A Vibrational Analysis of the Catalytically Important C4–H Bonds of NADH Bound to Lactate or Malate Dehydrogenase: Ground-State Effects[†,‡]

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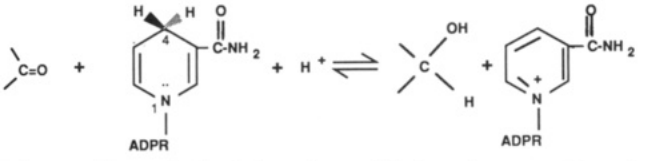
ABSTRACT: We have measured the frequency of the carbon–hydrogen stretching mode of the *pro-R* and *pro-S* C4–H bonds of NADH in solution and when bound to pig heart lactate (LDH) or mitochondrial malate (mMDH) dehydrogenases. This is achieved by specifically deuterating the C4 *pro-R* or *pro-S* hydrogens of NADH and determining the frequencies of the resulting C4–D stretches by Raman difference spectroscopy. We find that the frequencies of the two C4–D stretching modes for the two bonds are essentially the same for the unliganded coenzyme. On the other hand, the position of the *pro-S*-[4-^{2}H]NADH stretch shifts upward by about 23–30 cm^{-1} in its binary complex with either lactate or malate dehydrogenase relative to that observed in solution, while that for the bound *pro-R*-[4-^{2}H]NADH is relatively unchanged. The fact that the frequency of the *pro-R* hydrogen is not significantly affected during complex formation suggests that the rate enhancements for reaction of substrate with NADH brought about by both pig heart LDH and mMDH apparently do not involve either stabilization or destabilization of the *pro-R* hydrogen of NADH in enzyme–coenzyme binary complexes, in agreement with previous chemical studies. That these proteins are able to regulate the frequencies of the two C4–D bonds differentially, and hence the electronic distributions in these bonds, has important implications for the stereochemical reactions catalyzed by the NAD dehydrogenases, and this is discussed. We have studied a number of factors which can affect the C4–H stretch frequency by normal mode analyses of our Raman results based on semiempirical quantum mechanical calculations (MINDO/3, MNDO, and AM1). These factors include the interaction between the nicotinamide ring nitrogen and the ribose oxygen, the torsional angle of the amide arm, puckering of the ring, and the external charge or dipole modeled by a formaldehyde. Within the range of our study, the positions of the C4–D stretches may be understood as the result of two conformational changes of the nicotinamide ring that occur when NADH forms a binary complex with LDH or mMDH: the rotation of the amide group from a solution syn to anti in situ and the adoption of a "half-boat" of the dihydronicotinamide ring of NADH when bound to the two enzymes from an essentially planar solution structure. The estimated angle of the C4 ring carbon with respect to the other carbon atoms is around 15°, with the *pro-R* hydrogen at a pseudoaxial position and the *pro-S* hydrogen at a pseudoequatorial position. Our calculations also show that electrostatic interactions, as modeled by the interaction between the C–D bond and a point charge or a carbonyl dipole, can also be important in determining the C–D stretch frequency and differences between the *pro-R* and *pro-S* bond frequencies, although they apparently have no major effect in LDH or mMDH.

Lactate (LDH)[1] and malate (MDH) dehydrogenases catalyze the stereospecific transfer of a hydride, H$^-$, from the *pro-R* face (A-side) of NADH to the carbonyl carbon of pyruvate and oxaloacetate, respectively:



The position of the internal equilibrium between bound reactants and bound products for the reaction catalyzed by LDH is near unity for both the heart and muscle isozymes of LDH (Stackhouse et al., 1985; Burgner, unpublished results). On the other hand, the external equilibrium constant at pH 7 favors the NAD^+ and lactate side of the reaction by 5.6 kcal/mol. The 5.6-kcal shift in the position of the internal equilibrium *requires* that either reactant destabilization or product stabilization or some combination of both occurs in the ground state of the catalyzed reaction. This ground-state effect must be produced by noncovalent interactions between the ligands and the enzyme. One of us (J. W. Burgner) has argued elsewhere (Burgner & Ray, 1984a,c) that about one-half of this 5.6-kcal effect arises from either stabilization of bound NADH or destabilization of NAD^+ and the rest from a corresponding effect on lactate and pyruvate. It was also argued that all or at least most of this effect on the cofactor is present in its binary complex with LDH (Burgner & Ray,

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[‡] This paper is dedicated to the memory of Donald Sloan, who died during the course of this work. We deeply miss him as a friend and colleague.

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[1] Abbreviations: NADH, reduced β-nicotinamide adenine dinucleotide; [4-^{2}H]NADH, NADH deuterated at the C4 position; LDH, lactate dehydrogenase; mMDH, mitochondrial malate dehydrogenase; OMA, optical multichannel analyzer; 1-M-NH, *N*-methyl-1,4-dihydronicotinamide; DHFR, dihydrofolate reductase.

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1984c). Thus, it is possible that certain internal coordinates of NADH are modified in the binary complex ground state.

Two internal coordinates are important in the reaction coordinate for the reduction of ketones by NADH: the C=O bond of the substrate and the C4–H bond of the NADH coenzyme. Both these coordinates can be studied by vibrational spectroscopy. Recently, we showed that the carbonyl of pyruvate bound to LDH (as the pyruvate–NAD adduct; Burgner & Ray, 1984b) contains significantly more single bond character than does unbound pyruvate because of a strong hydrogen bond with the enzyme (Deng et al., 1989a). We presume that this polarization of the carbonyl reduces the height of the transition-state barrier sufficiently enough to account for the 10^6-fold rate effect (−8.4 kcal/mol) estimated for the interaction of the bound C=O with an active site histidine and an arginine (Burgner & Ray, 1984c; Deng et al., 1989a). The other coordinate, the C4–H coordinate, should provide some indication on the nature of the interaction of the enzyme with its coenzyme and the effect on the ground state. Thus, if NADH is destabilized in terms of the redox system, the C4–H bond should become more hydride-like (more easily transferred), and if it is stabilized, the bond should become more acidic (less easily transferred as a hydride).[2]

