Thermodynamics of Nicotinamide–Adenine Dinucleotide Addition to the Glyceraldehyde 3-Phosphate Dehydrogenases of Yeast and of Rabbit Skeletal Muscle. An Equilibrium and Calorimetric Analysis over a Range of Temperatures*

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ABSTRACT: The thermodynamics of the binding of nicotinamide–adenine dinucleotide (NAD) at pH 7.3 to the glyceraldehyde 3-phosphate dehydrogenases obtained from yeast and from rabbit skeletal muscle has been determined by fluorometric equilibrium titrations and by calorimetric titrations. The yeast enzyme was studied by both methods in the temperature range 5–40°, and the muscle enzyme in the range 5–25°. In both cases the equilibrium and calorimetric measurements were found to be entirely consistent with each other. The observations on the yeast enzyme indicate that under the experimental conditions used the four binding sites are identical and independent. At 25° the intrinsic thermodynamic parameters for each site have the values $\Delta G° = -7.4 \pm 0.1$ kcal mole^{-1}, $\Delta H = -12.4 \pm 0.5$ kcal mole^{-1}, and $\Delta S° = -16.8 \pm 1.7$ cal deg^{-1} mole^{-1}. An outstanding feature of the results is the extremely large change in apparent heat capacity, which amounts to -520 ± 20 cal deg^{-1} (moles of NAD bound)$^{-1}$, and is constant over the temperature range studied. As a result of the large negative value of ΔC_P, $\Delta S°$ is positive at 5° and negative at 25 and 40°. The data for the muscle enzyme make it evident, as observed by many other workers, that there is strong site interaction of an anticooperative character. Approximate apparent thermodynamic parameters for each site have been derived from the experimental data at 5 and 25°. For example, the ΔH values for sites 1, 2, 3, and 4 are, respectively, -16 ± 1, -12 ± 1, -12 ± 2, and -12 ± 2 kcal mole^{-1} at 5°, and -22 ± 1, -17 ± 1, -17 ± 2, and -14 ± 2 kcal mole^{-1} at 25°.

The sums of the enthalpy changes are known with much greater accuracy than the individual values, and amount to -51.8 ± 1.0 kcal mole^{-1} at 5° and -69.8 ± 1.0 kcal mole^{-1} at 25°. Thus at saturation, $\Delta C_P = -220 \pm 20$ cal deg^{-1} (moles of NAD bound)$^{-1}$. The data reported here cannot be rationalized in terms of the simplistic analyses of the thermodynamics of protein structure currently available. For example, the large decrease in apparent heat capacity associated with the binding of NAD to the yeast enzyme is suggestive of an important hydrophobic contribution to the binding; on the other hand, hydrophobic interactions at 25° are expected to result in minor changes in enthalpy and substantial increases in entropy, whereas the observed quantities are, respectively -12 kcal (moles of NAD bound)$^{-1}$ and -17 cal deg^{-1} (moles of NAD bound)$^{-1}$.

The glyceraldehyde 3-phosphate dehydrogenases of yeast and of skeletal muscle are homologous tetrameric enzymes that are qualitatively similar in most of their major properties (Perham and Harris, 1963; Velick and Furfine, 1963; Harris and Perham, 1965, 1968). However, they have exhibited contrasting behavior in their subunit interactions as expressed by NAD binding. The four binding sites of the yeast enzyme display positive homotropic cooperativity (Kirschner *et al.*, 1966) whereas those of the muscle enzyme are characterized by four widely divergent binding constants which can be interpreted in terms of negative cooperativity (Conway and Koshland, 1968). These opposing properties were observed by quite different methods and at widely different temperatures. The present report describes some of the thermodynamic parameters of the NAD binding interactions of the two enzymes in a common temperature range. Equilibrium binding measurements as a function of temperature were made with the very dilute protein solutions required for measurable dissociation equilibria of the strong complexes. For this purpose the quenching of the tryptophan fluorescence of the protein by bound NAD provided the speed and sensitivity required for work at elevated temperatures and at high dilutions. The temperature dependence of the equilibria of the muscle enzyme was complex and indicated the occurrence of unusual enthalpy and entropy parameters. Recent advances in flow microcalorimetry made it possible to measure the enthalpy changes in NAD binding directly as a function of temperature and to obtain calorimetric titration curves of both enzymes with NAD. In addition to providing direct measurements of ΔH, the new information provided by this approach was a measure of the ΔC_p terms, the difference in apparent heat capacities of reactants and products. This quantity was found to make a dominant contribution to the enthalpy parameters of the enzyme–NAD interactions.

Materials and Methods

Enzymes. The mammalian enzyme was prepared by minor modifications of previously published methods (Cori *et al.*, 1948) with the addition of EDTA and dithiothreitol to all

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solvents. In most instances the intermediate crystallization of aldolase was omitted in order to shorten the preparation time. The yeast enzyme was prepared by the method of Krebs (1955) modified by the substitution of high-speed centrifugations for the filtration steps. Rapid filtrations were employed when necessary to clarify the supernatant solutions. Although both enzymes are relatively stable as cold crystalline suspensions, they lose activity on storage at slow but measurable rate. The calorimetric ΔH values tend to decline with the age and activity of the enzymes. The results reported here were obtained with three to five-times-crystallized preparations that were no more than 2-weeks old. Both enzymes migrated as single bands, monitored by protein staining, in zone electrophoresis on cellulose acetate and in polyacrylamide gel. Both the holo and apo muscle enzymes yield multiple bands in electrofocusing measurements (Susor *et al.*, 1969; Velick, 1970). Similar results have been obtained with the pig muscle enzyme which has been fully sequenced and yields no evidence for inhomogeneities in primary structure (Harris, 1970). The basis of this behavior, which could significantly affect the interpretation of the binding equilibria, has not yet been established. The yeast enzyme is known to occur in multiple forms, documented most recently by Lebherz and Rutter (1967) and verified by sequence analysis (J. I. Harris and K. Kirschner, private communication). However the preparations that we have made from Fleischman's bakers' yeast have exhibited only one band on cellulose acetate and by electrofocusing.

