Vibrational Structure of NAD(P) Cofactors Bound to Three NAD(P) Dependent Enzymes: an Investigation of Ground State Activation

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The NAD(P) dependent dehydrogenases and reductases stereospecifically catalyze the transfer of a hydride ion from C4 of the dihydronicotinamide of the NAD(P) ring to the substrate. We have investigated the vibrational structure of the important C4−H coordinate for NAD(P)H and $NADP^+$ bound to three enzymes in binary and ternary (Michaelis mimics) complexes: the A-side specific lactate dehydrogenase (LDH) and dihydrofolate reductase (DHFR) and the B-side specific glycerol-3-phosphate dehydrogenase (G3PDH). This is achieved by specifically deuterating the C4 pro-R or pro-S hydrogens of the reduced ring or the C4 hydrogen of the oxidized ring, which results in a vibrational mode localized to the stretching motion of the labeled C4−H bond. We observed relatively minor changes in the stretch frequencies of the C4−H bonds showing that the electronic nature of the bond is not substantially modified by cofactor binding, a mechanism previously proposed to be involved in enzymic "activation" toward catalysis. However, from the observed band narrowing of the C4-D stretch band, it is clear that interactions at the active site in all three proteins greatly reduced the conformational flexibility of either the reduced or oxidized ring as the cofactor moves from solution to the binary complex or ternary complex, guiding the ring structure from the ensemble of structures accessible in solution toward a selected set. Moreover, as NAD(P)H binds to LDH or DHFR forming binary as well as ternary Michaelis mimic complexes, the pro-R hydrogen is brought to a pseudoaxial orientation, which is thought to be the proper geometry for the transition state of hydride transfer. Hence, ground state structural distortions imposed on the cofactor appear to populate preferentially the correct ring geometry for enzymic activity. Surprisingly, the mimics of their Michaelis complexes also contain a substantial second, presumably unproductive, population of the bound cofactor whereby the pro-S hydrogen is pseudoaxial. Unexpectedly, the geometry of NADH bound to G3PDH is nearly planar with the pro-R hydrogen slightly pseudoaxial. This would seem to be a poorly bound cofactor for catalysis although it may well be true that the transition state geometry for G3PDH is not that of LDH. How the results bear on various proposals concerning ground-state regulation of reactivity is discussed.

Introduction

The dehydrogenases (NAD-linked) and reductases (NADP-linked) catalyze the oxidation/reduction of various substrates facilitating the usually reversible hydride transfer from the C4 position of the dihydronicotinamide ring of NAD(P)H to substrate. Several hundred of these enzymes are known. As far as is known, all dehydrogenases display a marked stereospecificity. The so-called A-type dehydrogenases catalyze hydride transfer to and from the *re* face of the nicotinamide ring of NAD (pro-R specific) whereas B-type enzymes transfer to and from the *si* face (pro-S specific). Figure 1 shows a diagram of the nicotinamide moiety of NAD and its relationship to the bonded ribose. In general, enzymes that are found to transfer the pro-R hydrogen bind the nicotinamide ring in a trans conformation with respect to the ribose ring, the geometry shown in Figure 1, while pro-S transfer enzymes bind in the cis geometry. This correlation between the stereochemical transfer propensity of either the pro-R or pro-S hydrogen and the relationship of the ribose-nicotinamide ring torsional angle appears to come about because substrates bind after and on top of the nicotinamide ring. In this case, the correct face of the nicotinamide ring is brought into alignment with the substrate as dictated by geometry. In general, X-ray crystallographic studies of enzyme·

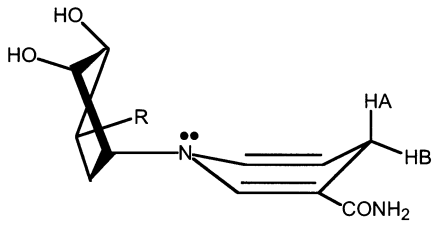

Figure 1. A molecular schematic of the nicotinamide headgroup and the bonded ribose moiety of NADH with the two groups in the trans geometry. The reduced NADH ring is shown as a boat structure with the pro-R C4 hydrogen (H_A) in the pseudoaxial geometry and the pro-S hydrogen (H_B) pseudoequatorial.

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cofactor·substrate ternary complexes show that the cofactor and substrate are tightly packed next to each other with the correct C4 hydrogen of the reduced nicotinamide ring pointed toward the hydride acceptor atom of substrate.

The electronic density in the C4−H bond, hence the force constant and frequency of the carbon−hydrogen stretch, is sensitive to the conformation of nicotinamide ring. To isolate the C4−H bond from other C−H bonds, either the C4 position of $NAD(P)^+$ can be deuterated or one of the C4 hydrogens of NAD(P)H can be stereospecifically deuterated. On the basis of vibrational normal-mode analysis of these compounds, it has been found that the force constant of this bond is most sensitive to the planarity of the nicotinamide ring.[1,2] Here, we report on Raman difference measurements of the stretching frequency of the C4-D bond of nicotinamide cofactors bound to two A-side enzymes, lactate dehydrogenase (LDH), and dihydrofolate reductase (DHFR), and the B-side enzyme, glycerol-3-phosphate dehydrogenase (G3PDH). Both protein NAD(P)H and $NAD(P)^+$ cofactor binary as well as ternary complexes prepared with inhibitors believed to be reasonable mimics of the Michaelis complex were examined. The present study extends considerably our previous work along these lines that investigated binary complexes of LDH and malate dehydrogenase with NADH.[1,2]

These studies determine the structures of the bound cofactors at the high resolution afforded by vibrational spectroscopy and, consequently, can be used to assess whether the ground-state structure of the bound cofactor is brought into an "activated" geometry or electronic state by binding to the enzymes. For example, the possibility of a strong protein induced distortion of the NAD^+ ring bound to LDH was inferred from binding equilibrium isotope effects of protonated, deuterated, and tritiated NAD^+ to LDH.[3] The predicted shift of over 100 cm^{-1} of the C4-D stretch upon binding based on the observed isotope effect would imply the formation of substantial positive charge at C4, and this was conjectured to have very important consequences for the catalytic strategy of LDH.[3] The present study directly measures shifts in the C4-D stretch and provides information about the source of the observed equilibrium isotope effect. In addition, the dihydronicotinamide ring of NAD(P)H, on the basis of computational studies, is proposed to adopt a boat shape where the transferred hydrogen occupies the axial position in those dehydrogenases studied thus far (both LDH and DFHR are included in these studies). These same studies also suggest that a boat conformation describes the geometry of the ring in the putative transition state.[4−8] Our measurements directly determine this geometrical distortion in situ. Also, variations in ring geometry so as to regulate reactivity[9−11] have also been invoked to explain the origins in specificity between A- and B-side enzymes. Perhaps with a few exceptions, the stereochemical outcome of the enzyme catalyzed reduction reaction can be correlated with the equilibrium constant, in solution, between ketone and NADH to form alcohol and NAD^+ for many or most of this enzyme class as suggested previously.[12,13]

