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Raman Spectroscopic Studies of NAD Coenzymes Bound to Malate Dehydrogenases by Difference Techniques†

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ABSTRACT: We report here on the Raman spectra of NADH, 3-acetylpyridine adenine dinucleotide, $APAD^+$, and a fragment of these molecules, adenosine 5′-diphosphate ribose (ADPR) bound to the mitochondrial (mMDH) and cytoplasmic (or soluble, sMDH) forms of malate dehydrogenase. We observe changes in the Raman spectrum of the adenosine moiety of these cofactors upon binding to mMDH, indicating that the binding site is hydrophobic. On the other hand, there is little change in the spectrum of the adenosine moiety when it binds to sMDH. Such observations are in clear contrast with those results obtained in LDH and LADH, where there are significant changes in the spectrum of the adenosine moiety when it binds to these two proteins. A strong hydrogen bond is postulated to exist between amide carbonyl group of NAD^+ and the enzyme in the binary complexes with both mMDH and sMDH on the basis of a sizable decrease in the frequency of the carbonyl double bond. The interaction energy for formation of a hydrogen bond is the same as found previously for LDH, and we estimate that it is 2.8 kcal/mol more favorable in the binary complex than in water. A hydrogen bond is also detected between the amide $-NH_2$ group of NADH and sMDH that is stronger than that formed in water and is of the same size as found in LDH. Surprisingly, the hydrogen bond to the $-NH_2$ group in mMDH is the same as that found for water. The strength of these hydrogen bonds likely contributes to the varying observed λ_{max} shifts in the near-UV absorbance of the reduced nicotinamide when it binds to these enzymes. The lack of increased hydrogen-bond strength in the binary complex of NADH with mMDH relative to that found in water is proposed as a partial explanation of the red-shift in λ_{max} found for reduced binary complex with mMDH, which is unusual for an A-side dehydrogenase. On the basis of the strength of the observed hydrogen-bond interactions between the acetyl moiety of $APAD^+$ and the enzyme in the binary complex, we estimate that the energy difference between the syn and anti conformers of the pyridinium ring of the bound cofactor is at least 7.3 kcal/mol in mMDH and substantially more than this in sMDH and LDH where stronger interactions with the amide $-NH_2$ can occur. This energy difference is sufficient, or nearly so, to determine the high degree of stereospecificity that is observed with these enzymes during transfer the *pro-R* hydrogen from NADH to substrate (LaReau et al., 1989).

To further our understanding of the changes in the bond orders of cofactors and substrates when they are transferred from the solvent to the more restrictive environment in the active site of a dehydrogenase, we extended our studies using sensitive Raman difference spectroscopy begun with ADH[1] and LDH (Callender et al., 1988; Chen et al., 1987; Deng et al., 1989a,b; Yue et al., 1984) to the mitochondrial and cytosolic forms of malate dehydrogenase. The Raman spectrum of the bound molecule is obtained by measuring the spectrum

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[1] Abbreviations: NADH, reduced β-nicotinamide adenine dinucleotide; mMDH, mitochondrial malate dehydrogenase; sMDH, cytoplasmic malate dehydrogenase; ADPR, adenosine 5′-diphosphate ribose; $APAD^+$, oxidized acetylpyridine adenine dinucleotide; OMA, optical multichannel analyzer; LADH, liver alcohol dehydrogenase; LDH, lactate dehydrogenase.

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of the enzyme/substrate complex and subtracting the spectrum of the enzyme itself. This results in the spectrum of the bound molecule and other molecular moieties that have been perturbed by substrate binding. Upon binding, the electronic nature of the bound cofactor or substrate is changed, often through noncovalent interactions. The modified electronic distribution results in modified vibrational force constants sometimes along quite well-defined and important coordinates. Thus, the changes observed in the vibrational spectra are a direct measure of how enzymes act upon their substrates. In general, the object of the specific noncovalent interactions between an enzyme and its substrate is either to accelerate bond making/breaking processes or to discriminate between various molecules, i.e., molecular recognition. Thus, a characterization of the origins and magnitudes of the interaction energies are of substantial importance.

The MDH enzymes reversibly catalyze the stereospecific transfer of a hydride ion from L-malate to NAD^+, forming oxalacetate and NADH (Straub, 1942). They are similar to LDH in many aspects. For example, they are homologous in both the coenzyme binding and the catalytic domains as judged by comparisons of both their amino acid sequences and their molecular structures (Birktoft et al., 1982). Each of these enzymes directly transfer a hydride ion, H^-, from the *pro-R* face (A-side) of NADH to the C2 carbon of a 2-ketoacetate, and they function via an ordered addition of reactants and release of products [cf., Adams (1987)]. Finally, both kinds of enzymes bind coenzymes with nearly the same affinity (Holbrook et al., 1975; Holbrook & Wolfe, 1972). However, there are differences in the properties of NADH bound to either mMDH or sMDH (or LDH). For example, the λ_{max} of NADH is red-shifted by about 13 nm when NADH is bound to mMDH but blue-shifted by about 5 nm when it binds to sMDH (or LDH) (Fisher et al., 1969).

Here, we report on the Raman spectra of NADH and NAD^+ and an active analogue of NAD^+, $APAD^+$, when bound to two the forms of malate dehydrogenase. To make band assignments to motions located on smaller components of the coenzymes, we have also obtained the spectrum of bound ADPR, a fragment of NAD that lacks the nicotinamide group. From these data, a number of conclusions on and a characterization of the interactions between the coenzymes and mMDH and sMDH can be reached. These are compared to that previously found for ADH and LDH.

