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Classical Raman Spectroscopic Studies of NADH and NAD^+ Bound to Lactate Dehydrogenase by Difference Techniques†

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Received July 25, 1988; Revised Manuscript Received September 16, 1988

ABSTRACT: The binding of the coenzymes NAD^+ and NADH to lactate dehydrogenase causes significant changes in the Raman spectra of both of these molecules relative to spectra obtained in the absence of enzyme. The molecular motions of the bound adenine moiety of both NAD^+ and NADH as well as adenine containing analogues of these coenzymes produce Raman bands that are essentially identical, suggesting that the binding of adenine to the enzyme is the same regardless of the nicotinamide head-group nature. We also have observed that the molecular motions of the bound adenine moiety are different from both those obtained when it is in either water, various hydrophobic solvents, or various other solvent compositions. Protonation of the bound adenine ring at the 3-position is offered as a possible explanation. Significant shifts are observed in both the stretching frequency of the carboxamide carbonyl of NAD^+ and the rocking motion of the carboxamide NH_2 group of NADH. These shifts are probably caused by hydrogen bonding with the enzyme. The interaction energies of these hydrogen-bonding patterns are discussed. The aromatic nature of the nicotinamide moiety of NAD^+ appears to be unchanged upon binding. Pronounced changes in the Raman spectrum of the nicotinamide moiety of NADH are observed upon binding; some of these changes are understood and discussed. Finally, these results are compared to analogous results that were recently reported for liver alcohol dehydrogenase [Chen et al. (1987) *Biochemistry 26*, 4776–4784]. In general, the coenzyme binding properties are found to be quite similar, but not identical, for the two enzymes.

Recent studies of enzyme catalysis [cf. Jencks (1975, 1980, 1986), Schowen (1978), Wolfenden (1976, 1978), Cook et al. (1981), Somogyi et al. (1984), Stackhouse et al. (1985), and, Burgner et al. (1987)] have focused on the extent that enzymes use noncovalent interactions to facilitate the making and breaking of covalent bonds and to perform stereospecific reactions. The energy and origin of these noncovalent interactions have been difficult to assess experimentally until recently. Raman spectroscopy is known for its ability to provide detailed information concerning such molecular properties of molecules and the interactions between molecules such as those that occur when a substrate binds at the active site of an enzyme. This has been particularly true in resonance Raman experiments of prosthetic chromophores contained in the active sites of proteins as in, for example, visual pigments, heme proteins, and some enzymes [reviewed in Carey (1982) and in papers found in Spiro (1987)]. The resonantly enhanced Raman spectrum of the colored prosthetic group so dominates the protein classical Raman spectrum that it is easily detected

† This work was supported by Grants GM35183 (City College) and RCMI RRO3060 (City College) from the National Institutes of Health and Grant 8616216 from The National Science Foundation (Purdue University).
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despite its small relative mass. Recently, we have shown that very good classical spectra of cofactors and substrates bound at the active site of an enzyme can be obtained by using ultrasensitive difference techniques (Yue et al., 1984; Chen et al., 1987). The classical Raman spectrum of the enzyme is subtracted from that of the enzyme–substrate binary complex. In Chen et al. (1987), we obtained the classical Raman spectra of NADH[1] when bound to liver alcohol dehydrogenase (LADH). Hence, Raman spectroscopy in combination with structural determinations and energy evaluations by computational methods may provide a powerful approach for assessing both the strength and the origin of the noncovalent (as well as covalent) interactions between enzymes and their substrates.

Here, we report on the Raman spectra of NADH and NAD^+ and fragments of these molecules bound to lactate dehydrogenase (LDH). This enzyme catalyzes the stereospecific oxidation of *l*-lactate by NAD^+ to pyruvate, forming the reduced coenzyme NADH (Warburg & Christian, 1936; Euler et al., 1936). Unlike LADH, there is no metal ion involved in the catalytic pathway of LDH, and NADH is not as tightly bound to LDH as LADH. A smaller blue shift (<5 nm) of the NADH absorption band at 340 nm is observed upon binding to LDH compared to that observed for LADH (15 nm) (Fisher et al., 1969). Moderately high resolution crystallographic studies of coenzyme complexes with LDH have been performed (White et al., 1976; Grau et al., 1981) that show similarities to and differences with the coenzyme binding pocket found in LADH (Eklund et al., 1981, 1984; Eklund & Brändèn, 1986).

In analyzing the difference spectra, we first compared data obtained from NADH, NAD^+, and a molecular fragment of these coenzymes, ADPR. From these comparisons, we assigned the observed bands to molecular motions located on one or another of the nicotinamide, adenine, and phosphate moieties of NADH and NAD^+. We found that pronounced changes in some of these molecular motions accompany the binding of the adenine and reduced nicotinamide moieties when NADH binds, as judged from the dramatic changes that occur in the Raman spectra between that of solution NADH and bound NADH. For instance, strong hydrogen bonding between the C=O and NH_2 moieties of the nicotinamide carboxamide group and the enzyme is observed, and aspects of the interaction energies can be estimated from the data. The aromatic nature of the oxidized nicotinamide moiety of NAD^+ is not significantly affected by binding. In addition, the data strongly suggest that the adenine moiety protonates when the coenzymes bind. The spectra of bound NADH and NAD^+ are compared to the analogous spectra obtained in our recent study on LADH. We find that the spectral features of both the adenine and nicotinamide moieties when NADH binds to either LDH or LADH are very similar, but with interesting differences.

Materials and Methods

NADH (100%) and NAD^+ (100%) were purchased from Boehringer Mannheim Co. (Indianapolis, IN), and ADPR was purchased from Sigma Chemical Co. (St. Louis, MO); they were used without further purification. Pig H4 LDH was prepared according to the procedure described in Burgner and Ray (1984) and stored at 4 °C in 2.6 M ammonium sulfate and 0.1 M phosphate, pH 7.2. Before use, LDH was dialyzed against 0.1 M phosphate buffer at pH 7.2 and 4 °C for several hours. After insoluable protein was removed by centrifugation, the enzyme solution was concentrated to about 1 mM, corresponding to a binding site concentration of 4 mM, by using a centricon centrifuge concentrator (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, using $\epsilon_{280} = 20\,000\ M^{-1}\ cm^{-1}$ for LDH, $\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$ for NADH, $\epsilon_{259} = 18\,000\ M^{-1}\ cm^{-1}$ for NAD^+, and $e_{259} = 15\,200\ M^{-1}\ cm^{-1}$ for ADPR. Since LDH contains four independent active sites, binary complexes of LDH were prepared by mixing a 1:<4 molar ratio of LDH to NADH, NAD^+, and ADPR. Since the concentration of the enzyme is significantly larger than the dissociation constants for each of the coenzymes and analogues used here (Stinson & Holbrook, 1973), typically better than 90% of the coenzymes (or analogues) were bound.

