Modification of mouse testicular lactate dehydrogenase by pyridoxal 5'-phosphate

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1. Mouse C₄ lactate dehydrogenase treated in the dark with pyridoxal 5'-phosphate at pH 8.7 and 25°C loses activity gradually; 1 mM-pyridoxal 5'-phosphate causes 83% inactivation, and higher concentrations of the reagent cause no further loss of activity. 2. The final extent of inactivation is very pH-dependent, greater inactivation occurring at high pH values. 3. Inactivation may be fully reversed by addition of cysteine, or made permanent by reducing the enzyme with NaBH₄. 4. The absorption spectrum of inactivated reduced enzyme indicates modification of lysine residues. Inactivation by 80% corresponds to modification of at least 1.8 mol of lysine/mol of enzyme subunit. 5. There is no loss of free thiol groups after inactivation with pyridoxal 5'-phosphate and reduction of the enzyme. 6. NAD⁺ or NADH gives complete protection against inactivation. Protection studies with coenzyme fragments indicate that the AMP moiety is largely responsible for the protective effect. Lactate (10 mM) gives no protection in the absence of added nucleotides, but greatly enhances the protection given by ADP-ribose (1 mM). Thus ADP-ribose is able to trigger the binding of lactate. 7. Pyridoxal 5'-phosphate also acts as a non-covalent inhibitor of mouse C₄ lactate dehydrogenase. The inhibition is non-competitive with respect to both NAD⁺ and lactate. 8. *K_m* values for the enzyme at pH 8.0 and 25°C, with the non-varied substrate saturating, are 0.3 mM-lactate and 6 μM-NAD⁺. 9. These results are discussed and compared with pyridoxal 5'-phosphate modification of other lactate dehydrogenase isoenzymes and related dehydrogenases.

There are three distinct types of lactate dehydrogenase (EC 1.1.1.27) in higher animals, the two major H and M somatic isoenzymes, and a third isoenzyme, designated C or X (Blanco & Zinkham, 1963), which is found uniquely in the testes. Synthesis of the C subunit occurs only in the spermatocyte, suggesting that C₄ lactate dehydrogenase must have a very specialized physiological function. Studies of the testicular enzyme have therefore centred on its catalytic properties as compared with other lactate dehydrogenase isoenzymes (e.g. Goldberg, 1977). C₄ lactate dehydrogenase is apparently well adapted to function in the direction of lactate oxidation (Battellino *et al.*, 1968; Hawtrey *et al.*, 1975). This property alone, however, cannot justify the existence of a separate testicular isoenzyme, since H₄ lactate dehydrogenase is similarly adapted for lactate oxidation. The C₄ isoenzyme is unique in having a broad substrate specificity (e.g. Blanco *et al.*, 1976). It can utilize as substrates 2-oxo and 2-hydroxy acids with carbon chains longer than those of pyruvate and lactate,

although the physiological significance of this broad specificity is not yet clear. It has been proposed that the enzyme uses such substrates in a trans-mitochondrial oxidation–reduction shuttle (Blanco *et al.*, 1976).

Chemical studies of mouse C₄ lactate dehydrogenase have been more limited. The amino acid composition has been determined (Goldberg, 1972; Wheat & Goldberg, 1975), and more recently a high-resolution crystallographic structure of the enzyme has been published (Musick & Rossmann, 1979a). The X-ray data have also been used to propose a tentative amino acid sequence (Musick & Rossmann, 1979b). The overall fold of the C₄ isoenzyme is very similar to previously studied structures of the other lactate dehydrogenase isoenzymes (Eventoff *et al.*, 1977). Goldberg (1972) has shown, however, that C₄ lactate dehydrogenase is much less susceptible to inactivation by thiol reagents than are the other isoenzymes.

Chemical modification with pyridoxal 5'-phosphate has been shown to inactivate pig M₄ lactate

dehydrogenase (Chen & Engel, 1975b), pig H₄ lactate dehydrogenase (Chen & Engel, 1975b; Jeckel, 1975) and dogfish M₄ lactate dehydrogenase (Chen & Engel, 1975e). There is some controversy as to whether inactivation involves reaction with amino or thiol groups. Inactivation of the pig M₄ isoenzyme has been claimed to be due to modification of a single lysine residue per subunit (Chen & Engel, 1975b), whereas inactivation of the pig H₄ isoenzyme has been claimed to involve only cysteine residues (Jeckel, 1975). The reaction between pyridoxal 5'-phosphate and lactate dehydrogenase C₄, which is much less susceptible to inactivation by thiol reagents, has now been examined.

