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# Modification of Pig $M_4$ Lactate Dehydrogenase by Pyridoxal 5′-Phosphate

## DEMONSTRATION OF AN ESSENTIAL LYSINE RESIDUE

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1. Pig $M_4$ lactate dehydrogenase treated in the dark with pyridoxal 5′-phosphate at pH 8.5 and 25°C loses activity gradually. The maximum inactivation was 66%, and this did not increase with concentrations of pyridoxal 5′-phosphate above 1 mM. 2. Inactivation may be reversed by dialysis or made permanent by reducing the enzyme with $NaBH_4$. 3. Spectral evidence indicates modification of lysine residues, and 6-*N*-pyridoxyl-lysine is present in the hydrolysate of inactivated, reduced enzyme. 4. A second cycle of treatment with pyridoxal 5′-phosphate and $NaBH_4$ further decreases activity. After three cycles only 9% of the original activity remains. 5. Apparent $K_m$ values for lactate and $NAD^+$ are unaltered in the partially inactivated enzyme. 6. These results suggest that the covalently modified enzyme is inactive; failure to achieve complete inactivation in a single treatment is due to the reversibility of Schiff-base formation and to the consequent presence of active non-covalently bonded enzyme–modifier complex in the equilibrium mixture. 7. Although several lysine residues per subunit are modified, only one appears to be essential for activity: pyruvate and $NAD^+$ together (both 5 mM) completely protect against inactivation, and there is a one-to-one relationship between enzyme protection and decreased lysine modification. 8. $NAD^+$ or NADH alone gives only partial protection. Substrates give virtually none. 9. Pig $H_4$ lactate dehydrogenase is also inactivated by pyridoxal 5′-phosphate. 10. The possible role of the essential lysine residue is discussed.

Lactate dehydrogenase (EC 1.1.1.27) is among the most thoroughly investigated nicotinamide nucleotide-linked dehydrogenases (for review, see Everse & Kaplan, 1973). The details of its catalytic mechanism are, nevertheless, not yet fully worked out, although a tentative mechanism has been proposed (Holbrook & Gutfreund, 1973). Various amino acid residues have been claimed to be essential for the catalytic activity, including cysteine (Di Sabato & Kaplan, 1963; Holbrook & Jeckel, 1967), tyrosine (Di Sabato, 1965), histidine (Woenckhaus *et al.*, 1969; Millar & Schwert, 1963; Holbrook & Ingram, 1973) and lysine (Yang & Schwert, 1970). These claims have been based mainly on studies of chemical modification of the enzyme. Several reports have directly implicated the imidazolium ring of a histidine residue (possibly residue 195 in the sequence based on the dogfish muscle enzyme) in the catalytic mechanism of lactate dehydrogenase (Winer & Schwert, 1958; Schwert *et al.*, 1967; Holbrook & Ingram, 1973; Holbrook & Gutfreund, 1973).

Pyridoxal 5′-phosphate has been used as a specific lysine-modifying reagent for various nicotinamide nucleotide-linked dehydrogenases, including bovine liver glutamate dehydrogenase (Anderson *et al.*, 1966; Piszkiewicz *et al.*, 1970; Piszkiewicz & Smith, 1971; Goldin & Frieden, 1972; Brown *et al.*, 1973; Chen & Engel 1975*a*), *Neurospora* glutamate dehydrogenase (Blumenthal & Smith, 1973; Gore *et al.*, 1973), horse liver alcohol dehydrogenase (McKinley-KcMee & Morris, 1972), glyceraldehyde 3-phosphate dehydrogenase (Ronchi *et al.*, 1969; Forcina *et al.*, 1971; Zapponi *et al.*, 1973), 6-phosphogluconate dehydrogenase (Rippa *et al.*, 1967), $NAD^+$-linked isocitrate dehydrogenase (Fan & Plaut, 1974), and 20α-hydroxysteroid dehydrogenase (Sato *et al.*, 1972). In all cases the chemical modification results in enzyme inactivation. Since these enzymes catalyse similar reactions (Colowick *et al.*, 1966; Everse *et al.*, 1971*b*), and have similar three-dimensional structures where these are known (Buehner *et al.*, 1973), it is possible that an essential lysine residue may be a common feature of their mechanisms (Chen & Engel, 1975*b*). It seemed worth pursuing this possibility in the case of lactate dehydrogenase, especially since Yang & Schwert (1970) had already observed that guanidination inactivates the enzyme.