Materials and Methods

Spectroscopy. Samples for the Raman spectroscopy were excited by laser lines from either an argon ion laser (Innova 70−5, Coherent) or a krypton ion laser (2000-CR, Coherent Radiation Inc., Palo Alto, CA), as detailed in the figure captions. Spectra were taken at 90° to the incident beam. Two separate spectrometers were used. System I. The first half of a Spex Model 1401 double monochromator was used to obtain the Raman spectra for the G3PDH enzyme. Scattering was detected by an optical multichannel analyzer (OMA) system model LN/CCD-1024 TKB/2 with ST-135 controller (Princeton Instruments Inc.) which was attached to the intermediate slit of the monochromator. Scattered excitation light was cut off by a Raman holographic edge filter (Physical Optics Corporation). System II. For the other enzymes, data were collected by a Macintosh computer (Apple, Cupertino, CA) interfaced with a CCD detector (Princeton Instruments model LN/CCD-1152UV with a ST-135 CCD controller) coupled to a Triplemate spectrometer (Spex Industries, Metuchen, NJ). All spectra were corrected for the nonidentical spectral response of the detector and calibrated against the known Raman lines of toluene. Separate spectra for enzyme and enzyme·inhibitor complexes in solution, approximate binding site concentration of a few millimolar, were measured using a special split cell (the volume of each side being about 30 ul) and a sample holder with a linear translator as previously described.[14,15] The spectrum from one side of the split cuvette is taken, the split cell is translated, and the spectrum from the other side is taken. This sequence is repeated until sufficient signal-to-noise is obtained. A difference spectrum is generated by numerically subtracting the sum of the spectra obtained from each side. In general, the two summed spectra do not subtract to zero, as judged by the subtraction of well know protein marker bands (for example, the amide-I, amide-III, and the 1450 cm^{-1} bands, the latter band being especially useful since it is generally not affected by protein conformational changes). These protein marker bands are determined from their bandwidths (generally much broader than those from spectra of bound substrates) and their characteristic positions. Hence, one summed spectrum is scaled by a small numerical factor, generally between 1.05 and 0.95, which is adjusted until the protein bands are nulled (see, e.g., ref 15). The same control procedures were performed on all the difference spectra results herein. A spectral calibration is done for each measurement using the known Raman lines of toluene, and absolute band positions are accurate to within ± 2 cm^{-1}.

Proteins. Pig H4 lactate dehydrogenase was purified from pig heart as previously described.[16] The samples were stored at −20 °C until needed for study. Before use, the sample was dialyzed against 50 mM phosphate buffer at pH 7.2. This enzyme, and the others, was prepared for Raman study by first removing insoluble protein by centrifugation and then concentrating to about 2 mM by use of a centricon centrifuge concentrator (Amicon, Lexington, MA).[17] *E. coli* DHFR was purified from strain CV634 containing the plasmid pCV29 by using a methotrexate affinity resin as previously described.[18] Rabbit muscle G3PDH was purchased from Boehringer Mannheim. Protein solutions were made up, precipitated by the addition of ammonium sulfate, and collected by centrifugation at 13000 rpm for 15 min. The precipitate was dialyzed against 10 mM Tris buffer at pH 7.4. Concentrations were determined by UV−vis absorption spectroscopy using the molar absorbance reported: LDH (a tetramer of four identical subunits), $e_{280} = 200000$ M^{-1} cm^{-1} (refs 19 and 20); DHFR (a dimer), $e_{280} = 31000$ M^{-1} cm^{-1} (ref 21); G3PDH (a dimer), $e_{280} = 48000$ M^{-1} cm^{-1} (refs 22 and 23).

Materials. NADH, NADPH, NAD^+, and $NADP^+$ were purchased from Boehringer Mannheim Co. (Indianapolis, IN). Concentrations of the cofactors were determined from their extinction coefficients: $e_{340} = 6220$ $M^{-1}cm^{-1}$ for NADH or NADPH and $e_{259} = 18000$ $M^{-1}cm^{-1}$ for NAD^+ ($NADP^+$).

Pro-R-[4-^{2}H]NADPH and pro-R-[4-^{2}H]NADH were prepared enzymatically according to published procedures,[1,24] and pro-S-[4-^{2}H]NADPH and pro-S-[4-^{2}H]NADH ($NADD_B$) were pre-

pared in a similar fashion.[1] [4-^{2}H]NADP$^+$ ([4-D]NADP$^+$) was prepared by the following modification of the San Pietro procedure.[25] About 40 mg of NADP$^+$ was dissolved in 1 mL of 1 M KCN prepared in 99.5% D_2O. To this pale yellow solution was added 8 μL of 10 N NaOD (the final pH was 11.4), and the resulting solution was incubated for 3 h at room temperature. After incubation, 5 mL of D_2O containing two millimolar equivalents of KH_2PO_4 were added, and the DCN was removed by bubbling nitrogen through the solution. The final pH was 7. The reaction product was purified by DEAE-sephacel column using nonlinear gradient of KCl from 0.0 to 0.5 M in 10 mM Tris, pH 7.0. The peak fractions with $OD_{258}/OD_{231} = 2.2$ were saved. The salt was removed by running the eluent from DEAE column through a Bio-gel P-2 column, and this eluent was lyophilized and stored in −80 °C. The purity of [4-^{2}H]NADP$^+$ was better than 95% as determined by the intensity of Raman band of NADP$^+$ at 1031 cm^{-1} which shifts down to 1018 cm^{-1} for [4-^{2}H]NADP$^+$.

