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

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ABSTRACT: We report the Raman spectra of reduced and oxidized nicotinamide adenine dinucleotide (NADH and NAD^+, respectively) and adenosine 5′-diphosphate ribose (ADPR) when bound to the coenzyme site of liver alcohol dehydrogenase (LADH). The bound NADH spectrum is calculated by taking the classical Raman difference spectrum of the binary complex, LADH/NADH, with that of LADH. We have investigated how the bound NADH spectrum is affected when the ternary complexes with inhibitors are formed with dimethyl sulfoxide (Me_2SO) or isobutyramide (IBA), i.e., LADH/NADH/Me_2SO or LADH/NADH/IBA. Similarly, the difference spectra of LADH/NAD^+/pyrazole or LADH/ADPR with LADH are calculated. The magnitude of these difference spectra is on the order of a few percent of the protein Raman spectrum. We report and discuss the experimental configuration and control procedures we use in reliably calculating such small difference signals. These sensitive difference techniques could be applied to a large number of problems where the classical Raman spectrum of a "small" molecule, like adenine, bound to the active site of a protein is of interest. The spectrum of bound ADPR allows an assignment of the bands of the bound NADH and NAD^+ spectra to normal coordinates located primarily on either the nicotinamide or the adenine moiety. By comparing the spectra of the bound coenzymes with model compound data and through the use of deuteriated compounds, we confirm and characterize how the adenine moiety is involved in coenzyme binding and discuss the validity of the suggestion that the adenine ring is protonated upon binding. The nicotinamide moiety of NADH shows significant molecular changes upon binding. We find that the aromatic nature of the NAD^+ nicotinamide ring is disrupted in the ternary complex LADH/NAD^+/pyrazole. We discuss various models which are consistent with the data and with the enzymatic mechanism of LADH. We finally note that the rather dramatic changes in the coenzyme molecular structure, that occur when NADH or NAD^+ binds, are not necessarily repeated at other dehydrogenase binding sites.

Nicotinamide adenine dinucleotides (NAD^+ and NADH)[1] are coenzymes for hundreds of oxidation-reduction reactions (Daziel, 1975). Their roles in enzymatic reactions have been under investigation for the last few decades by various techniques (Kaplan, 1960; Colowick et al., 1966; Hollis, 1967; Fisher et al., 1969; Schlessinger et al., 1975; Subramanian &

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[1] Abbreviations: NAD^+, oxidized β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide; LADH, liver alcohol dehydrogenase; ADPR, adenosine 5′-diphosphate ribose; AMP, adenosine 5′-monophosphate; NMN, β-nicotinamide mononucleotide; IBA, isobutyramide; Me_2SO, dimethyl sulfoxide; OMA, optical multichannel analyzer; fwhm, full width at half-maximum.

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Ross, 1977, 1978; Rizzo et al., 1987). A number of high-resolution X-ray crystallographic studies on various NAD-linked dehydrogenases and their binary and ternary complexes have been reported (Brändèn et al., 1975; Plapp et al., 1978; Eklund et al., 1981, 1984; Holbrook et al., 1975; Harris & Waters, 1976; Banaszak & Bradshaw, 1975; Rossman et al., 1975; Ohlsson et al., 1974). However, detailed understanding of the coenzyme molecular properties in situ and interactions between the coenzyme in the active site and surrounding amino acid residues can only be surmized or examined indirectly. Raman spectroscopy is very useful in providing information on such molecular properties and has been used successfully to study enzymatic reactions in some cases [see, e.g., Carey and Storer (1984)]. Recently (Yue et al., 1984b), we reported the Raman spectrum of NADH when bound to horse liver alcohol dehydrogenase (EC 1.1.1.1; LADH). Drastic differences were observed between the bound NADH spectrum and the NADH solution spectrum.

Raman spectroscopy is known for its ability to provide detailed information concerning the molecular properties of biologically interesting molecules. Resonance Raman spectroscopy has been particularly useful in elucidating the properties of prosthetic groups in the active sites of proteins as in, for example, visual pigments, hemoproteins, and others [see, e.g., Carey and Storer (1984)]. A requirement of this technique, however, is that the prosthetic group must absorb visible or near-ultraviolet light so as to be in resonance with the Raman exciting laser light. The resonance-enhanced Raman cross section of the prosthetic group is then much larger than the protein Raman spectrum and is, thus, easily obtained even though the prosthetic group is much smaller than the protein. Thus, resonance Raman studies are limited to proteins containing chromophoric groups. In principle, the Raman spectra of nonabsorbing small molecules at the active sites of proteins can be obtained by classical Raman spectroscopy by employing sensitive difference techniques. The protein/small molecule binary complex can be measured, and the protein spectrum subtracted. The resulting difference spectrum yields the Raman spectrum of the in situ small molecule and, possibly, protein changes brought about by the binding of the small molecule. There are clearly a large number of interesting biological problems which could be very usefully studied by such measurements. Enzyme–substrate interactions are obvious examples. The idea of using classical Raman difference spectroscopy to obtain changes in a molecular spectrum resulting from small perturbation has been previously proposed (Bodenheimer et al., 1972; Kiefer, 1973). However, apart from our recent study (Yue et al., 1984b), the technique has not been applied to the studies of proteins.

In our earlier study (Yue et al., 1984b), we employed sensitive difference techniques to obtain the Raman spectrum of bound NADH. We subtracted the Raman contribution of LADH from the binary LADH/NADH Raman spectrum. Here, we discuss in detail our methodology for obtaining these difference spectra and for assessing their accuracy. The difference between the binary complex and protein can be quite small, on the order of a few percent in the present study. Apart from shot noise, the effects of systematic factors, such as spectrometer drift, on such small differences must be assessed. The previous reports where Raman difference spectroscopy has been applied to biological molecular systems, like those of Rousseau and his colleagues (Shelnutt et al., 1979; Rousseau, 1981), used split rotating cells and showed how to obtain very accurate frequency differences between normal modes of two nearly identical samples. Unfortunately, this instrumentation is not particularly suitable for studies of the type described here. Rotating cells with photomultiplier detectors and photon counting techniques can avoid a number of systematic errors by quickly switching from one sample to another. The signals are gated, fed to a computer, and subtracted appropriately. It is a powerful technique when the Raman signal is fairly strong. However, the classical Raman spectra of proteins in solution are typically weak. It is necessary to use optically faster optical multichannel analyzer (OMA) detectors rather than photomultipliers in order to obtain spectra within a reasonable period of time and with sufficiently small shot noise (i.e., less than 1% of signal). OMA's, however, do not lend themselves easily to measuring light signals arising from switching quickly from one sample to another. This is because there is significant noise superimposed on the signal each time OMA detectors are read. OMA's are most properly used when the Raman signal is integrated and read as infrequently as possible. We show below how the effects of systematic errors can be estimated, under conditions where the two samples are infrequently changed. We show that problems associated with systematic errors can be sufficiently small, under 1%, to discern the spectrum of a protein-bound nucleotide from the protein/nucleotide binary spectrum.

