Dogfish M₄ Lactate Dehydrogenase: Reversible Inactivation by Pyridoxal 5'-Phosphate and Complete Protection in Complexes that Mimic the Active Ternary Complex

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Dogfish M₄ lactate dehydrogenase, like the corresponding pig enzyme, is inactivated by pyridoxal 5'-phosphate through modification of a single essential lysine residue. The activity is completely protected in the complexes E-NAD⁺-oxalate, E-NADH-oxamate and E-(NAD⁺-pyruvate adduct), but only partially protected in E-NAD⁺, E-NADH, E-NAD⁺-oxamate and E-NADH-oxalate.

Pig muscle M₄ lactate dehydrogenase (EC 1.1.1.27) is reversibly inactivated by the reaction of pyridoxal 5'-phosphate with an essential lysine residue (Chen & Engel, 1975b). This covalent modification is completely prevented by the addition of NAD⁺ and pyruvate, which form an adduct tightly bound at the active site of the enzyme (Arnold & Kaplan, 1974, and references cited therein). Identification of the essential lysine residue might give a clearer indication of its likely role, but lactate dehydrogenase from dogfish muscle is the obvious choice for such work, since it has been used for most of the structural studies (Adams *et al.*, 1973). It is important as a first step, therefore, to establish that the dogfish and pig lactate dehydrogenases react similarly with pyridoxal 5'-phosphate.

In this present study of the reaction of dogfish muscle lactate dehydrogenase with pyridoxal 5'-phosphate we have paid special attention to the extent of protection of the enzyme in its various ternary complexes.

Materials and methods

Dogfish (*Squalus acanthius*) M₄ lactate dehydrogenase, purified at the final stage by affinity chromatography on an AMP-Sepharose column (Lowe & Dean, 1974), was a gift from Dr. P. D. G. Dean, University of Liverpool, Liverpool, U.K. Coenzymes were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Lithium lactate and sodium oxamate were products of Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Sodium pyruvate and oxalic acid were supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other materials and procedures were as described previously (Chen & Engel, 1975b).

Results and discussion

Incubation of dogfish M₄ lactate dehydrogenase with 2mM-pyridoxal 5'-phosphate in 0.05M-sodium

pyrophosphate buffer, pH 8.5, at 25°C led to a gradual decline in catalytic activity, and within 20 min a steady value of residual activity, 20% of the initial value, was attained. Higher extents of inactivation could not be achieved by increasing the concentration of pyridoxal 5'-phosphate. Dialysis fully restored catalytic activity. When the modified enzyme was reduced with NaBH₄, however, the inactivation became irreversible. This is expected if the inactivation involves reaction of the ε-amino group of a lysine residue with the aldehyde function of the modifier to yield a Schiff base. The absorption spectrum of the enzyme after irreversible inactivation and dialysis indicates (Fischer *et al.*, 1963) that 3.8 mol of lysine/mol of enzyme subunit reacts in this way.

The effects of the chemical modification on the kinetic properties of the enzyme were studied. Initial rates of reaction in 0.1M-potassium phosphate buffer, pH 8, at 25°C were measured with varied NAD⁺ concentrations (22–220 μM with 5 mM-lactate) and with varied lactate concentrations (2–20 mM with 110 μM-NAD⁺). Within experimental error the apparent *K_m* values for NAD⁺ (330 μM) and lactate (38 mM) were identical for native enzyme and for enzyme irreversibly inactivated as described above and dialysed. The corresponding apparent maximum velocities of reaction were decreased fivefold by the chemical treatment. An enzyme sample dialysed without NaBH₄ reduction after treatment with pyridoxal 5'-phosphate gave the same kinetic constants as the native enzyme.

Qualitatively these results are very similar to those obtained with pig M₄ lactate dehydrogenase, although the extent of inactivation achieved in a single treatment with pyridoxal 5'-phosphate (70%) was somewhat less for the pig enzyme. The initial-rate measurements indicate that the chemical modification converts native enzyme into a totally inactive form.