Results

Deuterating a hydrogen on the nicotinamide ring will decrease its C−H stretch frequency by about 900 cm^{-1} and result in a normal mode whose motion is largely confined to the C−D stretch internal coordinate.[1] This C−D stretch will be essentially uncoupled from the other internal coordinates. The frequency of the C4-D stretch of NADH and NADPH is about 2110 cm^{-1}, while that for the C4-D stretch in NAD$^+$ or NADP$^+$ lies near 2300 cm^{-1}. This spectral region also contains some weak protein overtone bands. These bands were removed from the spectra by constructing a difference spectrum between protein·cofactor where the C4 carbon contained either a deuterium or hydrogen. The enzyme binding site concentrations used for these spectral studies generally were much higher than the dissociation constants for the various ligands used. Thus, when stoichiometric concentrations of cofactors and inhibitors are employed, they are practically all bound to the protein. Therefore, the difference spectrum is just the spectrum of the C−D stretch of the bound cofactor.

The NAD(P)$^+$ and NAD(P)H Spectra. Figure 2 (parts a−c) shows the C−D stretch band of pro-R[4-^{2}H]NADH (A-side labeled) in solution, in a binary complex with LDH and NADH, and in a ternary complex with the inhibitor oxamate, which is an analogue of pyruvate. The right side of Figure 2 shows the corresponding data for complexes made with B-side labeled cofactor, pro-S[4-^{2}H]NADH. The solution spectra (parts a and d) and the binary complex spectra (parts b and e) have been reported previously;[1] they are shown again so that comparisons can be made more easily with the herein newly obtained data of LDH and the other enzymes.

Figure 3 shows the analogous spectra of pro-R[4-^{2}H]NADPH and pro-S[4-^{2}H]NADPH in solution, in a binary complex with DHFR, and in a ternary complex with DHFR and the inhibitor methotrexate (MTX). Figure 4 shows the data for the G3PDH protein in binary complexes with pro-R[4-^{2}H]NADH and pro-S[4-^{2}H]NADH and in ternary complexes with an inhibitor, fructose-1,6-bisphosphate. Both narrowing as well as position shifts are observed when comparing spectra of NADH (or NADPH) in solution versus those for protein bound cofactors. The bandwidths (full width at half-maximum, $\Gamma_{1/2}$), deconvoluted from the instrument response bandwidth, are given in the figure captions.

Figure 5 shows the C−D stretch spectrum for [4-^{2}H]NAD$^+$ in solution, complexed with LDH, and complexed with LDH and the inhibitor oxalate. Figure 6 shows the [4-^{2}H]NADP$^+$

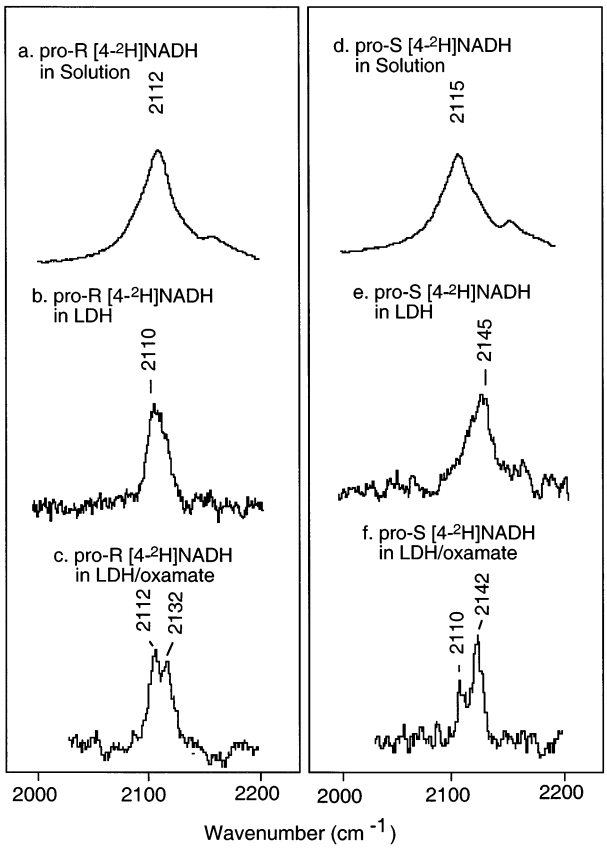

Figure 2. Raman difference spectra of (a) 100 mM pro-R [4-^{2}H]NADH at 4 °C, $\Gamma_{1/2} = 45$ cm^{-1}; (b) pro-R [4-^{2}H]NADH in LDH (LDH·NADH = 1.5/5 mM) at 4 °C, $\Gamma_{1/2} = 16$ cm^{-1} (c) pro-R [4-^{2}H]NADH complexed with LDH·oxamate (LDH·NADH·oximate = 1.5/5/5 mM) at 4 °C, $\Gamma_{1/2} = 6$ cm^{-1} for either the 2112 or 2132 cm^{-1} band; (d) 100 mM pro-S [4-^{2}H]NADH at 4 °C, $\Gamma_{1/2} = 45$ cm^{-1}; (e) pro-S [4-^{2}H]-NADH in LDH (LDH·NADH = 1.5/5 mM) at 4 °C, $\Gamma_{1/2} = 22$ cm^{-1}; (f) pro-S [4-^{2}H]NADH complexed with LDH·oxamate (LDH·NADH·oximate = 1.5/5/5 mM) at 4 °C $\Gamma_{1/2} = 6$ cm^{-1} for either the 2110 or 2124 cm^{-1} band. All spectra were obtained by subtracting the corresponding NADH spectrum taken under the same conditions. An approximately 100 mW of 488.0 or 514.5 nm argon ion laser line was used for the excitation. The resolution of the spectrometer was set at 6 cm^{-1} (full width at half-maximum).

spectrum in solution, complexed with DHFR, and complexed with DHFR·MTX. There is very little change in the spectra between the solution and binary complexes. The most dramatic difference in the ternary complexes is the substantial band narrowing of the C−D stretch band.