Materials and Methods

NADH (100%) and $APAD^+$ were purchased from Boehringer Mannheim Co. (Indianapolis, IN); ADPR was purchased from Sigma Chemical Co. (St. Louis, MO); all were used without further purification. Pig heart sMDH was purchased from Sigma Chemical Co. and then repurified by the procedure of Banaszak (1966). Pig heart mMDH was purchased from Calibiochem (La Jolla, CA) as a ammonium sulfate suspension and used without further purification. Just prior to the Raman experiment, the enzymes were dialyzed against 0.1 M phosphate buffer at pH 7.2 and 4 °C for several hours. After the insoluble protein was removed by centrifugation, the enzyme solution was concentrated to 4–5 mM (8–10 mN binding site concentration) with a Centricon centrifuge concentrator, Centricon 30 (Amicon, Lexington, MA).

The enzyme activity was measured before and after each Raman experiment; no significant activity loss was detected. Concentrations of enzyme and coenzymes were determined by UV–vis absorption spectroscopy, with ϵ_{280} = 19 000 M^{-1} cm^{-1} for mMDH, ϵ_{280} = 66 000 M^{-1} cm^{-1} for sMDH (Theorell & Langan, 1960), ϵ_{340} = 6220 M^{-1} cm^{-1} for NADH, ϵ_{259} = 18 000 M^{-1} cm^{-1} for $APAD^+$, and ϵ_{259} = 15 200 M^{-1} cm^{-1} for ADPR. Since both MDHs contain two independent active sites, binary complexes of MDH were prepared by using a 1:<2 molar ratio of MDH to either NADH, $APAD^+$, or ADPR. The concentrations of the enzymes are significantly larger than the dissociation constants for each of the coenzymes and analogues used here (Holbrook & Wolfe, 1972); so, greater than 90% of the coenzymes (or analogues) are bound.

A specially fabricated split-cell cuvette was used to hold the sample. The cuvette contained two 2.5- by 3-mm compartments with a 1-mm spacer separation. About 25 μL of the enzyme sample was loaded into one side of the split-cell cuvette, while the same amount of enzyme/substrate complex was loaded into the other side. The cuvette was transferred to a cuvette holder (maintained at 4 °C in a bath/circulator) for measurement. The translational motion of the cuvette holder, moving each side of the split-cell cuvette in and out of the Raman exciting laser beam, was achieved by a translator stage-stepping motor combination (model Unidex XI with ATS302 stages, Aerotech Inc., Pittsburgh, PA). This was interfaced to a Mac II computer (Apple Computer), which controlled the entire experiment. Positioning to within ±1 μm was achieved.

Raman spectra were obtained with an optical multichannel analyzer (OMA) system consisting of a Triplemate spectrometer (Spex Industries, Metuchen, NJ) and a model 1420 reticon solid-state detector system photodiode array and a model 1218 controller (EG&G, Princeton Applied Research, Princeton, NJ). The detector was interfaced to the Mac II computer that is also used for data manipulation and analysis. Typically, 80 mW of the 488-nm line or 40 mW of the 457.9-nm line from an argon laser (model 165, Spectra Physics, Mountain View, CA) was used to excite Raman scattering. Under these conditions, about 800 cm^{-1} (for the 488-nm line) or 1000 cm^{-1} (for the 457.9-nm line) can be detected simultaneously. The instrument was calibrated against known Raman peaks of toluene. Absolute band positions are accurate to within ±2 cm^{-1}, and relative band positions are somewhat more accurate, ±1.5 cm^{-1}. The slits were set to achieve a resolution of 8 cm^{-1}. None of the spectra presented here have been smoothed.

The procedures and controls that we use in obtaining sensitive Raman difference spectra between a protein and a protein/ligand binary complex have been discussed at length previously (Deng et al., 1989c; Yue et al., 1989). Differences as small as 0.1% can be detected.

Results

Figure 1 shows the difference spectra of NADH bound to mMDH (spectrum a), sMDH (spectrum b), and free in solution (spectrum c). The spectra of these binary complexes are dominated by the Raman intensities of the protein, and the contribution of the bound cofactor to these spectra ranges from 0.5 to 30% of the protein bands, depending on the cofactor band. Thus, the spectra of NADH bound to these enzymes are generated by subtracting the spectrum of the apoenzyme from that of the binary complex according to procedures described elsewhere (see Materials and Methods). Since we are able to measure Raman intensities for both the binary complex and the protein with an accuracy of 0.1%, extremely good signal-to-noise ratios are achieved for the difference spectra of the stronger bands. The intensity of those bands originating from the dihydronicotinamide moiety in the difference spectrum for mMDH (Figure 1a) is somewhat larger than those observed for NADH either free in solution or bound to sMDH (Figure 1b) or to other dehydrogenases