Using these difference techniques, we have extended our previous results on bound NADH by obtaining its Raman spectrum over a wider spectral range (350–1750 cm^{-1}), by examining the changes on the spectrum of bound NADH over a broad pH range, and by studies of deuteriated LADH/NADH complexes. We have also obtained the spectrum of ADPR bound to the active site of LADH. The purpose of this measurement is 2-fold. A protein conformational change accompanies the binding of NADH to LADH (Eklund et al., 1984; Brändèn et al., 1975). This conformational change does not occur when ADPR binds, but the interactions between the adenosine moiety and the protein are similar to that of NADH (Eklund et al., 1984; Yonetani, 1963; Theorell & Yonetani, 1964). By comparing the spectrum of bound NADH with that of bound ADPR, we are able to conclude that none of the major bands of the bound NADH spectrum result from this conformational change. Moreover, ADPR lacks NADH's nicotinamide moiety, and this is very useful in assigning bands in the bound NADH Raman spectrum to particular NADH moieties. We also report the Raman spectrum of bound NADH and NAD^+ in abortive ternary complexes with LADH inhibitors, IBA and Me_2SO for NADH and pyrazole for NAD^+.

Our results reveal that the adenine moieties of both NADH and NAD^+ (in addition to ADPR) are perturbed significantly upon binding. It is not clear, however, whether the adenine ring is protonated as suggested earlier by UV–vis difference spectroscopy (Fisher et al., 1967). The nicotinamide ring moiety of NADH is also modified upon binding. We discuss various molecular models which are consistent with the data and with the catalytic attributes of LADH. We also show, in agreement with previous reports (Theorell & Yonetani, 1963; Shore & Gilleland, 1970; Andersson et al., 1981; Eklund et al., 1982), that pyrazole interacts strongly with the nicotinamide moiety of NAD^+, forming a NAD–pyrazole adduct, when it binds to the binary complex of LADH/NAD^+.

Materials and Methods

NADH and NAD^+ were purchased either from Sigma Chemical Co. (grade III; St. Louis, MO) or from Boehringer Mannheim Co. (100%; Indianapolis, IN). LADH was purchased as a crystalline suspension in 10% ethanol from Boehringer Mannheim Co. Before use, the LADH suspension

was centrifuged at 4 °C at 6000*g* for 20 min to collect the crystals. The pellet was redissolved in 0.05 M buffer and then centrifuged again at 4 °C at 6000*g* for 10 min to remove denatured protein. The supernatant was then dialyzed overnight at least twice against 0.1 M buffer and then concentrated by using a collodion bag vacuum filtration apparatus (A. H. Thomas, Philadelphia, PA) or a centricon centrifuge concentrator (Amicon, Lexington, MA). The activity was measured by the method of Dalziel (1963). No enzyme preparation was used with an activity less than that reported by Dalziel. Deuteriated samples were prepared by repeatedly concentrating enzyme solutions diluted by D_2O/deuteriated buffer. Isobutyramide, dimethyl sulfoxide, and pyrazole were purchased from Sigma Chemical Co. and used without further purification. Concentrations of enzymes and coenzymes were determined spectroscopically, using $\epsilon_{280} = 3.57 \times 10^4$ M^{-1} cm^{-1} for LADH, $\epsilon_{340} = 0.622 \times 10^4$ M^{-1} cm^{-1} and $\epsilon_{259} = 1.44 \times 10^4$ M^{-1} cm^{-1} for NADH, $\epsilon_{259} = 1.52 \times 10^4$ M^{-1} cm^{-1} for ADPR, and $\epsilon_{259} = 1.8 \times 10^4$ M^{-1} cm^{-1} for NAD^+. LADH contains two independent active sites; thus, the binary complex of LADH/NADH was prepared by mixing a 1:<2 molar ratio of LADH to NADH, NAD^+, or ADPR. Depending on the pH, the ratio was adjusted so that at least 90% of the coenzyme or ADPR molecules were bound (DeTraglia et al., 1977; Yonetani, 1963; Theorell & Yonetani, 1964; Li & Vallee, 1964; Taniguchi et al., 1967). A typical concentration of LADH, employed in the Raman studies, was 1 mM. Ternary complexes with inhibitors were prepared by using excesses of the inhibitors as needed (Theorell & McKinley-McKee, 1961; Theorell & Yonetani, 1963; Shore & Gilledand, 1970). Samples were loaded into 3 mm × 3 mm fluorescence cuvettes. The cuvettes were transferred to a cuvette holder (maintained at 4 °C in a bath/circulator) for measurement. The LADH enzymatic activity was monitored before and after Raman measurement. Denaturation of the enzymes or their binary or ternary complexes was not observed during the Raman measurement. Solution spectra of NADH were obtained by using the methods of Yue et al. (1986).

Raman spectra were measured through the use of 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). Spectral lines were calibrated against known assignments of toluene. The detector was interfaced to an LSI-11/2 minicomputer (Digital Equipment Corp., Marlboro, CA) which was also used for analysis. All spectra shown in the following figures were obtained by using either the 488- or the 514.5-nm lines of an argon ion laser (Model 165, Spectra Physics, Mountain View, CA). Under these conditions, about 800 cm^{-1} can be detected simultaneously. Some spectra were also obtained with excitation at 413 and 468 nm with a krypton ion laser (Model 2000-CR, Coherent Radiation Inc., Palo Alto, CA). Band positions are accurate to ±2 cm^{-1}.

A typical measured 800 cm^{-1} segment of a spectrum of LADH/NADH required typically 1–2 h to obtain using the OMA system and 150 mW of laser power incident on the sample. The data presented herein are the result of many runs, each run obtained by integrating the incident Raman signal on the OMA detector for about 10 min. Details of the method of measurement and the procedures used to assess systematic error factors are more fully discussed under Results.

