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Investigation of the Nature of Enzyme-Coenzyme Interactions in Binary and Ternary Complexes of Liver Alcohol Dehydrogenase with Coenzymes, Coenzyme Analogues, and Substrate Analogues by Ultraviolet Absorption and Phosphorescence Spectroscopy†

S. Subramanian,* J. B. Alexander Ross,‡ Philip D. Ross, and L. Brand

ABSTRACT: The difference spectra of binary and ternary complexes of horse liver alcohol dehydrogenase with oxidized and reduced nicotinamide adenine dinucleotides, nicotinamide 1,N^6-ethenoadenine dinucleotide, and adenosine diphosphate ribose along with a number of substrate analogues have been measured. These spectra bear a very close resemblance to those obtained by perturbation of the coenzyme(s) and their analogues by acid, NaCl, dioxane, or *tert*-butyl alcohol. It is inferred that the coenzymes experience a combination of ionic and nonpolar environments at the adenine binding site of the enzyme. This is borne out by published X-ray crystallographic results. The phosphorescence spectra do not indicate the presence of ionized tyrosine in ternary complexes involving enzyme, coenzyme, and substrate analogues. The ultraviolet spectra can be explained as arising from the perturbation of the coenzyme chromophores upon binding to the enzyme without having to invoke tyrosine ionization.

The extensive X-ray crystallographic studies of liver alcohol dehydrogenase (LADH)[1] and several of its binary and ternary complexes (Brändén et al., 1975; Brändén, 1977; Eklund & Brändén, 1979) indicate that LADH undergoes a conformational change upon formation of complexes containing the nicotinamide adenine dinucleotide coenzymes, NAD^+ and NADH. In solution studies, Subramanian & Ross (1977, 1978) found that the thermodynamic parameters for the binding of NAD^+ and NADH to LADH differed dramatically from those obtained for the binding of NAD^+ and NADH to other dehydrogenases. In addition, the thermodynamic parameters for the binding of ADP-ribose to all the dehydrogenases studied (Subramanian & Ross, 1978), including LADH, were very similar. These results were also consistent with the occurrence of a conformational change in LADH upon binding NAD^+ or NADH but not ADP-ribose. Further thermodynamic studies (Subramanian & Ross, 1979) demonstrated that the binding of NAD^+ to LADH was accompanied by a proton dissociation from a zinc-bound water on the enzyme while no proton release was detected upon binding NADH to LADH in the pH range 6-8.6.

The tryptophan fluorescence of LADH is partially quenched upon binding of NAD^+ and NADH (Luisi & Favilla, 1970) as well as a number of other reagents (Theorell & Tatemoto, 1971). The quenching produced by NADH is usually attributed to energy transfer (Theorell & Tatemoto, 1971). The quenching by NAD^+ has been ascribed to both energy transfer and a conformational change by Abdallah et al. (1978). In the binary and ternary complexes of LADH with NAD^+, it was argued (Laws & Shore, 1978) that the fluorescence quenching could be due neither to resonance energy transfer nor to collisional quenching by virtue of the fact that the tryptophan residues are far from the active center of the enzyme. Tryptophans-314 which are in a hydrophobic milieu are reported not to change their environment as a result of the conformational change while tryptophans-15 already in an aqueous environment are reported to become more exposed to the solution (Eklund & Brändén, 1979). Hence, it was postulated by Laws & Shore (1978) that ternary complex formation causes a conformational change and a consequent ionization of tyrosine-286 at the surface of the enzyme. They further suggested that resonance energy transfer from Trp-314 to ionized Tyr-286 was responsible for the quenching of protein fluorescence.

It is the purpose of this communication to study the LADH-coenzyme interactions by ultraviolet difference

† From the Laboratory of Nutrition and Endocrinology (S.S.) and the Laboratory of Molecular Biology (P.D.R.), National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205, and the Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218 (J.B.A.R. and L.B.). *Received January 23, 1981.* This research was supported in part by NIH Grant GM 11632.

‡ Present address: Department of Laboratory Medicine, University of Washington, Seattle, WA 98195.

[1] Abbreviations used: LADH, liver alcohol dehydrogenase (EC 1.1.1.1); ϵNAD^+, nicotinamide 1,N^6-ethenoadenine dinucleotide; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; TFE, 2,2,2-trifluoroethanol; Me_2SO, dimethyl sulfoxide; IBA, isobutyramide; Pyr, pyrazole.

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spectroscopy and relate the spectral changes in the bound coenzyme to the spectral properties of the coenzymes in different solvent environments. A secondary motivation is to examine the interesting suggestion of Laws & Shore (1978, 1979) that Tyr-286 ionizes during ternary complex formation by studying the phosphorescence spectra of the enzyme and a few of the complexes.

