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

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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

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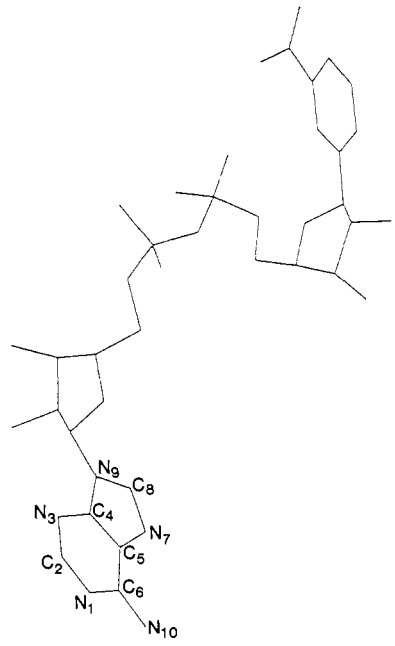

FIGURE 1: Schematic drawing of an NAD^+ molecule with the atom numbering used in this paper.

Fermi resonance doublet of tryosine was not found to be an accurate guide to tyrosine hydrogen bonding in UVRR spectra (Rava & Spiro, 1985b).

NAD^+ and NADH have absorption maxima at 260 nm, thus enabling the UVRR investigation of dehydrogenases to yield information relating to both the protein and the coenzyme. Raman spectra have been obtained for NADH bound to LADH (Chen et al., 1987; Yue et al., 1984), but in these NR spectra the NADH contribution to the holoenzyme spectrum is extremely small; only careful difference spectra show the spectrum of the bound coenzyme. In this paper, we demonstrate that UVRR spectroscopy can be a sensitive probe of coenzyme binding to dehydrogenases. The UVRR spectra of the holoenzymes of GAPDH, LADH, and YADH show coenzyme bands of equal intensity to the aromatic amino acid bands of the enzyme itself. In addition we have studied the effects of substrate binding to GAPDH by obtaining the UVRR spectrum of the acyl enzyme intermediate. We have not been able to repeat a previous observation of a unique band in the spectrum of the acyl enzyme, attributed to a thioester vibration (Chittock et al., 1987).

Experimental Procedures

GAPDH from rabbit muscle, LADH, YADH, NAD^+, and NADH were purchased from Sigma Chemical Co. and used without further purification. LADH and YADH were obtained in a crystalline form, and GAPDH was obtained as a suspension in a solution containing 4 M ammonium sulfate, 1 mM EDTA, and 4 mM mercaptoethanol. Specific activities for LADH and YADH were 2.1 and 310 units, respectively, where 1 unit is equal to 1 μmol of NADH released per minute per milligram of protein, at pH 8.8. GAPDH activity was determined by a single turnover experiment, which directly measures the number of active sites that are acylated per GAPDH tetramer present. The amount of NADH released when an excess of substrate glyceraldehyde 3-phosphate (GAP) was added to GAPDH at pH 6.0 in the absence of phosphate was measured spectrophotometrically, and assumed to be equivalent to the amount of acylation. Provided that dithiothreitol (DTT) was added to the system to ensure the reduced state of the active cystine thiol group, a high activity was observed. The number of acylated sites per GAPDH tetramer was found to be between 1.7 and 2.0. Although there is still some controversy, many other groups have found a maximum of two-site acylation of GAPDH under these conditions [Malhotra and Bernhard (1968) and references cited therein], so we assume GAPDH activity to be close to its maximum. GAPDH purity was checked by SDS–PAGE, and contaminating proteins were found in small amounts (<2%). GAP was prepared by hydrolysis of the ethyl acetal (Sigma). All samples for Raman spectroscopy were made up in 0.05 M tris(hydroxymethyl)methylamine buffer and adjusted to pH 6.0–6.5 (GAPDH, GAP) or to pH 7–7.5 (YADH, LADH). Concentrations of enzymes and coenzymes for Raman experiments were in the range 4×10^{-5} to 6×10^{-6} M and were determined from their UV absorptions with the absorption coefficients in Table I. Apoenzymes were prepared by incubation of the enzymes with activated charcoal at room temperature for 15–20 min. Holoenzymes of YADH and LADH were prepared by stoichiometric addition of NADH or NAD^+ to the apoenzymes so as to occupy half the coenzyme sites (i.e., two NADH per YADH, one NADH per LADH), and thus ensure complete binding of the coenzyme. NADH binding to LADH was confirmed spectroscopically by observing the shift of the nicotinamide absorption from 340 to 325 nm.