The bound NAD with which the mammalian enzyme crystallizes was removed by charcoal treatment. For this purpose, 0.5 to 1.0 g of sedimented crystals was dissolved in 5 ml of 1.0 M potassium phosphate (pH 6.7) and stirred with 1 g of acid-washed Norit A for 5 min at 30°. The suspension was then filtered by suction through a 1-mm thick pad of analytical grade Cellite (Johns Manville) on Whatman No. 1 filter paper. Initial A_{280}/A_{260} ratios of 1.05 were increased to 1.94 or higher. A second treatment with one third the amount of charcoal and a fresh filter brought the ratio of 2.05. Residual nucleotide determined by A_{260} measurements of perchloric acid filtrates was less than 0.03 mole of NAD equivalents/mole of protein subunit. Although the apo protein could sometimes be brought to a microcrystalline state by cautious ammonium sulfate addition at room temperature, it was routinely precipitated at 80% saturation of ammonium sulfate (pH 8.2), concentrated by sedimentation and stored as a thick amorphous suspension containing 2 mM EDTA and dithiothreitol at 3°. The yeast enzyme after three to four crystallizations still contained contaminating pigment and traces of NAD. One charcoal treatment followed by a final recrystallization removed all detectable traces of pigment and NAD.

Specific activities of both enzymes were measured in 0.1 mM NADH and 0.1 M imidazole (chloride) (pH 7.0) in the presence of a glyceryl 3-phosphate generating system consisting of 0.2 mM magnesium chloride, 0.2 mM ATP, 5 mM D-3-phosphoglycerate, and 20 μg ml^{-1} of 3-phosphoglycerate kinase crystallized from yeast. Under these conditions at 25°, the reaction rates were 22,000 and 19,000 moles of NADH oxidized per min per mole of enzyme (mol wt 144,000) for the yeast and muscle enzymes, respectively. Concentrations of the apo proteins were measured by absorbance at 280 nm using an absorption coefficient of 0.84 cm^2 mg^{-1} in cuvets of 1-cm light path, pH 7. The NAD concentrations were based upon enzymatic reduction to NADH.

Fluorescence Measurements. The binding of NAD was measured by the quenching of the fluorescence of the tryptophan residues of the protein excited at 300 nm and monitored at 350 nm (Velick, 1958; Velick *et al.*, 1960). For this purpose Aminco Bowman and Turner Model 210 spectrofluorometers were employed. Both instruments operate in the luminescence mode. In addition, the Model 210 can be operated in a mode in which the fluorescence is compared to a reference signal that is modulated by the energy of the transmitted light. Under these conditions, there is automatic correction of the fluorescence intensity for attenuation of incident light by small increments in absorbance produced by additions of nucleotide in a titration. Concentrated NAD solutions were added from a micrometer buret through a teflon capillary to 2 or 3 ml of enzyme solution. The solutions were stirred with a polyethylene or Teflon rod kept in the solution but out of the light path during measurement. Temperature was controlled ± 0.5° and monitored by a micro thermistor.

In experiments at enzyme concentrations several orders of magnitude larger than the NAD–enzyme dissociation constants and at 5 and 30°, the amplitudes both of the absorbance change and of the fluorescence quenching with the muscle enzyme are colinear with binding stoichiometry and with each other through ligand addition to 2.7 to 3 of the four binding sites of the protein. Binding at the fourth and weakest site has so far been measured only by equilibrium dialysis and by ultracentrifugal separation. In the simpler case of the yeast enzyme, the optical signals appear to serve as indicators of NAD binding at all four sites.

Equilibrium dialysis controls for the stoichiometry of NAD binding in dilute protein solutions were carried out with 10^{-6} M protein and [^{3}H]NAD at 3°. Stretched dialysis tubing was employed to accelerate the rate of equilibration which approached completion in 10 hr. The NAD was labeled by reduction with [^{3}H]ethanol and alcohol dehydrogenase and reoxidized by a dehydrogenase reaction of opposite stereospecificity, dihydroxyacetone phosphate and α-glycerophosphate dehydrogenase. The products were purified by DEAE-Sephadex chromatography. Radioactivity was measured in a Packard Model 3003 liquid scintillation counter, and protein concentrations in the presence of nucleotide were determined by the method of Lowry *et al.* (1951). A binding stoichiometry of 2.8 to 3 at the NAD concentration limit of the fluorescence quenching titrations was obtained at 3° but stability and time restrictions prevented application of this approach to the higher temperatures of interest. Ultrafiltration through Diaflo membranes (Amicon) offered the speed required of a separation method at higher temperatures but was complicated by nucleotide retention blanks and uncertain corrections. However, this method and dialysis equilibrium experiments with concentrated protein solutions confirmed the maximal binding stoichiometry of 4 at the high NAD concentration limits of the calorimetric titrations.