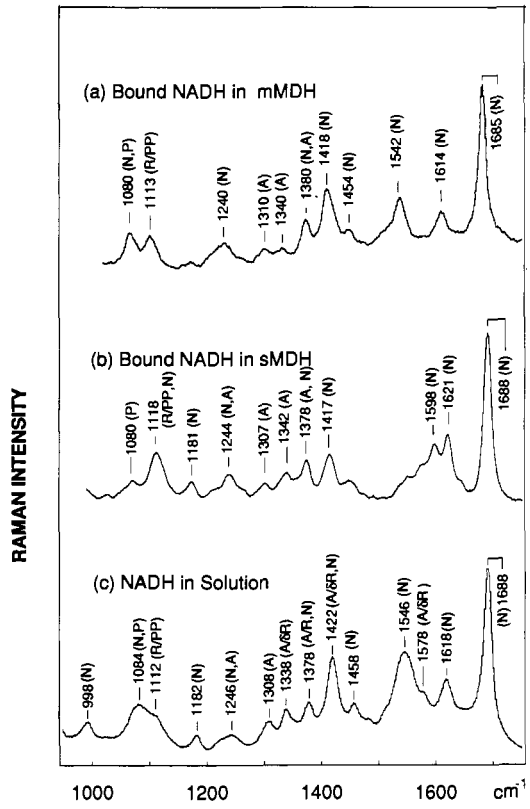

FIGURE 1: Raman spectra of (a) bound NADH in mMDH (mMDH/NADH = 4/6 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2; (b) bound NADH in sMDH (sMDH/NADH = 5/8 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2; and (c) NADH in solution (100 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2. About 40 mW of the 457.9-nm laser line was used to obtain spectra a and b. Spectrum c was obtained by using ~80 mW of the 488.0-nm laser line. Assignments of the solution peaks in spectrum c are from Yue et al. (1986); see the text for the assignments of the bound NADH peaks in MDHs: A = adenine; N = nicotinamide (or 3-acetylpyridine); P = phosphate; Pr = protein; PP = pyrophosphate; R(δR) = ribose; S = solvent; ? = unknown.

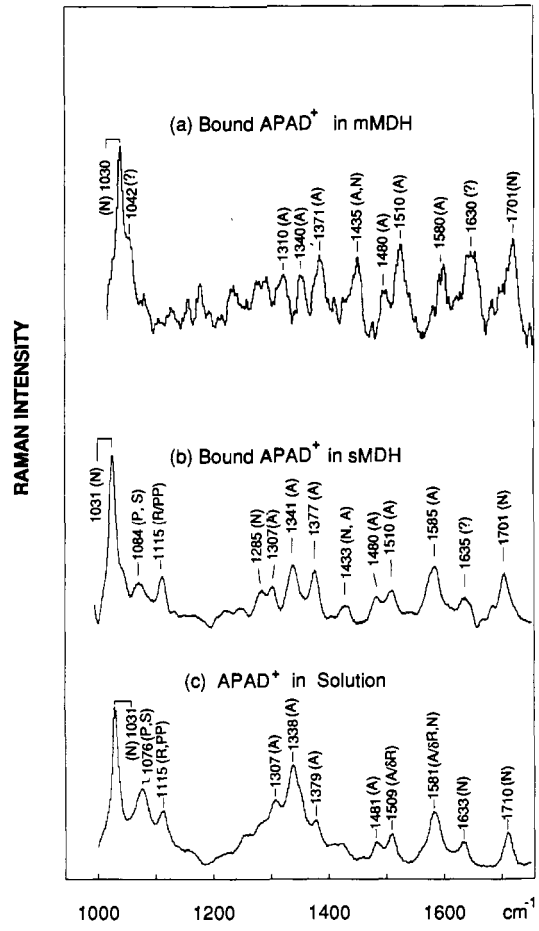

FIGURE 2: Raman spectra of (a) bound APAD⁺ in mMDH (mMDH/NAD⁺ = 4/6 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2; (b) bound APAD⁺ in sMDH (sMDH/APAD⁺ = 5/8 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2; and (c) APAD⁺ in solution (100 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2. All spectra in this figure were obtained by using ~80 mW of the 488.0-nm laser line. See the text and Figure 1 for the labeling scheme.

that we have measured [LADH, Chen et al. (1987); LDH, Deng et al. (1989c)]. This intensity effect is caused by a preresonance Raman effect that occurs when the 457.9-nm line used to excite the Raman scattering interacts with the 353-nm absorbance band of the mMDH/NADH complex whose absorption maximum is red-shifted at least 13 nm relative to that of NADH either in solution or bound to these other dehydrogenases. If the sample is irradiated with the 488-nm line, the preresonance effect diminishes as expected, yet the positions of the peaks remain the same.

Figure 2 shows the difference spectrum of APAD⁺ bound to mMDH (spectrum a), bound to sMDH (spectrum b), and free in solution (spectrum c). Difference spectra for these enzymes also were determined for binary complexes with NAD⁺ (data not shown), and the positions of those bands not associated with the pyridium ring are practically the same for both APAD⁺ and NAD⁺. The 3-acetylpyridium moiety of APAD⁺, like NAD⁺, shows few Raman active bands (compare Figures 2 and 3). We have denoted bands associated with the 3-acetylpyridine ring by the letter "N" in Figure 2. The aromatic ring mode (Bellamy, 1975), which is found at 1031 cm^{-1} in APAD⁺ and which is unaffected by binding, is also at 1031 cm^{-1} for NAD⁺ and is independent of whether NAD⁺ was free in solution or bound to mMDH or sMDH. Figure 3 shows the MDH/ADPR difference spectrum with mMDH (spectrum a) and sMDH (spectrum b) and the Raman spectrum of ADPR in solution (spectrum c).

As pointed out elsewhere (Chen et al., 1987; Deng et al., 1989c) and below, bands observed in the difference spectra can arise from a number of different sources with the most significant differences caused by the presence of the bound coenzyme or analogue. We showed previously (Yue et al., 1986) that the non-resonance Raman bands of NAD cofactors and analogues arise from molecular motions generally isolated to smaller moieties of the larger molecule. Peaks in Figure 1c are labeled as either adenine (A), nicotinamide or 3-acetylpyridine (N), ribose (R), phosphate (P), or pyrophosphate (PP). In some cases, a band is found to be largely located on a particular moiety, but its position and/or strength is somewhat dependent on a connecting group. For example, some of the peaks of adenine are influenced by the presence of the ribose in adenosine, and we have used the nomenclature A/δR to identify such peaks. Other peaks are strongly associated with two moieties, and these are labeled A/R. In some cases, an observed band contains contributions from two normal modes that happen to have the same or nearly the same frequency. For instance, the peak at 1422 cm^{-1} in Figure 1c is the sum of two bands, one located on the nicotinamide head group, which is responsible for about 90% of the total intensity, and the other on adenine, which contributes the remaining 10%. Such peaks are labeled by (N,A) to denote this degeneracy. Other sources of difference peaks have been documented elsewhere [cf. Deng et al. (1989c) and references therein].