Structural Interpretations. NMR studies have shown that there is a sizable difference in chemical shift between the pro-R and pro-S hydrogens of NADH in solution,[26] in contrast to the present Raman data, which indicate a very modest difference in frequency between the pro-R[4-^{2}H]NADH and pro-S[4-^{2}H]-NADH C−D stretch frequencies. The difference in chemical shifts between the pro-R and pro-S hydrogens have been interpreted as arising from boat structures induced by a ring stacking interaction between the dihydronicotinamide and adenine ring,[26] which brings one hydrogen into an pseudoaxial orientation and the other pseudoequatorial. The equilibrium distribution between stacked and unstacked conformations is near one.[26] The C4-D stretch frequency is sensitive to the planarity of the ring, and an axial deuteron will yield a stretch frequency that is lower than an equatorial deuteron (see below). The near equality of the pro-R[4-^{2}H]NADH and pro-S[4-^{2}H]-NADH stretch frequencies in solution suggest that the overall conformation of the reduced nicotinamide ring is essentially

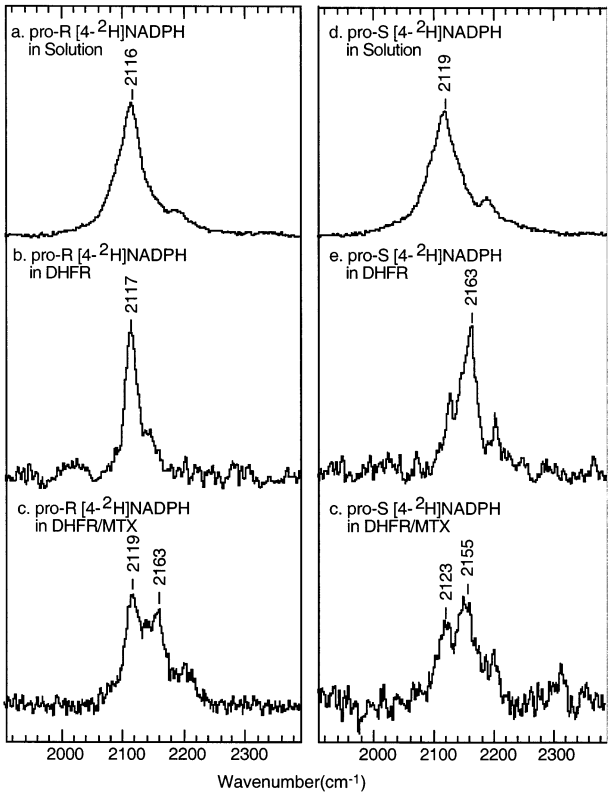

Figure 3. Raman difference spectra of (a) 100 mM pro-R [4-^{2}H]-NADPH at 4 °C, $\Gamma_{1/2} = 41$ cm^{-1}; (b) pro-R [4-^{2}H]NADHP in DHFR at 4 °C, $\Gamma_{1/2} = 21$ cm^{-1}; (c) pro-R [4-^{2}H]NADHP complexed with DHFR·MTX at 4 °C, $\Gamma_{1/2} = 25$ cm^{-1} for either the 2119 or 2163 cm^{-1} band; (d) 100 mM pro-S [4-^{2}H]NADPH at 4 °C, $\Gamma_{1/2} = 45$ cm^{-1}; (e) pro-S [4-^{2}H]NADPH bound to DHFR at 4 °C, $\Gamma_{1/2} = 25$ cm^{-1}; (f) pro-S [4-^{2}H]NADPH complexed with DHFR·MTX at 4 °C, $\Gamma_{1/2} = 18$ cm^{-1} for either the 2123 or 2155 cm^{-1} band. All spectra were obtained by subtracting the corresponding NADPH spectrum taken under the same conditions. Enzyme and ligand concentrations were about 4 mM. Laser excitation for b and e was the 568.2 nm line at about 120 mW, and the rest were taken with the 647.1 nm line running at 120 mW from a krypton ion laser. The resolution of the spectrometer was set at 6 cm^{-1} (full width at half-maximum).

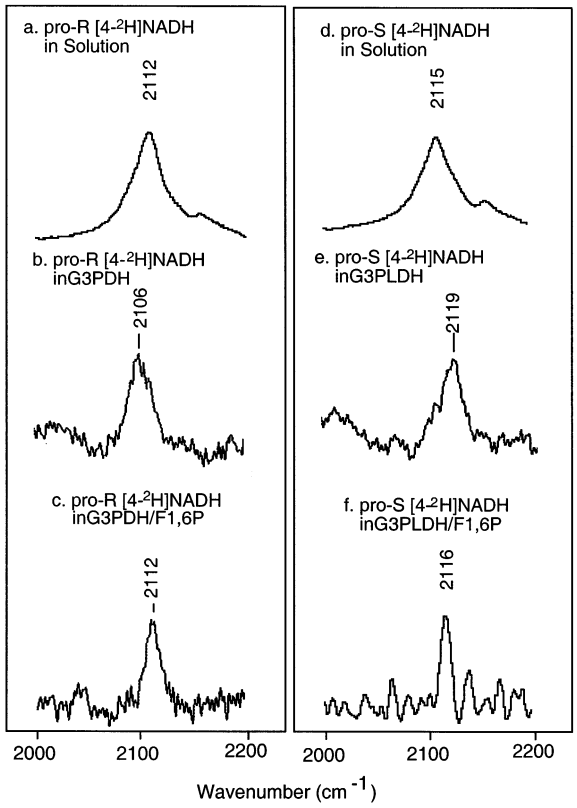

Figure 4. Raman difference spectra of (a) 100 mM pro-R [4-^{2}H]NADH at 4 °C, $\Gamma_{1/2} = 45$ cm^{-1}; (b) pro-R [4-^{2}H]NADH in G3PDH (G3PDH·NADH = 1.5/<3 mM) at 4 °C, $\Gamma_{1/2} = 24$ cm^{-1}; (c) pro-R [4-^{2}H]NADH complexed with G3PDH·fructose-1,6-diphosphate (G3PDH·NADH·fructose-1,6-diphosphate = 1.5/<3/30 mM) at 4 °C, $\Gamma_{1/2} = 11$ cm^{-1}; (d) 100 mM pro-S [4-^{2}H]NADH at 4 °C, $\Gamma_{1/2} = 45$ cm^{-1}; (e) pro-S [4-^{2}H]NADH in G3PDH (G3PDH·NADH = 1.5/<3 mM) at 4 °C, $\Gamma_{1/2} = 21$ cm^{-1}; (f) pro-S [4-^{2}H]NADH complexed with G3PDH·fructose-1,6-diphosphate (G3PDH·NADH·fructose-1,6-diphosphate = 1.5/<3/30 mM) at 4 °C, $\Gamma_{1/2} = 11$ cm^{-1}. All spectra were obtained by subtracting the corresponding NADH spectrum taken under the same conditions. An approximately 100 mW of 514.5 nm argon ion laser line was used for the excitation. The spectral resolution was 4 cm^{-1} (full width at half-maximum).