It should be noted that the quenching of protein fluorescence by NAD, in contrast to that by NADH, is not a common property of NAD enzymes and appears to be associated with the unique 260-nm absorption band of the glyceraldehyde 3-phosphate dehydrogenase–NAD complex. On the basis of nonenzymic model experiments, Cilento and Tedeshi (1961) have proposed that the 260-nm absorption band arises from a charge-transfer interaction between bound NAD and a tryptophan residue at the binding site. There is as yet no direct evidence that a tryptophan residue is appropriately situated for such an interaction and the possibility remains that the absorption band arises from interactions of other

types. In the latter event, the 260-nm band which overlaps the emission band of tryptophan fulfills the condition for fluorescence quenching through excitation energy transfer by the coupled oscillator mechanism. Our concern about colinearity of quenching and binding stoichiometry arises from the fact that quenching by the coupled oscillator mechanism is sensitive to the geometry of the complex, and hence to ligand-induced protein conformation changes, and is, therefore, not necessarily parallel with the saturation function.

Calorimetric Measurements. The enthalpy measurements were made in a flow modification of the Beckman Instruments Model 190 microcalorimeter which is a further improvement over the modification described by Sturtevant and Lyons (1969). In this device the two reactant solutions, supplied from two precision syringes driven at variable speeds by ten-position gear reduction boxes and synchronous motors, pass through 0.5-mm i.d. platinum tubes in good thermal contact with a massive cylindrical aluminum heat sink, and then through a simple mixing junction into 250 cm of platinum flow tubing in good thermal contact with a 10,000-junction thermopile. Heat evolved or absorbed in the flow tubing is rapidly exchanged with the heat sink through the thermopile, thus developing a voltage. The difference between the output of this thermopile and another very similar one not in thermal contact with the flow tubing is amplified and recorded by a strip-chart recorder fitted with a ball and disc integrator. With a rapid exothermic or endothermic process, a steady state of balance between heat evolution or absorption in the flow tubing and heat transfer with the heat sink is established within 4–5 min, and ordinarily flow is continued 4–8 min after reaching the steady state, the heat effect being evaluated by integration of the thermopile output during this steady-state period.

The calorimetric assembly is contained within a hermetically sealed brass cylinder immersed in a water bath controlled to ±0.005° or better, with approximately 5 cm of styrofoam insulation between the heat sink and the submarine cylinder. The syringes and syringe drives are at room temperature, connected to the calorimeter by Teflon tubing passing through the water bath. The apparatus has functioned well in the temperature range 5–45°.

A major limitation on the available sensitivity of the calorimeter is the residual instability of the base line. Precise thermostating of the calorimeter as described above, and enclosure of the microvolt amplifier in an oven regulated at about 35° to ±0.1°, has given sufficient stability so that a full-scale sensitivity of 3 μV is routinely employed. This corresponds to a heat effect of approximately 800 μcal min^{-1}. A heat change of 500 μcal min^{-1} or more can be evaluated with a precision of a few tenths of a per cent; a very small heat change, in the range of 5 μcal min^{-1}, can be estimated with a standard error of the mean of several determinations approaching 0.5 μcal min^{-1}. Useful flow rates range from 0.01 to 0.50 ml min^{-1}.

The instrument is calibrated by means of measurements on the heat of protonation of Tris and the heat of formation of water. The heat of ionization of Tris at 25° is taken to be 11.35 kcal mole^{-1} (Öjelund and Wadsö, 1969), and that of water at 25°, 13.37 kcal mole^{-1} (Hale *et al.*, 1963; Vanderzee and Swanson, 1963). For other temperatures the temperature coefficient of the heat of ionization of water given by Harned and Owen (1958) is applied to the 25° value given above.

The flow calorimeter is an extremely convenient instrument to use. All the thermal titrations reported in this paper at

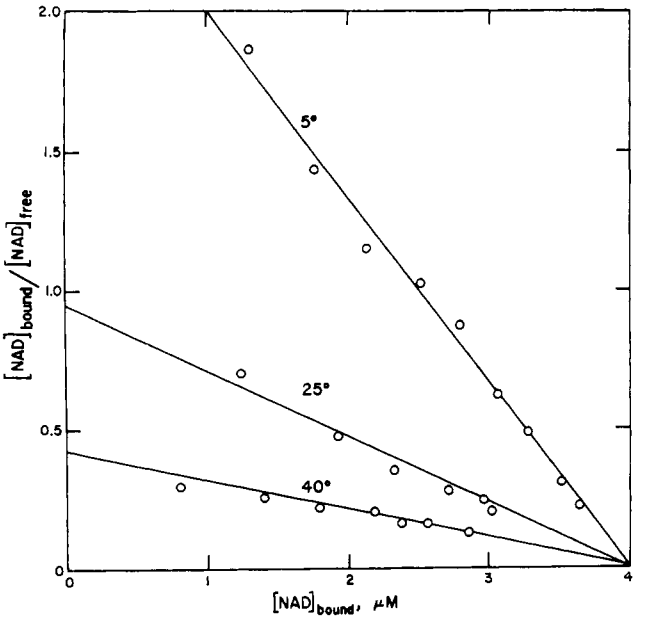

FIGURE 1: Fluorometric titration of yeast glyceraldehyde 3-phosphate dehydrogenase with NAD in 0.05 M potassium phosphate buffer (pH 7.34) containing 0.05 M KCl and 0.002 M EDTA. Enzyme concentration 1.0 × 10^{-6} M. Fluorescence quenching was measured at 350 nm, bandwidth 10 nm, excited at 300 nm.