Experimental Procedures

Materials. Horse liver alcohol dehydrogenase was obtained from Boehringer-Mannheim as an alcoholic suspension and was dialyzed extensively to remove alcohol. The removal of traces of ethanol followed the procedure described earlier (Subramanian & Ross, 1979). NAD^+ (alcohol free) and NADH were obtained from P-L Biochemicals. ADP-ribose, AMP, ϵNAD^+, and imidazole (low fluorescent) were Sigma products. Pyrazole, isobutyramide, 2,2,2-trifluoroethanol (gold label), dimethyl sulfoxide (spectral grade), and dioxane (spectral grade) were supplied by Aldrich; *tert*-butyl alcohol was analytical grade from J. T. Baker Chemical Co. Ethylene glycol (spectral grade) was from Eastman.

Methods. Enzyme concentrations were determined by using the absorption coefficient of 0.455 cm^2 mg^{-1} at 280 nm. Enzymatic activity was routinely determined for each preparation and found to be within the limits specified by the manufacturer. The concentrations of coenzymes and coenzyme analogues were determined by using standard extinction coefficients. Potassium phosphate buffer, 0.1 M, at pH 7.6 was used for enzyme–ligand mixing experiments, unless otherwise stated. Alcohol contamination in the enzyme was minimized by overnight incubation with excess NAD and subsequent extensive dialysis and charcoal treatment (Subramanian & Ross, 1979).

Difference Spectra. Difference spectra were obtained by using either a Cary 14 or Cary 219 spectrophotometer. A four-cell arrangement was used in most cases of formation of enzyme–ligand(s) complexes. In other cases, the difference spectra were measured by adding appropriate volumes of ligand solutions to the proper solvents. Matched split-compartment cells of 10-mm total path length were also used in some solvent perturbation experiments. All spectra were recorded at 25 ± 0.1 °C with thermostatic control. In general, volumes of 3 mL in each cell were used with enzyme concentrations in the range of 10–20 μN. The coenzymes and other substrate analogues, in most cases, were at concentrations well in excess of their respective dissociation constants, but within the limits of the optical constraints.

Phosphorescence Measurements. A conventional right-angle optical configuration was used for measuring phosphorescence spectra. Exciting light, provided by a 150-W high-pressure xenon arc (Canrad-Hanovia), was selected by a 0.25-m Jarrell-Ash monochromator set for a 3.2-nm band-pass. The samples were contained in 4-mm i.d. quartz tubes (Wilmad Glass), positioned vertically in a quartz Dewar flask containing liquid nitrogen. The Dewar was set in a black circular holder equipped with 5-mm-wide windows cut at 90°. Fluorescence and scattered light were eliminated by a rotating can chopper. Emission was detected through a second 0.25-m Jarrell-Ash monochromator set for a 6.6-nm band-pass by an EMI 9558 phototube (EMI-Gencom). The signal was amplified by electronics described elsewhere (Witholt & Brand, 1968) and processed by a voltage-to-frequency converter (Dymec 2210). The pulses were counted by a Hewlett-Packard 5326B timer–counter–DVM under control by a Hewlett-Packard minicomputer. The grating assembly of the emission monochromator was turned by a stepping motor also

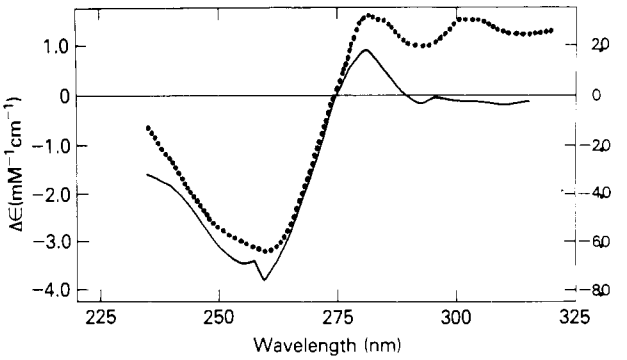

FIGURE 1: Difference absorption spectra of binary complexes of liver alcohol dehydrogenase with NAD (···) and ADP-ribose (—). (Scale) Right ordinate for NAD and left ordinate for ADP-ribose.

