NICOTINAMIDE ADENINE DINUCLEOTIDE IS ABOUT 44% FOLDED IN NEUTRAL AQUEOUS SOLUTIONS

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1. Introduction

By comparing the acidity constants of the adenine moiety of NAD with that of adenosine diphosphoribose, which lacks the nicotinamide ring of NAD, a simple analysis indicates that 44% of NAD is folded in neutral solution at 25°C and 0.15 M ionic strength. Decreasing temperature or ionic strength results in an increase in the percentage of folded NAD. For the overall folding interactions of NAD, $\Delta H = -3.1$ kcal .mol^{-1} and $\Delta S = 11$ kcal .mol^{-1} .degree^{-1}. The reduced dinucleotide NADH is folded to similar extents with and without adenine moiety protonation.

The conformation of nicotinamide adenine dinucleotide (NAD) in neutral aqueous solutions has been a subject of continuing discussion. Both nuclear magnetic resonance [1] and optical [2] spectroscopic results have been utilized to estimate the extent of folding or stacking of the two aromatic ends of the molecule. (The indicated recent references provide numerous further citations in the literature.) Arguments of varying degrees of sophistication and uncertainty have given rise to estimates ranging from virtually complete stacking to almost all molecules in an extended conformation. The majority of interpretations favor considerable folding or stacking.

In this communication we present a simple argument based on comparison of the acidity constants of the adenine moiety of NAD with that of adenosine diphosphoribose (ADPR), which possesses all the attributes of NAD except the aromatic nicotinamide group, and therefore is unable to form an intramolecular stack. The greater acidity of the adenine moiety in NAD is attributed to intramolecular folding in that molecule which has no counterpart in ADPR. Titration results are also reported for the reduced dinucleotide, NADH.

2. Materials and methods

The best quality β-NAD, β-NADH, and ADPR were found to be from Sigma Chemical Co. As determined by titration, the percentage purities were 98% and 95% for NAD and ADPR, respectively. The compounds contained some material titrating near pH 6 which was allowed for in the analysis. The acidity constants were determined from ⩾14 points on the titration curve with a greater number on the acid side of neutrality due to the impurities. The pH, required in the more acidic solutions, was found by calibrating the electrodes in order to take into account the activity coefficient of H^+ and characteristics of the electrodes [3]. The reported acidity constant logarithms are concentration constants. They may be converted to the more usual activity in H^+ constants by adding 0.080, 0.067, and 0.048 at 0.15, 0.06, and 0.015 ionic strengths, respectively. Ionic strength was maintained by KCl and the ligands titrated with KOH. Experiments in which ionic strength was maintained with $(CH_3)_4NCl$ and ligand titrated with $(CH_3)_4NOH$ gave closely similar results. Ligands were titrated at ~3 mM on a Radiometer PHM64 pH meter under a nitrogen atmosphere. NMR spectra were recorded on a Varian EM 390 spectrometer at 2.5 mM ligand.

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3. Results

The titration results may be interpreted in terms of the cyclic system:

$$\begin{tikzcd} HA_u \arrow[r, shift left, "K_1"] \arrow[d, shift left] & H^+ + A_u \arrow[l, shift left] \arrow[d, shift left, "K_f"] \\ HA_f \arrow[u, shift left, "K_{f'}"] \arrow[r, shift left] & H^+ + A_f \arrow[l, shift left, "K_2"] \arrow[u, shift left] \end{tikzcd}$$

where the acidity constants $K_1 = [H^+]\,[A_u]/[HA_u]$ and $K_2 = [H^+]\,[A_f]/[HA_f]$ refer to the unfolded and intramolecularly folded forms, respectively. The equilibrium folding constants $K_f = [A_f]/[A_u]$ and $K_{f'} = [HA_f]/[HA_u]$ refer to the basic and positively charged adenine moiety protonated at N(1), respectively. From the properties of a cyclic system, $K_1K_f = K_2K_{f'}$.

The acidity constant determined by titration of NAD is given by:

$$K_a = \frac{[H^+]\,([A_u] + [A_f])}{[HA_u] + [HA_f]} = K_1\frac{1 + K_f}{1 + K_{f'}} \qquad (1)$$

Since it cannot fold in the manner that is unique to NAD, the acidity constant of ADPR is given by K_1. Thus, as indicated in eq. (1) the ratio of acidity constants K_a/K_1 for NAD to ADPR provides a measure of intramolecular folding in NAD.

