to those of the whole virus.

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Raman Study of Reduced Nicotinamide Adenine Dinucleotide Bound to Liver Alcohol Dehydrogenase†

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ABSTRACT: We report the first Raman spectra of reduced nicotinamide adenine dinucleotide (NADH) when bound to an enzymatic active site, that of liver alcohol dehydrogenase (LADH). This was obtained by subtracting the Raman spectrum of LADH from that of the binary LADH/NADH complex. There are significant changes in the spectrum of bound NADH as compared to that in solution. The data indicate that both the nicotinamide moiety and the adenine moiety are involved in the binding. At least one of the two NH_2 moieties of NADH also participates.

Oxidized nicotinamide adenine dinucleotide (NAD^+)[1] and its reduced form (NADH) are coenzymes for hundreds of enzymatic oxidation–reduction reactions (Dalziel, 1975). The nature of the interaction of these cofactors with proteins is not understood. Some information on this interaction is available from X-ray crystallographic studies on several dehydrogenases (Brändén et al., 1975; Plapp et al., 1978; Eklund et al., 1981; Holbrook et al., 1975; Harris & Waters, 1976; Banaszak & Bradshaw, 1975; Rossman et al., 1975; Ohlsson et al., 1974). In these studies, electron-density differences were calculated to give the location of the ligand within the protein. Nevertheless, the nature of the interaction(s) between the ligand and protein can only be deduced and not studied directly with this technique. In contrast, Raman spectroscopy measures the vibrational normal modes and is sensitive to bonding changes that are likely to occur in binding of ligands to proteins. A

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[1] Abbreviations: LADH, liver alcohol dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; NAD^+, oxidized nicotinamide adenine dinucleotide; ADPR, adenosine diphosphate ribose.

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number of Raman studies on NAD^+ and NADH have been reported (Patrick et al., 1974; Forrest, 1976; Nishimura & Tsuboi, 1980; Barrett, 1980). Ultraviolet resonance Raman studies on NADH (Tsuboi, 1976; Bowman & Spiro, 1980) and its various fragments (Rodgers & Peticolas, 1980) provide the basis of further understanding of these cofactors.

We report here a Raman study of NADH when it is bound to liver alcohol dehydrogenase (LADH, EC 1.1.1.1). LADH catalyzes the oxidation of various primary and secondary alcohols to the corresponding aldehydes along with the reduction of NAD^+ (Sund & Theorell, 1963). It is known that when LADH binds NAD^+ or NADH, conformational changes in the enzyme occur (Subramanian & Ross, 1977, 1978; Luisi & Favilla, 1970; Theorell & Tatemoto, 1971; Shore et al., 1975; Eklund et al., 1981). It is also known that the adenine moiety is required for the binding of NADH to the enzyme (Hollis, 1967; Subramanian et al., 1981). X-ray crystallographic studies of binary complexes of LADH with various analogues of NAD^+ and ternary complexes involving NADH or NAD^+ (Abdallah et al., 1975; Plapp et al., 1978; Eklund et al., 1981) support the crucial role of the adenine moiety. It has been suggested that the nicotinamide moiety is also important (Hollis, 1967). Our data show directly that both the nicotinamide moiety and the adenine moiety are significantly involved when NADH binds to LADH.

Materials and Methods

NADH (grade III) and NAD^+ (grade III) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. LADH was purchased from Boehringer Mannheim Co. (Indianapolis, IN). The enzyme was dialyzed against buffer solution to remove ethanol and then concentrated as described previously (Yue et al., 1984). Concentration of the enzyme was determined spectroscopically, with $\epsilon_{280} = 3.57 \times 10^4\ M^{-1}\ cm^{-1}$. The concentration of NADH was calculated by adding a known amount of buffer into preweighed vials of NADH and checked spectroscopically. The binary complex of LADH/NADH was prepared by mixing a 1:2 molar ratio of LADH to NADH. Under these conditions, the formation of complex was almost 100% with no excess LADH or NADH (DeTraglia et al., 1977) and was verified spectroscopically by the shift of the 340-nm absorption peak of NADH to 325 nm. Samples were maintained at 4 °C in a circulator unless stated otherwise. Enzyme activity was monitored by the method of Dalziel (1963). No denaturation of the enzyme or its binary complex was observed during the Raman measurement. Most Raman spectra were obtained by an OMA system consisting of a Triplemate spectrometer (Spec Industries, Metuchen, NJ) and a solid-state detector system—Model 1420 water-cooled photodiode array and model 1218 controller (EG&G, Princeton Applied Research, Princeton, NJ). Spectral lines were calibrated against known lines of toluene. Some spectra were obtained with a Spex 1401 double spectrometer controlled by a Compudrive controller (CD2A, Spex Industries), a cooled RCA 31034 photomultiplier, and photon-counting electronics. Both systems were interfaced to a LSI-11 minicomputer (Digital Equipment Corp., Malboro, MA). Data were stored and analyzed on the LSI-11 minicomputer. A Coherent (Palo Alto, CA) Model CR-2000 krypton ion laser and a Spectra Physics (Mountain View, CA) Model 165 argon ion laser were used to produce monochromatic radiation.

