Binding of Reduced and Oxidized Nicotinamide Adenine Dinucleotide to Pig Heart Supernatant Malate Dehydrogenase†

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ABSTRACT: The association reactions of NADH and NAD+ with dimeric pig heart supernatant malate dehydrogenase (s-MDH) have been measured at pH 6 and 8 by calorimetric and fluorescence methods, and the thermodynamic parameters describing these reactions have been evaluated. Coenzyme binding is associated with the uptake of 0.55 mol of H^+/mol of NADH at pH 8 and 0.19 mol of H^+ at pH 6. No significant effect of NAD+ binding on proton binding was observed. Increase in ionic strength strongly affects the free energies of binding of NAD+ and NADH. No cooperativity was observed in the enthalpy or free energy changes for binding of NAD+ or NADH. The differences in free energy of binding of NAD+ and NADH and the effect of pH on binding of NADH are entropy based. These effects are interpreted as reflecting a small number of interactions within the active site that are predominantly ionic.

The binding of the coenzymes NAD+ and NADH to dehydrogenases is of interest because the rate-limiting step in the enzyme-catalyzed reactions is often a coenzyme dissociation process that may be coupled to a conformational change (Frieden & Fernandez-Sousa, 1975; Whitaker et al., 1974) and because the binding of NAD+ and NADH to multisubunit enzymes sometimes exhibits negative cooperativity (Velick et al., 1971), which is suggestive of an allosteric control mechanism.

In the case of pig heart supernatant malate dehydrogenase (s-MDH),[1] which is a dimer, there has been uncertainty about the existence of subunit cooperativity. The structure of a crystalline s-MDH–NAD+ complex, determined at 2.5 Å (Webb et al., 1973), showed that the occupancy by NAD+ of the binding site of one subunit was different from that for the other subunit. A study by Mueggler et al. (1975) showed that the binding of NADH to s-MDH was not cooperative except in the presence of excess L-malate. This result has been disputed recently by Lodola et al. (1978), who found no evidence for cooperativity in the binding of NADH in the presence or absence of L-malate.

In order to clarify the coenzyme binding properties of s-MDH in particular and of dehydrogenases in general, we have carried out calorimetric and fluorescence measurments of the binding of NADH and NAD+ to s-MDH at two pHs and have measured the proton uptake associated with these binding reactions. Because enthalpy changes are often more sensitive to changes in the chemistry of a process than are free energy changes, heat measurements can be particularly useful for probing the thermodynamics of an enzyme reaction.

Experimental Procedures

Materials. Pig heart supernatant malate dehydrogenase (s-MDH) was prepared by the method of Glatthaar et al. (1974). The s-MDH used in initial experiments was further purified by recrystallization twice at pH 5 (Wade, 1972). For most preparations, the acid precipitation and CM-cellulose steps of Glatthaar et al. (1974) were dropped, and chromatography on hydroxylapatite (Eberhardt, 1972) was added as a final step. The first two of the three peaks of activity to be eluted from the hydroxylapatite column were collected separately and used in the binding studies. We detected no difference in thermodynamic properties between them. All s-MDH solutions were treated with charcoal in buffer of ionic strength 0.3 immediately before use, in order to remove variable amounts of tightly bound UV-absorbing material. The s-MDH was stored as a precipitate in 90% saturated $(NH_4)_2SO_4$ solution of pH 7, with 1 mM Na_2EDTA and 1 mM β-mercaptoethanol. There was less than a 20% decrease in specific activity over a period of more than 1 year. All preparations of s-MDH yielded a single band of protein when subjected to disc gel electrophoresis by the procedure of Dietz & Lubrano (1967). A value of $E_{280}^{1\%} = 9.8$ for s-MDH was obtained from dry-weight determinations on deionized protein (Dintzis, 1952). The deionized protein is unstable. Molar concentrations were calculated by assuming a dimer of 73 500 daltons (Kar & Aune, 1974). All NAD+ and NADH (ChromatoPure Grade) were obtained from P-L Biochemicals. In proton-binding experiments, the coenzymes were used without further purification. Most calorimetric experiments and all fluorescence experiments were performed with NAD+ purified by the method of Stinson & Holbrook (1973) and with NADH purified by the method of Mueggler et al. (1975) or by a modification of the latter by using a linear gradient from 0.05 M Tris-HCl (pH 8), 0.1 M NaCl to 0.05 M Tris-HCl (pH 8), 0.2 M NaCl. Purified NAD+ solutions had an absorbance ratio (280/260) of 0.196–0.199, and purified NADH solutions had an absorbance ratio (260/340) of 2.25–2.28. Concentrations were determined by using ϵ_{340} 6320 for NADH (McComb et al., 1976) and ϵ_{259} 17 800 for NAD+ (Siegel et al., 1959). Reagent grade L-malate from Sigma and L-malic acid from Schwarz/Mann were used without further purification. All other chemicals were of reagent grade, and all solutions were prepared with deionized water.

