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Reversible Modification of Pig Heart Mitochondrial Malate Dehydrogenase by Pyridoxal 5'-Phosphate

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1. Pig heart mitochondrial malate dehydrogenase incubated with pyridoxal 5'-phosphate at pH 8.0 and 25°C gradually loses activity. Such inactivation can be largely reversed by dialysis or by addition of L-lysine or L-cysteine, and can be made permanent by $NaBH_4$ reduction. 2. Modification of malate dehydrogenase with pyridoxal 5'-phosphate at 35°C involves two phases, an initial inactivation which is reversible and a slower irreversible second stage. 3. The initial reaction between pyridoxal 5'-phosphate and malate dehydrogenase appears to involve reversible formation of a Schiff base with the ε-amino group of a lysine residue. 4. Inactivation of malate dehydrogenase by pyridoxal 5'-phosphate at 10°C involves only the reversible reaction. 5. At 10°C repeated cycles of treatment with pyridoxal 5'-phosphate and $NaBH_4$ reduction lead to a stepwise decline in residual activity. 6. Apparent K_m values for malate and NAD^+ are unaltered in the partially inactivated enzyme. 7. NAD^+ and NADH give only partial protection against pyridoxal 5'-phosphate inactivation. Substrates give no effect.

Pyridoxal 5'-phosphate has been proved to be a very useful and specific reagent in revealing essential lysine residues in a number of nicotinamide nucleotide-linked dehydrogenases (for references see Chen & Engel, 1975*c*) and various glycolytic enzymes (for references see Colombo & Marcus, 1974). In most of the previous cases studied, chemical modification by pyridoxal 5'-phosphate has resulted in reversible enzyme inactivation owing to the formation of a Schiff base between the aldehyde group of the modifier and the ε-amino group of a lysine residue on the enzyme. In view of the uniform susceptibility of a series of $NAD(P)^+$-linked dehydrogenases to reversible inactivation by pyridoxal 5'-phosphate, it seemed possible that an essential lysine residue might be a common feature of their mechanism (Chen & Engel, 1975*a*). Yost & Harrison (1971) reported, however, that the inactivation of pig heart mitochondrial malate dehydrogenase (EC 1.1.1.37) by pyridoxal 5'-phosphate is irreversible. The apparent contrast with the pattern of reversible modification obtained with other dehydrogenases led us to re-examine the reaction with malate dehydrogenase in greater detail. Although we have confirmed Yost & Harrison's (1971) finding that the enzyme is irreversibly inactivated by pyridoxal 5'-phosphate when incubated at 35°C, it appears that at lower temperatures the inactivation, like that of the other dehydrogenases, is reversible and due to lysine modification. Results and conclusions very similar to those reported here have been reported in another paper from Harrison's laboratory (Wimmer *et al.*, 1975).

A preliminary account of our work has appeared elsewhere (Chen & Engel, 1975*a*).

Materials and Methods

Pig heart mitochondrial malate dehydrogenase, coenzymes and oxaloacetic acid were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. The commercial enzyme preparation was extensively dialysed against 0.1 M-potassium phosphate buffer, pH 7.0, and stored in this buffer until required. The stored solution was further dialysed against 0.1 M-potassium phosphate of appropriate pH before each experiment. Enzyme concentration was determined spectrophotometrically from the absorption at 280nm ($E^{1\%}_{1cm} = 2.80$) (Thorne, 1962). L-Malic acid was a product of Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Assays of enzyme activity were carried out with a recording fluorimeter of the type described by Dalziel (1962). The routine assay mixture (6ml) contained 10mM-malate and 100μM-NAD^+ in 0.1 M-potassium phosphate buffer, pH 8.0 at 25°C.

For inactivation experiments, mitochondrial malate dehydrogenase was incubated in the dark with pyridoxal 5'-phosphate in 0.1 M-potassium phosphate buffer, pH 8.0, at 25°C, or as indicated in the text. At time-intervals, samples were removed for activity assays.

