

Stereospecificity of the Intramolecular Association of Reduced Pyridine Coenzymes[†]

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ABSTRACT: Proton magnetic resonance results are reported which focus primarily on the properties of the N4 methylene protons of the dihydropyridine ring in reduced pyridine coenzymes, analogues, and nucleotides. The N4 protons have been found to be nonequivalent in all the dinucleotides studied, but in mononucleotides they are chemical-shift equivalent within the limits of resolution, 0.002 ppm. The magnitude of the nonequivalence, $\Delta\delta_{AB}$, is found to be influenced by a number of other parameters besides the previously reported dependence on temperature. Nonpolar solvents such as methanol cause the nonequivalence to decrease. Increasing concentrations of NADH or NADPH or increasing concentrations of 5'AMP for a fixed concentration of NADH decrease the nonequivalence as a result of a preferential upfield shift of the N4A proton. Protonation of the adenosine 2'-phosphate in NADPH

results in a 0.036-ppm upfield shift of the N4B proton without affecting the N4A proton. In addition, the relative nonequivalence of the N4 protons in all the coenzyme analogues studied has been found to be the same; i.e., the N4B proton is upfield and the N4A proton is downfield. Finally, analysis of the vicinal coupling constants of the dihydropyridine ring indicates that at 22 °C $J_{4A-5} < J_{4B-5}$ in all the dinucleotides, whereas these coupling constants are equal in NMNH. The stereoselectivity observed in these experiments are interpreted in terms of a model where the reduced pyridine coenzymes exist as a rapid, dynamic equilibrium between an open, extended conformation and a specific, folded conformation in which the B face of a puckered dihydropyridine ring is associated with the adenine ring.

Pyridine coenzyme dependent dehydrogenases catalyze hydride transfers which are stereoselective with respect to both the coenzyme and the substrate (Fisher et al., 1953).¹ Stereoselectivity has also been observed in the nonenzymatic, chemical reactions of pyridine coenzymes.² Dithionite reduction of NAD⁺ in D₂O preferentially incorporates deuterium into the 4A (*pro-R*) position (Pullman et al., 1954), and

ferricyanide oxidation of the reduced coenzyme preferentially removes the 4A proton (San Pietro et al., 1955). These results, together with the observation of fluorescence transfer from the adenine to the dihydronicotinamide chromophores in NADH (Weber, 1957), prompted Velick (1961) to propose that the nonenzymatic, chemical stereoselectivity was a consequence of the solution conformation of the pyridine coenzymes. The stereoselectivity was attributed to a *specific* intramolecular association between the adenine and the B face of the pyridine ring.

Proton nuclear magnetic resonance (¹H NMR) investigations (Meyer et al., 1962; Jardetzky and Wade-Jardetzky, 1966; Hollis, 1969; Patel, 1969; Sarma and Kaplan, 1970a; Oppenheimer et al., 1971; McDonald et al., 1972; Sarma and Mynott, 1973; Oppenheimer and Kaplan, 1974; Czeisler and Hollis, 1975) have provided supporting evidence for an intramolecular association between the adenine and pyridine moieties. However, only in our preliminary report (Oppenheimer et al., 1971) has ¹H NMR evidence been presented which indicates a stereoselective association.

Specific vs. random intramolecular interactions of the dihydropyridine moiety can be distinguished by the preferential perturbation of one N4 methylene proton relative to the other N4 proton, since these protons lie above and below the plane of the ring unlike the N2, N5, or N6 protons. The absolute specificity of the interactions can be followed, since the absolute configuration of the N4 protons is known for NADH and NADPH (Cornforth et al., 1962). Studies can also be conducted on reduced coenzyme analogues, since a number of their absolute configurations have also been determined (Biellman et al., 1974; Oppenheimer and Kaplan, 1974) and their N4 proton resonances have been assigned by enzymatic deuterium labeling (Oppenheimer et al., 1971).

We report the results of investigations into the behavior, under a variety of conditions, of the chemical shifts and coupling constants of the N4 proton resonances of reduced pyridine nucleotides and coenzyme analogues. These data provide

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¹ Recently new ¹H NMR methods have been devised for determining this stereospecificity (Arnold et al., 1976) and a thorough review has been written on the subject (You et al., 1978), in which the stereospecificities of 124 oxidoreductases are summarized.