Assay of MDH Activity. The assay medium contained 0.18 mM NADH, 0.13 mM oxalacetate, 0.05 M Tris-HCl (pH 7.4), 0.25 M NaCl, 1 mM Na_2EDTA, 1 mM β-mercaptoethanol, and 1 mg/mL bovine serum albumin. Assays were done at 30 °C, and the reaction was monitored by the change in A_{340}. One unit of enzyme catalyzed the oxidation of 1 μmol of NADH/min. Purified s-MDH had a specific activity of 740 units/mg of protein.

Solution Preparation. A few experiments bearing on the stoichiometry of the reaction were done in low-salt buffer (0.05

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[1] Abbreviations and symbols used: s-MDH, pig heart supernatant malate dehydrogenase; LDH, pig heart lactate dehydrogenase; ΔH_{app}, measured heat effect for complex formation per mole of s-MDH active site; ΔH_{corr}, ΔH_{app} corrected for the heat of buffering of protons released or taken up by the protein in complex formation; $\Delta\gamma_{H^+}$, moles of protons taken up per mole of active site in complex formation.

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M potassium phosphate, pH 6.5). All other binding experiments were done in one of two buffer systems of ionic strength 0.3: (a) 0.05 M NaH_2PO_4, 0.25 M NaCl solution titrated with 0.05 M Na_2HPO_4, 0.15 M NaCl solution to pH 6 (pH 5.99 measured at 25 °C for experiments done at 10 °C); (b) 0.05 M Tris-HCl, 0.25 M NaCl solution titrated to pH 8 with concentrated NaOH (pH 7.58 for experiments done at 10 °C). Solutions of s-MDH, generally 5–10 mg/mL, were prepared by dissolving the $(NH_4)_2SO_4$ precipitate in buffer and dialyzing exhaustively against buffer at 4 °C. Stock solutions of NAD^+ and NADH were made by either dissolving the unpurified coenzyme in buffer or by adding appropriate amounts of buffer salts or NaCl or both to the pooled fractions of purified coenzyme, to obtain the desired pH and ionic strength. Stock solutions of L-malate were made by titrating 3 M malic acid solutions to the desired pH with concentrated NaOH.

Calorimetric Measurements. The steady-state heat flow was determined for continuous mixing of a stream of s-MDH with a stream of buffer containing NADH or NAD^+. Measurements were made with an LKB microcalorimeter, thermostated at 10 or 25 °C and calibrated by using either the internal heater or the heat of dilution of sucrose (Gucker et al., 1939). Flow rates were determined on the day of the experiment by weighing the amount of water pumped out of a vessel in 1 h. The combined flow rate for both solutions was about 20 mL/h. Small corrections were made for the heats of dilution of solutions containing more than about 1 mM coenzyme. s-MDH solutions did not give a measurable heat of dilution.

Fluorescence Titrations. Fluorescence titrations of s-MDH with NADH, performed to measure the equilibrium binding of the cofactor, were carried out with an Aminco-Bowman instrument thermostated at 25 ± 0.1 °C, with excitation at 340 nm and emission monitored at 440 nm. Correction was made for absorption of exciting light.

Proton Binding Measurements. The uptake of protons associated with the binding of NADH and NAD^+ to s-MDH was measured with a Radiometer Model 11 titrator, used as a pH-stat in conjunction with a Radiometer Model 26 pH meter and a mechanically driven 50-μL syringe. The s-MDH solution was dialyzed for several hours at room temperature against an unbuffered 0.3 M NaCl solution. Protein solution (3 mL) was placed in a water-jacketted vessel thermostated at 25 ± 0.2 °C, equipped with magnetic stirring and containing the pH electrode. Aliquots (50 μL) of concentrated CO_2-free NADH or NAD^+ solutions were added manually, and the amount of 0.01 N HCl needed to return the solution to the original pH was recorded. After each experiment, titrations of measured volumes of 0.01 M Tris were used to determine the number of equivalents of H^+ per unit response of the recorder. No attempt was made to remove CO_2 from the s-MDH solution. Base-line rates of acid uptake were recorded before and after coenzyme addition, allowing an appropriate correction to be made. s-MDH was saturated after addition of only one or two aliquots of NADH, so that subsequent aliquots served as a measure of any nonspecific proton effects associated with NADH addition. It was not practical to fully saturate s-MDH with NAD^+; thus, the association constant determined from the calorimetric measurements was assumed in calculating the proton change at saturation.

