Energetics of the Cooperative and Noncooperative Binding of Nicotinamide Adenine Dinucleotide to Yeast Glyceraldehyde-3-Phosphate Dehydrogenase at pH 6.5 and pH 8.5. Equilibrium and Calorimetric Analysis Over a Range of Temperature†

Carl W. Niekamp,‡ Julian M. Sturtevant,* and Sidney F. Velick

ABSTRACT: The binding of nicotinamide adenine dinucleotide (NAD^+) to yeast glyceraldehyde-3-phosphate dehydrogenase (GPDH) has been studied at pH 6.5 and 8.5, at 5, 25, and 40 °C, by calorimetry, fluorometry, spectrophotometry, equilibrium dialysis, and flow dialysis. As reported earlier for pH 7.3 (Velick S. F., Baggott, J. P., and Sturtevant, J. M. (1971), *Biochemistry 10,* 779), the binding is accompanied by enthalpy changes which become rapidly more negative as the temperature increases, with $\Delta C_p = -500$ to -750 cal deg^{-1} (mole of NAD^+ bound)$^{-1}$, and by entropy changes which also, as required by the large negative ΔC_p, become rapidly more negative with increasing temperature. The binding data at pH 6.5 can be fitted on the basis of either four identical noninteracting sites, or of four sites showing a small degree of negative cooperativity. The data at pH 8.5, particularly at 40 °C, require the introduction of positive cooperativity, as was previously shown by Kirschner et al. (Kirschner, K., Eigen, M., Bittman, R., and Voigt, B. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 1661), and can be equally well fitted on the basis of a sequential model (Adair, G. S. (1925), *J. Biol. Chem. 63,* 529) or a concerted model (Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88). It is proposed that the observed thermodynamic changes are largely the result of a hydrophobic effect due to a decrease in the exposure of nonpolar groups to the solvent, and of a tightening of the protein structure when the coenzyme is bound with concomitant decrease in the number of easily excitable internal degrees of freedom.

A previous report described the energetics of nicotinamide adenine dinucleotide (NAD^+)[1] binding to the glyceraldehyde-3-phosphate dehydrogenase (GPDH) of yeast over a range of temperatures (Velick et al., 1971). At each temperature, the data obtained in fluormetric and calorimetric titrations were fitted with theoretical curves calculated on the basis of four identical and noninteracting binding sites. The present study extends this work to the binding at pH 6.5 and 8.5 over the temperature range of 5–40 °C. Calorimetric titrations have been supplemented under the same conditions of pH and temperature with one or more of the following methods for determining binding constants: spectrophotometry, fluorometry, flow dialysis, and equilibrium dialysis.

In contrast to the noncooperative binding at pH 7.3, the binding of the coenzyme at pH 8.5 exhibits cooperativity, particularly at 40 °C, as demonstrated by a variety of equilibrium and kinetic measurements (Kirschner, 1971; Kirschner et al., 1971; von Ellenrieder et al., 1972). These investigators concluded that the cooperativity can be adequately interpreted in terms of the concerted transition model proposed by Monod et al. (1965). In a study by the method of equilibrium dialysis over a temperature range at pH 8.5, Cook and Koshland (1970) concluded that their results were best described by the classical scheme of four successive equilibria with both negative and positive cooperativity as expressed by the intrinsic equilibrium binding constants $K_1 < K_2 > K_3 > K_4$.

The study of ligand binding to multisubunit proteins in the past has been directed primarily at the determination of binding constants and free energies. Further insight into the nature of a binding reaction may follow from a knowledge of the enthalpic and entropic contributions to the standard free-energy change in the reaction. In addition, the fitting of the equilibrium binding data collected over a temperature range can be critically checked for internal thermodynamic consistency via the van't Hoff equation if calorimetric enthalpy data are obtained under the same conditions.

Materials and Methods

Materials. [*carbonyl*-^{14}C]Nicotinamide adenine dinucleotide, batch 10, with a specific activity of 50 mCi/mmol, was obtained from Amersham/Searle Corp. Nicotinamide [2,8-^{3}H]adenine dinucleotide, lot number 745-253, with a specific activity of 3.46 Ci/mmol, was purchased from New England Nuclear. Dithiothreitol was obtained from Calbiochem. Sephadex G-25 and DEAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals. Yeast 3-phosphoglyceric phosphokinase, yeast alcohol dehydrogenase, D-(−)-3-phosphoglyceric acid (disodium salt), NAD^+, NADH, and imidazole were purchased from Sigma Chemical Co. Aquasol, the liquid scintillation counting cocktail, was obtained from New England Nuclear. All other chemicals were reagent grade. Glass-distilled, deionized water was used throughout in making up solutions.

All pH measurements were made using a Radiometer pH meter (Model TT1a). Protein and coenzyme concentrations, enzyme activity assays, and spectrophotometric titrations were determined using a Cary, Model 14, spectrophotometer. The molecular weight and the 280-nm absorption coefficient at pH

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06520, and the Department of Biological Chemistry, University of Utah, Salt Lake City, Utah 84112. *Received July 28, 1976.* This research was supported by research grants from the National Institutes of Health (GM04725 and AM13115) and the National Science Foundation (GB36346X).

‡ Present address: Hanover College, Hanover, Indiana 47243.

[1] Abbreviations used: NAD^+, NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms, respectively; GPDH, yeast glyceraldehyde-3-phosphate dehydrogenase; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

7 and 25 °C used for apo-GPDH were 144 000 and 0.84 cm^2 mg^{-1}, respectively.

The preparation of GPDH from Fleischman's bakers' yeast has been described previously (Velick et al., 1971). The enzyme was stored as a slurry in 80% saturated ammonium sulfate at 5 °C. Just before use, it was centrifuged at 15 000 rpm for 20 min at 5 °C. The crystalline pellet was dissolved in 1 or 2 ml of 0.05 M phosphate (pH 6.5) or 0.05 M pyrophosphate (pH 8.5) buffer, both containing 2 mM EDTA and 0.5 mM dithiothreitol, and incubated for 1 h at 0 °C. The dithiothreitol and remaining traces of ammonium sulfate were removed from the enzyme solution by passage through a Sephadex G-25 column, which was previously equilibrated with the nitrogen-saturated elution buffer, 0.05 M phosphate, pH 6.5, or 0.05 M pyrophosphate, pH 8.5, both containing 2 mM EDTA. The active apoenzyme was kept at 0 °C in a nitrogen atmosphere or in an evacuated container. The enzyme activity was measured as previously described (Velick et al., 1971). The yeast 3-phosphoglyceric phosphokinase used in the assay was free of ammonium sulfate. GPDH preparations sometimes attained but never exceeded the specific activity of 155 IU (μmol of NADH reduced per min per mg of GPDH). The values for the 280 nm/260 nm enzyme absorbance ratio generally fell in the range of 2.1 ± 0.1, indicating very little residual-bound nucleotide (Kirschner et al., 1971).

The purchased NAD^+ was used without further purification. The NAD^+ concentrations were determined spectrophotometrically at 260 nm using a molar extinction coefficient supplied by Sigma Chemical Co. that ranged from 17 700 to 18 100 M^{-1} cm^{-1} depending on the particular lot number. The chemical purity of the NAD^+ was checked by converting NAD^+ to NADH with yeast alcohol dehydrogenase by the method of Colowick et al. (1951).

Equilibrium Dialysis. Equilibrium dialysis studies were performed at 5 °C. One-milliliter equilibrium dialysis cells with a membrane surface to cell volume ratio of 2 cm^{-1} were employed. High porosity Sartorius membrane filters, type SM 115-36, were purchased from Science Essentials Co. In order to reduce the equilibration time, magnetic stirring bars were placed on each side of the membrane and stirred by water-driven magnetic stirrers, which were immersed in a 5 °C water bath. Control experiments in the absence of enzyme showed the equilibration time to be about 7 h.