In the present paper we present studies of the modification of pig $M_4$ lactate dehydrogenase by pyridoxal 5′-phosphate, providing evidence that this enzyme indeed possesses a single essential lysine residue per subunit.

### Materials and Methods

Pig muscle lactate dehydrogenase and also the purified $M_4$ isoenzyme were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. The $M_4$ isoenzyme was also purified from the commercial muscle enzyme by chromatography on CM-cellulose (Pesce *et al.*, 1964). Its purity was further checked by

starch-gel electrophoresis as described by Shaw & Prasad (1970). Enzyme concentration was determined by absorption at 280nm ($E^{1\%}_{1cm} = 14.0$) (Jaenicke & Knof, 1968). The lithium L-lactate used was a product of Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K., and sodium pyruvate was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Activity assays were carried out with a recording fluorimeter of the type described by Dalziel (1962). The routine reaction mixture (4 or 6ml) contained 10mM-lactate and 180$\mu$M-$NAD^+$ in 0.05M-potassium phosphate buffer, pH8.0 at 25°C. For inactivation experiments, the enzyme (0.02mg/ml) was incubated in the dark at 25°C with pyridoxal 5'-phosphate in 0.05M-sodium pyrophosphate buffer, pH8.5 and, at timed intervals, 5$\mu$l portions were removed for activity assays. All other experimental procedures and chemicals used were as previously described for studies of glutamate dehydrogenase (Chen & Engel, 1974).

Spectra and measurements of the stoicheiometry of pyridoxal 5'-phosphate incorporation were recorded with a Cary 14 spectrophotometer. An extinction coefficient of $1.07 \times 10^4$ litre·mol$^{-1}$·cm$^{-1}$ at 327nm for the reduced Schiff-base adduct of pyridoxal 5'-phosphate and the $\varepsilon$-amino group of lysine (Fischer *et al.*, 1963) was used.

Sedimentation-velocity experiments were performed with a Beckman model E analytical ultracentrifuge run at 56000rev./min at 7°C with standard and wedge cells with Dural centre-pieces and schlieren optics.

6-*N*-Pyridoxyl-lysine was identified by subjecting the acid hydrolysate of reduced pyridoxal 5'-phosphate-modified $M_4$ lactate dehydrogenase to high-voltage electrophoresis on Whatman 3MM paper in a system containing pyridine–acetic acid–water (1:10:89, by vol.), pH3.5, for 60min at 2000V. A reference standard was prepared by treating $\alpha$-*N*-acetyl-lysine (Calbiochem Ltd., London W1H 1AS, U.K.) with pyridoxal 5'-phosphate and reducing with $NaBH_4$. All samples were hydrolysed in 6M-HCl at 110°C for 24h.

## Results

Incubation of $M_4$ lactate dehydrogenase with pyridoxal 5'-phosphate in 0.05M-pyrophosphate buffer, pH8.5, at 25°C led to a gradual decline in enzyme activity to a steady value reached in 30–60min (Fig. 1, inset). This final value was determined by the pyridoxal 5'-phosphate concentration, but, even with the highest concentrations, the residual activity at the end of incubation was never less than 30% of the original activity (Fig. 1).

Inactivation was fully reversed by extensive dialysis. Reduction with $NaBH_4$, however, rendered the inactivation irreversible. After reduction with $NaBH_4$ and dialysis, the partially inactive 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 an $\varepsilon$-amino group and pyridoxal 5'-phosphate (Fischer *et al.*, 1963). There was no absorption at wavelengths longer than 380 nm. With 65% inactivation, 3.2mol of pyridoxal 5'-phosphate were incorporated/mol of enzyme subunit.

The results shown in Fig. 1 raise a question as to whether the fully modified enzyme is 30–35% active, or whether the enzyme does not become fully modified under these conditions. The same issue arises in the study of the modification of glutamate dehydrogenase (Goldin & Frieden, 1972) and alcohol dehydrogenase (McKinley-McKee & Morris, 1972) by pyridoxal 5'-phosphate. In the former case it has been shown (Chen & Engel, 1975*a*) that the persistence of activity is due to the existence of an equilibrium between a non-covalent enzyme–modifier complex, which appears to be active because it dissociates rapidly, and the inactive covalently modified form. If the explanation for lactate dehydrogenase is similar, then it should