In this paper, the frequencies of the stretching modes are determined for both the *pro-R* and *pro-S* C4–H bonds. These frequencies are measured for NADH in solution and for NADH bound to pig heart LDH and mitochondrial malate dehydrogenase (mMDH). This is accomplished using NADH that was specifically deuterated at either the *pro-R* or *pro-S* C4 position. Deuteration lowers the frequency of the now C4–D stretch to near 2100 cm^{-1}, which is a vibrationally silent spectral region apart from some low-intensity overtone bands. Thus, the mode is essentially removed from the background of all other C–H stretches. Moreover, deuteration lowers the frequency of the C4–D stretch so that the mode is substantially uncoupled from all other vibrational modes and is, therefore, quite localized to the C4–D group. This simplifies considerably analysis of the results.

Substantial differences in frequency between the *pro-R* and *pro-S* stretches are observed for NADH bound to LDH and mMDH but not for NADH in solution. This suggests that these proteins, and presumably other dehydrogenases by extension, are able to regulate the chemical state of the C4–H bonds of the NADH in the ground state in ways not observed in the solution chemistry of NADH. Thus, the results bear on a number of important attributes concerning the enzymology of the NAD dehydrogenases, and we will consider these issues. In order to provide some basis for interpretations of our results (and future results on other NAD-dependent dehydrogenases in molecular terms), we also performed normal mode analyses based on semiempirical quantum mechanical calculations. The point of these calculations is to assess the importance of various factors on the strength of the C4–H bonds. By combining the results of these calculations with the known structures of a dehydrogenase from crystallographic studies, it is possible to understand how a particular enzyme may regulate the chemistry of the cofactor in situ.

Materials and Methods

Experimental. NAD^+ (100%) was purchased from Boehringer Mannheim Co. (Indianapolis, IN). [1,1-$^2H_2$]Ethanol (98%) and [1-^{2}H]glucose (98%) were purchased from Cambridge Isotope Laboratories (Woburn, MA). Enzymes used to make deuterium-labeled NADH at the C4 position of the nicotinamide ring (liver alcohol dehydrogenase, aldehyde dehydrogenase, and glucose dehydrogenase) were purchased from Sigma Chemical Co. (St. Louis, MO). They were used without further purification.

pro-R-[4-^{2}H]NADH was prepared enzymatically according to published procedures (Viola et al., 1979). *pro-S*-[4-^{2}H]-NADH was prepared by mixing 100 mg of NAD^+, 30 mg of [1-^{2}H]-glucose, and 50 units of glucose dehydrogenase in 5 mL of H_2O at pH 9 at room temperature for about 3 h. At the end point, which was determined on the basis of the 340-nm absorption, the reaction mixture was vortexed with several drops of CCl_4, centrifuged to remove the enzyme, and applied to a P2 (Bio-Rad, Richmond, CA; 3 × 40 cm) column. The fractions containing [4-^{2}H]NADH were combined and lyophilized to dryness and stored under argon. The purity of [4-^{2}H]NADH (*pro-R* or *pro-S*) was better than 95% as determined by NMR.

Pig H4 lactate dehydrogenase (LDH) was prepared according to the procedure previously described (Burgner & Ray, 1984b). The mMDH was purchased from Calbiochem (La Jolla, CA) and was further purified by chromatographing it on a DEAE A-50 (3 × 30 cm) column to remove a fluorescent material. About 100 mg of mMDH was loaded on the column equilibrated with 5 mM phosphate buffer at pH 6.7 and then eluded with 20 mM phosphate buffer at the same pH. The enzyme fractions were collected and stored as an ammonium sulfate precipitate until used for Raman experiments. For the Raman experiments, both LDH and mMDH were dialyzed against either 50 mM phosphate or 20 mM triethanolamine at pH 7.2 and then concentrated according to the procedure described (Deng et al., 1989b). The typical concentrations of LDH and mMDH were 1.5 mM (6 mN active site) and 4 mM (8 mN), respectively. For the binary complex experiments with either LDH or mMDH, a slightly less than stoichiometric amount of [4-^{2}H]NADH (*pro-R* or *pro-S*) was added in all cases. Under these conditions, more than 95% of the cofactors were bound.

Raman difference spectra were obtained by using one of the optical multichannel analyzer spectrometer systems described in detail (Yue et al., 1989). Typically, 100 mW of 488-nm or 514.5-nm laser line from an argon ion laser was used to excite Raman scattering. The instruments were calibrated against known peaks of the toluene spectrum. The band positions are accurate to ±2 cm^{-1}, and the spectrum resolution was 8 cm^{-1}. None of the spectra presented here have been smoothed.

Computational. N-Methyl-1,4-dihydronicotinamide is used as the model compound for the normal mode analysis of the C4–D stretching vibrations of [4-^{2}H]NADH. The normal modes were obtained using the NORCOR program from QCPE. The modified MINDO/3 program, which incorporated the Coulombic potential of point charges (Gilson et al., 1988), was used to calculate the geometry and initial force field. The force field was then scaled to reproduce the solution Raman spectra of [4-^{2}H]NADH in the C–D stretch region. The MNDO and AM1 methods (Dewar & Thiel, 1977; Dewar et al., 1985),

[2] It seems reasonable to suppose that there is a relationship between the ability of a C–H bond to donate a hydride ion and the force constant of the bond in that the force constant is a measure of the electronic distribution of the bond. However, we know of no quantitative relationship that has been established. On the basis of theoretical and experimental relationships between force constants, bond strengths, and charge associated with hydrogen found in X–H bonds, we think it very probable that a lower force constant, hence a lower X–H frequency, is associated with a larger hydride donor reactivity. For example, the X–H stretch force constant goes as follows in the series $SiH_4 < CH_4 < NH_4^+$, and this is matched by the electron density at the hydrogen as $SiH_4 > CH_4 > NH_4^+$ (Wiberg & Wendoloski, 1984).