Labeled [*carbonyl*-^{14}C]NAD^+ was tested for both chemical purity, as described above, and for radioactive purity. The radioactive purity was determined according to a modified procedure of Chaykin et al. (1965). The NAD^+ was eluted through a DEAE-Sephadex A-25 column with an elution buffer of 0.05 M glycine with a linear salt gradient ranging from 0 to 0.8 M NaCl. Both the chemical and radioactive purity of the [*carbonyl*-^{14}C]NAD^+ were found to be over 98%. The radioactive NAD^+ stock solution was prepared by dissolving the [*carbonyl*-^{14}C]NAD^+ in a solution containing unlabeled NAD^+. The resultant solution was found to be stable for several days when the solution was adjusted to pH 6 and stored in the refrigerator. The decomposition rate constant of NAD^+ is extremely low at pH's below 7 (Lowry et al., 1961).

Flow Dialysis. Nucleotide binding was measured over a temperature range by flow dialysis (Colowick and Womack, 1969). The apparatus was modified by the attachment of a Lucite tube to the upper chamber which allowed the flow dialysis cell to be submerged in a water bath along with the eluting buffer reservoir. The addition of unlabeled NAD^+ through the Lucite tube into the upper chamber of the dialysis cell was accomplished by means of a micrometer syringe. The radiochemical purity of the [*adenine*-2,8-^{3}H]NAD^+, stated by New England Nuclear to be 97%, was verified by paper chromatography on Whatman no. 1 paper, with the developing solvent isobutyric acid, concentrated ammonium hydroxide, 0.1 M EDTA, and water (66:1:1:32). The 3% freely diffusible impurities were corrected for in binding calculations. The following procedures and conditions were employed during an experiment. The enzyme concentration ranged from 48 to 54 μM. The enzyme solution was added to the upper chamber and allowed to come to temperature equilibrium, and the radioactive NAD^+ was then added. The flow rate of the elution buffer through the bottom chamber of the dialysis cell was approximately 9 ml/min. A Gilford fraction collector with a drop counter attachment was used to collect samples at a rate of three tubes per minute. The steady state was usually reached within 2 min after the addition of the unlabeled NAD^+ into the upper chamber. The duration of an experiment in which six points were obtained was 35–40 min. Additional points were sacrificed in each experiment in order to collect several tubes at steady state at each NAD^+ concentration. The time required for achievement of a steady state was checked as a function of temperature. It was shown that negligible amounts of radioactive NAD^+ were lost from the upper chamber during an experiment by obtaining the same steady state value both in the absence and presence of enzyme with excess unlabeled NAD^+.

Fluorescence Titrations. The binding of NAD^+ to GPDH was measured by the quenching of the fluorescence of the tryptophan residues of the enzyme excited at 300 nm and monitored at 350 nm, as previously described (Velick et al., 1971).

Spectrophotometric Titrations. The binding of NAD^+ to GPDH was determined by difference spectral titrations at 400 nM. The absorbance of the GPDH-NAD^+ complex at 400 nm was corrected for any background absorbance due to free NAD^+. The spectrophotometer cell compartments were thermostated and continually flushed with N_2 in order to minimize oxidation of the enzyme sulfhydryl groups. An entire spectral titration curve could be obtained within 10–20 min. The spectral data presented are the average of several spectral titration curves.

Thermal Titrations. The binding enthalpies were measured in a flow calorimeter based on the Beckman Instruments, Model 190, microcalorimeter (Velick et al., 1971). Enzyme and coenzyme solutions were flowed into the calorimeter at equal rates of 0.044 ml min^{-1} in most experiments, with occasional changes to half or twice the flow rates in order to check for completeness of the reaction. All appropriate corrections for heats of dilution and viscous heating due to flow were applied. The actual heat effects observed ranged from 10 to 500 μcal min^{-1}. The instrument was calibrated at 5, 25, and 40 °C by measurements of the heat of reaction of HCl with excess NaOH, using the values for the enthalpies of ionization of water as a function of temperature reported by Grenthes et al. (1970).

The small uptake of protons on the binding of NAD^+ and the small heats of ionization of the buffers employed lead to buffer corrections to the observed enthalpies not exceeding 0.25 kcal mol^{-1}. Such corrections were not applied.

Results

Enthalpy of Binding of NAD^+ to GPDH at Saturation. The most firmly established of our calorimetric data are the en-

TABLE I: Calorimetric Values for the Binding of NAD^+ to Yeast Glyceraldehyde-3-phosphate Dehydrogenase.

	pH 6.5		pH 7.3[a]		pH 8.5	
Temp (K)	ΔH_{max}	ΔC_p	ΔH_{max}	ΔC_p	ΔH_{max}	ΔC_p
278.2	−14.5		−7.6		−27.2	
		−2100		−2100		−3000
298.2	−56.4		−49.6		−87.6	
		−1900		−2100		−3100
313.2	−84.8		−80.4		−134	

[a] Results obtained by Velick et al. (1971). ΔH_{max}, kcal (mol of enzyme)$^{-1}$; ΔC_p, cal K^{-1} (mol of enzyme)$^{-1}$.

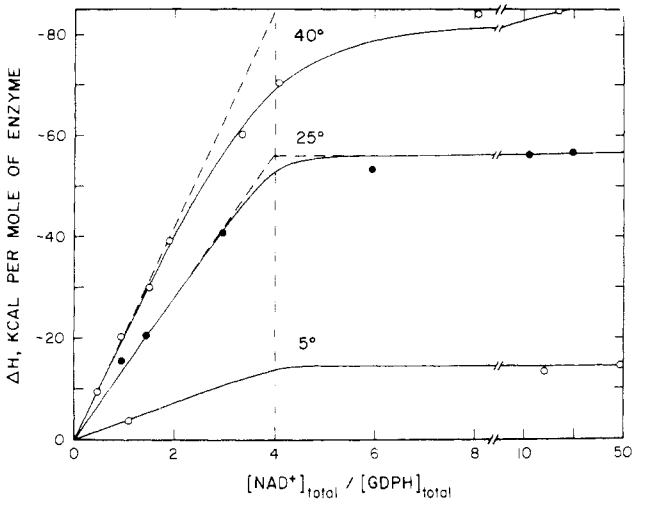

FIGURE 1: Thermal titrations of yeast GPDH with NAD^+ in 0.05 M phosphate, 2 mM EDTA buffer, pH 6.5, performed at 5, 25, and 40 °C. The enzyme concentration ranged from 20 to 52 μM. The curves were calculated using the values of K_i and ΔH_i given in Table II.

thalpy changes which result when the apoenzyme is saturated with the coenzyme:

$$GPDH + 4\ NAD^+ \rightarrow GPDH(NAD^+)_4 \qquad (1)$$

These quantities were determined at pH 6.5 and 8.5 at 5, 25, and 40 °C and are summarized in Table I, along with those reported by Velick et al. (1971) for the same system at pH 7.3.

Binding of NAD^+ at pH 6.5. The binding of NAD^+ to yeast GPDH was observed as a function of the concentration of coenzyme by calorimetry at 5, 25, and 40 °C, and by fluorescence quenching at 3 and 37 °C. As shown in Figures 1 and 2, all the binding curves at this pH appear to exhibit hyperbolic form.

Binding of NAD^+ at pH 8.5. The equilibrium dialysis and flow dialysis data at 5 °C are presented in a Scatchard plot (Figure 3). The nonlinear curve observed is of the form expected for a system exhibiting positive cooperativity. Extrapolation of the linear portion of the plot leads to a value of 3.2 mol of NAD^+ bound per mol of enzyme at saturation. Similar results were obtained from the 25 °C flow dialysis data. From equilibrium dialysis data, Cook and Koshland (1970) obtained values of 3.3 and 3.5 at 4 and 25 °C, respectively. von Ellenrieder et al. (1972) obtained a value between 3.0 and 3.2 at 20 °C without enzyme activity correction. They found their enzyme to be 74–81% active from assays of enzyme activity. Upon using the active enzyme concentration, a value of approximately four was obtained for the number of binding sites per molecule. Kirschner et al. (1971) utilized both enzyme activity and the extinction coefficient of the GPDH–NAD^+ complex at pH 8.5 to determine the active enzyme concentration at 40 °C.