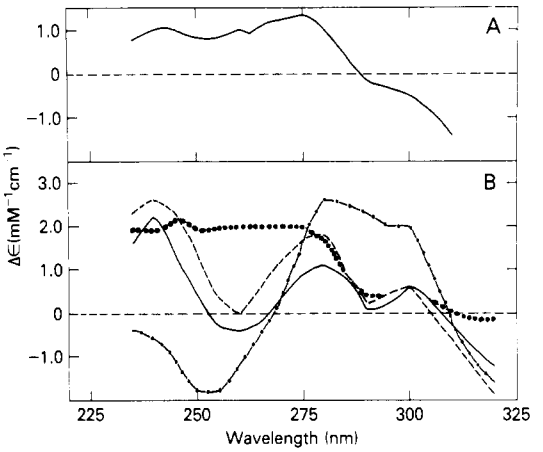

FIGURE 2: Difference absorption spectra of liver alcohol dehydrogenase–NADH binary complex (A) and LADH–NADH–X ternary complexes (B). The identity of X is (—) isobutyramide, (---) dimethyl sulfoxide, (···) imidazole, and (-·-·-) pyrazole.

controlled by the computer. At the end of each scan, the spectrum was displayed on a Hewlett-Packard 5401B multichannel analyzer. The data were then transferred to a Hewlett-Packard system 1000 computer for analysis and hard-copy output. The spectra were not corrected for the instrument response. However, the solvent background when above base line was subtracted from the spectra, especially for the spectra above pH 9.

Preparation of enzyme complexes and adjustments of pH were carried out at 0 °C in 0.1 M phosphate buffer prior to addition of an equal volume of ethylene glycol. The use of ethylene glycol for low-temperature protein spectra has been discussed elsewhere (Ross et al., 1980). Immediately after mixing with the ethylene glycol, the glasses were formed by plunging the quartz sample tubes into the liquid nitrogen. In all experiments, the LADH concentration prior to adding the ethylene glycol was 110 μN. The ternary complexes were formed with 120 μM coenzyme (NAD^+ or NaDH) and 5 mM inhibitor component (pyrazole, TFE, Me_2SO, or IBA), before mixing with the ethylene glycol. Experiments carried out at twice the inhibitor concentration gave identical but noisier spectra in the cases of TFE and Me_2SO.

Results

Difference Spectra. The difference spectra of several binary and ternary complexes of LADH and also of coenzymes and their analogues under different solvent conditions are shown in Figures 1–6. The conditions employed and the salient spectral features are also listed in Table I.

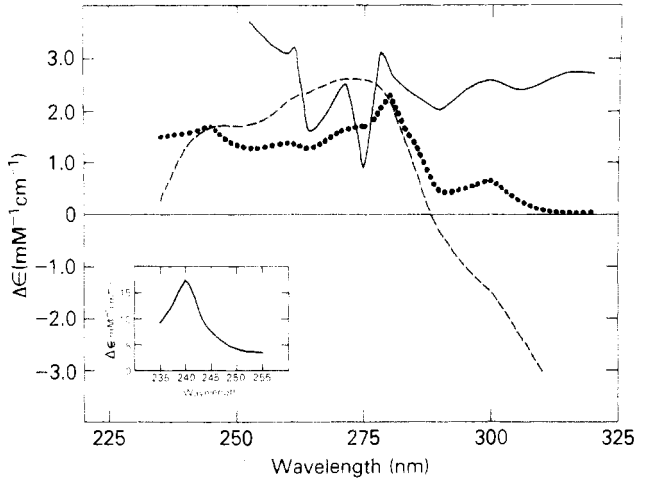

FIGURE 3: Difference absorbance spectra of ternary complexes of LADH: (—) LADH–ϵNAD–trifluoroethanol, (···) LADH–NAD–trifluoroethanol, (- - -) LADH–NADH–trifluoroethanol. (Inset) The short-wavelength region of the difference spectrum of LADH–ϵNAD–trifluoroethanol.

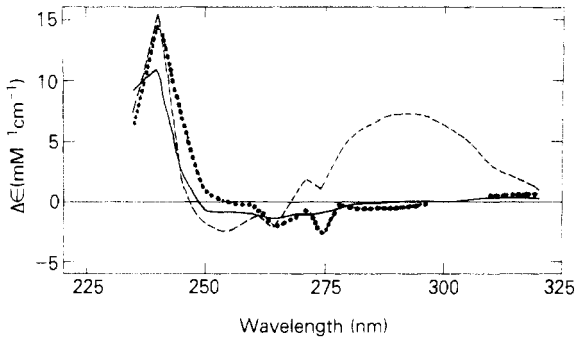

FIGURE 4: Difference spectra of binary and ternary complexes of LADH with ϵNAD: (—) LADH–ϵNAD, (···) LADH–ϵNAD–trifluoroethanol, (- - -) LADH–ϵNAD–pyrazole.