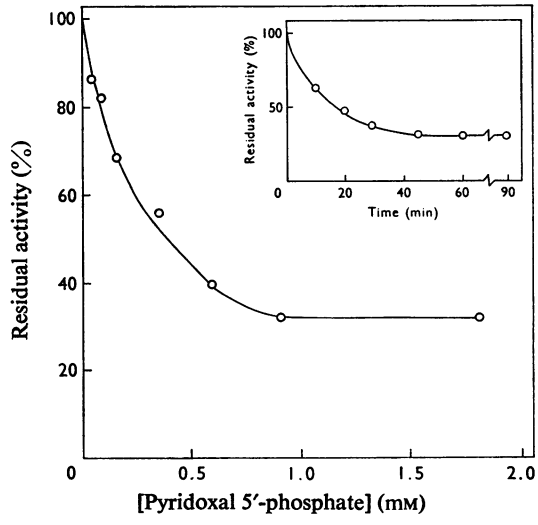


Fig. 1. *Effect of pyridoxal 5'-phosphate concentration on the final residual activity at equilibrium of pig $M_4$ lactate dehydrogenase*

Pig $M_4$ lactate dehydrogenase (0.02mg/ml) was incubated with various concentrations of pyridoxal 5'-phosphate at 25°C in 0.05M-sodium pyrophosphate buffer, pH8.5. Each point represents the activity obtained at equilibrium in such an incubation. The inset shows a time-course of inactivation, with 0.90mM-pyridoxal 5'-phosphate, typical of those from which the points on the main graph were obtained. In most cases the measurements plotted on the main graph were made after 1h of incubation.

Table 1. *Effect of repeated cycles of pyridoxal 5'-phosphate inactivation, $NaBH_4$ reduction and dialysis on pig $M_4$ lactate dehydrogenase*

Conditions: pig $M_4$ lactate dehydrogenase was incubated with 1.8mM-pyridoxal 5'-phosphate until the activity reached a steady value (60min) 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 cycle | Residual activity (%) | Molar ratio |
|---|---|---|
| First | 34 | 3.2 |
| Second | 17 | 4.8 |
| Third | 9 | 5.3 |

be possible to inactivate the enzyme further by a second cycle of pyridoxal 5'-phosphate treatment after reduction with $NaBH_4$ (Chen & Engel, 1975*a*). Table 1 shows the results of successive cycles of inactivation and the amount of pyridoxal 5'-phosphate incorporated at each stage. After three such cycles the residual activity was decreased to 9% of its original value.

Ultracentrifugation experiments were performed to establish whether chemical modification had resulted in gross structural changes. At enzyme concentrations of 2.3mg/ml, the native and reduced, partially modified enzyme gave identical sedimentation patterns ($s_{20,w} = 6.8$S). Clearly, modification by pyridoxal 5'-phosphate does not dissociate the tetrameric enzyme.

6-*N*-Pyridoxyl-lysine was identified in the acid hydrolysate of modified enzyme by high-voltage electrophoresis. A fluorescent spot was visible under u.v. light in the hydrolysate of the modified enzyme with a mobility identical with that of the authentic reference sample. Such fluorescent spots could be stained only faintly with ninhydrin. No corresponding spot was detected in the hydrolysate of the native enzyme. 6-*N*-Pyridoxyl-lysine moved towards the cathode slightly more slowly than free lysine.

Some kinetic measurements were made with the native $M_4$ lactate dehydrogenase and with reduced, partially modified enzyme. Fig. 2 shows that chemical modification had not altered the $K_m$ values for $NAD^+$ or lactate, but the $V_{max.}$ values for the modified enzyme were lower.

The effects of oxidized and reduced coenzymes and substrates on the final extent of modification were studied. The reversibility of the inactivation by pyridoxal 5'-phosphate offers the possibility of measuring a finite residual activity at equilibrium (Fig. 1). The advantages of 'equilibrium protection studies' in such a system have been discussed (Chen & Engel, 1974). Fig. 3 demonstrates that both $NAD^+$ and NADH protected $M_4$ lactate dehydrogenase against inactivation by pyridoxal 5'-phosphate. In neither case, however, was complete protection obtainable, even after saturation with coenzyme. Maximal protection was greater with NADH than with $NAD^+$, and was attained with a lower concentration. Lactate and pyruvate both gave slight protection, which we attribute to an ionic-strength

