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Some Effects of Environment on the Folding of Nicotinamide–Adenine Dinucleotides in Aqueous Solutions†

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ABSTRACT: Nicotinamide-adenine dinucleotide (NAD) and dihydronicotinamide-adenine dinucleotide (NADH) exist in solution in a folded form in which the two bases, adenine and nicotinamide or dihydronicotinamide, are stacked over each other. This study was undertaken to investigate the forces that cause NAD and NADH to fold. Several statistical methods for the analysis of data involving the variation of the base proton chemical shifts with temperature were examined. The statistical method yielding the best results was used to obtain the parameters in the folding model. These values were then used as the initial estimates for a direct least-squares fit to the nonlinear equation relating the chemical shifts and the temperature. The values of the parameters calculated in this manner depend upon which proton is used to make the estimate. From this it was concluded that the folding reaction is not a two-state process. This means that unless the proportion that the intermediate folded forms contribute to the experimental chemical shift is known, it is not possible to calculate the usual thermodynamic parameters from the variation of chemical shifts with temperature. Instead, what one obtains is a ratio of the sum of weighted concentrations of all forms which are not completely folded to the sum of weighted concentrations of all forms that are not completely unfolded. The "thermodynamic parameters" (*i.e.*, $\Delta H'$, $\Delta F'$, and $\Delta S'$) that one would calculate from this ratio are difficult to relate to the usual definitions of the equilibrium constant, ΔF, ΔH, and ΔS obtained from a two-state process. It is clear from the data, however, that NAD and NADH behave differently when their behavior in 7 M methanol or 1 M urea is compared to their behavior in water. For example, $\Delta H'$ and $\Delta S'$ for folding are smaller for NAD when the reaction proceeds in 1 M urea or 7 M methanol instead of water, but larger for NADH when the reaction occurs in 1 M urea instead of water.

It is now well established (Meyer *et al.*, 1962; Czerlinski and Hommes, 1964; Jardetzky and Wade-Jardetzky, 1966; Miles and Urry, 1968; Sarma *et al.*, 1968; Cross and Fisher, 1969; Cattrall *et al.*, 1969) that oxidized or reduced nictoinamide-adenine dinucleotide (NAD or NADH) can assume a conformation wherein the nicotinamide ring and the adenine ring of a single molecule are in (nearly) parallel planes. Jacobus (1971) has raised cogent questions about the arguments of others which Jacobus feels were constructed to be "appealing in light of research objectives." While it is certainly true that published chemical shift data alone "cannot be interpreted to define unambiguously the molecular geometry of pyridine dinucleotides," it is also true that the combination of nuclear magnetic resonance (nmr), ultraviolet, and fluorescence spectral data makes it highly probable that intramolecular stacking interactions exist between the adenine and the nicotinamide or 1,4-dihydronicotinamide rings of NAD or NADH. These intramolecular interactions are reflected by hypochromic and hyperchromic effects in the ultraviolet, the ability of adenine to transfer energy to the 1,4-dihydronicotinamide ring of NADH at rates in excess of 10^{11} transfers/sec (Scott *et al.*, 1970), and significant changes to higher field in the chemical shifts of the nmr spectrum of NAD or NADH. The nmr effect is due to the fact

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that as the bases approach the appropriate (nearly parallel) position near one another, the protons of one base are situated over the other base in such a manner that the ring currents induce large changes in the proton chemical shifts. If one increases the sample temperature (Jardetzky and Wade-Jardetzky, 1966), decreases the pH (Jardetzky and Wade-Jardetzky, 1966; Sarma *et al.*, 1968; Catterall *et al.*, 1969), or decreases the polarity of the solvent (Catterall *et al.*, 1969), the bases move apart, the intramolecular interactions are reduced, and the ring current effect is decreased. These events are reflected by large changes to lower field in the proton chemical shifts.

It is intuitively clear that there must be limits to the changes in chemical shifts to higher and lower fields. We shall refer to the chemical shift of the conformer wherein the bases are close enough that intramolecular repulsion prohibits further approach (*i.e.*, the "completely folded" conformer) as δ_c, and we shall refer to the chemical shift of the conformer wherein the bases are so far apart that there are no detectable intramolecular interactions (*i.e.*, the "completely unfolded" conformer) as δ_u. δ_c and δ_u can be estimated by extrapolation to low and high temperatures, and δ_u can be approximated from the chemical shifts of the individual protons of adenosine monophosphate (AMP) for the adenine ring protons and of nicotinamide mononucleotide (NMN) or reduced nicotinamide mononucleotide ($NMNH_2$) for the nicotinamide ring protons.

At a field strength of 51 kG only a single resonance appears for each of the protons except the C_4H of the 1,4-dihydronicotinamide ring of NADH. Even in that case the two signals exhibit identical temperature dependence and overlap to such an extent that they can be treated as one signal. For these reasons we can assume that the chemical shift of each right-handed helical conformer (Sarma and Kaplan, 1970b) will be equal to the chemical shift of each corresponding left-handed helical conformer. The equilibrium between the "completely folded" and the "completely unfolded" forms of NAD and NADH can be represented by

$$X_0 \rightleftarrows X_q \qquad \text{(I)}$$

$$X_0 \rightleftarrows X_1 \rightleftarrows \cdots \rightleftarrows X_q \qquad \text{(II)}$$

where X_q is the sum of the completely folded right- and left-handed helical molecules, each X_i ($i = 1, \ldots, q - 1$) represents the sum of each partially folded right- and left-handed form, and X_0 represents the completely unfolded molecules ($\delta_{X_q} = \delta_c$, and $\delta_{X_0} = \delta_u$).

Poland and Scheraga (1965) point out that apparent thermodynamic parameters for reaction II estimated from an observable that is an ensemble average do not possess the usual (*i.e.*, obvious) thermodynamic significance. However, such parameters do possess important thermodynamic significance (Lumry *et al.*, 1966).

It is customary to define an apparent equilibrium constant, K, in terms of an observable and the extrapolated values of the observable at high and low temperatures (Lumry *et al.*, 1966). When the observable is a chemical shift in a nuclear magnetic resonance spectrum, K is given by eq 1

$$\frac{\delta - \delta_m}{\delta_n - \delta} = \frac{X_n(\infty) + \sum_{m+1}^{n-1}(1 - a_i)X_i(\infty)}{X_m(\infty) + \sum_{m-1}^{n-1} a_i X_i(\infty)} = K \qquad (1)$$

if we assume a sufficiently rapid interconversion between the folded and unfolded helices (Czerlinski and Hommes, 1964). In eq 1 δ is the ensemble average chemical shift, the a_i are the weighting factors, $a_i = (\delta_i - \delta_n)/(\delta_m - \delta_n)$, and the δ_i are the individual chemical shifts for each X_i.

For reaction I the right-hand side of eq 1 is $X_n(\infty)/X_m(\infty)$ (*i.e.*, $(\delta_u - \delta)/(\delta - \delta_c)$ is the equilibrium constant for the overall intramolecular reaction). For reaction II the right-hand side of eq 1 is the ratio of the sum of a weighted average of the concentrations of all components with subscripts greater than m (but not greater than n) to the sum of a weighted average of the concentrations of all components with subscripts less than n (but not less than m). Similar results can be derived for other detection methods.

An environmental change that is sufficiently small that it would not effect the weighting factors in eq 1 can cause K to be different by altering the free energy of an elementary chemical reaction. Thus an effect of environment on δ_u from reactions I or II or on δ_c from reaction I should be due primarily to differences in solvent–dinucleotide intermolecular interactions. On the other hand, for reaction II K can also be different if these environmental perturbations change n and/or m. Thus δ_c from reaction II could be different in different environments if different minimum intramolecular distances between the nicotinamide and the adenine rings constitute what we refer to as the completely folded conformer. Consequently, if reaction I is correct, δ_c should be relatively unaffected by the folding environment; if reaction II is correct, δ_c could be quite different in different environments.