The LADH–NAD spectrum (Figure 1) differs from that obtained by Taniguchi et al. (1967) in relative extinctions in the 280- and 300-nm peaks. The small differences may be due to the different pH values employed in the two studies. Taniguchi et al. measured the spectrum at pH 10 while ours was obtained at pH 7.6. The difference spectrum for LADH–NADH was the same with or without 0.1 M IBA at pH 7.0 according to Taniguchi et al. (1967). In our study, we measured the sharper spectrum for LADH–NADH with 5 mM IBA which is almost identical with that obtained by Laws & Shore (1979). The LADH–NADH binary spectrum, however, was less sharp than that of the ternary complex. We have noticed this distinction in other ternary complexes (involving LADH–NADH) as well. Uncontested explanation cannot be offered for this difference with our current knowledge.

We also studied the difference spectra of ϵ-adenosine and ϵAMP in the solvent systems used for ϵNAD^+. The spectra obtained (not shown) were identical in shape with that of ϵNAD^+ in each solvent system, indicating that the spectral features arise mostly from ϵ-adenine ring perturbation.

Difference spectra for other adenine analogues such as purine, ribosylpurine, 6-(methylamino)purine, and 6-(dimethylamino)purine in 3 M NaCl vs. buffer displayed features similar to the 280-nm maximum and 255–260-nm minimum seen with NAD^+, NADH, and AMP, except for small shifts in the wavelengths of the maxima and minima (spectra not shown). This further indicates that the perturbation of the purine ring predominantly contributes to the observed difference spectra of LADH with coenzymes.

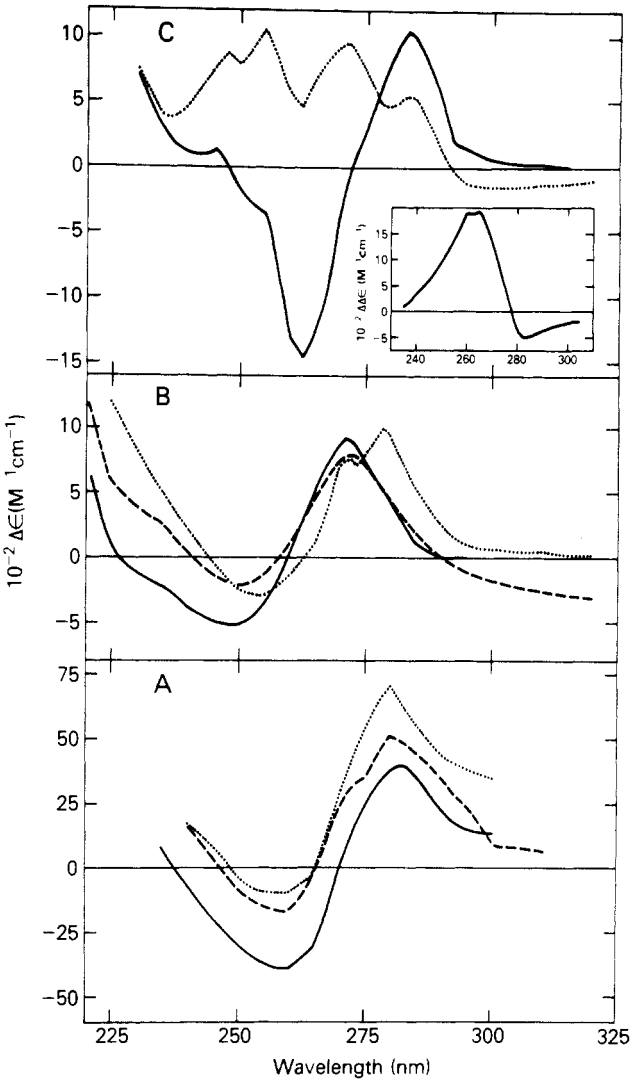

FIGURE 5: Difference spectra of NAD, NADH, ADP-ribose, and AMP arising from solvent perturbation. (A) Difference spectra in 96.8% dioxane–3.2% H_2O (v/v) vs. H_2O: (—) ADP-ribose, (- - - -) NADH, (···) NAD. The absorbance spectrum of each compound was recorded in each solvent system and the difference spectrum was obtained by a subtraction procedure. (B) Difference spectra in 4.8 M NaCl vs. 0.1 M potassium phosphate buffer, pH 7.4: (—) AMP, (·····) NAD, (- - - -) NADH. (C) Difference spectra in 0.01 N HCl (final pH 2.27) vs. water: (—) ADP-ribose, (···) NAD. The inset spectrum was obtained by algebraic subtraction of the ADP-ribose difference spectrum from the NAD difference spectrum.

The LADH–ADP-ribose complex in the CPK model, generated from the atomic coordinates, is shown in Figure 7. This figure is introduced in order to explore the effects of ionic interactions involved in LADH–coenzyme complexes.