Experimental

Crystalline mouse C₄ lactate dehydrogenase, originally prepared in the laboratory of Professor E. Goldberg (Northwestern University, Evanston, IL, U.S.A.), was generously given by Dr. W. D. L. Musick and Professor M. G. Rossmann (Purdue University, Lafayette, IN, U.S.A.). The enzyme was dialysed against 0.1 M-sodium phosphate buffer, pH 7.0, before use. An absorption coefficient of $1.58 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for rat lactate dehydrogenase (Anderson & Weber, 1965) was used for the mouse C₄ isoenzyme (Goldberg, 1972). For purposes of calculation, an enzyme subunit mol.wt. of 35 000 was used (Goldberg, 1972). The enzyme had a specific activity of $27 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ when assayed at pH 7 and 25°C with 0.7 mM-pyruvate and 200 μM -NADH.

NADH (grade 2), NAD⁺ (lithium salt), ADP and AMP were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. NADH, purified by the chromatographic procedure of Wenz *et al.* (1976), had an $A_{260}:A_{340}$ ratio of 2.33 and a residual A_{340} of 1.5% when treated with pyruvate and lactate dehydrogenase at pH 7.5. The A_{340} was subsequently used as a measure of NADH concentration. NAD⁺ was assayed enzymically with yeast alcohol dehydrogenase (Dalziel, 1961). ADP-ribose and NMN (P-L Biochemicals) were obtained from International Enzymes Ltd., Windsor, Berks. SL4 5NJ, U.K. A larger amount of ADP-ribose was prepared by treating NAD⁺ with NaOH (Kaplan *et al.*, 1951) and purifying the products by ion-exchange chromatography on DEAE-cellulose. Purity of the final product was checked by t.l.c. (Lindberg *et al.*, 1973) with commercial ADP-ribose as a standard. Concentrations of ADP-ribose, ADP and AMP were measured spectrophotometrically at 259 nm (P-L Biochemicals, 1961). NMN concentration was measured at 266 nm by using an absorption coefficient of $4.6 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (P-L Biochemicals, 1961).

L-(+)-Lactate (lithium salt) and guanidine hydro-

chloride (grade 1) were from Sigma Chemical Co., Poole, Dorset BH17 7NH, U.K. Pyridoxal 5'-phosphate and sodium pyruvate were products of Koch-Light Laboratories, Colnbrook, Bucks. SL3 0BZ, U.K. Buffer salts were of analytical grade, and deionized glass-distilled water was used throughout. Test tubes used for enzyme dilutions and incubations were boiled in 0.01 M-EDTA before use.

Activity assays were carried out with a recording fluorimeter of the type described by Dalziel (1962), which was calibrated with purified NADH. The routine reaction mixture (4 ml) contained 10 mM-lactate and 180 μM -NAD⁺ in 0.05 M-potassium phosphate buffer, pH 8.0, at 25°C. For inactivation experiments, the enzyme (0.07 mg·ml⁻¹) was incubated in the dark at 25°C in 0.05 M-sodium pyrophosphate buffer, pH 8.7. Twice the required final incubation volume was made up, and half used as a control. Pyridoxal 5'-phosphate was added to give the required final concentration, and an identical volume of water was added to the control. Ligands tested as protecting agents were included in both incubations. Samples (10 μl) were removed from both incubations at timed intervals for activity assays as described above. In order to obtain an accurate estimate of initial activity it was essential to allow for carry-over of pyridoxal 5'-phosphate. A final concentration of 2.5 μM -pyridoxal 5'-phosphate in the assay is sufficient to inhibit the enzyme by about 5% (see the Results and discussion section). The appropriate correction factor was therefore determined for each incubation by assaying the control at zero time in the presence and absence of pyridoxal 5'-phosphate at the final concentration expected in the assays of the experimental incubation.

Absorption spectra and spectrophotometric measurements of the stoichiometry of pyridoxal 5'-phosphate incorporation were recorded with a Cary 14 spectrophotometer (Cary Instruments, Monrovia, CA, U.S.A.). An absorption coefficient of $1.07 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 327 nm for the reduced Schiff-base adduct of pyridoxal 5'-phosphate and the ϵ -amino group of lysine (Fischer *et al.*, 1963) was used (see the Results and discussion section).

Free thiol groups were determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). The enzyme was denatured in 4 M-guanidine hydrochloride/0.05 M-Tris/HCl, final pH 8.3, to expose all the available thiol groups.

