Biochem. J. (1975) **149**, 627–635
Printed in Great Britain

Horse Liver Alcohol Dehydrogenase

A STUDY OF THE ESSENTIAL LYSINE RESIDUE

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

(*Received* 27 *January* 1975)

1. The inactivation of horse liver alcohol dehydrogenase by pyridoxal 5′-phosphate in phosphate buffer, pH8, at 10°C was investigated. Activity declines to a minimum value determined by the pyridoxal 5′-phosphate concentration. The maximum inactivation in a single treatment is 75%. This limit appears to be set by the ratio of the first-order rate constants for interconversion of inactive covalently modified enzyme and a readily dissociable non-covalent enzyme–modifier complex. 2. Reactivation was virtually complete on 150-fold dilution: first-order analysis yielded an estimate of the rate constant ($0.164\,min^{-1}$), which was then used in the kinetic analysis of the forward inactivation reaction. This provided estimates for the rate constant for conversion of non-covalent complex into inactive enzyme ($0.465\,min^{-1}$) and the dissociation constant of the non-covalent complex (2.8mM). From the two first-order constants, the minimum attainable activity in a single cycle of treatment may be calculated as 24.5%, very close to the observed value. 3. Successive cycles of modification followed by reduction with $NaBH_4$ each decreased activity by the same fraction, so that three cycles with 3.6mM-pyridoxal 5′-phosphate decreased specific activity to about 1% of the original value. The absorption spectrum of the enzyme thus treated indicated incorporation of 2–3mol of pyridoxal 5′-phosphate per mol of subunit, covalently bonded to lysine residues. 4. NAD^+ and NADH protected the enzyme completely against inactivation by pyridoxal 5′-phosphate, but ethanol and acetaldehyde were without effect. 5. Pyridoxal 5′-phosphate used as an inhibitor in steady-state experiments, rather than as an inactivator, was non-competitive with respect to both NADH and acetaldehyde. 6. The partially modified enzyme (74% inactive) showed unaltered apparent K_m values for NAD^+ and ethanol, indicating that modified enzyme is completely inactive, and that the residual activity is due to enzyme that has not been covalently modified. 7. Activation by methylation with formaldehyde was confirmed, but this treatment does not prevent subsequent inactivation with pyridoxal 5′-phosphate. Presumably different lysine residues are involved. 8. It is likely that the essential lysine residue modified by pyridoxal 5′-phosphate is involved either in binding the coenzymes or in the catalytic step. 9. Less detailed studies of yeast alcohol dehydrogenase suggest that this enzyme also possesses an essential lysine residue.

Pyridoxal 5′-phosphate has become a popular reagent for the selective modification of lysine residues (Means & Feeney, 1971), and many enzymes are reversibly inactivated by this reagent (see, e.g., references in Chen & Engel, 1975*b*). A universal feature, however, is the persistence of a small fraction (5–25%) of the activity, even after prolonged incubation with pyridoxal 5′-phosphate at saturating concentrations. Various explanations have been advanced. Thus Goldin & Frieden (1972) conclude that modification of the reactive residue, lysine-126, in bovine glutamate dehydrogenase (EC 1.4.1.3) decreases but does not abolish catalytic activity. Johnson & Deal (1970), discussing the residual activity of pyruvate kinase (EC 2.7.1.40) treated with pyridoxal 5′-phosphate, propose that unfolding unmasks non-essential residues which then compete with the remaining essential residues for the reagent. McKinley-McKee & Morris (1972) suggest that in horse liver alcohol dehydrogenase (EC 1.1.1.1) there may be two mutually exclusive sites for attack by pyridoxal 5′-phosphate.

For glutamate dehydrogenase, recent evidence (Chen & Engel, 1975*b*) suggests a simpler explanation of the residual activity. In the presence of pyridoxal 5′-phosphate at saturating concentrations there appears to be an equilibrium between Schiff base, only slowly dissociable and therefore apparently inactive, and a non-covalent complex which is rapidly dissociable and therefore active in the normal catalytic assay. In the present paper we have sought to establish whether the reaction between horse liver alcohol dehydrogenase and pyridoxal 5′-phosphate follows a similar mechanism to that proposed for glutamate dehydrogenase.

Experimental

Horse liver alcohol dehydrogenase was purchased as a crystalline suspension from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., and prepared for use as described by McKinley-McKee & Morris (1972). Enzyme concentration was determined by the method of Theorell & McKinley-McKee (1961). When its purity was checked by starch-gel electrophoresis, the enzyme was found to consist mainly of isoenzyme EE, with trace amounts of isoenzymes EE′, EE″ and ES′ (Pietruszko & Theorell, 1969). Catalytic activity was measured fluorimetrically (Dalziel, 1962) with a routine assay mixture containing 8.5mM-ethanol and 85μM-NAD^+ in 0.05M-potassium phosphate buffer, pH8 at 25°C, unless otherwise specified.

Most of the other procedures were similar to those described previously (Chen & Engel, 1975*b*). The rapidity of inactivation of alcohol dehydrogenase by pyridoxal 5′-phosphate at 25°C, however, hindered precise kinetic analysis. For most experiments, therefore, the enzyme was incubated with pyridoxal 5′-phosphate at 10°C. The micro-pipettes used for sampling were also cooled to 10°C to minimize warming during transfer to the assay mixture. A high recorder-chart speed [30cm (12in)/min] was also used to minimize reactivation occurring during assay.