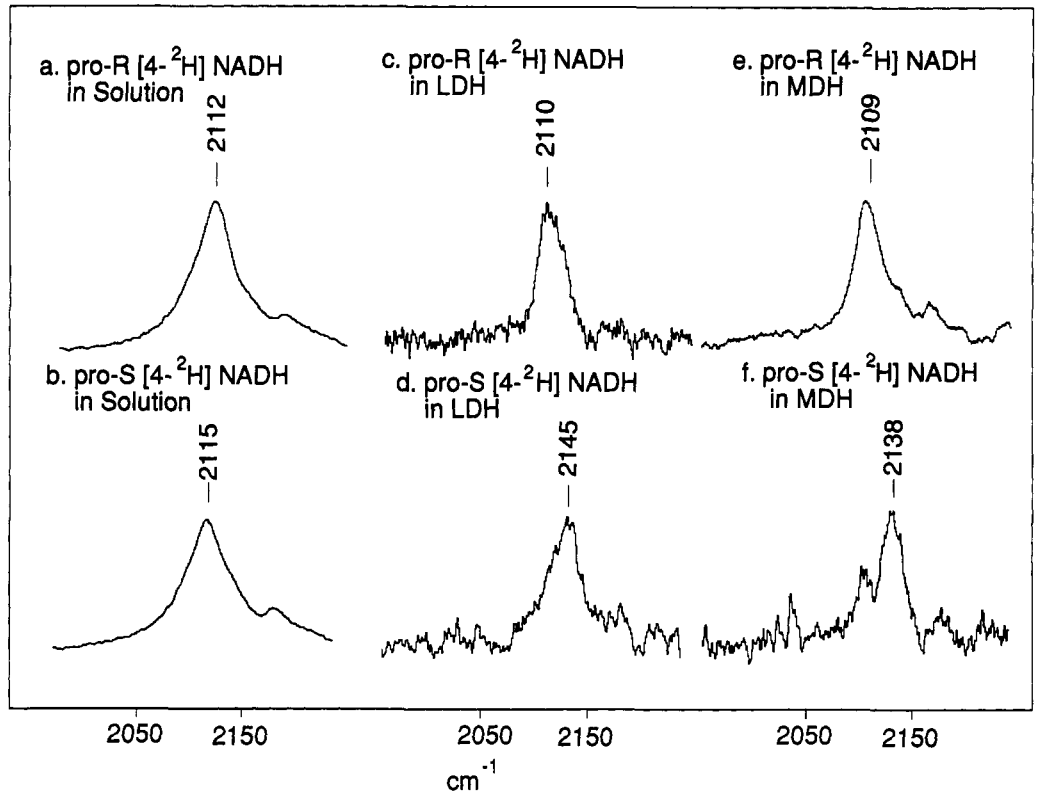


FIGURE 1: Raman difference spectra of (a) 100 mM *pro-R* [4-²H]NADH at 4 °C, (b) 100 mM *pro-S* [4-²H]NADH at 4 °C, (c) *pro-R* [4-²H]NADH in LDH (LDH/NADH = 1.5/5 mM) at 4 °C, (d) *pro-S* [4-²H]NADH in LDH (LDH/NADH = 1.5/5 mM) at 4 °C, (e) *pro-R* [4-²H]NADH in mMDH (mMDH/NADH = 4/7 mM) at 4 °C, and (f) *pro-S* [4-²H]NADH in mMDH (mMDH/NADH = 4/7 mM) at 4 °C. All spectra were obtained by subtracting the corresponding NADH spectra under the same condition. An approximately 100-mW 488.0- or 514.5-nm laser line was used for the Raman excitation.

implemented in the QCPE program AMPAC2.1, were also used to do similar calculations.

Results

Spectral Studies. The Raman spectra of [4-²H]NADH shown in Figure 1 are obtained by subtracting the spectrum of NADH (or enzyme–NADH complex) from that of [4-²H]NADH (or enzyme–[4-²H]NADH) using a Raman difference spectrometer (see Materials and Methods). Spectra a and b in Figure 1 show the Raman spectra for unliganded *pro-R*-[4-²H]NADH and *pro-S*-[4-²H]NADH, respectively, in the spectral region where C4–D stretching modes are found. Thus the broad bands at 2112 cm^{-1} for *pro-R*-[4-²H]NADH and 2115 cm^{-1} for *pro-S*-[4-²H]NADH can be assigned to the C4–D stretching modes for these two deuterions. The broadness of the bands for the unliganded NADH (Figure 1, spectra a and b) suggests that the nicotinamide ring has multiple conformations in solution.[3]

Spectra c and e in Figure 1 show the Raman spectra of *pro-R*-[4-²H]NADH bound to LDH and mMDH, respectively. The *pro-R* C4–D stretching frequency is effectively unchanged upon binding to either enzymes. In clear contrast, the *pro-S* C4–D stretching frequency shifts upward by 30 and 23 cm^{-1} for *pro-S*-[4-²H]NADH bound to LDH (spectrum d) and mMDH (spectrum f), respectively. Thus, both enzymes affect the two C4–H bonds of the dihydronicotinamide ring in a nonsymmetrical way. The difference in frequency between the two stretches is quite large.[4]

The most plausible explanation for this difference has to do with the conformation of the dihydronicotinamide ring. There is little energy difference between planar and shallow boat forms of this ring. For a planar ring, both C4 hydrogens are nearly chemically equivalent (Oppenheimer et al., 1978), and their two C4–D stretching frequencies should be nearly the same, which is the case in solution (Figure 1). On the other hand, if when the ring adopts a boat form, where one C4–H bond becomes axial while the other equatorial, the two hydrogens, hence the two stretches, should differ. Generally, the equatorial C–D frequency is somewhat higher than that of the axial bond; for example, it is about 20 cm^{-1} higher in the case of [2-²H_1]cyclopentene (Rafilipomanana et al., 1985). Thus, the observed broadness of the bands for unbound NADH in Figure 1 may result from a population of molecules with differing conformations which are associated with somewhat different boat angles that average to a planar structure. Each of these conformations will contribute to the Raman intensity