Data Analysis. Data from the thermal and fluorescence titrations were analyzed by assuming independent binding sites for the ligand, according to the equation:

$$X = \frac{X_{max}[L]_{free}}{K_d + [L]_{free}} \quad (1)$$

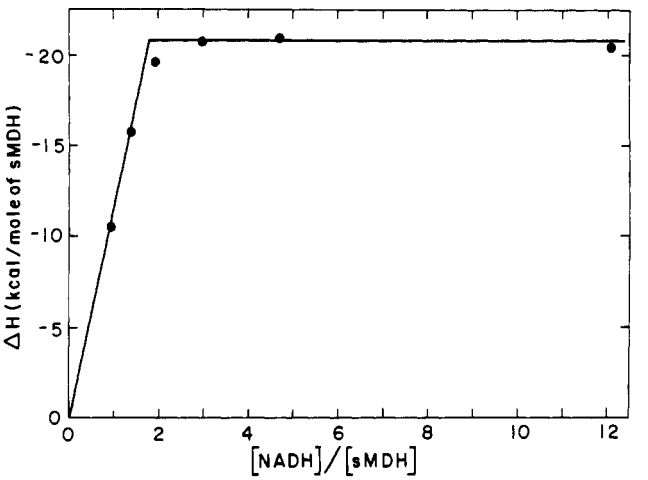

FIGURE 1: Thermal titration of s-MDH with NADH at 25 °C in 0.05 M potassium phosphate buffer (pH 6.5); [s-MDH dimer] = 3.6×10^{-5} M after mixing. Least-squares analysis of these data yielded an end point, indicated by the intersection of the solid lines, of 1.9 ± 0.1 NADH/s-MDH. This analysis also yielded $\Delta H_{max} = -11.3$ ± 0.6 kcal/mol of s-MDH monomer and $K_d = 0.4 \pm 0.6$ μM^{-1}.

where $[L]_{free}$ is the concentration of unbound ligand, X is either the change in heat content per mole of s-MDH monomer from the calorimetric experiments or the corrected fluorescence enhancement from the fluorescence experiments, X_{max} is the value of X at saturation, and K_d is the dissociation constant. A nonlinear least-squares fitting procedure (Nelder & Mead, 1965) was used to determine the values of K_d and X_{max} which gave the best fit to the data. Since only $[L]_{total}$ is known, the computer program used a Newton–Raphson approximation to calculate $[L]_{free}$ from $[L]_{total}$ and the current parameter estimates. For fits of the calorimetric and fluorescence titration data, the difference between $[L]_{total}$ and $[L]_{free}$ was 10% or less.

Results

Selection of Model. Because of conflicting reports in the literature, a primary concern was to determine the number of binding sites per s-MDH dimer and to measure the degree of cooperativity, if any, exhibited by NAD^+ or NADH in binding to s-MDH. Figure 1 shows the results of a calorimetric titration of s-MDH with NADH.[2] The binding of NADH is sufficiently strong that, at the concentrations of s-MDH used in these calorimetric measurements, essentially all added NADH is bound. Accordingly, the intersection of the straight line regions of Figure 1 corresponds to an end point of 1.9 ± 0.1 sites/s-MDH dimer, from the best least-squares fit to the data. This result confirms that both subunits bind NADH. The result also is a measure of the accuracy of the s-MDH concentration calculated from absorbance measurements. Because of the range of reported values of $E_{280}^{1\%}$, from 9.3 (Gerding & Wolfe, 1969) to 15 (Lodola et al., 1978), an independent check of binding site concentration was necessary. The origin of the variability in $E_{280}^{1\%}$ may be the tightly bound, UV-absorbing material mentioned in the Experimental Procedures section and removed by charcoal treatment in these experiments.

[2] In order to minimize electrostatic effects and variations in ionic strength, experiments were done routinely in buffers of ionic strength 0.3. However, because high salt was discovered to weaken coenzyme binding, some experiments were done in a low salt buffer (0.05 M potassium phosphate, pH 6.5), in order to sharpen the break in titrations with NADH (Figure 1) and to allow saturation at accessible NAD^+ concentrations (Figure 2).