To prepare a stable pyridoxal 5'-phosphate-modified enzyme, the native enzyme was incubated with pyridoxal 5'-phosphate at the appropriate concentration, and then reduced by adding from a freshly prepared solution of 40mM-$NaBH_4$ a volume just sufficient to decolorize the pyridoxal 5'-phosphate. The enzyme was then dialysed against 0.1 M-potassium phosphate buffer. Estimates of the stoicheiometry of incorporation of pyridoxal 5'-phosphate were based on an extinction coefficient of

1.07×10^4 litre·mol^{-1}·cm^{-1} at 327nm for the reduced Schiff base adduct formed with the ε-amino group of lysine (Fischer *et al.*, 1963).

All other experimental procedures and chemicals used were as described previously (Chen & Engel, 1975*b*,*c*).

Results

Pig heart mitochondrial malate dehydrogenase, when incubated with pyridoxal 5'-phosphate in 0.1M-potassium phosphate buffer, pH8.0, at 25°C, gradually lost activity (Fig. 1). Within 30min, the inactivation reached an apparent equilibrium. The extent of inactivation was determined by the pyridoxal 5'-phosphate concentration (Fig. 1, inset), but, even with the highest modifier concentration used, the extent of inactivation after 60min was less than 80%. If the incubation period was extended, there was a further slow decline in activity, so that after 8h only 5% of the initial activity remained. After 8h, however, even the control enzyme sample incubated in buffer without pyridoxal 5'-phosphate had lost 30–40% of activity (Fig. 2).

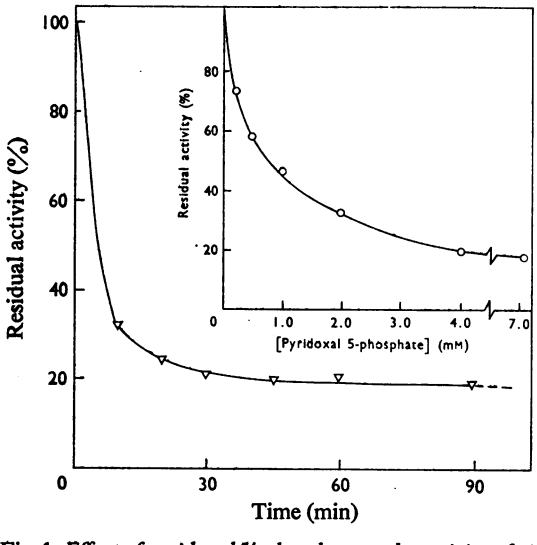

Fig. 1. *Effect of pyridoxal 5'-phosphate on the activity of pig heart mitochondrial malate dehydrogenase at 25°C*

Malate dehydrogenase (0.02mg/ml) was incubated with 4mM-pyridoxal 5'-phosphate in 0.1M-potassium phosphate buffer, pH8.0, at 25°C. Samples were removed at intervals for activity assays. The inset shows the effect of pyridoxal 5'-phosphate concentration on the residual activity of malate dehydrogenase. Each point represents the residual activity obtained after 60min of incubation with the indicated concentration of the modifier.

The reversibility of inactivation by pyridoxal 5'-phosphate was investigated as follows. An enzyme sample was incubated with 4.0mM-pyridoxal 5'-phosphate in 0.1M-potassium phosphate buffer, pH8.0, at 25°C. After 4h, such a sample was divided into two portions: one portion was dialysed against 0.1M-potassium phosphate, pH8, immediately, whereas the other was reduced with $NaBH_4$ before dialysis. After dialysis, the sample that had not been reduced possessed 80% of its initial activity, whereas the reduced sample remained only 16% active. The control sample incubated without pyridoxal 5'-phosphate was found to be 86% active. These results show that pyridoxal 5'-phosphate modification of mitochondrial malate dehydrogenase at 25°C is largely reversible. After reduction with $NaBH_4$ and dialysis, the partially inactivated enzyme displayed a typical protein absorption peak at 280nm and a second maximum at 327nm, which is characteristic of the reduced Schiff base formed between the ε-amino group of a lysine residue and pyridoxal 5'-phosphate (Fischer *et al.*, 1963). On the other hand, the non-reduced sample showed only the absorption peak at 280nm.