[3] On the basis of NMR studies [cf. Oppenheimer (1987)], about 30% of NADH appears to take on a "stacked" conformation in water, the dihydronicotinamide ring interacting with the adenine moiety of NADH, and the nicotinamide ring may be puckered in this conformation. However, the C4–D stretch bands of both *pro-R* and *pro-S* labeled NADH under "destacking" conditions (8 M urea at 50 °C or methanol or NADH that has been hydrolyzed with snake venom phosphodiesterase) are nearly the same as those obtained under the conditions shown in Figure 1 (in water at 4 °C). The band positions lie to within a couple of wavenumbers, and the band shapes are also similar (but not identical). It is clear from the rather large bandwidths of the C–D stretches that NADH exists as multiple conformations in solution. In a future publication, we shall analyze the small but reproducible differences between the stacked and more destacked conditions.

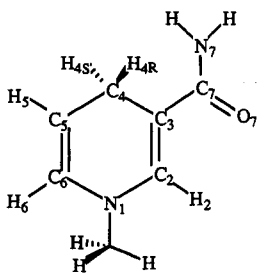
[4] There are a number of possible explanations for the smaller band at about 2115 cm^{-1} observed in the bound *pro-S*-[4-²H]NADH in MDH in Figure 1f. One is that NADH binds this enzyme in two different conformations, the minor conformation having a stretch at 2115 cm^{-1}; another is that this band is a combination mode of two lower frequency modes, and its intensity is enhanced by Fermi resonance (Rafilipomanana et al., 1985).

Table I: Empirically Derived Force Constants Associated with the C–D Stretch

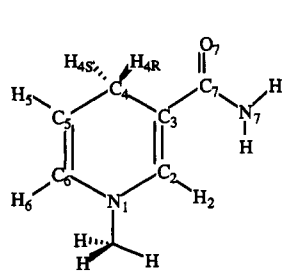
stretch (mdyn/Å)	
K_{C-D}	4.58
K_{C-C}	6.2
bend (mdyn Å/rad^2)	
K_{C-C-C}	1.34
K_{C-C-D}	0.52
stretch–bend (mdyn/rad)	
$K_{C-D,C-C-D}$	−0.09
$K_{C-D,C-C-C}$	−0.14
stretch–stretch (mdyn/Å)	
$K_{C-D,C-D}$	0.12
$K_{C-D,C-C}$	0.29
bend–bend (mdyn Å/rad^2)	
$K_{C-C-D,C-C-D}$	0.12

according to its population (heterogeneous broadening). Further, for the bound cofactor, the significantly higher stretching frequency for the *pro-S* C4–D stretch suggests that the ring assumes on average a boat conformation with the *pro-S* hydrogen equatorial and the *pro-R* axial. This boat conformation may be caused by the interaction between the N1 nitrogen and the ribose oxygen as suggested by the ab initio studies (Wu & Houk, 1991). However, the absence of a corresponding decrease in the stretching frequency of the *pro-R* bond compared to its solution frequency for the bound cofactor is bothersome, since such a shift is expected during the deformation of the ring (see below). In addition, other factors, like the orientation of the amide and the presence of an external dipole group, may also influence the stretching frequencies of the two C4–D bonds. For these reasons, we have performed normal mode analyses based on semiempirical quantum chemical calculations using MINDO/3, MNDO, and AM1 Hamiltonians on models of the cofactor.

Calculation of Scaling Factors. It has been shown that an appropriate scaling of force constants derived from semiempirical force field calculations yields vibrational frequencies that are comparable to those obtained with a scaled ab initio force field (Gilson et al., 1988). We adopt that approach here. Conformational studies on *N*-methyl-1,4-dihydronicotinamide (1-M-NH) at the ab initio SCF/STO-3G and SCF/3-21G



Model Compound I



Model Compound II

levels predict an absolute minimum at the planar syn conformation (Cummins & Gready, 1989). Recent NMR studies of nicotinamide and its analogs with fused C7=O7 syn or anti conformation also suggest that nicotinamide has an C7=O7 syn conformation in solution (Fischer et al., 1988). Therefore, model compound I, with a planar ring and the C7=O7 bond syn to N1, was used as our solution model to obtain the initial force field. We have also performed similar calculations using *N*-(hydroxylmethyl)-1,4-dihydronicotinamide as a model compound. The interaction between the N1 nitrogen and the hydroxyl oxygen does induce a small difference between the *pro-R* and *pro-S* C4–D stretch frequency. However, this difference is less than 5 cm^{-1} and unlikely to be a major factor to account for the difference observed in pig heart LDH and mMDH. Therefore, we have used 1-M-NH in the following normal mode analyses. Its geometry was fully optimized except for the three hydrogens on the methyl group and for the C7=O7 which was fixed in the syn orientation.

Table II: Calculated Changes in Key Parameters between Model Compounds I and II[a]

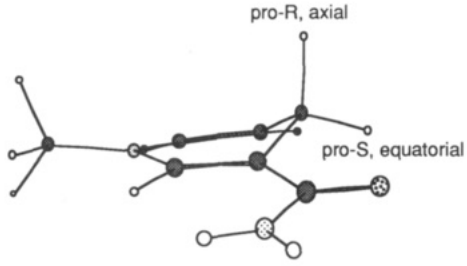
method	ΔH (kcal/mol)	ΔK_{C-D} (%)	$\Delta\nu_{C-D}$ (cm^{-1})	$\Delta\nu(K_{C-D})$ (cm^{-1})
MINDO/3	+2.0	+2.0	+22	+21
MNDO	+1.4	+1.2	+10	+12
AM1	+2.8	+1.2	+9	+12

[a] ΔH is the heat of formation change, ΔK_{C-D} is the C–D stretch force constant change, $\Delta\nu_{C-D}$ is the C–D stretch mode frequency change calculated by changing the entire force field, and $\Delta\nu(K_{C-D})$ is the C–D stretch mode change calculated by changing the C–D stretch force constant (K_{C-D}) alone.