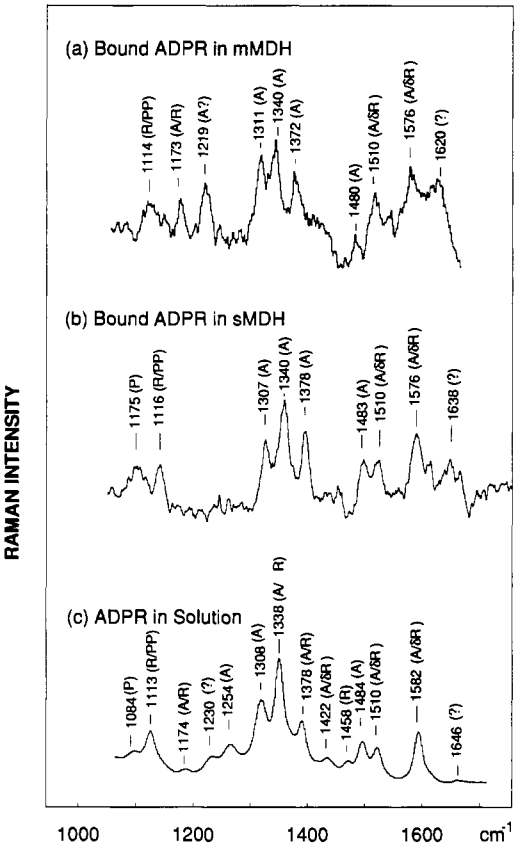

FIGURE 3: Raman spectra of (a) bound ADPR in mMDH (mMDH/ADPR = 4/6 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2; (b) bound ADPR in sMDH (sMDH/ADPR = 5/8 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2; and (c) ADPR in solution (80 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2. All spectra in this figure were obtained by using ~80 mW of the 488.0-nm laser line. See the text and Figure 1 for the labeling scheme.

The assignment of a band to a particular moiety of NADH, $APAD^+$, or a protein conformational change, or to a protein residue perturbed upon coenzyme binding is documented elsewhere (Deng et al., 1989a,c) and is briefly described here. Bands from nicotinamide or its analogues are easily assigned by comparing the spectra of bound NADH, NAD^+, $APAD^+$, and ADPR. The Raman spectra of oxidized and reduced forms of nicotinamide or its analogues differ markedly because of their different electronic structure (see Figures 1c, 2c, and 3c), and ADPR lacks the nicotinamide head group. Thus, bands that vary among the three spectra are safely assigned to the nicotinamide moiety (or its analogues). Having identified nicotinamide spectra produced by either analogue, most of the remaining bands can be assigned by a close correspondence to a peak in the solution spectra of NADH, $APAD^+$, and/or ADPR. The results of the assignments are shown in the figures. There are several peaks that we are currently unable to assign, and they have been labeled with a "?". These bands may well represent changes in protein structure that takes place when the cofactors bind.

Some of the bands associated with the nicotinamide group can be assigned to a vibrational mode of a local internal coordinate. For example, the rather broad peak at 1084 cm^{-1} in the NADH solution spectrum (Figure 1c) contains intensity arising from an amide, $-NH_2$, rocking motion [see Yue et al. (1986) and references therein]. The position of this $-NH_2$ rocking mode shifts to 1118 cm^{-1} when NADH binds to sMDH but only becomes sharper when NADH binds to mMDH. Corroborating this assignment is the observation that most of the intensity at 1118 cm^{-1} for the NADH in sMDH and at 1080 cm^{-1} for the NADH in mMDH as well as the intensity at 1084 cm^{-1} for unbound NADH disappears when the amide hydrogens are deuterated by dissolving the samples in D_2O [cf. Yue et al. (1986)]. The broad band at 1546 cm^{-1} in the solution NADH spectrum (Figure 1c) apparently is associated with the coupled C=C and C=O stretching vibrations of the reduced nicotinamide group (Deng et al., 1989c; Yue et al., 1986). This band shifts down to 1542 cm^{-1} when NADH is bound to mMDH and shifts up to 1598 cm^{-1} when NADH binds to sMDH. These shifts are qualitatively correlated with the shift in the near-UV absorption maximum of NADH when bound to the respective MDHs. The main 3-acetylpyridine peak at 1031 cm^{-1} in solution (Figure 3c) is an aromatic ring vibration (Bellamy, 1975) and is also observed in NAD^+. It seems reasonable to assign this band to the "triangle" vibration of the C2, C4, and C6 carbons of the aromatic ring, on the basis of an 11-cm^{-1} downshift when the nicotinamide C4 position is deuterated but unchanged when N1 is labeled with ^{15}N (our unpublished results). The band at 1710 cm^{-1} found in the solution spectrum of $APAD^+$ (Figure 3c) is due to the acetyl C=O stretch internal coordinate as verified by ^{18}O labeling (Deng et al., 1989a). This band shifts down 9 cm^{-1} when the cofactor binds to either mMDH or sMDH (Figure 2).