² Abbreviations used are: NAD⁺, nicotinamide adenine dinucleotide; NADP⁺, NAD⁺ phosphate; NADH, reduced NAD⁺; NADPH, reduced NADP⁺; (PyAl)AD⁺, pyridine aldehyde adenine dinucleotide; (AcPy)AD⁺, acetyl pyridine adenine dinucleotide; (TN)AD⁺, thionicotinamide adenine dinucleotide; (N-Met)NAD⁺, the N-methylnicotinamide analogue of NAD⁺; ϵ -NAD⁺, the 1,N⁶-ethenoadenine analogue of NAD⁺; NHD⁺, the hypoxanthine analogue of NAD⁺; (PyAl)ADH, (AcPy)ADH, (TN)ADH, (N-Met)NADH, N(H)DH are reduced (PyAl)AD⁺, (AcPy)AD⁺, (TN)AD⁺, (N-Met)NAD⁺, N(H)D⁺, respectively; NADH(OAc)₄, NADH with the four ribose hydroxyl groups acetylated; NMNH, reduced nicotinamide mononucleotide; NMND_{A,B}, NMNH with a random deuterium label at the nicotinamide 4 position; NADD_{A,B}, NADH with a random deuterium label at the nicotinamide 4 position; NADD_B, NADH with a deuterium at the nicotinamide 4B position; NMND_B, the pyridine mononucleotide of NADD_B; TSP, sodium trimethylsilylpropionate-2,2,3,3-*d*₄; DSS, 4,4-dimethyl-4-silapentane-5-sulfonate; TMAC, tetramethylammonium chloride; EDTA, (ethylenedinitrilo)tetraacetic acid; ¹H NMR, proton nuclear magnetic resonance; 5'AMP, 5'-adenosine monophosphate.

TABLE I: Chemical Shifts of the Dihydropyridine Protons.^a

	N2	N6	N5	N4		$\Delta\delta_{AB}^b$
Mononucleotides						
DHNR	7.157	6.118	5.009	3.075		<0.002
NMNH	7.166	6.239	5.036	3.075		<0.002
NMNH(OAc) ₂	7.139	6.252	5.045	3.055		<0.002
Dinucleotides						
				A	B	
NADH	6.934	5.963	4.750	2.739	2.636	0.102
NADPH	6.923	5.957	4.784	2.809	2.723	0.086
NADH(OAc) ₄	6.925	6.025	4.868	2.870	2.759	0.111
ϵ NADH	6.720	5.870	4.655	2.600	2.525	0.080
N(H)DH	6.986	6.057	4.882	2.898	2.830	0.059
(N-Met)NADH	6.868	6.007	4.741	2.661	2.525	0.136
(TN)ADH	7.636	6.057	4.968	2.818	2.718	0.100
(AcPy)ADH	7.280	6.000	4.930	2.573	2.532	0.041
(PyAl)ADH	7.093	6.002	4.984	2.648	2.602	0.045

^a Chemical shifts are in ppm from internal TSP and are reported to within 0.002 ppm. The concentrations are 50 mM, pD 8.5, at 22 °C.

^b $\Delta\delta_{AB}$ of less than 0.07 ppm was determined from the spectrum of the nonspecifically deuterium-labeled nucleotides.

compelling evidence for a stereospecific intramolecular association for a wide variety of 1,4-dihydropyridine dinucleotides. Furthermore, these data should help clarify the structure of pyridine nucleotides in solution; a topic which at present is subject to conflicting interpretations.

Experimental Section

Materials. The dinucleotides NAD⁺, NADH, NADP⁺, and NADPH were obtained from P-L Biochemicals and used without further purification. The analogues (PyAl)AD⁺, (AcPy)AD⁺, (TN)AD⁺, and (N-Met)NAD⁺ were synthesized by exchange of the nicotinamide moiety of NAD⁺ with the respective free bases by pig brain NADase (Kaplan and Ciotti, 1956). ϵ -NAD⁺ was prepared by the addition of 2-chloroacetaldehyde to NAD⁺ (Secrist et al., 1972), and N(H)D⁺ was prepared by incubation of NAD⁺ in dilute nitrous acid (Kaplan, 1957). The analogues were reduced to their corresponding dihydropyridines with yeast alcohol dehydrogenase. The pyridine mononucleotides were prepared by cleavage of the respective dinucleotides with snake venom phosphodiesterase according to the procedure of Kaplan and Stolzenbach (1957), and purified as outlined by Oppenheimer and Kaplan (1976). The ribosides were prepared by incubation of the mononucleotides in 0.1 M ammonium bicarbonate, pH 8.0, with alkaline phosphatase. Purity was determined from the UV and ¹H NMR spectra as well as paper chromatography.

Deuterium labels at the A or B position of the dihydropyridine ring were prepared enzymatically as previously outlined (Oppenheimer et al., 1971; Arnold et al., 1976). The labeled mononucleotide NMND_B was prepared from NADD_B by phosphodiesterase cleavage and separated from 5'-AMP on a DEAE-cellulose column as described above.

Spectral Determination. The ¹H NMR spectra were obtained on a Varian HR-220 nuclear magnetic resonance spectrometer equipped with a Nicolet Technology Fourier transform system. The samples were routinely lyophilized twice from 99.8% D₂O and then dissolved in 100% D₂O. The following internal standards were used as indicated: sodium trimethylsilylpropionate-2,2,3,3-*d*₄ (TSP), 4,4-dimethyl-4-silapentane-5-sulfonate (DSS), and tetramethylammonium chloride (TMAC). All samples also contained 1 mM EDTA in order to suppress line broadening by possible paramagnetic ion impurities.

