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COMPARISON OF LACTATE AND MALATE DEHYDROGENASES: FLUORESCENCE AND THERMODYNAMIC PROPERTIES[1]

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SUMMARY - Equilibrium, thermochemical, and time-resolved fluorescence measurements have been carried out in order to compare pig heart lactate dehydrogenase (LDH) and cytoplasmic malate dehydrogenase (MDH). The differences in the thermodynamic parameters for binding of NADH and NAD+ show the same pattern for both enzymes. The stronger binding of NADH is entropy-based, which can be understood as reflecting electrostatic interactions. The tryptophan fluorescence of MDH and LDH differ for the free enzymes and in quenching by NADH. The differences can be accounted for in terms of a single long-lived tryptophan residue present in LDH and not in MDH.

INTRODUCTION - Considerable structural and chemical information is available for lactate dehydrogenase (LDH) and cytoplasmic malate dehydrogenase (MDH). High-resolution crystal structure results have been obtained for both enzymes (1-3), amino acid sequences are available for several lactate dehydrogenases (4), and the sequence of pig heart cytoplasmic malate dehydrogenase is under investigation (5). LDH and MDH are homologous (6), and the reactions catalyzed by these enzymes are closely similar with regard to substrate and mechanism (1-2, 7). A careful examination of the correspondences between the enzymatic and other properties of LDH and MDH should be fruitful in identifying chemical features common to both enzymes and thus likely to be of particular importance.

Attention is focused in this paper on the differences in binding of the cofactors NADH and NAD+ and on the fluorescence behavior of the free proteins and the NADH complexes. Both MDH and LDH bind NADH ca 1000 times more tightly than NAD+. Cofactor release can be rate-determining in both directions of the

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enzyme reaction (1, 7), for which slow isomerizations of the binary complexes are important (1). Measurements of the steady-state fluorescence of LDH and MDH have defined a principal difference between these proteins, viz. the quenching of tryptophan fluorescence by NADH is linear for MDH (8-9) and nonlinear for LDH (10), which has been attributed to differences in quenching mechanism (8).

EXPERIMENTAL - Pig heart supernatant MDH was prepared by the method of Glatthaar et al. (11), modified by introduction of chromatography on hydroxylapatite. Charcoal treatment was used to remove tightly-bound UV-absorbing material. Pig heart LDH was prepared by the method of Reeves and Fimognari (12), with the enzyme being treated similarly with charcoal before use. NAD+ and NADH were obtained from P-L Biochemicals and for most experiments were further purified (9-10). Concentrations were determined spectrophotometrically, using the molar extinction coefficients ε_{340} = 6,320 for NADH, ε_{259} = 17,800 for NAD, ε_{280} = 194,000 for LDH, and ε_{280} = 72,000 for MDH. Solutions were of ionic strength 0.3, adjusted with NaCl, and contained 0.05 M sodium phosphate for pH near 6 or 0.05 M Tris chloride for pH near 8.

Heats of reaction were measured using an LKB flow microcalorimeter, with solutions pumped at ca 0.15 ml/min. Protein concentrations were 5 to 30 mg/ml. Appropriate corrections were applied for the heats of dilution of cofactor and salt solutions. There was no significant heat of dilution for enzyme solutions.

Steady-state fluorescence measurements were made with an Aminco-Bowman spectrofluorimeter or with a laboratory-built device. Equilibrium constants for binding of NADH were determined by fluorescence titrations. Corrections were applied for inner filter and related effects.

Excitation for time-resolved fluorescence measurements was with an ADP-doubled N_2 dye laser. The fluorescence decay data were analyzed assuming a two exponential decay: $I(t) = a_1 \cdot \exp(-t/\tau_1) + a_2 \cdot \exp(-t/\tau_2)$, where $I(t)$ is the fluorescence intensity at time t after excitation and a_1 and a_2 are scaling factors related to the number of emitters with lifetimes τ_1 and τ_2, respectively. The parameters a_1, a_2, τ_1, and τ_2 were determined by least squares fitting to an experimental decay curve, using convolution of the above equation with the experimental pulse shape and routines based on simplex or truncated Taylor's series methods. For further details on data reduction and experimental procedures, see Torikata et al. (13) and Johnson and Rupley (14).

