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Conformation of NADH studied by fluorescence excitation transfer spectroscopy

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Abstract

Dihydronicotinamide adenine dinucleotide (NADH), is known to stack in two limiting conformations. Surprisingly, previous experimental work on NADH has not clearly defined whether this folding and unfolding process can be described as first order (involving only two states) or whether one or more intermediates must be included in the description. In addition, a large disparity exists between reported equilibrium constants for the aqueous solution at room temperature. Using methanol as a denaturant, we have used fluorescence excitation transfer spectroscopy to probe the stacking/un-stacking equilibrium. Our results can be represented using a simple two state model. Furthermore, the mole fraction of aqueous NADH in the stacked configuration is significantly higher than previously reported. Using a thermodynamic two state model, we have determined $\Delta G^{o}(H_2O) = 8.8 \pm 1.4$ kJ mol^{-1}. From excitation energy transfer measurements the fraction of NADH in the folded form at 295 K was determined to be 0.55.

Keywords: NADH; Folding; Conformation; Fluorescence excitation transfer spectroscopy

1. Introduction

β-Nicotinamide adenine dinucleotide, NAD^+, and its reduced form β-dihdronicotinamide adenine dinucleotide, NADH, play an important role in the conversion of chemical energy to useful metabolic energy [1]. From its reduced form, NADH, is oxidized in a series of steps which lead to the reduction of molecular oxygen. From formal reduction potentials [2]

$$NADH + \frac{1}{2}O_2 + H^+ - NAD^+ + H_2O \qquad E^{\circ\prime} = 1.14\ V \qquad (1)$$

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corresponding to a free energy change of −220 kJ (−52 580 calories) with reduction occurring at the C-4 position of the nicotinamide moiety of the molecule (Fig. 1). Over 40 years ago, Weber observed an excitation energy transfer process where excitation of the adenine moiety of the molecule at 254 nm results in fluorescence of the dyhdronicotinamide and concluded that NADH exists as an intramolecular complex [3]. Shortly thereafter, Velick presented spectral evidence for the folded conformation and the denaturing effect of solvents and temperature [4].

To date, the most detailed work exploring the denaturing of NADH has been carried out using proton nuclear magnetic resonance spectroscopy, ^{1}H-NMR. McDonald et al. were able to determine basic thermodynamic parameters governing the folding by analyzing proton chemical shifts as a function of temperature in both aqueous, methanol and urea solutions [5]. For example, the equilibrium constant for $[NADH]_N/[NADH]_D$ in 7 M solution of methanol was determined to be 0.014 ± 0.002 and 0.600 ± 0.628 for the protons in the 2 and 4 position of the diydronicotinamide (NC-2 and NC-4), respectively. However, the equilibrium constant determined for urea and aqueous solutions showed little change for the NC-2 and NC-4 protons [$(0.31–0.37) \pm (0.06–0.1)$]. Statistically significant differences did exist between calculated changes in enthalpy and entropy. Because the calculated thermodynamic parameters varied depending on which proton was used in the analysis, McDonald et al. concluded that the folding reaction is not a two state process.

More recently, Rosei and Mosca examined the conformation of NADH by Raman spectroscopy [6]. While providing only qualitative information, the authors suggest their data are representative of a continuous conformational transition rather than a cooperative one. Finally, Couprie et al. examined fluorescence decays and rotational dynamics of NADH on the picosecond time scale. While their work focused on the feasibility of a UV storage ring free-electron laser, the short fluorescence lifetime of NADH was directly measured. The dihydronicotinamide was excited at 350 nm followed by monitoring of the fluorescence amplitudes at 460 nm. Two different temporal components were observed and used as a first approximation of the equilibrium constant. Results were in agreement with previous NMR work on the oxidized form of the dinucleotide, NAD^+ [7].