The difference spectra reported here contain contributions of the solvent to various degrees. The most significant contributions are a broad (110 cm^{-1} fwhm) peak at 1636 cm^{-1} due to water (1200 cm^{-1} for D_2O; 80 cm^{-1} fwhm) and two (pH-dependent) buffer lines near 990 and 1076 cm^{-1} (for pH 7.0) and 990 and 1022 cm^{-1} (for pH 9.6).

Results

Subtraction Procedures. We discussed here the techniques and controls we use in obtaining the classical Raman difference spectrum between a protein and a protein/ligand binary complex. It might seem unlikely that the Raman signal from a molecular moiety like adenine, molecular weight 135, could be discerned from a 40 000 molecular weight (per active site) protein like LADH. The respective classical Raman signals might be predicted to scale according to weight, suggesting that the Raman signal of adenine would be about 0.3% that of LADH. However, the classical Raman bands observed from proteins are broad compared to nucleotide bands. For example, the amide I protein band near 1668 cm^{-1} is some 100 cm^{-1} wide compared to the 10 cm^{-1} (or less) width of a typical adenine band (see data below). This suggests that the peak intensity ratio of nucleotide to protein bands would be some 10 times larger (3%) than predicted by simply scaling the relative molecular weights. As will be shown below, we find that adenine's classical Raman bands are on this order. Raman bands which we can assign to NADH's nicotinamide moiety are somewhat larger. The reduced nicotinamide moiety at the active site of LADH is characterized by a well-known absorption at 325 nm, which may result in some preresonance enhancement of nicotinamide's Raman bands. Modern detection equipment, such as that employed here, is capable of obtaining the Raman spectrum of a protein with a noise to signal ratio (due to shot noise) of greater than 1% within a couple of hours. Thus, it is clearly feasible to obtain the classical Raman signal from a protein-bound nucleotide (or other relatively small molecule) by employing sensitive difference techniques and by subtracting the protein spectrum from the binary complex spectrum.

The main experimental problem in measuring small differences in a spectrum is due to systematic factors. For example, varying ambient temperature or room vibration can lead to spectrometer drift. The power level of the laser light used to stimulate the Raman signal varies, leading to signal variation. Optical multichannel detection equipment of the type used in the present investigation is more immune to those factors than detectors based on photomultipliers since an entire spectral segment is measured at once. There is a tendency, therefore, for the entire spectral segment to be affected equally by systematic factors so that the production of spurious bands in the difference spectrum is minimized. Moreover, the influence of these factors on the difference spectra from two samples may be decreased by obtaining spectra in an interleaved ABBAA...BA fashion. Spectrometer drift will tend to affect the sum of the A measurements in the same way as the sum of the B measurements and subtract out of the difference provided the drift time is larger than the time used in individual A and B measurements.

Our procedure for assessing the influence of systematic factors, which might adversely affect the A − B difference spectrum, involves adding every other A spectrum in our ABBAA...BA sequence and subtracting this from 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.

The results for this procedure for a typical data set are given in Figure 1. This experiment made use of eight runs of the enzyme alone and eight runs of the binary LADH/NADH

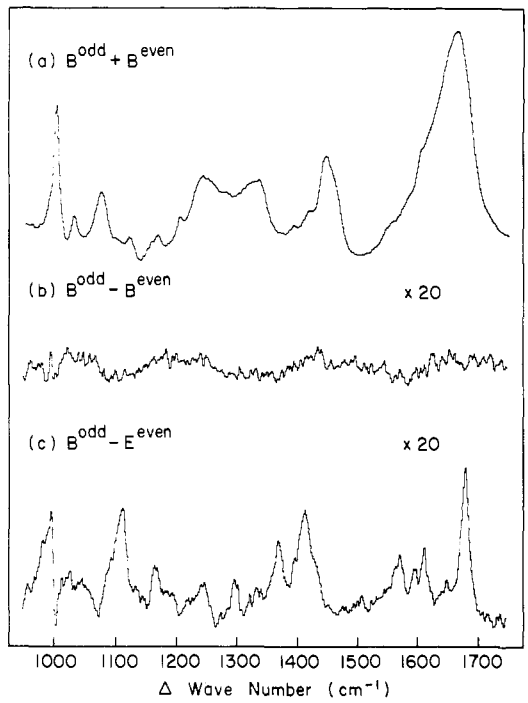

FIGURE 1: Sum and difference spectra of B^{even} (binary complex), B^{odd}, and E^{even} (enzyme) from a particular set of experiments as defined in the text. (a) $B^{odd} + B^{even}$; (b) $B^{oddd} - B^{even}$; (c) $B^{odd} - E^{even}$. The intensity scale of (b) and (c) is 20 times that of (a). Samples were in 0.1 M phosphate buffer, pH 6.0.

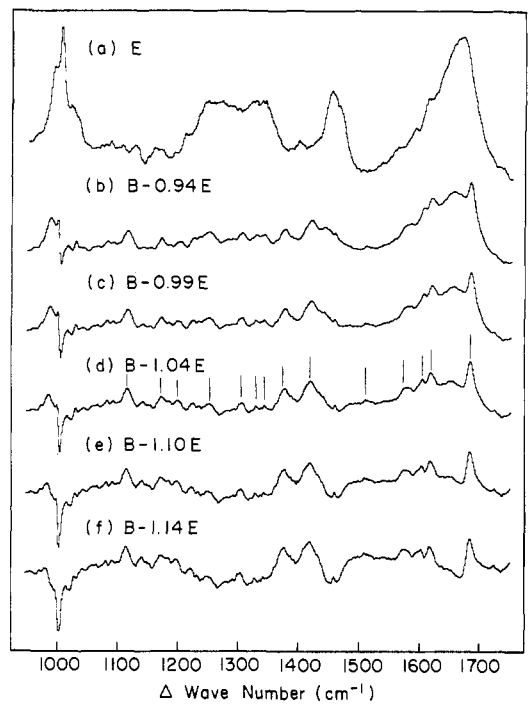

FIGURE 2: Difference spectra between the spectrum of the LADH/NADH complex (B) and that of LADH (E), $B - xE$, showing various different scaling factors, x, between $x = 0.94$ and $x = 1.14$. The optimal value of x is 1.04 (d) for this set of data. Peaks associated with bound NADH (see Figure 3a) are marked in (d) for this set of data. Samples were in 0.1 M pyrophosphate buffer, pH 9.6. Differences between the spectra in Figures 1a and 2a near 1000 cm^{-1} are due to pH-sensitive buffer bands.