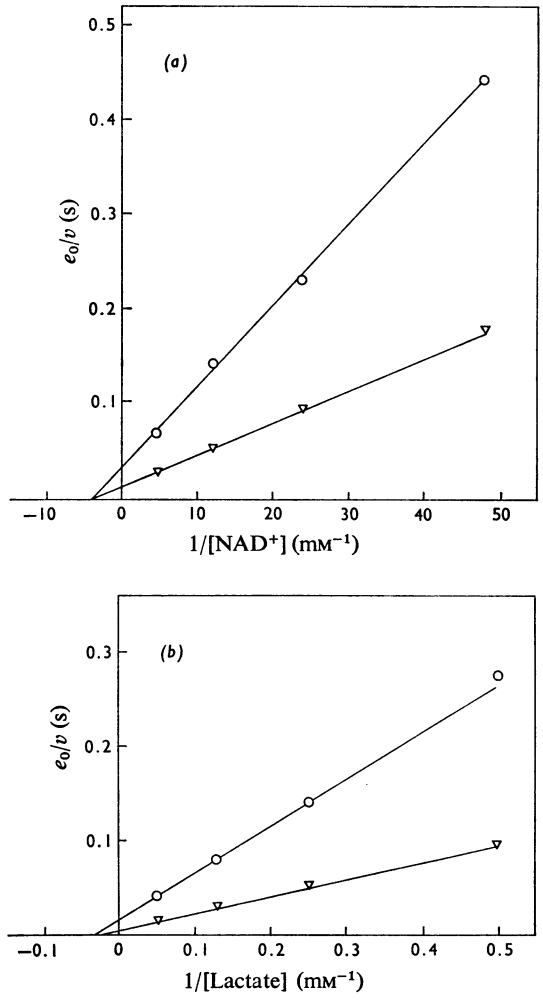


Fig. 2. *Lineweaver–Burk plots for lactate oxidation of native and reduced pyridoxal 5'-phosphate modified $M_4$ lactate dehydrogenase*

The initial rates were measured in duplicate (at least) at 25°C in 0.1M-potassium phosphate buffer, pH8.0, with (*a*) 104$\mu$M-$NAD^+$ and various concentrations of lactate, and (*b*) 4.98mM-lactate and various concentrations of $NAD^+$ with native enzyme (▽) and reduced partially modified enzyme (○). The modified enzyme was 65% inactivated.

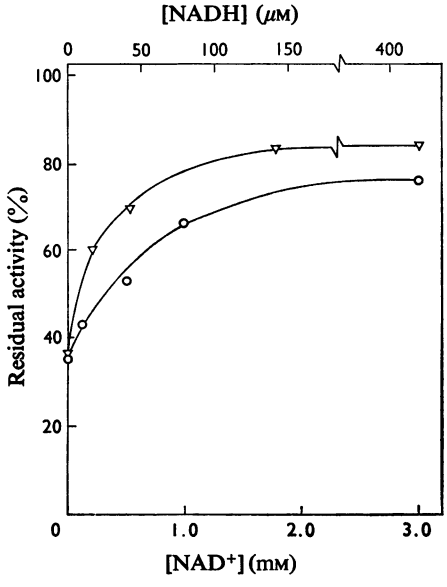


Fig. 3. *Concentration-dependence of the protection of pig $M_4$ lactate dehydrogenase by $NAD^+$ and NADH*

Each point represents the final activity obtained in an incubation as described in Fig. 1. Each incubation mixture contained 1.0mM-pyridoxal 5'-phosphate plus NADH (▽) or $NAD^+$ (○) at the concentrations indicated.

effect, since acetate gave identical results. In similar experiments with horse liver alcohol dehydrogenase (McKinley-McKee & Morris, 1972; Chen & Engel, 1975*b*), complete protection was given by the coenzymes, but none by the substrates.

At an NADH concentration (0.10mM) where maximal protection was achieved, supplementation with lactate gave no further increase in protection. The corresponding experiment with $NAD^+$ and pyruvate was impossible, because the enzyme lost activity very rapidly when incubated with $NAD^+$ and pyruvate in the absence of pyridoxal 5'-phosphate. It has been shown that an inactive abortive complex is readily formed when lactate dehydrogenase is incubated with $NAD^+$ and pyruvate or their analogues (Fromm, 1963; Gutfreund *et al.*, 1968; Everse *et al.*, 1971*a*; Di Sabato, 1968, 1971).