The relative activities of holo- and apo-GAPDH were monitored before and after laser irradiation by measuring the initial rate of NADH production during the reaction of enzyme with GAP and phosphate in the presence of excess NAD^+ at pH 7.5. Holo-GAPDH lost only 4% of activity after laser irradiation in the Raman experiments, whereas apo-GAPDH lost 16–20%.

UV radiation at wavelengths of 220, 240, or 260 nm was produced by frequency doubling the output from an excimer (Lumonics HX460)–pumped dye laser (Lambda Physik FL3002) using a β-barium borate crystal (Laser Lines, Banbury, U.K.). The laser pulse width was ca. 10 ns (FWHH). The Raman radiation was collected by conventional right-angle scattering in a Spex Triplemate spectrometer and detected with an OSMA intensified diode array (Spectroscopic Instruments, West Germany). Typically, enzyme spectra were collected from 600 to 1200 s, the dye laser being operated at 25–30 Hz and 1 mJ/pulse being loosely focused at the sample, so as to avoid saturation phenomena. The enzyme solutions were continuously flowed through a quartz capillary tube so as to present a new sample to each pulse of the laser. The acyl enzyme was flowed in the same manner, but a simple mixing device was employed so as to freshly form acyl enzyme shortly before laser irradiation.

Results and Discussion

Both NAD^+ and NADH have strong absorption maxima at 260 nm. These are of comparable magnitude to the 260-nm absorption of the aromatic amino acid residues of GAPDH, LADH, and YADH (see Table I). Thus, the 260-nm-excited RR spectra of the binary complexes should show features due to both the coenzyme and the enzyme aromatic amino acid residues. Figure 2 compares the RR spectra obtained for NAD^+ (Figure 2a), with those of holo- and apo-GAPDH (spectra b and c of Figure 2, respectively). Consistent with the fact that the 260-nm absorption bands of NAD^+ and NADH are largely due to the absorption of the adenine moiety, the 260-nm RR spectra of NAD^+ and NADH are seen to be virtually identical. The bands in the RR spectra are, with the exception of a small nicotinamide band at ca. 1030 cm^{-1}, assigned to adenine vibrations (Yue et al., 1986). The

Table I: Absorption Coefficients for Enzymes and Coenzymes in the Far-Ultraviolet

	$10^{-3}\epsilon$ (M^{-1} cm^{-1})		
	220 nm[a]	240 nm[a]	260 nm
NAD^+	11	10	17.8
NADH	12.7	7.8	14.9
GAPDH	2000	240	100[b]
LADH	800	50	20.4[c]
YADH		187	104[c]

[a] Values estimated from absorption spectra, using 260-nm absorption coefficients. [b] Values for holoenzyme with ca. two NAD^+ per tetramer, using ϵ_{280} = 138 000 M^{-1} cm^{-1} (3.25 NAD^+ per tetramer; Smith, 1966). [c] Values for apoenzyme using 280-nm absorption coefficients from Sund and Theorell (1963).