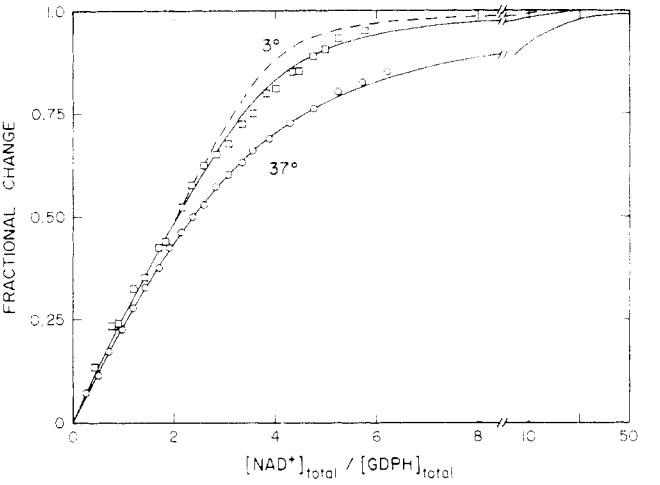

FIGURE 2: Fluorometric titrations at 3 and 37 °C for the binding of NAD^+ to yeast GPDH at pH 6.5. The enzyme concentration was 4.4 μM. The curves were drawn using the binding constants listed in Table II. The dashed curve at 3 °C was calculated with the values $K_1 = 0.076\ \mu M$, $K_2 = 0.15\ \mu M$, and $K_3 = K_4 = 0.30\ \mu M$, which are consistent in terms of the van't Hoff equation and the observed enthalpies (see Discussion) with the values obtained at 37 °C, whereas the solid curve at 3 °C provides a better fit using the values of $K_1 = 0.076\ \mu M$, $K_2 = 0.15\ \mu M$, and $K_3 = K_4 = 0.64\ \mu M$.

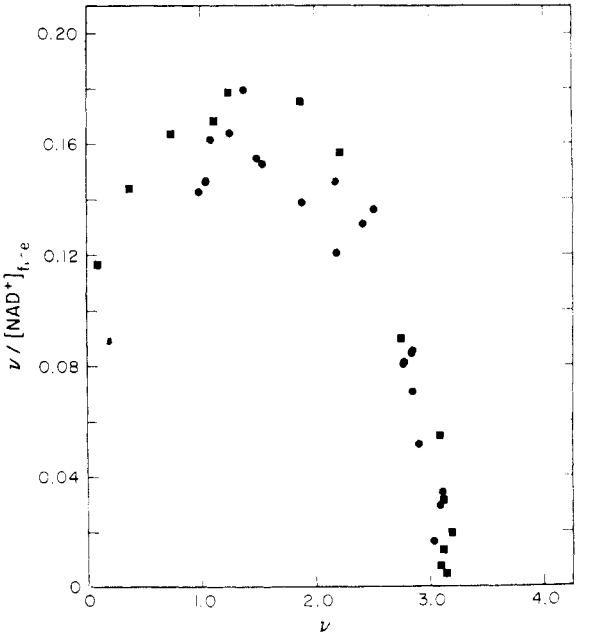

FIGURE 3: Equilibrium dialysis (●) and flow dialysis (■) results for the binding of NAD^+ to yeast GPDH at pH 8.5 and 5 °C. The data are presented as a Scatchard plot of $\nu/[NAD^+]_{free}$ vs. ν, where ν is the mol of NAD^+ bound/mol of enzyme.

Using the value 3.2 obtained from the equilibrium dialysis and flow dialysis results, the "active" enzyme concentration with respect to NAD^+ binding capacity, as opposed to catalytic ability, was taken to be 80% of the total enzyme concentration. As mentioned previously, the apoenzyme has an A_{280}/A_{260} value of 2.1 ± 0.1. The relationship of this ratio to the moles of NAD^+ bound can provide an estimate of the amount of

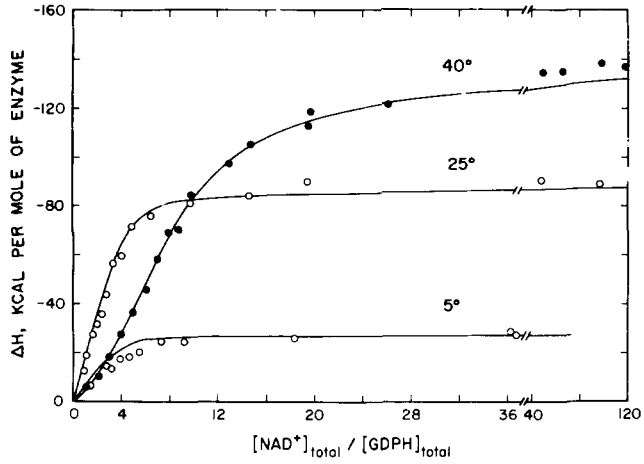

FIGURE 4: Thermal titrations of yeast GPDH with NAD^+ in 0.05 M pyrophosphate–2 mM EDTA buffer (pH 8.5) performed at 5, 25, and 40 °C. The enzyme concentration ranged from 39 to 41 μM. The theoretical curves are drawn using the values of K_i and ΔH_i given in Table IV.

NAD^+ bound to the apoenzyme (Kirschner et al., 1971). The residual amount of NAD^+ bound to the apoenzyme was approximated to be 0.025 mol of NAD^+ per mole of binding sites. In the remainder of this paper, all enzyme concentrations and data presented at pH 8.5 utilize the "active" enzyme concentration (80% of the total concentration) and assume the apoenzyme to be 97.5% nucleotide free.[2]

Thermal titration curves determined at 5, 25, and 40 °C are shown in Figure 4. The enthalpy change per mole of enzyme is plotted as a function of the ratio of the total concentration of NAD^+ to the total concentration of enzyme. The curves at 5 and 25 °C appear to be of normal hyperbolic shape, while that at 40 °C is definitely sigmoidal.

The binding of NAD^+ was studied by equilibrium and flow dialysis at 5 °C as mentioned above, and by spectrophotometry, flow dialysis, and fluorometry at all three temperatures. The results of these measurements are compared with the thermal data in Figure 5. In view of the wide range of enzyme concentrations used in various types of experiments, it was necessary to plot the data as functions of the concentration of unbound NAD^+, $[NAD^+]_{free}$. This quantity was directly observed in the equilibrium and flow dialysis experiments but had to be calculated for the fluorometric, spectrophotometric, and calorimetric data. This calculation is based on the assumptions that the enzyme, after its concentration is corrected as outlined above, binds four molecules of NAD^+ per molecule of enzyme, and that, except in the thermal titrations, there is a linear relation between the change in the experimental observable and the extent of binding. For the most part, the data collected by all five methods are in reasonable agreement and substantiate these assumptions, although, for example, colinearity of fluorescence signals and extent of binding does not occur in all protein-ligand systems (Archer et al., 1973). It should be mentioned that the data collected by the various methods are subject to more scatter, particularly where relatively tight binding exists, when plotted against $[NAD^+]_{free}$ rather than