Table I: Thermodynamics of Coenzyme Binding to Pig Heart Supernatant Malate Dehydrogenase

conditions	coenzyme	K_d (M^{-1})	ΔH_{app} (kcal/mol of monomer)	$\Delta\gamma_{H^+}$[k] (mol/mol of monomer)	ΔH_{corr}[j] (kcal/mol of monomer)
pH 6:[a] 10 °C	NADH		-7.9 ± 0.2[e] (3)[g]		
25 °C	NADH	(7.8 ± 0.2)[c] $\times 10^{-7}$ (6)[d]	-10.1 ± 0.5[c] (10)[g]	0.19	−10.1
10 °C	NAD$^+$	(6.9 ± 0.9)[e] $\times 10^{-4}$ (1)[f]	-5.0 ± 0.2[e,h]		
25 °C	NAD$^+$	(1.7 ± 0.3)[e] $\times 10^{-3}$ (2)[f]	-9.3 ± 0.7[e,h]	0.06	−9.3
pH 8:[b] 10 °C	NADH		-3.8 ± 0.2[e] (2)[g]		
25 °C	NADH	(6.9 ± 0.3)[e] $\times 10^{-6}$ (4)[d]	-5.7 ± 0.2[e] (8)[g]	0.55[i]	−12.0
25 °C	NAD$^+$	(2.3 ± 0.3)[e] $\times 10^{-3}$ (2)[f]	-7.1 ± 0.5[e,h]	0.02	−7.3

[a] 0.05 M $NaPO_4$, $\mu = 0.3$ with NaCl. [b] 0.05 M Tris-HCl, $\mu = 0.3$ with NaCl. [c] Standard error of the mean of parameter estimates from separate experiments. [d] Fluorescence titrations (number of experiments in parentheses). [s-MDH] ranged from 2.3 to 3.5 $\times 10^{-7}$ M at pH 6 and 1.1 to 2.5 $\times 10^{-6}$ M at pH 8. [e] Standard error of the parameter estimate from least-squares fitting of combined data from several experiments. [f] Thermal titrations (number of experiments in parentheses). [s-MDH] after mixing ranged from 8 $\times 10^{-5}$ to 1.5 $\times 10^{-4}$ M. [g] Determined by reaction with 2- to 20-fold excess NADH and extrapolated, if necessary, to saturation by using K_d from fluorescence titration. Number of measurements in parentheses. [h] Determined as one of the parameters from the fit of the thermal titrations. [i] Average value of three experiments. [j] Calculated by using $\Delta H = 0.25$ kcal/mol for $NaPO_4$ and 11.35 kcal/mol for Tris-HCl buffer (Izatt & Christensen, 1970). [k] Estimated uncertainty is ±0.05.

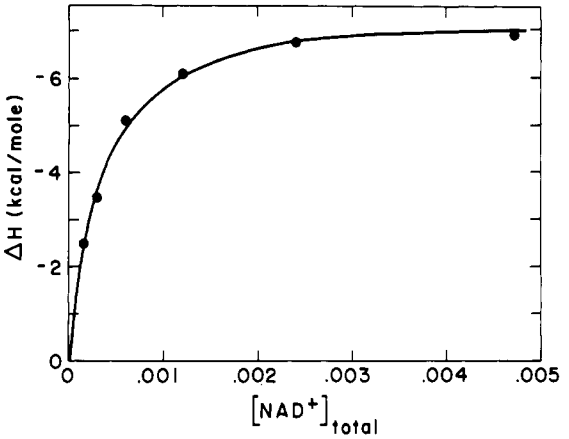

FIGURE 2: Thermal titration of s-MDH with NAD$^+$ at 25 °C in 0.05 M potassium phosphate buffer (pH 6.5); [s-MDH dimer] = 3.6 $\times 10^{-5}$ M. The solid line is the best least-squares fit of the data, with $\Delta H_{max} = -8.0 \pm 0.3$ kcal/mol of s-MDH monomer and $K_d = 0.28 \pm 0.02$ mM^{-1}.

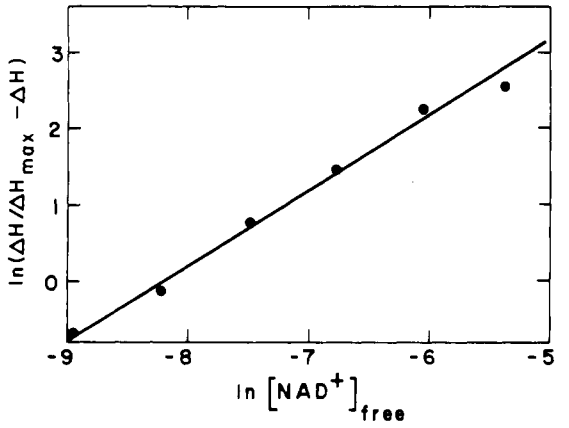

FIGURE 3: Hill plot of the data in Figure 2. The solid line has a slope of one.