complex. Defining the ith enzyme run as E_i and similarly the binary complex runs as B_i, the sequence can be defined as $B_1E_1E_2B_2B_3E_3E_4B_4B_5E_5E_6B_6B_7E_7E_8B_8$. Each run consisted of 10 min of integration time with 180 mW of the 488-nm line of an argon ion laser incident on the sample. Let $B^{odd} = B_1 + B_3 + B_5 + B_7$ and $B^{even} = B_2 + B_4 + B_6 + B_8$. Figure 1a shows the total binary spectrum, $B^{odd} + B^{even}$. The effective noise level and the effects of any systematic problems are shown in Figure 1b which contains the difference spectrum, $B^{odd} - B^{even}$. Note that the scale for Figure 1b is 20 times that of Figure 1a. On the same scale as Figure 1b, the binary spectrum minus the enzyme spectrum, $B^{odd} - E^{even}$, is plotted in Figure 1c. These results show clearly that the signal size is much larger than the spectral noise, whether due to shot noise or systematic errors. In this particular case, the noise level is represented in Figure 1b and is at least 5 times smaller than the difference signal of Figure 1c. Apart from shot noise, some small structure is evident near 1000 cm^{-1} in Figure 1b. This is due to a (minor) incomplete subtraction of the sharp 1003 cm^{-1} phenylalanine band of LADH (Figure 1a; Yue et al., 1984a). The difference spectra also contain Raman bands near 1000 cm^{-1} which arise from the phosphate buffers in our samples. Because of varying concentrations from sample to sample, these bands do not completely subtract out. Bands that are due to buffer lines and/or incomplete subtraction of LADH's strong phenylalanine peak are marked with an "S" in the data shown below. Similar noise levels (data not shown) are obtained by examining various other possible control subtractions, i.e., $E^{odd} - E^{even}$, $B_1 - B_7$, etc. It is clear that no fewer than four measurements, ABBA, should be performed. Fewer than four would not permit any satisfactory assessment of adverse systematic factors which might affect the subtractions.

We are unable to control exactly the signal levels, sample concentrations, and sample alignments to better than a few percent. Thus, we must introduce a factor, x, in forming the difference spectrum, i.e., $B - xE$. The value of this factor is generally close to 1 (within 10%) but is otherwise not known a priori. In our case, this factor is determined when bands, clearly assignable to the protein, subtract out in calculating various $B - xE$ spectra. Bands arising from the bound substrate are quite distinct and discernible from the protein bands in most cases. In general, protein bands are relatively broad, as discussed above, and their location and intensity patterns are known. It is, of course, quite possible that some protein conformational changes take place when substrate binds. Sharp bands may be observed in the difference spectrum which result from this conformational change. The assignment of difference spectral bands arising from bound NADH, rather than from LADH conformational changes when NADH binds, must be made on an experimental basis. This is described in a later section.

In Figure 2, we show a series of difference spectra, $B - xE$, where x is varied over a 10% range in order to assess the results of an over- or undersubtraction. It is evident from the figure which bands, in the difference spectra, can be assigned as arising from the presence of the bound NADH coenzyme and which bands are due to an over- or undersubtraction. These bands are marked in spectrum d of Figure 2. In our study, the δ-CH_2 protein band near 1450 cm^{-1} is particularly useful since the spectrum of NADH contains no bands near 1450 cm^{-1}. Moreover, it is known that δ-CH_2 protein bands are relatively insensitive to protein conformational changes.

Bound NADH and ADPR. Figure 3a shows the difference spectrum between the binary complex of LADH/NADH and LADH. The spectrum has been obtained by using the procedure discussed above and represents the pooled results over many different runs. The region between 1000 and 1750 cm^{-1} has been reported previously (Yue et al., 1984b) at pH 9.6. For this report, measurements were performed at pH 6.0, 7.0, and 9.6 and were repeated many times and at different ex-

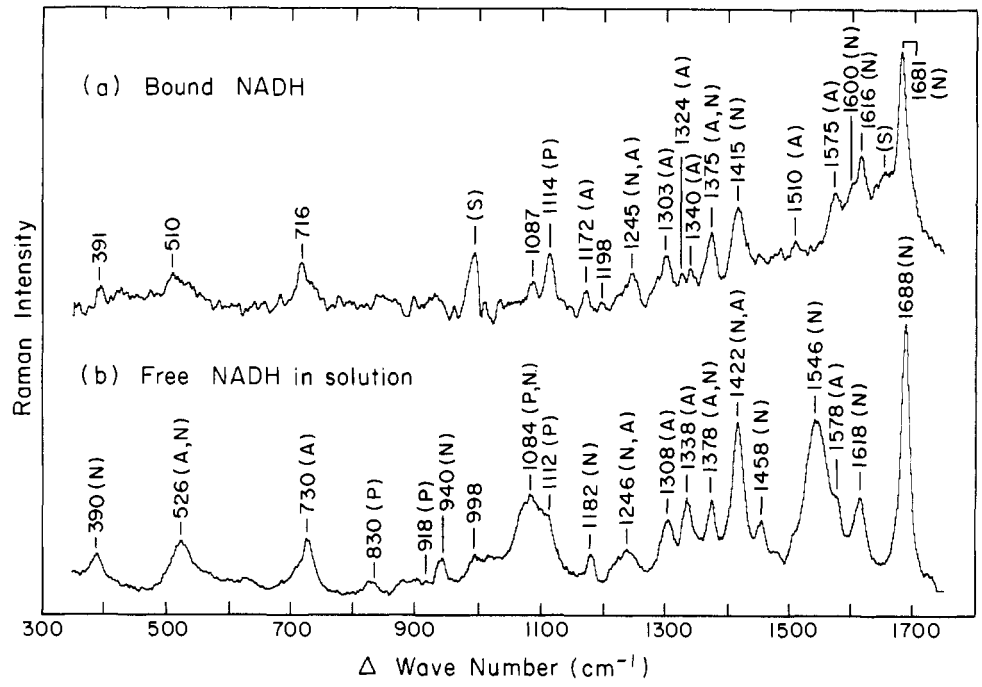

FIGURE 3: Raman spectra of (a) bound NADH (LADH/NADH = 1 mM/2 mM) at 4 °C in 0.1 M pyrophosphate buffer, pH 9.6, and (b) NADH in solution (67 mM) at room temperature in 0.1 M phosphate buffer, pH 7.0. Assignments of the solution peaks in (b) are from Yue et al. (1986); see text for assignments of the bound NADH peaks: A = adenine; P = phosphate; N = nicotinamide.

citation wavelengths (i.e., at 488 and 514.5 nm for NADH and at 413, 468, 488, and 514.5 nm for ADPR). The difference spectra were identical, and all peaks, even the very small ones that are labeled in the figure, were consistently observed. No pH dependence was observed. The Raman spectrum of NADH in solution is shown in Figure 3b for comparison [see e.g., Yue et al. (1986)]. The classical Raman bands in the solution spectra of NADH (or NAD^+) have been found to arise from molecular motion from one or another of the adenine (A), nicotinamide (N), or, to a very limited extent, phosphate (P) moieties of the larger molecule (Yue et al., 1986). These have been so identified in Figure 3b. Similar assignments are made in the data of Figure 3a based on the results below.

