Theoretical Evaluation of Conformational Preferences of NAD^+ and NADH: An Approach to Understanding the Stereospecificity of NAD^+/NADH-Dependent Dehydrogenases

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Abstract: Potential energy surfaces for rotations of ribose about the nicotinamide N–C bonds in NAD^+ and NADH have been evaluated from ab initio molecular orbital calculations with the 3-21G and 6-31G* basis sets. In the optimal conformation of NAD^+ the glycosidic C–O bond is near the plane of the nicotinamide ring. By contrast, the lowest energy conformer of NADH has the glycosidic C–O bond nearly perpendicular to the dihydronicotinamide ring. The redox potential of the NAD^+/NADH couple is a function of the ribose orientation. There is boat-like puckering of the 1,4-dihydropyridine ring, and the direction of the puckering is anti to the glycosidic bond. The geometry of the transition structure for hydride transfer is similar to that of NADH. When NADH is in the anti conformation, the transfer of the *pro-R* hydrogen is preferred, and when NADH is in the syn conformation, the transfer of the *pro-S* hydrogen is preferred.

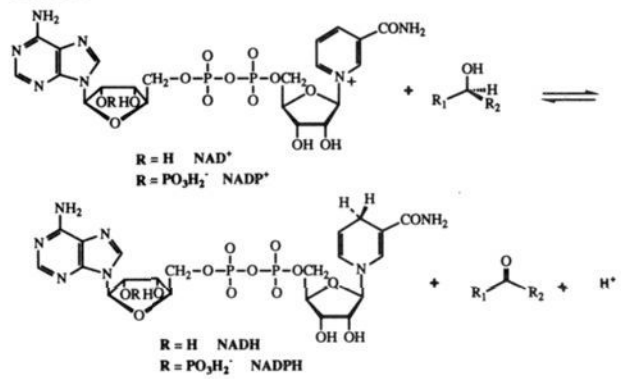
Introduction

The NAD^+/NADH coenzyme-dependent dehydrogenases are a ubiquitous class of enzymes which are fascinating for many reasons.[1,2] One area of interest is the mechanism of the enzymatic oxidations and reductions, as represented in Scheme I, for alcohol to aldehyde or ketone interconversions, including the geometrical features of the transition state. Both experiments and theoretical calculations have suggested that these reactions generally involve direct hydride transfer.[3,4] We have shown that the transition structures for simple hydride transfer reactions are usually bent with a syn arrangement of carbonyl and the π-system of the reductant.[5,6]

Another aspect of interest is the stereospecificity of the enzymes. There are two classes of dehydrogenases, those transferring the *pro-R* hydrogen from the 4-position of reduced nicotinamide cofactor, and those transferring the *pro-S* hydrogen.[7] The former are called A-specific enzymes, and the latter are B-specific enzymes.

X-ray crystal structures and NMR studies of several dehydrogenases with a bound NAD^+ have been reported.[8-15] In the A-specific enzymes, NAD^+ binds in the conformation with the nicotinamide glycosidic bond anti with respect to the amide group and in the B-specific enzymes NAD^+ binds in a syn conformation as shown by **1** and **2**, respectively. There is possibly one exception to this rule. These data are summarized in Table

Scheme I



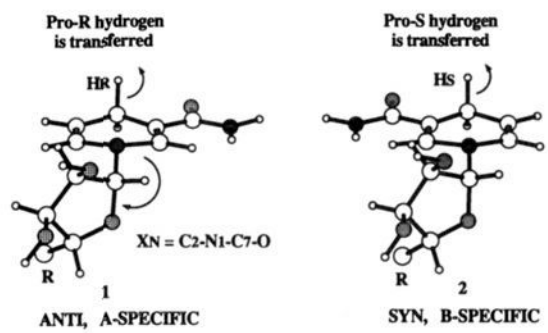


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Table I. Conformations of Ribose in DNA and RNA (with Respect to Bases) and in NAD^+ Free Acid, Li–NAD^+ (with Respect to Nicotinamide Ring), and Enzyme-Bound NAD^+ (NADH) in Some Dehydrogenases, from X-ray or NMR Data