The effect of $NAD^+$ and pyruvate on the inactivation of $M_4$ lactate dehydrogenase by pyridoxal 5'-phosphate was therefore approached in a different manner. The enzyme was incubated with a mixture of pyridoxal 5'-phosphate and protecting agents for 45min, and then reduced with $NaBH_4$. Enzyme activity was measured after extensive dialysis. Table 2 summarizes the results. As in the experiments shown in Fig. 3, it was found that $NAD^+$ (5mM) gave

Table 2. *Effect of $NAD^+$ and pyruvate on pyridoxal 5'-phosphate inactivation of $M_4$ lactate dehydrogenase*

Conditions: pig $M_4$ lactate dehydrogenase (1.0mg/ml) was incubated with 1.50mM-pyridoxal 5'-phosphate+protecting agent in 0.05M-sodium pyrophosphate buffer, pH8.5, at 25°C for 45min and then reduced with $NaBH_4$. Enzyme activity and the stoichieometry of incorporation of pyridoxal 5'-phosphate were determined after extensive dialysis.

| Conditions | Residual activity (%) | Molar ratio |
|---|---|---|
| Control | 97 | 0 |
| 1.5mM-Pyridoxal 5'-phosphate | 36 | 3.1 |
| 1.5mM-Pyridoxal 5'-phosphate+5.0mM-$NAD^+$+5.0mM-pyruvate | 102 | 2.4 |
| 1.5mM-Pyridoxal 5'-phosphate+5.0mM-$NAD^+$ | 82 | 2.7 |

partial protection against inactivation, and this was reflected in a decreased incorporation of pyridoxal 5'-phosphate. When, however, modification was carried out in the presence of both $NAD^+$ (5.0mM) and pyruvate (5.0mM), enzyme activity was completely protected. In the absence of any protecting agent, the enzyme was 64% inactivated with 3.1mol of pyridoxal 5'-phosphate incorporated/mol of enzyme subunit, whereby in the presence of $NAD^+$ and pyruvate only 2.4mol of modifier were incorporated and full enzyme activity was preserved. These values strongly suggest that although pyridoxal 5'-phosphate reacts with several residues in the enzyme, only one of these is essential for catalytic activity.

Since differences in the peptide 'maps' of the tryptic hydrolysates of pig heart and muscle isoenzymes, and also in their *C*-terminal sequences, have been reported (Wieland *et al.*, 1964; Mella *et al.*, 1969), it was decided to compare the effects of pyridoxal 5'-phosphate on the two isoenzymes. Limited experiments with the $H_4$ isoenzyme (Fig. 4) revealed an essentially similar pattern to that obtained with the $M_4$ isoenzyme. When $H_4$ lactate dehydrogenase was 75% inactivated, however, only 1.5mol of pyridoxal 5'-phosphate was incorporated/mol of H subunit. Anderson *et al.* (1974) reported that NADH protects $H_4$ lactate dehydrogenase against inactivation by *N*-heptylmaleimide, but no protective effect was noted in the inactivation of $M_4$ lactate dehydrogenase. We find that, like the $M_4$ isoenzyme, $H_4$ lactate dehydrogenase is partially protected both by NADH and by $NAD^+$ against inactivation by pyridoxal 5'-phosphate (Fig. 4).

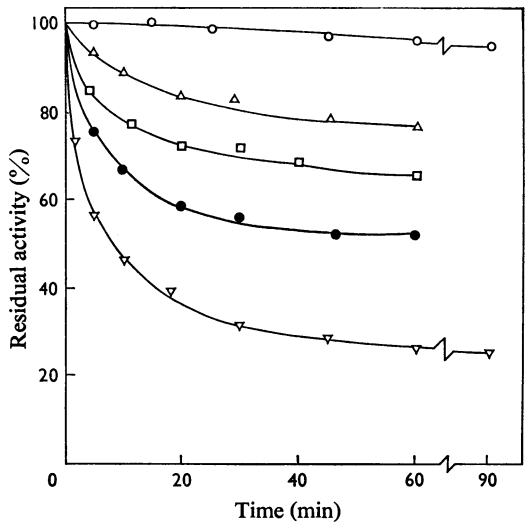


Fig. 4. *Time-courses of pyridoxal 5′-phosphate inactivation of pig $H_4$ lactate dehydrogenase and the effect of oxidized and reduced coenzyme*

All experimental conditions were as described in Fig. 1. Pig $H_4$ lactate dehydrogenase was incubated with 1.0mM-pyridoxal 5′-phosphate (▽), 0.10mM-pyridoxal 5′-phosphate (□), 1.0mM-pyridoxal 5′-phosphate+0.20mM-NADH (△) and 1.0mM-pyridoxal 5′-phosphate+1.0mM-$NAD^+$ (●). The control (○) contained only enzyme and buffer.