In each of the cases above, thermodynamic parameters were determined by thermal denaturation of NADH. McDonald et al. have pointed out that their work (and other ^{1}H-NMR studies)

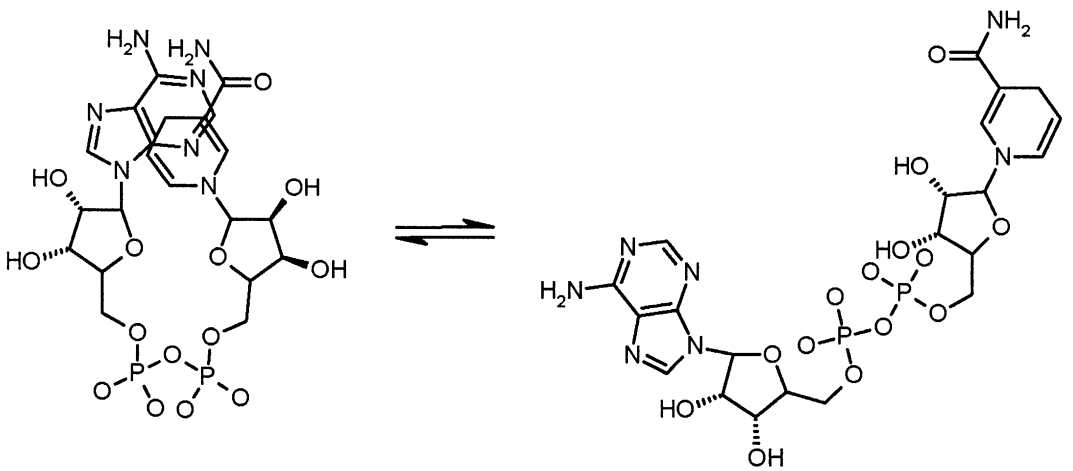

Fig. 1. β-NADH equilibrium exist between the folded and unfolded conformations. Efficient energy transfer only occurs between the two chromophores in the folded conformation.

only provides a determination of the apparent enthalpy change ($\Delta H'$) from the chemical shifts obtained at various temperatures. As mentioned above, work by Couprie et al. provides direct measurement of the fluorescence lifetimes of the dihydronicotinomide moiety; however, it is not clear how the two decay rates are related to the two chromophores as stated since the adenine moiety absorbs at 260 nm.

We have utilized fluorescence excitation transfer spectroscopy to probe the conformational changes of NADH. In the case where the adenine nucleotide is excited and emission from the dihydronicotinamide nucleotide is monitored, the transfer rate decreases by the sixth power of distance between the two nucleotides assuming a dipole–dipole interaction [8]. Comparable transfer between molecules would require concentrations on the order of 10^{-2} M. Working at 10^{-5}–10^{-6} M allows observation of only intramolecular energy transfer with the observed fluorescence dependent on the separation between the adenine and dihydronicotinamide nucleotides.

Experimental results are analyzed using the solvent denaturation model developed by Schellman where the equilibrium is treated as a two state system [9]. While not supplanting stoichiometric binding models, Schellman's work describes the interactions between macromolecules and solvent in a general thermodynamic context. The applicability of this model to proteins is demonstrated by a recent article by Jones [10]. In the case of NADH, knowledge of specific binding interactions within the molecular framework is limited and the thermodynamic model is particularly advantageous in describing the current system.

2. Experiment

All experiments were conducted using a commercial spectrofluorometer (SLM-AMINCO model 8100). Excitation was accomplished using a Xenon arc lamp and fluorescence was monitored by a PMT (Hamamatsu R928). Both excitation and emission monochromators were set for a 4 nm bandwidth. Excitation spectra are corrected for instrument response using Rhodamine B in ethylene glycol as a reference. Excitation spectra for the solvents were obtained to correct for background fluorescence of the buffer and methanol solutions. Room temperature measurements were carried out at 295 ± 1 K. Variable temperature studies were accomplished by circulating water at a preset temperature through the cell mounting block. Temperatures were measured directly during each scan using a K-type thermocouple.

