporation.

These experiments indicate that covalent modification of a single residue, lysine-126, in glutamate dehydrogenase does, after all, completely abolish activity. The prediction that $k_{-2} \ll k_{+2}$ may be directly tested by a study of re-activation by dilution. Fig. 3 shows that a sample of glutamate dehydrogenase, inactivated to the extent of 90% by incubation with 0.9mM-pyridoxal 5'-phosphate, gradually regained 92% of its initial activity on being diluted 100-fold into 0.1M-sodium phosphate buffer, pH7.0. The wide usable sensitivity range of the fluorimeter allowed assay of both the concentrated and diluted enzyme samples without difficulty.

Since the experimental indications are that $k_{-1} \gg k_{+2} > k_{-2}$ (Scheme 1) it is likely that, in the course of re-activation, slow breakdown of the Schiff base is followed by very rapid dissociation of pyridoxal 5'-phosphate from the non-covalent complex. If so, the re-activation may be expected initially to exhibit first-order kinetics with a rate limited by the value of k_{-2}. It must inevitably depart from such first-order kinetics as the reaction between free enzyme and pyridoxal 5'-phosphate becomes once again significant, but, since this is a second-order step, its maximum possible rate is decreased by a factor of 1×10^4 in the diluted solution.

First-order analysis of the results in Fig. 3 indeed gave a linear plot for the first few minutes (inset), and thus yielded an estimate of $0.048\,min^{-1}$ for k_{-2} from the slope. The validity of the analysis was checked by repeating the experiment with a different dilution (150-fold) and, therefore, different final pyridoxal 5'-phosphate concentrations. A similar value ($0.049\,min^{-1}$) of k_{-2} was obtained.

If the 'equilibrium' hypothesis represents a valid interpretation of the results, then the minimum attainable activity in a single cycle provides an estimate of $k_{-2}/(k_{+2}+k_{-2})$. Since an estimate of k_{-2} is available, an independent estimation of k_{+2} would provide a further test of the hypothesis. Pseudo-first-order analysis of time-courses of inactivation was therefore attempted.

There are two possible approaches to the first-order analysis of reversible reaction; the rigorous approach involves calculating the extent of progress towards equilibrium (Frost & Pearson, 1961); $\ln(A_0-A_e)/(A_t-A_e)$ is plotted against time, where A_0, A_e and A_t are respectively the concentration of starting material at time 0, at equilibrium (time $= \infty$) and at time t. The slope of the linear plot gives an effective rate constant equal to the sum of the two opposing rate constants. An alternative approach, an approximation, may be used (Glasstone, 1948) if the equilibrium lies far over to one side and/or if it is possible to make several reliable experimental measurements during the early course of reaction, before the reverse rate becomes significant. This approximation consists of analysing the approach to completion (100% conversion) rather than to equilibrium. One plots $\ln(A_0/A_t)$ against time, with A_0 and A_t defined as above. This approach has been described, for instance, by van't Hoff (1898), as used in the analysis of reversible ester hydrolysis. In contrast with the first method, for which all time-points should in theory lie on a straight line, for the second method one expects systematic deviation from the limiting straight line as the reverse reaction becomes more significant.

The advantage of the second method, if the conditions for the approximation are met, is that it gives an estimate of the forward rate constant independent of the estimate of the opposing constant. Since we wished to prove the existence of an equilibrium rather than to assume it, the second method appeared preferable. We established by computer simulation that, under the conditions of our experiments, one might indeed expect to obtain an estimate of the forward constant within a few per cent of the true value.

Fig. 4 shows plots of $\ln(A_0/A_t)$ against time for various concentrations of inactivator. As predicted these are initially linear, but deviate as reaction progresses.