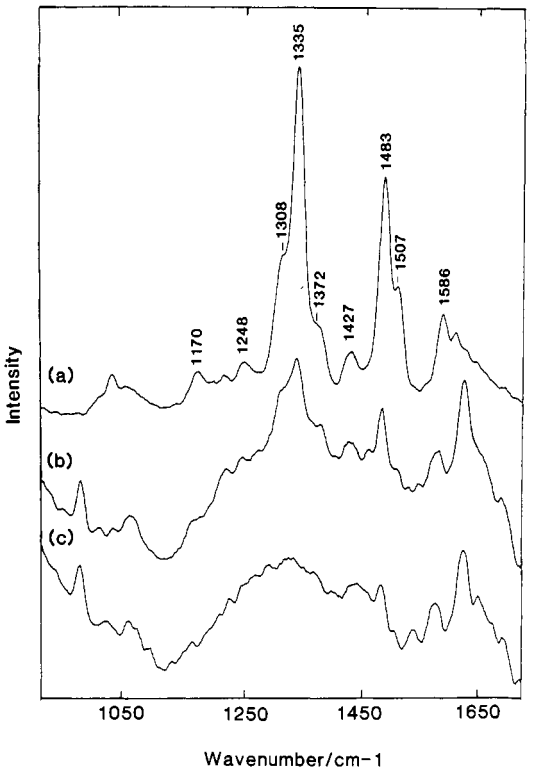

FIGURE 2: 260-nm-excited RR spectra of (a) NAD^+ (3×10^{-4} M, pH 6.8), (b) holo-GAPDH (2×10^{-5} M, pH 6), and (c) apo-GAPDH (2×10^{-5} M, pH 6).

spectrum of NAD^+ in Figure 2a is also closely similar to that of NADH excited at 273.0 nm (Rodgers & Peticolas, 1980). The relative enhancement of bands in the RR spectrum is dependent on the symmetry of the vibrations and their effectiveness in modeling the geometry of the excited state. Thus, the NR band at 730 cm^{-1} is weak in the RR spectrum, similar to the observation that the symmetric ring breathing mode of tryptophan is not resonance enhanced with excitation in this region (Rava & Spiro, 1985a). The wavenumber values for NAD^+ are listed in Table II. The holo-GAPDH spectrum (Figure 2b) shows, as expected, features due to NAD^+, tyrosine, and tryptophan. Comparison with the apoenzyme spectrum (Figure 2c) shows that the aromatic amino acid bands overlap with bands of NAD^+. Accurate wavenumber values for bound NAD^+ were determined from the (b) − (c) difference spectrum and are given in Table II. It can be seen (Figure 2c,b) that significant wavenumber shifts and relative intensity changes of certain NAD^+ bands result from the binding to GAPDH: in particular, the 1335-cm^{-1} band remains unshifted but lowered in intensity relative to neighboring bands at 1314 and 1377 cm^{-1}. The two latter bands appear

Table II: Wavenumber Values (cm^{-1}) for NAD^+ and NADH in Solution

free NAD^+ (NR)[a]	free NAD^+ (RR)	NAD^+·GAPDH	NADH·YADH	NADH·LADH
730 s[b]	730 w			
1032 vs	1030 w			
	1170 mw	ca. 1172 w	1160–1173 w	1166 mw
1254 mw	1248 mw			
1308 m	1308 m	1314 m	ca. 1304 sh m	ca. 1301 sh w
			1325 s	1325 s
1338 s	1335 s	1336 s	1335 s	1335 s
1378 m	1372 m	1378 m	1371 m	1369 m
1422 m	1427 mw	1422 m	ca. 1420 mw	1420 w
1484 mw	1483 s	1483 s	1480 s	1480 s
1510 mw	1507 m	1509 m	1505 m	1510 m
1580 m	1586 m	1590 m	1582 mw	1584 m
	1609 mw		ca. 1607 mw	1609 mw

[a] Taken from Yue et al. (1986). [b] w = weak, m = medium, s = strong, v = very, and sh = shoulder.

to be shifted from their positions in the free NAD^+ spectrum. The 1314-cm^{-1} band is shifted up from 1308 cm^{-1}; the 1377-cm^{-1} band is shifted up from its position at 1372 cm^{-1} in the free NAD^+ spectrum. The 1377-cm^{-1} band is better resolved from the 1335-cm^{-1} band due to both an upshift by 5 cm^{-1} and the decrease in width of the 1335-cm^{-1} band. The 1483- and 1509-cm^{-1} bands appear little changed, but the 1483-cm^{-1} band is lowered in intensity in the bound NAD^+ spectrum.