compound	conf	specificity	χ_n (deg)[a]	year and author	resolution (Å)	refinement *R*
DNA and RNA			−159	1962, Arnott		
NAD^+ (free acid)	anti		−150	1984, Parthasarathy	high	
Li–NAD^+ (free acid)	anti		−170	1981, Reddy	1.09	0.10
DMLDH, binary	anti	A	−134	1973, Chandrasekher	3.0	
DMLDH, ternary	anti	A	−106	1976, White	2.8	
BSLDH, ternary	anti	A	−124	1989, Piontek	2.8	0.26
(s)-lac-NAD^+–LDH	anti	A	−81	1981, Grau	2.7	
s-MDH 1	anti	A	−108	1973, Webb	2.5	
s-MDH 2	anti	A	−107	1973, Webb	2.5	
c-MDH 1	anti	A	−96	1989, Birktoft	2.5	0.17
c-MDH 2	anti	A	−92	1989, Birktoft	2.5	0.17
SDH	anti	A	−110	1984, Gronenborn	NMR	
DHFR	anti	A	−123	1982, Filman	1.7	0.15
LADH-NAD1	anti	A	−98	1984, Eklund	2.9	0.22
LADH-NAD2	anti	A	−101	1984, Eklund	2.9	0.22
Lobster, GAPDH						
red subunit	syn	B	43	1975, Moras	2.9	
green subunit	syn	B	51	1975, Moras	2.9	
B. stearothermophilus GAPDH						
subunit O	syn	B	80	1987, Skarzynski	1.8	0.18
subunit P	syn	B	81	1987, Skarzynski	1.8	0.18
subunit Q	syn	B	86	1987, Skarzynski	1.8	0.18
subunit R	syn	B	86	1987, Skarzynski	1.8	0.18
BLC	syn	B	66	1984, Fita	2.5	0.19
glutathione reductase	anti	B	−168	1989, Pai	3.0	

[a] χ_n's are in Eklund's definition,[12] that is, the dihedral angle C_2–N_1–C_7–O. Some χ_n's are calculated from the value of C_2–N_1–C_7–C in the original literature.

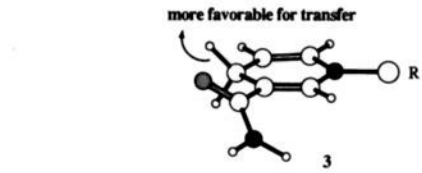
I. It has been suggested that this feature will be found in all of the dehydrogenases.[7]

Several hypotheses have been offered to explain why there are two distinct stereospecificities of the enzymes.[7,16] One proposal is that the stereospecificity of enzymes is a historical accident. Enzymes that have the same stereoselectivity are likely to have evolved from a single enzymatic progenitor and to have structural homogeneity. Since there are two families of enzymes with opposite stereospecificities, it has been suggested that all the dehydrogenases are derived from two precursors which happened to have different stereoselectivities, and the stereospecificity has been conserved during enzyme evolution.[7]

Schneider-Bernlöhr et al. proposed a correlation consistent with this interpretation for the stereospecificity of alcohol and polyol dehydrogenases: the zinc-containing family with higher subunit molecular weight is A-specific, and the family without zinc and lower subunit molecular weight is B-specific.[17]

Benner et al. proposed an alternative hypothesis: the stereoselectivity of the dehydrogenases is the result of evolution which selects enzymes to possess the maximum catalytic efficiency.[18,19] The Benner group identified a correlation between the thermodynamic reduction potential of the alcohol-carbonyl couple with the stereochemistry of the enzyme. Benner proposed that an enzyme is A-specific if the equilibrium constant of the reaction it catalyzes is larger than 10^{12}, but B-specific if the equilibrium constant of the reaction is smaller than 10^{12}. The correlation is a consequence of the following four hypotheses. (1) The *pro-R* hydrogen is transferred from a nicotinamide cofactor bound in the active site in an anti conformation; the *pro-S* hydrogen is transferred from a nicotinamide cofactor bound in a syn conformation. (2) *anti*-NADH is a weaker reducing agent than *syn*-NADH. (3) Optimal enzymes bind substrates so as to match or nearly match the free energies of bound intermediates; thus, each reaction step occurs with no change in free energy.[20] (4) Dehydrogenases have evolved to be optimal enzymes.[18] It was to test some of the chemical aspects of these hypothesis that we undertook this study.