Methylation of horse liver alcohol dehydrogenase with formaldehyde and $NaBH_4$ was attempted by the methods of Jörnvall (1973*a*) and Tsai *et al.* (1974). In experiments according to the latter method, $NaBH_4$ (2.5mg) was added to the enzyme (1mg/ml) in 1ml of 0.05M-sodium pyrophosphate buffer, pH9. Additions (10μl) of 9.25% (v/v) formaldehyde in the same buffer were made every 10min for 1h at 4°C. After the final addition of formaldehyde, the reaction mixture was kept in ice for 30min before being dialysed extensively. The control was treated in the same way except that formaldehyde was omitted. In the alternative procedure (Jörnvall, 1973*a*) enzyme (1mg/ml) was incubated at 4°C with 25mM-formaldehyde in 0.05M-sodium pyrophosphate buffer, pH8.5. After 30s, $NaBH_4$ was added and the mixture was kept cold for another 30min. After extensive dialysis, the enzyme activity was assayed. The methylated enzyme was treated with pyridoxal 5′-phosphate in the same way as the native enzyme.

Results

Fig. 1 shows that incubation of horse liver alcohol dehydrogenase with pyridoxal 5′-phosphate in phosphate buffer, pH8, at 10°C caused gradual loss of catalytic activity. A steady residual activity was reached within about 20min. The attainable inactivation increased with the concentration of pyridoxal 5′-phosphate to a maximum achieved with 1mM-modifier (Fig. 1, inset). With this concentration of pyridoxal 5′-phosphate the residual activity was 25–26% of the initial value. These results are qualitatively similar to those obtained by McKinley-McKee & Morris (1972), although the inactivation proceeded much faster at the higher temperature used in their study.

A reversible two-stage reaction for pyridoxal 5′-phosphate and enzyme (Scheme 1), as reported previously for glutamate dehydrogenase (Piszkiewicz & Smith, 1971; Chen & Engel, 1975*b*), is taken as a working model for the reaction with horse liver alcohol dehydrogenase. The evidence of McKinley-McKee & Morris (1972) suggests that the inactivation involves formation of a Schiff base, and it is provisionally assumed in what follows that a Schiff base is the end product of the two-stage reaction. Since pyridoxal 5′-phosphate was present in all cases in great molar excess over the enzyme, it was possible to attempt a pseudo-first-order analysis of the time-course of inactivation. Since the minimum residual activity obtained was approx. 25%, the ratio k_2/k_{-2} (Scheme 1) is approx. 3 (i.e. 75/25), in contrast with the situation for glutamate dehydrogenase, for which this ratio is 12–13 (Chen & Engel, 1975*b*). In the latter case, it was possible to adopt an 'initial-rate' approach by

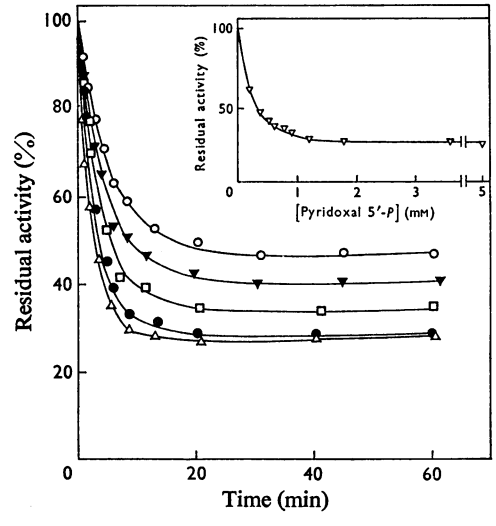

Fig. 1. *Time-courses of inactivation of horse liver alcohol dehydrogenase by pyridoxal 5′-phosphate*

Alcohol dehydrogenase (0.26mg/ml) was incubated at 10°C in 0.05M-potassium phosphate buffer, pH8, with pyridoxal 5′-phosphate at the following concentrations: 0.36mM (○), 0.50mM (▼), 0.90mM (□), 1.2mM (●), 3.6mM (△). Samples were withdrawn for assay at intervals. The residual activity at equilibrium in such incubations is plotted against pyridoxal 5′-phosphate concentration in the inset.

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

Scheme 1. *Proposed two-step mechanism for reaction of pyridoxal 5'-phosphate with horse liver alcohol dehydrogenase and bovine glutamate dehydrogenase*

making sufficient measurements in the early part of the time-course, before the back reaction became significant. With the much more evenly poised equilibrium shown in Fig. 1, this simplified analysis is not satisfactory, and the reversible first-order reaction must be treated as such. For a reversible reaction:

$$A \underset{k'}{\overset{k}{\rightleftharpoons}} B$$

it is readily shown (e.g. Frost & Pearson, 1961) that

$$-dA_t/dt = (k+k')-k'A_0 \quad (1)$$

where A_0 and A_t are the concentrations of A at time 0 and time t, and B is assumed to be initially absent. By integrating eqn. (1) and introducing A_e, the concentration of A at equilibrium, eqn. (2) may be derived:

$$\ln\left[\frac{A_0-A_e}{A_t-A_e}\right] = (k+k')t \quad (2)$$

Thus the process obeying apparent first-order kinetics is the approach to equilibrium, characterized by the effective rate constant $(k+k')$, rather than the approach to 100% conversion with a rate constant k, as for an effectively irreversible reaction.