Discussion

Adenine Binding. The Raman spectrum of the adenine group of NAD and its analogues change significantly in the spectral region between 1300 and 1400 cm^{-1} when bound to some dehydrogenases (Austin et al., 1989; Chen et al., 1987; Deng et al., 1989c). The Raman bands in this region are due to delocalized vibrations of the purine ring plus contributions from motions of the bound ribose group. Attempts to assign these bands by normal mode analysis are underway (Tsuboi et al., 1987). However, these bands are sensitive to internal configurations of adenosine as well as external perturbations (Deng et al., 1989c; Nishimura et al., 1986). On the basis of X-ray structural and kinetic studies, the adenine-binding site of NAD dehydrogenases is hydrophobic in nature and not particularly specific for adenine. This binding pocket for many dehydrogenases is strongly conserved and is an $\alpha\beta\alpha\beta\alpha\beta$ motif, which often is referred to as the Rossmann fold [cf. Schultz and Schirmer (1979)].

The Raman spectrum of the adenine moiety bound to mMDH differs slightly from that of adenine in solution (Figure 3a). The differences are similar to those found when 9-ethyladenine is transferred from water to a more hydrophobic environment (Deng et al., 1989c). The Raman spectrum of the bound adenine moiety in sMDH is effectively the same as that observed in water. The small changes in the adenine region of the spectrum produced by binding to malate dehydrogenases are consistent with a shift from a hydrophilic to a hydrophobic environment; and, in fact, the absence of change in this region observed with sMDH suggests that its adenine group is quite accessible to solvent.

In contrast, a marked change occurs in the 1300–1400-cm^{-1} region of the Raman spectrum for several adenine-containing cofactors and analogues bound to LDH and LADH; four bands replace the triplet found in solution (Austin et al., 1989; Chen et al., 1987; Deng et al., 1989c). We have conjectured previously that this latter result was produced by either protonation or formation of a strong hydrogen bond between the enzyme and one of the adenine ring nitrogens because of the proximity of an aspartate to N3 of the ring (Chen et al., 1987; Deng et al., 1989c).[2] A more detailed comparison of the

coenzyme-binding pockets of the various dehydrogenases provides some additional insight into the interactions that produce the different Raman patterns observed for the adenine moiety. The structures of the coenzyme pocket for several dehydrogenases, including sMDH (Birktoft et al., 1989b), LDH (Grau et al., 1981; Griffith & Rossmann, 1987), and LADH (Eklund et al., 1984), are known to moderate resolution. As pointed out above, these pockets are both similar and hydrophobic. One difference is the position of a strongly conserved aspartate in the adenosine-binding site of these enzymes, Asp-41 in MDH, Asp-53 in LDH, and Asp-223 in LADH. This Asp is buried in a hydrophobic environment and does not form an ion pair with any other enzyme residue in the binary complexes. But, it does interact with the coenzyme. One of its carboxylate oxygens forms a hydrogen bond with the 3′-oxygen atom of the adenosine ribose group of the cofactor in the crystal structure of sMDH and with the 2′-oxygen of this group with LDH and LADH [for a review, see Eklund and Brändén (1987)]. The other Asp carboxylate oxygen is about 3.4 Å from adenine N3 in both dogfish LDH[3] (Griffith & Rossmann, 1987) and LADH (Eklund et al., 1984) but significantly farther, at about 4 Å, in sMDH (Birktoft et al., 1989b). It is therefore reasonable to suppose that the interaction between adenine's N3 and this carboxylate oxygen is substantially weakened in sMDH compared to LDH and LADH. However, the affinity of LDH for NADH and NAD does not vary significantly in the pH range 5–9 (Holbrook & Stinson, 1973). Since the pK_a of the adenosine ring is about 3.9 with protonation occurring primarily at the N1 position (Moore & Underwood, 1969) and the pK_a of this aspartic acid (Asp-41) should also be near four, neither protonation of the adenine ring nor formation of an ion pair with LDH seems likely unless this interaction is small compared to other energies or the pK_a of either the adenine ring or the aspartate shifts upward by at least 6 units in both the binary and ternary complexes. Such large changes of pK_a for nitrogen on nitrogen-containing aromatic ring systems is uncommon but possible inside enzymes. For example, the N1 nitrogen on the pteridine ring of methotrexate (an inhibitor of dihydrofolate reductase) has a pK_a of 5.7 in solution. When methotrexate binds to the reductase, its pK_a shifts up to >10 (Cocco et al., 1981, 1983). X-ray crystallographic studies show that one of the oxygens of an aspartic acid residue (Asp-27) forms a strong hydrogen bond with N1 of the methotrexate. The distance between the oxygen and nitrogen is 2.8 Å, which is a typical distance for the hydrogen-bonded atoms (Bolin et al., 1982). When the Asp-27 is replaced by either asparagine or serine, the N1 nitrogen of methotrexate becomes unprotonated upon binding (Howell et al., 1986). A mutant LDH or LADH with aspartate being replaced by asparagine would help to clarify this uncertainty.

Another interpretation of the Raman spectra of adenosine bound to LDH and LADH is that the protein/nucleic acid complex takes on multiple conformations. If each complex yields a different adenosine spectrum in the 1300–1400-cm^{-1} region, the measured spectrum would contain more bands than the model spectra, as observed. For example, although the distance between the aspartic acid oxygen and N3 of adenine inside LADH and dog fish LDH is slightly longer than the normal hydrogen-bonding distances [2.4–3.0 Å, cf. Olovsson and Jonsson (1976)], it is possible that the adenine is in an equilibrium of protonated and unprotonated states in these two enzymes. Another more likely possibility, we believe, is that the adenosine cavity varies in its solvent accessibility, with one conformation being quite free of water, or containing specifically placed structural water molecules, and the other containing disordered solvent. The vibrational modes of adenosine in the 1300–1400-cm^{-1} region respond to solvent polarity and hydrogen-bonding patterns (Deng et al., 1989c). In this regard it should be noted that the cavity is at the surface of the protein so that solvent entry is not impeded by substantial protein structure.