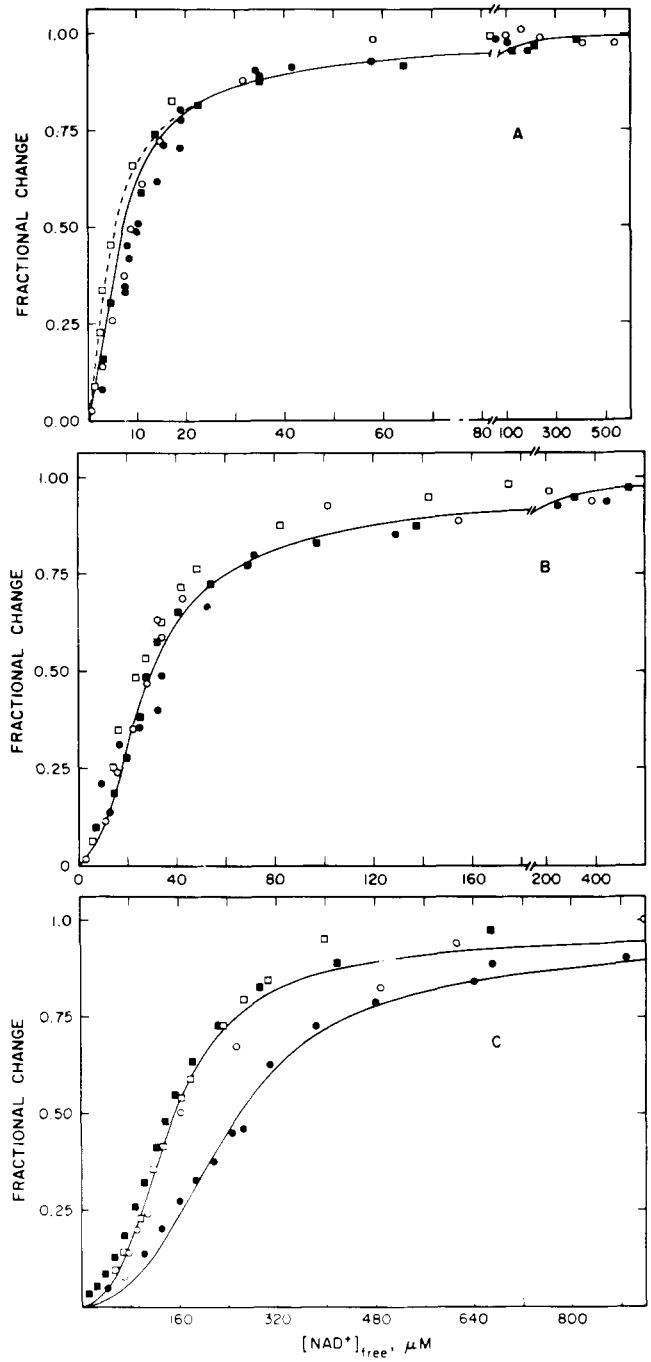

FIGURE 5: The binding of NAD^+ to yeast GPDH at pH 8.5 (A) 5 °C; data obtained by equilibrium dialysis (●), flow dialysis (○), spectrophotometry (■), and fluorometry (□). (B) At 25 °C; data obtained by flow dialysis (○), spectrophotometry (■), fluorometry (□), and calorimetry (●). (C) At 40 °C; data obtained by flow dialysis (○), spectrophotometry (■), fluorometry (□), and calorimetry (●). The fractional change of the experimental observable is plotted against the free NAD^+ concentration. The equilibrium and flow dialysis data are plotted against the observed free NAD^+ concentration, while the calorimetric, fluorometric, and spectrophotometric data are plotted against calculated values of this concentration. The enzyme concentration ranges were 3.5, 18–40, 26–36, 38–43, and 39–41 μM for fluorometric, spectrophotometric, equilibrium dialysis, flow dialysis, and thermal titrations, respectively. The theoretical curves were generated using the values listed in Table III.

$[NAD^+]_{total}$. The calculated values of $[NAD^+]_{free}$ are determined from the difference of two large numbers, $[NAD^+]_{total}$ and $[NAD^+]_{bound}$, where the bound NAD^+ concentration is evaluated as $4.0\,Y[GPDH]_{total}$, and Y is the fractional change

[2] The enzyme concentration was not corrected as explained here in the interpretation of the data at pH 6.5, nor in earlier work at pH 7.3 (Velick et al., 1971). In a single equilibrium dialysis experiment performed at pH 6.5, 5 °C, the Scatchard plot indicated 3.7–4.0 mol of NAD^+ bound/mol of enzyme at saturation. Mockrin et al. (1975) have recently reported a similar comparison between pH 7.5 and 8.5; they attribute the apparent inability of the enzyme to bind 4 mol of NAD^+ to negative cooperativity.

TABLE II: Apparent Thermodynamic Parameters for the Binding of NAD^+ to Yeast GPDH at pH 6.5. Model: $K_1 > K_2 > K_3 = K_4$ and $\Delta H_1 = \Delta H_2 = \Delta H_3 = \Delta H_4$.

Property	Temp (°C)	1st Site	2nd Site	3rd and 4th Sites	Total[c]
K[a] (μM)	3	0.076	0.15	0.30 (0.64)[b]	
	5	0.08	0.16	0.31	
	25	0.22	0.43	0.87	
	37	0.66	1.3	2.6	
	40	0.91	1.8	3.6	
$\Delta G°$ (kcal mol^{-1})	3	−9.0	−8.6	−8.2 (−7.8)[b]	−34.1 (−33.3)[b]
	5	−9.0	−8.7	−8.9	−34.3
	25	−9.1	−8.7	−8.3	−34.3
	37	−8.8	−8.4	−7.9	−33.0
	40	−8.7	−8.2	−7.8	−32.5
ΔH (kcal mol^{-1})	3	−2.6	−2.6	−2.6	−10.3
	5	−3.6	−3.6	−3.6	−14.5
	25	−14.1	−14.1	−14.1	−56.4
	37	−19.8	−19.8	−19.8	−79.1
	40	−21.2	−21.2	−21.2	−84.8
$\Delta S°$ (cal K^{-1} mol^{-1})	3	23	22	20 (19)[b]	86 (83)[b]
	5	20	18	19	71
	25	−17	−18	−20	−74
	37	−36	−37	−38	−150
	40	−40	−41	−43	−170
ΔC_p (cal K^{-1} mol^{-1})	15	−520	−520	−520	−2100
	32.5	−470	−470	−470	−1900

[a] All values are presented as binding values with the exception of the intrinsic association equilibrium constants, K, values for which are given as dissociation constants. [b] The values in parentheses are the values used to obtain a better fit (shown as a solid line in Figure 2) to the fluorometric data at 3 °C. [c] The weighted mean standard deviation for fitting all the thermal and fluorometric data is 2.3%. Using the better fit at 3 °C reduces the weighted mean standard deviation to 1.6%.

in the experimental observable. The experimental error in the determination of the fractional change can be such that the error in determining $[NAD^+]_{bound}$ is greater than the value of $[NAD^+]_{free}$, in which case $[NAD^+]_{free}$ is experimentally indistinguishable from zero (Weber and Anderson, 1965).

Discussion

Use of the van't Hoff Equation in Analyzing Binding Data. Calorimetric data seldom lead to binding constants as accurate as those based upon the more specific observations afforded by spectrophotometric, fluorometric, or equilibrium dialysis methods. This is particularly true in the present multisite case at pH 8.5, where the enthalpy increments for the binding of NAD^+ at successive sites are not equal. However, the calorimetric enthalpies are of importance in arriving at equilibrium constants based upon other kinds of data taken over a range of temperatures. Such equilibrium constants must be mutually consistent in terms of the van't Hoff equation. If we use dissociation equilibrium constants and association enthalpies, and assume ΔC_P to be independent of temperature, the integrated form of the van't Hoff equation is

$$\ln \frac{K_{T_2}}{K_{T_1}} = \frac{1}{R}\left(\frac{\Delta H_{T_2}}{T_2} - \frac{\Delta H_{T_1}}{T_1}\right) - \left(\frac{\Delta C_p}{R} \ln \frac{T_2}{T_1}\right) \quad (2)$$

NAD^+ Binding at pH 6.5. The binding curves observed at pH 6.5 all appear to be of hyperbolic form, and linear extrapolation of the initial slopes of the thermal titration curves, measured at relatively high enzyme concentration, intersects the enthalpy limits at the point corresponding to the binding of 4 mol of NAD^+/mol of GPDH (Figure 1). It might therefore be concluded that the four binding sites are identical and noninteracting and have equal binding enthalpies. In fact, all of the data of pH 6.5 can be reasonably well fitted on this basis. However, a slightly better fit was obtained over the whole temperature range on the assumption of negative cooperation between sites such that $K_1 > K_2 > K_3 = K_4$ and $\Delta H_1 = \Delta H_2 = \Delta H_3 = \Delta H_4$, where K_i is the dissociation constant and ΔH_i the enthalpy of binding for the ith site. The dissociation constants at 3, 5, 25, and 40 °C in Table II were calculated from values obtained by fitting the fluorometric results at 37 °C by means of the van't Hoff equation with $\Delta C_p = -0.50$ kcal deg^{-1} (mol of NAD^+ bound)$^{-1}$, and were then shown to be reasonably consistent with the data at these temperatures. A somewhat better fit to the data at 3 °C was obtained by setting $K_3 = K_4 = 0.64$ μM instead of 0.30 μM.