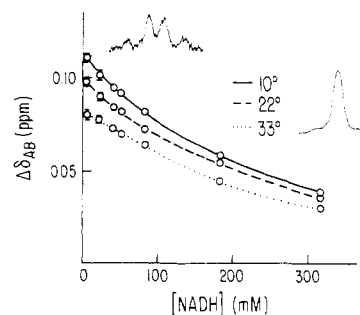


FIGURE 1: The dependence of the chemical-shift nonequivalence of the N4 protons on the concentration of NADH. The results for three temperatures are shown: 10 (—), 22 (---) and 33 °C (···).

Results

Assignments. The chemical shifts for the dihydronicotinamide ring protons of NMNH, NADH, NADPH, and the reduced NAD analogues are given in Table I. The specific assignments of the N4 protons of NADH and NADPH have been previously reported (Oppenheimer et al., 1971), as have those for the N4 protons of (AcPy)ADH, (TN)ADH, and (PyAl)ADH (Oppenheimer and Kaplan, 1974). The latter assignments have been determined by the acid-catalyzed rearrangement reaction of the enzymatically prepared, specifically labeled, reduced coenzymes. The N4 protons of NADH(OAc)₄ were assigned by acylation of the specifically labeled dinucleotide. The remaining dihydropyridine protons have been assigned by comparison with the resonances of NADH and by homonuclear spin-decoupling experiments.

Chemical Shift Nonequivalence of the N4 Methylene Protons. One of the most striking features of the ¹H NMR spectrum of NADH at high field is the observable nonequivalence of the N4 methylene protons. This nonequivalence is a property found in all the reduced dinucleotides we have studied but is not observed in NMNH (see Table I). The data on the chemical shifts of the N4 protons can be summarized as follows: (1) The N4 methylene protons are unresolved, $\Delta\delta_{AB} < 1$ Hz at 220 MHz, in all the mononucleotides and mononucleosides we have studied. (2) The N4 methylene protons are nonequivalent in all the dinucleotides we have observed, regardless of alterations in the N3 substituent, the purine ring, or the ribose hydroxyl groups (acylation). (3) The relative

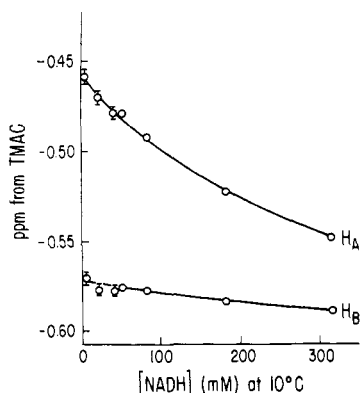


FIGURE 2: The dependence of the individual N4 proton chemical shifts on the concentration of NADH at 10 °C. This demonstrates the preferential upfield shift of the N4A proton.

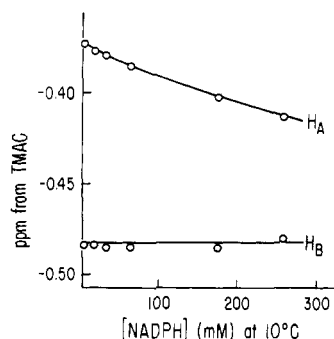


FIGURE 3: The dependence of the individual N4 proton chemical shifts on the concentration of NADPH at 10 °C. This demonstrates the similar but smaller preferential upfield shift of the N4A proton in NADPH relative to NADH (Figure 2).

chemical shifts of the N4 protons are maintained in all the analogues and chemically modified coenzymes we have observed; the B proton is upfield relative to the A proton. (4) The chemical-shift nonequivalence of the N4 protons as well as their upfield shift relative to the mononucleotide decreases with either increasing temperature (Patel, 1969; Sarma and Kaplan, 1970a) or increasing concentrations of methanol (nonpolar solvent).

Concentration Effects. Increasing the concentration of NADH has a large effect on the chemical-shift nonequivalence of the N4 methylene protons³ as can be seen in Figure 1. The coalescence of the AB pattern is caused by the preferential, 0.1-ppm, upfield shift of the N4A proton relative to the N4B proton as shown in Figure 2. A parallel phenomenon is also observed in NADPH (Figure 3), although the magnitude of the effect is smaller than that observed in NADH.