Nicotinamide Binding. Two bands in the spectrum of unliganded APAD$^+$ arise from the 3-acetylpyridinium moiety of APAD$^+$. The first of these is the dominant 1031-cm^{-1} band, which is quite characteristic of aromatic six-membered rings such as the pyridinium cation [for normal mode analysis, see Bellamy (1975) and Tsuboi et al. (1987)]. This band is also located at 1031 cm^{-1} for NAD$^+$ and PAAD$^+$ (unpublished results). The frequency of this band is unaffected during formation of a binary complex between APAD$^+$ and either sMDH or mMDH; thus, we conclude that the aromatic character of the oxidized moiety of the nicotinamide head is unaffected by binding. The position of this aromatic ring mode also does not vary (within 1–2 cm^{-1}) for these oxidized cofactors during formation of binary complexes with LDH (Deng et al., 1989a).

The other band associated with the unligand 3-acetylpyridinium moiety of APAD$^+$ is at 1710 cm^{-1} in Figure 2c, which is characteristic of acetyl C=O stretches. This band shifts 28 cm^{-1} upon labeling the acetyl oxygen with ^{18}O (Deng et al., 1989a). A simple reduced mass calculation yields a shift of 41 cm^{-1} for a "pure" C=O diatomic molecule, which is within reasonable agreement with that observed. When APAD$^+$ is bound to either MDH, this band red-shifts about 9 cm^{-1} to 1701 cm^{-1} (Figure 2a,b) and this band also shifts down about 30 cm^{-1} when the cofactor is labeled with ^{18}O (data not shown). On the basis of the frequency decrease of the bound carbonyl moiety, we conclude that the carbonyl bond of the bound acetyl moiety has less double bond character. This decrease in frequency almost certainly has its origins in a difference in strength of the hydrogen bond between the carbonyl of APAD$^+$ and solvent for the unliganded molecule and enzyme for the bound cofactor. Both experimental studies (Thijs & Zeegers-Huyskens, 1984) and recent theoretical calculations (Latajka & Scheiner, 1990) suggest that the frequency of a carbonyl, C=O, stretch follows a Badger–Bauer-like rule in that the stretching frequency is directly proportional to the strength of hydrogen bond between the carbonyl and a nearby proton donor. A stronger hydrogen bond yields a down-shifted C=O stretch. The susceptibility of a particular carbonyl to polarization by hydrogen bonding

[2] The Raman spectrum of the related purine nucleotide, guanosine, contains bands in the 1300–1400 cm^{-1} region that are quite sensitive to the torsional angle of the glycosidic bond (Nishimura & Tsuboi, 1986). A similar effect also may occur for the adenosine bands. However, this change in angle cannot be responsible for the changes observed in the Raman spectrum for adenosine bound to the MDHs relative to those observed for either LDH or LADH. The torsional angle for the adenosine glycosidic bond is −96° and −114°, −108°, −103°, and −89° in LADH (subunit A), LADH (subunit B), LDH, and sMDH (subunit A) and sMDH (subunit B), respectively, very nearly the same [cf. Birktoft et al. (1989b)]. Yet the Raman spectra of bound adenosine moiety in the first two enzymes are virtually the same (Deng et al., 1989c) but differ substantially from the third (present work).

[3] We have observed the new adenine band at 1325 cm^{-1} when either NAD or its adenine-containing analogues are bound to pig heart LDH (Deng et al., 1989c). The distance between N3 and the oxygen of Asp-53 for an analogue of the ternary complex bound to pig heart LDH is about 4 Å on the basis of the X-ray structure (Grau et al., 1981). However, this structure is not particularly well refined, and a smaller distance is possible. It is also possible that the structure of this complex is somewhat different than for those complexes used for Raman measurements.

depends somewhat on the internal coordinates that make up the specific normal mode and the degree that close local groups may affect the electronic distribution of the C=O moiety. For a well-localized carbonyl like that of acetone, it is found that each kcal/mol change in interaction energy corresponds to a shift of 2 cm^{-1} in the stretching frequency of the C=O stretch (Latajka & Scheiner, 1990; Thijs & Zeegers-Huyskens, 1984). A better model for 3-acetylpyridine is likely to be acetophenone, where the correlation is 3.2 cm^{-1} for each kcal/mol of interaction energy (Latajka & Scheiner, 1990). Thus, the -9-cm^{-1} shift observed above is consistent with a formation of a stronger hydrogen-bond interaction between the acetyl carbonyl moiety to MDH relative to this group hydrogen bonded to water. The hydrogen bond is 2.8 kcal/mol stronger in situ if acetophenone is used as the model (or 4.5 kcal/mol if acetone is used as the model). Essentially the same shift in the C=O frequency is observed for $APAD^+$ bound to LDH (Deng et al., 1989a). Thus, we conclude that the strength of the carbonyl hydrogen bond for these three proteins is quantitatively the same. X-ray studies on sMDH show the nicotinamide carbonyl hydrogen bonded to a bound water molecule, which is in turn hydrogen bonded to other residues (Birktoft et al., 1989a). The structure of mMDH is not sufficiently refined to determine directly the identity of the hydrogen-bond donor to the carbonyl group. However, both the conformation of NAD^+ bound to sMDH and the side chains of sMDH making direct hydrogen bonds with the cofactor are conserved in mMDH (Birktoft et al., 1989a). Thus, we predict that a bound water molecule is likely the source of the hydrogen-bond donor in mMDH.