Results

Figure 1 shows the Raman spectrum of LADH and its binary complex with NADH. It is clear that most of the

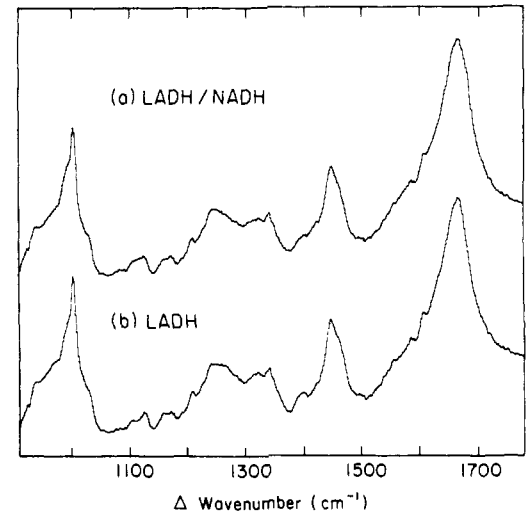

FIGURE 1: (a) Raman spectrum of the binary complex LADH/NADH in 0.1 M pyrophosphate-buffered solution (pH 9.6). Concentrations were (LADH) 1 mM and (NADH) 2 mM. (b) Raman spectrum of LADH. Concentration was 1.3 mM. Samples were maintained at 4 °C. Laser line was 488 nm with incident power of 100 mW, and the resolution was 6 cm^{-1}.

Raman scattering of the binary complex is due to LADH (Yue et al., 1984). In fact, the intrinsic Raman cross section of NADH is only a few percent of that of LADH; the strongest peak of NADH in solution at 1688 cm^{-1} is about 10% of the 1450-cm^{-1} peak of LADH. In order to obtain the Raman spectra of bound NADH, difference spectra were calculated. It is known that binding of NADH quenches the fluorescence of LADH (Luisi & Favilla, 1970; Theorell & Tatemoto, 1971). High pH can also quench fluorescence (Wolfe et al., 1977). At pH 9.60, the Raman spectrum of LADH and that of the binary complex have only slightly different fluorescence backgrounds. The backgrounds were removed, and any difference in concentration or collection optics between the two samples was corrected by comparing the peaks at 1450 cm^{-1} (Lippert et al., 1976). The 1450-cm^{-1} peak, which is due to the α-CH_2 vibration, is a convenient reference because it is relatively insensitive to different secondary and tertiary structures of the protein. In addition, there is no peak in the Raman spectra of NADH in buffer solution around this region. The resulting difference spectrum is shown in Figure 2b. There is a small residual broad peak around 1650 cm^{-1} due to different contributions of water in the original spectra. Figure 2a shows a typical Raman spectrum of NADH in buffer solution for comparison.

The difference spectrum is due to either bound NADH or changes in LADH spectra upon NADH binding. There is no obvious negative peak, which can only be due to LADH changes. It is known that Raman spectroscopy is sensitive to a few percent changes in protein structure (Yu et al., 1974; Kint & Tomimatsu, 1979). Changes in protein structure will appear as changes in the Raman spectrum in the amide I and/or amide III vibration at 1645–1680- and 1230–1300-cm^{-1} regions, respectively. That there are no negative peaks nor obvious "new" peaks in these regions suggests that the change in the structure of LADH upon NADH binding is small. The difference spectrum in Figure 2b is due, probably entirely, to bound NADH.