5, 25, and 40° were run in less than 4 days of intensive work. At the protein concentrations employed in the present work and flow rates of 0.04 ml min^{-1} from each syringe which were used throughout, the total heat effects observed during approximately 5 min of flow during the steady-state ranged from near zero (yeast enzyme at 5°) to a maximum of 1 mcal (yeast enzyme at 40°, saturating NAD). A conservative estimate of the calorimetric precision is 0.5–1.0 kcal mole^{-1} for the experiments with the yeast enzyme, and 1.0–2.0 kcal mole^{-1} for those with the muscle enzyme which was used at half as high a concentration. The major sources of error are believed to arise from the states of the proteins, which are subject to numerous perturbations that are difficult to control.

Results

Yeast Enzyme. FLUOROMETRIC TITRATIONS. The titration curves observed with the yeast enzyme are hyperbolic at 4, 25, and 40°. The linear plots for 1.0 × 10^{-6} M enzyme in Figure 1, which are based on the simple mass action law in the form

$$\frac{[\mathrm{NAD}]_{\mathrm{bound}}}{[\mathrm{NAD}]_{\mathrm{free}}} = 4K[\mathrm{E}]_0 - K[\mathrm{NAD}]_{\mathrm{bound}} \qquad (1)$$

where the quantities in brackets are concentrations, $[E]_0$ being the total enzyme concentration, and K is the association constant, establish that the binding of NAD can be described at each temperature in terms of a single intrinsic dissociation constant for four equivalent and independent sites. These results obtained at pH 7.3 are in accord with those of Kirschner *et al.* (1966), who could detect cooperative binding of NAD kinetically or by equilibrium titration only at a more alkaline pH.

CALORIMETRIC TITRATIONS. The protein concentrations employed for calorimetry were 46 × 10^{-6} M at 5 and 25°

TABLE I: Thermodynamic Parameters for the Binding of NAD to Yeast Glyceraldehyde Phosphate Dehydrogenase at pH 7.3.

	Fluorometric Results[a]		Calorimetric Results[b]			
Temp (°K)	K (M^{-1})	$\Delta G°$, kcal (moles of NAD)$^{-1}$	ΔH, kcal (moles of NAD)$^{-1}$	ΔC_p, cal deg^{-1} (moles of NAD)$^{-1}$	$\Delta G°$,[c] kcal (moles of NAD)$^{-1}$	$\Delta S°$,[d] cal deg^{-1} (moles of NAD)$^{-1}$
278.2	6.55×10^5	−7.40	−1.9	−525		+19.9
298.2	2.36×10^5	−7.33	−12.4	−517	−7.43	−16.8
313.2	1.05×10^5	−7.19	−20.1		−6.99	−41.6

[a] Equilibrium titration of 10^{-6} M enzyme. [b] Calorimetric titration of 46–56 $\times$ 10^{-6} M enzyme in 0.05 M potassium phosphate buffer–0.05 M KCl–0.002 M EDTA (pH 7.32). [c] Calculated by means of eq 2 and 3. [d] Based on the mean of the fluorometric and calorimetric values for $\Delta G°$.

after mixing with an equal volume of NAD solution in the calorimeter, and 56 $\times$ 10^{-6} M in the experiments run at 40°. The results are plotted in Figure 2. At these enzyme concentrations, initial increments of nucleotide are bound stoichiometrically. Short linear extrapolations of the initial slopes intersect the enthalpy limit at points corresponding to a stoichiometry of 4 moles of NAD bound per mole of protein. It is inferred from this result that the enthalpy change on ligand addition is the same for each of the four sites.

There is a strong and linear dependence of ΔH on temperature corresponding to a decrease of 2080 cal deg^{-1} (moles of enzyme)$^{-1}$. Thus ΔC_p for the reaction, the difference in the apparent heat capacities of the reactants and products, is −520 cal deg^{-1} mole^{-1} for each site. At 5° the maximum enthalpy change at saturation can be measured with confidence but the magnitudes of the observed enthalpy changes were too small to allow the initial slope of the curve to be determined accurately.

The thermodynamic parameters for NAD binding to the yeast enzyme are listed in Table I. Both ΔH and $\Delta S°$ exhibit large temperature dependence, corresponding to the large negative ΔC_p. $\Delta S°$ undergoes a change of sign at about 18°. $\Delta G°$ is seen to be nearly independent of temperature.

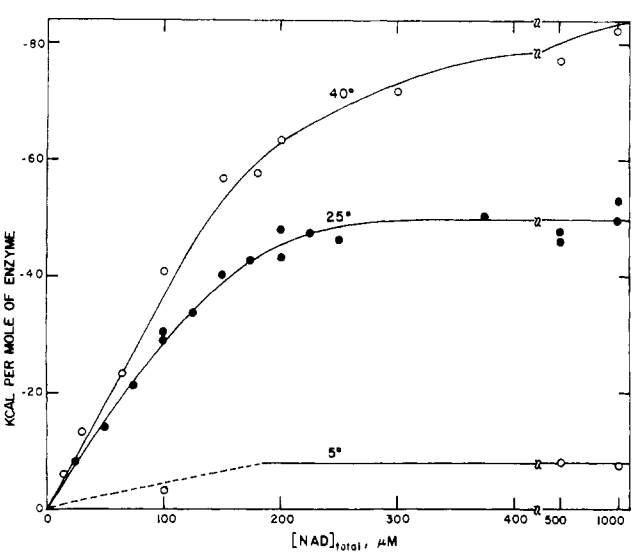

FIGURE 2: Thermal titration of yeast glyceraldehyde 3-phosphate dehydrogenase with NAD in 0.05 M potassium phosphate buffer (pH 7.32) containing 0.05 M KCl and 0.002 M EDTA. Enzyme concentrations 46 $\times$ 10^{-6} M at 5° and 25°, 56 $\times$ 10^{-6} M at 40°. The curves at 25 and 40° are drawn using the values of K and ΔH given in Table I.