For reaction II K will not be the same when different methods of detection are used if n and/or m are different, or if the weighting of the $X_i (n \geq i \geq m)$ is different (Lumry *et al.*, 1966). If some of the steps are not detectable by a particular method, the free-energy change of the overall detected reaction is changed by an amount equal to the free-energy change of the steps that are not detected. This would lead to different values of K obtained from different detection methods for reaction II, but not for reaction I. Similarly, if the weighting of the X_i differs in different detection systems, then the various methods of detection will result in preferential information about the weighted aspects of the overall reaction. Lumry and associates (1966) have suggested using these facts as a basis for a test to distinguish between reactions I and II; they could also be used to isolate various aspects of complex processes.

Statistical Methods

Jardetzky and Wade-Jardetzky (1966) suggested a method for the rough approximation of the order of magnitude (not an accurate value) of the enthalpy, entropy, and free-energy changes due to folding of NAD *via* reaction I from nmr chemical shifts. Recently these methods were applied to NADH by Sarma and Kaplan (1970a). So far, however, no suitable statistical procedures that include proper fitting methods and calculations of the variances of the desired parameters have been reported. In this section we discuss several procedures that do provide information about the reliability of the estimated parameters.

Lumry and associates (1966) have pointed out that the gas constant times the slopes of van't Hoff plots of ln K *vs.* $1/T$ does not equal the overall enthalpy change for reaction II, and eq 1 details this fact for the type of experiments reported here. However, as long as one is aware of this limita-

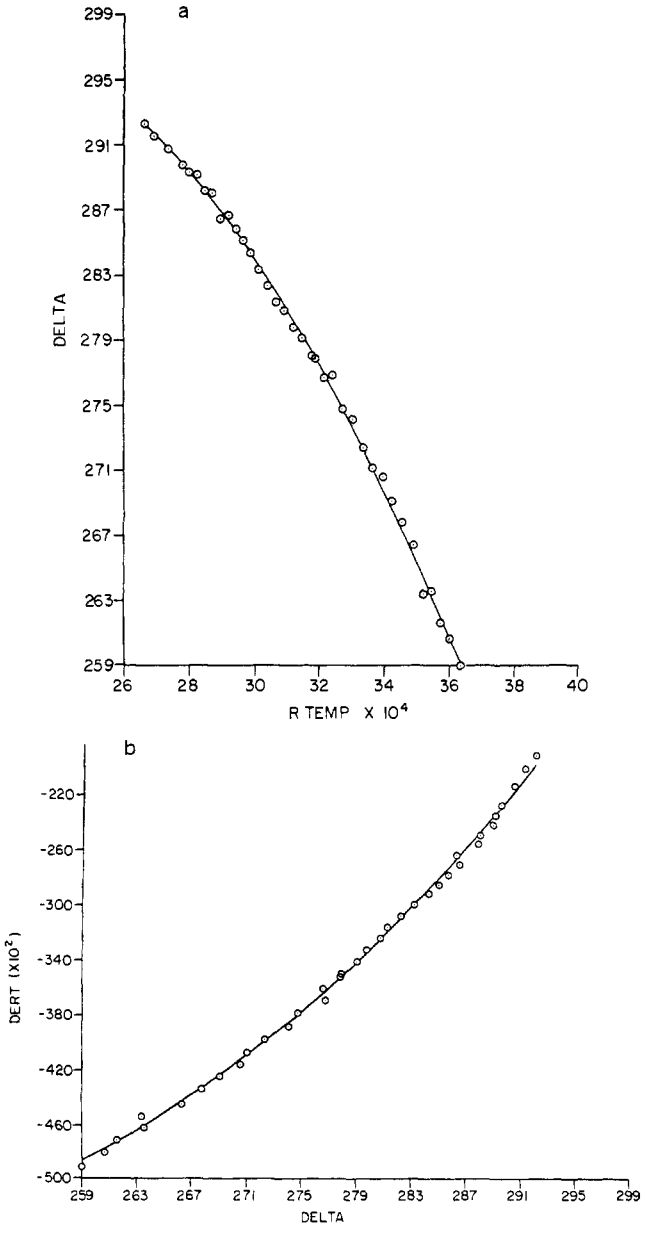

FIGURE 1: Chemical shifts obtained with the aid of a 100-Mcycle nmr machine are from the proton associated with C-4 in the reduced nicotinamide ring of NADH. The experiments were done with 0.1 M β-NADH in 0.1 M phosphate buffer (pH 7.0). (a) Graph of chemical shifts observed experimentally (circles) or calculated from the least-squares fit to eq 4 (continuous line) *vs.* the reciprocal of the absolute temperature. (b) Graph of derivatives calculated from eq 5 (circles) or from the least-squares fit to eq 6 (continuous line) *vs.* the experimentally determined chemical shifts obtained at different temperatures.

tion, significant thermodynamic information can be deduced from observations of the temperature dependence of ensemble averages. For example, we can obtain an idea of the relationship between the overall equilibrium constant and K for the case wherein the δ_i in eq 1 are evenly spaced between δ_n and δ_m. Then if $K = 1$, $X_n(\infty) = X_m(\infty)$, and K is the overall equilibrium constant for reaction II. For $X_n(\infty) < X_m(\infty)$, $K > X_n(\infty)/X_m(\infty)$; for $X_n(\infty) > X_m(\infty)$, $K < X_n(\infty)/X_m(\infty)$. A practical consequence of this result is that apparent enthalpy changes calculated from K will be larger than the enthalpy change calculated from $X_n(\infty)/X_m(\infty)$. If we differentiate the natural logarithm of K and substitute the result into the van't Hoff relation, we should denote the enthalpy change as $\Delta H'$ because we do not know *a priori* if K is the equilibrium constant for the overall folding reaction.

$$\frac{\partial\delta}{\partial\left(\frac{1}{T}\right)} = \frac{\Delta H'}{R(\delta_c - \delta_u)}(\delta - \delta_u)(\delta_c - \delta) \qquad (2)$$

Equation 2 provides a basis for the estimation of an apparent enthalpy change ($\Delta H'$) from the chemical shifts obtained at various temperatures. An apparent free-energy change ($\Delta F'$) can be calculated at each temperature from the usual relationship between the free energy, the temperature, and K; an apparent entropy change ($\Delta S'$) can be obtained from the usual relationship between the entropy, the enthalpy, the free energy, and the temperature.

In order to use eq 2 we must either integrate it, or obtain an estimate for $\partial\delta/\partial(1/T)$ for each experimental point. Integration is straightforward and yields

$$\delta = \frac{\delta_u + \delta_c e^{\Delta S'/R} e^{-\Delta H'/RT}}{1 + e^{\Delta S'/R} e^{-\Delta H'/RT}} \qquad (3)$$

The difficulties with using eq 3 are that it contains an additional parameter (which is not independent) and that methods for fitting δ and T require *a priori* estimates of δ_u, δ_c, $\Delta H'$, and $\Delta S'$. On the other hand, estimates for $\partial\delta/\partial(1/T)$ can be easily obtained by fitting δ and $1/T$ to an appropriate polynomial, differentiating the polynomial with respect to $1/T$, and calculating the derivative. In practice we found that a quadratic in $1/T$ served as an appropriate polynomial

$$\delta = A\left(\frac{1}{T}\right)^2 + B\left(\frac{1}{T}\right) + D \qquad (4)$$

The fit to eq 4 was accomplished by the usual least-squares procedure (see, for example, Draper and Smith, 1966). The cross-product matrix involved in the fit tended to be nearly singular so it was computed and inverted in double precision. Table IA and Figure 1a illustrate the excellent fit of the quadratic.