As for several other dehydrogenases (see references cited in Chen & Engel, 1975b) the likely explanation of the persistence of activity even after incubation with pyridoxal 5'-phosphate at saturating concentrations is that the inactive species is in equilibrium with

a rapidly dissociable enzyme-pyridoxal 5'-phosphate complex. If so, a second treatment with pyridoxal 5'-phosphate after reduction and dialysis should further inactivate the enzyme (Chen & Engel, 1975a). Three successive treatments of dogfish M_4 lactate dehydrogenase with 2mM-pyridoxal 5'-phosphate decreased the activity to 19.6%, 4.2% and finally 1.5% of its initial value. The value of 4.2% is very close to 19.6% of 19.6% (i.e. 3.8%). Such a geometric progression of inactivation is theoretically predicted if a single essential lysine residue is modified in each inactivated enzyme subunit. This pattern has also been observed in the reactions of pyridoxal 5'-phosphate with bovine glutamate dehydrogenase and horse alcohol dehydrogenase (Chen & Engel, 1975c), but is less closely followed in the case of pig M_4 lactate dehydrogenase, implying, perhaps, interference by other non-essential lysine residues (Chen & Engel, 1975b).

The substrates, lactate and pyruvate, and their inhibitory analogues, oxalate and oxamate, at concentrations up to 10mM, give no protection against inactivation when included in incubations of dogfish M_4 lactate dehydrogenase with pyridoxal 5'-phosphate (Table 1). Even at saturating concentrations, the coenzymes NAD^+ and NADH gave only partial protection, resulting in residual activities of 55.8 and

75.5% respectively at equilibrium (Table 1). These findings closely match those for the pig M_4 enzyme, and their significance is discussed in our earlier paper (Chen & Engel, 1975b).

It is known that lactate dehydrogenase undergoes a marked conformational change in the transition from a binary enzyme-coenzyme complex to a ternary complex (Rossmann *et al.*, 1972). It was therefore decided to investigate whether the essential lysine residue is more completely protected in ternary than in binary complexes. Table 1 shows that inactivation by 2mM-pyridoxal 5'-phosphate was completely prevented by 0.5mM-NADH with 8mM-oxamate, and by 4mM- NAD^+ with 8mM-oxalate. On the other hand, oxamate did not augment the protection given by NAD^+ alone, nor did oxalate augment that given by NADH alone. Kinetically, oxalate and oxamate behave as analogues of lactate and pyruvate respectively (Winer & Schwert, 1959; Novoa *et al.*, 1959; Novoa & Schwert, 1961). Thus it appears that the essential lysine residue is fully protected only in complexes that mimic the productive ternary complex.

Rossmann *et al.* (1972) have shown that crystals of enzyme-NADH-oxamate and of enzyme- NAD^+ -oxalate are isomorphous with those of the complex formed by the enzyme with the inhibitory pyruvate- NAD^+ adduct, but not with crystals of unliganded enzyme. In all three complexes the loop of residues 100-120 is folded down. One might therefore expect complete protection by the pyruvate- NAD^+ adduct in our system. The degree of protection by this compound, however, cannot be ascertained simply by assaying the equilibrium mixture, because the adduct itself inactivates the enzyme. Consequently the extent of inactivation by pyridoxal 5'-phosphate in the presence of the adduct can only be determined by reducing the equilibrated inactivation mixture with $NaBH_4$ and dialysing. Under these conditions only the inactivation by the pyruvate- NAD^+ adduct is reversed. Table 2 shows the results of a protection study of this type. Absorption measurements at 327nm (Fischer *et al.*, 1963) allow correlation of the extent of inactivation with the stoichiometry of incorporation of pyridoxal 5'-phosphate in the presence and in the absence of protecting agents.