Phosphorescence Spectra. Figure 8 shows the phosphorescence spectra of LADH, excited at 280 (labeled 1) and 295 nm (labeled 2), in phosphate buffer at pH 7.6, 9.3, and 10.4. Comparison of these spectra, taking special note of the 295-nm excitation, reveals that the protein phosphorescence has a marked pH dependence. Spectra obtained from the model compounds *N*-acetyltryptophanamide and *N*-acetyltyrosine confirmed the well-known observation that whereas the emission profile of the indole group is independent of pH in this range, the phosphorescence characteristics of the phenol group change dramatically upon ionization [cf. reviews by Weinryb & Steiner (1971) and Longworth (1971)]. Furthermore, the molar extinction coefficient of tyrosine is insignificant at 295 nm ($\sim$15 M^{-1} cm^{-1}) compared with that

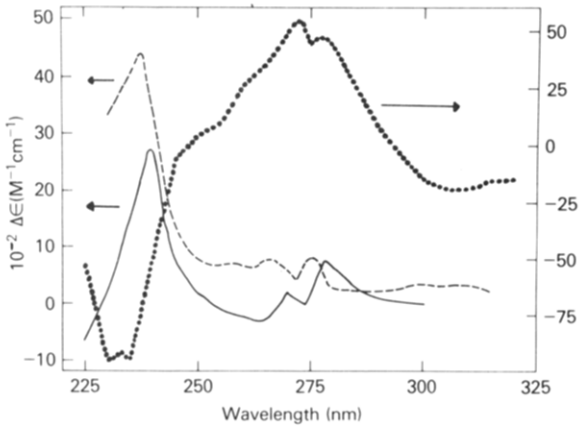

FIGURE 6: Solvent perturbation difference spectra of εNAD: (—) difference spectrum in 4.8 M NaCl vs. 0.1 M K phosphate buffer, pH 7.4; (···) difference spectrum in 0.01 N HCl, pH 2.27, vs. 0.1 M phosphate buffer, pH 7.4; (---) difference spectrum in 50% *tert*-butyl alcohol–50% 0.1 M phosphate buffer (v/v) vs. 0.1 M phosphate buffer. The ordinate scale for each spectrum is indicated.

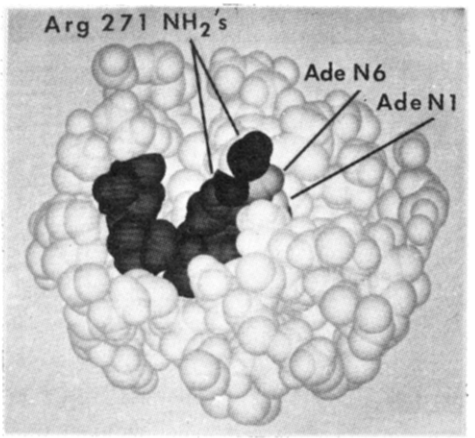

FIGURE 7: CPK model of LADH–ADP-ribose complex generated from the atomic coordinates. The adenine N6 and N1 atoms and the NH_2 groups of arginine-271 of the enzyme are indicated.

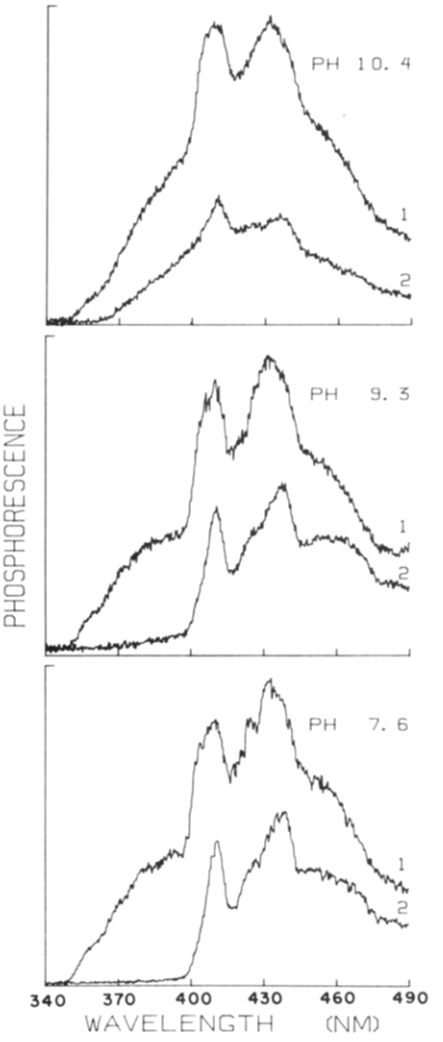

FIGURE 8: Phosphorescence spectra of liver alcohol dehydrogenase at three different pH values. The excitation wavelengths were 280 and 295 nm for spectra labeled 1 and 2, respectively.