Others have considered the influence of the 3-amide group of nicotinamide on the stereospecificity of the reaction. MINDO/3 and STO-3G calculations were performed for the hydride transfer from 3-carbamido-1,4-dihydropyridine to cyclopropenium cation and to hydroxymethyl cation.[21] Donkersloot et al. found from calculations that the transition state of hydride transfer is assisted by the amide dipole.[21] As shown in **3**, the easier delivery of



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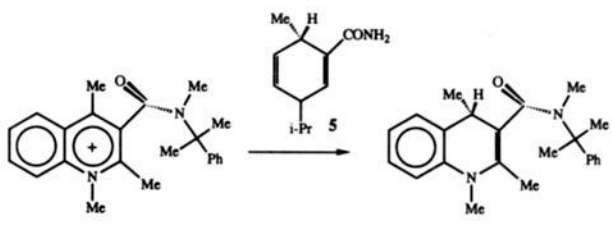

hydride occurs on the same face as the carbonyl group. This implies that A-specific dehydrogenases bind NAD^+ and NADH in conformations in which the carbonyl bond is directed to the *pro-R* face, while an enzyme is B-specific if it binds NAD^+ and

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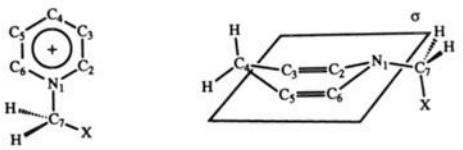
NADH in conformations in which the carbonyl bond is directed toward the *pro-S* face. This argument gains some support from Ohno's observation that the optically active NAD^+ model compound, **4**, is reduced by **5** to give highly stereoselective formation of **6**.[22] The favored reaction course involves hydride transfer to the face near the carbonyl oxygen. However, Ohno et al. also found that when the reducing agent was an ionic hydride such as BH_4^- or AlH_4^-, the outcome is not consistent with the Donkersloot hypothesis. In general, this hypothesis has received little attention, probably because of the lack of evidence for the carbonyl out-of-plane distortion from X-ray studies of enzyme-bound NAD^+ crystal structures. It should be pointed out that the amide-directing effect found in Donkersloot's calculations is most likely caused by electrostatic interaction between the carbonyl oxygen and the hydride acceptor, which was chosen to be a carbenium cation. Whether such an effect exists when the hydride acceptor is a neutral species needs to be investigated further.

Although Benner's correlation has been criticized,[23,24] it intrigued us because some of the chemical aspects could be tested by theoretical calculations. For example, why do A-specific enzymes bind the NAD^+ in the anti conformations, and B-specific enzymes bind the NAD^+ in the syn conformations? Is *anti*-NADH a weaker reducing agent than *syn*-NADH? To answer these questions, we carried out calculations to evaluate the rotational potential energy surface about N_1–C_7 bonds in model systems, **7**–**10**. The calculations indicated that the potential



7 X=OH
8 X=CH_3
Φ = C_2-N_1-C_7-X

9 X=OH
10 X=CH_3
Φ = σ/N1-C_7-X

energy surfaces for NAD^+ and NADH are quite different. The redox potential of NAD^+/NADH coenzyme couple is dependent upon the conformation of ribose about the N_1–C_7 bond. Here we present these results and discuss the connection of these results with the stereospecificities of the NAD^+/NADH-dependent dehydrogenases.

Results and Discussion

Ab initio molecular orbital calculations were carried out with Pople's GAUSSIAN82 and GAUSSIAN86 programs.[25] Geometrical optimizations were performed with the 3-21G basis set and energetics were also evaluated with the 6-31G* basis set.

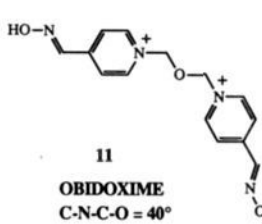
1. Rotational Potential for NAD^+ Models. *N*-Hydroxymethylpyridinium ion (**7**) and *N*-ethylpyridinium ion (**8**) were optimized with the restriction of the pyridinium ring to planarity. It has been shown by Raber et al. that the out-of-plane distortion of the pyridinium ring is very destabilizing.[26] The ethyl group in **8** is restricted in the eclipsed conformation to model the ribose structure, while the hydroxyl group in **7** is fixed anti to the N_1–C_7 bond. Optimization of **7** gave a structure with the C–O bond coplanar with the pyridinium ring (Φ = 0°), and optimization of **8** gave a perpendicular structure (Φ = 90°). The potential energy surfaces for rotation about the N_1–C_7 bonds were studied by optimization of **7** and **8** with the C_2–N_1–C_7–R dihedral angle fixed at various values. The calculated relative energies of these conformations are given in Table II.