planar. Hence, we reported previously that the stacking interaction has only a very small affect on the C4-D stretch frequency, since the positions of stacked and unstacked NADH bands lie within a couple of wavenumbers of each other and since the band shapes are also similar.[1]

The C4-D stretch frequency of the reduced nicotinamide ring is sensitive to a number of structural factors. We have previously performed quantum mechanical ab initio and semiempirical calculations as well as studies on model systems to sort out the relative importance of the various contributions.[1,2] These findings are summarized below.

The orientation of the amide group affects the C4-D stretch frequency of the reduced nicotinamide ring. A rotation of the C═O bond from cis with respect to the C4-D bonds to trans will raise *both* C4-D stretches by about 10−20 cm^{-1}. The orientation of the amide group in solution[27] is probably cis and trans for NAD(P)H bound to each of enzymes studied here and probably all other NAD(P)H dependent enzymes as well. Hence, we expect that the average frequency of the two C4-D stretches will increase as NAD(P)H binds to the three proteins. The dependence of the C4-D stretch on the orientation of the amide does not affect significantly the difference in frequency between the two stretch modes.

Nearby polarizing groups, either atoms carrying partial charges or dipoles, can affect the frequency of one C4-D mode more than another depending on the proximity of the electrostatic interaction. From the X-ray structure of LDH complexed with NADH, there are no nearby charged groups that should have a noticeable effect on the stretch frequencies. In DHFR, we noted previously[1] that the oxygen of Ala-97 carbonyl is within 3.3 Å of the C4 carbon on the nicotinamide ring and is on the pro-S side. This was estimated to yield a ca. 22 cm^{-1} upward shift of the pro-S C4-D stretch relative of the pro-R C4-D stretch.

The most important structural factor contributing to the difference between the two C4-D stretch frequencies is the formation of a nonplanar ring. The two bonds have equal force constants for a planar symmetrical reduced nicotinamide ring and, hence, equal stretching frequencies. This degeneracy is removed, however, as the ring is deformed. For example,[1] if the C4 carbon is out of the plane of the ring by 15°, forming a pseudoboat structure, the stretching frequency of the axial bond was calculated to decrease by about 5−14 cm^{-1} while the equatorial bond increased by 11−18 cm^{-1}, which yields a difference between the two frequencies of 16−32 cm^{-1}. The results of these calculations are consistent with vibrational studies on other bent ring systems.[28]

Structures of the Reduced Nicotinamide Ring in the Binary Complexes. The pro-R and pro-S C4-D stretch frequen-

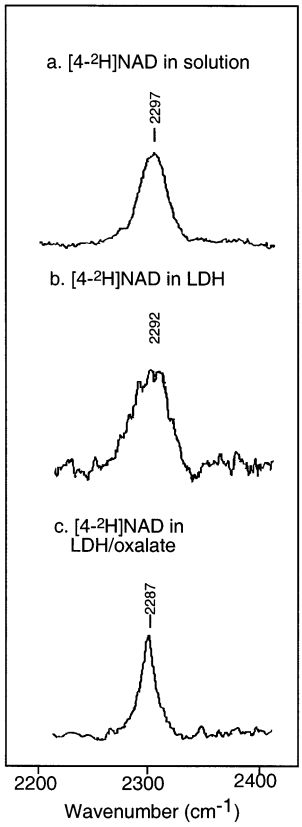

Figure 5. Raman difference spectra at 4 °C of [4-^{2}H]NAD$^+$ (a) in aqueous solution, $\Gamma_{1/2} = 25$ cm^{-1}; (b) bound to LDH binary complex, $\Gamma_{1/2} = 28$ cm^{-1}; (c) bound to LDH ternary complex with oxalate, $\Gamma_{1/2} = 8$ cm^{-1}. All spectra were obtained by subtracting the corresponding NAD$^+$ spectra under the same conditions. Enzyme concentrations were about 1.5 mM and ligands about 5 mM. An approximately 100 mW of 488.0 or 514.5 nm laser line was used for the excitation. The resolution of the spectrometer was set at 6 cm^{-1} (full width at half-maximum).

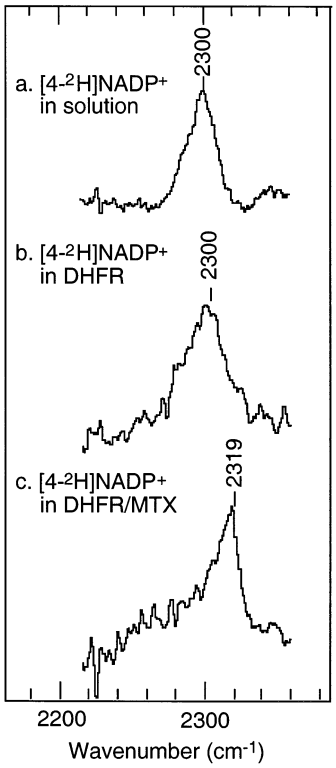

Figure 6. Raman difference spectra at 4 °C of [4-^{2}H]NADP$^+$ (a) in aqueous solution, $\Gamma_{1/2} = 20$ cm^{-1}; (b) bound to DHFR binary complex, $\Gamma_{1/2} = 20$ cm^{-1}; (c) bound to DHFR ternary complex with MTX, $\Gamma_{1/2} = 15$ cm^{-1}. All spectra were obtained by subtracting the corresponding NADP$^+$ spectra under the same conditions. Enzyme concentrations were about 4 mM. Laser excitation was 568.2 nm, 120 mW. The resolution of the spectrometer was set at 6 cm^{-1} (full width at half-maximum).