LADH undergoes a conformational change when NADH (or NAD^+) binds (Eklund et al., 1984). Thus, the difference spectrum of Figure 3a may arise from changes in the protein structure as well as from the presence of the bound substrate. Since the most prominent spectral features observed in the Figure 3a difference spectrum are clearly positive peaks (with one possible exception at 1003 cm^{-1}; see discussion below) and many can be associated with features found in the spectrum of NADH in solution (compare Figure 3a with Figure 3b), there appears to be little influence on the difference spectrum due to the protein conformational change upon binding. We may, however, determine directly whether any of the peaks in the difference are due to protein conformational changes and, at the same time, help assign the difference spectrum peaks to either nicotinamide or adenine moieties by measurements on the binary LADH/ADPR complex.

ADPR lacks NADH's nicotinamide head and has been shown, in X-ray crystallographic studies (Eklund et al., 1984), to bind to LADH in the same way as NADH in that the adenosine moiety is in the same relative position to the amino acid residues of LADH. Moreover, the binding of ADPR does not induce a conformational change in LADH. Thus, the LADH/ADPR − LADH difference spectrum can show no difference bands due to a protein conformational change. Figure 4a shows the ADPR difference spectrum. For comparison, the Raman spectrum of ADPR in solution is shown in Figure 4b. The bands at 1576, 1510, 1375, 1340, 1324, 1303, 1245, and 1172 cm^{-1} in Figure 3a can be clearly assigned to the adenine moiety since they appear essentially at the same positions and relative intensities (except for the 1375 and possibly 1245 cm^{-1} bands) in the bound ADPR spectrum of Figure 4a. The 1375 and 1245 cm^{-1} bands in the bound NADH spectrum of Figure 3a are relatively more intense than

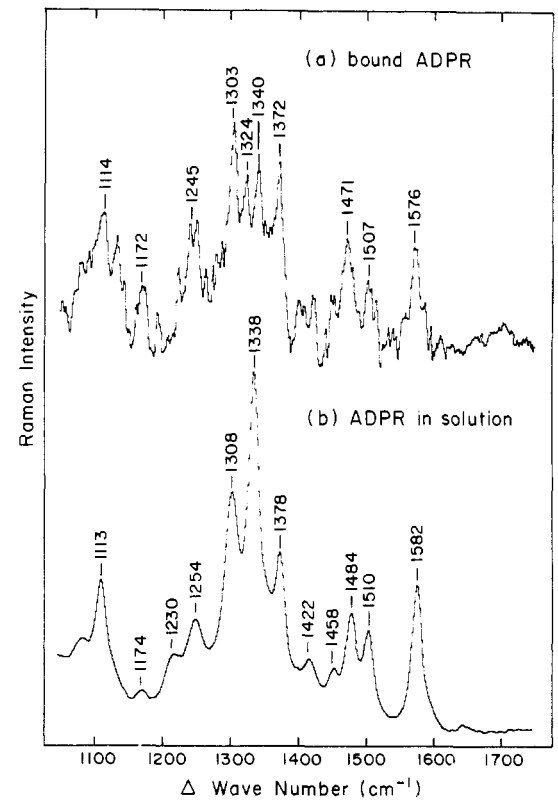

FIGURE 4: Raman spectra of (a) bound ADPR (LADH/ADPR = 1 mM/1.8 mM) at 4 °C and (b) ADPR in solution (80 mM) at room temperature. Both were in 0.1 M pyrophosphate buffer, pH 9.6.

in the bound ADPR data of Figure 4a. These bands very likely also contain a contribution from a mode located on the nicotinamide moiety. This is true of the solution data (Figure 3b). We have labeled bands in Figure 3a as adenine bands as a result of these correspondences. The 1114 cm^{-1} band in Figure 3a is found in the bound and solution spectra of ADPR (Figure 4). This peak has been assigned to a ribose mode with a pyrophosphate contribution (Yue et al., 1986). We have labeled this peak in Figure 3a as a phosphate mode on the basis of this close correspondence. We note that the relatively intense bands at 1681, 1616, 1600, and 1415 cm^{-1} found in NADH's difference spectrum (Figure 3a) are absent in Figure 4a. These correspond quite closely to bands found in the solution spectrum of NADH which have been previously assigned to reduced nicotinamide (Figure 3b). It seems quite reasonable to assign those lines in Figure 3a to NADH's nicotinamide head rather than to protein conformational changes on this basis.

In obtaining the difference spectra for NADH and ADPR, we have observed consistently a negative peak at ca. 1003 cm^{-1}. There is a very strong peak in the spectrum of LADH at 1003 cm^{-1} due to phenylalanine. The negative peak may be an artifact of subtraction. However, it is also possible that the negative peak is real and shows that 1 or more of the 18 phenylalanine residues are involved in binding in such a way that the 1003 cm^{-1} mode is perturbed.

Deuteriation Studies. The triplet marker bands at 1308, 1338, and 1378 cm^{-1} in the solution spectrum of Figures 3b and 4b are readily assigned to the adenine component of NADH [see, e.g., Yue et al. (1986)]. This triplet is quite modified when NADH binds. Either the central 1338 cm^{-1} band (Figure 3b) disappears or its intensity is greatly reduced in the bound spectrum (Figure 3a). In addition, new smaller bands appear in the bound NADH spectrum which are assigned to the adenine moiety, e.g., the 1324 and 1172 cm^{-1} bands. We have observed earlier (Yue et al., 1986), in titration studies of NAD^+ or ADPR, that the adenine triplet marker bands are dramatically changed when the sample pH is below about 4. These results are consistent with a protonation of the adenine ring with a pK of 3.9 (Moore & Underwood, 1969).[2] It has also been suggested that the adenine moiety is protonated when NADH or NAD^+ binds to LADH (Fisher et al., 1967).