The reversibility of the reaction between pyridoxal 5'-phosphate and mitochondrial malate dehydrogenase was further confirmed by the addition of lysine to the enzyme during the course of inactivation. Fig. 3 shows that, after 20min of incubation at 25°C with 4mM-pyridoxal 5'-phosphate at pH8.0, addition of L-lysine (final concentration 9.8mM) caused a gradual recovery of enzyme activity (from 25 to 80%).

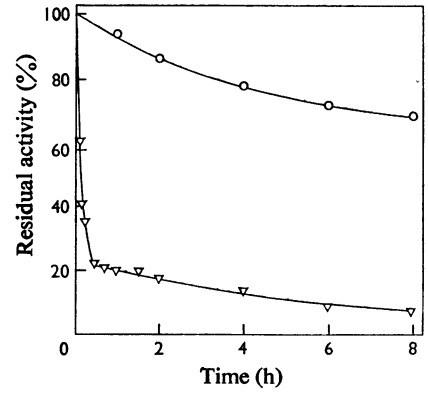

Fig. 2. *Extended incubation of pig heart mitochondrial malate dehydrogenase with pyridoxal 5'-phosphate at 25°C*

Malate dehydrogenase (0.02mg/ml) was incubated with 4mM-pyridoxal 5'-phosphate in 0.1M-potassium phosphate buffer, pH8.0, at 25°C, as described in Fig. 1: incubation with 4mM-pyridoxal 5'-phosphate (▽), control (○).

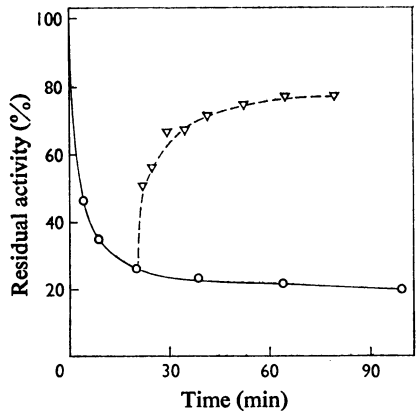

Fig. 3. *Reversal by* L-*lysine of the inactivation of pig heart mitochondrial malate dehydrogenase by pyridoxal 5′-phosphate at 25°C*

Malate dehydrogenase (0.03mg/ml) was incubated with 4mM-pyridoxal 5′-phosphate as described in Fig. 1 (○) and assayed at intervals. After 20min, 0.5ml of incubation mixture was removed and mixed with 10μl of 0.5M-L-lysine. The enzyme activity of this sample was then also assayed at intervals (▽).

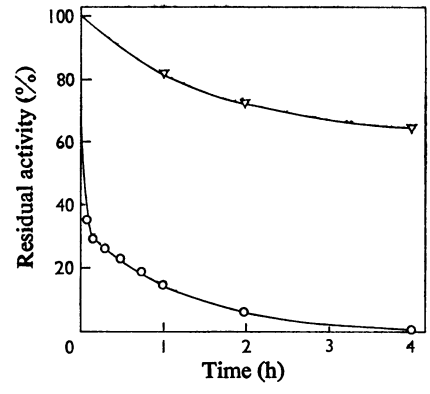

Fig. 4. *Inactivation of pig heart mitochondrial malate dehydrogenase by pyridoxal 5′-phosphate at 35°C*

Malate dehydrogenase (0.3mg/ml) was incubated with 4mM-pyridoxal 5′-phosphate in 0.1M-potassium buffer, pH7.5, at 35°C. At timed intervals, samples were removed for activity assays: control (▽), +pyridoxal 5′-phosphate (○).