Addition of 5'AMP to a fixed NADH concentration also induces a preferential upfield shift of the N4A proton with the concomitant coalescence of the AB pattern (Figure 4). However, increasing the 5'AMP concentration against a fixed concentration of NMND_{A,B}, on the other hand, results in an equal upfield shift of both the N4 protons; i.e., no observable

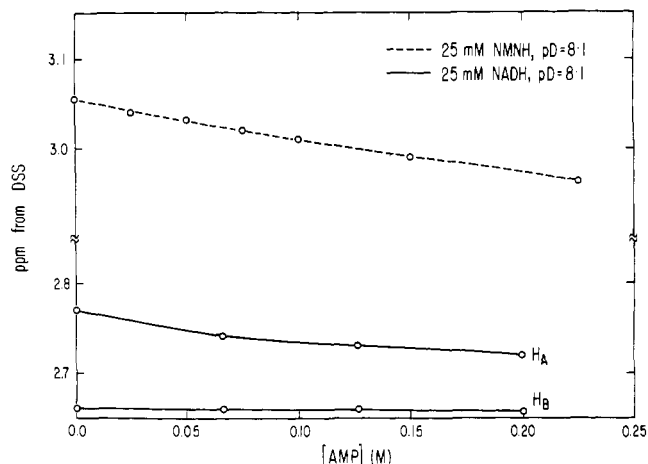


FIGURE 4: The effect of increasing concentration of 5'AMP at 22 °C on the chemical shifts of the N4 protons of 25 mM NMND_{A,B} (---) and 25 mM NADH (—).

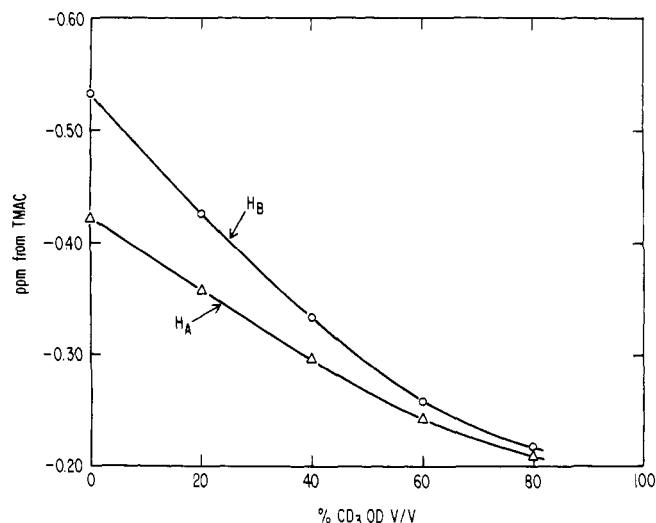


FIGURE 5: The dependence of the chemical shifts of the individual N4 protons of NADD_{A,B} on methanol concentration.

nonequivalence is induced in the N4 protons upon association with 5'AMP.

Methanol Effects. Methanol, a known destacking agent, has been observed to decrease the intramolecular association between the dihydronicotinamide and adenine rings in NADH, as monitored by fluorescence (Freed, 1967) and perturbation of chemical shifts in ¹H NMR spectra (Hollis, 1969; McDonald et al., 1972). Increasing methanol concentrations also cause a decrease in the chemical-shift nonequivalence of the N4 protons, as shown in Figure 5. Extrapolation to 100% methanol would indicate that the chemical shifts of the N4 protons should be nearly identical.

NADPH pH Effects. Protonation of the 2'-phosphate on the adenosine moiety of NADPH causes downfield shifts in the resonances of the adenine A1', A2', A3', A2 and A8 protons. These shifts are similar to those reported for 2'AMP (Schweizer et al., 1967). In addition, we find that protonation of the 2'-phosphate in NADPH also causes a preferential, 0.036 ppm, upfield shift for the N4B proton as shown in Figure 6, whereas the other dihydronicotinamide protons, including the N4A proton, change by less than 0.006 ppm. In fact, the shift of the N4B proton is comparable to that of the A3' proton. This result indicates that the N4B proton is both proximal to, and directed toward, the adenine 2'-phosphate. Such an in-

³ McDonald et al. (1972) and Sarma and Mynott (1973) have observed upfield shifts of the adenine moieties with increasing NADH concentration, an effect attributed to adenine-adenine association. These investigators, however, reported the absence of any significant effects of concentration on the proton spectra of the dihydronicotinamide moiety. The failure of these investigators to observe the preferential 0.1-ppm concentration-induced upfield shift of the N4A proton is a consequence of coalescence of the N4 methylene resonances at the lower frequency of 100 MHz, utilized in these studies.

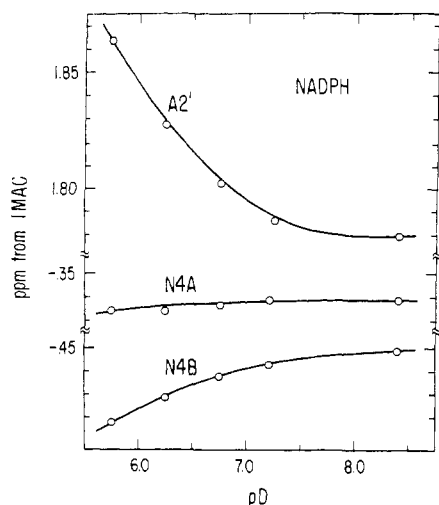


FIGURE 6: The dependence on pH of the chemical shifts of the individual N4 protons of NADPH. Note the preferential shift of the N4B proton which clearly parallels the titration of the 2'-phosphate.