In Scheme 1, the assumption is made that the initial formation of the intermediate complex and its dissociation are much faster than the first-order interconversion of intermediate complex and Schiff base (Kitz & Wilson, 1962; Piszkiewicz & Smith, 1971; Chen & Engel, 1975*b*). Eqn. (2) can therefore be modified to deal with the reaction under present consideration as shown in eqn. (3):

$$\ln\left[\frac{A_0-A_e}{A_t-A_e}\right] = k_{app.}t = \left[\left(\frac{k_2[\text{pyridoxal 5'-phosphate}]}{(k_{-1}/k_{+1})+[\text{pyridoxal 5'-phosphate}]}\right)+k_{-2}\right]t \quad (3)$$

where A_0, A_t and A_e represent the enzyme activity initially, at time t, and at equilibrium respectively, and $k_{app.}$ is the value of the apparent pseudo-first-order rate constant for any given concentration of pyridoxal 5'-phosphate. In eqn. (4) the expression for $k_{app.}$ is rearranged in a linear form:

$$\frac{1}{k_{app.}-k_{-2}} = \frac{1}{k_{+2}}\left[1+\frac{k_{-1}}{k_{+1}[\text{pyridoxal 5'-phosphate}]}\right] \quad (4)$$

Thus in contrast with cases in which the breakdown of the Schiff base is negligible during the early stages of inactivation, it is not possible here to evaluate k_{+2} and k_{-1}/k_{+1} merely by plotting $1/k_{app.}$ against [pyridoxal 5'-phosphate]$^{-1}$. In the present case, k_{-2} must be first evaluated, and then $1/(k_{app.}-k_{-2})$ may be plotted against [pyridoxal 5'-phosphate]$^{-1}$.

Fortunately direct estimation of k_{-2} is possible. Preliminary experiments showed that activity could be fully restored by either dialysis or extensive dilution, and indeed the progress curves in assays of partially inactive enzyme showed some tendency to curve upwards. An attempt was therefore made to estimate k_{-2} by first-order analysis of the time-course of reactivation. Dilution displaces the equilibrium between the intermediate complex and free enzyme (Scheme 1). If, as seems likely, $k_{-1} \gg k_{+2}$, the initial phase of reactivation is rate-limited by the conversion of Schiff base into intermediate complex governed by the rate constant k_{-2}. The experiment proved less easy than the equivalent procedure for glutamate dehydrogenase (Chen & Engel, 1975*b*), because, even at 10°C, the reactivation of horse liver alcohol dehydrogenase is much faster than that of glutamate dehydrogenase at 25°C. Fig. 2 shows the cumulative results of three separate experiments. The first-order plot (Fig. 2 inset) shows some scatter but nevertheless allows estimation of k_{-2} (0.164 min^{-1}) from the slope. This estimate was then used in the kinetic analysis of the inactivation process.

Fig. 3 shows plots according to eqn. (2) of $\ln[(A_0-A_e)/(A_t-A_e)]$ against time. Most are reasonably good straight lines, although they inevitably show some scatter as equilibrium is approached. The slopes of these lines give a value of $k_{app.}$ for each concentration of pyridoxal 5'-phosphate. Fig. 4 is a plot, based on eqn. (4), of $1/(k_{app.}-k_{-2})$ against [pyridoxal 5'-phosphate]$^{-1}$. This plot is linear for high modifier concentrations but shows significant deviation from linearity at low pyridoxal 5'-phosphate concentrations, where k_{-2} becomes dominant; possibly this indicates inaccuracy in the estimation of k_{-2}. The line in Fig. 4 clearly does not pass through the origin, indicating that there is a kinetically significant enzyme–modifier complex formed before inactivation. Estimates of k_{-1}/k_{+1} (2.8×10^{-3} M) and k_{+2} (0.465 min^{-1}) were obtained from Fig. 4, but their accuracy, especially that of the estimate of k_{-1}/k_{+1}, depends on the reliability of the value for k_{-2}.

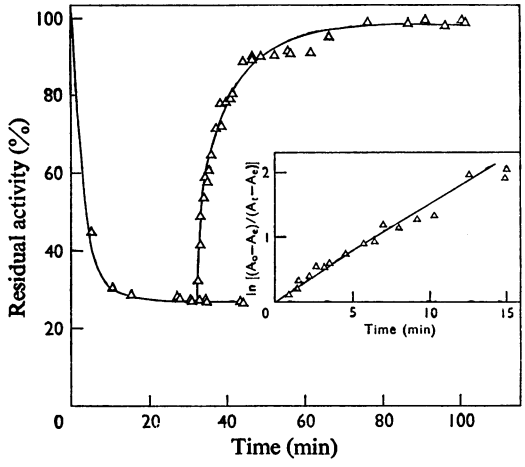

Fig. 2. *Reactivation of horse liver alcohol dehydrogenase by dilution after inactivation by pyridoxal 5'-phosphate*

Alcohol dehydrogenase (1 mg/ml) was treated with 1.8 mM-pyridoxal 5'-phosphate under the conditions of Fig. 1. After 30 min, the mixture was diluted 150-fold with 0.05 M-potassium phosphate buffer, pH 8, and assayed at intervals. The plot shows the combined results of three such experiments. The inset shows a first-order plot of the reactivation process.

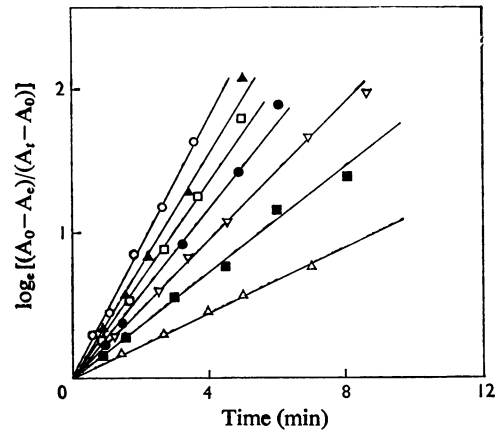

Fig. 3. *Pseudo-first-order analysis of the inactivation of liver alcohol dehydrogenase by pyridoxal 5'-phosphate*

Conditions were as in Fig. 1. For details of the method of analysis see the text. The pyridoxal 5'-phosphate concentrations were 0.18 mM (△), 0.36 mM (■), 0.60 mM (▽), 1.2 mM (●), 1.8 mM (□), 3.6 mM (▲), 5.0 mM (○).