The X-ray structures of holo-GAPDH (Buehner et al., 1974; Leslie & Wonacott, 1984; Skarzyński et al., 1987) show NAD^+ to be in an open, extended conformation when bound to the enzyme. This is in contrast to the generally accepted solution conformation, where the open form is in equilibrium with a folded (stacked) conformer (Gruber et al., 1975; Oppenheimer et al., 1978). To confirm that the changes observed were not simply an effect of this conformational change, the RR spectrum of NADH in solution was recorded at 75 °C, where the equilibrium lies in favor of the open conformation. No significant differences were observed between the RR spectrum and that in Figure 2a, in agreement with previous NR data (Yue et al., 1984). We conclude, therefore, that the changes observed are due to either (a) a more specific conformational change, (b) adenine ring protonation, as suggested by other work (Chen et al., 1987), or (c) specific hydrogen bonding between enzyme and coenzyme, and other medium effects.

The X-ray structures and detailed conformational parameters have been determined for Li^+·NAD^+ (Reddy et al., 1981), NAD^+ bound to GAPDH (Skarzyński et al., 1987), and NADH bound to LADH (Eklund et al., 1984) and lactate dehydrogenase (LDH) (White et al., 1976). In Li^+·NAD^+ crystals, both the adenine and the nicotinamide heterocycles are in anti orientations. This conformation is also approximately assumed by the coenzyme in LADH and LDH, which are "A-type" dehydrogenases. However, in GAPDH the nicotinamide ring is in a syn orientation: a difference of a 180° rotation about the glycosidic bond of the nicotinamide ring. GAPDH is thus different from the other dehydrogenases, being a "B-type" dehydrogenase. In solution, there is evidence from NMR that NAD^+ exists with its nicotinamide ring in a 2:1 ratio of syn and anti conformations (Birdsall et al., 1975). Accordingly, as suggested in (a) above, this specific conformational change could explain the observed spectral differences. From this point of view, it is then interesting to compare the RR spectra of NADH bound to B-type dehydrogenases with the holo-GAPDH RR spectrum. The UVRR difference

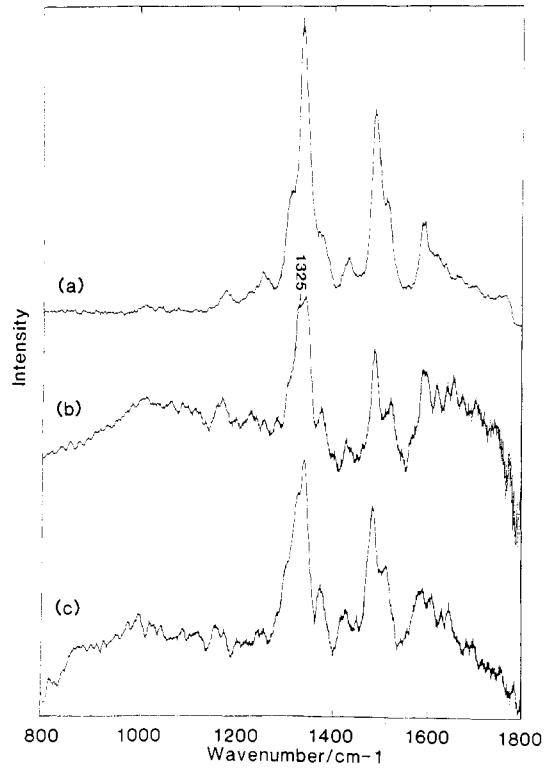

FIGURE 3: 260-nm-excited (a) RR spectrum of NADH (8×10^{-4} M, pH 7.5), (b) difference RR spectrum of NADH bound to LADH (4.3×10^{-5} M, pH 7.5), and (c) difference RR spectrum of NADH bound to YADH (NADH 4.8×10^{-5} M, pH 7.5). In (b) and (c) the difference spectrum is the holoenzyme spectrum minus the apoenzyme spectrum.