Table II. 3-21G and 6-31G* Relative Energies (kcal/mol) of *N*-(Hydroxymethyl)pyridinium Ion (**7**) and *N*-Ethylpyridinium Ion (**8**) with Different C_2–N_1–C_7–X Dihedral Angles

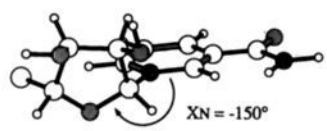
	7		8	
C–N–C–O(C) (°)	3-21G	6-31G*	3-21G	6-31G*
0	0.0	0.3	4.5	4.7
20	0.3	0.3	3.7	3.9
30	0.8	0.0		
40	1.2	0.2	1.8	1.9
60	2.7	0.1	0.4	0.5
80	4.0	0.3	0.1	0.1
90	4.2	0.2	0.0	0.0

The calculated energies of **7** are strongly basis-set dependent. The 3-21G basis set strongly favors the coplanar structure. The energy increases steadily as the dihedral angle Φ increases from 0° to 90°. The perpendicular structure (Φ = 90°) is disfavored by 4.2 kcal/mol. However, the 6-31G* basis set indicates a flatter potential, with the conformation of Φ = 30° being slightly favored over the others. Such drastic basis-set dependence has been noted before for analogue allyl alcohol and ethers.[27] While the 3-21G basis set gives a preference of eclipsed conformation (C=C—C—O = 0°), a skew conformation (C=C—C—O ≈ 120°) is calculated to be more stable with the 6-31G* basis set. It has been shown that the 6-31G* basis set gives a reliable description of the potential surface of allylic ethers even without geometrical optimizations at this level.[28] The 3-21G basis set is known to exaggerate electrostatic interactions, but this problem is corrected with the 6-31G* basis set.[28]

In the case of **7** and analogues, there is no experimental rotational barrier for direct comparison. X-ray crystal structures of N-substituted pyridinium ions indicate that a glycosidic bond tends to be near the plane of pyridine ring.[29] Van Havere et al. reported that the C–N–C–O dihedral angles in obidoxime chloride (**11**)



11
OBIDOXIME
C-N-C-O = 40°



12
OPTIMAL CONFORMATION OF NAD⁺

is about 40°.[29] NMR studies and STO-3G ab initio molecular orbital calculations indicate that benzyl alcohol prefers a conformation with the benzylic C–O bond out of the phenyl ring by about 40°,[30] while *N*-fluoromethylbenzene prefers a planar structure.[31] We suggest that the glycosidic bond prefers to be near the plane of pyridine ring electronically, since this allows hyperconjugative donation by the CH_2 group into the electron-deficient pyridinium ring; the C–O bond at the same time tends to be perpendicular sterically. Thus, the net barrier to rotation about the N_1–C_7 bond is quite small.

The methyl group in *N*-ethylpyridinium ion (**8**) is calculated to prefer to be perpendicular to the pyridine ring, presumably for steric reasons. The potential energy surface is similar with both the 3-21G and 6-31G* basis sets. The coplanar conformation is significantly less stable. The calculated 4.7-kcal/mol destabilization of the coplanar conformation with respect to the perpendicular conformation is likely somewhat overestimated, because

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Table III. 3-21G and 6-31G* Relative Energies (kcal/mol) of *N*-(Hydroxymethyl)-1,4-dihydropyridine (**9**) and *N*-Ethyl-1,4-dihydropyridine (**10**) with Different σ/N_1-C_7-X Dihedral Angles

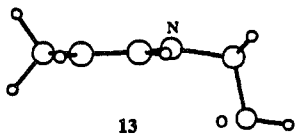
	9		10	
σ/N_1-C_7-X[a]	3-21G	6-31G*	3-21G	6-31G*
10	2.6	5.8	6.8	6.8
30	1.7	4.1	3.6	3.0
50	1.1	2.7	1.3	1.2
70	0.4	1.0	0.4	0.7
90	0.0	0.0	0.0	0.0

[a] This dihedral angle is somewhat different from $C_2-N_1-C_7-X$ because of the pyramidalization at N_1.

of the constraint of the methyl group in an eclipsed conformation. Ethylbenzene has the methyl in the perpendicular conformation, and the rotational barrier is only about 1.3 kcal/mol.[32] In this case, the ethyl group is in staggered conformation, and the steric interaction between the methyl group and C_2-H in the coplanar conformation is relatively small. However, NAD^+, owing to the constraint of the ribose five-membered ring, the $C_\alpha-C_\beta$ bond is partially eclipsed. The constraint of the methyl in eclipsed conformation in our NAD^+ model exaggerates the steric interaction. The real barrier to the rotation is expected to be between that of ethylbenzene and our calculated value, and is likely to be in the range of 2–4 kcal/mol.