The solid curves in Figure 1 were calculated on the basis of four identical, noninteracting sites, with the equilibrium constants and enthalpies given in Table I. The mutual consistency of the fluorometric binding data and the calorimetric results can also be shown by calculating $\Delta G°$ at 25 and 40° from the value at 5° by means of the Gibbs–Helmholtz equation. In its integrated form, for a reaction with temperature-independent ΔC_p, this equation is

$$\frac{\Delta G_1}{T_1} - \frac{\Delta G_2}{T_2} = \Delta H_0\left(\frac{1}{T_1} - \frac{1}{T_2}\right) - \Delta C_p \ln \frac{T_1}{T_2} \qquad (2)$$

where temperatures are expressed in °K. ΔH_0 is given by

$$\Delta H = \Delta H_0 + T\Delta C_p \qquad (3)$$

and has the value ΔH_0 = 143.08 ± 0.03 kcal (moles of NAD bound)$^{-1}$. Values of ΔG^0 calculated in this way are listed in Table I, and are seen to be in satisfactory agreement with the fluorometric values. (It should be noted here that it is entirely permissable to take the calorimetric ΔH values as standard-state quantities since they are determined at very low concentrations.)

Muscle Enzyme. EQUILIBRIUM TITRATIONS. In order to obtain measurable dissociation of the high-affinity complexes of the muscle enzyme, the fluorescence quenching titrations were carried out with 10^{-7} M protein. A set of titrations over a range of temperatures is plotted in Figure 3. The curves approach a plateau at NAD concentrations above 10^{-5} M. Equilibrium dialyses of dilute protein against tritium-labeled NAD established the stoichiometry under these conditions as 3 ± 0.2 moles of NAD bound per mole of protein. Dissociation at the first and strongest binding site is measurable at all but the lowest temperature. Binding at the fourth site was not measured fluorometrically.

The major features of the results are the extreme elongation of the curve at 2.5°, the strong temperature dependence of the equilibria, and the increase in the slopes of the curves as the temperature is raised. At least three equilibrium constants are required to fit a theoretical curve to the experimental points at 2.5°. These constants and sets of apparent constants fitted to the curves at higher temperatures are listed in Table II. Included for comparison are the results obtained by

TABLE II: Apparent Association Constants for the Binding of NAD to the Glyceraldehyde 3-Phosphate Dehydrogenase of Rabbit Skeletal Muscle.

Temp (°C)	K_1 (M^{-1})	K_2 (M^{-1})	K_3 (M^{-1})	K_4 (M^{-1})
2.5[a]	$\geq 3 \times 10^{10}$	2.4×10^8	3.0×10^6	
3.0[b]	$>10^{11}$	$>10^9$	3×10^6	4×10^4
11[a]	2.5×10^8	1.1×10^7	2.2×10^6	
19[a]	4.3×10^7	5.0×10^6	1.8×10^6	
28[a]	2.7×10^6	2.7×10^6	2.7×10^6	(2.8×10^4)[c]
36[a]	1.5×10^6	1.5×10^6	1.5×10^6	

[a] Fluorescence quenching measurements at pH 7.4 in 0.09 M KCl–0.01 M potassium phosphate buffer–0.002 M EDTA–10^{-7} M enzyme. [b] Equilibrium dialysis of 10^{-5} M enzyme–0.1 M sodium pyrophosphate (pH 8.5) (Conway and Koshland, 1968). [c] Ultracentrifugal separation, 25°, pH 7.5, 10^{-5} M enzyme (de Vijlder and Slater, 1969).

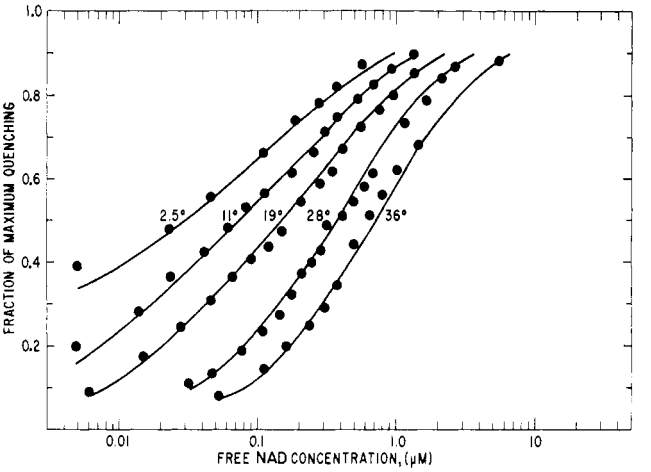

FIGURE 3: Fluorometric titration of the three high-affinity NAD binding sites of glyceraldehyde 3-phosphate dehydrogenase of rabbit skeletal muscle in 0.01 M potassium phosphate buffer (pH 7.4) containing 0.09 M KCl and 0.001 M EDTA. Enzyme concentration 1.4×10^{-7} M. The curves are generated by the equilibrium constants listed in Table II.

Conway and Koshland (1968) at 3°. These workers used relatively high concentrations of unresolved native complex and based their estimates on NAD removal in a stepwise series of equilibrium dialyses. A value for the fourth site at 25°, obtained by de Vijlder and Slater (1968) using ultracentrifugal separation and charcoal-treated apo enzyme, is also included.