$\partial\delta/\partial(1/T)$ can now be calculated for each temperature from the derivative of eq 4

$$\frac{\partial\delta}{\partial\left(\frac{1}{T}\right)} = 2A\left(\frac{1}{T}\right) + B \qquad (5)$$

Substitution from eq 5 into eq 2 yields

$$2A\left(\frac{1}{T}\right) + B = -\frac{\Delta H'}{R(\delta_c - \delta_u)}(\delta - \delta_u)(\delta_c - \delta) \qquad (6)$$

It is clear from eq 6 that the calculated derivatives should be fit to a quadratic in the chemical shifts.

$$\frac{\partial\delta}{\delta\left(\frac{1}{T}\right)} = E\delta^2 + F\delta + G \qquad (7)$$

TABLE I

	A. Results of Fit to eq 4	
	Variance of δ: 0.17255	
$A = -1.53479 \times 10^7$	$B = 6.26184 \times 10^4$	$D = 2.34410 \times 10^2$
	Variance–Covariance Matrix	
A	B	D
A 9.18784×10^{11}	-5.80504×10^9	9.09823×10^6
B -5.80504×10^9	3.67393×10^7	-5.076795×10^4
D 9.09823×10^6	-5.76795×10^4	9.07146×10^1
	Correlation Coefficients	
A 1.000000000000000	−0.999155000246770	0.996578377157050
B −0.999155000246770	1.000000000000000	−0.999120225276847
D 0.996578377157050	−0.99912022527684	1.000000000000000
	B. Results of Fit to eq 7	
	Variance of $\partial\delta/\partial(1/T)$: 1.52929×10^5	
$E = 1.13218 \times 10^1$	$F = -5.37644 \times 10^3$	$G = 5.84366 \times 10^5$
	Variance–Covariance Matrix	
E	F	G
E 0.57275	-3.16515×10^2	4.36727×10^4
F -3.16515×10^2	1.74957×10^5	-2.41470×10^7
G 4.36727×10^4	-2.41470×10^7	3.33360×10^9
	Correlation Coefficients	
E 1.000000000000000	−0.999868776221447	0.999465998892918
F −0.999868776221477	1.000000000000000	−0.999863869696349
G 0.999465998892918	−0.999863869696349	1.000000000000000

where $E = \Delta H'/R(\delta_c - \delta_u)$, $F = \Delta H'(\delta_c + \delta_u)/R(\delta_c - \delta_u)$, and $G = \Delta H'\delta_c\delta_u/R(\delta_c - \delta_u)$.

The least-squares fit of eq 7 was also obtained in double precision. For subsequent use the variance–covariance matrix of the three coefficients was obtained. As frequently happens in polynomial regression, the parameters were highly correlated. This indicates that the region of acceptable fits in the parameter space is long in some direction. Thus, care should be taken to get estimates of the variability of the derived quantities. Table IB and Figure 1b illustrate this fit.

We now use the values of E, F, and G and the method of propagation of error (see, for example, Kendall and Stuart, 1963) to obtain δ_c, δ_u, and $\Delta H'$ and their variances

$$\delta_c = \frac{-F - r}{2E} \pm \frac{[F^4 - 4EF^2G + 2E^2G^2 + F(F^2 - 2EG)r]V(E) + E^2(E^2 + Fr - 2EG)V(F) + 2E^4V(G)}{2E^4r^2} + \frac{2E[F(3EG - F^2) + (EG - F^2)r]C(E,F) + 2E^2(F^2 - 2EG + Fr)C(E,G) - 2E^3(F + r)C(F,G)}{2E^4r^2} \quad (8)$$

$$\delta_u = \frac{-F + r}{2E} \pm \frac{[F^4 + 2E^2G^2 - 4EF^2G + F(2EG - F^2)r]V(E) + E^2(F^2 - 2EG - Fr)V(F) + 2E^4V(G)}{2E^4r^2} + \frac{2E[F(3EG - F^2) + (E^2 - EG)r]C(E,F) + 2E^2(F^2 - 2EG - Fr)C(E,G) + 2E^3(r - F)C(F,G)}{2E^4r^2} \quad (9)$$

$$\Delta H' = \frac{Rr}{2E} \pm \frac{R^2[(F^4 + 4E^2G^2 - 4EF^2G)V(E) + E^2F^2V(F) + 4E^4V(G) + 2EF(2EF - F^2)C(E,F) + 4E^2(F^2 - 2EG)C(E,G)]}{4E^4r^2} - \frac{R^24E^3FC(F,G)}{4E^4r^2} \quad (10)$$

where $r = (F^2 - 4EG)^{1/2}$; $V(E)$, $V(F)$, and $V(G)$ are the variances of E, F, and G; $C(E,F)$, $C(E,G)$, and $C(F,G)$ are the covariances of E and F, E and G, and F and G. The values, variances, and partial derivatives of δ_c, δ_u, and $\Delta H'$ estimated from the data in Figure 1a and the calculations indicated in Table I and Figure 1b are listed in Table IIA.[1]

Since δ_u is the type of constant that can be estimated from independent experiments, we have devised an alternative method for the estimation of δ_c and $\Delta H'$. This second method utilizes the independently estimated δ_u and assumes that an *a priori* estimate of δ_u is correct.

Since we know δ_u, we may calculate δ_c two ways

$$\delta_{c_1} = -(\delta_u + F/E) \quad (11)$$

$$\delta_{c_2} = G/\delta_u E \quad (12)$$

Since in general δ_{c_1} and δ_{c_2} will not be exactly the same (they could be quite different), we need to devise a method of choosing the best one for δ_c. Alternatively, if it is appropriate

[1] The equations for these partial derivatives may be obtained by writing directly to C. W.

TABLE II

A. Estimation of δ_c, δ_u, and $\Delta H'$ from the data in Figure 1
$\delta_c = 168.4 \pm 5.6$ $\delta_u = 306.5 \pm 0.4$ $\Delta H' = -3108 \pm 73$

Partials of	With respect to E	F	G
δ_c	18.1540	0.0107785	0.000639947
δ_u	−60.975	−0.196110	−0.000639947
$\Delta H'$	−1487.63	−6.84342	−0.028820

B. Estimation of δ_c and $\Delta H'$ from $\delta_u = 305.3$ and the data in Figure 1

$$\delta_c = 169.6 \pm 5.2 \qquad \Delta H' = -3056 \pm 88$$

C. Estimation of δ_c, δ_u, and $\Delta H'$ from $\Delta S' = -15.70$, the data in Figure 1, and the initial estimates for δ_c, δ_u, and $\Delta H'$ listed in A, above

$$\delta_c = 164.9 \pm 19.8 \quad \delta_u = 306.4 \pm 0.2 \quad \Delta H' = -3088 \pm 79$$

D. Estimation of δ_c, $\Delta H'$, and $\Delta S'$ from $\delta_u = 305.3$, the data in Figure 1, and initial estimates for δ_c and $\Delta H'$ listed in B, above, and the initial estimate for $e^{\Delta S'/R}$ equal to 0.001985

$$\delta_c = 169.1 \pm 39.2 \qquad \Delta H' = -3059 \pm 219$$
$$e^{\Delta S'/R} = 0.001857 \pm 0.0000145$$

E. Estimation of δ_c and $\Delta H'$ from $\Delta S' = -16.94$ and $\delta_u = 305.3$, the data in Figure 1, and the initial estimates for δ_c and $\Delta H'$ listed in B, above

$$\delta_c = 164.1 \pm 32.8 \qquad \Delta H' = -3034 \pm 183$$

to do so, we may wish to obtain a weighted average of δ_{c_1} and δ_{c_2} to use as the best estimate of δ_c

$$\delta_c = w\delta_{c_1} + (1 - w)\delta_{c_2} \quad 0 \leq w \leq 1 \tag{13}$$

We obtain w in eq 13 by minimizing the variance of δ_c. If there is no minimum of the variance of δ_c for $0 \leq w \leq 1$, we choose $w = 0$ if the minimum occurs for $w < 0$, and $w = 1$ if the minimum occurs for $w > 1$. Our calculations seem to indicate that either δ_{c_1} or δ_{c_2} is a better estimate for δ_c than any nontrivial linear combination of the two. However, the computation of w is not entirely in vain because without it we are unable *a priori* to tell whether δ_{c_1} or δ_{c_2} is the better estimate.