Because NAD$^+$ does not fluoresce, there are few solution studies of the binding of this nucleotide to s-MDH. The calorimeter is well suited for measuring NAD$^+$ binding. Figure 2 shows the results of a calorimetric titration of s-MDH with NAD$^+$. The Hill plot in Figure 3 shows that NAD$^+$ binds with no detectable cooperativity under these conditions. Since this conclusion is based on a thermal titration, one can conclude that both subunits bind NAD$^+$ not only with the same ΔG but also with the same ΔH.

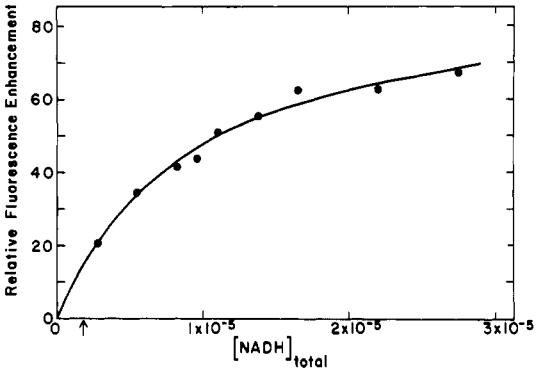

FIGURE 4: Fluorescence titration of s-MDH with NADH at 25 °C in 0.05 M Tris-HCl, 0.25 M NaCl (pH 8.0); [s-MDH dimer] = 9.2 $\times 10^{-7}$ M. The solid line is the best least-squares fit of the data, with $\Delta F_{max} = 88.8$ and $K_d = 7.9 \pm 1.0$ μM^{-1}.

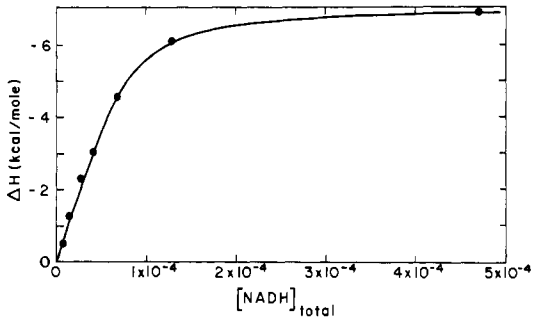

FIGURE 5: Thermal titration of s-MDH with NADH in the presence of 0.15 M sodium malate; 10 °C, 0.05 M sodium phosphate buffer (pH 6.12), 0.23 M NaCl; [s-MDH dimer] = 3.85 $\times 10^{-5}$ M. Solid line is the best least-squares fit of the data, with $\Delta H_{max} = -7.1 \pm 0.1$ kcal/mol of s-MDH monomer and $K_d = 9.6 \pm 1.0$ μM^{-1}.

Fluorescence titrations of s-MDH with NADH were performed to determine the equilibrium constants reported in this paper. The data from one of these titrations are plotted in Figure 4, along with the best least-squares fit to the data by assuming independent binding sites. We confirm the results of the fluorescence titration studies of Mueggler et al. (1975) and Lodola et al. (1978), which gave no evidence of cooperativity.

Figure 5 shows the results of a thermal titration of s-MDH with NADH in the presence of 0.15 M sodium malate at pH 6.1. Under these conditions there is no significant deviation of the data from a fit by assuming two independent binding sites. Similar results were obtained at pH 8 at 10 °C.

Table II: Thermodynamic Parameters for Coenzyme Binding to Pig Heart Supernatant Malate Dehydrogenase, 25 °C, Ionic Strength 0.3

pH	coenzyme	K_d (M^{-1})	$\Delta G°$ (kcal/mol)	$\Delta H°$[a] (kcal/mol)	$\Delta S°$ (cal/K mol)	$\Delta C_p°$[b] (cal/K mol)
6	NADH	7.8×10^{-7}	−8.30 ± 0.02	−10.1 ± 0.5	−6 ± 1	−150 ± 50
8	NADH	6.9×10^{-6}	−7.01 ± 0.03	−12.0 ± 0.8	−17 ± 3	−130 ± 30
6	NAD^+	1.7×10^{-3}	−3.76 ± 0.10	−9.3 ± 0.7	−19 ± 3	−290 ± 60
8	NAD^+	2.3×10^{-3}	−3.59 ± 0.07	−7.3 ± 1.1	−12 ± 4	

[a] Corrected for the heat of buffering associated with the uptake of protons during binding. The uncertainties given for the pH 8 values reflect this contribution. [b] Calculated from measured ΔH values without correction for heat of buffering.