All NADH solutions were prepared fresh daily. β-NADH (Sigma, 98%), Methanol (spectrophotometric grade, 99.9%) and Tris[hydroxymethyl]aminomethane hydrochloride (Trizma hydrochloride, Sigma, 99%) were used as received. β-NADH was refrigerated when not in use. Buffer solutions (0.01 F) were prepared by dissolving an appropriate amount of the Trizma hydrochloride and adjusting the pH to 7.3 ± 0.1 using a 0.05 or 0.1 M NaOH solution. Stock solutions of β-NADH were prepared by dissolving 1.2–12.5 mg of NADH in 50 ml of the buffered solvent; 1.00–2.00 ml aliquots of the stock solution were then used to prepare final solutions that had a NADH concentration of 0.70–85 μM and a percent volume methanol of 0–90%. Methanol concentrations were determined using the reported density of 0.7914 g/ml [11].

3. Results

Free energy was determined using the linear free energy model outlined by Schellman [9]. Originally developed to treat solvent denaturation of macromolecules with cooperative structures, the generalized thermodynamic treatment is used to examine interactions between macromolecules and solvent in contrast to stoichiometric binding theories [12]. Assuming only two states and ignoring activity coefficients, the equilibrium between the folded (native, N) and unfolded (denatured, D) states of NADH is given by

$$K = \frac{[\mathrm{NADH}]_\mathrm{D}}{[\mathrm{NADH}]_\mathrm{N}} \tag{2}$$

Using a molar scale, the chemical potential of NADH in solution is expressed by

$$\mu_j = \mu_j^0 + RT\ln C_j + RTb_j \tag{3}$$

where μ_j^0 is the chemical potential of the reference state, C_j is the molarity and RTb_j is the excess free energy. Using standard notation for the above system: $j = 1$, 2, 3 for the principal solvent, water; solute under investigation, NADH; and denaturant, methanol, respectively.

In solution, the chemical potential of the native and denatured states of NADH are equal,

$$\mu^0_{\mathrm{NADH}}(\mathrm{N}) + RT\ln[\mathrm{NADH}]_{\mathrm{N}} + RTb_{\mathrm{NADH}}(\mathrm{N}) = \mu^0_{\mathrm{NADH}}(\mathrm{D}) + RT\ln[\mathrm{NADH}]_{\mathrm{D}} + RTb_{\mathrm{NADH}}(\mathrm{D}) \tag{4}$$

Defining $\Delta\mu^0 = \mu^0_{\mathrm{NADH}}(\mathrm{D}) - \mu^0_{\mathrm{NADH}}(\mathrm{N})$, $\Delta b_{\mathrm{NADH}} = b_{\mathrm{NADH}}(\mathrm{D}) - b_{\mathrm{NADH}}(\mathrm{N})$ and using Eq. (2) above, Eq. (4) can be expressed as

$$\Delta\mu = \Delta\mu^0 + RT\Delta b_{\mathrm{NADH}} \tag{5}$$

Using the linear free energy model outlined by Schellman, the excess free energy is represented by a Taylor series in all solute concentrations

$$b_j = \sum_{k>1} b^0_{Jk} + \sum_{k,n>1} b^0_{jkn} C_k C_n \tag{6}$$

In the limit of low concentrations of NADH and with the assumption that b_2 is linear in the concentration of the denaturant, $k = 3$, then

$$b_2 = b^0_{2\,3} C_3 \tag{7}$$

Substituting Eq. (7) into Eq. (5)

$$\Delta\mu = \Delta\mu^0 + RT\Delta b^0_{2\,3} C_3 \tag{8}$$

When $[\mathrm{NADH}]_{\mathrm{N}} = [\mathrm{NADH}]_{\mathrm{D}}$, $C_3 = C_m$ and $\Delta\mu = 0$ and

$$\Delta\mu^0 = -RT\Delta b^0_{2\,3} C_m \tag{9}$$

To further simplify the notation we can group $-RT\Delta b^0_{2\,3}$ into a single constant m so that