Based on these conformational preferences, we expect that the optimal conformation of *anti*-NAD^+, or of *syn*-NAD^+, is the one shown by **12**, in which the C–C bond is nearly perpendicular to the pyridine ring, and the C–O bond is near the plane of the ring, in agreement with experimental observations. As shown in Table I, the average dihedral angle of the glycosidic bond of ribose with the plane of the bases in DNA and RNA is about 20°.[33] The glycosidic bond in free acid NAD^+ and lithium complexed NAD^+ is about 30° and 10°, respectively, with respect to the nicotinamide ring.[34,35] The calculations indicated that there is a significant conformational preference, and this is in agreement with the reported 4.5-kcal/mol barrier to rotation of this bond of NAD^+ in solution.[36]

2. Rotational Potential for NADH Models. *N*-(Hydroxymethyl)-1,4-dihydropyridine (**9**) and *N*-ethyl-1,4-dihydropyridine (**10**) were studied in the same way as **7** and **8**, except that the dihydropyridine ring was not restricted to coplanarity. The relative energies of the optimized conformations are given in Table III. Both molecules prefer the perpendicular conformations, as shown by **13** and **14**.



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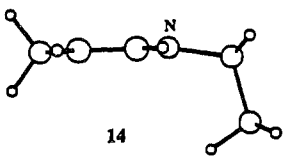
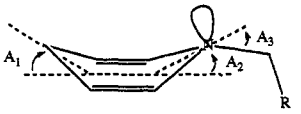


14

The results are in agreement with recent X-ray crystal structures of *N*-(methoxymethyl)-1,4-dihydronicotinamide and *N*-propyl-1,4-dihydronicotinamide.[37,38] The C–O bond in the former deviates from the perfect perpendicular position by 12° and the C–C bond in the latter by 10°,[37] probably because of the existence of the 3-amide group.

Table IV. Direction of Ring Puckering and Nitrogen Pyramidalization of 1,4-Dihydropyridine as a Function of Orientation of R[a]



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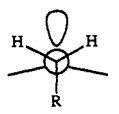

16

	R = OH			R = Me		
σ/N_1-C_7-R[b]	A_1	A_2	A_3	A_1	A_2	A_3
90	3	5	14	−0.6	0.4	10
70	4	5	11	−1	−0.5	3
50	5	5	1	−3	−3	−11
30	2	2	−7	−5	−5	−17
10	0.7	0.3	−5	−3	−3	−8

[a] All angles are in degrees. [b] As defined in **9** and **10**.

The energies for the rotation of N_1-C_7 bond of **9** are similar by the 3-21G and the 6-31G* calculations. The energy increases steadily as the C–O bond is rotated from the perpendicular position to the eclipsed conformation. The C–O bond prefers to be perpendicular to the dihydropyridine ring because of the anomeric effect; since C–O is a much better acceptor than C–H, there is stabilization with the σ^*_{C-O} orbital when it is anti to the amine lone pair.[39,40] In addition, the electrostatic and steric interactions between the oxygen lone pairs and the double bonds are minimized in the perpendicular conformation.

The calculated potential energy surface for the *N*-ethyldihydropyridine (**10**) is similar to that of **9**. The magnitude of the rotational barrier is not basis-set dependent. The calculated perpendicular preference for the methyl group is smaller than that of the hydroxyl group in **9** (except for very small Φ), and this preference is primarily due to steric effects. The destabilization for the conformations with the C–C bond near the pyridine ring plane is most likely overestimated once again because of the conformational constraint of the ethyl group.

While both C–C and C–O bonds tend to be perpendicular to the pyridine ring, the optimal conformations of *anti*- and *syn*-NADH are expected to be similar to **17** and **18**, respectively, in which the C–O bond is nearly perpendicular to the dihydropyridine ring, since it has larger perpendicular preference than the C–C bond.

3. Direction of Puckering of 1,4-Dihydronicotinamide Ring of NADH and Pyramidalization of the Ring Nitrogen as Functions of Ribose Orientation. Raber et al. have reported that 1,4-dihydropyridine is planar.[26] However, the out-of-plane distortion of the ring is relatively easy. Calculations on structures having 10° and 20° folding to a boat conformation indicate energy increases of 0.3 and 1.4 kcal/mol, respectively. This ring flexibility is similar to that of 1,4-dihydrobenzene.[41] This indicates that the ring puckering can be induced easily by substitution at N_1. Indeed, our calculations indicated that both *N*-(hydroxymethyl)-1,4-dihydropyridine (**9**) and *N*-ethyl-1,4-dihydropyridine (**10**) are nonplanar. Geometry optimizations with constraint of the dihedral angle, Φ, lead to one conformation regardless of which direction the ring is puckered in the starting geometry. The directions of ring puckering and nitrogen pyramidalization are collected in Table IV. Positive angles are defined in **15**.