Table I lists the frequencies of bound NADH and NADH in solution. Several obvious changes can be seen when NADH binds to LADH as compared to that in solution. The most prominent change is the disappearance of the major peak in the solution spectrum at 1545 cm^{-1}. The 1338-cm^{-1} band also

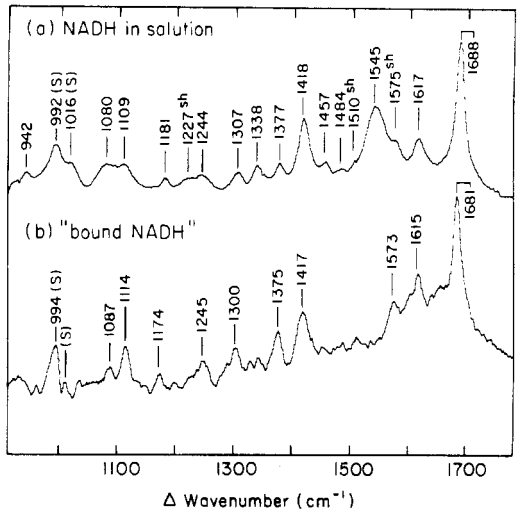

FIGURE 2: (a) Raman spectrum of NADH in 0.1 M pyrophosphate-buffered solution (pH 9.6). Concentration was 32 mM. Conditions are as in Figure 1. (b) Difference spectrum of spectra a and b of Figure 1 showing basically bound NADH. The peak around 1685 cm^{-1} was arbitrarily adjusted to be about the same height in both panels. (S) Peak from solvent/buffer.

Table I: Principal Peak Frequencies of NADH in Solution and When Bound to LADH

bound NADH (cm^{-1})	NADH in solution (cm^{-1})	bound NADH (cm^{-1})	NADH in solution (cm^{-1})
	942	1417	1418
1087	1080		1457
1114	1109		1484
1174	1181		1510 (sh)
	1227 (sh)[a]		1545
1245	1244	1573	1575 (sh)
1300	1307	1615	1617
	1338	1681	1688
1375	1377		

[a] sh, shoulder.

has apparently disappeared. It is well-known that the 1338-cm^{-1} band is due to a ring mode of the adenine moiety (Lord & Thomas, 1967; Lafleur et al., 1972; Rodgers & Peticolas, 1980). The disappearance of this band supports the fact that the adenine part of NADH is directly involved in binding. Similarly, the broad 1545-cm^{-1} band has been assigned to the nicotinamide moiety (Rodgers & Peticolas, 1980; Bowman & Spiro, 1980). The fact that this band is missing in NAD^+ supports this assignment (C. L. Martin et al., unpublished results). The amide protons can be exchanged by deuterons by suspending NADH in D_2O buffer overnight. The peak at 1545 cm^{-1} shifts to 1559 cm^{-1} under these conditions, indicating that the carboxamide group of the nicotinamide is also involved in this normal mode. The disappearance of the 1545-cm^{-1} band shows clearly that the nicotinamide moiety is directly involved in the binding of NADH by LADH.

The broad feature around 1080 cm^{-1} in the spectra of NADH in solution has been assigned to the NH_2 rock (Bowman & Spiro, 1980). NADH contains two NH_2 groups, one with the nicotinamide ring and another with the adenine ring. As expected, the band shifts to around 900 cm^{-1} upon deuteration of NADH (Bowman & Spiro, 1980; C. L. Martin et al., unpublished results); the peak at 1109 cm^{-1}, which is not assigned to the NH_2 groups, remains unchanged. The solution spectrum peak at 1080 cm^{-1} of Figure 2a shifts to 1087 cm^{-1} with reduced intensity and appears to narrow upon forming the binary complex, Figure 2b. It is thus likely that at least one of the two NH_2 groups in NADH is directly involved in binding, disrupting the rocking motion of NH_2.

There are other changes in the relative intensities of several bands. For example, the relative intensity of the 1418- and 1688-cm^{-1} bands of NADH in solution remains relatively unchanged upon binding, as do those of the 1307- and 1377-cm^{-1} bands. However, the ratio of the 1418- (1688-) cm^{-1} to 1307- (1377-) cm^{-1} band is considerably reduced when NADH binds to LADH.