Table I lists those force constants important in determining the C4–D stretch frequencies which were obtained by a fit to the observed spectra. The values of this force field were guided by the quantum mechanical derived set with regard to the sign and relative size of the off-diagonal elements. The C4–D stretch mode frequency calculated by this set of force constants is 2110 cm^{-1}. Force constant scale factors are then calculated as the ratio between the MINDO/3, MNDO, or AM1 force constants and the empirically derived set. These are then used to scale the calculated force constants later. By varying the force field values, we found, as expected, that the C4–D stretch frequency depends almost solely to its stretch force constant, which suggests that the C4–D stretch mode is quite well represented by the internal stretching motion of the C4 hydrogen. Other force constant or coupling constant changes have only minor effects on the C4–D stretch mode. Therefore, we believe reasonable results on the C4–D stretch mode can be obtained even on a molecule which is not fully relaxed, either because of internal constrains or fixed external perturbations. Apparently, this methodology may not be applicable for a complete normal mode analysis.

Effects of Rotation of the Amide Group. The conformation of the amide group of NADH bound to LDH or MDH is anti (Birktoft et al., 1982; Holbrook et al., 1975). Accordingly, the effect on the C4–D stretch frequency by this change of the geometry was determined by using model compound II, with C7=O7 anti to the N1 of the ring. The results of calculations based on the MINDO/3, MNDO, and AM1 methods are given in Table II where all quantities are relative to those calculated for the C7=O7 syn conformation. The heat of formation increases by between 1 and 3 kcal/mol during C7=O7 syn to anti conformational change, which is in agreement with ab initio SCF/STO-3G and 3-21G calculations (Cummins & Gready, 1989). This relatively small difference in the heat of formation between C7=O7 syn and anti implies that the amide can adopt either orientation in enzymes, being easily stabilized by the appropriate positioning of H-bond donor and acceptors. The data in Table II also indicate that the force constant for the C4–D stretch increases by 2.0%, 1.2%, and 1.2%, and the calculated changes in the C4–D stretch mode are 20, 10, and 9 cm^{-1}. This is a relatively large change. Thus, we suggest that the orientation of the amide is an important determinant of the C4–D stretch frequency.

Effects of Dihydronicotinamide Boat Formation. Model compound III with C7=O7 anti and the C4 carbon bent out of the ring plane by various angles was used in the calculations. The results of the calculations on the model compound with the C4 carbon bent out of the ring plane by 15° are shown



Model Compound III

in Table III. Here, the heat of formation only increases by about 1 kcal/mol compared with model compound II; this value is consistent with the 0.3 and 1.4 kcal/mol increase for a 10° and 20° angle for C4 bending, respectively, obtained by ab initio calculations (Wu & Houk, 1991). The calculated stretching force constant for the equatorial bond increases by 1.1–2.0% and that for the axial bond decreases by 0.7–1.8%, which corresponds to a stretching frequency increase of 11–18 cm^{-1} and decrease of 5–14 cm^{-1}, respectively, compared with those of model compound II. The smaller absolute shift of the axial C4–D frequency compared with that of the equatorial C4–D stretch is due to the difference in geometry between the axial and equatorial hydrogens; normal mode calculations with equal force constants for the two C4–D bonds yield this ca. 5-cm^{-1} difference. Thus, this comparison between model compounds III and I (planar ring, C7═O7 syn) indicates that the equatorial C4–D stretch increases by 20–40 cm^{-1} while the axial C4–D stretch increases by only 4–8 cm^{-1}. Thus, these two conformational changes, C7═O7 syn to anti and planar to boat, independently affect the C4–D stretch frequency, and their effects are essentially additive. We also note that the bending of N1 out of the ring plane is only about one-fourth of that for the C4 on the basis of our semiempirical calculations. For one calculation, where the C4 carbon was forced to bend about 25° out of plane, the energy optimizations resulted in the N1 nitrogen bending only about 5–8°, depending on the particular semiempirical method used.

Effects of Nearby Polarizing Groups. To study the effect of electrostatic interactions on the frequency of the C4–D stretch frequency, we have considered two possibilities. The first of these is an external point charge positioned 4.2 Å away from the carbon along one of the C4–H bonds. The MINDO/3 calculations using model compound I show that a half-positive charge will cause the C4–D stretching frequency to decrease by about 100 cm^{-1} and that a half-negative charge will produce the opposite shift. While neither LDH nor MDH contains nearby negative charges, this calculation shows that such charge groups, which induce a substantial field gradient, can have a large effect on the stretching frequency of the C–H bond.