According to Scheme 1, the minimum residual activity attainable in the presence of saturating concentrations of pyridoxal 5'-phosphate should be (initial activity) $\times k_{-2}/(k_{+2}+k_{-2})$, reflecting the fraction of readily dissociable, intermediate complex in equilibrium with the inactive Schiff base. The value obtained from the estimates of k_{+2} and k_{-2} is 24.5%, in close agreement with the measured minimum, 25–26% (Fig. 1).

The analysis of rates of inactivation and reactivation may be compared with an equilibrium analysis (Chen & Engel, 1975*b*) of the extents of inactivation achieved with various concentrations of pyridoxal 5'-phosphate. The activity at equilibrium is given by eqn. (5):

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

where X is the fractional activity of the Schiff base, here assumed to be zero, and R is the fractional residual activity at equilibrium in the presence of a given concentration of pyridoxal 5'-phosphate. A plot of [pyridoxal 5'-phosphate]$^{-1}$ against $1/(R-1)$ should be linear, with a slope of $k_{+1}k_{+2}/k_{-1}k_{-2}$ and an abscissa intercept of $1+k_{-2}/k_{+2}$. The reciprocal of this abscissa intercept gives the maximum achievable fractional inactivation. McKinley-McKee & Morris (1972) have also used this plot, basing it, however, on different premises. In confirmation of their finding, Fig. 5 shows a linear plot of our results.

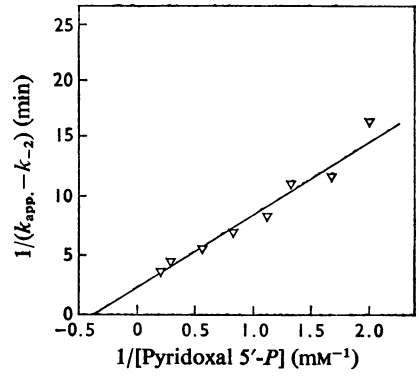

Fig. 4. *Estimation of the true first-order rate constant for covalent inactivation of horse liver alcohol dehydrogenase by pyridoxal 5'-phosphate*

$(k_{app.}-k_{-2})^{-1}$ is plotted against the reciprocal of the concentration of pyridoxal 5'-phosphate. The estimate of k_{-2} is obtained from the slope of the line in Fig. 2. The estimates of $k_{app.}$, the apparent pseudo-first-order rate constant for inactivation corresponding to each concentration of pyridoxal 5'-phosphate, are obtained from the slopes of the lines in Fig. 3. For the detailed justification of this plot, see the text.

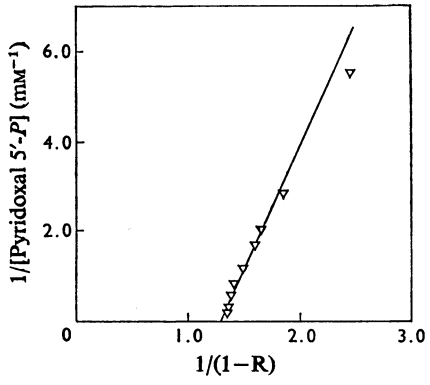

Fig. 5. *Equilibrium analysis of the inactivation of horse liver alcohol dehydrogenase by pyridoxal 5'-phosphate*

The plot shows the dependence of the residual activity, R, on the concentration of pyridoxal 5'-phosphate. R, which is expressed as a fraction of the original activity, was measured at equilibrium in incubations of alcohol dehydrogenase with pyridoxal 5'-phosphate under the conditions of Fig. 1. For the theoretical basis of this plot, see the text.

Table 1. *Effect on horse liver alcohol dehydrogenase of repeated cycles of treatment with pyridoxal 5'-phosphate*

Horse liver alcohol dehydrogenase (2.45mg/ml) was incubated at 10°C in 0.05M-potassium phosphate buffer, pH8, with 3.6mM-pyridoxal 5'-phosphate until the activity reached a steady value (35min) and was then reduced with freshly prepared ice-cold 40mM-$NaBH_4$. Specific activity was determined before and after dialysis against 0.05M-potassium phosphate buffer, pH8. The whole procedure was then repeated. The stoicheiometry of incorporation of pyridoxal 5'-phosphate at each stage was determined after reduction with $NaBH_4$ by measuring the E_{327} (Fischer *et al.*, 1963).

Number of cycles	Residual activity (%) Before reduction	Residual activity (%) After reduction	Pyridoxal 5'-phosphate incorporated (mol/mol of subunit)
1	25.5	25.8	2.05
2	6.4	6.0	2.50
3	0.9	0.9	3.00

From the slope and the intercept of this line, k_{+2}/k_{-2} may be determined as 3.4, and the dissociation constant for the intermediate complex, k_{-1}/k_{+1}, as 6×10^{-4}M. The considerable discrepancy between this value for the dissociation constant and the previous estimate may reflect the relative inaccuracy of the kinetic analysis.