Yost & Harrison (1971) reported that the inactivation of malate dehydrogenase by pyridoxal 5′-phosphate at 35°C was not reversed by exhaustive dialysis nor by the addition of lysine or β-mercaptoethanol. Fig. 4 illustrates our results obtained under their conditions. When malate dehydrogenase was incubated with 4mM-pyridoxal 5′-phosphate in 0.1M-potassium phosphate buffer, pH7.5, at 35°C, the enzyme activity declined very quickly in the first 20min and then more slowly until the activity was almost completely lost after 4h, in agreement with the finding by Yost & Harrison (1971). Under these conditions, however, the control sample was also unstable, retaining only 60% of its activity after 4h. This is in marked contrast with the stability in the control reported by Yost & Harrison (1971).

Our original experimental conditions differed from those of Yost & Harrison (1971) with regard to pH as well as temperature. We have no results for pH8 and 35°C, but a preliminary survey of pH-dependence at 25°C showed that inactivation was slower and less extensive at pH7.5 than at pH8. We therefore attribute the increased rate of inactivation shown in Fig. 4 to the higher temperature.

The reversibility of inactivation at 35°C was investigated as follows: an enzyme sample incubated with pyridoxal 5′-phosphate for 4h as above (residual activity = 0.5%) was divided into two portions, one being reduced with $NaBH_4$. Both portions were dialysed against 0.1M-potassium phosphate buffer and assayed. The residual activities (Table 1) were 0.4 and 0.7% of the initial value in the reduced and unreduced samples respectively. The inactivation under these conditions is clearly irreversible, as stated by Yost & Harrison (1971). Spectrophotometric measurements made with the unreduced sample after dialysis also confirmed the presence of a new absorption maximum at 325nm with significant absorption extending to 450nm.

The dependence of the reversibility of inactivation at 35°C on the time of incubation was studied. During an incubation of malate dehydrogenase with 4mM-pyridoxal 5′-phosphate in 0.1M-potassium phosphate buffer, pH7.5, at 35°C, samples (0.2ml) were withdrawn at intervals and mixed with 10μl of 1.0M-cysteine before assay. Fig. 5 shows partial recovery of activity in the early stages of incubation. The extent of such recovery decreased with time of incubation. Addition of lysine gave similar results.

Since the reversible reaction is a more prominent feature of the inactivation of malate dehydrogenase by pyridoxal 5′-phosphate at 25°C than at 35°C, it was decided to lower the temperature of incubation further. At 10°C, with 4mM-pyridoxal 5′-phosphate in 0.1M-potassium phosphate buffer, pH8.0, the activity of the enzyme declined to 40% of the initial value and was then stable for up to 10h. The control was also relatively stable under these conditions. The inactivated sample was 86% active after dialysis (cf. control), but if it was first reduced with $NaBH_4$ it remained only 40% active after dialysis. This suggested that, at the lower temperature, the modi-

Table 1. *Reversibility of pyridoxal 5'-phosphate inactivation of mitochondrial malate dehydrogenase at 35°C*

Mitochondrial malate dehydrogenase was incubated with 4.0mM-pyridoxal 5'-phosphate in 0.1M-potassium phosphate buffer, pH7.5, at 35°C, as in Fig. 4. At the times indicated, the sample was divided into two portions, one being reduced with $NaBH_4$. After dialysis of both samples the residual activity was measured.

Time of incubation (min)	Residual activity (%)	
	Not reduced	Reduced
20	72.6	23.8
240	0.7	0.4

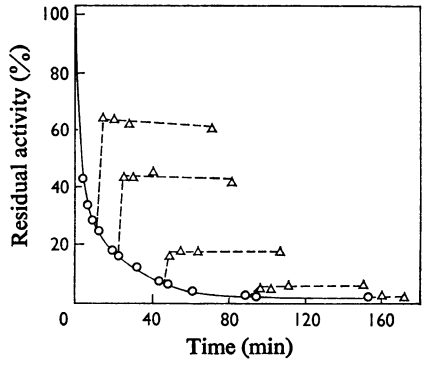

Fig. 5. *Reversal by L-cysteine of the inactivation of malate dehydrogenase at 35°C by pyridoxal 5'-phosphate*