spectra of NADH bound to LADH (apoenzyme spectrum subtracted from holoenzyme spectrum) and of NADH bound to YADH are shown in spectra b and c of Figure 3. The RR spectrum of free NADH is shown for comparison (Figure 3a). In the case of YADH, only ca. 40% of the NADH present is enzyme bound; consequently, the spectrum shows a superposition of features due to bound and free coenzyme. The wavenumber positions are listed in Table II. At the concentration and pH employed, NAD^+ has a low affinity for YADH. Thus, as expected, the difference spectrum of NAD^+ in the presence of YADH (not shown) was found to be indistinguishable, although of poorer quality, from that of free NAD^+.

The spectra in Figure 3b,c show similar features that are different from the spectrum of the free coenzyme, but also different from that of the GAPDH-bound coenzyme. We can now consider how the syn/anti difference in the nicotinamide orientation in the A- and B-type enzymes might explain the spectral differences. Assuming the solution spectrum is that of a 2:1 mix of syn and anti conformers, then in the simplest view a superposition of 1A + 2B type bound coenzyme spectra should produce a "free" coenzyme spectrum. This accounts for at least one band in the spectrum, namely that at 1372 cm^{-1}, which shifts down by 2–3 cm^{-1} when NADH is bound to LADH but shifts up by 5 cm^{-1} when it is bound to GAPDH. It does not account for the large 1325-cm^{-1} band observed in the NADH spectrum when it is bound to LADH and YADH, however, nor for the down shift of the 1425–1429-cm^{-1} band in all the bound coenzyme spectra. We conclude, therefore, that although the anti/syn difference can account for some of the differences in the bound coenzyme spectra, it is obviously only at most a contributing factor in determining the coenzyme spectrum.

The RR spectrum of NADH bound to LADH presented here contrasts with that reported for NR spectra, which showed the complete disappearance of the 1335-cm^{-1} band and the appearance of a very weak feature at 1325 cm^{-1} (Yue et al., 1984; Chen et al., 1987). The resonance enhancement factors which result in the 1335-cm^{-1} band, being by far the strongest feature in the RR spectra, are, of course, absent from the NR spectra, making these less sensitive to certain types of change which arise from environmental effects. However, in both NR and RR data there is clear agreement that significant changes consequent upon the binding of coenzyme to enzyme are found in the 1335-cm^{-1} band. In both sets of data, the 1335-cm^{-1} band is reduced in relative intensity, and a new band appears at 1325 cm^{-1}. In the NR investigation, the disappearance of the 1335-cm^{-1} band was tentatively interpreted as an indication of protonation at N_7 of the adenine ring. This was based on the assignment of the 1335-cm^{-1} band by analogy with a normal coordinate analysis of 9-methyladenine as being predominantly a C_8N_7–N_7C_5 stretching mode, with some contribution from C_8H and C_2H in-plane deformations (Tsuboi et al., 1973). The NR spectrum of NAD^+ below pH 4, where the adenine ring is protonated, is indeed different from that obtained above pH 4 (Yue et al., 1986). These spectral differences are now, however, the same as those observed in the NR spectrum when NADH binds LADH. Moreover, N_7 is only protonated in solution at pH 2 or lower, since it has a pK_a of ca. 2, whereas N_1 has a pK_a of ca. 3.8, making it a more likely candidate for protonation. Thus, the previous suggestion of N_7 protonation of the adenine ring would appear to be incorrect. Although the RR spectra in Figures 2b and 3b,c do not support adenine protonation, they do nevertheless bear some resemblance to the protonated NAD^+ spectrum. The alternative of strong hydrogen bonding to adenine N_1 could well produce spectral features similar to those observed on full N_1 protonation. It is also possible that N_7 hydrogen bonding could produce similar spectral changes. The most recent normal coordinate analyses (Tsuboi et al., 1987; Majoube, 1985) indicate that C–N_1 vibrations contribute to the intensities of both the 1483- and 1338-cm^{-1} bands. In the analysis of Majoube, C–N_7 vibrations also contribute to the intensities of both bands, but Tsuboi only implicates N_7 involvement in the 1338-cm^{-1} vibration. The spectral changes observed could thus be due to hydrogen bonding to N_1 and perhaps also to N_7. The lack of adenine spectral changes in the UVRR spectrum of $[poly(dA\text{-}dT)]_2$, where the adenine hydrogen bonds via N_1 to thymine in the Watson–Crick pair (Fodor & Spiro, 1986), is perhaps surprising in the light of the above interpretation.