Results and discussion

Incubation of mouse C₄ lactate dehydrogenase with pyridoxal 5'-phosphate in 0.05 M-pyrophosphate buffer, pH 8.7, at 25°C led to a gradual decline in enzyme activity to a steady value reached

in 60–90 min (Fig. 1a). This final value was dependent on the pyridoxal 5'-phosphate concentration, but, even with the highest concentrations, the residual activity at the end of incubation was never less than 17% of the original activity (Fig. 1b). This pattern of inactivation has been reported for several other dehydrogenases, and the question arises as to whether the fully modified enzyme is partially active, or whether the enzyme does not become fully modified under the conditions used. For glutamate dehydrogenase, Chen & Engel (1975a) have shown

clearly 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 on dilution in the assay, and the inactive covalently modified form. Further studies with alcohol dehydrogenase (Chen & Engel, 1975c) and pig M_4 lactate dehydrogenase (Chen & Engel, 1975b) suggest that this mechanism of modification by pyridoxal 5'-phosphate might be more widely applicable. It is thus also the probable explanation for persistence of activity in mouse C_4 lactate dehydrogenase.

Fig. 2 shows that inactivation was fully reversed by the addition of excess cysteine, which reacts with pyridoxal 5'-phosphate to produce a stable thiazolidine (Buell & Hansen, 1960; Mackay, 1962). Reduction with $NaBH_4$, however, rendered the inactivation irreversible. This clearly suggests that inactivation is caused by reversible reaction with amino groups to form Schiff bases. After reduction with $NaBH_4$ and dialysis, the partially inactive enzyme displayed a typical protein absorbance peak at 280 nm and a second maximum at 325 nm, which is characteristic of the reduced Schiff base formed between an ϵ -amino group and pyridoxal 5'-phosphate (Fischer *et al.*, 1963). There was no absorbance at wavelengths longer than 380 nm. With 80% inactivation, 1.8 mol of pyridoxal 5'-phosphate was incorporated/mol of enzyme subunit. This ratio of incorporation is very similar to that obtained with H_4 lactate dehydrogenase (Chen & Engel, 1975b) of

Fig. 1. Effect of pyridoxal 5'-phosphate concentration on the inactivation of mouse C_4 lactate dehydrogenase. Mouse C_4 lactate dehydrogenase ($0.07 \text{ mg} \cdot \text{ml}^{-1}$) was incubated in the dark at pH 8.7 and 25°C with various concentrations of pyridoxal 5'-phosphate, and activity was followed with time. (a) Final concentrations of (▲) 0.1 mM-, (□) 0.25 mM-, (●) 0.5 mM- and (△) 1.0 mM-pyridoxal 5'-phosphate were used. The control (○) contained only enzyme and buffer. (b) Percentage residual activity after 90 min incubation is shown plotted against pyridoxal 5'-phosphate concentration.

Fig. 2. Reversal by L-cysteine of the inactivation of mouse C_4 lactate dehydrogenase by pyridoxal 5'-phosphate

Mouse C_4 lactate dehydrogenase was incubated with 1 mM-pyridoxal 5'-phosphate as described in the Experimental section (○). At 15 and 90 min, 0.2 ml samples were removed, mixed with $10 \mu\text{l}$ of 1.0 M-cysteine, and incubated. Enzyme activity was then assayed (▲).

1.5 mol/mol of subunit for 75% inactivation, but is considerably lower than the 4.8 mol/mol of subunit for 83% inactivation found with M_4 lactate dehydrogenase (Chen & Engel, 1975b). Clearly more non-specific modification occurs with the M_4 iso-enzyme. These absolute values for pyridoxal 5'-phosphate incorporation must be subject to some doubt, however, as there have been reports (Blackburn & Schachman, 1976; Paech & Tolbert, 1978) indicating that the absorption coefficient of the reduced Schiff-base adduct between pyridoxal 5'-phosphate and the ϵ -amino group of lysine in free solution may not be generally applicable to modified lysyl residues in proteins. The apparent absorption coefficient at 325 nm may be decreased as much as 2-fold in a protein environment, and thus estimates of the number of lysine residues modified based on the published absorption coefficient provide only a minimum value. A comparison of the spectra of 6-*N*-pyridoxyl-lysine in water and in 50% (v/v) dioxan indicates that this may be due to the hydrophobicity of the local environment (K. G. Gould & P. C. Engel, unpublished work).

Inactivation of mouse C_4 lactate dehydrogenase by pyridoxal 5'-phosphate is markedly pH-dependent (Table 1). With a fixed concentration of pyridoxal 5'-phosphate, the extent of inactivation at equilibrium is greater at high pH values than at low pH values.