On the basis of sedimentation equilibrium analysis of dilute protein solutions in experiments of long duration, Hoagland and Teller (1969) concluded that the holo and apo enzyme establish tetramer–dimer equilibria with apparent dissociation constants, at 3°, of about 10^{-7} and 10^{-6} M, respectively. Gel filtration experiments by Constantinides and Deal (1969) indicated that a more extensive dissociation promoted by ATP and low temperatures is reversed by warming in a time-dependent reaction. In both cases the dimeric form is enzymically inactive. The extent of agreement between the fluorometric results with dilute protein and the results with concentrated protein by dialysis equilibrium indicates that subunit dissociation in the present case was not a dominant factor in influencing the NAD binding at low temperatures. Removal of bound NAD and fluorometric titration of the apo enzyme was done rapidly without significant loss of enzyme activity. We are therefore inclined to attribute the fluorometric results, as well as the calorimetric results reported in the next section, at all temperatures primarily to properties of the tetrameric protein. The progressive decrease of the binding constants with increasing temperature is accompanied by a convergence of the three high-affinity K's. The fourth and weakest binding site is slightly temperature dependent but remains distinctly separated from the others over the temperature range examined.

An apparent average enthalpy change in NAD complex formation at the three high-affinity sites of the apo muscle protein can be estimated from a van't Hoff plot, log K *vs.* $1/T$, taking the reciprocal midpoints of the curves in Figure 3 as measures of an average binding constant. Such a plot is approximately linear and the slope corresponds to an apparent ΔH of $-16{,}000$ cal/mole of NAD bound. The greater temperature dependence of the first two binding constants relative to the others, as seen by inspection of Figure 3 and Table II, leads one to expect that the first 1 or 2 equiv of NAD bound would be associated with larger enthalpy changes. The calorimetric measurements described in the next section give the magnitudes and distribution of the ΔH's directly at protein concentrations sufficiently high to eliminate concern about contributions from subunit dissociation.

CALORIMETRIC TITRATIONS. Enthalpy measurements were made at enzyme concentrations of 25×10^{-6} M at 5 and 25°. Measurements at higher temperatures were omitted to avoid the possibility of denaturation and precipitation of the relatively unstable apo protein and resultant obstruction of the capillary flow system. The results are plotted in Figures 4 and 5. As in the case of the yeast enzyme, ΔH is not constant but increases with temperature. The value of ΔC_p at saturation of the enzyme by NAD is -900 cal deg^{-1} (moles of enzyme)$^{-1}$, about 45% of the value for the yeast enzyme but still of considerable magnitude. Unlike the yeast enzyme,

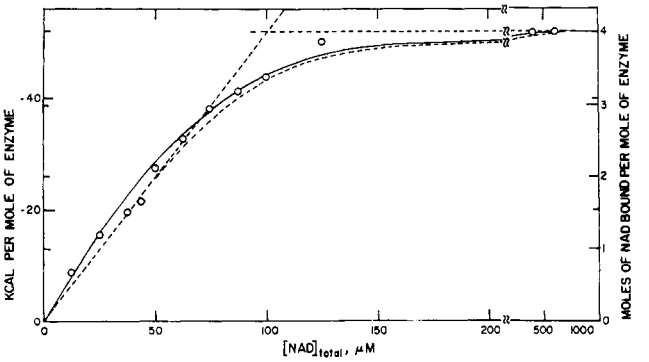

FIGURE 4. Thermal titration of glyceraldehyde 3-phosphate dehydrogenase of rabbit skeletal muscle with NAD in 0.05 M potassium phosphate buffer (pH 7.32) containing 0.05 M KCl and 0.002 M EDTA, at 5°. Enzyme concentration 25×10^{-6} M. The solid curve gives values of enthalpy changes calculated according to the equilibrium constants and enthalpies listed in Table III, and the dashed curve gives values of the calculated extent of binding.

TABLE III: Apparent Thermodynamic Parameters for the Binding of NAD to the Glyceraldehyde 3-Phosphate Dehydrogenase of Rabbit Skeletal Muscle at pH 7.3.

	5°			25°		
Binding Site	$\Delta G°$, kcal (moles of NAD)$^{-1}$	ΔH, kcal (moles of NAD)$^{-1}$	$\Delta S°$, cal deg^{-1} (moles of NAD)$^{-1}$	$\Delta G°$, kcal (moles of NAD)$^{-1}$	ΔH, kcal (moles of NAD)$^{-1}$	$\Delta S°$, cal deg^{-1} (moles of NAD)$^{-1}$
1	−12.2	−16.0	−13.5	−8.72	−22.0	−40.4
2	−11.4	−12.4	−3.4	−8.72	−16.7	−22.6
3	−7.0	−11.7	−13.0	−8.72	−16.7	−22.6
4	−6.0	−11.7	−24.1	−5.79	−14.4	−29.2

there is a substantial ΔH of complex formation at 5°. Moreover, the initial points of the thermal titration do not increase linearly but follow a curved course from the origin.