The other model, of interest for LDH and MDH, involves the effect of a nearby dipole, such as a carbonyl. This interaction was modeled by moving a formaldehyde molecule, initially positioned at infinity with its C═O bond approximately collinear with one of the C4–H bonds and with the oxygen nearest the hydrogen, toward one of the C4–H hydrogens in model compound I. The change in C4–D stretching frequency as a function of distance was calculated using the MNDO and AM1 methods, and these are given in Table IV. First, we note that both methods predict that there will be little change in the C4–D stretch frequency of the C–D bond which is *not* along the C4–H···O line (changes are not given in the table). Second, we note that both methods also produce quite consistent results as long as the oxygen is moved no closer than to within 4.5 Å of the C4 carbon. At this distance, the heat of formation has changed little and the effect on the C–D stretching frequency is small, 5 cm^{-1} or less. For shorter distances, the MNDO calculations predict a much stronger (and unfavorable) interaction between nicotinamide and formaldehyde than those from AM1 when the external carbonyl oxygen is moved to within 4 Å of the C4 carbon. For example, the heat of formation increase is calculated to be 5.0 kcal/mol at 3.3 Å with MNDO as compared to −0.5 kcal/mol at the same distance with AM1. These values correspond to an increase in the C–D stretching frequency of 85 cm^{-1} with the MNDO method as compared to only 22 cm^{-1} with the AM1 method (see Table IV). We suggest that the AM1 calculation is probably more reliable in predicting changes in the C4–D stretching frequency shifts for the following reason. In X-ray structural studies of DHFR–NADPH complexes, the oxygen of the Ala 97 carbonyl is only 3.3 Å away from C4 carbon and is on the *pro-S* side of the nicotinamide ring (Filman et al., 1982). We have recently determined that the frequency of the *pro-R* stretching mode remains unchanged (at 2114 cm^{-1}) relative to solution values, but the *pro-S* stretching mode shifts upward to 2163 cm^{-1} (Zheng, Kraut, and Callender, unpublished observations), which is a considerably larger shift than observed with either LDH or mMDH. This extra 17-cm^{-1} upward shift in the *pro-S* C4–D stretching mode observed when NADPH binds to DHFR as compared to that observed when NADH binds to LDH is closer to the 22-cm^{-1} frequency difference between the two C–D stretching modes estimated by the AM1 method than to the 85-cm^{-1} difference estimated by MNDO method. From this result, it seems that the MNDO method may overestimate the interaction between the nicotinamide and external formaldehyde. Thus, it seems clear that a carbonyl moiety further than 4.5 Å from the C4–H, as in LDH, should have only a minor effect on the C4–D stretch frequency.

We also calculated the effect of the carbonyl of the amide of nicotinamide on the two C4–D stretches in a situation where the C═O group was aligned parallel to one of the C–H bonds. This was simulated with model compound II by constraining the amide to an angle of 35° from the plane of the ring so that

Table III: Calculated Changes in Key Parameters between Model Compounds II and III[a]

method	ΔH (kcal/mol)	ΔK_{C-D} (%)		$\Delta\nu_{C-D}$ (cm^{-1})		$\Delta\nu(K_{C-D})$ (cm^{-1})	
		eq	axial	eq	axial	eq	axial
MINDO/3	+1.2	+2.0	−1.8	+18	−14	+19	−14
MNDO	+1.0	+1.4	−0.7	+15	−5	+15	−4
AM1	+0.7	+1.1	−0.7	+11	−5	+9	−4

[a] ΔH is the heat of formation change, ΔK_{C-D} is the C–D stretch force constant change, $\Delta\nu_{C-D}$ is the C–D stretch mode frequency change calculated by changing the entire force field, and $\Delta\nu(K_{C-D})$ is the C–D stretch mode change calculated by changing the C–D stretch force constant (K_{C-D}) alone. eq = equatorial.

Table IV: Calculated Changes in ΔH and C–D Stretch Frequency by a Nearby Carbonyl[a]

method	C═O···C4 distance (Å)	ΔH (kcal/mol)	$\Delta\nu$ (cm^{-1})
MNDO	4.5	+0.2	+5
	3.7	+0.4	+24
	3.3	+5.0	+85
AM1	4.5	−0.2	−2
	3.7	−0.6	−8
	3.3	−0.5	+22

[a] ΔH is the heat of formation change, and $\Delta\nu$ is the C–D stretch mode frequency change calculated by changing the entire force field. Calculations were performed on model compound I.

the amide oxygen is closer to one of the C4–D bonds. This has a very small effect on the C4–D frequencies compared to the results given for the strictly anti conformation in Table II. The calculated stretching frequency of the C4–D bond closer to the oxygen of the amide is consistently higher than that of the other for all three methods but by only a few inverse centimeters. Thus the differences between the two C4–D stretching modes are small, being about one-fifth or less than the differences produced by bending the C4 carbon 15° out of the ring plane (Table III).

Summary of Our Normal Mode Analyses Based on Semiempirical Quantum Mechanical Methods. We have used three semiempirical methods (MINDO/3, MNDO, and AM1) to probe several factors which may influence the C4–H stretch frequency of dihydronicotinamide. Two of those factors, namely, the orientation of the amide and the interaction between N1 nitrogen and the oxygen on the ribose, are unlikely to have a major effect on the observed difference between *pro-R* and *pro-S* C4–D stretch frequencies when NADH is bound to pig heart LDH and mMDH. The external dipole or charge as well as the ring puckering, which can be induced by hydrophobic interactions, can in principle account for the two C4–D stretch frequency differences. That the average of the two C4–D stretches is higher in situ compared to that found in solution may be understood in terms of an anti conformation of NADH's amide group in situ compared to a syn conformation found in solution. We should note here that we have not exhausted all possible factors which may affect C4–H stretch frequency. For example, bound solvent molecules and the protein environment as a whole are not included in this study.

Discussion

We have obtained the Raman spectra of NADH specifically deuterated at either the *pro-R* or *pro-S* position of the dihydronicotinamide C4 carbon both unliganded and bound to either LDH or mMDH. For the unbound cofactor, the frequency of both C–D stretches is virtually the same, with maxima in the range 2112–2115 cm^{-1}. This result suggests that the *pro-R* and *pro-S* C–H bonds are quite symmetrical in their force constants. Hence, the electron density near the *pro-R* and *pro-S* hydrogens is nearly the same. Formation of a binary complex of NADH with either LDH or mMDH produces a substantial change in the dihydronicotinamide ring that causes one of the two C–D bonds to become significantly stronger than the other. On the basis of linear energy correlations between molecular charge density for C–H bonds and stretching frequency (Gussoni et al., 1984; Wiberg & Wendoloski 1984), we suggest that the chemical potential of the two hydrogens toward hydride transfer differs substantially.[2] We discuss below on the basis of semiempirical quantum mechanical calculations how the protein environment might bring about such a difference in frequency for the two stretches. That the protein is able to affect the C4–D stretch frequency seems to have important mechanistic implications for these two enzymes and perhaps to nicotinamide cofactor dependent dehydrogenases as a whole. We discuss this as well.