$$\Delta\mu^0 = m\,C_m \tag{10}$$

The fraction of NADH in the unfolded state is given by

$$\theta = \frac{K_{\mathrm{eq}}}{1 + K_{\mathrm{eq}}} \tag{11}$$

From the relationship between free energy and the equilibrium constant

$$\theta = e[-m(C_m - C)/RT\ ]/1 + e[-m(C_m - c)/RT\ ] \tag{12}$$

m and C_m are determined by a nonlinear least-squares of the denaturation curve. Experimentally, the fraction of NADH in the unfolded state is defined as

$$\theta = \frac{I_{\mathrm{N}} - I}{I_{\mathrm{N}} - I_{\mathrm{D}}} \tag{13}$$

where I, I_{N}, and I_{D} are the fluorescence intensity at methanol concentration C, fluorescence intensity of NADH in its native (folded) state and the fluorescence intensity of the denatured (unfolded) NADH, respectively.

A sample of experimental data showing the change in fluorescence intensity as a function of methanol concentration is presented in Fig. 2a. The two chromophores of NADH have absorption bands at approximately 260 nm for adenine and 340 nm for dihydronicotinamide nucleotides. In this work, the adenine nucleotide is excited at 260 nm; emission from the dihydronicotinamide nucleotide is monitored from 300 to 500 nm. The denaturation curve for NADH is shown in Fig. 2b. Analysis were carried out over four concentrations between 1.4 and 14.1 μM with no significant variations between individual data sets. For analysis, the experimental results from the different concentrations were combined and used to calculate an average. The uncertainty at each

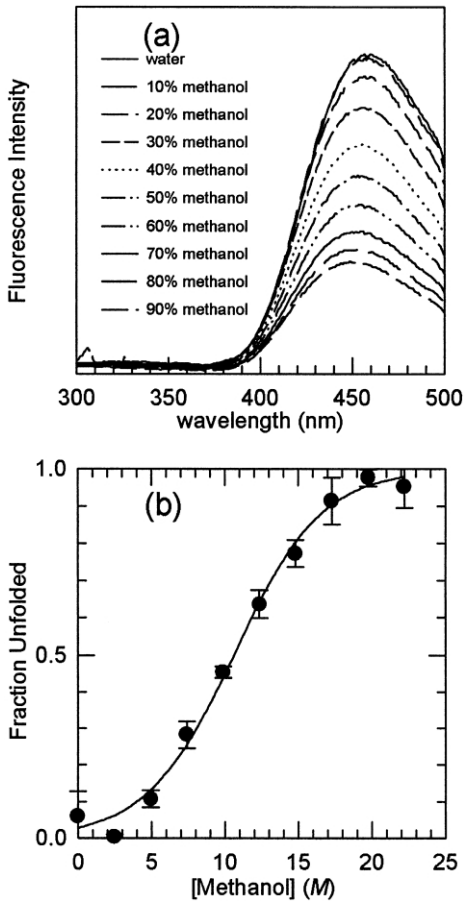

Fig. 2. (a) Fluorescence spectra showing the decrease in intensity as the methanol concentration increases. Excitation wavelength was 260 nm. (b) Denaturation curve of NADH. The line is a best fit to Eq. (11) in the text.

point along the denaturation curve was determined at the 95% confidence level. The uncertainties for each parameter in free energy were determined by computing the standard deviation for the non-linear least-squares fit of the data to Eq. (12). The standard deviation is taken as representative of the overall experimental uncertainty (approx. 15%). Unless otherwise indicated, the reported uncertainty is $\pm 2\ \sigma$.

Excitation spectra, corrected for instrument response, were recorded and energy transfer efficiency determined by taking the ratios of fluorescence intensities at 260 and 340 nm. This ratio was then corrected for direct absorption of dihydronicotinamide at 260 nm [13]. These results at 295 K and 277 K are presented in Fig. 3a–c. Results show approximately 70% and 90% of the excitation energy is transferred when the NADH is in its native state at 295 K and 277 K with a