About 30 μL of the samples is loaded into 3 × 3 mm fluorescence cuvettes, and the cuvettes were transferred to a cuvette holder (maintained at 4 °C in a bath/circulator) for measurement. The cuvette holder could take two cuvettes at the same time, and the translational motion to move each 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 LSI-11/2 minicomputer (Digital Equipment Corp., Marlboro, MA), which controlled the entire experiment. Positioning to within ±1 μm was possible.

Raman spectra were measured by using an optical multichannel analyzer (OMA) system, which consisted 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 an LSI-11/2 minicomputer (Digital), which was also used for data manipulation and analysis. Typically, 110 mW of 488-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} can be detected simultaneously. The instrument was calibrated against known assignments of the toluene spectrum. 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 classical Raman difference spectra between a protein and a protein/ligand binary complex have been discussed at length previously (Chen et al., 1987). We have found that the Raman signal of a molecular fragment like adenine is around 3% of that of a 40000 molecular weight protein. Thus, noise signals, which may arise from systematic factors like spectrometer drift as well as from simple shot noise, must be kept to less than around 0.5% of the protein Raman signal. Our spectrometer is capable of collecting sufficient Raman intensities from a protein in about 2 h so as to yield a noise to signal ratio due to shot noise of 0.5% or better. Several precautions were taken to minimize systematic errors. The entire spectrometer system, including the exciting laser, was mounted on a vibration-free table (Model RS-58-12, Newport Corp., Fountain View, CA). Ambient room temperature was controlled to within ±1.5 °C and relative humidity to within

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

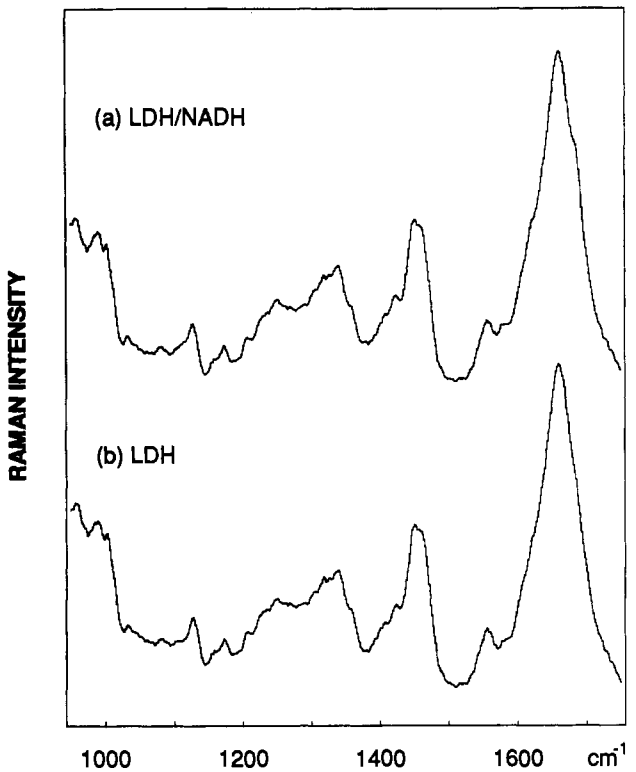

FIGURE 1: Raman spectra of (a) LDH/NADH (LDH:NADH = 1:2.5 mM) binary complex and (b) LDH at 4 °C in 0.1 M phosphate buffer, pH 7.2.

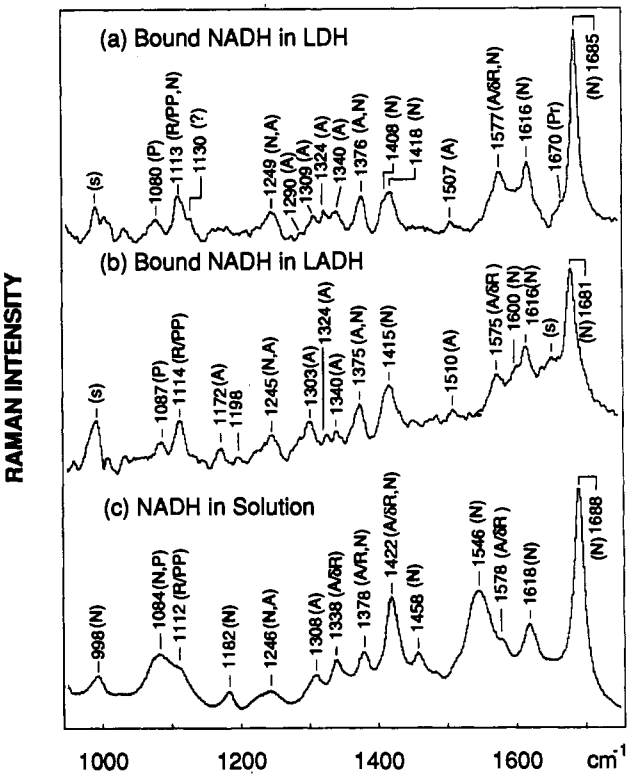

FIGURE 2: Raman spectra of (a) bound NADH in LDH (LDH:NADH = 1:2.5 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2, (b) bound NADH in LADH (LADH:NADH = 1:2 mM) at 4 °C in 0.1 M pyrophosphate buffer, pH 9.6, and (c) NADH in solution (70 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2. Assignments of the solution peaks in (c) are from Yue et al. (1986); assignments of the bound NADH peaks in LADH (b) are from Chen et al. (1987); see text for the assignments of the bound NADH peaks in LDH (a). A = adenine; N = nicotinamide; P = phosphate; Pr = protein; PP = pyrophosphate; R(δR) = ribose; S = solvent; ? = unknown.

±3%. Sample positioning is especially critical. The instrument response in both signal intensity and wavelength repeatability is very sensitive to the positioning of the focused exciting laser beam within the sample and reference cells with respect to the spectrometer optical path. The Aerotech positioning equipment accuracy of ±1 μm mentioned above is adequate to ensure proper alignment and repeatability. Our procedure for assessing the influence of systematic factors, which might adversely affect an A − B difference spectrum, involves taking interleaved spectra in an ABBA...BA sequence, adding every other A spectrum and subtracting this from the sum of the remaining A spectra. This is repeated for the B spectra. In such a subtraction, the resulting A − A or B − B difference should result only in simple shot noise. Typical results from such procedures are provided in Chen et al. (1987). Also, test runs where both the A and B cuvettes contained the same samples were performed. Generally, we found that systematic error factors were no larger than 0.2% of the protein peak Raman signal.