The combination of NAD^+ (4mM) and pyruvate (4mM) completely protected dogfish M_4 lactate dehydrogenase against inactivation by 4mM-pyridoxal 5'-phosphate (Table 2). Formation of the pyruvate- NAD^+ adduct under these conditions was established in separate experiments in which the enzyme was incubated with pyruvate and NAD^+ in the absence of pyridoxal 5'-phosphate. In preserving the 80% of the activity that would otherwise have been lost, the adduct also protected approx. 0.8 mol of lysine/mol of enzyme subunit. This again implies that modification of a single essential lysine residue per subunit is

Table 1. *Effects of coenzymes and substrates or substrate analogues on the equilibrium position of the inactivating reaction between dogfish M_4 lactate dehydrogenase and pyridoxal 5'-phosphate: activity measured without $NaBH_4$ reduction*

Conditions were as follows. Dogfish M_4 lactate dehydrogenase (0.02mg/ml) was incubated with 2mM-pyridoxal 5'-phosphate at 25°C in 0.05M-sodium pyrophosphate buffer, pH 8.5, with the additions listed. At timed intervals 5 μ l samples were taken for activity assays. The residual activity after 60min, when the inactivation reaction had clearly reached equilibrium, is given below.

Addition	% of initial activity remaining at equilibrium
None	20.5
10mM-Lactate	20.5
10mM-Pyruvate	19.5
10mM-Oxalate	22.1
10mM-Oxamate	20.3
1mM- NAD^+	50.5
4mM- NAD^+	54.2
4mM- NAD^+ + 8mM-oxamate	55.8
4mM- NAD^+ + 8mM-oxalate	100.0
0.1mM-NADH	74.6
0.5mM-NADH	75.5
0.5mM-NADH + 8mM-oxamate	100.0
0.5mM-NADH + 8mM-oxalate	74.8
0.5mM-NADH + 8mM-lactate	75.5

Table 2. *Effects of coenzymes, substrates and substrate analogues on the reaction between dogfish M₄ lactate dehydrogenase and pyridoxal 5'-phosphate: activity and stoichiometry of lysine modification measured after NaBH₄ reduction*

Conditions were as follows. Dogfish M₄ lactate dehydrogenase (1mg/ml) was incubated with 4mM-pyridoxal 5'-phosphate and the protecting agents indicated in 0.05M-sodium pyrophosphate buffer, pH8.5, at 25°C for 30min, reduced with NaBH₄ and dialysed. The number of lysine residues modified was estimated from the optical absorbance at 327nm (see the text). A control sample incubated as described but in the absence of pyridoxal 5'-phosphate retained full activity.

Added protecting agent	Residual activity (%)	Lysine residues modified (mol/mol of enzyme subunit)
None	19.6	3.8
4mM-NAD ⁺	50.7	3.5
4mM-NAD ⁺ + 4mM-pyruvate	98.3	3.0
4mM-NAD ⁺ + 4mM-oxamate	55.9	3.4
1mM-NADH	76.2	3.3
1mM-NADH + 4mM-oxamate	101.6	2.9
8mM-Pyruvate	20.2	3.8

responsible for the inactivation by pyridoxal 5'-phosphate.

The other results in Table 2 confirm those of Table 1 with respect to the lack of protection by pyruvate, the partial protection by NAD⁺ and NADH, and the complete protection by NADH plus oxamate. In all cases there was approximately a one-to-one relation between the number of active subunits protected and the decrease in the number of lysine groups modified.

In conclusion, it appears that in dogfish M₄ lactate dehydrogenase, as in the pig M₄ enzyme, the modification of a single lysine residue by pyridoxal 5'-phosphate abolishes catalytic activity. This residue is not indispensable for coenzyme binding, since

neither coenzyme protects it completely. On the other hand, the formation of ternary complexes analogous in structure to the productive ternary complex involved in catalysis prevents modification of the essential lysine residue. This is consistent with a catalytic role for the essential residue or an involvement in substrate binding, but other interpretations are also possible (Chen & Engel, 1975b). Although the modifier reacts with other, non-essential, lysine residues, the possibility of specific and complete protection of the essential residue should facilitate its labelling and identification. This in turn should permit a better-informed guess as to the function of the essential lysine residue.

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