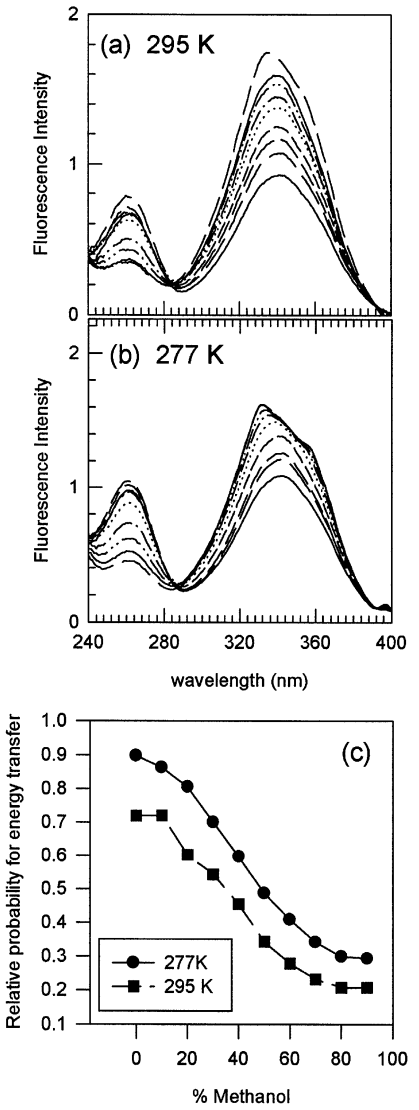

Fig. 3. (a) Fluorescence excitation spectra for NADH at 295 K; (b) fluorescence excitation spectra for NADH at 277 K. [NADH] = 1.7 μM for both data sets. Spectra use the same % methanol v/v labels as Fig. 2. (c) Relative probability for energy transfer as a function of % methanol v/v.

NADH concentration of 1.7 μM. This reaches a minimum at 80% methanol, v/v (approx. 20 M methanol). Temperature and concentration dependent measurements show that the energy transfer efficiency is dependent on temperature and concentration of NADH. While the temperature dependence has been observed in early fluorescence work by Velick and is the basis of most NMR studies to date, the results presented here are the first to show the excitation transfer efficiency approach unity as the temperature decreases to ~273 K. Finally, careful examination of the concentration dependence revealed the low concentrations necessary to maximize the energy transfer. This result was surprisingly low, approximately 1 μM.

4. Discussion

Large macromolecular structures such as proteins and polypeptide chains show sharp transitions between the native and denatured state indicative of a cooperative transition. In contrast, the denaturation curve for NADH with methanol is broad with the fraction of molecules unfolded changing slowly as denaturant is added. A similar, broad transition is also observed for low molecular weight, polypeptide chains [14]. At low and high methanol concentrations the change in fluorescence intensity does not change within the experimental uncertainty of the measurements and we assume these points represent the completely folded and unfolded forms of NADH respectively. We have fit the experimental data to Eq. (12) which lead to $C_m = 10.63 \pm 0.42$ M and $m = 828.5 \pm 104.6$ J l^{-1} mol^{-2}. Thus, the change in free energy of unfolding in the limit of low concentrations of solute and no denaturant is 8.81 ± 0.43 kJ mol^{-1}. It is important to note that this observation measures the relative change in folding. That is, at room temperature and at concentrations above approximately 1–2 μM, a fraction of the NADH exists in the unfolded form. The fluorescence excitation transfer technique probes only the folded NADH. Determination of the overall equilibrium constant was accomplished using the measured energy transfer efficiency. Upper limits were set using the energy transfer efficiency at 275 K (i.e. 1) for the folded conformation while limits for the unfolded conformation were determined from energy transfer efficiency measurements at 80–90% v/v methanol. Our value for the fraction of NADH folded (X_{Folded}) is larger than those determined by ^{1}H-NMR and fluorescence lifetime measurements. A comparison of our results determined above with those previously published are given in Table 1.