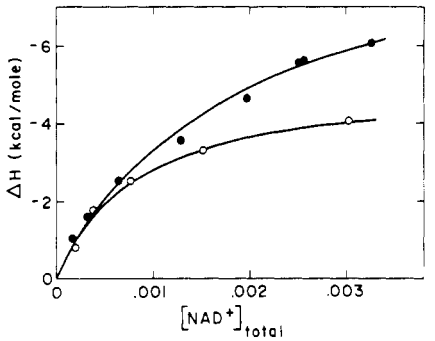

FIGURE 6: Thermal titrations of s-MDH with NAD^+ in 0.05 M sodium phosphate buffer (pH 6.0), ionic strength = 0.3. (●) Data from two titrations at 25 °C with [s-MDH dimer] of 4×10^{-5} and 7.6×10^{-5} M after mixing. (○) Data from one titration at 10 °C with [s-MDH dimer] = 6.5×10^{-5} M. The solid lines are the best least-squares fits of the data; the values of the parameters from these fits are included in Table I.

Malate at 0.15 M concentration is sufficient to saturate in ternary complex formation at the higher NADH concentrations of Figure 5 (Mueggler et al., 1975). The limiting value of ΔH is 3 kcal more positive than in the absence of malate (Table I). These data indicate that the enthalpy of binding of malate to the binary complex is small and positive.

Thermodynamics of Coenzyme Binding. The following experiments were performed to define values of the thermodynamic parameters for interaction of NAD^+ and NADH with s-MDH.

The binding of NAD^+ to s-MDH at pH 6 and 8 and at 10 and 25 °C was measured by thermal titration. The best-fit values of K_d and ΔH_{app} are listed in Table I. Typical data, from experiments done at pH 6, are displayed in Figure 6. The binding of NAD^+ to s-MDH under these conditions is weak, and, despite the relatively high concentrations of s-MDH used, the concentration of bound NAD^+ was less than 10% of $[NAD+]_{total}$. The highest concentration of NAD^+ used was 0.003 M, the concentration of the purified coenzyme eluted from the ion-exchange column. At 25 °C, 0.003 M NAD^+ saturates 60% and, at 10 °C, 80% of the s-MDH binding sites.

At the concentrations of s-MDH needed to perform flow microcalorimetric measurements, most of the NADH added is bound, even when the enzyme is not fully saturated, making the calculation of $[NADH]_{free}$ and, thus, the equilibrium constant subject to large errors. Because of this fact, determinations of the association constant for NADH binding to s-MDH were made by fluorescence titrations, with calorimetric measurements being used to determine the enthalpy of binding. Fluorescence titrations were done at pH 6 and 8 and at 25 °C. The value of K_d giving the best fit to the data was determined for each titration. The average value of K_d for each pH is listed in Table I. Data from a typical fluorescence titration are shown in Figure 4. For all titrations, at least 75% of the s-MDH binding sites were filled at the highest NADH concentrations used. The enthalpy of binding of NADH to s-MDH was determined by mixing excess NADH with s-MDH in the flow microcalorimeter. The values of ΔH_{app} are listed in Table I.

Protein–ligand binding reactions often involve a significant release or uptake of protons. Table I contains the results of experiments (described in the Experimental Procedures section) that measured the number of moles of protons taken up from solution per mole of coenzyme bound ($\Delta\gamma_{H^+}$). Table I also gives values of ΔH_{corr}, which is ΔH_{app} corrected for the heat of buffering associated with the proton uptake. This correction is not significant except for NADH binding at pH 8, where ΔH_{corr} is more than twice ΔH_{app}.

The values of the equilibrium binding constants for NAD^+ and NADH obtained in this work can be compared with literature values for pig heart s-MDH. The only reports for NAD^+ are of indirect measurements made at low ionic strength and pH 7.5 to 8, which gave K_d = 585 μM^{-1} (Holbrook & Wolfe, 1972) and 600–1000 μM^{-1} (Frieden & Fernandez-Sousa, 1975), to be compared with K_d = 280 μM^{-1} measured by the calorimetric experiments of Figure 2 for pH 6 and low ionic strength. Lodola et al. (1978) have measured the binding of NADH at pH 8, 25 °C, and high ionic strength, obtaining values of K_d = 9–11 μM^{-1}, to be compared with the value K_d = 6.9 μM^{-1} found in this work (Table II). There are many measurements of NADH binding at ionic strength 0.05–0.1 (Holbrook & Wolfe, 1972; Mueggler et al., 1975; Frieden & Fernandez-Sousa, 1975; Lodola et al., 1978), which gave constants falling in the range 0.17–0.6 μM^{-1} at pH near 6 and 0.8–1.5 μM^{-1} at pH near 8. There have been no reports of the heats of reaction of s-MDH with NAD^+ or NADH.