The X-ray structure of holo-LADH to 2.9-Å resolution (Eklund et al., 1984) does in fact indicate that both N_7 and N_1 are hydrogen bonded to water molecules that are, in the case of N_1, also hydrogen bonded to the enzyme. This X-ray analysis also highlighted the close proximity of a positive charge (Arg-271) near the adenine ring. The particular spectral changes observed in Figure 3b could thus be due to the presence of this charge in the nonpolar environment, and not due to specific hydrogen bonding. We can immediately discount this explanation on inspection of the YADH primary structure. YADH does not have a positively charged residue in the position of Arg-271 (Jornvall et al., 1978), so the presence of the 1325-cm^{-1} shoulder (in Figure 3c) cannot be due to this charge effect.

The spectrum of NAD^+ bound to GAPDH is considerably less perturbed than that of NADH bound to LADH. This can be interpreted as an indication of less extensive and/or

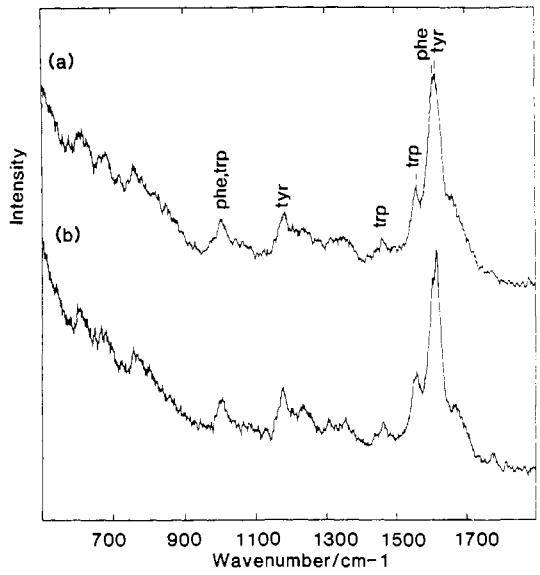

FIGURE 4: 220-nm-excited RR spectra of (a) holo-GAPDH (6×10^{-6} M, pH 6) and (b) apo-GAPDH (6×10^{-6} M, pH 6).