In order to explore the possibility that the adenine's ring is protonated upon binding to LADH, we have measured the bound NADH spectrum in D_2O/deuteriated buffer. This is shown in Figure 5, which compare protonated and deuteriated spectra of bound NADH. A peak at 1340 cm^{-1} appears in the bound deuteriated NADH spectrum compared to the protonated spectrum. In addition, the protonated NADH (Figure 5a) band at 1415 cm^{-1} moves to 1425 cm^{-1} in the deuteriated NADH spectrum, with an apparent increase in intensity. This peak is known to be associated with the nicotinamide moiety and was similarly upward shifted for NADH

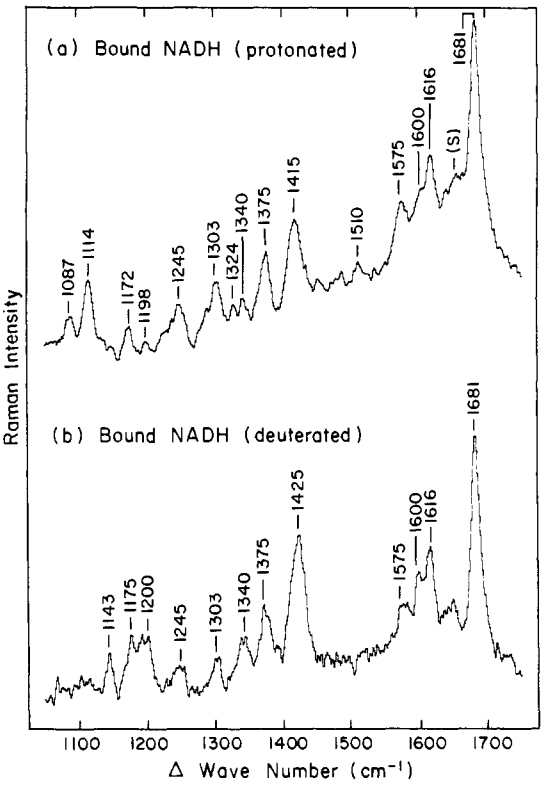

FIGURE 5: Raman spectra of (a) bound NADH (LADH/NADH = 1 mM/2 mM) in 0.1 M pyrophosphate buffer, pH 9.6, and (b) bound NADH (LADH/NADH = 0.9 mM/1.4 mM) in deuteriated 0.1 M pyrophosphate buffer, pD 9.0; both were at 4 °C.

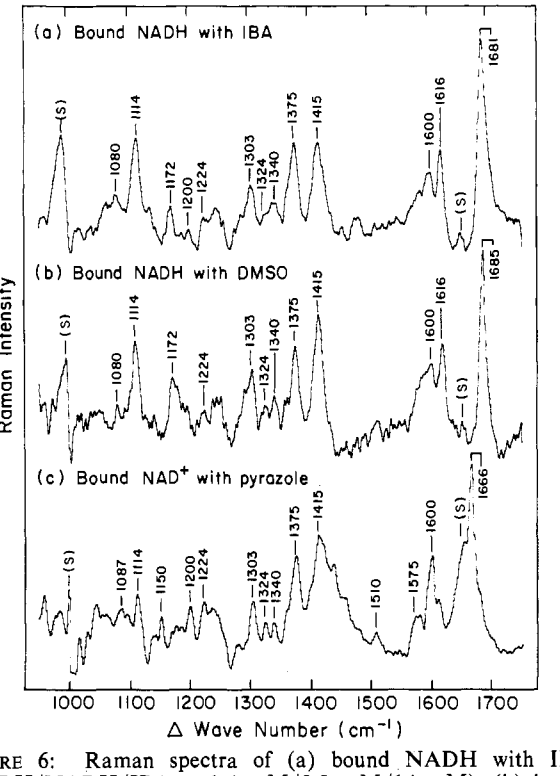

FIGURE 6: Raman spectra of (a) bound NADH with IBA (LADH/NADH/IBA = 1.4 mM/2.8 mM/14 mM), (b) bound NADH with Me_2SO (LADH/NADH/Me_2SO = 1.0 mM/2.0 mM/105 mM), and (c) bound NAD^+ with pyrazole (LADH/NAD^+/pyrazole = 1.1 mM/2.0 mM/5.0 mM); all were in 0.1 M pyrophosphate buffer, pH 9.6, at 4 °C.

in solution upon deuteriation (Yue et al., 1986).

Effects of Inhibitors. We examined the extent to which certain inhibitors affect the Raman spectrum of the bound coenzymes. IBA and Me_2SO form very tight ternary com-

[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 obtained similar absorption spectra of ADPR (or NAD^+) under high salt solutions (4 M NaCl), in 50% *tert*-butyl alcohol and other solvents. We, on the other hand, observed no change in the Raman spectrum or ADPR (or NAD^+) in 4 M NaCl or in 50% *tert*-butyl alcohol solutions, relative to that in dilute solution at pH >5. Furthermore, major changes in the spectrum of ADPR in D_2O/deuteriated buffer can be seen only at a pD below 4. We believe that the many changes in the Raman spectrum of the adenosine moiety upon lowering the pH value from 5 to 3 are due to protonation of the adenine.

plexes with LADH/NADH. Figure 6a and Figure 6b show the spectra of NADH in the ternary LADH/NADH/IBA and LADH/NADH/DMSO complexes, respectively. Pyrazole binds tightly to the LADH/NAD^+ complex, and Figure 6c shows the spectrum of bound NAD^+ in the LADH/NAD^+/pyrazole complex. These spectra were generated in the same way as described above. The spectrum of LADH was subtracted from each ternary complex spectrum. The inhibitor concentration is in excess over the coenzyme in each case. The Raman spectra of free IBA are relatively weak, and its solution spectra did not contribute to the spectrum of Figure 6a. The spectra of Me_2SO and pyrazole in solution were present to an extent, and their respective solution spectra have been subtracted out of Figure 6b,c.