From the estimated values of k_{-1}/k_{+1} and k_{+2}/k_{-2} it may be calculated that, under the conditions of the reactivation experiment (Fig. 2), 99% of the initial

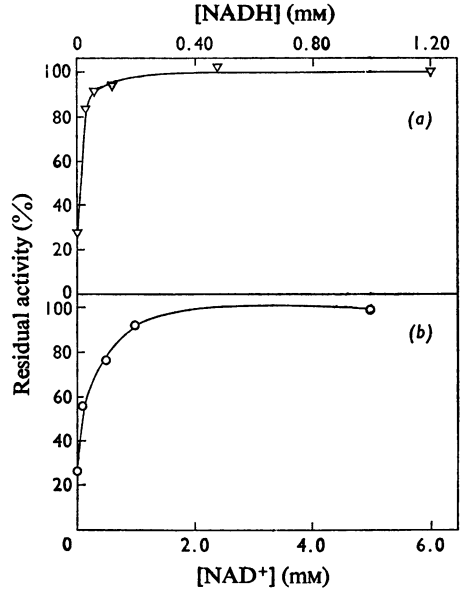

Fig. 6. *Protection of horse liver alcohol dehydrogenase by coenzymes*

Each point represents the activity of the enzyme at equilibrium in an incubation under the conditions of Fig. 1. Each incubation mixture contained, in addition to 3.6mM-pyridoxal 5'-phosphate, NADH (*a*, ▽) or NAD^+ (*b*, ○) at the concentration indicated.

activity should have been regained. The mean from the three experiments was in close agreement with this value. This is not a stringent test of the constants, but it does indicate the absence of secondary irreversible changes.

McKinley-McKee & Morris (1972) found that repeated cycles of treatment with pyridoxal 5'-phosphate, followed by reduction with $NaBH_4$ and dialysis, led to complete inactivation of horse liver alcohol dehydrogenase. We have confirmed their finding. As shown in Table 1, each cycle decreased starting activity by a similar fraction, so that, after three cycles, the activity was only about 1% of its original value. Under our conditions, however, the total incorporation of pyridoxal 5'-phosphate was only 2–3mol/mol of subunit, in contrast with the earlier finding of 6–7mol/mol of subunit. It appears that, at the lower temperature used in our work, less non-specific labelling of lysine residues occurs. Borohydride treatment alone does not significantly decrease activity under the conditions used.

The effects of NAD^+, NADH and substrates on the modification of horse liver alcohol dehydrogenase by pyridoxal 5'-phosphate were studied as previously described for glutamate dehydrogenase

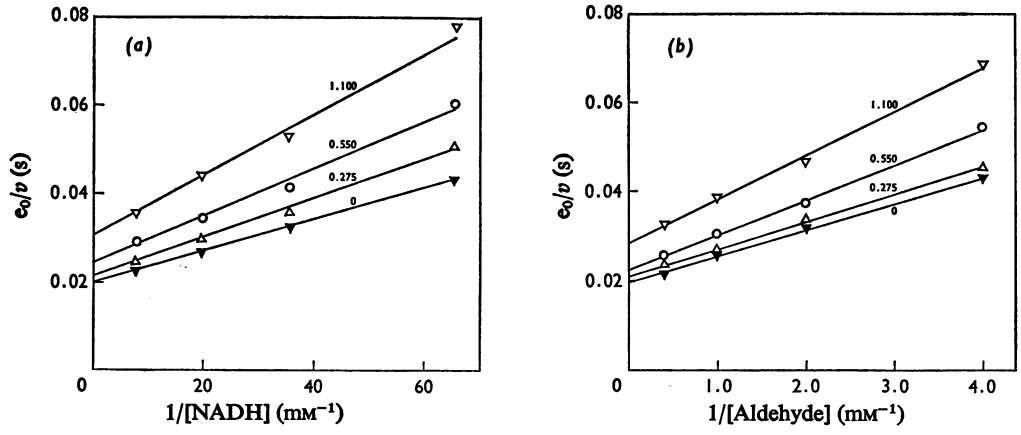

Fig. 7. *Inhibition of horse liver alcohol dehydrogenase by pyridoxal 5'-phosphate in steady-state initial-rate experiments*

The Figure shows Lineweaver–Burk plots of initial rates in 0.05M-potassium phosphate buffer, pH8, at 25°C. Reactions were initiated by the addition of 10μl of alcohol dehydrogenase (0.12mg/ml) to a reaction mixture (3ml) containing pyridoxal 5'-phosphate at the concentrations (mM) indicated, and (*a*) 2.5mM-acetaldehyde with various concentrations of NADH, (*b*) 0.12mM-NADH with various concentrations of acetaldehyde. Each point represents the mean of three measurements.

(Chen & Engel, 1975*d*), advantage being taken of the possibility, for a reversible reaction, of equilibrium analysis (Chen & Engel, 1974). In the studies of protection by NADH, the concentration of NAD^+ in the assay mixture was increased to 0.38mM to combat product inhibition by NADH transferred with the sample. Likewise, when acetaldehyde was used as a protecting agent, the ethanol concentration in the assay mixture was raised to 42.5mM. If the covalently modified enzyme is completely inactive, the measured extent of inactivation of a given sample should be independent of the assay conditions. This was found to be the case.

Fig. 6(*a*) shows the residual activity of the enzyme at equilibrium in the presence of different concentrations of NADH and 3.6mM-pyridoxal 5'-phosphate. In contrast with the partial protection observed in the studies of glutamate dehydrogenase, NADH at a concentration of 0.5mM protected alcohol dehydrogenase completely. This confirms the observation of McKinley-McKee & Morris (1972). In the same way the inactivation was prevented by oxidized coenzyme, NAD^+ (Fig. 6*b*). In contrast with the coenzymes, however, the substrates, acetaldehyde (up to 10mM) and ethanol (up to 50mM) failed to offer any protection.