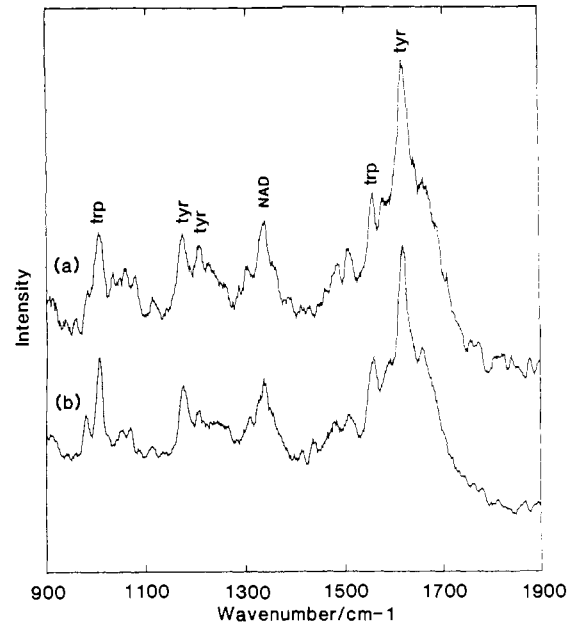

FIGURE 5: 240-nm-excited RR spectra of (a) GAPDH (2×10^{-5} M) with NAD^+ (1.2×10^{-4} M) at pH 6.0 and (b) GAPDH (2×10^{-5} M) with NAD^+ (1.2×10^{-4} M) and GAP (ca. 10^{-4} M) at pH 6.0. In both (a) and (b) a small amount of dithiothreitol (DTT) was added to the GAPDH to restore full enzyme activity (see Experimental Procedures).

weaker hydrogen bonding between coenzyme and enzyme. An X-ray structure of holo-GAPDH at 1.8-Å resolution (Skarzyński et al., 1987) indicated hydrogen bonding to N_1 and NH_2 (N_{10}) of adenine. Weaker hydrogen bonding to N_1 might explain the reduced effects observed on the GAPDH-bound NAD^+ spectrum. However, it is also equally possible that the considerably different environment experienced by NAD^+ when bound to GAPDH causes the difference between the coenzyme spectra when bound to the different enzymes. LADH is seen to provide a generally more hydrophobic adenosine binding environment for its coenzyme than GAPDH. Consistent with this, the coenzyme spectrum when bound to GAPDH resembles the solution spectrum more closely than does the LADH-bound coenzyme spectrum.

Structures of apo- and holo-GAPDH from thermophilic bacteria to 2.4-Å resolution give evidence for NAD^+-induced sequential conformational changes in the overall tertiary and quaternary structure (Leslie & Wonacott, 1984). In this context, it is interesting to investigate effects of NAD^+ binding on the RR spectrum of GAPDH. The 260-nm-excited RR spectrum does not show any major effects of NAD^+ binding on the enzyme bands of GAPDH, but the NAD^+ spectrum swamps the smaller protein bands in the holo-GAPDH spectrum. A more satisfactory way to observe the holo-GAPDH RR spectrum without interference from NAD^+ bands is to record the RR spectrum at 220 nm, where the GAPDH absorption far exceeds that of NAD^+ (Table I). The 220-nm-excited RR spectra of holo- and apo-GAPDH are shown in spectra a and b of Figure 4. The spectra are remarkably similar, showing bands at 1001 (phenylalanine), 1010 sh (tryptophan), 1178 (tyrosine), 1231, ca. 1355, 1460, and 1555 cm^{-1} (tryptophan) and a broad feature with components at 1605 and 1613 cm^{-1} (phenylalanine and tyrosine). There is a slight difference in the contribution of the water band at ca. 1640 cm^{-1} which distorts the intensities of these two bands.

The similarity of the two spectra in Figure 4 may be interpreted as showing that the aromatic amino acid residues (14 phenylalanine, 9 tyrosine, and 2 tryptophan residues per subunit in the pig muscle enzyme) are not significantly perturbed by the conformational changes accompanying coenzyme binding. The phenylalanine bands have not, however, previously been found to be sensitive to environmental changes in studies of other proteins, so that the lack of major spectral changes here may not be significant. Two phenylalanine residues (Phe-34 and Phe-99) clearly do contribute to the hydrophobic character of the adenine binding pocket. Correlations between Raman band intensities and hydrogen bonding on solvent exposure of tyrosine and tryptophan residues in proteins have been well documented for NR spectra (Clark & Hester, 1986). UVRR studies of α-lactalbumin and insulin have shown that these NR correlations do not always hold in the resonant spectra (Rava & Spiro, 1985b). Equally, new hydrogen-bonding or environment-sensitive bands might be expected to be revealed. Although UVRR studies of free amino acids have been thorough, UVRR studies of proteins are still at a rather preliminary stage. Thus, the fact that the observed tyrosine and tryptophan bands in the spectra in Figure 4 do not differ may also be interpreted as an indication of lack of sensitivity in the RR spectra. However, only the average tyrosine or tryptophan state is shown. Since there are nine tyrosine and two tryptophan residues in each GAPDH subunit, changes accompanying coenzyme binding would have to be substantial before changes could be accurately pinpointed in the RR spectra. In fact, tyrosine and tryptophan residues are not found at the coenzyme binding site, nor have they been implicated as being an integral part of the conformational change observed by the X-ray study.