It must be emphasized that a uniquely valid fit of experimental binding data of limited accuracy cannot be obtained, especially in a case such as this where several binding sites are involved. The constants listed in Table II should give a means for predicting with reasonable accuracy certain properties of GPDH under conditions of concentration or temperature where measurements were not made. However, the support that they provide for the occurrence of negative cooperativity at pH 6.5 is marginal.

The large variation with temperature of both ΔH and $T\Delta S°$ at pH 6.5, corresponding to the large negative ΔC_p of approximately −2000 cal deg^{-1} (mol of enzyme)$^{-1}$, is evident in Table II. The thermodynamic parameters reported by Velick et al. (1971), for the binding of NAD^+ to GPDH at pH 7.3 where independent and identical sites were found, are similar to those listed in Table II. The fact that $\Delta G°$ is relatively little affected by temperature is to be expected from the fact that the first derivative of $\Delta G°$ with respect to temperature is $-\Delta S°$ and, thus, does not directly involve ΔC_p.

Hill Plots of Dialysis Data at pH 8.5. Hill plots of the flow dialysis data at 5, 25, and 40 °C and of the equilibrium dialysis

data at 5 °C at pH 8.5 give Hill coefficients, n_H, of 1.6, 1.8, and 2.1 at 5, 25, and 40 °C, respectively (Figure 6). These n_H values compare with 1.8 at 20 °C (von Ellenrieder et al., 1972) and 2.3 ± 0.2 at 40 °C (Kirschner et al., 1971). Values greater than unity are clear indication that positive site cooperation occurs in this system at pH 8.5.

Binding of NAD^+ at pH 8.5: Sequential Model. Plots of all the binding data at 5 and 25 °C against the total ligand concentration, $[NAD^+]_{total}$, have apparent hyperbolic shapes. The equilibrium dialysis, flow dialysis, spectrophotometric and fluorometric data agree within experimental error with the predictions based upon a model of four identical noninteracting sites having dissociation constants of 4.9 μM at 5 °C and 27 μM at 25 °C. The thermal data are represented by these equilibrium constants together with the binding enthalpies of −6.6 and −21.9 kcal (mol of NAD bound)$^{-1}$ at 5 and 25 °C, respectively. The enthalpies and binding constants are mutually consistent in terms of the van't Hoff equation. Using the values at 5 °C and a ΔC_p of −740 cal K^{-1} (mol of NAD^+)$^{-1}$, the van't Hoff calculated dissociation constant, 15.2 μM at 20 °C, is in satisfactory agreement with the value of 14.1 μM reported by von Ellenrieder et al. (1972) for 20 °C. It is significant that binding data, which are fitted well by hyperbolic curves, can also be fitted within experimental uncertainty to sigmoidal curves, as shown in Figure 5A,B which will be discussed below.

The thermal titration curve at pH 8.5 and 40 °C shown in Figure 4 is definitely sigmoidal in shape. The most general equation which can be applied here (Adair, 1925) involves four unequal equilibrium constants and four unequal enthalpies. Since the apoenzyme is known to be composed of four identical subunits, the most reasonable model with eight adjustable parameters includes cooperative and/or anticooperative site interactions and the statistical factors appropriate for tetrahedral symmetry. With this model, there are originally four sites with intrinsic dissociation constant K_1 and enthalpy of binding ΔH_1. After one of the sites has been occupied, there remain three identical sites with parameters K_2 and ΔH_2, then two sites with K_3 and ΔH_3, and finally one site with K_4 and ΔH_4. Setting $A_i = [L]/K_i$, where [L] is the free ligand concentration, the fraction of sites occupied is given by

$$Y = \frac{A_1 + 3A_1A_2 + 3A_1A_2A_3 + A_1A_2A_3A_4}{Z} \quad (3)$$

and the enthalpy change per mol of enzyme by

$$\Delta Q = (4\Delta Q_1 + 6\Delta Q_2 + 4\Delta Q_3 + \Delta Q_4)/Z \quad (4)$$

In these equations,

$$Z = 1 + 4A_1 + 6A_1A_2 + 4A_1A_2A_3 + A_1A_2A_3A_4 \quad (5)$$

and

$$\Delta Q_i = \sum_{j=1}^{i} \left(\Delta H_i \prod_{k=1}^{i} A_k \right) \quad (6)$$

The thermal data for 40 °C in Figure 4 can be well fitted with intrinsic dissociation constants 1000, 300, 70, and 30 μM and corresponding enthalpies −9.0, −13.0, −19.0, and −93.0 kcal mol^{-1}. With eight adjustable parameters it is, of course, out of the question to obtain a uniquely satisfactory fit, and, indeed, various of the above parameters can be changed, as will be seen below. The spectrophotometric, fluorometric, and flow dialysis data at 40 °C can be fitted moderately well with the same set of equilibrium constants, with a weighted mean standard deviation of ±3.3%.

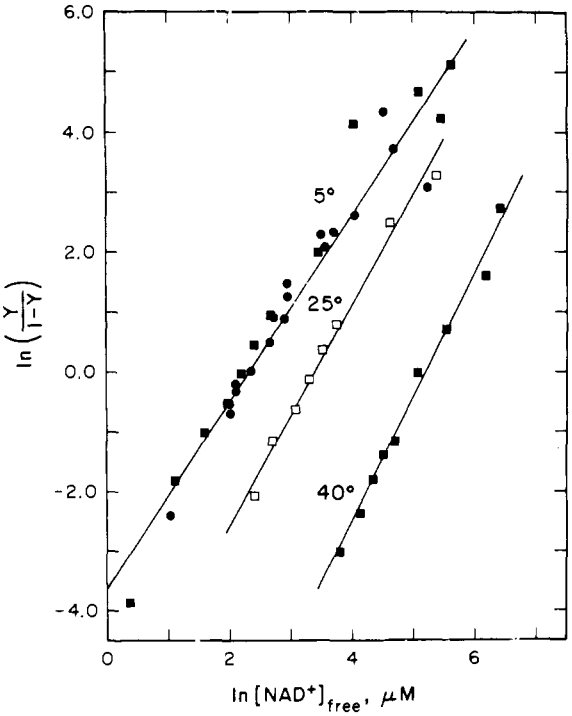

FIGURE 6: Hill plots of flow dialysis (□, ■) data at 5, 25, and 40 °C and equilibrium dialysis (●) data at 5 °C for the binding of NAD^+ to yeast GPDH at pH 8.5. Y is the fractional extent of binding.

Two points need emphasis here. In the first place, as noted above, the equilibrium constants and enthalpies at all three temperatures must be mutually consistent in terms of the van't Hoff equation. This requirement very greatly limits the range of parameters permissible in the curve-fitting process. Secondly, the data in Figure 5C suggest that some of the properties employed may be nonlinear functions of the coverage. The 40 °C flow dialysis data, which provide a direct linear measure of the extent of binding, definitely indicate that the thermal titration does not provide a linear measure. Such a conclusion is not so definitive for the spectrophotometric and fluorometric data, which agree fairly well with the flow dialysis results. It is evident that large differences in the individual site enthalpies at 40 °C must be included.

Although the dissociation constants and enthalpies given above led to a good fit of the 40 °C data, they could not be satisfactorily extended to include the data at 5 and 25 °C. As shown in Figure 5A,B, the various types of data at 4 and 25 °C can be fitted in a thermodynamically consistent manner with a model of four identical sites having site interactions such that $K_1 < K_2$, $k_3 = K_4$, the equilibrium constants and enthalpies being given the values listed in Table III. The dissociation constants at 5 °C were selected to give the best fit for the equilibrium and flow dialysis data, since these data alone give an unambiguous measure of the binding. The individual site enthalpies, $\Delta H_1 \neq \Delta H_2 = \Delta H_3 = \Delta H_4$, with $\Delta H_{total} = \Sigma \Delta H_i$, at 5 and 25 °C were then adjusted to give acceptable fits for the thermal, spectrophotometric, fluorometric, and flow dialysis data at 25 °C.