TABLE II: Dihydropyridine $J_{4,5}$ Vicinal Coupling Constants.^a

	$^3J_{4A,5}$	$^3J_{4B,5}$
NMNH	(3.5)	3.5
NADH, 25 °C	3.1	3.9
NADH, 70 °C	3.4	(3.5)
NADPH	3.1	(3.9)
(AcPy)ADH	2.9	4.0
(TN)ADH	3.1	(3.8)
(PyAl)ADH	3.0	(3.7)

^a Coupling constants measured from the N5 proton in the specifically labeled nucleotide. Values in parentheses are calculated by subtracting the value of $J_{4,5}$ in the specifically labeled nucleotide from the sum of the $J_{4,5}$ coupling constants determined from the unlabeled nucleotide. The values are accurate to within 0.1 Hz.

teraction should be purely electrostatic and should have a $1/r^2$ distance dependence (Buckingham, 1960). It should also be pointed out that this effect is unique to NADPH, since the chemical shift for the corresponding protons in NADH are invariant over the same range of pD.

Coupling Constants of the Dihydropyridine Protons. The vicinal coupling constants, $^3J_{4,5}$, for NMNH, NADH, NADPH, and a number of reduced NAD analogues are listed in Table II. These data show that, unlike the vicinal coupling constants in NMNH which are identical, the vicinal coupling constants for the dinucleotides are dissimilar. In all the dinucleotides, $^3J_{4B,5}$ is greater than $^3J_{4A,5}$. Increasing temperature diminishes the difference between the two vicinal coupling constants for the dinucleotides, averaging them toward the value of 3.5 observed for NMNH. It should be noted that vicinal coupling constants are minimal when the bonds of the coupled protons are orthogonal and are maximal when they are coplanar (Karplus, 1959, 1963). A qualitative analysis of the difference in coupling constants in the dinucleotide indicates that the dihedral angle, 4A-5, is displaced toward 90° and the dihedral angle, 4B-5, is displaced toward 0°. In other words, the N4A proton moves more axial and the N4B proton moves more equatorial with respect to the nicotinamide ring.

This conformational analysis is further substantiated in NADH by the differences observed in the values of the long-range coupling constants $^5J_{4A,6}$ and $^5J_{4B,6}$. Vinylic coupling

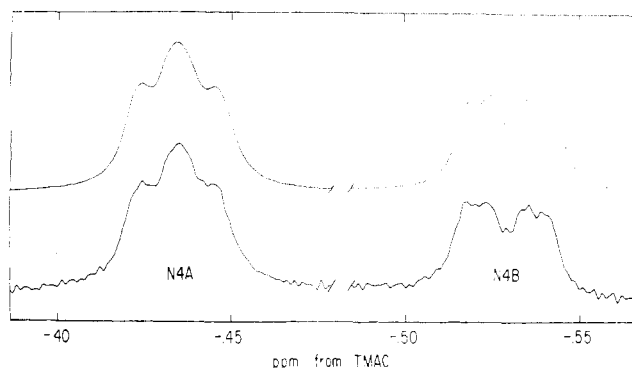


FIGURE 7: The N4 proton resonances obtained from spectra of specifically labeled NADD_A and NADD_B shown on the bottom and the computer simulation of these spectra shown on the top. The values of the coupling constants used for the simulation were $J_{4A,5} = 3.1$, $J_{4A,6} = 2.0$, $J_{4B,5} = 3.9$ and $J_{4B,6} = 1.5$ Hz. The value of $J_{4A,2}$ and $J_{4B,2}$ was found to be less than 0.5 Hz and the deuterium coupling to the remaining N4 proton (expected to be 3.1 Hz) was unobservable because of the rapid relaxation of the deuterium (Lee et al., 1974).

is maximal when the protons are orthogonal and minimal when they are coplanar (Barfield, 1964). Thus, the observation that $^5J_{4A,6}$ is 2.0 Hz whereas $^5J_{4B,6}$ is 1.5 Hz is consistent with a conformation in which the N4A proton is displaced axial and the N4B proton is displaced equatorial in the dinucleotides. In NMNH these coupling constants are 1.7 Hz. The difference in coupling constants is clearly observable (Figure 7), especially in the N4 deuterium-labeled dinucleotides because of the elimination of second-order effects due to the strong geminal coupling.

Discussion

The difference in chemical-shift properties of the N4 protons between mononucleotides and dinucleotides can be understood if the general causes of nonequivalence in diastereomerically related protons are considered. Two diastereotopic protons are, in principle, chemical-shift nonequivalent because they cannot be related by any symmetry operation; thus, their chemical and/or magnetic environments are dissimilar (Mislow, 1966). In general, to observe separate resonances for a mixture of diastereomers or diastereotopically related groups requires that the chemical or magnetic anisotropy generated by the chiral centers must be greater than the line width of the resonances in question. If the chiral center is distant or nearly symmetrical with respect to the protons in question, then the anisotropy of the magnetic environment may not be sufficient to produce resolution.