In the reaction of GAPDH with its natural substrate, GAP, the product 1,3-diphosphoglycerate is formed with reduction of NAD^+ to NADH. The reaction mechanism involves the formation of an acyl enzyme intermediate, which is fairly stable in the absence of nucleophiles. The acyl enzyme, formed by reaction of a cysteine thiol group at the active site, contains a thioester bond which absorbs at ca. 240 nm. Figure 5 compares the 240-nm-excited UVRR spectrum of GAPDH in the presence of an excess of NAD^+ (a), with the corresponding spectrum of the GAPDH acyl enzyme (b), formed by mixing GAPDH with GAP (see Experimental Procedures).

The spectra show bands due to tryptophan and tyrosine vibrations of GAPDH and also due to the free NAD^+ in solution. (Free NAD^+ in solution is >6 times in excess of

NAD^+ bound to GAPDH.) No peaks are observed that might be attributable to thioester vibrations. The previous report assigning a ca. 1590-cm^{-1} band to a thioester carbonyl stretching vibration has not been verified here (Chittock et al., 1987). However, some changes in relative intensities of some tryptophan and tyrosine bands are observed on forming the acyl enzyme. The 1206-cm^{-1} band of tyrosine decreases in intensity relative to other tyrosine bands at 1174 and 1620 cm^{-1}. The 1007- and 1557-cm^{-1} bands of tryptophan appear to increase slightly in relative intensity on forming the acyl enzyme. A band at 1508 cm^{-1}, which overlaps with a band of NAD^+, has been assigned to a tyrosine photodecomposition product (Johnson et al., 1986). The low intensity of this band relative to that of the two tyrosine bands at 1174 and 1620 cm^{-1} in our spectra indicates that photodecomposition under our experimental conditions is not a major problem. A UVRR study of insulin in monomeric and dimeric forms attributed a general decrease in the tyrosine signal to increased solvent exposure of tyrosine residues (Rava & Spiro 1985a). However, we observe only a decrease in the 1206-cm^{-1} tyrosine band relative to the 1174-cm^{-1} band on forming the GAPDH acyl enzyme. X-ray crystallography has nothing to contribute to this discussion, since no X-ray study of a ternary complex of GAPDH has been reported. From the X-ray structures of holo-GAPDH, Tyr-311, which is conserved in all dehydrogenases, is present in the active site pocket. The structure of lobster GAPDH at 2.9-Å resolution indicated hydrogen bonding between Tyr-311 and the essential histidine-176 residue (Moras et al., 1975), while the structure of bacterial GAPDH at 1.8-Å resolution indicated no such hydrogen bonding of the Tyr-311 residue (Skarzyński et al., 1987). The RR data show that one or more tyrosine residues are undergoing some change on forming the acyl enzyme. However, we are unable to determine whether it is the active site tyrosine that is being perturbed or whether it is a more general structural change (similar to that observed on coenzyme binding), affecting one or more of the remaining eight tyrosine residues.

Added in Proof

We acknowledge a preprint from Prof. R. Callender (Deng et al., 1989) wherein a previous assignment of NR difference spectra is revised in favor of a proposed protonation of N_3 of the adenine ring of enzyme-bound NADH.

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Registry No. GAPDH, 9001-50-7; ADH, 9031-72-5; NAD, 53-84-9; NADH, 58-68-4; GAP, 142-10-9.

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