Proceeding further with this model, a good fit was obtained at 40 °C for the thermal data, but a distinctly poorer fit for the spectrophotometric, fluorometric, and flow dialysis data. To achieve the better fit of these data shown in Figure 5C, it was necessary to select dissociation constants, listed in the second row for 40 °C in Table III, differing from the van't Hoff consistent values in the first row by an average of 0.40 kcal (mol

TABLE III: Apparent Thermodynamic Parameters for the Binding of NAD^+ to Yeast GPDH at pH 8.5. Model: $K_1 < K_2 = K_3 = K_4$ and $\Delta H_1 \neq \Delta H_2 = \Delta H_3 = \Delta H_4$.

Property	Temp (°C)	1st Site	2nd, 3rd, and 4th Sites	Total[c]
K[a] (μM)	5	14 (45)[b]	4.8	
	25	300	17	
	40	7300	120	
		(5800)	(53)	
ΔH (kcal mol^{-1})	5	−17.6	−3.2	−27.2
	25	−33.6	−17.9	−87.3
	40	−45.6	−29.6	−134.4
$\Delta G°$ (kcal mol^{-1})	5	−6.2 (−5.5)[b]	−6.8	−26.5 (−25.8)[b]
	25	−4.8	−6.5	−24.3
	40	−3.1	−5.6	−19.9
		(−3.2)	(−6.1)	(−21.6)
$\Delta S°$ (cal K^{-1} mol^{-1})	5	−41 (−43)[b]	13	−3 (−5)[b]
	25	−97	−38	−210
	40	−140	−77	−370
		(−140)	(−75)	(−360)
ΔC_p (cal K^{-1} mol^{-1})	15	−800	−740	−3000
	32.5	−800	−780	−3100

[a] All values are presented as binding values with the exception of the intrinsic equilibrium constants, K, values for which are given as dissociation constants. [b] The value in parentheses at 5 °C gives a better fit for all data collected at this temperature (Figure 5A). The second row of values at 40 °C (in parentheses) was used to fit the fluorometric, spectroscopic, and flow dialysis data. [c] The weighted mean standard deviation for fitting the spectral, fluorometric, equilibrium dialysis, flow dialysis, and thermal data over the temperature range is ±6.7%. Using the values given in parentheses, the weighted mean standard deviation for fitting all the data is ±2.7%.

of ligand bound)$^{-1}$ in terms of free energy. Although it is probable that, if the assumption of linearity in the observed absorbance and fluorescence changes were relaxed, it would be possible to find a thermodynamically consistent set of dissociation constants which obviate the necessity for a separate set of constants for these data, this seems to us to be an exercise in numerology which would not significantly enhance our understanding of the system. The same statement applies to the many additional forms of the general Adair formulation which might be applied.

The sequential model, which we have arrived at here, with the first site having different properties from the other three, is similar to the concerted model discussed in the next section, in that it can be considered that binding the first molecule of ligand changes all four sites to have the properties given in Table III for the 2nd, 3rd, and 4th sites. On this view, which amounts to setting $K_T \gg K_R$ (eq 8 and 9) in the concerted model, the thermodynamic parameters for the conversion of the enzyme from its initial to its final form are as follows: ΔH = −14.4, −15.7, −16.0 kcal mol^{-1}; $\Delta G°$ = +0.4 (+1.3), +1.7, +2.5 (+2.9) kcal mol^{-1}; $\Delta S°$ = −54(−56), −59, −63(−65) cal K^{-1} mol^{-1} at 5, 25, 40 °C, respectively; and ΔC_p = −60, −20 cal K^{-1} mol^{-1} at 15 and 32.5 °C, respectively. A difficulty in this interpretation is that $\Delta G°$ = +0.4 kcal mol^{-1} at 5 °C implies that only two-thirds of the enzyme is in the "initial" form before the addition of NAD^+, so that the first value of ΔH given above would be −22 kcal mol^{-1}, which is rather far out of line with the values for this quantity at 25 and 40 °C. This difficulty does not arise if $\Delta G°$ has the value, +1.3 kcal mol^{-1}, given in parentheses.

Binding of NAD^+ at pH 8.5: Concerted Model. The results of the extensive studies by Kirschner and his colleagues of the binding of NAD^+ to yeast GPDH have been interpreted in terms of the concerted allosteric model proposed by Monod et al. (1965). In this section, we consider the application of this model to our data.

In the concerted model, each subunit can exist in either the T form with lower ligand affinity or the R form with higher ligand affinity, but in any one molecule all subunits must be in the same form. The following equilibria are thus involved for the apoprotein containing n subunits:

$$T_0 \rightleftarrows R_0;\ L_0 = \frac{[T_0]}{[R_0]};\ \Delta H_0 \tag{7}$$

$$T_{i+1} \rightleftarrows T_i + L;\ \left(\frac{i+1}{n-i}\right) K_T = \frac{[T_i][L]}{[T_{i+1}]};\ -\Delta H_T \tag{8}$$

$$R_{i+1} \rightleftarrows R_i + L;\ \left(\frac{i+1}{n-i}\right) K_R = \frac{[R_i][L]}{[R_{i+1}]};\ -\Delta H_R \tag{9}$$

In these expressions T_i and R_i represent species with i molecules of ligand, L, bound; L_0 is the equilibrium constant and $-\Delta H_0$ the enthalpy change for the isomerization of R_0 to T_0; K_T and K_R are intrinsic dissociation constants; and ΔH_T and ΔH_R are binding enthalpies. It is assumed that in either allowed form of the protein all sites are identical and noninteracting.

The equilibrium constants and enthalpies listed in Table IV were obtained on the basis of the concerted model and the van't Hoff equation. In fitting the spectral and fluorometric data, it was assumed that no change takes place in the T_0 to R_0 conversion, and that the change per site occupied is the same for both the T and R forms. Curves constructed with these constants are almost identical with those given in Figure 5 for the sequential model. There is certainly no basis in our data for a choice between the concerted model, with five adjustable parameters (L_0, K_T, K_R, ΔH_0, $\Delta H_T = \Delta H_R$), and the sequential model with four adjustable parameters (K_1, $K_2 = K_3 = K_4$, ΔH_1, $\Delta H_2 = \Delta H_3 = \Delta H_4$).

It was found, as in the case of the sequential model, that somewhat better fits of the noncalorimetric data were obtained using equilibrium constants that are not strictly consistent with the observed enthalpies according to the van't Hoff equation. These values are given in parentheses in Table IV, and corre-

TABLE IV: Apparent Thermodynamic Parameters for the Binding of NAD^+ to Yeast GPDH at pH 8.5. Concerted Model.

Property	Temp (°C)	$T_0 \rightarrow R_0$[a]	R Form	T Form	Total
L_0 for $R_0 \rightleftarrows T_0$	5	3.5	4.1 (5.5)[b]	100 (140)[b]	
K_R, K_T (μM)	25	12	17	420	
	40	60	100 (60)	2600 (1500)	
ΔH (kcal mol^{-1})	5	−4	−5.8	−5.8	−27.2
	25	−16	−17.9	−17.9	−87.6
	40	−25	−27.3	−27.3	−134.2
$\Delta G°$ (kcal mol^{-1})	5	0.69	−6.86 (−6.69)[b]	−5.09 (−4.91)[b]	−26.7 (−26.1)[b]
	25	1.47	−6.51	−4.61	−24.6
	40	2.55	−5.73 (−6.05)	−3.70 (−4.05)	−20.2 (−21.7)
$\Delta S°$ (cal K^{-1} mol^{-1})	5	−17	3.8 (3.2)[b]	−2.6 (−3.2)[b]	−1.8 (−4.0)[b]
	25	−59	−38	−45	−210
	40	−88	−69 (−68)	−75 (−74)	−360 (−360)
ΔC_p (cal K^{-1} mol^{-1})	15	−600	−610	−610	−3000
	32.5	−600	−630	−630	−3100

[a] All values in the table are presented as binding values with the exception of the intrinsic equilibrium constants, K_R and K_T, whose values are given as dissociation constants. All values for the T_0–R_0 isomerization are given for $T_0 \rightarrow R_0$ direction with the exception of the L_0 values, which are given for the $R_0 \rightarrow T_0$ direction. [b] The weighted mean standard deviation for fitting the spectral, fluorometric, equilibrium dialysis, flow dialysis, and thermal data over the temperature range is ±5.4%. The values given in parentheses at 5 and 40 °C deviate slightly from van't Hoff consistency, and lead to a weighted mean standard deviation of ±2.7%.

spond in terms of free energy to an average difference of 0.26 kcal mol^{-1} from the van't Hoff consistent model.