Since, in Scheme 1, the slow first-order interconversion is preceded by a rapid equilibration with pyridoxal 5'-phosphate, the observed forward rate

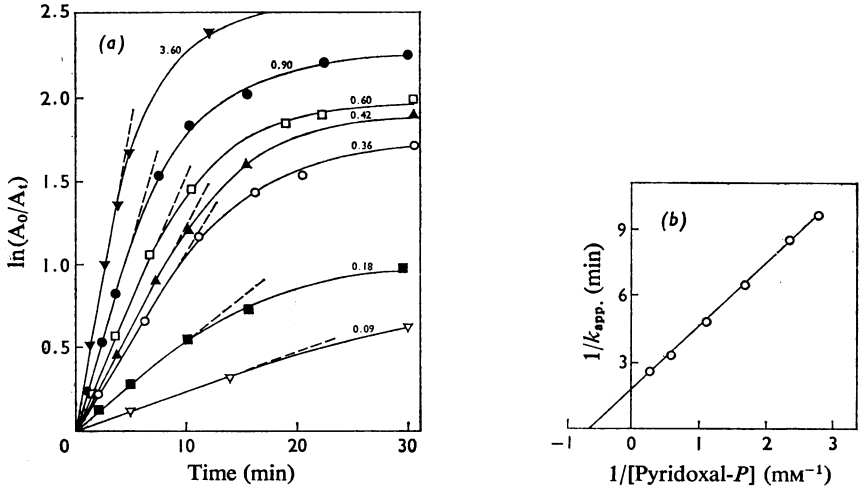

Fig. 4. *Pseudo-first-order kinetic analysis of inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate*

(*a*) Pseudo-first-order plots of inactivation of glutamate dehydrogenase with various pyridoxal 5-'phosphate concentrations (mM concentrations as indicated on curves). Conditions as in Fig. 1. (*b*) Double-reciprocal plot of pseudo-first-order rate constants for inactivation of glutamate dehydrogenase against pyridoxal 5'-phosphate concentration. The plotted values are from Fig. 4(*a*).

constant for inactivation is an apparent constant; Piszkiewicz & Smith (1971*b*) showed by applying the Michaelis & Menten (1913) assumption that:

$$\frac{1}{k_{app.}} = \frac{1}{[\text{pyridoxal 5'-phosphate}]}\frac{k_{-1}}{k_{+1}k_{+2}} + \frac{1}{k_{+2}} \quad (1)$$

This predicts that a plot of $1/k_{app.}$ against 1/[pyridoxal 5'-phosphate], the equivalent of a Lineweaver–Burk plot, should be linear with an ordinate intercept of $1/k_{+2}$ and a slope of $k_{-1}/k_{+1}k_{+2}$.

Values of $k_{app.}$ were obtained from the slopes of the lines in Fig. 4(*a*) and replotted as a reciprocal plot (Fig. 4*b*). This plot is linear, as predicted, and the estimates of k_{+2} and k_{-1}/k_{+1} are 0.588 min^{-1} and 1.59 mM respectively.

The estimate of k_{+2} can now be combined with the independent estimate of k_{-2} from the reactivation experiment to give:

$$\frac{k_{-2}}{k_{+2}+k_{-2}} = 0.074$$

This predicts, therefore, that a single cycle of treatment with a saturating concentration of pyridoxal 5'-phosphate should decrease activity to 7.4% of its initial value. This corresponds closely to the minimum values actually measured (Fig. 1).

The analysis up to this point makes no use of the extent of catalytic activity reached at equilibrium with sub-saturating concentrations of pyridoxal 5'-phosphate. These residual activities are determinable with great accuracy, since they are stable, and are amenable to analysis as follows. With Scheme 1 as a starting point, it may be calculated that:

$$e = \mathrm{E}\left\{1+\frac{k_{+1}}{k_{-1}}\left(1+\frac{k_{+2}}{k_{-2}}\right)[\text{pyridoxal 5'-phosphate}]\right\}$$

where e and E are the concentrations of total and free enzyme respectively. If it is assumed that the Schiff base (Scheme 1) is totally inactive, and that the non-covalent complex dissociates very rapidly and completely on dilution into an assay mixture, the fractional residual activity, R, at equilibrium is given by:

$$R = \frac{1+\frac{k_{+1}}{k_{-1}}[\text{pyridoxal 5'-phosphate}]}{1+\frac{k_{+1}}{k_{-1}}[\text{pyridoxal 5'phosphate}]\left(1+\frac{k_{+2}}{k_{-2}}\right)} \quad (2)$$