Malate dehydrogenase (0.03mg/ml) was incubated with 4mM-pyridoxal 5'-phosphate in 0.1M-potassium phosphate buffer, pH7.5 at 35°C (○). During the incubation, samples (0.2ml) were removed at timed intervals, mixed with 10μl of 1.0M-cysteine and incubated at 35°C. Enzyme activity was then assayed (△).

fication of malate dehydrogenase by pyridoxal 5'-phosphate involves only the reversible phase. It has been shown that the steady residual activity observed in the modification of various enzymes by pyridoxal 5'-phosphate is due to the existence of an equilibrium between a rapidly dissociable non-covalent enzyme–modifier complex and the inactive covalently modified form (Chen & Engel, 1975*b*,*c*). If this is also the reason for the persistence of 40% of the initial activity when malate dehydrogenase is modified at 10°C, it should be possible to inactivate the enzyme further by a second cycle of pyridoxal 5'-phosphate treatment after reduction with $NaBH_4$ (Chen & Engel, 1975*b*). Table 2 shows the results of repeated cycles of inactivation and the amount of pyridoxal 5'-phosphate incorporated at each stage. After three cycles of such

Table 2. *Effect of repeated cycles of pyridoxal 5'-phosphate inactivation, $NaBH_4$ reduction and dialysis on mitochondrial malate dehydrogenase at 10°C*

Pig heart mitochondrial malate dehydrogenase was incubated with 4.0mM-pyridoxal 5'-phosphate in 0.1M-potassium phosphate buffer, pH8.0, at 10°C, as described in Fig. 4, until the activity reached a steady value (90min), and was then reduced with $NaBH_4$. After dialysis, the same procedure was repeated. After each cycle the stoicheiometry of incorporation of pyridoxal 5'-phosphate was determined from the absorption at 327nm and the specific activity was measured.

No. of cycles	Residual activity (%)	Pyridoxal 5'-phosphate incorporated (mol/mol of enzyme subunit)
First	43.4	0.7
Second	28.9	1.4
Third	16.7	2.0

treatment, the residual activity was decreased to 16% of its original value. Treatment with $NaBH_4$ in the absence of pyridoxal 5'-phosphate was without effect. It should be noted that with this enzyme, like lactate dehydrogenase (Chen & Engel, 1975*c*) but unlike glutamate dehydrogenase (Chen & Engel, 1975*b*), successive cycles of treatment do not produce equal fractional inactivation. This could mean either that modification of other residues interferes with modification of the lysine residue of primary interest (cf. McKinley-McKee & Morris, 1972), or that modification of this residue does not abolish activity entirely.

In pursuit of the latter possibility we examined the effect of modification by pyridoxal 5'-phosphate on the kinetic parameters of malate dehydrogenase. An enzyme sample was inactivated to the extent of 80%, with 1.7mol of pyridoxal 5'-phosphate incorporated per mol of enzyme subunit, by treatment with 4mM-pyridoxal 5'-phosphate at pH8.0 for 20min at 25°C. Figs. 6(*a*) and 6(*b*) show that the apparent K_m values for NAD^+ and for malate were unaltered by this treatment, whereas the apparent $V_{max.}$ values were decreased. This suggests that the modified enzyme molecules are probably totally inactive.

The effects of oxidized and reduced coenzymes and substrates on the modification of malate dehydrogenase by pyridoxal 5'-phosphate were studied as described previously for lactate dehydrogenase and glutamate dehydrogenase (Chen & Engel, 1974, 1975*c*). The advantage of equilibrium protection analysis of a reversible inactivation (Chen & Engel, 1974) is that one needs only to measure the final stable extent of inactivation. NAD^+ and NADH both protect malate dehydrogenase against pyridoxal 5'-phosphate modification (Figs. 7*a* and 7*b*), but in neither case was full protection achieved