We may now calculate $\Delta H'$ from

$$\Delta H' = RE(\delta_u - \delta_c) \tag{14}$$

The values and variances of δ_c and $\Delta H'$ estimated from the assumed value of $\delta_u = 305.3$, the data in Figure 1a, and the calculations indicated in Table I and Figure 1b are listed in Table IIB.

We mentioned in connection with eq 3 that $\Delta S'$ is not an independent parameter. For reaction 11

$$\Delta S' = \frac{\Delta H'}{T} + R \ln \frac{\delta_u - \delta}{\delta - \delta_c} \tag{15}$$

It is possible, therefore, to use the values of δ_c, δ_u, and $\Delta H'$ calculated from eq 8, 9, and 10 to obtain one estimate of $\Delta S'$, and to use the value of δ_c calculated from eq 13, the value of δ_u obtained from independent experiments, and the value of $\Delta H'$ calculated from eq 14 to obtain a second estimate of $\Delta S'$. We have used the first estimate of $\Delta S'$ together with the values of δ_c, δ_u, and $\Delta H'$ calculated from eq 8, 9, and 10 as the initial estimates for a three-parameter least-squares fit to eq 3; similarly, we have used the second estimate of $\Delta S'$ and the value of δ_u obtained from independent experiments together with the values of δ_c and $\Delta H'$ calculated from eq 13 and 14 as the initial estimates for both a two- and three-parameter least-squares fit to eq 3. In both cases, the Marquardt compromise (see, for example, Draper and Smith, 1966, p 272), was used to obtain the least-squares fit. The results of these calculations are summarized in Table IIC–E.

Of the two methods suggested in this section, the first seems to provide estimates of $\Delta H'$, δ_u and δ_c with slightly lower variances than were obtained by the second method. This result is not unexpected in view of the fact that δ_u used in the second method is the chemical shift obtained from a mononucleotide in buffer whereas the extrapolated δ_u was obtained for a series of different solvents. However, when the results of either method are used as the initial estimates for a least-squares fit directly to eq 3, little or no refinement of the fit is observed. In what follows, therefore, we use the first method to obtain rough estimates for $\Delta H'$, δ_u, and δ_c. These estimates are then used as initial guesses for a least-squares fit to eq 3. Any great discrepancy between the results of the two methods and the fit to eq 3 should be interpreted as an indicator of a probable systemic error.

Materials and Methods

The nmr spectra were obtained on a Varian HA-100 spectrometer equipped with a 15-in. magnet and a variable-temperature probe accommodating 12-mm diameter sample tubes. When necessary, the signal to noise ratio for dilute samples was improved with the aid of a Technical Measurements Corp. Model C-1024 time-averaging computer used in conjunction with a voltage controlled oscillator for scanning the spectra. Resonance positions were determined by interpolation. The precision of the shift measurements determined directly (±0.1 Hz) and from the time averaged spectra is ±0.2 Hz. All shifts were measured with respect to sodium 3-(trimethylsilyl)propanesulfonate used as an internal standard (a product of Merck Chemical Co., purchased from Brinkmann Instruments, Inc., Westbury, N. Y.). Sample temperatures were measured using a digital thermistor probe. The precision of these measurements is ±0.1°.

NAD, NMN, and the disodium salts of NADH and NMNH were purchased from Sigma Chemical Co. and 5′-AMP was purchased from the Nutritional Biochemical Corp. These were used without further purification. Deuterium oxide, 99.8% isotopically pure, is from Diaprep, Inc., and methanol-d_4 is from NMR Specialties, Inc.

Deuterated phosphate buffer was prepared by lyophilizing 0.1 M orthophosphate buffer previously adjusted to the correct pH, exchanging the residue with D_2O, relyophilizing, and finally diluting to the original volume with D_2O. A similar procedure was followed in preparing urea-d_4.

The reported solution pH's are the direct readings from the pH meter.

Results

The base proton chemical shifts of 0.1 M NAD and 0.1 M NADH were measured as a function of temperature in

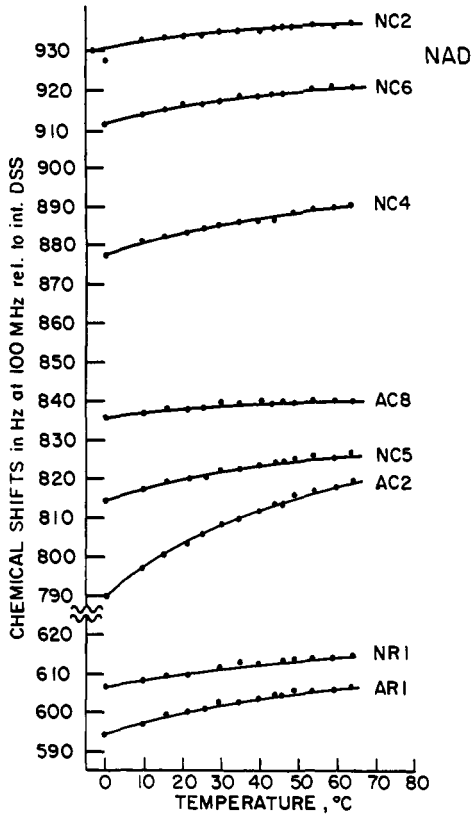

FIGURE 2: Temperature dependence of 0.1 M NAD proton chemical shifts in D_2O with 0.1 M phosphate (pH 7.0) and 0.0025 M silapentanesulfonate.

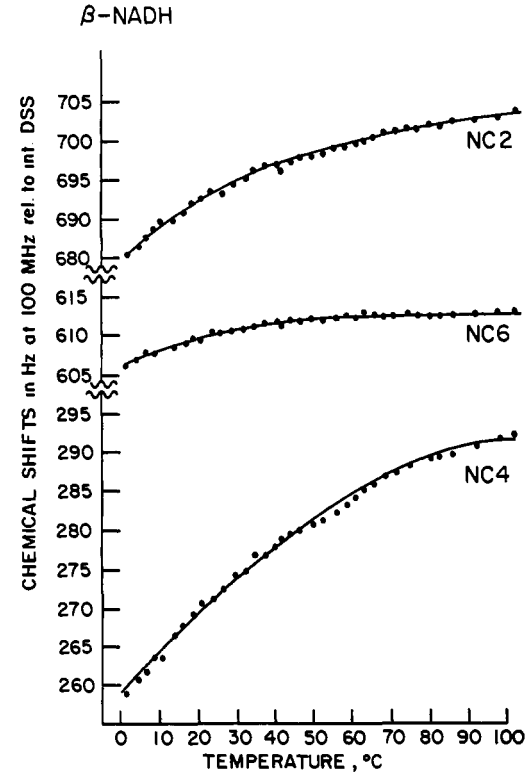

FIGURE 3: Temperature dependence of NADH proton chemical shifts in D_2O with 0.1 M phosphate (pH 7.0) and 0.0025 M silapentanesulfonate.

pH 7.0, 0.1 M orthophosphate buffer. The results for NAD and NADH appear in Figures 2 and 3, respectively. For NAD, the adenine C_2H chemical shift undergoes the largest change over the observed temperature range followed by the nicotinamide C_4H, C_5H, the adenine ribose C_1', NC_6H, $NC_1'H$, NC_2H, and AC_8H in that order. The curves all appear to be hyperbolic though presumably they would be sigmoidal over a wider temperature range were the observable range not restricted by the fact that the samples freeze at about 0° and begin to decompose rather rapidly at temperatures above 80° as evidenced by the loss of signal intensity. Except for the high-temperature sample decomposition, the variation of the chemical shifts with temperature is completely reversible in that the chemical shift at any temperature may be reproduced regardless of whether that temperature is approached from a higher or lower value.