The addition of IBA and Me_2SO to the LADH/NADH complex has very little if any effect on the bound NADH spectrum. Pyrazole, complexed with LADH/NAD^+, produces a bound spectrum that resembles the spectrum of NADH. The peak at 1681 cm^{-1} in the binary complex data of NADH (Figure 3a) is replaced by a similar band at 1666 cm^{-1} upon the formation of the NAD^+/pyrazole ternary complex where a peak at 1415 cm^{-1} remains. It is known that pyrazole binds to the enzyme but also associates with the nicotinamide moiety of bound NAD^+. For example, X-ray crystallography (Eklund et al., 1982) shows that the N2 nitrogen atom of pyrazole is bound directly to the active-site zinc atom and the N1 pyrazole nitrogen is within 2 Å of nicotinamide's C4 position. Shore and Gilleland (1970) also suggested that pyrazole binds to the nicotinamide ring moiety of NAD^+ and not the carboxamide. There is, moreover, a substantial similarity in the absorption spectrum of LADH/NADH (λ_{max} = 325 nm; see above) compared to LADH/NAD^+/pyrazole (λ_{max} = 290 nm; Theorell & Yonetani, 1963; D. Chen and R. Callender, unpublished results) in a region assigned to the nicotinamide moiety. We also find that the addition of excess pyrazole to a solution of LADH/NADH resulted in no changes to the bound Raman spectrum of NADH (data not shown).

Discussion

We have recently shown [Yue et al., 1986; see also Rodgers and Peticolas (1980) and Bowman and Spiro (1980)] that most of the Raman bands of NADH and NAD^+ can be assigned to motions associated with the adenine (as influenced by the bonded ribose) and the nicotinamide moieties of these molecules. Little is known about the actual atomic motions that constitute these normal modes at present (Tsuboi et al., 1973). These molecules are moderately large, and it will take a great deal of systematic study to understand their Raman spectra fully. Nevertheless, some specific structural information has been obtained from the present experiments. We discuss how our results bear on what is known concerning the structure and mechanism of action of liver alcohol dehydrogenase.

Adenine Binding. The binding of NADH or NAD^+ to LADH appears to be, at least, a two-step process. The binding of the AMP moiety of the coenzyme is apparently followed by a rather significant protein conformational change [see, e.g., Eklund and Brändén (1986) for a review]. This has been deduced by a variety of structural and binding studies which suggest that the adenosine binding site must be the recognition side for coenzyme binding. For example, coenzyme analogues, including ADPR, have similar coenzyme–enzyme interactions (Kvassman et al., 1981; Samama et al., 1977, 1981; Cedergren-Zeppezauer et al., 1982; Eklund et al., 1984). The cleft between the AMP and NMN domains is open in the apoenzyme, permitting easy entrance of the nicotinamide ring. After coenzyme binding, the protein conformational change that occurs upon formation of the holoenzyme results in a closed form so that the coenzyme's nicotinamide head is pushed deeper into the enzyme. UV–vis absorption studies (Fisher et al., 1967; Subramanian et al., 1981) showed significant changes in adenine's 260-nm absorption band upon coenzyme or ADPR binding. All these results suggest that the protein tightly binds the adenine moiety. It has been suggested that the adenine moiety is protonated upon binding (Fisher et al., 1967) as the changes in adenine's 260-nm absorption band upon binding are similar to those found when adenine in solution is titrated to low pH.

The environment of the adenine part of NAD^+ and NADH bound to LADH has been determined by X-ray diffraction at 2.9-Å resolution. The site is largely hydrophobic, not particularly specific for adenine, and can accommodate many other hydrophobic groups (Einarsson et al., 1974; Biellman et al., 1979; Eklund et al., 1984). However, the polar groups of Asp-223 and Arg-271 (which form an ion pair with Asp-273) are very close to the adenine moiety. It is possible that a stable rearrangement of ion pairs or hydrogen bonding is formed at the adenine binding site consisting of these residues and/or hydrogen-bonded water molecules with bound adenine.

Our Raman results on bound ADPR show that a significant molecular effect takes place when adenine binds to LADH, as adenine's strong 1338 cm^{-1} mode disappears. This mode has been assigned by Tsuboi and his colleagues (Tsuboi et al., 1973) to be mainly due to stretching motions of the adenine's C5–N7 and C8–N7 bonds. The other observed bands in adenine's solution spectrum, particularly the 1308 and 1378 cm^{-1} bands, are assigned to be due to mainly C–H bending motions mixed with carbon–carbon and carbon–nitrogen stretching motions other than C5–N7 and C8–N7. Thus, it is feasible that the Raman data can be interpreted as resulting from a specific interaction on N7 of the adenine ring. X-ray studies suggest that N7 is hydrogen bonded to a water molecule when bound to LADH (Eklund et al., 1984). Such hydrogen bonding may be enough to disrupt this mode completely. However, it is not clear that these normal mode calculations have enough reliability to warrant conclusions of such molecular detail. Studies employing isotopically labeled adenine are needed for more certain understanding of adenine's normal mode structure and its behavior when bound to LADH.

Unfortunately, our studies on deuteriated bound NADH are unable to confirm or exclude adenine protonation upon binding. The spectrum of deuteriated bound NADH contains the strong adenine 1340 cm^{-1} band found in the solution spectrum (compare Figures 4b and 5b). This suggests to us that the adenine moiety is loosely bound to deuteriated LADH compared to tightly bound in protonated samples. One would expect more dramatic changes in adenine's spectral features from exchange of a hydrogen by a deuteron in a protonated adenine ring. For example, the 1329 cm^{-1} band found in protonated adenine in solution at low pH (Yue et al., 1986) moves to 1342 cm^{-1}, and the relative intensity of the 1411 cm^{-1} band is halved in D_2O solutions at low pD (D. Chen and R. Callender, unpublished data). It should be noted that NADH is clearly bound to LADH in D_2O since the strong NADH 1546 cm^{-1} nicotinamide band which is absent in the bound (protonated) NADH spectrum (Figure 3a) is still absent (Figure 5b). It is possible that dissolving LADH/NADH in D_2O/deuteriated buffer results in some protein conformational changes and a looser protein–adenine binding affinity.