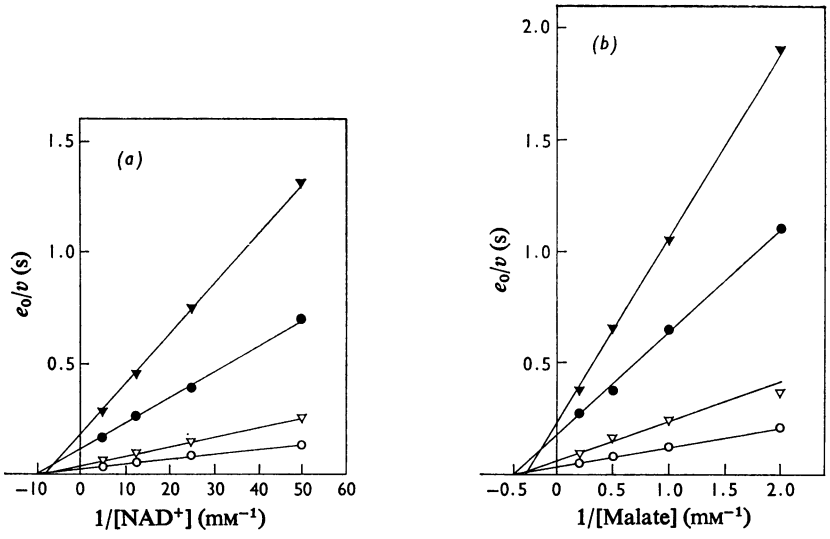

Fig. 6. *Double-reciprocal plots for malate oxidation catalysed by native and modified malate dehydrogenase*

The initial rates were measured spectrophotometrically in duplicate (at least) at 25°C in 0.1M-potassium phosphate buffer, pH8.0, with (*a*) 5.0mM-malate (○, ●) or 2.0mM-malate (▽, ▼) and various concentrations of NAD^+, and (*b*) 101 μM-NAD^+ (○, ●) or 49 μM-NAD^+ (▽, ▼) and various concentrations of malate: native enzyme (○, ▽), reduced modified enzyme (●, ▼). The modified enzyme was 80% inactivated with pyridoxal 5′-phosphate (see the text), reduced with $NaBH_4$ and dialysed, and contained 1.7mol of pyridoxal 5′-phosphate incorporated/mol of enzyme subunit. e_0 is the molar concentration of enzyme subunits [mol.wt. 35000 (Thorne & Kaplan, 1963)].

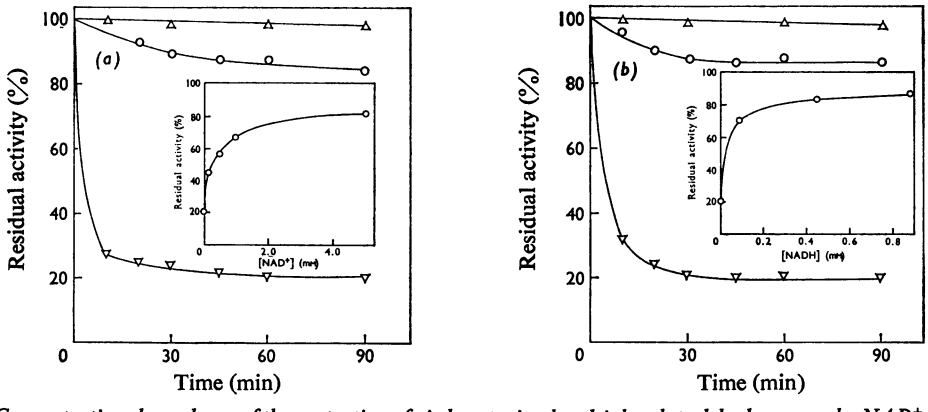

Fig. 7. *Concentration-dependence of the protection of pig heart mitochondrial malate dehydrogenase by NAD^+ and NADH*

(*a*) Malate dehydrogenase (0.03mg/ml) was incubated with 4mM-pyridoxal 5′-phosphate as described for Fig. 1 in the absence (▽) or presence (○) of 5mM-NAD^+; (△) control experiment with 5mM-NAD^+ but no pyridoxal 5′-phosphate. The inset shows the residual activity obtained after 90min of incubation with 4mM-pyridoxal 5′-phosphate in the presence of different concentrations of NAD^+ as indicated. (*b*) Malate dehydrogenase was incubated as described for Fig. 7(*a*) above in the absence (▽) or presence (○) of 0.89mM-NADH; (△) control experiment with 0.89mM-NADH but no pyridoxal 5′-phosphate. The inset is similar to that in Fig. 7(*a*), showing results obtained with different concentrations of NADH instead of NAD^+.