The thermodynamics of NAD complex formation with the mammalian enzyme is obviously complicated and the data presently available are insufficient for a detailed analysis. However, the qualitative and some of the quantitative features of the results may be seen in outline. Enthalpy limits at both experimental temperatures are obtained at NAD concentrations sufficiently high to require contributions from all four binding sites. Lines are therefore drawn in Figures 4 and 5 from the origins to the points on the plateaus corresponding to a stoichiometry of four binding sites or 100×10^{-6} M NAD. Binding curves at both temperatures based on equilibrium constants obtained from the fluorometric titrations are given in the figures and may be used to estimate rough values for the enthalpy increments for each site. At the high protein concentrations employed the binding constants are sufficiently large to make complex formation virtually stoichiometric during addition of the first 2 molar equiv of NAD. At 5°, for example, the enthalpy increments for the first two sites are −16.0 and −12.4 kcal mole^{-1}, leaving a total of −23.4 kcal mole^{-1} for the remaining two sites. The calorimetric data are consistent with an equal distribution of heats, or −11.7 kcal mole^{-1} each, for sites 3 and 4.[1]

The enthalpy increments estimated in this way are listed in Table III. The values for the first and second sites at both temperatures are probably reliable to ±1 kcal mole^{-1} or better, while those for the third and fourth sites are subject to greater uncertainty, perhaps as much as ±2 kcal mole^{-1}. The solid curves in Figures 4 and 5 are calorimetric titration curves calculated on the basis of the thermodynamic parameters given in Table III. It is seen that the fluorometric and calorimetric data are entirely consistent with each other. Although the parameters evaluated in this simplified treatment must be interpreted with caution, they indicate the significant trends. As in the case of the yeast enzyme, the binding interactions are strongly exothermic and are associated with decreases in the enthalpy of the system. Whereas the four binding sites of the yeast enzyme behave as equivalent and independent units, those of the muscle enzyme appear to respond to ligand addition by conformational changes that are propagated nonuniformly to other members of the tetramer by a process which is strongly temperature dependent.

There are numerous chemical manifestations of conformational transitions of the dehydrogenase that are induced by NAD binding. A recent example of particular interest is the observation of Eiselle and Wallenfels (1970) that NAD binding by both the yeast and muscle enzymes inverts the stereoselective rates of protein–thiol group alkylation by D- and L-iodopropionates. The NAD concentration dependence of the effect with the yeast enzyme can be rationalized by the allosteric two-state model, whereas that of the muscle enzyme is complex and indicates multiple and possibly discontinuous transitions.

In a search for other temperature-dependent parameters of NAD binding, titrations of the apo muscle enzyme were monitored by circular dichroism measurements at 7 and 27° from 200 to 400 nm. No detectable change as a function of NAD concentration was observed in the strong negative band centered at 217 nm which is considered sensitive to changes in helicity. However, linear changes in amplitude as a function of NAD concentration were observed in the

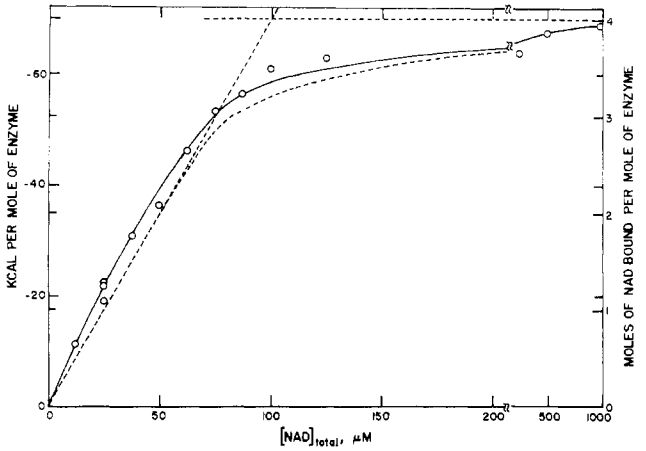

FIGURE 5: Thermal titration of glyceraldehyde 3-phosphate dehydrogenase of rabbit skeletal muscle with NAD in 0.05 M potassium phosphate buffer (pH 7.32) containing 0.05 M KCl and 0.002 M EDTA, at 25°. Enzyme concentration 25×10^{-6} M. The solid curve gives values of enthalpy changes calculated according to the equilibrium constants and enthalpies listed in Table III, and the dashed curve gives values of the calculated extent of binding.

[1] A preliminary report of work with the muscle enzyme (Velick *et al.*, 1970) was based upon data obtained with an earlier less stable version of the calorimeter by a pulsed rather than steady-state method in which protein and ligand concentrations were varied concurrently. The results in Table I of that communication do not in fact deviate widely from the present findings. However the extrapolation to zero enthalpy change for ligand addition to the fourth binding site was clearly not warranted.

several bands above 250 nm which are attributable to side chain Cotton effects of the aromatic amino acid residues. The apparent stoichiometry limit in these titrations at both temperatures was 3 rather than 4 moles of NAD bound per mole of protein at free NAD concentrations up to 100×10^{-6} M. Similar results at a single temperature have been obtained by de Vijlder and Harmsen (1969), who also observed that NAD addition produces an increment in molar ellipticity of the enzyme that correlates with the characteristic 360-nm absorption band of the complex. The molar ellipticity, like the 360-nm absorption band, appears to respond only to NAD addition at the three high-affinity binding sites. Whether or not this behavior is an intrinsic property of the maximally active native enzyme remains to be established.

Discussion

The most extensive thermodynamic analyses of protein conformational changes deal with the reversible denaturations of small, relatively stable monomeric proteins under rather extreme conditions of solvent, pH, and temperature. For example, the thermal unfolding of chymotrypsinogen (Brandts, 1964; Jackson and Brandts, 1970) and of ribonuclease A (Tsong *et al.*, 1970) are associated with large positive values of ΔH, ΔS, and ΔC_p. The negative parameters in the response of the dehydrogenases to NAD addition correspond, in these terms, to a renaturation process. Although the thermodynamic parameters in the two types of system on a molar protein basis are of comparable magnitude, the parameters of the dehydrogenase reactions are one-fifth to one-tenth those of the protein denaturations on a protein weight basis. Correspondingly, the dehydrogenases under the experimental conditions used in the present work do not exhibit any extensive unfolding as measured by optical and hydrodynamic properties. To the extent that changes in conformation occur, they are subtle and are presumably amplified by subunit reorientations. The two-state transition of the yeast enzyme at pH 8.5 has been reported, for example, to involve a small volume contraction and an increase in asymmetry as deduced from small-angle X-ray scattering (Durchschlag *et al.*, 1970).