of tyrosinate (~2300 M^{-1} cm^{-1}) (Wetlaufer, 1962). The emission maximum of *N*-acetyltyrosine at pH 7, excited at 280 nm, was at 395 nm, and the blue edge of onset was at ~350 nm. No phosphorescence was observed upon excitation at 295 nm. At pH 10, with excitation at 295 nm, a red-shifted spectrum was obtained which had its origin at about 360 nm and its maximum at about 405 nm. The blue edge of the pH 10.4 LADH spectrum, with 295-nm excitation, is just above 360 nm; at pH 7.6 the phosphorescence intensity is zero until 387 nm. On the basis of the phosphorescence characteristics of *N*-acetyltyrosine, we assign the LADH emission having its origin at 360 nm to ionized tyrosyl residues. Wide slits were used on the emission monochromator to enhance our detection of tyrosinate emission, and hence in the spectra shown in Figure 8 (and in Figure 9) the tryptophan emission is not resolved into two classes (Purkey & Galley, 1970). With an emission band-pass of 3.3 nm, we were able to separate the contributions of the buried and exposed tryptophans. Assuming that tyrosyl residues are approximately 55% ionized at pH 10.4, we calculate from the intensity of the 385-nm emission that at pH 9.3 we are detecting 7 ± 3% ionization (worst case estimate). The signal-to-noise ratio for the 385-nm phosphorescence intensity at pH 9.3 was 5:2.

The phosphorescence spectra for three ternary complexes of LADH at pH 7.6 are shown in Figure 9. The LADH–NADH–Me_2SO ternary complex also gave results similar to those shown for the LADH–NADH–IBA complex. The qualitative similarity of the LADH–NAD^+–TFE results to those of the NADH complexes deserves comment. Although TFE is an inhibitor, it appears from the phosphorescence results that there was reduction of the nicotinamide ring in the low-temperature complex. In every complex studied, the total emission was quenched compared with that of the free enzyme. In no case, when 295-nm excitation was employed and the pH was near neutrality, was there a significant increase in the phosphorescence intensity at 385 nm.

Discussion

High-resolution X-ray crystallographic studies of several dehydrogenases complexed with NAD^+ indicated that, in general, the coenzyme binds to the dehydrogenases in an extended conformation, as opposed to the solution conformation of the coenzyme being an equilibrium mixture of extended and folded conformations. The adenine and nicotinamide binding sites on the enzymes consist of predominantly nonpolar residues, and the pyrophosphate backbone of the coenzyme is held onto the surface of the enzymes usually by ionic and H-bond interactions with basic groups on the enzymes (Rossman et al., 1975).

The binding of NAD^+ or NADH to LADH is accompanied by conformational changes in the enzyme, but the binding of ADP ribose does not elicit a conformational change in LADH (Branden et al., 1975). The enzyme residues in contact with

Table I: Difference Spectral Features[a] of Several Complexes of LADH and of Coenzymes and Their Analogues Perturbed by Solvents

enzyme complex[b] or ligand	concn[c] of LADH and/or ligands	spectral features[d]	cf. Figure
LADH–ADP-ribose	20 μM LADH 134 μM ADP-ribose	max 281 min 255–260	1
LADH–NAD	10 μM LADH 57 μM NAD	max 300–305, 281 min 260	1
LADH–NAD–TFE	10 μM LADH 27 μM NAD 5 mM TFE	max 300, 280, 245 min 290, 265, 255	3
LADH–NADH	20 μM LADH 20 μM NADH	max 275, 243 shallow depression 250–260	2A
LADH–NADH–(X), (X) = IBA, imidazole, Me_2SO, Pyr	10 μM LADH 22 μM NADH 5 mM X	max 300, 280, 240–245 min 250–260	2B
LADH–NADH–TFE	10 μM LADH 22 μM NADH 5 mM TFE	max 270–275, 245	3
LADH–ϵNAD	10 μM LADH 33 μM ϵNAD	max 240 other features not prominent	4
LADH–ϵNAD–TFE	10 μM LADH 33 μM ϵNAD 5 mM TFE	max 300, 278, 271, 240 min 290, 275, 264	3, 4
LADH–ϵNAD–Pyr	10 μM LADH 25 μM ϵNAD 1.65 mM Pyr	max 290–295, 271, 240 min 274, 263, 255	4
NAD			
pH difference spectrum	83 μM NAD	max 283, 272, 255, 247.5 min 278, 262, 250, 236	5
dioxane–H_2O difference spectrum	28.6 μM NAD	max 280 min 255–260	
NaCl-buffer difference spectrum	83 μM NAD	max 278.5, 272 min 255	
ADP-ribose			
pH difference spectrum	32.4 μM ADP-ribose	max 283 min 262.5 shoulders at 255, 245	5
dioxane–H_2O difference spectrum	50 μM	max 283 min 260	
AMP			
NaCl-buffer difference spectrum	54.4 μM AMP	max 272 min 250 shoulder at 235	5
NADH			
dioxane–H_2O difference spectrum	31.5 μM NADH	max 280 min 260 shoulder at 273	5
NaCl-buffer difference spectrum	63.5 μM NADH	max 272 min 250 shoulder at 235	
ϵNAD			
pH difference spectrum	6.8 μM ϵNAD	max 273 min 230–235 shoulder at 245, 260, 277	6
tert-butyl alcohol buffer difference spectrum	56.4 μM ϵNAD	max 275, 266, 237.5 min 272	
NaCl-buffer difference spectrum	26.6 μM ϵNAD	max 278, 270, 239 min 274, 264	