(insets to Figs. 7*a* and 7*b*), even with saturating concentrations of coenzymes. This partial protection contrasts with lack of protection by the substrates malate (up to 25mM) or oxaloacetate (up to 5.0mM).

The mode of action of pyridoxal 5′-phosphate on malate dehydrogenase was further explored by using pyridoxal 5′-phosphate as an instantaneous inhibitor of the steady-state initial rate. Figs. 8(*a*) and 8(*b*)

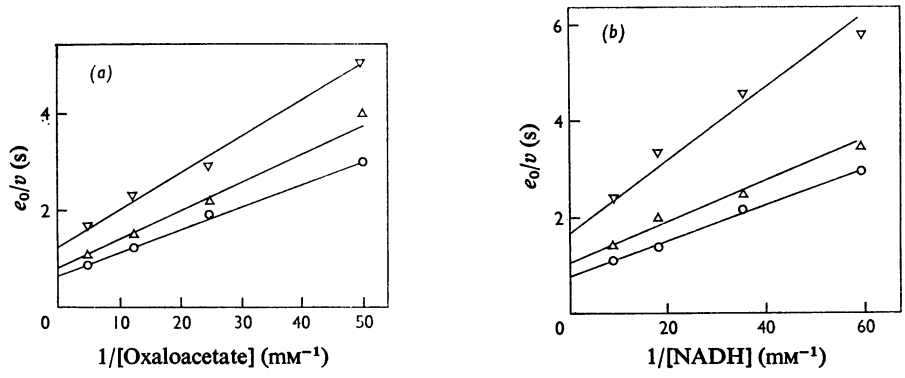

Fig. 8. *Inhibition of pig heart mitochondrial malate dehydrogenase by pyridoxal 5'-phosphate in steady state initial-rate experiments*

The Figure shows double-reciprocal plots of initial-rates in 0.1 M-potassium phosphate buffer, pH 8.0, at 25°C. The significance of e_0/v is as in Fig. 6. Reactions were initiated by the addition of 10 μl of malate dehydrogenase (0.025 mg/ml) to a reaction mixture (2 ml) containing: (*a*) 112 μM-NADH with various concentrations of oxaloacetate, (*b*) 100 μM-oxaloacetate with various concentrations of NADH: ○, no pyridoxal 5'-phosphate; △, +0.2 mM-pyridoxal 5'-phosphate; ▽, +0.8 mM-pyridoxal 5'-phosphate. Each point represents the mean of three measurements.

show that pyridoxal 5'-phosphate behaves as a non-competitive inhibitor with respect to both NADH and oxaloacetate.

Discussion

The observation by Yost & Harrison (1971) that modification of mitochondrial malate dehydrogenase with pyridoxal 5'-phosphate can lead to irreversible inactivation is confirmed in the present study. Irreversible inactivation occurred after incubation of the enzyme with modifier at high temperature (35°C) for long periods of time (4h or more). We have also shown, however, that the modification involves two phases, an initial inactivation, which can be reversed either by dialysis or by the addition of L-lysine or L-cysteine, and a slower, irreversible, second stage. The initial reaction between pyridoxal 5'-phosphate and malate dehydrogenase may involve reversible Schiff-base formation with the ε-amino group of a lysine residue, as suggested by the absorption spectrum. The slower irreversible reaction may involve conversion of a thiol group into a thiazolidine (Yost & Harrison, 1971).