The large positive ΔC_p that is observed in reversible protein denaturation is currently attributed to the exposure of hydrophobic groups to the solvent and a resulting increase in the order of the neighboring water molecules (see, for example, Tanford, 1970). In these terms, the large negative ΔC_p in the complex formation of NAD with the dehydrogenases would be attributed to a change in protein structure which diminishes exposure of hydrophobic groups to the solvent and increases intramolecular hydrophobic bonding. A transition of this type would stabilize the protein and is consistent with the observation that the first-order rate constant of thermal inactivation of the protein is decreased by an order of magnitude in the presence of NAD concentrations sufficient to saturate the binding sites (Furfine and Velick, 1965).

It may be noted that the positive $\Delta S°$ in the denaturation of chymotrypsinogen and other proteins and the negative $\Delta S°$ of NAD complex formation with the dehydrogenases are both of opposite sign from the contributions that would be expected to result, respectively, from the immobilization and release of solvent water proposed to account for the observed ΔC_p values. It would thus appear that other sources of enthropy change in these systems must occur. One possibility would be an increase in the excitation of intramolecular degrees of freedom of the protein with increasing temperature which is frozen out by renaturation or by ligand addition. This effect and related processes determine the sign of the observed $\Delta S°$ over most of the temperature range examined. However a reversal in the sign of $\Delta S°$ in NAD complex formation with the yeast enzyme is observed at low temperatures (Table I), indicating that there is a positive entropy source in the binding reaction.

Thermodynamic contributions arising from environmental changes of ionized or ionizable groups have not been explored. The measurements in this investigation have been made in a pH region where the association constants are maximal and exhibit a minimal pH dependence. By contrast, the pronounced positive cooperativity of NAD binding by the yeast enzyme (Kirschner *et al.*, 1966) occurs at pH 8.5 where the net charge on the protein is high (Velick and Hayes, 1953) and where the binding measurements by Stockell (1959) indicate a very strong dependence of K upon pH. A preliminary report of the thermodynamic parameters of NAD binding by the yeast enzyme at 40°, pH 8.5, has appeared (Kirschner and Schuster, 1970). These calculations were based upon the temperature dependence of relaxation amplitudes in equilibrium perturbation kinetics and provide assignments for both the R and the T forms of the protein. The numbers differ markedly for the two forms. Parameters for the R form, which presumably predominates under our experimental conditions, are in the range of those reported here for 40° but do not include a measure of the ΔC_p.

The yeast and skeletal muscle enzymes are clearly homologous in their gross physical features and catalytic properties but exhibit numerous differences in amino acid composition and hence in primary structure. With respect to their pyridine nucleotide binding properties, they differ from other dehydrogenases in exhibiting a higher affinity for NAD than for NADH, and from each other in the magnitudes of the association constants. The large differences in the ΔH of NAD addition, particularly at 5°, are further indications of intrinsic structural differences.

The importance of solvation as a major factor in determining the dynamic behavior of proteins in solution is well recognized. Thermodynamic data of the present type provide a necessary but relatively nonspecific type of information on this point. Some other properties of the hydration shell of a glyceraldehyde 3-phosphate dehydrogenase have recently been studied by Aune and Timasheff (1970).

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Properties of an Insoluble Form of Trypsin*

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ABSTRACT: Insoluble trypsin preparations containing as high as 95% of the original soluble tryptic activity toward a small substrate have been prepared by attaching the enzyme onto aminoethylcellulose with the use of glutaraldehyde. These preparations are quite resistant to autolysis and suspensions of them are stable at room temperature for days or in the cold for months. Many properties of the insoluble trypsins, such as pH optima, activity in urea, and inhibition by certain low molecular weight compounds, are qualitatively similar to those of soluble trypsin. The main differences between the two forms of the enzyme, besides their relative stabilities, are the lack of complete inhibition of the insoluble form by excess diisopropyl phosphorofluoridate; the incomplete inhibition of the insoluble trypsin by protein inhibitors and an 80–100% reactivation by slow oxidation of insoluble trypsins which had been inactivated by reduction of the disulfide bonds. Preliminary studies of the action of pronase on insoluble trypsin indicate that a significant degradation of the trypsin molecule can occur without loss of enzymatic activity.

There are two main reasons for studying insoluble enzymes. According to Crook (1968) these are: first, to determine alterations in properties which result when enzyme molecules are immobilized on an insoluble matrix; and, second, to provide convenient insoluble catalysts which can be readily manipulated and easily removed from the reaction mixture. A further application is the possibility of determining the amino acid residues and conformation of the active center of an enzyme where this area has been relatively fixed in place. An additional application has resulted from the work presented here. The insoluble form of trypsin described in this paper is extremely resistant to autolysis and thus modification and degradation reactions can be performed at neutral or slightly basic pH values without the necessity of adding Ca^{2+} ions or inhibitors which sometimes are incompatible with other components in the reaction mixture. The stability of other forms of insoluble trypsin has been discussed in the literature (Weetall, 1970; Epstein and Anfinsen, 1962a,b; Epstein *et al.*, 1962; Levin *et al.*, 1964).

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