The van't Hoff consistent equilibrium constants at 40 °C given in Table IV are the same as those given by Kirschner et al. (1971), and, as a matter of fact, served as the starting point in our data-fitting efforts. The constants listed for 25 °C are similar to those reported by von Ellenrieder et al. (1972) for 20 °C.

It should be reemphasized here that the information content of the experimental data given in this paper is quite inadequate for the establishment of a uniquely valid model for the binding of NAD^+ to GPDH.

Temperature Dependence of Saturation Enthalpies. The temperature dependence of the enthalpy changes caused by saturation of the apoenzyme with NAD^+ leads to large negative values for ΔC_p, the change in heat capacity. These amount to −2000, −2100 (Velick et al., 1971), and −3000 cal deg^{-1} (mol of enzyme)$^{-1}$ at pH 6.5, 7.3, and 8.5, respectively (Table I). The uncertainty in these values is approximately ±100 cal deg^{-1} (mol of enzyme)$^{-1}$, and to within this limit ΔC_p is independent of temperature. Nearly the same values are observed at pH 6.5 and 7.3, whereas a significant increase is noted at pH 8.5 where there is an obvious change in the character of the binding with the appearance of positive cooperativity.

Various sources for a decrease in heat capacity may be suggested (Kauzmann, 1959). (a) A decrease in the exposure of hydrophobic groups to the solvent, resulting from the binding of NAD^+, would give a negative contribution to ΔC_p, since the specific heat of water ordered around nonpolar groups is higher than that of bulk water. This effect should lead to an increase in entropy, whereas, particulary at 25 and 40 °C, we have observed substantial entropy decreases. (b) An increase in the exposure of electrostatic charges, for example, by the breaking of "salt linkages", or internal ion pairs, would be expected to lead to decreases in both apparent heat capacity and entropy. For example, the ionization of carboxylic acids at 25 °C is accompanied by $\Delta S° \approx -20$ and $\Delta C_p \approx -45$ cal K^{-1} mol^{-1} (Edsall and Wyman, 1958). (c) A decrease in the number of readily excitable internal degrees of freedom in the apoprotein and/or the coenzyme would result in a negative ΔC_p. It is interesting in this connection to note that the value ΔC_p = 2000 cal deg^{-1} mol^{-1} corresponds to the loss of a minimum of 1000 easily excitable internal degrees of freedom, since the upper limit to the contribution to the heat capacity of such a degree of freedom is R (=2 cal deg^{-1} mol^{-1}). The fact that the holoenzyme is more stable with respect to thermal denaturation than the apoenzyme suggests that this source of negative ΔC_p may be significant. Furthermore, this source of heat capacity decrease should lead also to an entropy decrease, by virtue of the relation

$$\Delta S = \int_0^T \frac{\Delta C_p}{T} dT$$

if ΔC_p remains negative over a sufficient fraction of the integration interval. (d) The possibility should be included that the apoenzyme and/or the holoenzyme exists as a temperature-dependent equilibrium of more than one form, and that the nonvanishing ΔC_p reflects the interconversion of these forms. (e) Using buoyant-density measurements, Sloan and Velick (1973) studied the effect of the binding of NAD^+ on the preferential hydration of yeast GPDH at pH 7.4 and 8.5. At pH 7.4 and 25 °C, a −15.6% change in the preferential hydration of the apoenzyme was observed in the formation of the holoenzyme. This change, which releases approximately 500 water molecules per enzyme molecule, corresponds to a 6.3% volume contraction of the solvated GPDH on binding the coenzyme. The effect of the binding of NAD^+ on low-angle x-ray scattering by yeast GPDH at pH 8.5 and 40 °C revealed similar results; Durchschlag et al. (1971) found a volume decrease amounting to 7% at saturation. On the assumption that the partial specific volume, $\bar{v}_1$, for holoenzyme is the same as that measured for the apoenzyme, these authors concluded that the extent of hydration of the protein decreased by 0.07 g of solvent per g protein. This value is essentially the same as the change in preferential hydration values reported by Sloan and Velick (1973), which are 0.073, 0.075, and 0.030 g of H_2O per

g of protein at 25 °C (pH 8.5), 25 and 5 °C (pH 7.4), respectively. These decreases are similar to the values observed by Petrochenko and Privalov (1973) for the desorption of water by lysozyme, serum albumin, and egg albumin in their respective temperature regions of thermal unfolding. If this change in hydration is taken as the sole source of ΔC_p, the specific heat of the "bound" water lost on addition of NAD^+ is calculated to be 1.25 cal deg^{-1} g^{-1}, which appears to be a reasonable value. However, it should be added that, as in the case of the hydrophobic effect discussed above, a decrease in hydration of the protein would be expected to lead to an increase in entropy instead of the observed decrease.

The preceding qualitative discussion would not be materially altered if unitary entropies, obtained by deducting from the observed entropies the cratic term of 8.0 cal K^{-1} mol^{-1} for each site, were employed. Of the various effects considered here as explanations of the large negative values of ΔC_p, we believe those outlined in a and c above are most likely to make the major contributions.

Large negative heat-capacity changes have been reported for several cases of ligand binding to globular proteins. A number of these cases are summarized by Sturtevant (1977). The fact that negative values of ΔC_p have been found for a wide variety of ligands suggests that the sources of these changes are associated more with changes in the proteins than in the ligands.

The large negative values of ΔC_p, whatever their sources, mean that both ΔH and $\Delta S°$ are strong functions of the temperature. As is evident in Tables II and III, this means, in the present case, that at all three values of the pH, the apparent thermodynamic drive for the reaction changes with increasing temperature in the direction of being increasingly enthalpic in character.

Structural Considerations. The amino acid sequence of the yeast GPDH has been determined by Jones and Harris (1972) and the three-dimensional structure of lobster GPDH has been derived from x-ray crystallography by Buehner et al. (1974) and Moras et al. (1975). Although there is only a two-thirds identity of the amino acid sequences of the two enzymes, the residues which appear to be involved in binding NAD^+ are identical in the two molecules. It is therefore likely that the structures of the binding sites in the yeast and lobster muscle enzymes are quite similar. The identified interactions that contribute to the binding include hydrogen bonds, possible hydrophobic forces, an electrostatic interaction, and the thiol interaction with the nicotinamide moiety, probably a charge-transfer complex (Kosower, 1956), which is the source of the spectral band used in the spectrophotometric titrations.

It is usually considered that hydrogen bonds do not make large contributions to the thermodynamics of processes of the present type because new hydrogen bonds formed between ligand and protein, for the most part, replace previously existing bonds with the solvent or within the protein or ligand molecules. The formation of a new covalent or charge-transfer complex could make a substantial contribution to the enthalpy change in the binding process, but it is difficult to see how either of these could, in themselves, account for the large changes in heat capacity and entropy. As discussed earlier, the hydrophobic interactions which occur would be expected to lead to a negative value for ΔC_p, but a positive value of $\Delta S°$ rather than the observed negative value.

It thus appears that no combination of the interactions between the protein and the ligand indicated by the x-ray data is able to lead directly to a qualitative understanding of the observed thermodynamic data. At the present time, it seems likely that a major portion of the observed changes triggered or induced by NAD^+ binding are associated with a general tightening of the protein structure with the concomitant loss of numerous internal degrees of freedom. This would be expected to lead to decreases in both heat capacity and entropy. Such a change, as expressed in the volume contractions cited earlier, could involve numerous changes in atomic positions too small to be detected by x-ray analysis at a resolution of 2.5–3 Å. It is consistent not only with the stabilization of the protein against thermal denaturation, but also with the large effect of NAD^+ in promoting specific refolding of the protein after urea denaturation (Deal, 1969).