The possibility of covalent modification of cysteine residues has been ruled out by determining the number of free thiol groups under denaturing conditions before and after reaction with pyridoxal 5'-phosphate, reduction with NaBH_4 , and dialysis. In both cases the enzyme has 4.2 thiol groups/subunit, in good agreement with amino acid analy-

sis (Wheat & Goldberg, 1975), which indicates 4.23 residues of cysteine/subunit of mol.wt. 35 000.

The effects of coenzymes and coenzyme fragments on the final extent of inactivation were studied. The reversibility of the modification 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). Table 2 shows that both NADH and NAD^+ give complete protection against inactivation of C_4 lactate dehydrogenase by pyridoxal 5'-phosphate. This contrasts with the reported partial protection given by coenzyme with pig M_4 lactate dehydrogenase (Chen & Engel, 1975b). Protection studies with coenzyme fragments (Table 2) show that the adenine-containing half of the coenzyme molecule is responsible for the protective effect. NMN gives no protection whatsoever, whereas ADP-ribose, ADP and AMP all give substantial protection against inactivation. In protection studies of malate dehydrogenase inactivation by pyridoxal 5'-phosphate, Wimmer *et al.* (1975) also concluded that the ADP moiety of the coenzyme is responsible for any protective effect. NMN and AMP apparently are able to bind simultaneously to the enzyme (Table 2), in spite of the unfavourable proximity of the two negatively charged phosphate groups. As expected if the enzyme follows a compulsory-order mechanism,

Table 1. *Effect of pH on inactivation of mouse C_4 lactate dehydrogenase by pyridoxal 5'-phosphate*

Lactate dehydrogenase ($0.07 \text{ mg} \cdot \text{ml}^{-1}$) was incubated with 1 mM-pyridoxal 5'-phosphate at 25°C in 0.05 M buffers of various pH values. Sodium pyrophosphate buffer was used at pH 8.7, sodium phosphate at lower pH values. Assays were performed as described in the Experimental section. Values of activity at equilibrium were obtained from full time-course studies, and are averages of several values at equilibrium. Controls showed no more than 5% loss of activity during 90 min incubation.

Incubation pH	Activity at equilibrium (60–90 min) (% of initial)
7.05	60.0
7.55	45.5
8.0	33.3
8.7	17.8

Table 2. *Effects of coenzymes, coenzyme fragments and substrate on the equilibrium position of the inactivating reaction between mouse C_4 lactate dehydrogenase and pyridoxal 5'-phosphate*

Lactate dehydrogenase ($0.07 \text{ mg} \cdot \text{ml}^{-1}$) was incubated with 1 mM-pyridoxal 5'-phosphate and the various protecting agents, as described in the Experimental section. The residual activity after 90 min, when the inactivation reaction had clearly reached equilibrium, is given. All incubations were duplicated, with a maximum variability of 4% in the final activity measured at equilibrium.

Added protecting agent	Activity remaining at equilibrium (% of initial)
None	16–19
1 mM-NADH	99.8
1 mM-NAD ⁺	97.0
1 mM-ADP-ribose	77.3
4 mM-ADP-ribose	82.2
4 mM-ADP	70.9
4 mM-AMP	67.3
4 mM-NMN	16.4
10 mM-lactate	17.9
3 mM-AMP + 3 mM-NMN	80.8
1 mM-ADP-ribose + 10 mM-lactate	97.2

with coenzyme binding first, lactate gives no protection (Table 2). Lactate (10mM) and ADP-ribose (1mM) together, however, give essentially complete protection. This clearly implies the formation of a ternary enzyme-ADP-ribose-lactate complex, and thus indicates that the binding of the hydroxy acid substrate can be triggered by coenzyme fragments and does not require the complete NAD⁺ molecule.

As a consequence of the rapid formation of a non-covalent enzyme-modifier complex before covalent Schiff-base formation, pyridoxal 5'-phosphate acts as an 'instantaneous' non-covalent inhibitor of C₄ lactate dehydrogenase, as well as an inactivating agent. At pH 8.0 and 25°C, a concentration of 25 μ M-pyridoxal 5'-phosphate is sufficient to produce marked inhibition, and this concentration does not interfere with the fluorimetric assay. Inhibition appears to be simple non-competitive ($V_{\max}$ altered, but not K_m) with respect to lactate (Fig. 3a). With NAD⁺ as the varied substrate, both K_m and $V_{\max}$ are altered (Fig. 3b).