Results

Spectra. Figure 1 shows the Raman spectrum of LDH and its binary complex with NADH. Most of the Raman scattering of the binary complex is due to the enzyme, although a shoulder is clearly observed near 1680 cm^{-1} in the binary complex spectrum that is not seen in the LDH data. While the Raman spectrum of LDH is not analyzed in detail here, it is worth noting some of its most prominent features [see, e.g., Carey (1982) for a discussion of protein bands]. The strong broad protein band near 1660 cm^{-1} is the amide I band that arises from polypeptide backbone amide C=O stretching motions. The frequency of an amide I mode is dependent on hydrogen-bonding environment and hence protein secondary structure. For example, a frequency near 1650 cm^{-1} is suggestive of the relatively strong hydrogen-bonding environment found in α-helical structure, while frequencies near 1670 and 1665 cm^{-1} are typically found in antiparallel β-pleated sheet and disordered structures. The amide III region, also sensitive to secondary structure, lies from 1230 to 1300 cm^{-1} and is due to polypeptide backbone N–H in-plane bending and C–N stretching motions. The bands at 1340 and 1450 cm^{-1} and the γ-CH_2 and δ-CH_2 bands, respectively. These vibrations are relatively insensitive to the secondary structure of the protein.

Using previously described procedures (Chen et al., 1987; see Materials and Methods), we calculated the difference spectrum, shown in Figure 2a, between the LDH/NADH binary complex and LDH (parts a and b of Figure 1, respectively). As we noted above, the band intensities of the Raman spectrum of NADH are about 3–10% that of LDH. All the labeled peaks in Figure 2a have been consistently observed in at least 10 different experiments. For comparison, the difference spectrum between the binary complex of liver alcohol dehydrogenase (LADH) with NADH and LADH [from Chen et al. (1987)] is shown in Figure 2b, and the spectrum of NADH in solution [from Yue et al. (1986)] is shown in Figure 2c.

Bands observed in the difference spectra may arise from any of a number of differences between the protein binary complex and the protein itself. The most significant differences are caused by the presence of the bound coenzyme. We found previously (Yue et al., 1986) that the classical Raman bands of NADH (or NAD^+) arise from molecular motions generally located on smaller moieties of the larger molecule. Peaks may be labeled as either adenine (A), nicotinamide (N), ribose (R), phosphate (P), or pyrophosphate (PP). We have so labeled

all the peaks in the NADH data of Figure 2c in this way. 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. Some of the peaks of adenine are influenced by ribose binding to form adenosine, for example. We use the nomenclature A/δR to identify such peaks. Other peaks are strongly associated with two moieties, and these are labeled like A/R. In some cases an observed band contains contributions from two normal modes. The peak at 1422 cm^{-1} in Figure 2c is an example of the sum of two bands, one located on the nicotinamide head group (responsible for about 90% of the total intensity) and the other on adenine (the remaining 10%). We have labeled this peak (N,A) to denote this degeneracy and similarly for the other degenerate bands.

Bands in the difference spectra can also arise from changes in the secondary structure of the protein that occur when the coenzyme binds. As we have pointed out above, the positions of some protein bands are sensitive to secondary structure, and LDH undergoes a conformational change when NADH binds [cf. Holbrook et al. (1975)]. Another possibility that may result in the observation of a peak in the difference spectrum is when an interaction between NADH and surrounding protein moiety significanty affects a mode of the protein moiety. In these latter cases, a trough of intensity in the difference spectrum, corresponding to the unperturbed position of the Raman active band, would be observed among with a concomitant increase in intensity at the perturbed position.

In our previous work on NADH and complexes of NADH with LADH (Yue et al., 1986; Chen et al., 1987), we found that the peaks observed in the difference spectrum between LADH/NADH and LADH in Figure 2b arose from bound NADH and could be assigned to NADH moieties as is the case for the solution spectrum of NADH in Figure 2c. We have labeled the peaks in Figure 2b on the basis of these results. We observed no major bands that might arise from the protein conformational change which occurs when NADH binds to LADH to form perturbed protein modes. The assignments were based on the observations that most prominent features observed in Figure 2b were positive peaks and could be associated often with features found in the NADH spectrum in solution (Figure 2c). Moreover, the corresponding peaks to those in Figure 2b due to the adenine moiety were conserved in the bound ADPR spectrum (see Figure 4b). ADPR binding, unlike NADH binding, does not induce a conformational change when it binds to LADH (Eklund et al., 1984). Thus, the LADH/ADPR − LADH difference spectrum cannot contain bands due to a protein conformational change. Since no band disappeared in the bound ADPR spectrum relative to the bound NADH spectrum (apart from bands that could be clearly identified as nicotinamide), none of the bands in the bound NADH spectrum can arise from the conformational change.

Using a similar analysis, presented below, we find that some of the bands in the LDH/NADH with LDH difference spectrum in Figure 2a contain features due to the protein conformational change that accompanies NADH binding to LDH. We labeled such peaks Pr (for protein). As indicated above, it is also possible that the binding of NADH disrupts and/or forms strong interactions with protein moieties surrounding the bound NADH molecule, and some of the bands in Figure 2a may arise from motions located primarily on one or another of these protein moieties. However, we have not identified, conclusively, any bands that can be identified as such peaks, but a few peaks could not be assigned as either coenzyme peaks or bands arising from protein structural changes either.

From parts a and c of Figure 2, it is evident that the Raman spectrum of NADH bound to LDH differs markedly from the solution NADH spectrum. The binding of the nicotinamide moiety is clearly accompanied by the disappearance of the intense 1546-cm^{-1} band and an intensity decrease or disappearance in the 1458-cm^{-1} band found in the solution spectrum. The peak at 1084 cm^{-1} found in the solution spectrum consists of two bands, one arising from phosphate (ca. 30% of the intensity) and one arising from the rocking motion of the nicotinamide NH_2 moiety (ca. 70% of the intensity) (Yue et al., 1986). When the coenzyme binds to either LDH or LADH, there is a substantial loss in relative intensity at 1084 cm^{-1} and a concomitant increase in intensity at 1114 cm^{-1} which arises from the nicotinamide head group (see below). Thus, the rocking frequency of the nicotinamide NH_2 moiety appears to move from 1084 cm^{-1} in solution to 1114 cm^{-1} when bound to the coenzyme binding pocket. Also the major nicotinamide band at 1688 cm^{-1} found in the solution spectrum is downshifted by 3 cm^{-1} to 1685 cm^{-1} when NADH binds to LDH. The binding of the adenine moiety is indicated by a number of very pronounced changes as well; these are more clearly seen in the spectrum of bound ADPR given below.