[a] Potassium phosphate at 0.1 M and pH 7.6 was the medium for the enzyme–ligand complexes; the conditions of perturbation of coenzymes and analogues by pH, organic solvents, or salt are detailed in the figure legends. [b] In the LADH–NADH binary complex and in all ternary complexes, the conditions are such that the enzyme is fully complexed. For the LADH–ADP-ribose complex, a dissociation constant of 16 μM (Taniguchi et al., 1967) was used to calculate the extent of complexation. For the LADH–NAD complex, about 50% of the enzyme was complexed based on an interpolated dissociation constant of 65 μM (Tanguchi et al., 1967). No estimate of the dissociation constant for ϵNAD from LADH–ϵNAD is available. It was assumed to have the same K_d as in ternary complexes. To this extent, the LADH–ϵNAD binary spectrum should not be considered quantiatively accurate. [c] Concentration of LADH is expressed in subunits (active sites). [d] The numbers represent wavelength in nanometers (nm).

ADP ribose have been described (Eklund et al., 1976). The extent of conformational changes that occur in the enzyme upon formation of LADH–NADH–Me_2SO ternary complex (and by inference other ternary complexes involving the coenzymes) is described by Eklund & Brändén (1979) to be limited to the catalytic domains of the two subunits rotating and narrowing the cleft between the catalytic and coenzyme-binding domains.

It has been observed that the interaction of ADP-ribose with LADH produces spectral chhanges similar to those produced upon protonation of the adenine ring in ADP ribose (Fisher et al., 1967). The peaks at 280 nm and the negative troughs around 260 nm are present in LADH–ADP-ribose and other complexes studied, indicating that these arise from the interaction of adenine ring with LADH. The peaks in the 295–305-nm region have been attributed to the interaction of nicotinamide moiety of NAD^+ with the catalytic zinc (Taniguchi et al., 1967; Theorell & Yonetani, 1964) since these peaks are not seen in the LADH–ADP-ribose complex.

A peak at 240 nm in the difference spectrum of LADH–NADH complex (Figure 2A) is also accentuated in the LADH–NADH–X ternary complexes (Figure 2B). No peak at 240 nm is seen with either LADH–ADP-ribose or LADH–NAD^+ binary complexes, but a slight shoulder is discernible (Figure 1). The 240-nm peak was attributed to an ionized tyrosine by Laws & Shore (1979), but as will be evident from the discussion that follows, it most probably arises from a perturbation of the adenine ring.

A cursory examination of the difference spectra (Figure 5) of coenzymes and analogues under different conditions reveals that those conditions which cause a conformational change in the coenzyme or change the environment drastically also cause ultraviolet spectral changes; these changes, recognizably, have a general resemblance to those caused by coenzyme binding to LADH. It is known that neutral salts, nonpolar solvents like dioxane, and low pH conditions unstack dinucleoside phosphates (Johnson & Schleich, 1974). The difference spectra of NAD^+, and ADP-ribose obtained at low pH vs. neutral pH (Figure 5C), show a hyperchromic effect of the adenine chromophore in NAD^+ (inset of Figure 5C), presumably due to intramolecular unstacking of the NAD^+ molecule upon protonation. However, ADP-ribose is not a