It is possible to distinguish (Chen & Engel, 1975*d*) between the roles of pyridoxal 5'-phosphate as a long-term 'inactivator', and as an 'inhibitor' of the steady-state initial rate of the enzyme-catalysed reaction over a time-scale within which Schiff-base formation is negligible. Instantaneous inhibition merely reflects rapidly reversible formation of the intermediate enzyme–modifier complex. The Lineweaver–Burk plots shown in Figs. 7(*a*) and 7(*b*) demonstrate clearly that pyridoxal 5'-phosphate used as an inhibitor in this way is non-competitive with respect to both NADH and acetaldehyde.

The kinetic properties of the native and pyridoxal 5'-phosphate-modified enzyme (74% inactivated and reduced with $NaBH_4$) were compared. From the Lineweaver–Burk plots shown in Figs. 8(*a*) and 8(*b*) the apparent K_m values for NAD^+ and ethanol were approximately estimated. The native and modified enzymes gave identical K_m values for NAD^+ (10^{-5}M) and for ethanol (3.65×10^{-4}M). The chemical modification was reflected, however, in decreased values of $V_{max.}$. These findings reinforce the view that modification abolishes the activity of the molecules affected.

Enhancement of the activity of horse liver alcohol dehydrogenase by modification of lysine residues has been reported (Plapp, 1970; Jörnvall, 1973*a*; Tsai *et al.*, 1974). If pyridoxal 5'-phosphate modifies a strictly essential residue, modification of the same residue by other reagents should abolish activity. To ascertain whether the activating agent, formaldehyde, reacts with the residue that is modified by pyridoxal 5'-phosphate with loss of activity, alcohol dehydrogenase was treated first with formaldehyde and borohydride and then with pyridoxal 5'-phosphate.

In our hands the method of Tsai *et al.* (1974), in which portions of formaldehyde are added to an

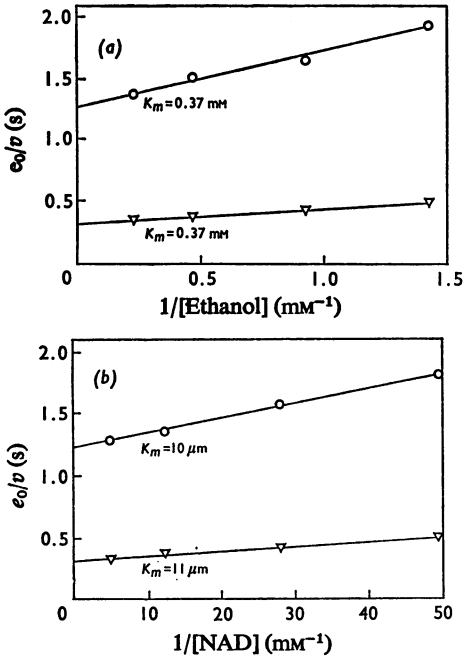

Fig. 8. *Comparison of the kinetic behaviour of alcohol dehydrogenase before and after chemical modification by pyridoxal 5′-phosphate*

The modified enzyme was inactivated to the extent of 74% by incubation with pyridoxal 5′-phosphate, reduced with $NaBH_4$, and dialysed. The Lineweaver–Burk plots show the results of spectrophotometric initial-rate measurements in 0.05M-potassium phosphate buffer, pH8, at 25°C with (*a*) 0.134mM-NAD^+ and ethanol concentration varied, (*b*) 14mM-ethanol and NAD^+ concentration varied. ▽, Native enzyme; ○, modified enzyme.

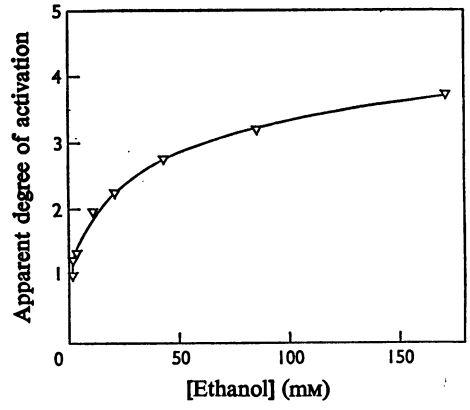

Fig. 9. *Dependence on ethanol concentration of the apparent degree of activation of horse liver alcohol dehydrogenase after methylation*

Alcohol dehydrogenase was methylated by treatment with formaldehyde and $NaBH_4$, as described in the text. Enzyme activity was assayed with reaction mixtures containing 0.05M-potassium phosphate buffer, pH8, at 25°C, 85μM-NAD^+ and ethanol at various concentrations.

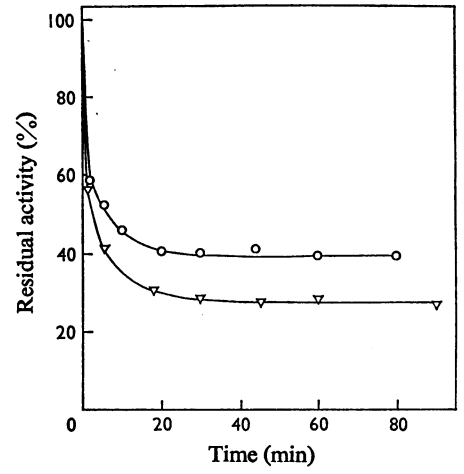

Fig. 10. *Time-courses of inactivation of native and methylated liver alcohol dehydrogenase by pyridoxal 5′-phosphate*

Samples of native alcohol dehydrogenase (▽) and methylated enzyme (○) were incubated with 3.6mM-pyridoxal 5′-phosphate under the conditions of Fig. 1.

enzyme solution already containing $NaBH_4$, yielded completely inactive enzyme. The much lower enzyme concentration (1mg/ml) used in our experiments was the only obvious difference. Addition of bovine serum albumin to make up the total protein concentration to that (25mg/ml) used by Tsai *et al.* (1974) did not prevent the inactivation. Activation was achieved, however, by using the procedure of Jörnvall (1973*a*), in which formaldehyde is added first to the enzyme and allowed to react before the addition of borohydride. Under our routine assay conditions, this resulted in a 60% increase in activity, confirming the results of Jörnvall (1973*a*). Tsai *et al.* (1974) claimed that methylation partially desensitizes the enzymes to substrate inhibition by ethanol. We have confirmed this and shown (Fig. 9) that it accounts for the apparent discrepancy between the results of Jörnvall (1973*a*) and the much larger degree of activation claimed by Tsai *et al.* (1974). Our activated preparation, when assayed as described by Tsai *et al.* (1974) with 0.17M-ethanol and 0.34mM-NAD^+ in 0.05M-pyrophosphate, pH9, was indeed four to five times as active as the native enzyme.