Discussion

X-ray diffraction analysis has shown that a crystalline s-MDH–NAD^+ complex contained coenzyme distributed asymmetrically between the subunits of the dimer (Glatthaar et al., 1972). Weininger et al. (1977) suggested that this asymmetry is caused by interactions between neighboring s-MDH molecules in the crystal. The solution data presented in this paper support the above explanation or some similar one not based upon cooperativity in the binding of NAD^+.

Evidence suggesting cooperativity has been reported for the binding of NADH (Cassman & Englard, 1966; Mueggler et al., 1975) and for certain other properties of s-MDH (e.g., histidine reactivity; Holbrook et al., 1974). Lodola et al. (1978) concluded on the basis of fluorescence measurements that there is no cooperativity in the binding of NADH, even in the presence of malate, i.e., under conditions used by Mueggler et al. (1975). The data obtained in the present work confirm the absence of cooperativity in the equilibrium binding of NADH in the presence or absence of malate and show, furthermore, that there is no cooperativity reflected in the enthalpy change for NADH binding.

To summarize, the data presented in this paper were analyzed in terms of a model in which the s-MDH dimer has two independent binding sites. No equilibrium or thermal properties that were measured are in conflict with this assumption. Thus, cooperativity is displayed neither in the free energy nor in the enthalpy of binding of NAD^+ or NADH to s-MDH.

Values of the standard thermodynamic parameters describing the binding of NAD$^+$ and NADH to s-MDH at 25 °C and at pH 6 and 8, based upon the values given in Table I, are listed in Table II.

There is strong pH dependence of the binding of NADH to s-MDH. The free energy of binding is 1.3 kcal/mol more positive at pH 8 than at pH 6. The value of $\Delta\gamma_{H^+}$ of 0.55 at pH 8 and the smaller value at pH 6 are in accord with the dependence of the free energy of binding on pH. In contrast, the enthalpy of binding is 3 kcal/mol more negative at pH 8 than at pH 6. The weaker binding at the higher pH thus reflects a substantial change in the entropy of binding. In this regard, there is no conformation change between pH 6 and pH 8, as measured by the steady-state and time-resolved fluorescence properties (T. Torikata et al., 1978, unpublished results) of the free enzyme and the enzyme–NADH complex. The similar ΔC_p values observed for pH 6 and pH 8 also support the conclusion that the conformation of the enzyme–NADH complex is the same at the two pHs. Thus, the entropy-based difference between the binding of NADH at pH 6 and pH 8 cannot be attributed to changes in the extent of hydrophobic contacts or in protein conformation.

A partial interpretation of the crystal structure data for s-MDH (Banaszak & Webb, 1975) has indicated a histidine residue at the active site. Holbrook et al. (1974) have found a histidine residue to be essential for s-MDH activity. It is tempting to ascribe to an active-site histidine residue the effect of pH on the free energy of NADH binding, the opposite effect of pH on the enthalpy of binding, and at least a contribution to the large difference in free energy of binding, comparing NAD$^+$ and NADH. In any event, the proton uptake associated with NADH binding requires that there be one or more groups of pK near 8 within the enzyme active site.

The enthalpy change given in Table II for binding of NADH at pH 8 is for the process

$$0.55\ H^+ + \text{NADH} + \text{s-MDH} = \text{s-MDH}(0.55\ H^+)\text{–NADH}$$

The partial protonation of one or more groups of the enzyme, probably with ΔH pronotation of −5 to −10 kcal/mol, must make a contribution to the total enthalpy change for the above process. Because this contribution cannot be assessed without additional information, the comparisons made below consider principally data for NADH binding at pH 6, where the change in protons bound is small.

There is a striking effect of ionic strength on the binding of NADH and NAD$^+$ to s-MDH. The data of Figure 2 for binding of NAD$^+$ in 0.05 M sodium phosphate buffer of pH 6.5 give K_d = 280 μM^{-1} and ΔH = −8.0 kcal/mol, to be compared with K_d = 1700 μM^{-1} and ΔH = −9.3 kcal/mol, measured in phosphate buffer of pH 6 and ionic strength 0.3 (Table I). The data of Figure 1 and Table I give for the binding of NADH for the same buffers, respectively, the values K_d = 0.4 μM^{-1}, ΔH = −11.3 kcal/mol and K_d = 0.78 μM^{-1}, ΔH = −10.1 kcal/mol. The value of K_d for NADH at pH 6.5 is a calorimetric determination and, because of the high enzyme concentration, is uncertain. The value of Holbrook & Wolfe (1972), K_d = 0.17 μM^{-1}, is likely correct. In this regard, change in ionic strength from 0.3 to near 0.75 weakens binding of NADH by approximately ten times (compare the data of Figure 5 and Table I). Thus, the effect of ionic strength is comparable for both NAD$^+$ and NADH, with an increase in ionic strength from approximately 0.1 to 0.3 making the free energy of binding more positive by 1 kcal/mol. Increase in ionic strength decreases slightly the enthalpy of NAD$^+$ binding and increases slightly the enthalpy of NADH binding; these changes are close to the limit of experimental error.