Several of the bands in the spectrum of NADH that arise from the reduced nicotinamide group are understood with respect to their normal modes. For instance, the amide $-NH_2$ rocking mode is found at 1084 cm^{-1} in unbound NADH (Figure 1c). The rocking frequency of this group shifts to 1118 cm^{-1} when NADH binds to sMDH (Figure 1b) but remains unchanged in mMDH (Figure 1a). A shift of approximately 30 cm^{-1} occurs when NADH binds to LDH (Deng et al., 1989) like that found for sMDH. We have speculated elsewhere that this effect is caused by an increased hydrogen-bond interaction between the enzyme and the $-NH_2$ moiety relative to the interaction between the amide and water (Deng et al., 1989c). X-ray structural studies show the amide moiety interacts with the side chain of Asn-130 in sMDH (Birktoft et al., 1989a) and of Ser-163 in LDH (Griffith & Rossmann, 1987). Unfortunately, as pointed out above, the structure of mMDH is not well refined, and the presence of a hydrogen-bond donor in the vicinity of the amide has not been determined (Birktoft et al., 1989a). Yet, as also pointed out above, both the active site residues in contact with the cofactor and the structure of the cofactor found for sMDH are conserved in mMDH. Thus, we suggest that a weaker hydrogen bond between the amide $-NH_2$ and Asn-130 will be found for mMDH than is found in the binary complexes with sMDH and LDH.

The presence of a hydrogen bond between an amide group and acceptor is expected to "stiffen" the $-NH_2$ rocking mode and increase its frequency on the basis of the well-known observation that hydrogen bonding decreases the N–H (or O–H) stretching frequencies while increasing their bending frequencies. For example, water dissolved in carbon tetrachloride has two stretching modes at about 3755 and 3650 cm^{-1} and a bending mode at 1595 cm^{-1} (Vinogradov & Linnel, 1971). In an aqueous environment, the H–O–H stretching bands are centered around 3425 and 3275 cm^{-1}, a downward shift of more than 300 cm^{-1}, while the H–O–H bending mode shifts upward by 50 cm^{-1} to 1645 cm^{-1}. The quantitative linear relationship between the N–H (or O–H) stretch frequencies and hydrogen-bonding interaction energy seems to exist for certain systems within certain ranges [cf. Hadzi and Bratos (1976)]. Even though the correlation between the hydrogen-bond energy and the N–H bending frequency shift or more directly on the NH_2 rocking mode is much less studied, we can still make a crude estimate of about 2–4 kcal/mol energy is involved for the 30-cm^{-1} frequency increase of the $-NH_2$ bending mode when NADH binds to sMDH and LDH, on the basis of the energy-stretch frequency and stretch-bending frequency correlation studies previously reported (Drago & Epley, 1969; Novak, 1974; Nozari & Drago, 1970; Pullin & Werner, 1965). By the same argument, the hydrogen bonding between the protein and the $-NH_2$ moiety in mMDH is not much different than that in solution.

Three bands in the 1500–1700-cm^{-1} region of the spectrum of NADH are highly coupled reduced nicotinamide C=C and C=O stretching modes. One change in the NADH spectrum upon binding to either MDH is an intensity decrease and a frequency shift in the 1546-cm^{-1} band of the solution spectrum (Figure 1c). This band shifts down to 1542 cm^{-1} and up to 1598 cm^{-1} for the mMDH (Figure 1a) and sMDH (Figure 1b) binary complexes, respectively. Such frequency shifts in these stretching modes should be associated with the shifts in the λ_{max} of near-UV absorbance maximum of NADH, since changes in λ_{max} likely are caused by changes in the double bond polarization of the dihydronicotinamide moiety (Maggiora et al., 1969). Polarization of a double bond will result in a decrease in the electron density of these bonds and a concomitant decrease of the bond order, which will cause a decrease in the frequency of its stretching vibration. The downward shift of the band at 1546 cm^{-1} for unliganded NADH to 1542 cm^{-1} in the binary complex with mMDH is expected on the basis of the red-shift of the 340-nm band for this complex ($\Delta\lambda_{max}$ = 13 nm). When NADH binds to sMDH, the 1546-cm^{-1} band shifts to 1598 cm^{-1}, which also is consistent with the blue-shift of its λ_{max} ($\Delta\lambda_{max} = -5$ nm). A correlation between λ_{max} of the near-UV band and the direction of frequency shift for the 1546-cm^{-1} band also holds in our studies of NADH bound to LADH ($\Delta\lambda_{max} = -15$ nm; $\Delta\nu$ = 1600 cm^{-1}) and LDH ($\Delta\lambda_{max} = -2$ nm; $\Delta\nu$ = 1577 cm^{-1}; Chen et al., 1987; Deng et al., 1989c). However, a linear correlation clearly does not exist between stretching frequency and λ_{max} of the near-UV band of NADH. But this absence may not be particularly surprising in general as the two measured quantities respond to local perturbations quite differently. Also, changes in both the ground and excited states affect the position of electronic absorption maxima, while only changes in the ground state affect the positions of vibrational bands.

Several factors contribute to the position of λ_{max} for NADH. One is the torsional angle between the carboxamide plane and the plane of the dihydropyridine. The carbonyl of the amide may point either toward (cis) or away from (trans) the ring nitrogen with the plane of the amide roughly parallel to that of the ring. The electronic density within various bonds of the reduced ring is likely to be a function of this angle, which will regulate the position of the absorption maximum of the reduced nicotinamide ring. For instance, the results of a self-consistent field molecular orbital calculation predicts that the λ_{max} of the cis form should be about 14 nm higher than that of the trans form (Maggiora et al., 1969). This prediction of a blue-shifted absorption maximum for the trans relative to that for the cis form is reinforced by a recent ab initio cal-