Pyramidalization at the ring nitrogen is induced in most of the conformations. The pyramidalization reaches a maximum in the conformations which have a perpendicular C_7-R or C_7-H bond. The direction of pyramidalization is always anti to the perpen-

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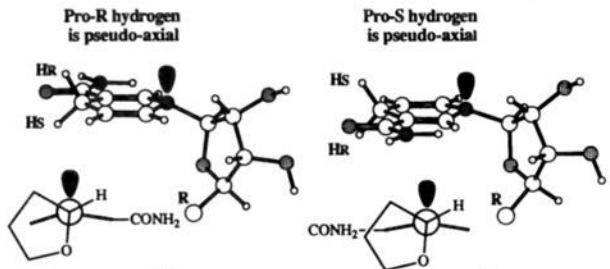
dicular bond, so that a partial staggering about the N_1–C_7 bond is achieved, as shown by **16**. Such pyramidalization is similar to that induced by allylic bonds in alkene and carbonyl systems,[42] which is generally rationalized by the avoidance of closed-shell repulsion as in ethane.[43]

Ring puckering is, in general, coupled with pyramidalization at the ring nitrogen atom. It is striking that the puckering caused by the C–O bond is always in the sense to induce staggering as shown in **15** and the puckering caused by the C–C bond is in the opposite direction (negative A_1 and A_2). It is also interesting that the puckering is largest when the C–O bond is near the perpendicular position ($\Phi = 90°$) or the C–C bond is nearly coplanar with the ring ($\Phi = 0°$).

In NADH, the C–O and C–C bonds are arranged simultaneously above and below the nicotinamide ring, respectively; consequently, the puckerings caused by the two bonds reinforce each other. Therefore, if the NADH is bound in the anti conformation in an enzyme-active site, the nicotinamide ring should be puckered in the direction shown in **17**, in which the *pro-R* hydrogen is at



17 ANTI-NADH **18** SYN-NADH

the pseudo-axial position. If the NADH is bound in the syn conformation, the nicotinamide ring should be puckered as shown in **18**, which has a pseudo-axial *pro-S* hydrogen. It is interesting that **17** and **18** not only represent optimal conformations for *anti*- and *syn*-NADH, respectively, but they also possess maximal ring puckering.

NMR studies of NADH indicate that the 1,4-dihydronicotinamide ring is in a boat conformation.[44] It has been generally assumed that this ring puckering is related to the chemical and biochemical reactivity of NADH.[45] Namely, the pseudo-axial hydrogen is more reactive than the pseudo-equatorial one. Therefore, *anti*-NADH should favor the transferring of the *pro-R* hydrogen, and *syn*-NADH the *pro-S* hydrogen.

Of course, the ring puckering induced by the N-substituents is very small. Such ring puckering is not even observable by X-ray analysis of crystals of N-substituted 1,4-dihydropyridine compounds.[37] Nevertheless, the ring puckering and pyramidalization at the ring nitrogen does have a significant energetic consequence according to the calculations. For example, the conformation derived by 180° rotation about the N_1–C_7 bond starting from the conformation of **13** is calculated to be 2.2 kcal/mol less stable than **13**, and 0.7 kcal/mol destabilization is introduced by rotating the N_1–C_7 bond by 180° starting from the conformation of **14**.

4. NAD⁺/NADH Redox Potential as a Function of the Conformation of Ribose with Respect to the Nicotinamide Ring. NAD^+ and NADH have different inherent conformational preferences. The glycosidic C–O bond in the NAD^+ is most stable when it is near the plane of the nicotinamide ring (**12**), while the same bond in the NADH is most stable when it is perpendicular to the nicotinamide ring (**17** or **18**). It is reasonable to consider that in the enzyme active site, NAD^+, NADH, and the transition state for hydride transfer are all likely to be bound in nearly identical conformations. Therefore, *NAD^+ and NADH binding energies, and the NAD^+/NADH redox potential will be dependent upon the orientation of the ribose with respect to the nicotinamide ring.*

Table V. Dissociation Constants (μm) for the Binary Complex E–NAD^+ and E–NADH[a]

enzyme	$K_{E\text{-}NAD}$	$K_{E\text{-}NADH}$
liver alcohol dehydrogenase	266	0.3
yeast alcohol dehydrogenase	350	11
malate dehydrogenase	280	1.0
lactate dehydrogenase	220	0.6
glutamate dehydrogenase	500	2–20
α-glycerophosphate dehydrogenase	9.2	0.02

[a] From Table IV, p 41 of ref 46. $K_{E\text{-}NAD} = [NAD^+][E]/[NAD^+ \cdot E]$.