RESULTS AND DISCUSSION - Thermodynamics. Table I contains pairs of values of the thermodynamic parameters for cofactor binding to MDH and LDH, given in that order. The third row of values of Table I are differences in thermodynamic parameters, calculated as those for NADH binding less those for NAD+ binding. The results given are for pH 6, where no large change in protons bound is associated with cofactor binding. At pH 8 binding of NADH to MDH is associated with uptake of 0.55 moles H+/mole NADH, compared with 0.19 at pH 6 (14). Proton effects for

TABLE I. COMPARISON OF PROPERTIES OF PIG HEART LDH AND CYTOPLASMIC MDH.[a]

Thermodynamics	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/K mol)	ΔCp (cal/K mol)
NADH	-8.3, -7.7	-10.1, -9.7	- 6.0, - 6.7	-150, -200
NAD+	-3.8, -4.6	- 9.3, -8.6	-18.5, -13.4	-290, -150
(NADH-NAD+)	-4.5, -3.1	- 0.8, -1.1	+12.5, + 6.7	+140, - 50

Fluorescence Lifetimes	a_1	τ_1 (ns)	a_2	τ_2 (ns)
free enzyme	0.7, 0.55	1.0, 1.0	0.3, 0.45	4.4, 6.55
NADH complex	0.7, 0.75	0.8, 0.55	0.3, 0.25	2.0, 2.3

[a]The first value of each pair is for MDH, the second for LDH. All measurements were in 0.05 M sodium phosphate buffer of pH 6.0 and ionic strength 0.3, adjusted with NaCl, except the fluorescence measurements for free LDH, for which 0.05 M sodium acetate buffer of ionic strength 0.3 was used. Estimated uncertainties, assuming no error in enzyme concentrations, are: ΔG, .2 kcal/mol; ΔH, .3 kcal/mol; ΔCp, 40 cal/K mol; a_i, 0.1; τ_1, 0.2 ns; τ_2, 0.1 ns. Values of a_i are normalized to unit sum.

LDH are smaller than for MDH but also are significant at pH 8. The enthalpy values of Table I were corrected for the small heats of proton transfer to buffer at pH 6. Calorimetric measurements for pig heart LDH (15), mitochondrial MDH (16, 17), and rabbit muscle and beef heart LDH (16, 17), have given ΔH values between -6.1 and -10.2 kcal/mol for NAD+ and -6.9 and -12.1 kcal/mol for NADH binding at 25 °C. Differences between the values for LDH in the literature and those of Table I can be understood as reflecting buffer effects, which were not measured in the previous work. The free energy values of Table I are in agreement with recent literature values for LDH (10) and MDH (7-9, 18), after allowing for the differences in pH and ionic strength of the various measurements.

It is remarkable that the 3-5 kcal/mol difference in free energy of binding of NADH and NAD+ is entropy-based, with the enthalpy change being less than half the size of the free energy effect. Furthermore, NADH binding to MDH is weaker at pH 8 by 1 kcal/mol (14), and this is also an entropy-based effect, with the difference in ΔH being of opposite sign to that in ΔG.

We believe that the effect of pH on NADH binding to MDH and the differences in binding of NADH and NAD+ have origin in the same or similar chemistry. It is important that these differences in thermodynamics are not a reflection of substantial differences in contacts between cofactor and enzyme. This has been shown by crystallographic analysis of the complexes of LDH with NADH and NAD+ (1), on the one hand, and by the steady-state and time-resolved fluorescence properties of the NADH-MDH complex, which are identical at pH 6 and 8, including the quenching of fluorescence by NADH (13).