NADH exists in two possible configurations in polar solvent (Meyer et al., 1962; Catterall et al., 1969; Cross & Fisher, 1969; Sarma & Kaplan, 1970; Oppenheimer et al., 1978). At 22 °C, there is a minimum of 30–40% of the folded form in solution where the adenine ring is stacked with the nicotinamide ring (Oppenheimer et al., 1978). At higher temperature or in nonpolar/destacking solvent such as methanol, NADH exists mainly in an open form whereas LADH-bound NADH exists entirely in the open form (Eklund et al., 1981). Raman spectra of NADH at 25 and 75 °C (where there is no stacking) were measured, and they are essentially the same. Thus, the observed stacking of NADH in solution has only a minimal effect on the Raman spectrum.

We also have attempted to measure the Raman spectra of bound NAD^+. Although the equilibrium constant of NAD^+/LADH is comparable to that of NADH/LADH at pH 9.6 (DeTraglia et al., 1977), measurement of Raman difference spectrum is hindered by the fact that the intrinsic Raman cross section of NAD^+ is only about one-fifth of that of NADH at 488-nm excitation.

Discussion

The adenine moiety is known to be important for the binding of NADH to LADH. NADH fragments without the nicotinamide ring can bind to LADH, and X-ray crystallographic studies (Abdallah et al., 1975; Plapp et al., 1978; Eklund et al., 1981) showed that adenine binds in a cleft in the coenzyme binding domain of LADH. Difference ultraviolet spectra (Subramanian et al., 1981) and NMR study (Hollis, 1967) also indicated that the adenine moiety is perturbed by binding.

The role of the nicotinamide moiety of NADH in binding has been studied less extensively. There is no clear indication from X-ray studies that the nicotinamide ring interacts with specific amino acid residues on LADH. However, Hollis (1967) has suggested that both adenine and nicotinamide rings are bound in the binary complex. By monitoring the NMR spectra of protons in NADH in D_2O buffer, he concluded that the nicotinamide moiety is affected by binding. In addition, Subramanian & Ross (1977, 1978) studied the calorimetry of NADH and NAD^+ binding for various dehydrogenases and found that there are large entropy changes only when NADH or NAD^+ binds to LADH. They noted further that binding of ADPR, a fragment without the nicotinamide part, to LADH does not result in a large entropy change. Furthermore, LADH forms orthorhombic crystals normally, but the binary LADH/NADH complex (and almost all ternary complexes of LADH/NADH with various substrates or inhibitors) cannot form orthorhombic crystals. In contrast, many NADH fragments, which do not contain nicotinamide, form orthorhombic crystals with LADH (Brändén, 1965; Zeppezauer et al., 1967). It is thus possible that the nicotinamide moiety is promoting the activity of LADH by forcing a conformational change on the enzyme (Biellmann & Jung, 1971).

Moreover, Samama et al. (1977) has showed that the carboxamide group of the nicotinamide moiety is crucial for the correct binding of NAD^+ to LADH. X-ray studies indicated that the analogue of NAD^+ without the carboxamide group binds LADH with the adenine moiety in the same way

as NAD^+ but the pyridine moiety is in a different position, further away from the active site. Studies on other dehydrogenases have also revealed some interactions of the carboxamide group with an amino acid residue on the enzyme, possibly with a histidine group (Webb et al., 1973; Adams et al., 1973). Woenckhaus et al. (1973) studied the binding of the analogue [3-[3-(bromoacetyl)pyridinyl]propyl]adenosine pyrophosphate to yeast alcohol dehydrogenase and showed a direct bonding between the carboxymethyl group and a histidine residue.

The Raman spectrum of bound NADH indicates that both adenine parts and nicotinamide parts are directly involved when NADH binds to LADH. The 1338-cm^{-1} peak associated with the adenine moiety and the 1545-cm^{-1} peak due to the nicotinamide moiety, found in solution spectrum of NADH, disappear in the LADH/NADH complex. In addition, at least one of the two NH_2 groups is also perturbed. Since the 1545-cm^{-1} peak is sensitive to the carboxamide, its disappearance is also consistent with a direct involvement of the carboxamide group. Raman measurements on NADH fragments and isotopically substituted NADH are in progress in order to understand these and the other spectral features and, hence, to investigate the nature of the binding.

Registry No. NADH, 58-68-4; LADH, 9031-72-5.

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