While there are pronounced changes in the Raman spectrum of nicotinamide when NADH binds to LDH, the observed nicotinamide bands are very much the same for NADH bound to either LDH or LADH, as can be seen by comparing parts a and b of Figure 2. There are, however, some differences. The major nicotinamide band lies at 1688 cm^{-1} (Figure 2c) in the solution spectrum, at 1685 cm^{-1} for NADH in LDH, and at 1681 cm^{-1} for NADH in LADH. The exact assignment of this mode is unclear at the present time, but it very likely contains major contributions from C=O and C=C stretching motions (Bowman & Spiro, 1980; Rodgers & Peticolas, 1980; Chen et al., 1987; Bajdor et al., 1987). It is thus reasonable to suppose that differences in the position of this dominant mode can be correlated with the λ_{max} of the near-UV $\pi\pi^*$ absorption band of NADH. In solution, this band is found at 340 nm, blue shifting to ~335 and 325 nm when NADH binds to LDH and LADH, respectively (Fisher et al., 1969). The intensity of the nicotinamide 1415-cm^{-1} band found in NADH in LADH (Figure 2b) appears to be reduced somewhat in NADH in LDH (Figure 2a), apparently concomitant with the formation of a shoulder at 1408 cm^{-1}. It is reasonable to suppose that the observed 1415-cm^{-1} band found in the NADH in LADH spectrum contains intensity from two degenerate normal modes; this degeneracy appears to be somewhat lifted when NADH bands to LDH. The 1600-cm^{-1} feature found in the NADH in LADH (Figure 2b) is not observed in the NADH in LDH spectrum (Figure 2a); likewise, there appears somewhat more intensity at 1577 cm^{-1} in the NADH in LDH spectrum as compared to the NADH in LADH spectrum due to nicotinamide.

Figure 3a shows the difference spectrum of LDH/NAD^+ with LDH. The spectrum of NAD^+ in solution is shown in Figure 3b. The nicotinamide moiety of NAD^+ shows very few Raman active bands, as is clear from the solution data. The main nicotinamide peak at 1033 cm^{-1} is unaffected when NAD^+ binds to LDH.

Figure 4 shows the LDH/ADPR difference spectrum with LDH (Figure 4a) and, for comparison purposes, the analogous difference spectrum of ADPR bound to LADH (Figure 4b) and the Raman spectrum of ADPR in solution (Figure 4c). This bound ADPR spectrum is of interest for two reasons.

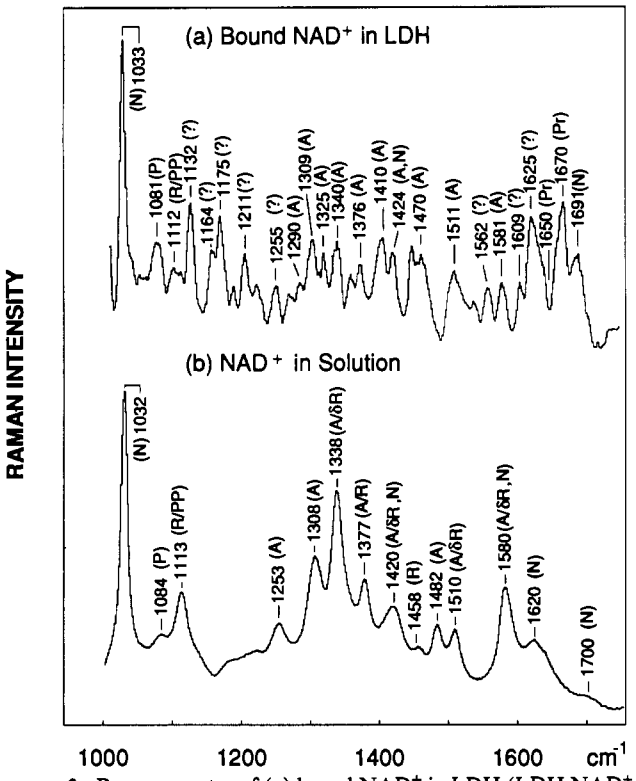

FIGURE 3: Raman spectra of (a) bound NAD^+ in LDH (LDH:NAD^+ = 1.5:5 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2, and (b) NAD^+ in solution (80 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2. See text and Figure 2 for the labeling scheme.

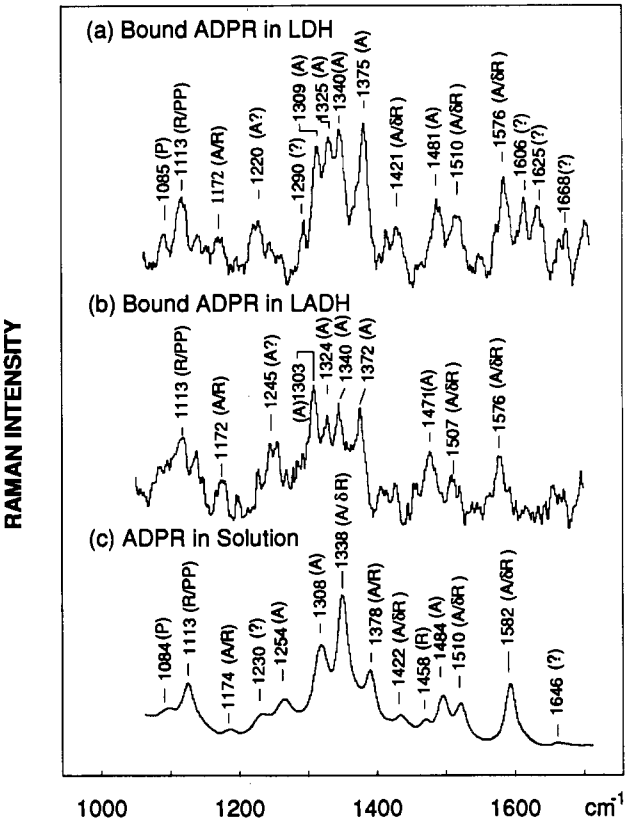

FIGURE 4: Raman spectra of (a) bound ADPR in LDH (LDH:ADPR = 1:2.5 mM) at 4 °C in 0.1 M phosphate buffer, pH 7.2, (b) bound ADPR in LADH (LADH:ADPR = 1:1.8 mM) at 4 °C in 0.1 M pyrophosphate buffer, pH 9.6, and (c) ADPR in solution (80 mM) at 4 °C in 0.1 M pyrophosphate buffer, pH 9.6. See text and Figure 2 for the labeling scheme.