The chiral centers of the D-ribose and the groups substituted on the ribose preclude any planes of symmetry for the dihydropyridine ring; thus, the N4 protons are diastereotopic in all reduced pyridine nucleotides. However, any nonequivalence of the N4 protons in the mononucleotides must result from the chiral centers of the ribose. The absence of resolved N4 protons in the 1,4-dihydropyridine mononucleotides/sides indicates that the magnetic anisotropy generated by these chiral centers is insufficient to resolve the N4 resonances. Thus, the resolution of the N4 protons in the NADH as identifiable resonances at 220 MHz (Patel, 1969; Sarma and Kaplan, 1970a) must be related to the anisotropy generated by the adenine moiety.

The chemical-shift equivalence of the N4 protons in the mononucleotides/sides and their nonequivalence in the dinucleotides (Table I) are difficult to rationalize on the basis of a linear, extended conformation as proposed by Jacobus (1971) and further discussed by Zens et al. (1976). The largest

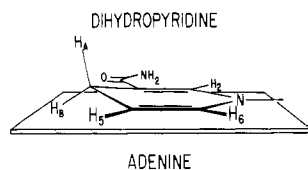


FIGURE 8: The structure of NAD(P)H in the folded form. Note that such folding produces both a preferential upfield chemical shift and an equatorial puckering of the B proton.

changes in chemical shift between the mononucleotides/sides and the dinucleotides occur for the N4 protons. Studies of the mononucleotides/sides show these protons to be invariant to perturbations arising within the contiguous ribose-phosphate moiety (Oppenheimer and Kaplan, 1976). Furthermore, in any linear conformation of the dinucleotide the N4 protons are the most distant from the adenine moiety.

Aromatic solvent induced shifts, i.e., transient, nonspecific collisional complexes with the adenine moiety, as favored by Ellis et al. (1973) can, in a general way, account for the chemical-shift behavior of the pyridine protons. However, we have shown a high degree of stereoselectivity in the dinucleotide adenine/dihydropyridine intra- as well as intermolecular interactions. At the same time, there is no evidence for such selectivity in the association of the mononucleotides; i.e., the N4 protons of NMND_{A,B} do not become nonequivalent with increasing 5'AMP concentration, even though the N4 protons show a substantial upfield shift. Such information is not consistent with a model of random, nonspecific collisional complexes for the reduced pyridine dinucleotides. In addition, recent intramolecular ¹H NMR relaxation studies indicate a close *average* proximity between the adenine and dihydronicotinamide rings, consistent with a base-stacking model (Oppenheimer et al., 1978).

The ¹H NMR results can be best rationalized by a model in which the dinucleotide exists as a rapid equilibrium between an open, extended conformation and a single, folded conformation where the *B* face of the dihydropyridine ring is toward the adenine (see Figure 8). In the open form, the magnetic environment is similar to that of the free mononucleotides. The intramolecular magnetic interactions are weak and have little effect on the chemical shift of the N4 protons, thus giving rise to the observed chemical-shift equivalence under unfolding conditions, e.g., high temperature or nonpolar solvents. In the folded form, the adenine and pyridine ring are juxtaposed in parallel-plane base stacking. Aromatic ring current shielding from the adenine generates the necessary anisotropic magnetic environment to account for the nonequivalence of the N4 protons possibly modified by diamagnetic shielding induced by ring puckerings (Oppenheimer et al., 1971). Finally, the specific shielding of the N4B proton upon protonation of the 2'-phosphate in NADPH is strongly supportive of a specific intramolecular interaction.

The observed chemical shifts of the N4 protons represent a time-averaged weighted sum of the contributions from the folded form in which they are nonequivalent and the open form in which they are equivalent. At high temperature or in a more nonpolar solvent, the rings destack and the proton resonances shift downfield and approach the chemical shifts of the mononucleotides as has been previously observed (Jardetzky and Wade-Jardetzky, 1966; Patel, 1969; Sarma and Kaplan, 1970a; McDonald et al., 1972). At the same time the N4 protons also approach chemical-shift equivalence and the vicinal coupling constants become similar.