cies of NADH and NADPH in solution (Figures 2 and 3) are nearly equal (and equal to each other as expected), suggesting that the reduced ring of the cofactor adopts a planar conformation on average. Both bands are unusually broad (bandwidth of 41−45 cm^{-1}) compared to the bandwidth of other C−D stretch mode such as the NADH C2-D stretch mode (bandwidth of 17 cm^{-1}, unpublished data). Such band broadening suggests that the ring adopts multiple conformations, where each conformer yields a somewhat different stretching frequency. According to our earlier normal mode studies,[1] the bandwidth is best explained structurally as arising from the ring adopting a range of boat conformations. Assuming that the entire width of the C4-D stretch of either NADH or NADPH comes from an ensemble of nonplanar rings, the observed 41−45 cm^{-1} implies a variation of just under ±7° for the C4 carbon above and below the plane formed by the C2, C3, C5, and C6 carbons. This is in line with calculations of the energy difference between planar and boat forms, which show that bringing the C4 carbon out of the ring plane by 15° only costs about 1 kcal/mol.[1,29]

When NADH binds to LDH, the C4-D stretch of the pro-R C4-D stretch lies 35 cm^{-1} below that of the pro-S C4-D stretch while the average of the two shifts up by 14 cm^{-1} from the average frequency measured for the unbound cofactor (Figure 2). As discussed and analyzed in our previous work,[1] there are no real polarizing protein atoms or moieties near the C4 atom of NADH bound to the active site of LDH. Hence, part of this shift in frequency is assigned to the rotation of the amide of the unbound dihydronicotinamide ring from a cis carbonyl relative to the C4 found in solution to a trans carbonyl in the binary complex and the rest assigned to those interactions between the cofactor and enzyme that stabilize a boat conformation for the dihydronicotinamide ring by bringing the C4 carbon out of the plane by about 15°. In the bound binary structure, the lower frequency of the pro-R C4-D has been assigned to a pseudoaxial conformation while the higher frequency of the pro-S C4-D corresponds to a pseudoequatorial position. [1,2] The C4-D stretch bandwidths are reduced by over a factor of 2 upon binding to LDH (see Figure 2). This indicates that the enzyme restricts the conformational freedom of the ring and perhaps also the orientational freedom of the amide. Since the band shape of the C4-D stretches are asymmetric, it is possible that there is a small amount of reduced nicotinamide in a different conformation in which the pro-S C4-D is at the pseudoaxial position and the pro-R C4-D at the pseudoequatorial position.

The frequency pattern of the two C4-D stretch modes of NADPH bound to DHFR is much the same as that found for LDH·NADH (compare Figures 2 and 3). The major difference is that the frequency of pro-S stretching mode in DHFR·NADPH is somewhat larger than that found for LDH·NADH (46 cm^{-1} compared to 35 cm^{-1}). As previously analyzed,[1] most of the difference between the two C4-D stretch modes arises from a deformation of the nicotinamide ring bringing C4 out of plane with some or much of the extra 9 cm^{-1} shift due to a interaction between the pro-S C4-D bond with the close by oxygen of Ala-97's carbonyl. Like NADH in LDH, the pro-R C4 deuteron is pseudoaxial and the pro-S deuteron is pseudoequatorial, and their C−D stretch bandwidths are also reduced by about a factor of 2 upon binding to DHFR. Modeling of the nicotinamide moiety bound to DHFR on the bases of crystallographic data

also suggests that the dihydronicotinamide ring adopts a pseudoboat structure.[30]

The frequency pattern for the C4-D is quite different when NADH binds to the B-side enzyme, G3PDH. First, little change in the average frequency is measured for the two stretching modes although the bandwidths are reduced by a factor of 2. This is not consistent with the prediction of normal-mode analysis for the situation when the amide carbonyl of the dihydronicotinamide ring changes from cis to trans to the C4 upon forming binary complex with G3PDH from Leishmania mexicana.[31] It is unclear why the average frequency did not change unless the structure of the protein from L. mexicana differs from the rabbit muscle protein used in this study, not likely in our opinion. Another possibility is that electrostatic interactions with both C4-D bonds could effectively counter the shift in average frequency. There is very little splitting between the pro-R and pro-S C4-D stretch frequencies, just 13 cm^{-1} compared to 46 cm^{-1} for DHFR·NADH and 35 cm^{-1} for LDH·NADH. This could be due to a small distortion of the planar nicotinamide ring. If so, like the two A-side proteins, the pro-R C4 deuteron is brought to a slight pseudoaxial geometry and the pro-S deuteron to pseudoequatorial. Another possibility for the small difference between the two frequencies is that the oxygen of an active site serine residue is close to C4 and points toward the pro-R side of the ring while a water molecule is close to C4 from the B face.

Structures of the Oxidized Nicotinamide Ring in the Binary Complexes. The C4-D stretch bandwidth of oxidized nicotinamide in solution is about 2-fold narrower than that of the reduced nicotinamide. This indicates the conformational freedom of the oxidized nicotinamide in solution is much more limited compared the reduced form. The C4-D stretch mode is essentially unaffected when $NAD(P)^+$ binds to either LDH or DHFR. Neither the position nor the bandwidth is changed by binding. Such results indicate that the constraints on the oxidized nicotinamide imposed by the enzyme in the binary complexes are not strong enough to reduce the C4-D bandwidth. In the case of $NADP^+$ and DHFR, this is easily understood because the nicotinamide moiety is probably not bound in the binary complex, as is found in the X-ray crystal structures.[32]

Structures of the Nicotinamide Ring in the Ternary Complexes. The two ternary complexes for A-side enzymes show very similar patterns in the C4-D stretch spectral region. The C−D stretch mode splits into two peaks with much narrowed bandwidths compared to that of the major conformer in the binary complex. The results can be best interpreted in terms of two conformations of the nicotinamide rings, each with very restricted freedom of deformation. One conformer is similar to the conformer found in the respective binary complex where the pro-R C4 deuteron is brought to a pseudoaxial and the pro-S deuteron in pseudoequatorial geometry. In the other conformer, the pro-R C4 deuteron is pseudoequatorial and the pro-S deuteron is pseudoaxial. In the ternary complex data involving G3PDH, only a single band is observed and, hence, only a single conformer. The nearly equal frequencies for C4 deuteron of NADH bound in the G3PDH ternary complex suggest that the ring is essentially planar. For all three ternary complexes, the bandwidths of the C4-D stretch modes have narrowed by a factor of 2 from those of the binary complexes (see Figures 2−4), indicating the flexibility of the nicotinamide is further limited upon binding of the inhibitor.