First, it is very useful in assigning the bands to particular moieties since ADPR lacks the nicotinamide moiety of NADH but induces the same conformational change in LDH upon binding as does NADH (Grau et al., 1981). Second, the bands arising from adenine and other moieties are more easily observed, as the more intense nicotinamide bands are absent.

There are clearly a number of very pronounced changes in the spectrum of adenine when it binds to either LDH or LADH. The intense adenine solution band at 1338 cm^{-1} is absent in the bound data, and several new bands, the 1325-cm^{-1} band, for example, appear in the bound spectra. In general, the spectra of bound ADPR in LDH and in LADH closely resemble each other with, however, important differences. For example, the band at 1245 cm^{-1} in ADPR in LADH (Figure 4b) appears to have moved to 1220 cm^{-1} in the ADPR in LDH spectrum (Figure 4a). Also new bands at 1290, 1606, and 1625 cm^{-1} appear in the ADPR in LDH spectrum compared to the ADPR in LADH spectrum. We have consistently observed a somewhat smaller intensity of the 1309-cm^{-1} peak relative to other nearby bands in the spectrum of ADPR bound to LDH compared to the apparently similar, but relatively more intense, band at 1303 cm^{-1} in the ADPR in LADH spectrum.

As discussed below, the adenine binding pocket in LDH and LADH is largely hydrophobic. In order to test whether or not the change from a hydrophilic to a hydrophobic environment is responsible for the rather dramatic changes in the Raman spectrum of the adenine moiety of the coenzymes upon binding to either LDH or LADH, we measured the Raman spectrum of 9-ethyladenine in various solvents including H_2O, propanol, chloroform, Methyl Cellosolve and *p*-dioxane. The Raman spectrum of 9-ethyladenine is quite close to that of adenosine and serves as a reasonable model compound for the adenosine moiety of NADH, NAD^+, etc. (Yue et al., 1986). The Raman spectra of 9-ethyladenine in all these solvents are very similar to each other. Relative to the H_2O spectrum, the spectrum of 9-ethyladenine in chloroform is found to be the most changed. In Figure 5, we show the Raman spectra of 9-ethyladenine in water and in chloroform. As can be seen, there are very few differences between these two spectra. Hence, the change from a hydrophilic to a hydrophobic environment is quite unlikely to be responsible for the Raman spectral changes that accompany binding of the adenine moiety to either LDH or LADH.

Band Assignments. The assignment of a band to a particular moiety of NADH or NAD^+, to a protein conformational change, or to a protein residue perturbed upon coenzyme binding is accomplished as follows. Nicotinamide bands are easily assigned by comparing the bound NADH, NAD^+, and ADPR spectra. X-ray crystallographic studies suggest that the protein structure is nearly the same for binary complexes of these three molecules with LDH (Chandrasekhar et al., 1973; Grau et al., 1981). The Raman spectra of oxidized and reduced nicotinamide differ markedly because of their very different electronic structure (Yue et al., 1986; see Figures 2c and 3c), and ADPR lacks the nicotinamide head group. Thus, bands that vary among the three bound spectra are safely assigned to the nicotinamide moiety. We note one assignment in particular and that concerns the band at 1113 cm^{-1} in the NADH in LDH and the NADH in LADH spectra of parts a and b of Figure 2. The relative intensity of this band is clearly higher than found in the NADH solution spectrum (Figure 2c) or the bound and solution spectra of NAD^+ (Figure 3). This can be seen by comparing the band intensities of 1084 and 1113 cm^{-1}. The 1084-cm^{-1} band arises from a phosphate mode, and the 1113-cm^{-1} band arises from a ribose pyrophosphate mode. In addition, the rather broad peak at

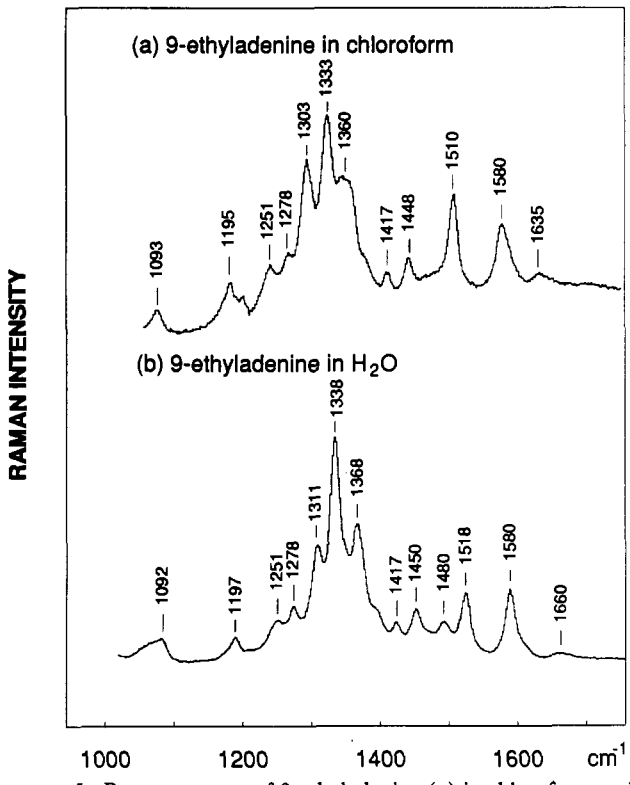

FIGURE 5: Raman spectra of 9-ethyladenine (a) in chloroform and (b) in H_2O at 4 °C and 100 mM concentrations.

1084 cm^{-1} in the NADH solution spectrum (Figure 2c) contains intensity arising from the nicotinamide NH_2 rocking mode (Bowman & Spiro, 1980; Rodgers & Peticolas, 1980; Yue et al., 1986). The contribution of the NH_2 rocking mode to signals at 1084 cm^{-1} disappears when NADH binds to LDH or LADH. The frequency of the NH_2 rocking mode seems to have moved to 1113 from 1084 cm^{-1} upon NADH binding. Corroborating this assignment is the observation that most of the intensity at 1113 cm^{-1} disappears in the NADH in LDH (data not shown) and in the NADH in LADH (Chen et al., 1987) spectra for samples suspended in D_2O, which deuteriates the NH_2 hydrogens, as occurs at 1084 cm^{-1} for NADH in D_2O (Bowman & Spiro, 1980; Rodgers & Peticolas, 1980; Yue et al., 1986).