The thermodynamic data of Table I cannot be explained by possible changes in hydrogen-bonding or hydrophobic interactions. With regard to the latter, there is no substantial difference between the NAD+ and NADH complexes in exposure of nonpolar groups to solvent. The heat capacity values (Table I) for NADH and NAD+ binding show no pattern, comparing MDH and LDH, and are relatively small, indicating the absence of the hydrophobic contribution and supporting the description of stronger binding of NADH as entropy-based.

A satisfactory explanation of the differences in cofactor binding may be found in charge interactions. Support for electrostatic interactions of macromolecules being entropy-based comes from the observations of entropy-based binding of ions to carbonic anhydrase (19) and to nucleotides and nucleic acids (20). Crystallographic and sequence studies of LDH (1, 4) have identified charged groups of the protein near the substrate and the nicotinamide ring, including a histidine and several arginine side chains. There is a ring of negative charges of aspartate and glutamate residues, for which the environment is altered when a loop of the LDH polypeptide chain moves during ternary complex formation (4). Crystallographic results for MDH show that the conformation of the bound NAD+ is similar to that for LDH (2).

The identification of the same pattern of thermodynamic values for MDH and LDH emphasizes the mechanistic importance of the difference in strength of binding of cofactors. Studies on model dihydronicotinamide reactions have shown the sensitivity of reaction rate to charge environment (21).

Fluorescence. Steady-state fluorescence measurements have shown that MDH and LDH differ in tryptophan emission and its quenching by NADH (8, 10). Time-resolved fluorescence measurements allow a detailed interpretation of these differences. Table I gives values of the decay parameters of equation (1) for LDH and MDH, as the free enzymes and the complexes with NADH.

The principal points describing the properties of the free enzymes are, first, the ratio of the pre-exponential factors (a_2/a_1) is ca 0.5 for MDH and 1 for LDH. Secondly, there is a particularly long-lived emitter in LDH of ca 8 ns lifetime that is absent from MDH. The 8 ns emitter, detected by analysis of the long-time decay, is seen in the value of τ_2 for LDH, which is 1.5 times that for MDH.

With regard to the effect of NADH on the tryptophan emission, the lifetime results show, first, that the short-lived emitters are not significantly affected by cofactor binding. Secondly, the fluorescence decay properties are the same for LDH and MDH when NADH is bound. Thirdly, the dependence of the decay parameters

a_2 and τ_2 on extent of complexation with NADH is linear for MDH and nonlinear for LDH. The nonlinear dependence of the parameters for LDH reflects strong (static) quenching of the 8 ns component. Thus the binding of NADH to one subunit of the LDH tetramer quenches the fluorescence of the tryptophan with 8 ns lifetime both within the same subunit and in one adjacent subunit.

These results can be described by a relatively simple model. There is only one tryptophan of LDH that can be subject to inter-subunit quenching, judging from the distances determined by the crystallographic results (1). This residue is Trp-248, which must have 8 ns emission lifetime and must be strongly quenched by binding of NADH. LDH has six tryptophan residues. Two of the remaining five are moderately long-lived of ca 4 ns lifetime, and three are short-lived of ca 1 ns lifetime. The differences in both the steady-state and the time-resolved results for MDH and LDH are explained if Trp-248 of LDH is not present in MDH, which has five tryptophan residues. Since this emitter is strongly quenched by NADH, the close similarity of the decay properties of the NADH complexes of MDH and LDH is explained. Deletion of the 8 ns contribution to the long-lifetime averaged emission properties (a_2 and τ_2 of Table I) gives similar values for MDH and LDH.

The point to be emphasized is that there is near identity of the fluorescence properties of LDH and MDH, if allowance is made for the contribution of one tryptophan that emits with 8 ns lifetime in LDH but does not emit in MDH. The general structural homology between MDH and LDH seen in the crystallographic results (6) is extended by the observation of similarity in a sensitive physical property, the time-resolved fluorescence.

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