culation on 1-methylnicotinamide at the SCF/STO-3G and SCF/3-21G levels (Cummins & Gready, 1989). X-ray crystallographic studies of sMDH (and LDH) suggest that nicotinamide is in the trans conformation when it binds to these two enzymes (Birktoft et al., 1989b; Griffith & Rossmann, 1987). Recently, a NMR study on several reduced nicotinamide analogues with a fixed trans or cis C=O configuration confirmed that the amide configuration of nicotinamide is cis in solution (Fischer et al., 1988), which is consistent with the theoretical prediction (Maggiora et al., 1969). The change of the amide orientation from cis to trans upon binding to LDH and sMDH should contribute to observed blue-shift of nicotinamide λ_{max}. Another factor that affects the position of λ_{max} is the type and strength of the hydrogen bonds between NADH's carboxamide group and the enzyme relative to those in solution. Previous studies suggest that hydrogen bonding to the C=O moiety of the amide would result in a red-shift (Maggiora et al., 1969), while hydrogen bonding to the NH_2 of the amide would affect a blue-shift (Fischer et al., 1988). Both mMDH and sMDH, as well as LDH, form stronger hydrogen bonds with the C=O group of the amide of $APAD^+$ than does water (see below). On the other hand, the interaction between $-NH_2$ group of NADH and either sMDH or LDH is stronger than with mMDH, possibly substantially stronger, as discussed below. Thus, this increased net interaction is consistent with the observed blue-shift in λ_{max} for NADH bound to either sMDH or LDH relative to that for mMDH.

One fascinating feature of the pyridine-dependent dehydrogenases is that the transfer of the hydride ion to substrate occurs stereospecifically [cf. Fersht (1985)]. Dehydrogenases transfer either the *pro-R* or the *pro-S* C4 hydrogen. Stereospecific transfer can show a high degree of fidelity. For example, an error rate of less than 1 part in 10^8 has been determined for the reaction catalyzed by LDH (LaReau et al., 1989). Structural studies show that (of those measured) *pro-R* enzymes bind the nicotinamide ring in the anti conformation about the glycosidic bond while *pro-S* bind the ring in the syn conformation. This suggests that simple geometrical considerations may be, at least partially, responsible for the stereospecific nature of these enzymes. These conformations apparently present the nicotinamide ring to substrate in such a way that dictates the specific stereochemistry. An important question then is what factors keep the nicotinamide ring in either a syn or anti conformation.

We have suggested previously that the hydrogen-bonding patterns that we observe for the amide moiety of NAD are sufficiently strong to be a major factor in determining the observed fidelity of hydride transfer (Deng et al., 1989a). It can be estimated that the "energy difference" between the transfer of the *pro-R* hydrogen compared to the *pro-S* hydrogen is greater than 10.4 kcal/mol during catalysis by LDH, based on the application of an Arrhenius analysis of measured error rates (LaReau et al., 1989; V. Anderson, personal communication). Assuming that the 10.4 kcal/mol "energy difference" can be related to the energy difference between the anti and syn conformations, we need to look for specific interactions that discriminate between these two. A molecular mechanics analysis of the active site of LDH suggests that the cavity containing the nicotinamide head group has sufficient space that both the syn and anti conformations are essentially equally accessible (V. Anderson, personal communication). X-ray studies suggest that the syn conformation places the amide in a hydrophobic environment while the anti conformation places it so that hydrogen bonds can form between the amide and enzyme (Grau et al., 1981; Griffith & Rossmann, 1987).

The Raman results provide an opportunity to estimate the strengths of the hydrogen bonds between protein and the coenzyme's amide moiety. The 2.8 kcal/mol interaction energy between the carbonyl of $APAD^+$ and either LDH, sMDH, or mMDH, estimated above, is the difference in the interaction energy between this group in water and bound at the active sites of these enzymes. We have used model compounds containing carbonyls to estimate the magnitude of the interaction energy between the amide carbonyl and water. Such a value should provide an estimate of the effect of transferring an amide carbonyl from an aqueous solvent to a hydrophobic environment. We find, for example, that acetone's carbonyl shifts 10 cm^{-1} for acetone in water compared to neat acetone. This represents a change in hydrogen-bonding interaction of 4.5 kcal/mol, according to the Badger–Bauer correlation discussed above. Thus a reasonable estimate for the strength of the hydrogen bond of the nicotinamide's carbonyl in the coenzyme-binding site of either LDH, sMDH, or mMDH is 7.3 kcal/mol (4.5 + 2.8 kcal/mol). At the present time, we are unable to quantify the hydrogen-bond interaction between the $-NH_2$ amide moiety and enzyme. However, additional 3.1 kcal/mol is well within our estimate of 2–4 kcal/mol for the strength of a hydrogen bond between the $-NH_2$ amide moiety and the enzyme.[4] Alternatively, Anderson and LeReau estimated a minimum "energy difference" of 8.2 kcal/mol in experiments on $PAAD^+$, where the $-NH_2$ has been replaced by a hydrogen to test the importance of the $-NH_2$ group (LaReau and Anderson, personal communication). This value is satisfyingly close to the 7.3 kcal/mol difference in hydrogen-bond energy estimated above for a carbonyl in the syn and the anti conformation at the active site of these enzymes. It is interesting to note that the results here then predict that the error rates of sMDH and mMDH should differ, if the presence or absence of hydrogen bonds is essentially responsible for the stereospecific transfer, because mMDH has a weaker $-NH_2$ hydrogen bond than sMDH. We are currently performing this experiment.

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[4] We want to point out here that several assumptions have been used in this estimation. First, we have assumed that the hydrogen-bond strength between the carbonyl bond of either reduced or oxidized nicotinamide (as well as its acetyl analogue) and the protein are similar. Second, we have assumed that the C=O and $-NH_2$ hydrogen-bond energies are additive. In general, it seems likely that there may be a correlation term, especially for the highly coupled system like nicotinamide.

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