Having identified the nicotinamide bands, most of the remaining bands can be assigned by a close correspondence to a peak in the solution spectra of NADH, NAD$^+$, and/or ADPR or to a peak found in the spectrum of ADPR bound to LADH; the peaks in these spectra have been assigned previously (Yue et al., 1986; Chen et al., 1987). Thus, in the bound ADPR data of Figure 4a, the 1309-, 1325-, 1340-, 1375-, 1421-, 1481-, 1510-, and 1576-cm^{-1} peaks are adenine-related peaks, the 1085-cm^{-1} peak is a phosphate mode, and the 1113-cm^{-1} peak is assigned to a ribose pyrophosphate mode. The 1220-cm^{-1} peak in Figure 4a has the same relative intensity as the 1245-cm^{-1} peak found in the ADPR bound to LADH spectrum (Figure 4b) and the 1254-cm^{-1} band in the solution spectrum of ADPR (Figure 4c). In the latter two cases, this band has been assigned to adenine, and we tentatively assign the 1220-cm^{-1} band in Figure 4a to adenine. We generally do not have sufficient data to label an adenine peak, for example, as having a small contribution from the bound ribose, a A/δR peak, in the bound spectra as we do in the case of solution spectra. Thus, the adenine-labeled peaks may or may not also contain a contribution from molecular motions of the bonded ribose. We have consistently observed a peak at 1670 cm^{-1} in the bound NAD$^+$ spectrum data of Figure 3a. We also appear to observe an intensity trough at 1650 cm^{-1}. For this reason and because NAD$^+$ has no bands in this frequency region while this is the amide I region (see Figure 1), we tentatively assign the 1670-cm^{-1} peak and the 1650 cm^{-1} trough to changes in the amide I protein bands that accompany the binding of NAD$^+$. As mentioned above, the amide I bands are sensitive to protein conformational changes. We have labeled these bands Pr peaks for this reason. A similar pattern is observed in the NADH in LDH spectrum of Figure 2a. There are several peaks that we are currently unable to assign, and they have been labeled with a question mark.

Discussion

Adenine Binding. Our Raman results on the adenine moiety indicate that substantial molecular changes accompany its binding to LDH. The Raman spectrum of bound adenine differs markedly with that of adenine in solution. The strong 1338-cm^{-1} solution band disappears upon binding and is replaced by bands at 1325 and 1340 cm^{-1}. Also, the intensity of the 1308- and 1378-cm^{-1} solution bands seem to change upon binding, and the 1254-cm^{-1} solution band either disappears or, as we have tentatively suggested above, moves to 1220 cm^{-1} when the adenine moiety binds to LDH. There are also several bands in the spectrum of bound ADPR (Figure 4a), which we are unable to assign specifically to adenine and which maybe protein bands (see above), but it is equally likely that one or more of these bands may be new adenine bands not found in the solution spectrum.

While the Raman spectrum of adenine bound to LDH differs considerably with its solution spectrum, its spectrum is quite similar to that of adenine bound to LADH. There are, however, differences, and these can be seen by comparing the ADPR data in parts a and b of Figure 4. For instance, the band at 1309 cm^{-1} in the LDH data (Figure 4a) probably has the same origins as the peak at 1303 cm^{-1} in the LADH data (Figure 4b). However, the 1303-cm^{-1} peak is not only slightly shifted but has somewhat higher relative intensity to that at 1309 cm^{-1}. The 1254-cm^{-1} peak found in the solution data (Figure 4c) appears to be found at 1245 cm^{-1} in the LADH spectrum (Figure 4b) and at 1220 cm^{-1} in the LDH spectrum (see Results). The 1471-cm^{-1} peak, an adenine band, found in the ADPR in LADH spectrum shifts to 1481 cm^{-1} in the ADPR in LDH spectrum. Also, one or more of the 1290-, 1606-, 1625-, and 1668-cm^{-1} bands found in the spectrum of ADPR in LDH may be associated with adenine, and there is no corresponding band in the ADPR in LADH spectrum.

The environments of the adenine moiety of NAD$^+$ bound to LDH (Grau et al., 1981) and to LADH (Eklund et al., 1984) have been determined by X-ray diffraction to moderate resolution. The adenine binding pocket is very similar for both enzymes, the site being generally hydrophobic in both cases. However, a strongly conserved residue among dehydrogenases in the adenosine binding site is an aspartate, Asp-223 in LADH and Asp-53 in LDH. In the binary complex, this Asp is buried in a hydrophobic environment and does not form an ion pair with any enzyme residue. However, it does interact with the coenzyme. One of the side-chain oxygens of the Asp forms a hydrogen bond with the 2′-oxygen atom of the NAD$^+$ or NADH adenosine ribose in the crystal structure of both LADH and LDH. The other Asp oxygen is close to adenine's N3, being 3.9 Å away in LADH and 3.3 Å away in LDH. In LADH, another polar group, Arg-271 (which forms an ion pair with Asp-273), is also near the adenine ring. The adenine binding site is close to the protein surface with adenine's NH_2 moiety at the boundary. The adenine ring is somewhat more

buried in LADH than in LDH. For example, the calculated (Eklund & Brändén, 1986) solvent accessibility of N6 (the NH_2 moiety nitrogen) is 14 and 30 Å^2 in LADH and LDH, respectively, compared to its solution value of 55 Å^2. On the other hand, only N1, N6, N7, and C8 are accessible to solvent to any degree in both enzymes.

The changes in the Raman spectrum of adenine when NADH or NAD^+ binds to either LDH or LADH are difficult to explain solely on the basis of a change from a hydrophilic to a hydrophobic environment. Experiments on 9-ethyladenine, which is a structural analogue of adenosine and has a Raman spectrum very close to that of adenosine (Yue et al., 1986), shows an almost identical Raman spectrum in water and in chloroform (Figure 5) and in a variety of other solvents (see Results). Moreover, we have shown previously that there is no change in the Raman spectrum of ADPR under high-salt conditions (4 M NaCl) or in 50% *tert*-butyl alcohol–water solutions (Chen et al., 1987). As the polar group of Arg-271 found in the LADH adenine binding site is not conserved in LDH, this arginine cannot be an essential factor in the normal mode pattern of adenine bound to LADH since the spectrum of LADH-bound adenine is very close to that of LDH-bound adenine.