The association of 5'AMP with NMND_{A,B} does not show

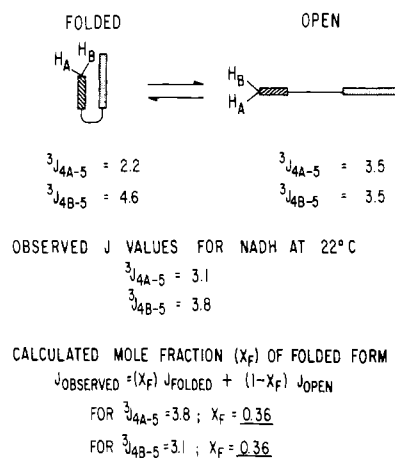


FIGURE 9: An illustration of the calculated percent folding of NADH using the deformation of the ${}^3J_{4-5}$ coupling constants. See text for details.

any preferential shielding of the N4 protons, indicating that the base-stacking interaction is not intrinsically asymmetric. It follows then that the association constants for the A and B sides NMNH/AMP dimers must be nearly equal. Based on the lack of preferential shifts of the N4 protons in the mononucleotides, we can argue that the asymmetric interaction observed in the dinucleotides is a consequence of the conformational constraints imposed in a negative sense. That is, the specificity of interaction is determined by the steric hindrance associated with other possible conformations. It is clear that the ribose hydroxyl groups do not actively participate in maintaining the observed folded conformation, since acylation of these groups does not cause substantial changes in the chemical-shift nonequivalence. The decreased sensitivity to concentration of the N4 protons of NADPH relative to NADH is consistent with this analysis, since the 2'-phosphate would be expected to hinder adenine-dihydronicotinamide dimer formation.

It is also clear that the N3 substituent on the pyridine ring does not alter the specificity of the intramolecular association, since the relative chemical shifts of the nonequivalent N4 protons in all the dinucleotide analogues are identical. In all cases, the N4A proton is downfield from the N4B proton.

The population of the specifically folded conformation can be qualitatively calculated from the observed perturbations in the coupling constant using the following assumptions (see Figure 9): (1) The reduced dinucleotide exists in a rapid (on the ¹H NMR time scale) exchange between a unique folded form, B side against the adenine, and a fully extended open form. (2) The vicinal coupling constants for the open form, ${}^3J_{4A-5}$ and ${}^3J_{4B-5}$, both equal 3.5 Hz as observed in NADH at 73 °C or NMNH. (3) The limiting value of 2.2 Hz for ${}^3J_{4ax-5}$ is assumed in the fully folded form based on the value of ${}^3J_{4-5}$ in the nicotinamide-pyruvate adduct where the dihydronicotinamide ring is constrained by the equatorial N4 pyruvate substitution so that the remaining N4 proton is axial (Arnold and Kaplan, 1974). (4) The limiting value of 4.6 Hz for ${}^3J_{4eq-5}$ is assumed from the value of ${}^3J_{4-5}$ in the nicotinamide-cyanide adduct where the N4 cyanide is axial and the N4 proton is equatorial (Arnold, 1975).

The choice of 2.2 and 4.6 Hz for the limiting values of these coupling constants agrees well with our assumption as to the uniformity and specificity of the distortion. Their average, 3.4 Hz, is in good agreement with the observed average of 3.5 Hz. Using the assumptions above, the degree of NADH folding can

be calculated based on the observed $^3J_{4-5}$ coupling constants as outlined in Figure 9. Such calculations, which are admittedly qualitative, yield similar populations of 36% based upon either value of $^3J_{4B-5}$ or $^3J_{4A-5}$. Thus, the *minimum* population of the specifically folded form is in the range of 30–40% at 22 °C, a value comparable to that determined for the total population of all folded forms [solvent perturbation, 40–50% folded (Cross and Fisher, 1969); proton magnetic resonance, ~24% folded (McDonald et al., 1972) and ~36% folded (Sarma and Kaplan, 1970b)]. The qualitative agreement of these calculations with ours adds strong support to our specific B-side model and clearly precludes the possibility of nearly equal populations of A-side folded forms in the reduced pyridine coenzymes.⁴ Furthermore, the value of $^3J_{4B-5}$ is greater than $^3J_{4A-5}$ in NADPH and all other analogues we have studied, suggesting the general nature of this specific interaction (Table II).

The conformational analysis of the dihydropyridine ring, however, cannot distinguish the origin of the puckering. It is possible that the dihydropyridine ring exists in two states, an A-side puckered and a B-side puckered form, which rapidly librate. In this case, interaction with the adenine ring would block the A-side puckered form, thus favoring the B-side puckered form. Alternatively, the dihydropyridine ring may normally exist in a planar conformation and interaction with the adenine ring physically deforms the dihydropyridine ring to the B-side puckered form.

These results, while being consistent with a single folded form model, cannot rule out by themselves the unlikely possibility that there are contributions from more than one folded conformation such as the P and M helical model of Sarma and Mynott (1973). Any such proposal for multiple conformations, however, is constrained by the fact that the *B face of the dihydronicotinamide ring must be against the adenine ring*. The results preclude the proposal that there are contributions from *syn* and *anti* torsional isomers of the dihydropyridine ring with the same folded backbone conformation. Instead, either one conformer predominates or there are two very different backbone conformations. The latter possibility is less likely in view of the properties of tetraacylated NADH.