Factors Influencing the C4–D Stretch. Intuitively, a C–D stretch mode should be an isolated (localized) mode, since no other vibrational mode has a frequency close to it. Our normal mode analysis on the C4–D stretch mode confirms this intuitive suggestion. We have found that the C4–D stretching mode of [4-^{2}H]NADH is localized to such an extent that its frequency is governed almost solely by its stretch force constant (i.e., it is insensitive to the changes of other force constants or structures used in the normal mode calculations within a reasonable limit; this is seen from Tables II and III).

The importance of molecular conformations and interactions that might influence the frequency of the C4–H modes has been considered by semiempirical quantum mechanical analyses. These include the effect of twisting of the amide torsional angle, puckering of the dihydronicotinamide ring, and positioning of a dipole near the *pro-S* hydrogen. For instance, our semiempirical calculations (Table II) indicate that the C4–D bond is about 2% stronger when the amide is in the anti as opposed to syn conformation with both C4–D bonds being affected similarly (about 10–20 cm^{-1}). While some difference between the frequencies of the two C4–D stretches is possible for an out-of-plane amide rotation, this factor is, however, unable to explain the near 30-cm^{-1} difference between the *pro-R* and *pro-S* C4–D stretch frequencies observed for bound NADH.

Another factor influencing the C4–D stretching frequency of [4-^{2}H]NADH is the bending of the C4 carbon out of the ring plane, forming a distorted boat structure as shown in model compound III. The calculations suggest that a ca. 15° bend in the ring would account for the about 30-cm^{-1} difference between the two C4–D stretches. Since these quite sizable distortions of the ring cost relatively little energy (see Results), any number of low-energy interactions could easily achieve this distortion.

One possibility is the "reverse anomeric effect". Here the interaction between the electron lone pair on nitrogen and the antibonding orbital of the sugar oxygen–carbon bond has been postulated to force the nicotinamide ring into a boat form (Nambiar et al., 1983). Recent ab initio calculations using *N*-(hydroxymethyl)-1,4-dihydropyridine as a model compound at the 3-21G level have confirmed that this interaction does result in a boat form of the pyridine ring (Wu & Houk, 1991). However, the predicted out-of-plane bending of the C4 carbon is only about 3–5°, well below the estimated angle required to produce our Raman observations. We have performed similar calculations on both *N*-(hydroxymethyl)-1,4-dihydronicotinamide and *N*-(hydroxymethyl)-1,4-dihydropyridine using the AM1 method. The predicted bending of the C4 carbon is 3–4° for *N*-(hydroxymethyl)-1,4-dihydropyridine, which is similar to that obtained by ab initio 3-21G calculations. However, this bending is only about 1.5° for *N*-(hydroxymethyl)-1,4-dihydronicotinamide, presumably because of the influence of the amide arm, and the difference between the two C4–D stretch frequencies is less than 5 cm^{-1}. Thus, on the basis of these calculations, the ribose oxygen–N1 nitrogen interaction is probably not directly responsible for the observed C4–D stretch frequency difference in pig heart LDH and mMDH.

Some form of noncovalent interactions between the protein and the cofactor could also produce the ring distortion. For example, the X-ray studies show that, in the cavity containing the dihydronicotinamide ring, there are 14 atoms from 7 amino acid residues within about 5 Å from the C4 carbon; three carbon atoms from Ile 249 that approach the C4 from the *pro-S* side of the nicotinamide are within 4 Å in dog fish LDH (Griffith & Rossmann, 1987). The hydrogen bonds that form between the amide carbonyl and $-NH_2$ moieties and the protein, with a total energy of about 10.3 kcal/mol estimated in our recent study (Deng et al., 1991), are strong enough to hold the ring quite firmly against Ile 249 to produce the ring distortion.

Another factor that could cause the difference in the two C4–D stretching modes is an external charge or dipole closer to one C4–D bond than to the other. However, this type of

interaction is unlikely the major factor for the observed 23–30-cm^{-1} difference between the *pro-R* and *pro-S* stretch modes when NADH is bound to LDH or mMDH for the following reasons. First, on the basis of the X-ray crystallographic studies, there are no negative external charges close to NADH's dihydronicotinamide ring in LDH and MDH. Second, the nearest carbonyl oxygen found in dogfish LDH is about 5.2 Å (Thr 245) from the C4 carbon of nicotinamide (Griffith & Rossmann, 1987) and about 4.5 Å in cytoplasmic MDH (Ser 241; Birktoft et al., 1989). Moreover, they are almost on the same plane of the nicotinamide ring. Therefore, the frequency difference between the C4–D stretch modes caused by an external carbonyl should be small according to our calculations (less than 5 cm^{-1}; Table IV).