A plausible explanation of the pronounced changes in the adenine spectrum when it binds, in view of the above, is that the adenine ring protonates and forms an ion pair with the carboxylate of Asp-53 in LDH and Asp-223 in LADH. Protonation of the adenine was first proposed by Fisher et al. (1967) to explain changes in adenine's 260-nm absorption band upon binding,[2] which are very similar to, but not identical with, those found when adenine in solution is titrated to low pH. We have found that major changes in the Raman spectrum of adenine bands (in, e.g., ADPR), particularly in the 1300–1400-cm^{-1} region, accompany protonation of adenine in solution (Yue et al., 1986), and these changes show a major isotope effect when experiments are performed in D_2O. While the observed spectral changes upon protonation of adenine in solution are qualitively the same as those found when the adenine ring (in NADH, NAD^+, or ADPR) binds to either LDH or LADH, the specific patterns are distinctly different. Thus, the results indicate that the adenine tautomer found in the coenzyme cleft would not be the same as that for adenine in solution at low pH under protonating conditions. It seems quite reasonable to suppose, because of the close distances involved, that the dehydrogenases' site-conserved aspartate donates a proton to adenine's N3 and forms a stable salt bridge. There is just about the correct distance (see above) to place a H^+ ion between Asp (COO^-) and adenine N3 as determined from the X-ray crystallographic data. In solution, adenine's N1 nitrogen protonates with a $pK \approx 3.9$ (Moore & Underwood, 1969). Unfortunately, studies of bound NADH and ADPR in deuteriated samples are unable to confirm or exclude whether adenine is protonated when it binds. In both LADH (Chen et al., 1987) and LDH (unpublished results), the Raman spectra of deuteriated samples contain the strong 1338-cm^{-1} band found in adenine solution spectra. This represents a large isotopically induced spectral change upon deuteriation as would be expected. However, the data can be interpreted as resulting from a loosely bound, or solvent-accessible, unprotonated adenine ring in deuteriated samples compared to more tightly bound, solvent-restricted bound ring in protonated samples (Chen et al., 1987).

Although unlikely in view of the pronounced changes that take place in adenine's vibrational spectrum when it binds to enzyme and the small changes that take place in different solvent types, it is also possible that the adenine group forms a very strong hydrogen bond in the binding site, sufficiently strong to cause the spectral changes. The pK_a's of the Asp groups mentioned above could shift substantially upward with the acid forming a strong hydrogen bond with N3 of the adenine ring. Alternatively, it is also possible that the solution structure of the protein relaxes sufficiently enough to accommodate a water molecule between adenine's N3 and the Asp, and this water molecule forms a strong hydrogen bond.

Nicotinamide Binding. The spectrum of NAD^+ contains few bands assigned to the nicotinamide moiety. The dominant 1033-cm^{-1} band is unchanged when NAD^+ binds to LDH (Figure 3), and such a sharp band near 1000 cm^{-1} is characteristic of aromatic rings. For example, benzene's spectrum contains an intense band near 991 cm^{-1}, which has been assigned to a ring breathing mode. N-Methylated pyridine, the simplest analogue for oxidized nicotinamide, also has an intense band at 1029 cm^{-1} (Chen et al., 1987). Thus, the aromatic nature of the nicotinamide moiety of NAD^+ apparently is unchanged upon binding.

On the basis of X-ray structural determinations, hydrogen bonding between the apoprotein and the nicotinamide carboxamide moiety has been proposed for binary and ternary complexes of LDH and LADH. We are able now to observe these interactions directly. For instance, the weak broad peak at 1700 cm^{-1} found in the solution spectrum of NAD^+ is probably due to the stretching motion of the amide C=O group of NAD^+ (Yue et al., 1986), and it is reasonable to suppose that the 1691-cm^{-1} band found in the bound spectrum is also the C=O stretching mode because of its high frequency (Figure 3).[3] The band is likely to sharpen when NAD^+ binds to the enzyme because of the more homogeneous bonding pattern found in the protein active site relative to that found in solution. A 9-cm^{-1} shift in the frequency, between the bound and unbound amide C=O stretching motion resulting from different hydrogen-bonding patterns, is very reasonable. For instance, Asher et al. (1977) have obtained an empirical correlation between the stretching frequency of a carbonyl bond and electrostatic interactions of the C=O moiety with nearby charged groups in studies of nonactin solid complexes with various cations. On the basis of this correlation, a shift of 9 cm^{-1} between the values for the bound and unbound carbonyl stretching frequencies implies a favorable electrostatic interaction between the carbonyl moiety and its protein environment of 7 kcal/mol relative to that found in water. We wish to emphasize that this is not the energy of the hydrogen bond formed between the carbonyl group and the enzyme. For example, another component of the hydrogen-bond energy arises from polarizing the C=O double bond, which results

[2] Subramanian et al. (1981) have suggested that the change in the absorption spectrum of the adenosine moiety at low pH relative to high pH is due to a change in the ionic and/or polar environment rather than protonation of the adenine ring. They observed changes in adsorption spectra of ADPR in various solutions, like high-salt and *tert*-butyl alcohol water mixtures, similar to those found by Fisher et al. (1967). We have previously shown, however, that the Raman spectrum of ADPR is virtually unaffected by these solvent changes (Chen et al., 1987). Clearly, the Raman spectrum is a much more sensitive probe of the question of adenine ring protonation.

[3] We have recently obtained the Raman spectrum of the cofactor acetylpyridine adenine dinucleotide ($APAD^+$) in solution and bound to LDH. In this molecule NAD^+'s carboxamide group is replaced by an acetyl group, and a clearly identified, as assigned through ^{18}O labeling studies, C=O stretching mode is observed. This mode lies at 1710 cm^{-1} for $ADPR^+$ in solution and shows a 10-cm^{-1} downward shift when bound to LDH. These data essentially prove the assignment of the NAD^+ 1700-cm^{-1} mode to the carbonyl stretch.

in its observed downward shift in frequency, and this is a negative term. Nevertheless, it is clear that the interaction between the carbonyl group and the enzyme is more favorable than that between it and water for NAD^+ in solution. In addition, was have also shown under Results that the amide NH_2 rocking mode, found at 1084 cm^{-1} in solution, shifts to 1114 cm^{-1} when NADH binds to LDH or LADH. This 30-cm^{-1} increase in frequency is in the correct direction for an increased hydrogen-bonding interaction at the coenzyme binding pocket relative to solution interactions. An increased and/or more directional electrostatic interaction between the NH_2 moiety and the apoenzyme found at the coenzyme binding pocket compared to that found in solution would be expected to increase the frequency of the rocking motion. At the moment, we have no way of estimating the energy of this hydrogen bonding at the binding pocket. A 30-cm^{-1} change in the rocking mode frequency is, however, rather sizable.[4] Our studies are on pig heart LDH where X-ray structural data have not been refined sufficiently to determine the hydrogen-bonding atoms. In dogfish LDH (M. G. Rossmann, Purdue University, personal communication), the amide hydrogens appear to hydrogen bond to the backbone carbonyl of residue 136, and the carbonyl oxygen appears to bind to a water molecule, which may be complexed to other protein moieties, as determined by crystallographic studies. A similar pattern is observed in LDH from *Bacillus stearothermophilus*.