Nicotinamide Binding. Upon binding, the nicotinamide moieties of both NADH and NAD^+ show major molecular changes as deduced from the changes in Raman spectra. The

most pronounced change in the NADH spectrum is the disappearance of the solution spectrum's nicotinamide 1546 cm^{-1} band (Figure 3b) when NADH binds. Apart from these changes, the 1688 and 1182 cm^{-1} bands in the solution spectrum appear to shift downward slightly to 1681 and 1172 cm^{-1}, respectively. There are some suggested assignments of these bands, but no studies, at this time, can unambiguously assign these bands to particular molecular motions. For example, it has been suggested (Bowman & Spiro, 1980) that the 1546 cm^{-1} band in the NADH solution spectrum arises from a ring C=C (out-of-phase) stretching motion. This assignment is based on an analogy with 1,4-cyclohexadiene spectral studies. Indeed, C=C stretching motions lie in the ca. 1500–1600 cm^{-1} region. However, the 1546 cm^{-1} mode is somewhat upshifted upon deuteriation of the amide's protons (Bowman & Spiro, 1980; Yue et al., 1984b), suggesting at least some influences of the carboxamide moiety. The intense solution NADH band at 1688 cm^{-1} has been assigned to carbonyl stretching of the carboxamide group by many different groups (Patrick et al., 1974; Forrest et al., 1976; Barrett, 1980; Nishimura & Tsuboi, 1980; Bowman & Spiro, 1980). Its unusually high intensity could result from a strong coupling between the C=O group and conjugated ring C=C bonds, both of which would undergo large bond length changes in the ground to excited electronic state transition (Bowman & Spiro, 1980). Rodgers and Peticolas (1980) have questioned a strictly carbonyl assignment of the 1688 cm^{-1} band based on a number of observations, the most cogent being the insensitivity of this band to solvent and $-NH_2$ deuteriation. They suggested that this band could result from a ring mode possibly with a contribution from –C=O stretching.

We can contribute to the assignment of the ca. 1688 cm^{-1} band by noting that a Raman study (unpublished data) of a compound, H_2NADH, where the nicotinamide C5–C6 double bond of NADH has been reduced shows no 1688 cm^{-1} Raman band at all (it is also missing in NAD^+). The highest band in H_2NADH lies at 1632 cm^{-1} and is significantly broader and with diminished relative intensity than the 1688 cm^{-1} band of NADH. Moreover, the strong 1681 cm^{-1} band found in the binary complex, LADH/NADH (Figure 3a), appears to be shifted to 1666 cm^{-1} in the ternary complex LADH/NAD^+/pyrazole data (Figure 6c). As discussed below, it seems reasonable to ascribe this to the formation of a NAD^+–pyrazole adduct with covalent bond formation occurring between C4 of NAD^+ and N2 of pyrazole. All these results certainly suggest that ring C=C stretching is involved with the 1688 cm^{-1} mode. We find it plausible that this mode is a highly coupled vibration extending over the whole conjugated π-electron structure of NADH, i.e., extends over the nicotinamide ring –C=C– and, perhaps, also the carbonyl –C=O moiety. Clearly needed for a proper interpretation of our data is experimental work involving isotopically labeled NADH compounds and associated normal mode calculations. Such work is in progress in our laboratory.

While the exact nature of the molecular binding properties of the nicotinamide structure must await further study, it is clear that the nicotinamide moiety undergoes a substantial change. One or both of two general mechanisms may be operating to cause such changes. It has been suggested that strain is an important factor in the high efficiency of enzymes (Jencks, 1969). In this regard, Cook et al. (1981) have proposed possible bent chair conformations for the reduced nicotinamide rings of NADH to explain secondary isotope effects found in kinetic experiments of LADH. Certainly such conformations would have a marked effect on the normal modes of nicotinamide of the type observed here. A second general mechanism is the possible effects of electrostatic interactions between coenzyme and nearby enzyme amino acid residues. X-ray studies (Eklund et al., 1984) suggest hydrogen bonding between nicotinamide's carboxamide group with the peptide backbone of Val-292, Ala-317, and Phe-319. This hydrogen bonding is consistent with the disappearance of the nicotinamide $-NH_2$ rocking mode at 1084 cm^{-1} (Yue et al., 1986) in the solution spectrum when NADH binds (Figure 3). A number of other potentially charged groups are also near the nicotinamide moiety.

Our results indicate that NAD^+ and pyrazole form a covalently bound adduct in the LADH/NAD^+/pyrazole ternary complex. The Raman spectrum of solution NAD^+ is characterized by an intense narrow 1032 cm^{-1} band which arises from oxidized nicotinamide [see, e.g., Yue et al. (1984b)]. Such a sharp intense 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. A similar intense mode at 1029 cm^{-1} is observed (unpublished result) with N-methylated pyridine, the simplest analogue for oxidized nicotinamide. The absence of this mode in the data of Figure 6c shows that the aromatic nature of the NAD^+ nicotinamide ring is completely disrupted in the LADH/NAD^+/pyrazole complex. Moreover, the spectrum of this complex strongly resembles that of bound NADH, particularly with regard to the 1666 and 1415 cm^{-1} bands (Figure 6c) compared to the 1681 and 1415 cm^{-1} bands (Figure 3a) which we have assigned to the nicotinamide moiety of bound NADH. It would seem reasonable to suggest that the bonding in the NAD^+/pyrazole ternary complex takes place at the C4 position of the nicotinamide ring. Indeed, X-ray crystallographic studies at 2.9-Å resolution show that the distance between pyrazole N1 and nicotinamide C4 to be very close, 2 Å (Eklund et al., 1982).

NAD^+ is more tightly bound to the LADH/pyrazole complex than to LADH, and this suggests that the LADH/NAD^+/pyrazole complex is a model for the transition state of the enzyme-catalyzed reaction (Theorell & Yonetani, 1963). Such a concept coupled with the above results is in agreement with the generally accepted pathway for catalysis by dehydrogenases. Hydride transfers occur from substrate to the C4 position of the NAD^+ nicotinamide ring. During this transfer, the aromatic nature of oxidized nicotinamide would be disrupted, presumably, with substantial positive charge developing at C4. This could result in a structure for NAD^+ which would resemble NADH.

Lastly, we would like to point out that the rather dramatic changes in molecular structure that occur when NADH binds to liver alcohol dehydrogenase are not necessarily repeated in other NAD-linked dehydrogenases. For example, preliminary results in our laboratory concerning coenzymes binding to yeast alcohol dehydrogenase indicate much fewer differences between solution and bound coenzymes. This implies that much smaller molecular perturbations are imposed by this dehydrogenase on the bound coenzymes.

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Registry No. NAD, 53-84-9; NADH, 58-68-4; ADPR, 20762-30-5; LADH, 9001-60-9; Me_2SO, 67-68-5; IBA, 563-83-7; pyrazole, 288-13-1.

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