One significant conformational feature of enzyme-bound NAD^+ and NADH is that the glycosidic C–O bond is nearly perpendicular to the nicotinamide ring regardless of whether they are in syn or anti conformation, as indicated by the near $\pm 90°$ χ_n values in Table I, with exception of glutathione reductase. Although these X-ray values of χ_n are only approximate owing to the limited resolution of the determinations, improved structural refinements appear to give χ_n values for bound NAD^+ or NADH that are closer to $\pm 90°$.[9,12,15] *Thus, NAD^+ binds in the enzyme-active site in inherently unfavorable conformations, while NADH binds in favorable conformations.*

In support of this, we note that it has been established that most dehydrogenases indeed bind NADH more strongly than they bind NAD^+, as shown by dissociation constants in Table V for some typical dehydrogenases.[46] Thus, the binding energy for NADH is about 2–4 kcal/mol higher than that of NAD^+. It is tempting to invoke an electrostatic interpretation of this phenomenon, because NAD^+ bears an additional positive charge on the nicotinamide ring. However, there is no clear evidence that this difference in binding energy is caused by electrostatic effect, based on X-ray crystal structures.[47]

5. Relationship between Stereospecificity of Dehydrogenases and the Conformations of NAD^+ and NADH. Why do dehydrogenases bind NAD^+ and NADH in the conformations with the glycosidic bond nearly perpendicular to the nicotinamide plane? One possibility is that such a binding conformation is a consequence to match or nearly match the free energies of bound intermediates, as in Benner's third hypothesis. This hypothesis was proposed in a seminal paper by Knowles and Albery in 1976.[20] It has been suggested to be a general rule for enzyme evolution.[48,49] Indeed, many types of enzymatic reactions have been observed to have internal equilibrium constants near unity. Recent detailed analyses by Benner et al. suggest that, in most cases, the internal equilibrium constant for a kinetically optimized enzyme is closer to unity than the "standard-state" equilibrium, but not necessary equal to unity.[50-52]

The equilibrium for the reactions represented by Scheme I is strongly toward the left-hand side. In order to match the free energy of bound intermediates, the dehydrogenases must bind the substrates on the right-hand side more strongly than the substrates

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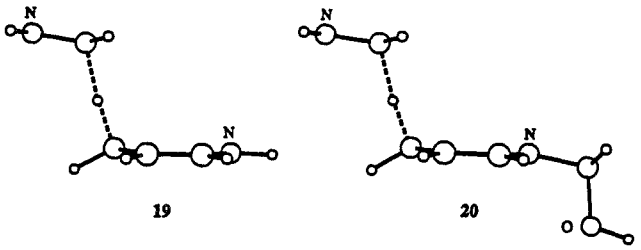
Table VI. Conformational Energies (kcal/mol) about the Rotation of N_1–C_7 Bond in the Transition Structure Model of Hydride Transfer, **20**

Lp-N-C-O (°)	3-21G	6-31G*
0	2.1	1.4
30	1.2	1.2
60	0.0	1.9
120	0.8	3.5
150	0.4	1.2
180	0.3	0.0

on the left-hand side. One possible way is to bind NAD^+ in a disfavorable conformation and to bind NADH in a favorable conformation.

Another possibility is that the conformations with near-perpendicular glycosidic C–O bond with respect to the nicotinamide are most favorable for the transition state of the hydride-transfer step, as proposed by Benner.[18] Since the puckering of the 1,4-dihydropyridine ring is the largest when the glycosidic C–O bond is nearly perpendicular, qualitatively such conformations should also facilitate the transfer of a pseudo-axial hydrogen.[53] We have previously reported that the transition structure of hydride transfer from 1,4-dihydropyridine to methyleniminium ion is in a boat-like conformation which resembles that of NADH.[5] We have also explored the conformational preference of the glycosidic bond in the transition state of hydride transfer. We started from the anti transition structure of the hydride-transfer reaction of 1,4-dihydropyridine with methyleniminium ion (**19**) and replaced the



19 **20**

N–H with a hydroxymethyl group. The hydroxymethyl group was first optimized with the constraint of OH anti-periplanar to the transferring hydrogen with the 3-21G basis set as shown in **20**. The C_2–N_1–C_7–O dihedral angle was varied and energies were evaluated with the 3-21G and 6-31G* basis sets. The relative energies are summarized in Table VI.