Hence, on re-arrangement:

$$\frac{1}{[\text{pyridoxal 5'-phosphate}]} = \frac{k_{+1}k_{+2}}{k_{-1}k_{-2}}\left(\frac{1}{1-R}\right) - \left(1+\frac{k_{+2}}{k_{-2}}\right)\frac{k_{+1}}{k_{-1}} \quad (3)$$

A plot of 1/[pyridoxal 5'-phosphate] against $1/(1-R)$ should therefore give a straight line with a slope of $k_{+1}k_{+2}/k_{-1}k_{-2}$ and an ordinate intercept of

$$-(1 + k_{+2}/k_{-2})(k_{+1}/k_{-1}).$$

The intercept on the abscissa, $1+(k_{-2}/k_{+2})$, is the reciprocal of the maximum extrapolated inactivation, corresponding to infinite modifier concentration. Fig. 5 shows a plot of 1/[pyridoxal 5'-phosphate] against $1/(1-R)$ for the experiments shown in Fig. 1 and other similar ones. It is indeed a good straight line. It should be noted, however, that such linearity is not in itself conclusive evidence for the validity of Scheme 1. McKinley-McKee & Morris (1972) show a similar plot for liver alcohol dehydrogenase, but have based their derivation on different premises.

The abscissa intercept in Fig. 5 is of course in agreement with the minimum activity of 6–7% observed in Fig. 1, since it merely presents the same result in a different manner. What is somewhat surprising, however, is that the slope, 1.74×10^4 M^{-1}, when combined with the estimate of k_{+2}/k_{-2} from the abscissa intercept, yields a value of 0.90 mM for k_{-1}/k_{+1}, the dissociation constant for the non-covalent complex. This is more than 40% lower than the value estimated from the pseudo-first-order analysis of inactivation. We have no explanation for this discrepancy except that it may be inherent in the relative inaccuracy of the kinetic analysis as compared with the measurement of static equilibrium activity. Pseudo-first-order inactivation plots according to the more rigorous (Frost & Pearson, 1961) method for a reversible reaction were linear for longer than the plots

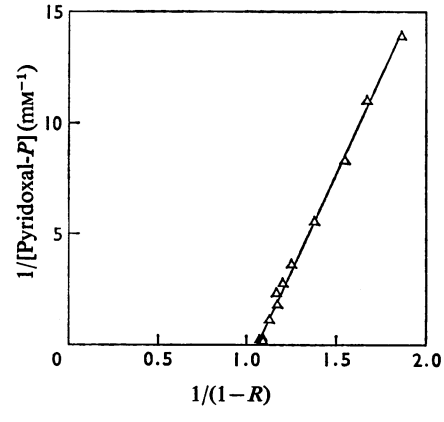

Fig. 5. *Double-reciprocal plots of fractional inactivation against pyridoxal 5'-phosphate concentration*

For significance of the plot see the text.

shown in Fig. 4(*a*), but still showed some tendency to curve. This might reflect a small systematic error, possibly in the determination of k_{-2}.

Equilibrium calculations provide one further check on internal consistency and the absence of significant irreversible changes additional or secondary to the reactions of Scheme 1. Eqn. (2) allows the calculation of activity at equilibrium after re-activation as well as inactivation. Thus for the experiment shown in Fig. 5, the residual pyridoxal 5′-phosphate concentration after dilution was 9μM. Eqn. (2) predicts that re-activation should restore the activity to 91% of its initial value. The observed value was 92%.

Discussion

In the present study we have confirmed the finding (Piszkiewicz & Smith, 1971*b*) that pseudo-first-order analysis of rates of inactivation of glutamate dehydrogenase by pyridoxal 5′-phosphate gives results consistent with a two-stage inactivation, in which initial, rapid, reversible binding is followed by much slower covalent-bond formation, also reversible (Scheme 1). It is noteworthy that the process shows no signs of subunit interactions.