Excitation spectra also show the presence of an isobestic point as the methanol concentration increases. The presence of an isobestic point is indicative of a two state system and further justify the use of the model by Schellman. Isobestic points arise when $2 + N$ species are present and N independent relations exist between the concentrations of these species and the parameter being varied [15]. If one chooses to represent the current equilibrium with multiple intermediates, all relations are dependent solely on the methanol concentration; therefore, the isobestic point is expected for the overlapping bands of the NADH. Previous results by Scott et al. [13] reported energy transfer efficiency of 0.42 are low compared

Table 1
Comparison of equilibrium constants and fraction of NADH folded[a]

	McDonald et al. (1972) (7 M methanol)	McDonald et al. (1972) (water)	Openheimer et al. (1978)	Couprie et al. (1994) (20°C)	Current work
X_{Folded}	0.9864 NC-2 0.6253 NC-4	0.2368 NC-2 0.2439 NC-4	0.36 NC-4	0.32	0.55

[a] NC-*n*: protons on dihydronicotinomide moiety.

with those obtained here even when considering the concentration of 10 μM used in their experiments. Our results agree qualitatively with the relative probability of energy transfer as a function of methanol concentration reported by Freed et al. [16] where normalized fluorescence intensities were used in the analysis ([NADH] = 10^{-3} M).

One additional feature of the excitation spectra presented here not previously reported is the additional structure associated with the dihydronicotinomide band which becomes visible at the highest methanol concentration, 90% methanol, v/v (22.2 M methanol). The observed band is due to transitions associated with the amide group on this mononucleotide. It is believed that the observed spectral changes are due to changes in hydrogen bonding associated with the amide functional group and its associated free rotation.

We believe that the inconsistency between our results and those presented in the literature is due to the wide range of NADH concentrations used in the various studies to date. Only Oppenheimer et al. reported significant effects of concentration on the proton spectra of the dihydronicotinamide moiety and have explained the absence of this observation for earlier NMR work. Data from Oppenheimer et al. are typically presented

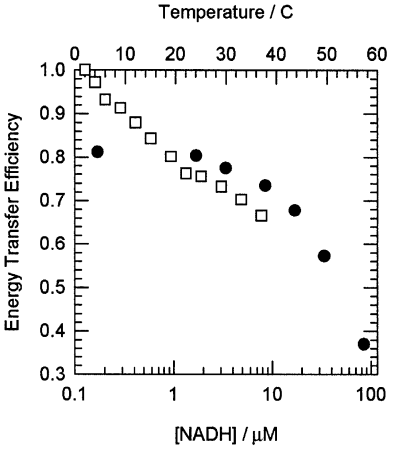

Fig. 4. Temperature (□) and concentration (●) dependence for NADH. [NADH] = 1.0 μM for the temperature dependent study.

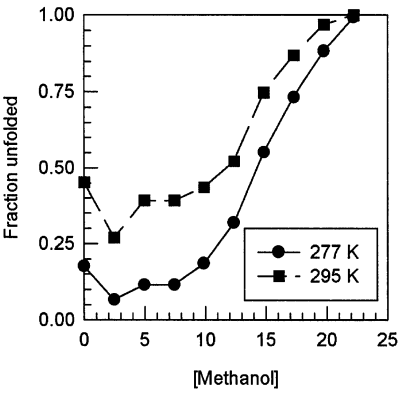

Fig. 5. Fraction unfolded as a function of methanol concentration setting the upper limit for energy transfer at unity as observed near 275 K.

in the range of 10–50 mM. If the energy transfer efficiency measurements presented in Figs. 4 and 5 can be taken as an indication of the concentration required to isolate intramolecular processes, then further work at low concentrations, approximately 1 μM, or further extrapolation from higher concentration results is required to examine the equilibrium between the folded and open conformations via NMR. Furthermore, population of the specifically folded conformation was calculated with the assumption that the protons at the C-4 position of the dihydronicotinomide are in an axial and equatorial arrangement when NADH is in the folded configuration. Since we observe nearly complete excitation energy transfer at 275 K, a more appropriate comparison with the fluorescence excitation transfer results presented here would be to calculate the mole fraction of the folded form from observed J coupling constants at low concentrations and $\sim$273 K.

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