It is generally agreed that negligible folding occurs in adenine protonated NAD, where both the adenine and nicotinamide moieties are positively charged. Thus $K_{f'} \simeq 0$ in the above scheme and the measured acidity constant ratio, $K_a/K_1 = 1 + K_f$. Results of the acidity constant determinations for NAD (pK_a) and ADPR (pK_1) are presented in table 1 at several temperatures and ionic strengths. Also presented are the calculated equilibrium constant for folding K_f and the percentage of NAD molecules that are folded in neutral aqueous solutions. The titrations were performed at $\sim$3 mM ligand, well below the onset of intermolecular interactions [4].

The estimated standard deviations are $\leqslant \pm 0.003$ in pK_a and pK_1 except for pK_a at 5°C where it is ± 0.007 and at 0.015 M ionic strength where it is ± 0.005. These values propagate the resultant standard deviations in K_f indicated in table 1. From the temperature dependencies $\Delta H_a = -3.2$ kcal .mol^{-1} for pK_a and $\Delta H_1 = -4.6$ kcal .mol^{-1} for pK_1. From the temperature variation of the equilibrium constant for folding K_f, the enthalpy of folding $\Delta H_f = -3.1$ kcal .mol^{-1} and $\Delta S_f = -11$ cal .mol^{-1} .degree^{-1}. An error analysis suggests that the standard deviations in the ΔH values are $< \pm 0.1$ kcal .mol^{-1}.

A comparison was made of the chemical shifts of the adenine H(2) and H(8) protons in the NMR spectra of NAD and ADPR. For the adenine moiety protonated forms each kind of proton exhibits the same chemical shift in the two compounds to within 0.02 ppm. In neutral solutions the chemical shift of both the H(2) and H(8) protons is shifted 0.07 ppm upfield in NAD relative to where it is found in ADPR. The nicotinamide portion peaks occur about 0.10 ppm upfield in neutral solution compared to adenine protonated NAD.

For reduced nicotinamide dinucleotide, NADH, at 25°C and 0.15 ionic strength, $pK_a = 3.88 \pm 0.01$. This value is only slightly less than that of ADPR under the same conditions. Since for this molecule the dihydropyridine ring is uncharged we cannot set $K_{f'} \simeq 0$ as was done for NAD. The near equality of the pK_a and pK_1 values in this system implies that $K_f = K_{f'}$. Thus for NADH the basic and adenine protonated forms are about equally folded to some

Table 1
Acidity constant logarithms for NAD (pK_a) and ADPR (pK_1)

T (°C)	I (M)	pK_a	pK_1	K_f	% folded
5	0.15	3.816	4.147	1.14 ± 0.04	53
25	0.15	3.648	3.898	0.78 ± 0.02	44
40	0.15	3.533	3.741	0.61 ± 0.02	38
25	0.06	3.677	3.948	0.87 ± 0.02	46
25	0.015	3.718	4.048	1.14 ± 0.03	53

undetermined extent. It is unlikely that NADH with a non-planar, non-aromatic dihydropyridine ring is more folded than NAD. Thus the K_f and percent folded values in table 1 may be taken as maximum values for NADH.

4. Discussion

The results presented in table 1 indicate that NAD is 44% folded in neutral aqueous solutions at 0.15 ionic strength and 25°C, and that the fraction folded increases with decreasing ionic strength and with decreasing temperature. The pK_1 value of ADPR and the pK_a value of NAD correspond to weighted averages over all of their conformations. By the nature of the analysis the equilibrium constant for folding K_f refers to a weighted average over all conformations realized by NAD but not by ADPR. NMR and other spectroscopic results [1,2] suggest that some degree of intramolecular stacking may occur in some of the folded conformations.

The enthalpy of folding of $\Delta H_f = -3.1$ kcal .mol^{-1} and $\Delta S = -11$ cal .mol^{-1} .degree^{-1} agrees with expectations for intramolecular folding with some degree of favorable interactions between the two kinds of aromatic rings. Because ΔH_f refers to an ensemble average over all intramolecularly-folded forms in dynamic equilibrium, it should not be assigned to any specific folded or stacked conformation.

The titration analysis responds to longer range interactions than do the spectroscopic methods. Thus the K_f values and percent folded forms of table 1 represent maximum values likely to be observed by any method under the conditions of the experiment. Because the nicotinamide portion of NAD bears a positive charge that is not present in the model compound ADPR, there is an electrostatic effect that alone should increase the acidity of the protonated adenine moiety. Due to the distance between and the screening of the two charged centers in the unfolded conformations this electrostatic effect should be negligible or nearly so. Such an electrostatic effect would be expected to produce a downfield shift in the H(2) and H(8) protons of the adenine moiety of NAD compared to ADPR. That no downfield chemical shift is observed even in the adenine protonated species suggests that the electrostatic effect is indeed negligible. To the extent that it does operate it will reduce K_f, again indicating that the values in table 1 are maximum values.

References

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