Small shifts are observed for the C4-D stretch of $NAD(P)^+$ in the ternary complexes made with LDH and DHFR compared to the respective binary complexes, but it is hard to discern any pattern since the shift is positive in one protein and negative in the other. On the other hand, the obvious changes in the ternary complexes are the 2-fold or more narrowing of the C−D stretch mode compared to that in the binary complexes. Such results indicate that the deformation of the oxidized ring is also further limited upon binding of the inhibitor, similar to that in the ternary complexes with the reduced cofactor.

Discussion

The Raman spectra were determined for unbound NADH, specifically deuterated at either the pro-R or pro-S C4 carbon of the dihydronicotinamide, NAD^+, deuterated at the C4 carbon of nicotinamide, and NADPH and $NADP^+$, deuterated in a likewise manner. Spectra were also measured for these cofactors bound to three proteins: two pro-R specific enzymes, lactate dehydrogenase and dihydrofolate reductase, and a pro-S specific protein, glycerol-3-phosphate dehydrogenase. Finally, spectra were determined for ternary complexes with inhibitors that, to a first approximation, mimic the Michaelis complexes of these three proteins.

It has been suggested that the frequencies of the dihydronicotinamide and nicotinamide C4−H stretch, which are a function of bond force constant and energy of the bond, could be related to hydride transfer rates.[1] For NAD^+, a consideration of simple resonance structures suggests that increased positive change on the C4 carbon could occur upon formation of a enzyme binary complex and increases in C4 reactivity of the bound NAD^+ toward nucleophiles are known to occur.[40] In addition, binding isotope studies on LDH using C4-D and C4-T indicate that the C4−H bond becomes less stiff when NAD^+ binds to the enzyme. A decrease in the stretching frequency of the C4−H would be consistent with these observations.[3,33] Unfortunately, the results of model systems do not bear out such simple concepts. We have performed measurements on a series of NAD^+ analogue N-alkylnicotinamides whose rates of reduction by cyanoborohydride in aqueous solution differ by 3 orders of magnitude[34] but found only small changes in their C4-D stretch frequencies (on the order of 1 cm^{-1}; unpublished data). On the NADH side, there also appears to be very little relationship between homolytic bond energy and the equilibrium constant for hydride transfer over a series of NADH model compounds.[35] Since the C4-D stretch frequency is expected to scale as the homolytic bond energy, these findings indicate little direct relationship between frequency and activity. In addition, large shifts in the frequency of the C4-D stretch are not observed when NAD(P)H binds to the enzymes studied thus far and these shifts are generally not larger than the heterogeneously broadened band of the cofactor's solution spectrum. This suggests that the large rate enhancements accomplished by NAD(P)H dependent enzymes, which can approach 10^{15}-fold,[40] do not reside in the enzymes ability to activate C4−H in the ground state by changing the electronic nature of the C4−H bond.

Alternatively, enzymes in accelerating bimolecular reactions perhaps hold the reactants in sufficiently close proximity that the reaction occurs more efficiently than in solution. First proposed and labeled as the "proximity" effect[36] and developed further over the years (e.g., ref 37), this concept recently has been developed quantitatively using numerical simulations.[38,39] These computational studies emphasize bringing the substrates from the ground state to a state closely resembling the structure of the transition state that is called the "near attack conformation" (NAC's). Several states may be accessible from the ground-state enzyme·substrate complex with some states approaching the NAC structure and, hence, be poised for bond breaking/

making to occur while others are not catalytically productive. The enzymatic rate enhancement is then proportional to the fraction that the complex spends in the NAC conformation.

That "freezing" in of specific conformations occurs and is of importance for the catalytic mechanism of the NAD(P) linked enzymes is supported by both theoretical and experimental results. For LDH, Burgner and Ray[40] have estimated that immobilization of the reactants (of both cofactor and substrate) at the active site of LDH contributes at least 1000-fold (ca. 4.2 kcal/mol) to the lowering of the transition state barrier for the hydride transfer reaction between cofactor and substrate. In molecular dynamics calculations on LDH, we have shown that motions of the protein atoms, for almost every atom, are more limited in the putative Michaelis complex than in the binary LDH·NADH complex.[41] Recently, we have shown that there are no detectable motions on the 10 ns−10 ms time scale between the bound pyruvate substrate and key active site residues of LDH in temperature jump relaxation studies.[42] Using molecular dynamics simulations, substates in the NAC conformation were estimated to represent a mole fraction of more than 50% in the active site of LDH,[4,38] an especially high fraction among the few enzymes that have been so studied. Similar calculations for DHFR yielded a mole fraction of 20%[38] or 43%.[8]

The present results, in agreement with the above, show that the conformational flexibility of the nicotinamide ring is greatly reduced when NAD(P)H or $NAD(P)^+$ binds to form protein·cofactor binary complexes in most cases and is reduced further in ternary protein·cofactor·inhibitor complexes in all cases. This can be deduced from the changes of the heterogeneously broadened bandwidths of the C4-D stretch modes of the cofactor upon binding to binary and ternary complexes. The reduction in bandwidth of NADH from its solution value for $\Gamma_{1/2}$ of 45 cm^{-1} to 16 cm^{-1} in the binary complex with LDH, for example, to just 6 cm^{-1} in the ternary complex, as shown in Figure 2, is brought about by a decrease in the freedom of thermo-motion around a specific cofactor conformation. Similar changes in bandwidth were found when the reduced cofactor binds to DHFR and G3PDH. Bandwidth narrowing is also found when $NAD(P)^+$ binds to LDH and DHFR although this effect is largely confined to the ternary complexes. Hence, a decrease in the thermo-motion around the productive cofactor conformation in the ternary complexes is perhaps a general phenomena for dehydrogenases.