References

Adair, G. S. (1925), *J. Biol. Chem. 63,* 529.

Archer, B. G., McGuire, T. C., and Krakauer, H. (1973), *Biochemistry 12,* 2151.

Buehner, M., Ford, G. C., Moras, D., Olsen, K. W., and Rossman, M. G. (1974), *J. Mol. Biol. 90,* 25.

Chaykin, S., Dagani, M., Johnson, L., Samli, M., and Bataille, J. (1965), *Biochim. Biophys. Acta 100,* 351.

Colowick, S. P., Kaplan, N. O., and Ciotti, M. M. (1951), *J. Biol. Chem. 191,* 447.

Colowick, S. P., and Womack, F. C. (1969), *J. Biol. Chem. 244,* 744.

Conway, E. J., and Downey, M. (1950), *Biochem. J. 47,* 355.

Cook, R. A., and Koshland, Jr., D. E. (1970), *Biochemistry 9,* 3337.

Cornish-Bowden, A., and Koshland, Jr., D. E. (1975), *J. Mol. Biol. 95,* 201.

Deal, W. C. (1969), *Biochemistry 8,* 2795.

Durchschlag, H., Puchwein, G., Kratky, O., Schuster, I., and Kirschner, K. (1971), *Eur. J. Biochem. 19,* 9.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, New York, N.Y., Academic Press, Chapter 8.

Grenthes, I., Ots, H., Ginstrup, O. (1970), *Acta Chem. Scand. 24,* 1067.

Jones, G. M. T., and Harris, J. I. (1972), *FEBS Lett. 22,* 185.

Kauzmann, W. (1959), *Adv. Protein Chem. 14,* 1.

Kirschner, K. (1971), *J. Mol. Biol. 58,* 51.

Kirschner, K., Eigen, M., Bittman, R., and Voigt, B. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 1661.

Kirschner, K., Gallego, E., Schuster, I., and Goodall, D. (1971), *J. Mol. Biol. 58,* 29.

Kosower, E. M. (1956), *J. Am. Chem. Soc. 78,* 3497.

Lowry, O. H., Passonneau, J. V., and Rock, M. K. (1961), *J. Biol. Chem. 236,* 2756.

Mockrin, S. C., Byers, L. D., and Koshland, D. E., Jr. (1975), *Biochemistry 14,* 5428.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12,* 88.

Moras, S. C., Olsen, K. W., Sabesau, M. N., Buehner, M., Ford, G. C., and Rossman, M. G. (1975), *J. Biol. Chem. 250,* 9137.

Petrochenko, S. I., and Privalov, P. L. (1973), *Biofizika 18,* 555.

Sloan, D. L., Samuelson, G. L., Ailion, D. C., and Velick, S. F. (1973), *J. Biol. Chem. 248,* 5424.

Sloan, D. L., and Velick, S. F. (1973), *J. Biol. Chem. 248,* 5419.

Sturtevant, J. (1977), *Proc. Natl. Acad. Sci. U.S.A.* (submitted).

Velick, S. F., Baggott, J. P., and Sturtevant, J. M. (1971), *Biochemistry 10*, 779.
von Ellenrieder, G., Kirschner, K., and Schuster, I. (1972), *Eur. J. Biochem. 26*, 220.
Weber, G., and Anderson, S. R. (1965), *Biochemistry 4*, 1942.

Kinetics of Product Inhibition during Firefly Luciferase Luminescence†

John J. Lemasters* and Charles R. Hackenbrock

ABSTRACT: A theoretical and experimental analysis is made of the kinetics of product inhibition during firefly luciferase luminescence. Equations for competitive, noncompetitive, and uncompetitive inhibition are derived which are useful in determining inhibitory mechanism when the product inhibitor, or its concentration, is unknown and not subject to direct experimental manipulation. Comparisons of experimental data with predictions based upon the three inhibitory models show that product inhibition during luciferase luminescence is noncompetitive with respect to both luciferin and ATP as substrates. The competitive and uncompetitive models are inconsistent with experimental data. These findings provide the basis for using luminescence to measure ATP concentration continuously in in vitro biological systems such as isolated mitochondria.

The bioluminescent enzyme, firefly luciferase, catalyzes the following reactions (cf. McElroy and DeLuca, 1973):

$$E + LH_2 + Mg^{2+} + ATP \leftrightarrow E \cdot LH_2AMP + Mg^{2+} + PP_i \quad (1)$$

$$E \cdot LH_2AMP + O_2 \rightarrow E + CO_2 + AMP + \text{product} + \text{light} \quad (2)$$

$$E + L + Mg^{2+} + ATP \leftrightarrow E \cdot LAMP + Mg^{2+} + PP_i \quad (3)$$

The initial activation step (reaction 1) is the formation of enzyme-bound luciferyl adenylate (LH_2AMP).[1] This enzyme complex reacts with molecular oxygen (reaction 2) to produce light, AMP, CO_2, and a product of luciferin now identified as the decarboxyketo derivative, oxyluciferin (Suzuki and Goto, 1971). Luciferase also catalyzes the activation of dehydroluciferin (L) to form enzyme-bound dehydroluciferyl adenylate (LAMP) which cannot subsequently react to produce light (reaction 3). Dehydroluciferin is a competitive inhibitor of luciferin in reaction 1.

Firefly luciferase luminescence may be used for the accurate and sensitive measurement of ATP (cf. Strehler, 1968). Addition of ATP to a solution containing firefly luciferase, luciferin, oxygen, and divalent cation results in light production whose intensity maximum is proportional to ATP concentration in accordance with the Michaelis–Menten equation. The intensity maximum, or flash height, lasts only a few seconds and is followed by an exponential decay that may last for several hours. This decay does not result from the consumption of substrate but is due to specific inhibition by a product of the luminescence reaction, presumably oxyluciferin (McElroy and Seliger, 1961). Several derivatives of luciferin have been shown to be competitive inhibitors of luciferin and noncompetitive inhibitors of ATP in the luminescence reaction (Denburg et al., 1969). More recent studies by Goto et al. (1973) indicate that oxyluciferin is also a competitive inhibitor of luciferin. However, Gates and DeLuca (1975) have questioned the simple competitive role of oxyluciferin in luminescence decay since excess amounts of luciferin do little to impede the rapid decrease of luminescence during the ongoing reaction. Since our original interest was in measuring ATP continuously in suspensions of metabolically active mitochondria (Lemasters and Hackenbrock, 1973), it became necessary to investigate the continuous kinetics of luminescence, especially the mechanism and kinetics of product inhibition. In this communication we extend our previous findings to show that product inhibition during the ongoing luminescence reaction is noncompetitive with respect to both luciferin and ATP, and that such inhibition is inconsistent with predictions based on either the competitive or uncompetitive inhibitory models.

Methods

Synthetic luciferin (LH_2) and purified luciferase were obtained from Du Pont Corp., Instrument Division. One unit of enzyme equaled 1.6–1.9 μg of protein (Lowry et al., 1951). Luciferase luminescence was detected by a Brice-Phoenix light scattering photometer, Model 2000-D, from a conventional 1-cm light-path cuvette holding 2–3 ml of constantly stirred reaction medium. Light tight additions were made with microliter syringes through a rubber stopper in the lid of the photometer compartment. The luminescence signal was recorded on a strip chart recorder. Statistical estimates of kinetic constants, K_s and V_{max}, obtained from flash height data were calculated by the method of Wilkinson (1961) for each series of experiments. Reaction velocities were expressed in arbitrary light units equaling the millivolt output of the photometer. This arbitrary light unit was not equivalent for different experimental series because of variations in photomultiplier tube voltage from one series of experiments to the next. The sub-

†From the Department of Cell Biology, The University of Texas Health Science Center at Dallas, Southwestern Medical School, Dallas, Texas 75235. *Received January 20, 1975.* This work was supported by Research Grant BMS 75-20141 from the National Science Foundation and Research Grants CA 17065-01 and RR 05426-13 from the National Institutes of Health.

[1]Abbreviations used: LH_2, luciferin; L, dehydroluciferin; LH_2AMP, luciferyl adenylate; LAMP, dehydroluciferyl adenylate; E, luciferase.