The sensitivity of the free energy of binding to ionic strength suggests that electrostatic interactions contribute importantly to the total free energy of binding of both NAD$^+$ and NADH, which is not surprising considering the chemistry of the cofactors. In view of the sensitivity of NAD$^+$ binding to ionic strength but not to pH change, the groups responsible for the sensitivity of NADH binding to pH are likely to be ones different from those involved in the interactions reflected in the ionic strength dependence.

The free energy of binding of NAD$^+$ to s-MDH is 4.5 kcal/mol more positive than for binding of NADH, the enthalpy of binding is only 1 kcal/mol more positive, and the heat capacity change is relatively small and negative for binding of both cofactors. The different thermodynamics for binding of NAD$^+$ and NADH cannot be understood satisfactorily without structural data for the two complexes, and this information is not yet available for s-MDH. Lactate and malate dehydrogenases are, however, closely homologous by both structural (Rossman et al., 1975) and enzymological criteria. High-resolution crystal structures of LDH and its complexes with cofactors have been described (Holbrook et al., 1975). Examination of the thermodynamic parameters $\Delta G°$, $\Delta H°$, and ΔC_p for binding of NAD$^+$ and NADH to LDH (experiments at pH 7 and ionic strength near 0.3 by Schmid et al., 1976; experiments at pH 6 and pH 8 and ionic strength 0.3 by C. C. O'Neal and J. A. Rupley, unpublished observations) shows that the values for LDH follow the pattern described above for s-MDH at pH 6. This similarity between the chemistries of the enzyme–cofactor complexes of LDH and s-MDH is additional evidence for homology and supports the use of the LDH structural information for interpretation of the thermodynamic results for s-MDH. Crystal structure analysis for lactate dehydrogenase shows that NAD$^+$ and NADH bind similarly (Holbrook et al., 1975). Thus, the above pattern of thermodynamic values cannot be attributed to additional hydrophobic interactions present in the complexes with NADH but absent in those with NAD$^+$. To the extent that the complexes with NAD$^+$ and NADH show no overall difference in structure, the differences in thermodynamics likely reflect a few interactions. The similar entropy-based character of the pH effect on NADH binding to s-MDH suggests that this behavior and the differences between the binding of NAD$^+$ and NADH may be linked to the same or to similar elements of the active site.

Electrostatic interactions of macromolecules have been found, in the binding of ions, to show entropy-based thermodynamics (Henkens et al., 1969; Rialdi & Biltonen, 1975). Changes in electrostatic interactions can be put forward as an explanation of the effects of pH and of cofactor oxidation on the thermodynamics of binding. In this regard, the crystallographic and sequence results for LDH (Holbrook et al., 1975; Eventoff et al., 1977) have located cationic and anionic groups near the nicotinamide and substrate binding regions of the active site.

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Purification and Subunit Structure of Nicotinamide Adenine Dinucleotide Specific Isocitrate Dehydrogenase from *Neurospora crassa*†

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ABSTRACT: *Neurospora crassa* nicotinamide adenine dinucleotide specific isocitrate dehydrogenase (EC 1.1.1.41) has been purified to homogeneity by the criteria of disc gel electrophoresis and sedimentation equilibrium. Purification of the enzyme is facilitated by the presence of phenylmethanesulfonyl fluoride and by the use of a ribose-linked adenosine 5′-monophosphate affinity column. The enzyme appears to be composed of nonidentical subunits of molecular weights 42 800 and 38 300 as estimated by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. From the intensity of each band and the native molecular weight, it is concluded that the enzyme is composed of either six or eight subunits, three or four of each type, respectively. The availability of pure enzyme will allow clarification of the structure of the enzyme by ligand binding studies.

The regulatory NAD^+ [1]-specific isocitrate dehydrogenase from *Neurospora crassa* has been suggested to bear at least three types of site: a substrate site specific for *threo*-D_s-isocitrate, NAD^+, and a divalent metal cation, and two regulatory sites, one specific for citrate, isocitrate, and structurally related molecules and a second specific for adenine nucleotides (Sanwal et al., 1963, 1965; Sanwal & Stachow, 1965; Sanwal & Cook, 1966). These conclusions have been based exclusively on results obtained from initial velocity kinetic studies. The regulatory characteristics of the enzyme,

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[1] Abbreviations used: IDH, isocitrate dehydrogenase; AMP, adenosine 5′-monophosphate; NAD^+, nicotinamide adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

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