The temperature dependence of the proton chemical shifts for the monomers, NMN, $NMNH_2$, and AMP, was studied to provide an estimate for the unfolded chemical shift, δ_u, of the dinucleotides and to reveal any specific moiety interaction with solvent. The results for 0.1 M NMN and 0.1 M $NMNH_2$ in pH 7.0, 0.1 M orthophosphate buffer appear in Figure 4, while those for 0.1 M AMP in the same buffer appear in Figure 5. The base proton chemical shifts for NMN are independent of temperature while those of $NMNH_2$ exhibit a very small decrease with increasing temperature. AMP, on the other hand, exhibits a rather large increase of chemical shift with increasing temperature, which over the experimental temperature range is almost as large as that observed over the same range for the adenine protons of NAD and NADH. T'so *et al.* (1969) have shown that dinucleotides such as ApA dimerize. The fact that the temperature dependence of the AMP chemical shifts is nearly as large as the corresponding shifts in NAD and NADH indicates that NAD and NADH form dimers under the experimental conditions employed here.

The dependence of the NAD proton chemical shifts on NAD concentration appear in Figure 6. The AC_2H and AC_8H shifts show a large variation with changes in concentration. This result is consistent with our previous report (Catterall *et al.*, 1969) that the AC_2H and AC_8H of NAD and NADH shift when these dinucleotides are diluted from 0.05 M to 5 mM; the result is also consistent with the data in Figure 5 which clearly indicates adenine–adenine intermolecular stacking in the 0–70° temperature range. Both the AC_2H and AC_8H shifts in Figure 6 reach a limiting value at 5.5° when the NAD concentration is about 1 mM; at 48.8° a limiting value is reached when the NAD concentration is about 8 mM. This indicates that the adenine–adenine intermolecular dissociation for NAD is complete at these combinations of temperature and dinucleotide concentration. The nicotinamide proton shifts show essentially no concentration dependence. This result is consistent with our previous report (Catterall *et al.*, 1969) that the shifts of the NC_4H of NAD and NADH are not concentration dependent and it is not inconsistent with our statement that the NC_5H, NC_2H, and NC_6H of NAD and the NC_2H of NADH show a *slight upfield* chemical shift when either dinucleotide is diluted from 0.05 M to 5 mM. Our previous statement was based on shifts of +1.8, +2.2, and +1.1 cycles for NC_5H, NC_2H, and NC_6H of NAD and +0.9 cycle for NC_2H of NADH. Since these shifts should be accurate to within about 1 cycle, they are of marginal significance in the present context. They are quite small, for example, compared to the shifts we observe when

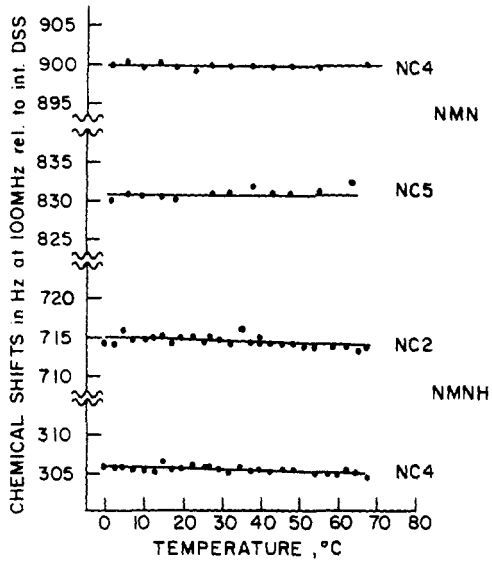

FIGURE 4: Temperature dependence of the 0.1 M NMN and 0.1 M $NMNH_2$ proton chemical shifts in D_2O with 0.1 M orthophosphate (pH 7.0) and 0.0025 M silapentanesulfonate.

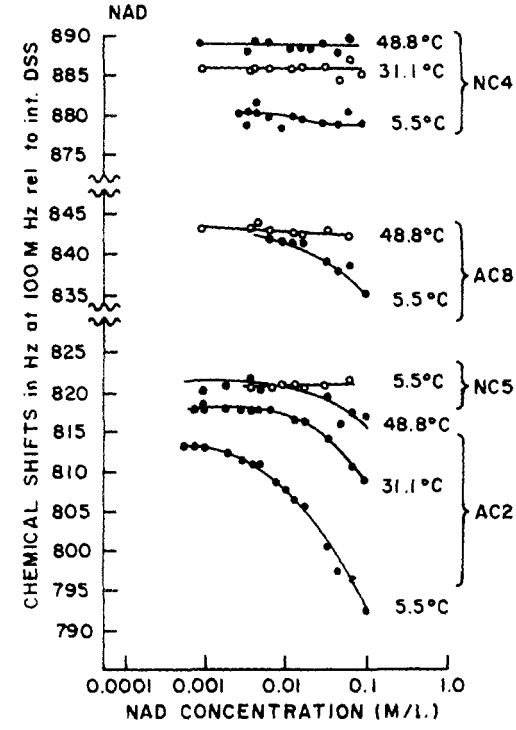

FIGURE 6: Dependence of NAD proton chemical shifts on NAD concentration at different temperatures in D_2O with 0.1 M orthophosphate (pH 7.0) and 0.005 M silapentanesulfonate.

the temperature is increased from 0° to 80°. The results in Figure 6 are also consistent with the concentration independence of the nicotinamide protons of NMN illustrated in Figure 4.

Since the nicotinamide ring of NAD or NMN is positively charged, one does not expect intermolecular interactions between nicotinamide rings for these nucleotides. Furthermore, since the 1,4-dihydronicotinamide ring of NADH or NMNH, is not aromatic, one would not necessarily expect to detect intermolecular interactions between 1,4-dihydronicotinamide rings by nmr chemical shifts. A practical consequence of this situation is that the unfolding of intramolecular interactions between the adenine and nicotinamide rings of NAD can be studied independently of intermolecular adenine–adenine or adenine–nicotinamide interactions. Furthermore, since 1,4-dihydronicotinamide–1,4-dihydronicotinamide intermolecular interactions either do not occur or do not result in significant chemical shifts, the unfolding of intramolecular interactions between the adenine and 1,4-dihydronicotinamide rings of NADH can be studied independently of intermolecular adenine–adenine, adenine–1,4-dihydronicotinamide, or 1,4-dihydronicotinamide–1,4-dihydronicotinamide interactions. In this paper we obtain chemical shifts for NC_5H and NC_4H of 0.1 M NAD and NC_4H and NC_2H of 0.1 M NADH (where dimerization of these dinucleotides is occurring). However, since the chemical shifts of these protons are independent of dinucleotide concentration in the 1 mM to 0.1 M concentration range, we obtain the same chemical shifts that we would observe at 1 mM (where dimerization of the dinucleotides is not detectable). It seems reasonable to assume that this should also be true for media where there is *less* stacking (*e.g.*, for aqueous methanol and aqueous urea). Thus, we are able to study the intramolecular reaction independently of the intermolecular interaction and compare the results we obtain in various environments that promote unstacking.