Incubation of such methylated enzyme with pyridoxal 5'-phosphate revealed that it was still subject to inactivation, although to a lesser extent than the native enzyme. Fig. 10 shows the time-courses of inactivation of native and methylated alcohol dehydrogenase by pyridoxal 5'-phosphate (3.6mM). The activity of the native enzyme declined to 25% of the initial value, compared with 41% for methylated enzyme.

Discussion

In considering the inactivation of alcohol dehydrogenase by pyridoxal 5'-phosphate it is important to establish whether the loss of activity results from the modification of a single essential residue, or whether the effect is less specific and less complete. If the first alternative is to be entertained, an explanation must be found for the persistence of a significant fraction of the activity after prolonged incubation with the modifier at saturating concentrations. According to the scheme of McKinley-McKee & Morris (1972) the residual activity is determined by the relative affinities for pyridoxal 5'-phosphate of two groups, one an essential lysine residue, the other a non-essential residue, which react with the modifier in a mutually exclusive manner. Our alternative hypothesis involves a two-stage reaction at a single site (Scheme 1). Residual activity is attributed to the presence of an intermediate complex in equilibrium with the Schiff-base adduct.

The use of a low temperature in the present studies has allowed kinetic as well as equilibrium analysis of the inactivation reaction. The evidence of saturation kinetics (Fig. 4) provides a strong argument for a two-stage reaction in which a second-order process is followed by a first-order transformation. Positive evidence in favour of the single-site hypothesis comes from the agreement between the observed minimum activity (Fig. 1) and the value predicted according to Scheme 1 from estimates of k_{+2} and k_{-2} obtained by kinetic analysis of inactivation and reactivation.

The stepwise inactivation on repeated cycles of treatment with pyridoxal 5'-phosphate and $NaBH_4$ (Table 1) is difficult to explain on the basis of the 'two-site' hypothesis. If both sites were lysine residues, and their reactions with pyridoxal 5'-phosphate were mutually exclusive, the second cycle of treatment would be without effect. Recognizing this difficulty, McKinley-McKee & Morris (1972) suggested that the second site may be an arginine residue, or even the zinc atom. The latter suggestion cannot be entirely excluded, but the first is difficult to reconcile with their finding of a single new peak in the amino acid analysis of the modified protein, corresponding to 6-*N*-pyridoxyl-lysine. On the basis of the equilibrium hypothesis, on the other hand, the stepwise decrease in activity is to be expected. The Schiff base should be reduced by borohydride, and pyridoxal 5'-phosphate, either free or non-covalently bound, is also reduced and thereby rendered incapable of further inactivating the enzyme. After dialysis, the second cycle should decrease activity by the same proportion as the first. Thus, since the first cycle, under our conditions, leaves 25–26% active enzyme, the second cycle should leave 25–26% of that remainder, i.e. 6–7%. The observed value (Table 1) was 6.4%.

The finding (Figs. 8*a* and 8*b*) that partial modification alters only $V_{max.}$ values and not K_m values indicates a decrease in the concentration of catalytically active enzyme and suggests again that the residual activity is due to the persistence of native enzyme.

Recent reports (Plapp, 1970; Jörnvall, 1973*a*; Tsai *et al.*, 1974) that non-specific lysine-modifying reagents, notably formaldehyde, enhance the activity of horse liver alcohol dehydrogenase seemed, at first sight, difficult to reconcile with the view that the enzyme contains a highly reactive essential lysine residue. In the present experiments, however, formaldehyde-activated alcohol dehydrogenase was still susceptible to inactivation by pyridoxal 5'-phosphate (Fig. 10). This suggests that different lysine residues are modified by the two treatments. The argument rests, however, on the unproven assumption that the activation by formaldehyde is complete under our conditions.

A preliminary experiment with yeast alcohol dehydrogenase showed that this enzyme is also inactivated by pyridoxal 5'-phosphate. In view of the limited extent of the homology between the amino acid sequences for the two alcohol dehydrogenases (Jörnvall, 1973*b*), this indication that the essential lysine residue has been conserved during evolution is highly significant.

We conclude that horse liver alcohol dehydrogenase, like ox liver glutamate dehydrogenase, reacts reversibly with pyridoxal 5'-phosphate in two stages, initial formation of a rapidly dissociable and presumably non-covalent complex followed by reaction to give a Schiff base. A single essential lysine residue is probably involved, but its complete modification, leading to complete inactivation, requires several cycles of treatment each followed by fixation with borohydride, and entails the incorporation of 1–2mol of pyridoxal 5'-phosphate per mol of subunit elsewhere in the protein.

The studies of protection and steady-state inhibition were undertaken in the hope of obtaining some clues as to the role of the essential lysine residue. The non-competitive inhibition (Figs. 7*a* and 7*b*) means that neither NADH nor acetaldehyde at saturating concentrations can displace the inhibitor from its non-covalent complex with the enzyme.