The differences in enzymatic activity of NAD analogues clearly do not result from any gross differences in the specificity of their intramolecular association. A qualitative interpretation of the vicinal coupling constants, J_{4-5} , for the analogues (Table II) in addition to the temperature dependence of their chemical shifts indicate that the degree of folding of the various analogues does not differ strongly. In no case have we observed for physiologic conditions a population of folded form in excess of 50%. Instead, the reduced dinucleotides all exist predominantly as the open forms at 37 °C, that are rapidly interconverting with a specific folded form. It remains to be determined what influence the dynamic interconversion between folded and open forms might have upon enzyme binding.

It is evident from the comparison of the chemical-shift properties of the N4 protons in the dinucleotides and the mononucleotides that the chemical-shift nonequivalence is unobservable in the absence of an asymmetric interaction. Thus, the observed nonequivalence of the N4 protons upon binding in the active site of the dehydrogenases (Lee et al., 1974) is indicative of an asymmetric association with the protein, especially since the coenzyme is unfolded.

Finally, the proposal that the N4 position of dihydropyridine

rings in general are puckered when folded against bulky groups such as adenine (or by inference the amino acid residues in an active site) provides an attractive mechanism by which enzymes may utilize some of their binding energy to lower the transition-state free energy of enzymatic reactions. Upon binding of NADH to a dehydrogenase, one of the N4 protons may move toward an equatorial position and the other toward an axial position. As a result, the C–H bond of the equatorially displaced proton would approach an sp^2 – $1s$ hybrid orbital which has a bond energy of ca. 109 kcal/mol, whereas the C–H bond of the axially displaced proton would approach a weaker $2p$ – $1s$ bond (ca. 80 kcal/mol). Thus, such distortion can be envisioned as providing a mechanism whereby dehydrogenases can activate the desired N4 proton for transfer, by specifically weakening the C–H bond of the hydride to be transferred.

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⁴ Any A-side folding would cancel the effects induced by B-side folding, thus substantially reducing the calculated percent of a specific B-side folded form.

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Assignment of the ¹³C Nuclear Magnetic Resonance Spectrum of Aqueous Ganglioside G_{M1} Micelles†

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ABSTRACT: This article describes the natural-abundance Fourier-transform carbon-13 nuclear magnetic resonance spectrum, at 67.88 MHz, of aqueous micelles of bovine brain ganglioside G_{M1} of purity greater than 99%. Assignments are given for every carbon nucleus in the molecule, on the basis of a comprehensive study of the relevant mono-, di-, tri-, and polysaccharides, including several containing sialic acid (5-acetamido-3,5-dideoxy-D-glycero-D-galacto-nonulopyranosonic acid), and phospho-, sphingo-, and glycosphingolipids. These assignments represent an extension of the ¹³C nuclear magnetic resonance data from monosaccharides and lipids to complex oligosaccharides and glycolipids. They also form the basis for interpretation of spectral perturbations induced in

G_{M1} by titration with paramagnetic europium(III). The single sialic acid in G_{M1} was found to be α-glycosidically linked in the oligosaccharide from considerations of its unique anomeric chemical shift. The sialic acid carboxyl and glyceryl side chain, along with additional ligands donated by the 2-acetamido-2-deoxy-β-D-galactopyranoside and terminal β-D-galactopyranoside residues in the oligosaccharide portion of G_{M1}, were found to be intimately involved with cation binding. It is proposed that the higher affinity, compared with monomeric sialic acid, of G_{M1} for cations may result from these additional oligosaccharide groups, which may effectively compete for water ligands in the metal cation coordination sphere.

Gangliosides are membrane glycosphingolipids containing a hydrophilic oligosaccharide portion glycosidically linked to a hydrophobic ceramide, which presumably anchors the ganglioside molecule in the membrane. Several classes of gangliosides are differentiated by the number and position of attachment of one or more sialic acid residues, giving rise to mono-, di-, and trisialogangliosides, denoted as G_M, G_D, and G_T, respectively (Svennerholm, 1964). The presence of the

sialic acid confers a negative charge on the oligosaccharide portion of the molecule at physiological pH, which engenders a strong interaction with cations of biological significance, like calcium (Abramson et al., 1972).

Although gangliosides have been known to be constituents of plasma membranes for a number of years, their function has only recently begun to be understood. With their hydrophilic oligosaccharide portions projecting out of the membrane, gangliosides are well suited to act as receptors for biological signal molecules (Fishman & Brady, 1976). Recently, gangliosides have been implicated as membrane receptors for a variety of endogenous and exogenous proteins, including hormones (Aloj et al., 1977, and references cited therein) such as thyrotropin, luteinizing hormone, and follicle-stimulating hormone (Ledley et al., 1976), interferon (Kohn et al., 1976), and the bacterial toxins elaborated by *Vibrio cholerae* (Moss et al., 1977, and references cited therein) and *Clostridium tetani* (Helting et al., 1977; Ledley et al., 1977). The specificity of the protein-ganglioside interaction is determined by the carbohydrate pattern of the ganglioside, each protein binding

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