Do these enzyme induced structural changes of the substrate (analog) in the dihydronicotinamide moiety lie on the reaction coordinate for hydride transfer? Our results suggest that the geometry of the reduced nicotinamide ring is constrained near the geometry expected for transition state of a hydride transfer, at least for LDH. In solution, the width of C4-D stretch band of the reduced nicotinamide ring likely arises from a mixture of pseudo-boat conformers with the C4 carbon either above or below the remainder of the ring. The binary complex of NAD(P)H with either LDH or DHFR seems to have fewer conformers available to the reduced ring suggesting that binding energy is used to select a nonplanar geometry. For LDH·NADH, computational studies suggest a boat geometry for the ring with the C4 approximately 15° out of plane and with the pro-R proton in a pseudoaxial position. In the ternary complexes, enzyme·cofactor·inhibitor, there are two conformers with nearly equal populations for both LDH and DHFR. Both are boat forms of the ring. One conformer is quite similar in geometry to that found in the binary complex where the pro-R C4 deuteron is pseudoaxial and the pro-S deuteron is in a pseudoequatorial geometry. The second conformer has the pro-R C4 deuteron pseudoequatorial and the pro-S deuteron pseudoaxial. The second is likely an analogue of a nonproductive ternary complex since it is in a wrong geometry for chemistry to occur.

It is often proposed that a specific C4−H of NAD(P)H is 'activated' toward hydride transfer by bringing the transferring hydrogen to an axial position. Originally, this was proposed because an axial attack of a hydride ion on the nicotinamide ring would involved the smallest shift of atoms.[5] Wu and Houk[6] suggested on the basis of gas phase ab initio calculations that the geometry of the transition state involves a boat conformation with the developing hydride pseudoaxial. However, the surrounding protein matrix may affect the correct geometry[7] as can the hydride acceptor atom (nitrogen found in DHFR versus carbon as in LDH).[6] Thus, it is important to consider the entire system. Molecular dynamics calculations[4] show that the reduced ring of NADPH bound to DHFR is predicted to adopt a modest boat structure in the transition state bringing the pro-R hydrogen axial, in agreement with the present experimental findings of the ground state of bound NADPH, although combined ab initio molecular dynamics calculations[7] find a planar transition state structure for NADPH in DHFR. No direct calculations exist for G3PDH, but the reduced ring bound to glyceraldehyde 3-P dehydrogenase, which binds the ring of cofactor cis to the ribose moiety[43] like G3PDH, is calculated to adopt close to a planar transition state structure.[4] This is the ground-state structure we have found for NADH bound to G3PDH.

For LDH, the molecular dynamics and semiempirical quantum mechanical calculations of Almarsson and Bruice[4] find that the pro-R hydrogen adopts a pseudoaxial geometry and attribute the calculated decreased activation enthalpy toward hydride transfer from boat forms to an increased π-character of the C4−H(axial) bond allowing greater overlap throughout the trajectory. In these calculations, it was found that a puckering of the dihydronicotinamide ring to bring the C4 and N1 out of plane by 15° and 5°, respectively, resulted in a net kinetic advantage of 4−5 kcal/mol. Our structural findings of NADH bound to LDH·oxamate are in outstanding agreement with these calculation. In the major population of the putative LDH·NADH·oxamate Michaelis complex, the pro-R hydrogen adopts a pseudoaxial geometry of just the predicted geometry.[44] On the other hand, we find two well populated conformers, and the second conformer has the pro-R hydrogen equatorial, predicted to be relatively nonproductive. This is in disagreement with the calculations of Almarsson and Bruice who found that the bulky isoleucine group on the pro-S side of the reduced nicotinamide ring forced the pro-R hydrogen axial about 97% of the time.

As reviewed above, it has been supposed (apparently incorrectly) that $NAD(P)^+$ activation could be brought about by an enzymic induced distortion whereby positive charge accumulates on C4, making the oxidized ring a better acceptor of the negatively charged hydride ion. That an enzyme could produce a significant charge rearrangement was in agreement with a study showing a substantial isotope effect on the binding of deuterated and tritiated NAD^+ to LDH.[3] If the entire isotope effect was expressed as a shift in the C4−H stretch frequency upon binding, the calculated stretching frequency decrease of ca. 100 cm^{-1} upon binding implies an increased positive charge at C4. However, the present results make it clear that the isotope effect must originate elsewhere, perhaps from out of plane bending modes, since no such frequency shift occurs when NAD^+ binds to LDH.

Finally, Benner and his colleagues[9−11] pointed out a correlation between the geometric arrangements of substrate and

cofactor at the active sites of dehydrogenases and reactivity of substrate. For a solution $K_{eq} < 10^{-11}$ M, the pro-R hydrogen is transferred on the enzyme in most cases while for $K_{eq} > 10^{-11}$ M, it is the pro-S hydrogen (K_{eq} = [ketone][NADH][H^+]/[alcohol][NAD^+]). They argued that the conformation of NAD(P)H is such that it is a weaker reducing agent when bound to pro-R proteins versus pro-S proteins in order to explain this correlation. This conjecture is based on the observation that the internal equilibrium constants on the enzyme between substrate and product are often close to unity while the solution equilibrium constant varies widely among the reactions that are catalyzed by the dehydrogenases and reductases. Since pro-R enzymes typically bind cofactor in the trans geometry while pro-S bind in the cis arrangement, this led to an attempt to understand reactivity based on the torsional angle between the nicotinamide ring and its bonded ribose. It was argued that the regulation of reactivity could be brought about through the donation of electron density from the lone pair on the N1 of the dihydronicotinamide ring into the adjacent antibonding orbital of the sugar-carbon bond from the ring to the bonded ribose group. Such interactions would make the pro-R hydrogen at the 4-position axial when the relationship between the dihydronicotinamide ring and the adjacent ribose group of the cofactor is in the trans geometry (see Figure 1) and equatorial when the cofactor adopts the cis geometry.[11] It is clear from our data that other interactions between the apoprotein and cofactor, particularly steric effects, overwhelm this "reverse anomeric effect". G3PDH binds NADH in a cis conformation, but the dihydronicotinamide ring is basically planar for G3PDH·NADH and not the predicted pro-S pseudoaxial geometry. If the ability to reduce a substrate is correlated with bringing the appropriate hydrogen to an axial geometry, our results show just the opposite behavior from that predicted, since the pro-R proteins bind NAD(P)H with the pro-R hydrogen in a pseudoaxial geometry while the only pro-S enzyme binds it in a planar conformation. On the other hand, recent theoretical studies (e.g., refs 4 and 7) seem to indicate that the NAC structure for the NAD(P)H linked enzymes varies with regard to the planarity of the nicotinamide ring.

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