We have also confirmed the observation by Goldin & Frieden (1972) that some activity remains even after prolonged incubation of glutamate dehydrogenase with pyridoxal 5′-phosphate at saturating concentrations. Asymmetric reaction of the two halves of the enzyme hexamer, as found with trinitrobenzenesulphonate (Coffee *et al.*, 1971) and fluorodinitrobenzene (di Prisco, 1971), might have accounted for such residual activity, but measurement of the number of molecules of pyridoxal 5′-phosphate per subunit, combined with the known specificity of the reagent for lysine-126 (Piszkiewicz *et al.*, 1970), seem to preclude this possibility. In agreement with other workers (Anderson *et al.*, 1966; Piszkiewicz *et al.*, 1970; Goldin & Frieden, 1972) we find after one cycle of inactivation 0.85–1.1 mol of pyridoxal 5′-phosphate incorporated/mol of enzyme subunits.

Another possibility, suggested by Goldin & Frieden (1972), is that full covalent modification of lysine-126 in all subunits fails to abolish activity. This has now been ruled out. It is clear that a single treatment with pyridoxal 5′-phosphate can never produce complete covalent modification of lysine-126, because the second step in Scheme 1, the intramolecular conversion of a Michaelis complex into a Schiff base, is much more readily reversible than previously appreciated. The rate constant, k_{-2}, for the hydrolysis of the Schiff base is not negligible in comparison with k_{+2}, the opposing rate constant. This has been shown in two ways: (i) by direct estimation of k_{-2} from an analysis, hitherto unattempted, of the rate of re-activation by dilution; (ii) by the demonstration that removal of the Schiff base from the equilibrium by reduction with borohydride renders the remaining active enzyme susceptible to further inactivation by a second treatment with pyridoxal 5′-phosphate.

In the light of this experimental evidence, there appears now to be little doubt that the Schiff base is totally inactive and that the integrity of lysine-126 is essential for catalytic activity. This confirms the conclusions of Holbrook & Jeckel (1969) and Holbrook *et al.* (1973) based on the stoicheiometry of inactivation with irreversible reagents.

It might be thought surprising that the equilibrium does not lie further towards the Schiff base. Malcolm (1971) measured the equilibrium constants for formation of a Schiff base between pyridoxal 5′-phosphate and various aliphatic and alicyclic amines in free solution. The value for *n*-butylamine, which seems most closely comparable with lysine, is 384M^{-1}. The equilibrium constant for the first-order interconversion of E–[pyridoxal 5′-phosphate] and E′–[pyridoxal 5′-phosphate] determined in the present study (k_{+2}/k_{-2}) is only 16. It could be argued therefore that the effective concentration of bound pyridoxal 5′-phosphate in relation to lysine-126 must be of the order of 0.04M. Such a low value suggests that in the Michaelis complex the modifier may not be in a favourable orientation for reaction with lysine-126. It is conceivable, for instance, that pyridoxal 5′-phosphate binds at the periphery of the active site, and that the mobile side chain of lysine-126 may only react with it when it swings in the right direction. The strength with which pyridoxal 5′-phosphate is initially bound to the enzyme is largely determined by the presence of the phosphate moiety, which presumably interacts with a group of opposite charge on the enzyme surface; pyridoxal, which lacks the phosphate, inactivates glutamate dehydrogenase without formation of a kinetically detectable non-covalent complex (Piszkiewicz & Smith, 1971*a*). On the basis of studies of the NADP-linked glutamate dehydrogenase from *Neurospora crassa*, Gore *et al.* (1973) have suggested that the rate of inactivation by pyridoxal 5′-phosphate at saturating concentrations may be limited by the rate of interconversion of two conformational states of the enzyme. The first-order rate constant for the conformational transition, detected by changes in protein fluorescence, corresponds closely to the extrapolated first-order constant for inactivation by pyridoxal 5′-phosphate (0.12min^{-1} at 21°C and pH 7.6). Clearly a process of this type could provide an alternative explanation for the low value of k_{+2}/k_{-2} in the inactivation of the bovine enzyme.

It remains to be established how blockage of lysine-126 abolishes the catalytic activity of glutamate dehydrogenase. An allosteric effect cannot be entirely excluded, but it seems more probable that lysine-126 is in the vicinity of the active site. The modification must in any case prevent either the binding of substrate(s) or successful subsequent catalysis.

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