## Discussion

The results reported above indicate that pig $M_4$ lactate dehydrogenase is inactivated by reaction with pyridoxal 5′-phosphate. The conclusion that this is due to modification of lysine residues is supported both by the spectral evidence, and more directly by the detection of 6-*N*-pyridoxyl-lysine in the acid hydrolysate of reduced, inactivated enzyme. The reversibility of the inactivation provides strong evidence that the loss of activity is not a result of the modification of cysteine or histidine residues.

Activity cannot be completely abolished by a single treatment with the modifier, but, as for ox liver glutamate dehydrogenase (Chen & Engel, 1975*a*), this may be explained by an equilibrium between a non-covalent enzyme–modifier complex and a covalent Schiff base. Both forms reactivate on dilution or dialysis, but at very different rates: the non-covalent complex dissociates so rapidly that it appears as active enzyme in routine catalytic assays, whereas the covalently modified enzyme dissociates so slowly that it appears to be inactive in such assays. As predicted on the basis of this model (Chen & Engel, 1975*a*), successive cycles of treatment with pyridoxal 5′-phosphate followed by $NaBH_4$ reduction led to further inactivation of $M_4$ lactate dehydrogenase (Table 1) accompanied by increased incorporation of the modifier. Similar stepwise decline in activity has also been observed in studies of the inactivation by pyridoxal 5′-phosphate of liver alcohol dehydrogenase (McKinley-McKee & Morris, 1972), pyruvate kinase (Johnson & Deal, 1970) and phosphoglucose isomerase (Schnackerz & Noltmann, 1971). In the present case, however, successive cycles did not diminish the residual activity by equal percentages: for example, activity after one cycle was 34% of the original, but after two cycles, instead of being 34% of 34% (i.e. approx. 12%), it was 17%. This might mean that the fully modified enzyme is, after all, partially active, or that modification of the lysine residue of primary interest is hindered by the modification of other reactive lysine residues. It is clear (Table 1) that more than one lysine residue/subunit reacts with the modifier. Nevertheless, of the reactive residues, only one appears to be involved in the catalytic activity; incubation with pyruvate and $NAD^+$ preserved the activity of 0.64mol/mol of enzyme by protecting 0.70mol of lysine/mol. Within experimental error this suggests a one-to-one relationship. It is impossible to judge from our results whether the remaining incorporation of pyridoxal 5′-phosphate is due to labelling of a small number of exceptionally reactive lysine residues, or to more random labelling of many residues.

Our contention, that a single cycle of chemical modification produces total inactivation of some of the enzyme rather than partial inactivation of all of the enzyme, is also supported by the kinetic results showing decreased values of $V_{max.}$, but unchanged $K_m$ values for $NAD^+$ and lactate under the conditions of assay (Fig. 2).

The loss of enzyme activity cannot be attributed to a gross change in quaternary structure, since the sedimentation pattern of modified lactate dehydrogenase was identical with that of native enzyme. We therefore conclude that inactivation by pyridoxal 5′-phosphate involves a more direct disturbance of the functional integrity of the active site.

Our findings confirm some earlier suggestions. Rippa (1970) referred to inhibition of lactate dehydrogenase by pyridoxal 5′-phosphate, and Johnson & Deal (1970), who used lactate dehydrogenase as a coupling enzyme in their study of pyruvate kinase, also commented on the interference by pyridoxal 5′-phosphate. Yang & Schwert (1970) suggested the possible presence of an essential lysine residue in lactate dehydrogenase, but their inference, based on the results of extensive guanidination of the enzyme, was inevitably more tentative than the present conclusions. Pfleiderer *et al.* (1968), by contrast, found that 14 of the 24 ε-amino groups of pig heart lactate dehydrogenase could be acetylated without loss of activity. It is possible that the structure of

pyridoxal 5'-phosphate is such as to direct the reagent to some of the lysine residues that do not react with *N*-acetylimidazole.