Implications on the LDH- and mMDH-Catalyzed Reactions. LDH and MDH are similar in many aspects (Birktoft et al., 1982), especially since each of these enzymes directly transfers a hydride ion, H^-, from the *pro-R* face of NADH to the C2 carbon of a 2-keto acid and functions via an ordered addition of cofactor and substrate, where the first step involves binding of the cofactor. We find that the frequency of the *pro-R* C4–D stretch is essentially unaffected when NADH binds to LDH or mMDH. This suggests that the bond strength of the *pro-R* is essentially the same when bound to these proteins as it is in solution. This is in agreement with the results of Burgner and Ray (1984a), who found that the rate of decomposition of CN^- from NAD-CN (the adduct of NAD^+ with CN^-) was the same for NAD-CN in solution as it was for NAD-CN at LDH's active site. It was also found that the enzyme discriminated against *pro-S* addition even for an unbound reactant such as CN^-. This is consistent with the observed frequency difference between the *pro-R* and *pro-S* C4–D stretches, which suggests that the bond strengths, and hence the chemical potential, of the two protons differ in situ, although steric effects could account for this differential reactivity equally well. The C–H stretching frequency is linearly correlated with bond length for some 40 compounds where the C–H bond lengths were determined by the microwave method (McKean, 1984). McKean's correlation shows that each 10-cm^{-1} increase in the C–H stretching frequency ($\sim$7-cm^{-1} C–D stretch) corresponds to a 0.001-Å shortening of the bond length. Thus, the 29–35-cm^{-1} higher frequency in the *pro-S* stretching mode over that for *pro-R* when the cofactor is bound to mMDH or LDH corresponds to a 0.004–0.005-Å shorter bond length. Such a trend, as pointed out earlier, is consistent with a hydrogen that is more acidic and less hydride-like.

Our results here suggest that the reactivity of the two C4 hydrogens differ, possibly by a large amount when bound to LDH and MDH. The difference in frequency between the *pro-R* and *pro-S* C4–D stretches for NADH bound to these enzymes is significant, since it is larger than the bandwidths of the corresponding bands measured for NADH in solution as shown in Figure 1. This immediately suggests that the average conformation of bound NADH and its associated oxidation–reduction potentials are unlike any found in solution. We have attempted to rationalize these frequency shifts in terms of specific interactions observed between NADH and the active sites of these enzymes. According to our calculations (see Results), some of the possible factors that may be responsible for regulating the frequency of the C4–D stretch, and the associated chemistry of the proton, are the interaction between the ribose oxygen and the N1 nitrogen, the torsional angle of the amide arm, the "boat" angle of the NADH ring, and protein dipoles and charged groups that are close to the C4 protons. While the calculations suggest that the major cause for the difference between the two C4–D stretches for NADH in LDH and mMDH is that the dihydronicotinamide ring adopts a pronounced half-boat conformation, forcing the *pro-R* proton into the axial orientation and the *pro-S* proton into the equatorial orientation, all of these putative determinants may be present to more or less degree in other NAD dehydrogenases.

Thus, it is quite conceivable that the active sites of this class of enzymes might well be able to achieve a substantial variation in the chemical potential of bound NADH by varying the electron density of the C4 hydrogens. This may be of importance to the issue of how the NAD dehydrogenases, as a enzyme class, are able to catalyze oxidation–reduction reactions between substrate–product pairs whose external equilibrium constants with NADH and NAD^+ span some 10 orders of magnitude [or ca. 14 kcal/mol; cf. Westheimer (1987)]. Assuming that the corresponding internal equilibrium constants are near 1 (Knowles & Albery, 1977; Stackhouse et al., 1985), substantial regulation of the oxidation–reduction potentials of the reacting molecules is necessary. Moreover, it seems clearly possible that the *pro-S* or the *pro-R* bonds may be affected quite differently across the class of NAD dehydrogenases. However, we suggest for both LDH and mMDH that the chemical potential of the *pro-R* hydrogen is not significantly changed in the binary complex. This clearly indicates that the differences in ground state suggested for various LDH binary complexes arise not from stabilization of NADH but from destabilization of NAD^+ [see the introduction and Burgner and Ray (1984c)].

Finally, one of the remarkable properties of the NAD dehydrogenases is their stereospecific transfer of the hydride ion from either the *pro-R* or *pro-S* face of the dihydronicotinamide face of NADH. In LDH, the error in the fidelity of transfer from the *pro-R* side compared to the *pro-S* side is smaller than 1 part in 10^8 (LaReau et al., 1989). From a structural studies point of view, it is known that *pro-R* enzymes bind the nicotinamide ring in the anti conformation about the glycosidic bond with the *pro-R* proton pointing toward the substrate binding pocket while *pro-S* enzymes bind the ring in the syn conformation with, now, the *pro-S* proton pointing toward the substrate. This prompted the early suggestion that simple geometrical considerations, one proton is closer to the substrate than the other, are responsible for the stereospecific nature of these enzymes.

This concept suggests two fairly stringent requirements. One is that the nicotinamide ring binds in the correct geometry, or remains in the correct geometry once bound, within the error rate of hydride transfer. This means a difference in the equilibrium constant between syn and anti of 1 part in 10^8, or 10.4 kcal/mol, for LDH. In a previous study (Deng et al., 1991), we have estimated that the hydrogen-bonding interaction between NADH's amide arm and the protein is about 10.3 ($\pm$1.0) kcal/mol in the anti conformation. Since the residues on the syn side of NADH in situ are hydrophobic so that the hydrogen-bond interaction for this conformation is presumably essentially zero, this analysis suggests that the cavities of these proteins have been designed to achieve a syn–anti equilibrium constant of 1 part in 10^8 or so based simply on these electrostatic considerations.

A second requirement suggested by this geometrical view is as follows. As is well-known, atoms in proteins are not located at fixed points but rather have substantial motion about their equilibrium points. It is not unusual for a protein atom to attain a root-mean-square deviation of 0.5 Å. Since the carbonyl carbon of substrate analogs lies about 3.2 Å from

the C4 carbon of NADH in X-ray crystallographic studies of ternary complexes of LDH (Griffith & Rossmann, 1987), it seems possible that the *pro-S* hydrogen positions itself from time to time within a distance close enough to react to the substrate's carbonyl carbon more often than 1 part in 10^8. If this is true, and an extensive set of molecular dynamical calculations could estimate this more quantitatively, it would then be required that the *pro-S* hydrogen be a poorer hydride donor than the *pro-R* hydrogen at LDH's coenzyme site. This is again consistent with inference drawn above that the *pro-S* hydrogen forms a stronger bond with NADH's C4 carbon than does the *pro-R* hydrogen.[2]

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