There are two obvious interpretations of the concentration dependence of the adenine protons and the concentration independence of the nicotinamide protons of the same molecule. (1) The intramolecular folding reaction is unaffected by the dimerization reaction; or (2) intermolecular interactions between adenines cause unfolding, but the unfolding is exactly compensated for by intermolecular interactions between adenine and nicotinamide rings. We cannot distinguish between these two possibilities yet, but it is interesting to note that concentration independence need not rule out an intermolecular interaction even when the intermolecular interac-

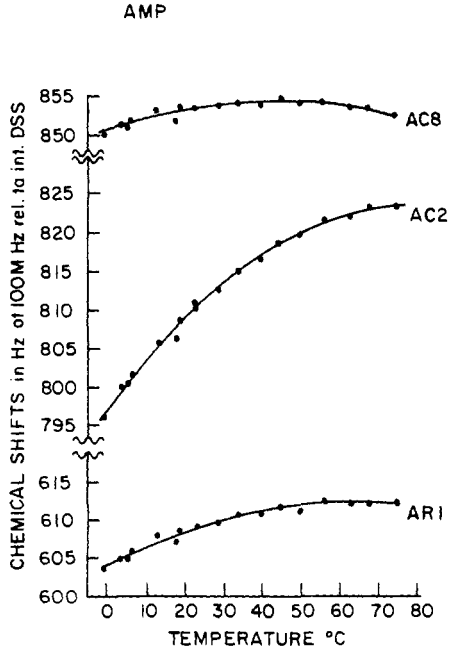

FIGURE 5: Temperature dependence of 0.1 M AMP proton chemical shifts in D_2O with 0.1 M orthophosphate (pH 7.0) and 0.0025 M silapentanesulfonate.

TABLE III

	Hz at 100 MHz					cal/Mole		
	δ_u	δ_c	$\delta_{298.2}$	$(\delta_u)_n$	K	$\Delta F'$	$-\Delta H'$	$-\Delta S'$ (eu/Mole)
					NAD			
C-4	896.1 ± 0.3	840.2 ± 15.7	833.864	(900)	0.2802 ± 0.107	755 ± 477	3928 ± 231	15.7 ± 2.37
C-5	833.6 ± 0.3	768.2 ± 27.3	820.411	(831)	0.2526 ± 0.138	815 ± 465	3512 ± 322	14.5 ± 2.64
					U-NAD			
C-4	894.0 ± 0.2	864.4 ± 13.9	889.507		0.1790 ± 0.107	1019 ± 817	5094 ± 364	20.5 ± 3.96
C-5	831.0 ± 0.3	796.6 ± 14.0	824.460		0.2347 ± 0.129	858 ± 328	4124 ± 328	16.7 ± 3.55
					M-NAD			
C-4	894.0 ± 0.1	858.9 ± 3.7	889.272		0.1557 ± 0.0223	1102 ± 171	7068 ± 91	27.4 ± 0.879
C-5	831.0 ± 0.2	796.0 ± 6.0	826.109		0.1624 ± 0.0390	1077 ± 291	6222 ± 143	24.5 ± 1.46
					β-NADH			
C-2	706.4 ± 0.2	651.2 ± 8.4	693.332	(715)	0.3102 ± 0.0666	693 ± 258	4672 ± 130	18.0 ± 1.30
C-4	306.4 ± 0.2	164.9 ± 19.7	271 887	(306)	0.3226 ± 0.0613	670 ± 227	3088 ± 112	12.6 ± 1.14
					U-NADH			
C-2	708.0 ± 0.3	622.6 ± 8.4	695.625		0.3747 ± 0.104	581 ± 337	4015 ± 166	15.4 ± 1.69
C-4	309.9 ± 0.2	177.6 ± 19.9	276.688		0.3352 ± 0.0693	648 ± 248	2663 ± 119	11.1 ± 23
					M-NADH			
C-2	708.2 ± 0.2	672.8 ± 3.2	701.278		0.01381 ± 0.00195	2538 ± 168	5354 ± 81	26.5 ± 0.836
C-4	335.8 ± 0.2	200.1 ± 88.6	284.953		0.5992 ± 0.628	303 ± 2223	1517 ± 612	6.1 + 9.50 −6.1

tion causes a change in the property actually being measured.

The bulk magnetic susceptibility of the solvent will change with changes in temperature and with it the chemical shifts of all substances dissolved in it, even in the absence of other effects. Since changes in the magnetic susceptibility will change all the shifts by the same amount, compensation for this effect was made by measuring the shifts relative to a small amount of sodium 2,2-silapentanesulfonate dissolved in the sample. The silapentanesulfonate also provided the lock signal used for maintaining a constant magnetic field. In order to determine whether or not the silapentanesulfonate, a detergent-like molecule, had any effect on the folding reaction of NAD, the dependence of the NAD shifts on silapentanesulfonate concentration were measured over a range of two orders of magnitude. The results appear in Figure 7. The silapentanesulfonate concentration had no effect on the NAD chemical shifts at silapentanesulfonate concentrations ranging from 0.2 to 20 mM. The normal concentration, 5 mM, used in these experiments does not perceptibly effect the system.

The chemical shift data in Figures 2 and 3 were used in the numerical method outlined earlier to calculate the thermodynamic parameters for the unfolding reaction; the results are tabulated in Table III. $\Delta H'$ for folding is about −5 kcal/mole, $\Delta S'$ is about −15 eu/mole and the equilibrium constant based upon reaction I would be about 0.2, indicating that about 15% of the dinucleotide would be is in the folded form. The extrapolated values for δ_u agree fairly closely with the values for the monomers. In NAD solutions containing methanol or urea $-\Delta H'$ estimated from the NC_5H is less than $-\Delta H'$ estimated from the NC_4H. In buffer alone or buffer containing methanol or urea $-\Delta H'$ estimated from the NC_2H of NADH is greater than $-\Delta H'$ estimated by following NC_4H. These differences, which are several standard deviations apart, are taken as evidence that the unfolding reaction is not a two-state process.

Jardetzky and Wade-Jardetzky (1966) measured the chemical shifts that occur in the nuclear magnetic resonance spectrum of NAD when the temperature is increased, and Sarma and Kaplan (1970a) reported similar experiments for NADH. Both workers used reaction I as their model for the intramolecular folding reaction, and both calculated the enthalpy change from van't Hoff plots by assuming that $(\delta - \delta_m)/(\delta_n - \delta)$ equals $X_n(\infty)/X_m(\infty)$. However, Chan and Nelson

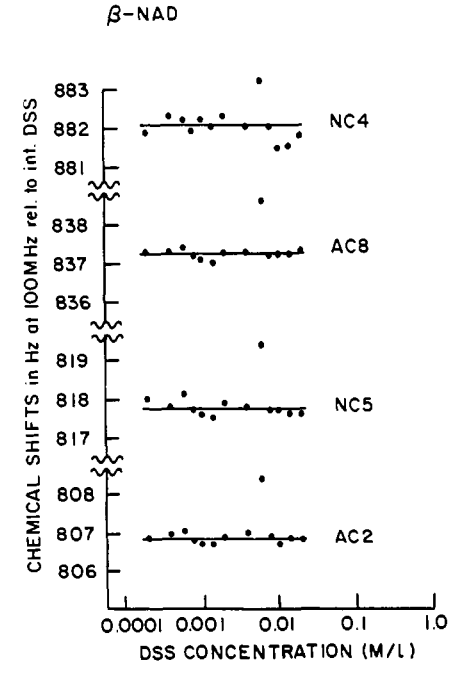

FIGURE 7: Dependence of 0.05 M NAD proton chemical shifts on silapentanesulfonate concentration in D_2O with 0.1 M orthophosphate (pH 7.0).