Modification for relatively short periods, or at a lower temperature (10°C), involved only Schiff-base formation as judged from the ready reversibility of the modification and from the spectral analysis. Similar conclusions have also been reached by Wimmer *et al.* (1975), although their temperature range did not extend far enough to eliminate the irreversible reaction completely.

The reduced, partially modified, malate dehydrogenase, with 20% residual activity and 1.7 mol of pyridoxal 5'-phosphate bound/mol of enzyme subunit, was normal with respect to the K_m values for malate and NAD^+ (Fig. 6). Only the rate of malate oxidation was decreased. Thus it seems likely that the partial loss of catalytic activity on modification by pyridoxal 5'-phosphate is attributable to a decrease in the concentration of active enzyme, resulting in a mixture of fully active and completely inactive enzyme (Colombo & Marcus, 1974; Chen & Engel, 1975*b*). It appears therefore that in mitochondrial malate dehydrogenase, as in several other nicotinamide nucleotide-linked dehydrogenases (for references see Chen & Engel, 1975*c*), the modification of lysine residues with pyridoxal 5'-phosphate abolishes the catalytic activity.

The protection experiments demonstrated that NAD^+ and NADH both confer on malate dehydrogenase only partial protection against modification by pyridoxal 5'-phosphate (Fig. 7). It may be concluded that the ε-amino groups involved in the modification are not essential for coenzyme binding. The failure of the substrates to give any protection is consistent with the compulsory-order mechanism of mitochondrial malate dehydrogenase at pH 8 (Raval & Wolfe, 1962*a*,*b*; Silverstein & Sulebele, 1969), in which substrate will only bind to the binary enzyme–coenzyme complex and not to free enzyme. Wimmer *et al.* (1975) also report protection of malate dehydrogenase by its coenzymes against inactivation by pyridoxal 5'-phosphate, but they did not study the dependence of this effect on coenzyme concentration.

Steady-state inhibition experiments (Fig. 8) showed that pyridoxal 5'-phosphate is non-competitive

with respect to both NADH and oxaloacetate. Munkres (1970) observed similar behaviour in a study of mitochondrial malate dehydrogenase from *Neurospora*. Such non-competitive behaviour suggests that the non-covalent enzyme–modifier complex can still bind the catalytic reactants. The partial nature of protection (Fig. 7) suggests that this is also true for the covalently modified enzyme. It is arguable that binding, although possible, may be imperfect and unproductive. Equally, however, the chemical modification may interfere with catalysis rather than binding of the coenzymes. Wimmer *et al.* (1975) have shown clearly, in protection studies at 37°C with nicotinamide and ADP, that it is the latter moiety of the coenzyme which is responsible for protection. It cannot be assumed, however, that such studies of protection against irreversible inactivation can be extrapolated to lower temperatures. A discrepancy between our results and theirs is apparent in the studies of steady-state inhibition, for they find competition between pyridoxal 5′-phosphate and NAD^+. Their studies, however, were at pH10.6, and it is again risky to attempt a direct comparison between the two sets of results.

Kinetic studies have shown that two groups on the enzyme surface of malate dehydrogenase with p*K* values of approx. 7 and 9 are involved in the catalytic function (Raval & Wolfe, 1962*c*; Cassman, 1967), and that during malate oxidation the participating group with p*K* about 7.0, possibly an imidazole, is unprotonated and the second group, with p*K* of about 9, presumably a lysine group, is protonated. These proposals are strikingly similar to those made for lactate dehydrogenase by Winer & Schwert (1958, 1959). Various workers have shown that histidine residues are essential for the catalytic activity of malate dehydrogenase (Anderson, 1970; Anderson & Rabin, 1970; Holbrook *et al.*, 1974). The precise role of lysine residues in mitochondrial malate dehydrogenase remains uncertain. Electrostatic, steric or allosteric effects caused by pyridoxal 5′-phosphate incorporation could explain the present results, but, equally, a mechanism involving the co-operation of histidine and lysine residues may be envisaged, as suggested (Chen & Engel, 1975*c*) for lactate dehydrogenase.

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