The partial nature of protection against pyridoxal 5'-phosphate by $NAD^+$ and NADH indicates (Engel & Chen, 1975) that the modified enzyme can still accommodate the coenzyme. It therefore seems unlikely that the essential lysine residue is directly involved in the binding of coenzyme. The failure of substrates to give significant protection is consistent with the compulsory-order mechanism, in which there is no direct formation of binary enzyme–substrate complexes (Takenaka & Schwert, 1956; Zewe & Fromm, 1962). By contrast, glutamate dehydrogenase, which follows a random-order mechanism (Engel & Dalziel, 1970; Silverstein & Sulebele, 1973), is protected against pyridoxal 5'-phosphate by NADH and also by 2-oxoglutarate (Engel & Chen, 1975).

Although $NAD^+$ alone gives only partial protection to lactate dehydrogenase, $NAD^+$ and pyruvate together completely protect the enzyme against inactivation by pyridoxal 5'-phosphate (Table 2). It has been suggested that the ternary abortive complex enzyme–$NAD^+$–pyruvate [strictly speaking it is a binary complex between the enzyme and a covalent pyruvate–$NAD^+$ adduct (Arnold & Kaplan, 1974)], which is formed under these conditions, is analogous to the active transition-state complex (Everse *et al.*, 1971*a*). Since the protection of a single lysine residue/subunit in this complex completely prevents inactivation by pyridoxal 5'-phosphate, it is reasonable to suggest that this essential lysine residue is involved either in binding of the substrate to the enzyme–coenzyme complex, or in a subsequent catalytic step. The long list of $NAD(P)^+$-linked dehydrogenases that are susceptible to inactivation by pyridoxal 5'-phosphate (see the introduction) includes some that have uncharged substrates. This might therefore indicate that a catalytic role for the lysine is more likely, if indeed there is a common role for lysine in these enzymes.

On the basis of their kinetic studies of lactate dehydrogenase Winer & Schwert (1958, 1959) have suggested that a group of p$K$ approx. 7 may act as source and sink for the proton involved in the overall reaction, and that another group with p$K$ 9.3, possibly a lysine residue, may be involved in the binding of the pyruvate carboxyl group. Holbrook & Ingram (1973) have obtained evidence that an essential histidine residue with p$K$ 6.8 has to be protonated in order that the binding of substrates or substrate analogues may occur.

The precise function of the essential lysine in the $M_4$ isoenzyme (and apparently also the $H_4$ isoenzyme) of pig lactate dehydrogenase remains uncertain. Mechanisms involving the co-operation of histidine and lysine residues may be envisaged. Such a mechanism has been proposed for phosphoglucose isomerase (Dyson & Noltmann, 1968). As yet, no three-dimensional structure is available for the pig lactate dehydrogenases. The basic mechanism might, however, be expected to be very similar in lactate dehydrogenases from different vertebrates, and limited studies of dogfish $M_4$ lactate dehydrogenase (S.-S. Chen & P. C. Engel, unpublished work) show that this also is reversibly activated by pyridoxal 5'-phosphate. There are two known lysine residues in the vicinity of the active centre of dogfish $M_4$ lactate dehydrogenase. The first, lysine-145, is over 1 nm from the position of the bound coenzyme (Adams *et al.*, 1973*a*,*b*). This residue is partially excluded from solvent by the 'isomerization' of the enzyme–coenzyme complex, but corresponding structural changes do not occur in horse liver alcohol dehydrogenase (Brändén *et al.*, 1973) or glyceraldehyde 3-phosphate dehydrogenase (Buehner *et al.*, 1973). The second lysine, residue 250, interacts directly with the carboxyamide substituent of the nicotinamide ring of the coenzyme (M. G. Rossmann, personal communication). Again no corresponding lysine residue has been found in alcohol dehydrogenase, although here the position of the nicotinamide ring is less certain.

There must now be some doubt as to the postulated common role of lysine in the NAD(P)-linked dehydrogenases (Chen & Engel, 1975*b*). The possibility cannot be ruled out that some or all of these 'essential' lysine residues are remote from the active site(s), but that their modification locks the enzyme(s) in an inactive conformation. Alternatively, their modification, like that of the 'essential' cysteine residue 165, of dogfish $M_4$ lactate dehydrogenase, may result in steric hindrance of the catalysed reaction(s) (Adams *et al.*, 1973*a*). A third possibility is that the lysine residue(s), though not in the active centre, may be involved in a charge-relay system essential to the proper functioning of the enzyme(s). Clearly, in lactate dehydrogenase, identification of the essential lysine residue within the overall primary sequence will be essential in further defining its role.

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