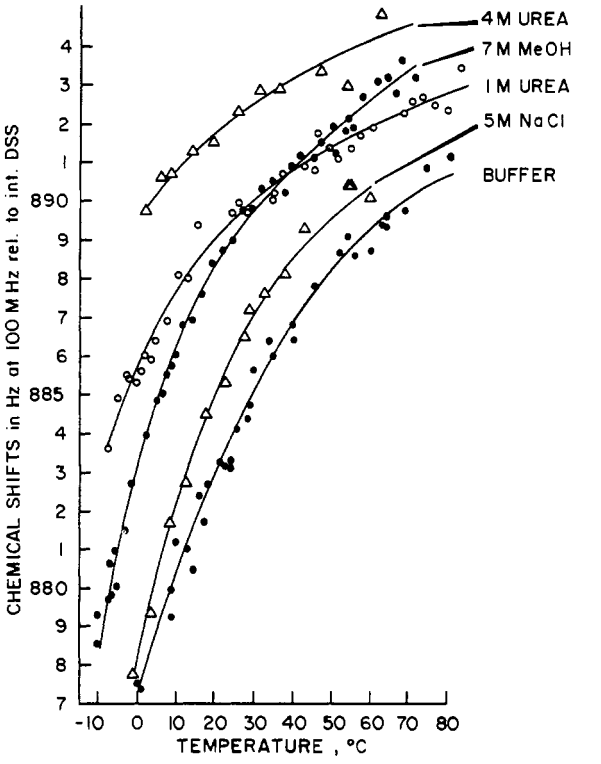

FIGURE 8: Comparison of the temperature dependence of the NC_4H chemical shifts of 0.1 M β-NAD in 7.3 M d_4-methanol in D_2O, 1.0 M d_4-urea in D_2O, each solution containing 0.1 M orthophosphate (pH 7.0) and 0.0025 M silapentanesulfonate.

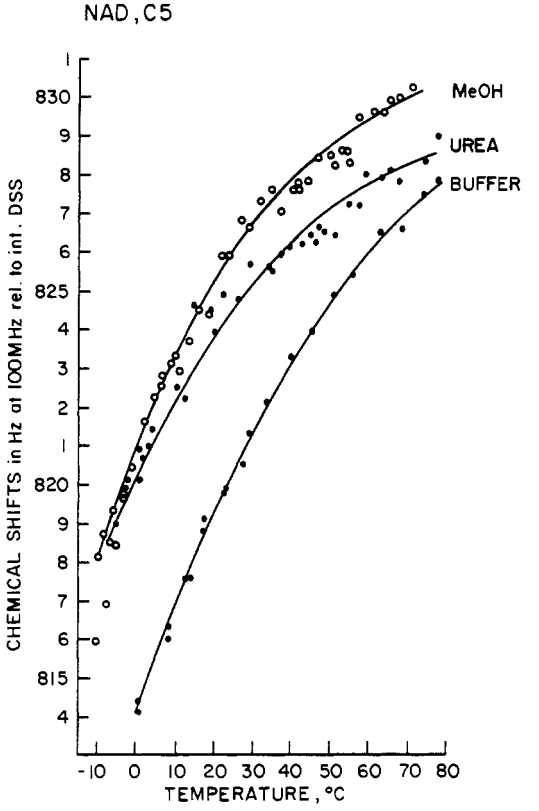

FIGURE 9: Comparison of the temperature dependence of the NC_5H chemical shifts of 0.1 M β-NAD in 7.3 M d_4-methanol in D_2O, 1.0 M d_4-urea in D_2O, each solution containing 0.1 M orthophosphate (pH 7.0) and 0.0025 M silapentanesulfonate.

(1969) argue convincingly for the use of reaction II for stacking reactions of this type, and the results reported here confirm this view. Since there really is no evidence to support the choice of reaction I, it seems likely that the apparent enthalpy change of about -5 kcal/mole reported for the folding of NAD (Jardetzky and Wade-Jardetzky, 1966) or NADH (Sarma and Kaplan, 1970a) as well as the apparent enthalpy changes reported here are maximum values of the enthalpy change for the overall reaction detected by chemical shifts.

In order to determine the nature and relative importance of the forces involved in NAD and NADH folding, the reaction was studied in 7 M methanol and 1 M urea. The results for 0.1 M NAD and 0.1 M NADH in 7 M methanol-d_4 (pH 7.0) 0.1 M orthophosphate buffer) appear in Figures 8 and 9. The results for the unfolding of 0.1 M NAD and 0.1 M NADH in 1 M urea (pH 7.0)–0.1 M orthophosphate buffer in D_2O appear in Figures 10 and 11. The chemical shift *vs.* temperature curves again appear to be hyperbolic. The chemical shifts of NAD and NADH at most temperatures are greater in methanol or urea than in buffer alone indicating that the dinucleotides are more unfolded in the urea or methanol than in buffer at the same temperature. The data in Figures 8, 9, 10, and 11 were used to calculate the thermodynamic parameters for the folding reaction in methanol or urea. The results appear in Table III. The mixed solvents change the behavior of NAD and NADH only modestly but there are interesting trends. As already mentioned, both NAD and NADH appear to be more unfolded in the mixed solvents than in the buffer alone, judging from the fact that the chemical shifts are at lower fields in the mixed solvents at any given temperature. As seen in Table III, however, the calculated values of $\Delta F'$ and K do not reflect the greater degree of unfolding in all cases, *i.e.*, for NAD, K decreases in going from buffer to urea while for NADH it increases. This reflects a relatively large error in our estimate of K.

Discussion

Our results on the thermodynamics of unfolding of NAD and NADH are in substantial agreement with those obtained earlier by Jardetzky and Wade-Jardetzky (1966) in that our parameters are within the relatively large estimated error of their results. However, our method of analysis allows an objective estimate to be made of the variance in the estimates of the thermodynamic parameters. It is particularly interesting that the values obtained for $\Delta H'$ differ depending on which proton chemical shift is followed and that these differences are outside the experimental error. Table III illustrates that in the case of NAD in solutions containing urea or methanol, $-\Delta H'$ obtained from the C_4H shifts is consistently larger than $-\Delta H'$ obtained by following the C_5H shifts. Similarly for NADH in buffer alone or in solutions containing urea or methanol, the C_2H shifts give consistently larger $-\Delta H'$ values than the C_4H. Our interpretation of these differences is that the two-state model (reaction I) is not adequate to describe the temperature dependence of NAD and NADH nmr spectra but rather that intermediate, partially unfolded states exist in appreciable concentrations in the thermal transition range for both dinucleotides. Not inconsistent with this interpretation are our calculated values of δ_u and δ_c for each proton in the three different solvent environments. The δ_u estimates differ by less than 2 Hz (the NADH C_4H in

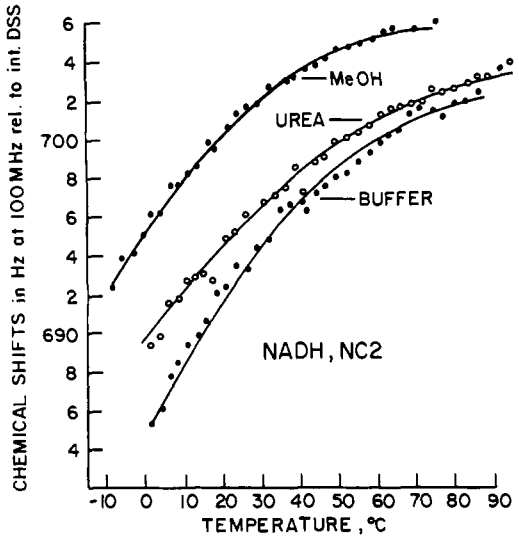

FIGURE 10: Comparison of the temperature dependence of the NC_2H chemical shift of 0.1 M β-NADH in 7.3 M d_4-methanol in D_2O, 1.0 M d_4-urea in D_2O, and D_2O, each solution containing 0.1 M orthophosphate (pH 7.0) and 0.0025 M silapentanesulfonate.