The 6-31G* calculations indicate that the conformational preference is similar to that in the NADH. That is, the C–O prefers to be anti-periplanar to the transferring pseudo-axial hydride. The structure with the C–O syn-periplanar ($\Phi = 0°$) to the transferring hydrogen is 1.4 kcal/mol less stable. When the hydroxyl group deviates more from the perpendicular positions ($\Phi = 0°$ and 180°), the energy increases. However, the syn structure becomes more stable than the anti structure ($\Phi = 60°$ versus $\Phi = 120°$).

Although further studies are needed in order to understand better the remarkable feature of perpendicular conformation of the glycosidic bond of NAD^+ in dehydrogenase bound state, the transition structure model calculations do suggest that the conformations with the C–O nearly perpendicular are most favorable for the hydride transfer transition state. It is also apparent that in such conformations the preferred transferring hydrogen is the one anti-periplanar to the C–O bond. That is, if the NAD^+ and NADH are bound in the anti conformation, the transferring of the *pro-R* hydrogen is more favorable, and if the NAD^+ and NADH are bound in the syn conformation, the *pro-S* hydrogen is favorably transferred.

However, the calculations also suggest that the above correlation is reversed if the NAD^+ and NADH are bound in the conformations with the glycosidic bond near the nicotinamide ring and the C_α–C_β bond is nearly perpendicular. That is, transfer of the *pro-S* hydrogen is more favorable than that of the *pro-R* hydrogen if the NAD^+ and NADH are in the anti conformation. This can explain why glutathione reductase is B-specific despite the fact that it binds NADH in an anti conformation, since the χ_n value of bound NADHP is −168° (Table I).[54] Whether such a relationship between enzyme-bound NAD^+/NADH conformation and stereospecificity is general needs further experimental tests. Oppenheimer has suggested that when syn yields *pro-S* and anti yields *pro-R*, the coenzyme must bind to the enzyme first, and the substrate must subsequently bind on top. This is a result of the known geometry of the active site. If, instead, the coenzyme were to bind on top of the substrate, then the opposite stereospecificity would result.[23]

Another interesting hypothesis by Benner was that A-specific dehydrogenases are weaker reducing agents then B-specific dehydrogenases. We have not yet explored the effect of the 3-amide group of nicotinamide on the conformational preference of ribose. X-ray structures of *N*-(methoxymethyl)-1,4-dihydronicotinamide and *N*-propyl-1,4-dihydronicotinamide revealed that both the methoxymethyl and propyl groups in the two compounds adopt the conformations with larger than 90° C_2–N_1–C_7–X dihedral angle (102° and 100°, respectively),[37] as if to avoid repulsive interaction with the 3-amide group. Such interactions can certainly influence the conformational preference of NAD^+ and NADH, causing differentiation in redox potential between the syn- and anti-bound NAD^+/NADH coenzyme couple.

Conclusion

While NAD^+ prefers to have the glycosidic C–O bond nearly coplanar with the nicotinamide ring, the glycosidic bond in NADH is most stable when perpendicular to the nicotinamide ring. Therefore, the binding energy of NAD^+ and NADH and the NAD^+/NADH redox potential are functions of the conformation of ribose with respect to the nicotinamide ring in the enzyme-active site. While the direction of puckering of NADH is always anti to the glycosidic bond, the puckering is most significant when the glycosidic bond is nearly perpendicular to the nicotinamide ring. The conformational preference of the ribose in the hydride-transfer transition state appears to be similar to that in NADH. We suggest that there is a general tendency for NAD^+/NADH-dependent dehydrogenases to bind NAD^+ and NADH in the conformations with nearly perpendicular glycosidic bonds. This feature of binding is at least partially responsible for the weaker binding energy of NAD^+ with respect to NADH. We also suggest that it is this feature of binding that is responsible for the generalization of anti/A specificity and syn/B specificity. Our calculations support Benner's functional explanation of the stereospecificity of NAD^+/NADH-dependent dehydrogenases.

Acknowledgment. We are grateful to the National Science Foundation for financial support of this research. We thank Professor Steven A. Benner for discussions and encouragement and Professor Michael G. Rossmann for sending us a preprint of a paper summarizing X-ray studies of LDH crystal structures.

(53) A quantitative evaluation needs full geometrical optimizations of transition structures, and this will be studied.

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