Fig. 3 also shows that mouse C₄ lactate dehydrogenase has a very low K_m for NAD⁺ of 6 μ M under the conditions used. Indeed, only the sensitivity of the fluorimeter made the experiment feasible. The K_m for lactate, 0.3mM, is also lower than those of the other isoenzymes under similar conditions. During these experiments, apparent substrate inhibition by lactate was observed at concentrations above 1mM.

The difference in $V_{\max}$ values for the two sets of data in Fig. 3 reflects this inhibition. C₄ lactate dehydrogenase has been reported as not showing substrate inhibition by lactate (Blanco *et al.*, 1976), in agreement with its proposed physiological function in lactate oxidation. These experiments were performed at pH 9 and the higher temperature of 37°C. In view of the known effect of temperature on K_m values of mouse C₄ lactate dehydrogenase (Goldberg, 1972; Wheat & Goldberg, 1975), rate measurements with high lactate concentrations were repeated at 37°C. Fig. 4 shows that, whereas at 25°C marked substrate inhibition occurs with lactate concentrations greater than 1mM, at 37°C under the same conditions no substrate inhibition is observed up to 10mM-lactate, and the apparent K_m for lactate is increased nearly 5-fold. This example clearly illustrates the hazards of extrapolating kinetic properties of an enzyme determined *in vitro*, under one set of conditions, to predict behaviour *in vivo* under different conditions.

Alcohol dehydrogenase, malate dehydrogenase and the three isoenzymes of lactate dehydrogenase all catalyse similar reactions, and their subunits have similar three-dimensional structures. In all cases, the kinetics of inactivation by pyridoxal 5'-phosphate indicate the formation of a non-covalent enzyme-modifier complex before covalent modification. As a consequence of complex-formation, pyridoxal 5'-phosphate acts as an inhibitor for alcohol dehydro-

Fig. 3. Inhibition of mouse C₄ lactate dehydrogenase by pyridoxal 5'-phosphate in steady-state initial-rate experiments. Lineweaver-Burk plots are shown of initial rates in 0.1M-phosphate buffer, pH 8.0 at 25°C, with (a) 190 μ M-NAD⁺ and varied concentrations of lactate in the presence (●) and absence (▲) of 25 μ M-pyridoxal 5'-phosphate, and (b) 10mM-lactate and varied concentrations of NAD⁺ in the presence (○) and absence (△) of 25 μ M-pyridoxal 5'-phosphate. The rates were measured in duplicate and averaged. Values of e_0/v were calculated from values of v (μ M $\cdot$ s⁻¹) and enzyme-subunit concentration, e_0 (μ M).

Fig. 4. Effect of temperature on substrate inhibition of mouse C₄ lactate dehydrogenase by lactate. Conditions used were as described for Fig. 3. Initial-rate measurements were made in duplicate with 190 μ M-NAD⁺ and varied concentrations of lactate at (a) 25°C (O) and (b) 37°C (Δ). Values of e_0/v were calculated as in Fig. 3.

genase (Chen & Engel, 1975c), malate dehydrogenase (Chen & Engel, 1975d) and all three lactate dehydrogenase isoenzymes (K. G. Gould & P. C. Engel, unpublished work). In all cases inhibition is non-competitive with respect to both coenzyme and substrate, suggesting that the initial binding of pyridoxal 5'-phosphate is not within the active site. Bound pyridoxal 5'-phosphate subsequently reacts reversibly with a lysine residue to form a Schiff base, and this causes enzyme inactivation. With malate dehydrogenase (Yost & Harrison, 1971; Wimmer *et al.*, 1975) and H₄ lactate dehydrogenase (Jeckel,

1975) there is the possibility of secondary, irreversible reaction of the Schiff base with a thiol group to form a thiazolidine derivative.

Although the initial binding site for pyridoxal 5'-phosphate probably does not lie within the coenzyme-binding site, it is clear that in the lactate dehydrogenase isoenzymes the reactive lysine residue is not available for modification in ternary complexes (Chen & Engel, 1975e). There appears to be a good correlation between complete protection against inactivation by pyridoxal 5'-phosphate and the formation of those ternary complexes in which the mobile loop is known to be folded inwards, enclosing the active site. In view of the evidence (Musick & Rossmann, 1979a) that the C₄ isoenzyme very readily adopts the 'loop-down' conformation even in the absence of coenzyme, it is noteworthy that for this isoenzyme complete protection against inactivation by pyridoxal 5'-phosphate is attainable in binary enzyme-coenzyme complexes. Further studies are now required to identify the 'essential' lysine residue in lactate dehydrogenase in order to clarify the basis of inactivation by pyridoxal 5'-phosphate.

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