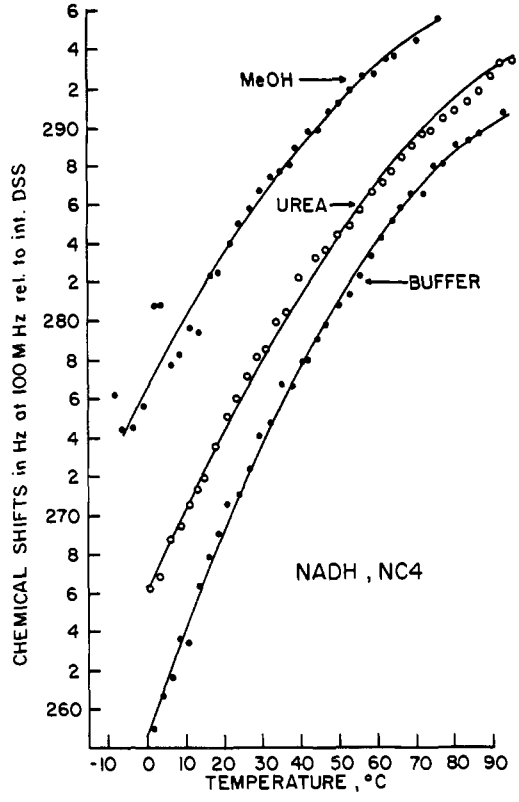

FIGURE 11: Comparison of the temperature dependence of the NC_4H chemical shift of 0.1 M β-NADH in 7.3 M d_4-methanol in D_2O, 1.0 M d_4-urea in D_2O, and D_2O, each solution containing 0.1 M orthophosphate (pH 7.0) and 0.0025 M silapentanesulfonate.

methanol is an exception. For unexplained reasons, perhaps due to a systemic error or unknown origin, all of the parameters for this case seem to be out of line.). As expected from the position of the equilibrium, the δ_c values show large variations. Unfortunately, it is impossible to use these values of δ_c to rule out a two-state transition between the same folded and unfolded conformations in each solvent. Different values for δ_c in different solvents would be possible, of course, only if the completely folded conformations were to differ depending on the solvent or if some of the X_i with lower subscripts are not formed (or are present to a lesser extent) in the different environments.

At least two plausible mechanisms have been proposed to account for the interaction of water with solutes. Experiments on the solubility of small nonpolar molecules in water indicate that exposure of these molecules to solvent leads to an entropy loss due to the ordering of water in the vicinity of the solute (Frank and Evans, 1945). This is accompanied by a small heat loss analagous to the formation of ice. The second mechanism involves the formation of a cavity in the solvent to accommodate the solute molecule (Sinanoglu and Abdulhur, 1965). Associated with the cavity is an amount of energy proportional to the surface area of the cavity and the bulk surface tension of the solvent. An unfolded dinucleotide requires a total cavity surface area greater than that required for a folded molecule with the result that the folded form is favored by the resulting decrease in the solvent energy.

In principle these two mechanisms can be distinguished experimentally on the basis of the thermodynamic parameters for the unfolding reaction in different solvent systems. The addition of solutes such as urea or methanol to water destabilized the folded form of a dinucleotide. If the solute destabilizes the folded form of a dinucleotide by interfering with the formation of structured solvent, then the enthalpy and entropy change for the folding reaction should be greater in the mixed system. If, on the other hand, the main effect of the solute is to lower the surface tension of the solvent, the enthalpy and entropy change for the dinucleotide folding reaction should be greater in buffer alone. In the first case the amount of structured water around the unfolded dinucleotide has been reduced by the solute. When the dinucleotide refolds there is less structured water to "melt" and take up heat, so that the evolved heat is greater than in the absence of solute. In the second case, the amount of energy invested in the cavity around the unfolded dinucleotide is less than in the absence of the solute. When the dinucleotide refolds and the cavity surface shrinks, there is proportionally less energy released than in the absence of the solute.

As can be seen in Table III, $-\Delta H'$ and $-\Delta S'$ for the folding reaction are greater for NAD, but smaller for NADH in 1 M urea than in buffer. One might be tempted to conclude that for NAD, the surface tension is important while for NADH the structuring of solvent plays a dominant role in the folding reaction. Unfortunately, it may only be concluded that NAD behaves oppositely from NADH. Correlation of the entropy and enthalpy changes with the surface tension is only suggested by these data. If the changes in the surface tension alone could explain the data, then one would expect that $-\Delta H'$ for folding of NAD would be smaller, not greater, in urea than in the methanol, since the surface tension of the urea solution is less than that of the methanol solution. Perhaps this question may be resolved by studying the folding reaction in one solvent system at various solute concentrations to discover the effect of varying the surface tension on the thermodynamic parameters. In this way, the possibility of introducing unknown complicating factors by switching solvent systems can be eliminated.

It is interesting to speculate that the difference in properties between NAD and NADH may play some important role in enzymic reactions involving these cofactors. The only part of the molecule which differs between NAD and NADH is the

pyridine ring. Perhaps once NAD or NADH has done its job and is oxidized or reduced, its properties are changed sufficiently to reduce its affinity for the binding site on the enzyme. This could contribute to the efficiency of the catalyzed reaction.

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Chemical Evidence for Conformational Differences between the Red- and Far-Red-Absorbing Forms of Oat Phytochrome†

Stanley J. Roux*

ABSTRACT: The observation that glutaraldehyde inhibits the photoreversibility of the red-absorbing form of phytochrome (Pr) more than that of the far-red-absorbing form (Pfr) suggested that Pr and Pfr might have different reactive sites available to the aldehyde. An approach to evaluating this suggestion was made by performing amino acid analyses on highly purified phytochrome which had been reacted with glutaraldehyde as Pr, as Pfr, and while cycling between Pr and Pfr under constant red light (here called Pcy). Results of the analyses indicated that of the 27 lysine residues (per 60,000 molecular weight) in the control phytochrome, 13 in Pr and Pcy had reacted with glutaraldehyde compared to only 11 in Pfr under the conditions employed. These results were repeated several times with a standard deviation of less than 3%. The data suggest that there are two more aldehyde-accessible lysines in Pr and Pcy than in Pfr and are consistent with the hypothesis that the Pr → Pfr transformation involves a protein conformation change along with a change in the chromophore group. Similar experiments, with trinitrobenzenesulfonic acid (TNBS) as the reagent, were performed with results numerically different from the glutaraldehyde experiments but still consistent with the conformational change hypothesis. Peptide map analyses of TNBS-reacted and trypsin-digested Pr and Pfr were also consistent with the same hypothesis.

Phytochrome is a blue-green biliprotein widely distributed throughout the plant kingdom. The action spectra of several light-induced morphological changes in plants suggest that they are controlled by phytochrome and so this protein is thought to play a key role in plant development.

Phytochrome has two spectrally differing forms; one, Pr[1], has an absorption maximum at about 667 nm, and the other, Pfr, has a maximum at about 725 nm. These forms are interconverted, through short-lived intermediates, by irradiation with light of the appropriate wavelength.

$$\mathrm{Pr} \underset{\text{far-red light } (ca.\ 725\ \text{nm})}{\overset{\text{red light } (ca.\ 665\ \text{nm})}{\rightleftharpoons}} \mathrm{Pfr}$$

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[1] The abbreviations used are: Pr, the red-absorbing form of phytochrome; Pfr, the far-red-absorbing form of phytochrome; TNBS, 2,4,6-trinitrobenzenesulfonic acid; HA, hydroxylapatite; TNP, trinitrophenyl.