Conversely, therefore, in this complex pyridoxal 5'-phosphate does not prevent the binding of coenzyme or substrate. In the protection experiments, however, complete protection by coenzymes is observed (Figs. 6*a* and 6*b*). This contrasts with the partial protection by coenzymes found with other NAD^+-linked dehydrogenases (Ronchi *et al.*, 1969; Chen & Engel, 1975*a*,*c*,*d*), and means that, when the modifier is covalently attached to the essential lysine residue, the coenzyme can no longer be bound. This alone is, of course, sufficient to explain the total inactivation of the enzyme by pyridoxal 5'-phosphate. It would also, however, be consistent with a more general mechanism for the nicotinamide nucleotide-linked dehydrogenases (Chen & Engel, 1975*a*) in which an essential lysine residue is involved in orientation of the coenzyme or in catalysis.

Unlike the coenzymes, the substrates, acetaldehyde and ethanol, do not protect alcohol dehydrogenase against modification by pyridoxal 5'-phosphate. These results, which contrast with those for glutamate dehydrogenase (Chen & Engel, 1975*d*) are consistent with the different mechanisms of these two dehydrogenases. Liver alcohol dehydrogenase obeys a compulsory-order mechanism (Theorell & Chance, 1951; Dalziel, 1963), forming binary complexes with only the coenzymes. Glutamate dehydrogenase, however, follows a random-order mechanism (Engel & Dalziel, 1970; Silverstein & Sulebele, 1973); hence the protection by both NADH and 2-oxoglutarate.

Despite the clues given by the experiments discussed above, confident assignment of a role to the 'essential' lysine modified by pyridoxal 5'-phosphate will depend on identification of this residue within the primary sequence and three-dimensional structure of horse liver alcohol dehydrogenase.

Note Added in Proof (Received 13 May 1975)

Since this work was completed, two papers have appeared that have an important bearing on the subject (Sogin & Plapp, 1975; Dworschack *et al.*, 1975). These workers have modified horse liver alcohol dehydrogenase at pH8.4 and 25°C with pyridoxal, causing activation, and with pyridoxal 5'-phosphate, causing partial inactivation, after first acetimidylating about 80% of the lysine residues. They have identified the lysine residue involved in activation and inactivation as lysine-228. There are, however, important differences between their detailed kinetic findings and ours, and, since their experimental procedure also differs from ours, it cannot be assumed that the residue modified under our conditions is also lysine-228.

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 also by financial assistance to S.-S. C. from the British Council and the Lee Foundation, Singapore.

References

Chen, S.-S. & Engel, P. C. (1974) *Biochem. J.* **143**, 569–574
Chen, S.-S. & Engel, P. C. (1975*a*) *Biochem. Soc. Trans.* **3**, 80–82
Chen, S.-S. & Engel, P. C. (1975*b*) *Biochem. J.* **147**, 351–358
Chen, S.-S. & Engel, P. C. (1975*c*) *Biochem. J.* **149**, 107–113
Chen, S.-S. & Engel, P. C. (1975*d*) *Biochem. J.* **149**, 619–626
Dalziel, K. (1962) *Biochem. J.* **84**, 244–254
Dalziel, K. (1963) *J. Biol. Chem.* **238**, 2850–2858
Dunn, M. F. (1974) *Biochemistry* **13**, 1146–1151
Dworschack, R., Tarr, G. & Plapp, B. V. (1975) *Biochemistry* **14**, 200–203
Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T. & Bränden, C.-I. (1974) *FEBS Lett.* **44**, 200–204
Engel, P. C. & Dalziel, K. (1970) *Biochem. J.* **118**, 409–419
Fischer, E. H., Forrey, A. M., Hedrick, J. L., Hughes, R. C., Kent, A. B. & Krebs, E. G. (1963) in *Chemical and Biological Aspects of Pyridoxal Catalysis* (Snell, E. E., Fasella, P. M., Braunstein, A. & Rossi-Fanelli, A., eds.), p. 543, Pergamon Press, Oxford
Frost, A. A. & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd edn., p. 186, London, Macmillan
Goldin, B. R. & Frieden, C. (1972) *J. Biol. Chem.* **247**, 2139–2144
Johnson, J. S. & Deal, W. C., Jr. (1970) *J. Biol. Chem.* **245**, 238–245
Jörnvall, H. (1973*a*) *Biochem. Biophys. Res. Commun.* **51**, 1069–1076
Jörnvall, H. (1973*b*) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2295–2298
Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245–3249
McKinley-McKee, J. S. & Morris, D. L. (1972) *Eur. J. Biochem.* **28**, 1–11
Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp. 132–134, Holden-Day, San Francisco
Pietruszko, R. & Theorell, H. (1969) *Arch. Biochem. Biophys.* **131**, 288–298
Piszkiewicz, D. & Smith, E. L. (1971) *Biochemistry* **10**, 4544–4552
Plapp, B. V. (1970) *J. Biol. Chem.* **245**, 1727–1735
Ronchi, S., Zapponi, M. C. & Ferri, G. (1969) *Eur. J. Biochem.* **8**, 325–331
Silverstein, E. & Sulebele, G. (1973) *Biochemistry* **12**, 2164–2172
Sogin, O. C. & Plapp, D.V. (1975) *J. Biol. Chem.* **250**, 205–210
Theorell, H. & Chance, B. (1951) *Acta Chem. Scand.* **5**, 1127–1144
Theorell, H. & McKinley-McKee, J. S. (1961) *Acta Chem. Scand.* **15**, 1797–1810
Tsai, C. S., Tsai, Y.-H., Lauzon, G. & Cheng, S. T. (1974) *Biochemistry* **13**, 440–443