Upon binding to either LDH or LADH, the Raman spectrum of the reduced nicotinamide moiety of NADH shows some very interesting changes (Figure 2). We have already discussed that the shift in frequency of the NADH 1688-cm^{-1} solution band to 1685 cm^{-1} for NADH in LDH to 1681 cm^{-1} for NADH in LADH and correlated these changes to λ_{max} under Results. The largest change in the NADH spectrum is the disappearance of the solution spectrum's 1546-cm^{-1} band (Figure 2c) upon binding. This occurs in both the LDH spectrum and the LADH spectrum. It has been suggested (Bowman & Spiro, 1980) that the 1616- and 1546-cm^{-1} bands are the in-phase and out-of-phase C=C stretches, respectively, of the reduced nicotinamide moiety by analogy with 1,4-cyclohexadiene bands at 1639 and 1680 cm^{-1} (Stidham, 1965; Bajdor et al., 1987). The lowered frequencies could be due to conjugation and coupling to the carboxamide C=O bond. The 1546-cm^{-1} band is upshifted somewhat upon deuteriation of the amide's protons (Yue et al., 1986).

It is difficult to rationalize how the 1546-cm^{-1} band could disappear upon NADH binding. Bowman and Spiro (1980) speculate that the large relative intensity of this mode compared to the 1616-cm^{-1} band could result from the specific nature and direction of the transition dipole moment of NADH's 340-nm absorption band. This near-UV $\pi\pi^*$ band likely extends from the reduced ring moiety to the carboxamide group. As we have seen above, the carboxamide moiety is strongly hydrogen bonded to the enzyme active site, and these interactions together with others "fix" the orientation of the nicotinamide ring with respect to the carboxamide group. It is conceivable that a rotation about the ring–carboxamide bond could modulate the intensity of the out-of-phase 1546-cm^{-1} band by varying the relative direction of the ring and carboxamide transition dipole moments, which together sum coherently to form the total transition dipole moment. Thus, the particular orientation of this angle found at the active site could result in a marked decrease in the intensity of the 1546-cm^{-1} band. It is clear that studies of isotopically labeled nicotinamide compounds coupled with theoretical studies estimating the intensities of the observed coenzymes bands are needed so that occurate assignments of the modes and their strengths may be made. This work is currently under way.

Registry No. NADH, 58-68-4; NAD^+, 53-84-9; LDH, 9001-60-9; ADPR, 20762-30-5; 9-ethyladenine, 2715-68-6.

[4] It must be remembered that these are not the only interactions between the nicotinamide head of NAD^+ with its protein environment. Other interactions between the charged ring and its hydrophobic binding pocket must more than offset these favorable ones since both LDH and LADH bind NAD^+ less favorably than ADPR.

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An Ultraviolet Resonance Raman Study of Dehydrogenase Enzymes and Their Interactions with Coenzymes and Substrates†

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Received June 3, 1988; Revised Manuscript Received September 19, 1988

ABSTRACT: Ultraviolet resonance Raman (UVRR) spectra, with 260-nm excitation, are reported for oxidized and reduced nicotinamide adenine dinucleotides (NAD^+ and NADH, respectively). Corresponding spectra are reported for these coenzymes when bound to the enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and liver and yeast alcohol dehydrogenases (LADH and YADH). The observed differences between the coenzyme spectra are interpreted in terms of conformation, hydrogen bonding, and general environment polarity differences between bound and free coenzymes and between coenzymes bound to different enzymes. The possibility of adenine protonation is discussed. UVRR spectra with 220-nm excitation also are reported for holo- and apo-GAPDH (GAPDH·NAD^+ and GAPDH alone, respectively). In contrast with the 260-nm spectra, these show only bands due to vibrations of aromatic amino acid residues of the protein. The binding of coenzyme to GAPDH has no significant effect on the aromatic amino acid bands observed. This result is discussed in the light of the known structural change of GAPDH on binding coenzyme. Finally, UVRR spectra with 240-nm excitation are reported for GAPDH and an enzyme–substrate intermediate of GAPDH. Perturbations are reported for tyrosine and tryptophan bands on forming the acyl enzyme.

The binding of the coenzyme nicotinamide adenine dinucleotide in both its oxidized and reduced forms (NAD^+ and NADH) to dehydrogenases has been studied extensively by a variety of methods (Sund, 1977; Gronenborn & Clore, 1982; Leslie & Wonacott, 1984; Chen et al., 1987). The X-ray structures of alcohol dehydrogenases from liver (LADH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and lactate and malate dehydrogenase have provided substantial information about bound NAD^+ conformation, bonding, and environment, highlighting the general similarity of coenzyme binding domains and coenzyme conformation throughout these enzymes (Rossmann et al., 1975). Comparison of holo- and apoenzyme (NAD^+–enzyme complex and enzyme alone, respectively) structures has also pinpointed gross protein structure changes on binding coenzyme (Leslie & Wonacott, 1984; Eklund et al., 1984). The structure and atom numbering system for NAD^+ are given in Figure 1.

Raman and resonance Raman (RR) spectroscopies have been increasingly used over the last 2 decades in the study of biological systems [for a recent review, see Clark and Hester (1986)]. Heme proteins, flavoproteins, rhodopsins, and photosynthetic systems have all been investigated by RR spectroscopy, using lasers in the visible and near-ultraviolet regions. However, many enzymes, including GAPDH and alcohol dehydrogenases, do not contain chromophores in the visible or near-UV, although in some cases artificial substrates have been used to provide suitable chromophoric labels (Carey, 1982). The recent development of far-ultraviolet lasers has initiated a new growth in the use of RR spectroscopy of biological systems. Aromatic amino acids absorb in the wavelength regions 250–280 and 200–230 nm, and the amide group absorbs at ca. 190 nm, so all proteins can be investigated by UVRR spectroscopy. Although many of the bands that are present in normal (nonresonance) Raman (NR) spectra can also be observed in UVRR spectra, they do not always retain the same sensitivity to environment and bonding. The UVRR spectra of the individual aromatic amino acids have now been established (Rava & Spiro, 1985a; Asher et al., 1986), but since UVRR spectra have been obtained for only a small number of proteins, identification of structure–spectra relationships is still at a preliminary stage. Secondary structure estimates have been made from the RR amide II band, with 192-nm excitation, whereas the amide I band is comparatively weak in the RR spectrum and not an accurate guide to secondary structure (Copeland & Spiro, 1987). Similarly, in the study of insulin with 200-nm excitation, the commonly used

† We thank the SERC for financial support.
‡ University of York.
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0006-2960/89/0428-1533$01.50/0 © 1989 American Chemical Society