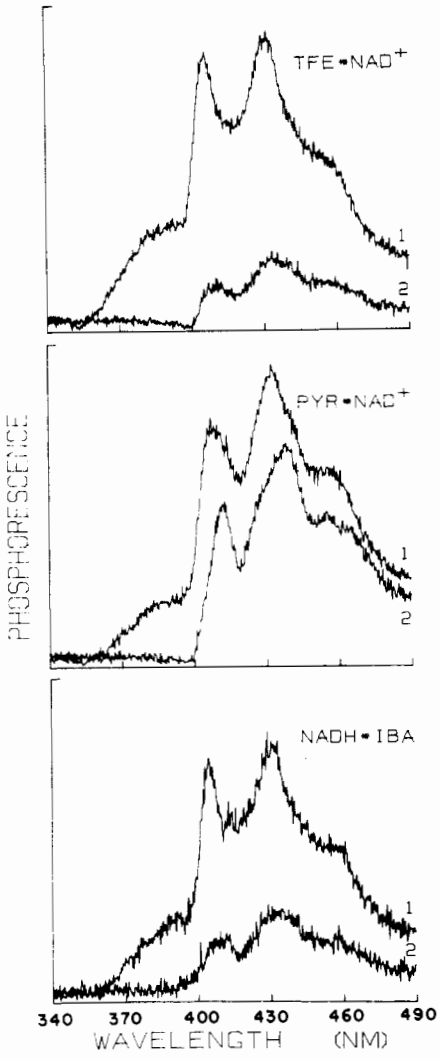

FIGURE 9: Phosphorescence spectra of ternary complexes of LADH. Spectra labeled 1 and 2 in each frame correspond to excitation wavelengths of 280 and 295 nm, respectively.

stacked molecule, and hence the spectral changes in ADP-ribose must be a direct result of the protonation of the adenine ring. The resemblance of the LADH–ADP-ribose difference spectrum to that of the ADP-ribose protonation difference spectrum prompted Fisher et al. (1967) to speculate that some protonated group of the enzyme must be interacting with the adenine ring. They mentioned arginine, lysine, or cysteine as possible candidates for a proton transfer. However, further examination of the coenzyme and its analogues in organic solvents and NaCl points out that the effects produced by protonation of the adenine ring can also be produced by salt and dioxane (Figure 5, B and A). The difference spectra in 4.8 M NaCl have almost the same features as those at low pH. AMP, which has no capability for intramolecular stacking, at low enough concentration to avoid intermolecular stacking also displays the 271-nm positive peak coupled with the 250-nm trough. NAD^+ and NADH may be unstacked by salt, as shown by higher extinctions for these two molecules in salt solutions. In addition, AMP and NADH exhibit a shoulder at 235 nm resembling that obtained in binary and ternary complexes of LADH at 240 nm. It is conceivable that the resemblance of the salt spectrum to the low pH spectrum has a common source, viz., a proton or a cation (Na^+). The difference spectra of NAD^+, NADH, and ADP-ribose in 96.8% dioxane–3.2% water (v/v) vs. water (Figure 5A) also have spectral features very similar to the pH perturbation and salt perturbation spectra. Moreover, even though dioxane could unstack NAD^+ and NADH, the fact that the dioxane-perturbed ADP-ribose difference spectrum has the same shape as those of NAD^+ and NADH strongly argues for the perturbation of the adenine ring by the solvent environment being mainly responsible for the spectral changes.

Thus, we have seen that the difference spectra of NAD^+, NADH, ADP-ribose, and AMP in low pH, dioxane, or 4.8 M NaCl measured vs. H_2O or neutral pH buffer are very similar to each other. The simplest expanation for this observation is that the diverse solvent environments perturb transitions common to all these molecules in a similar manner. Two π–π^* transitions and one n–π^* transition have been ascribed to the 260-nm absorption of adenine (Miles et al., 1969; Voelter et al., 1968). A perturbation involving the nonbonding electrons whether by a change in pH or by a solvent rearrangement in the vicinity of the nonbonding electrons could cause the observed spectral changes. Recently, Frechet et al. (1979) studied the thermal perturbation spectra of several monomeric nucleic acid residues and obtained difference spectra with positive peaks at 283 nm and negative minima around 250–260 nm, similar to the solvent perturbation spectra of NAD^+, NADH, and ADP-ribose that we have measured. By comparing the thermal perturbation spectra with solvent perturbation of the same compounds, these authors concluded that the spectral changes can be attributed to hydration changes of the bases in the nucleotide. Water can perturb the interaction of the nonbonding electrons of nitrogen and oxygen atoms with the π-electron system. Dehydration, whether induced by higher temperature or by substitution with an organic solvent, cation, or a proton, could cause changes in such interactions. Specifically, a proton is known to bind to N1 of adenine at low pH. Similarly, at high salt concentrations, Na^+ could interact with the nonbonded electrons of N1, and likewise dioxane could replace water in the vicinity of N1 of the adenine ring in dioxane–water mixtures. The result of all this may be to displace the water bonded to N1 and thereby modify the contribution to the difference spectrum which was influenced previously by the hydrated water.

It is not necessary to invoke a protonation of adenine in the bound ADP-ribose (Fisher et al., 1967) to explain the similarity of the low-pH ADP-ribose spectrum to that of ADP-ribose bound to LADH. In fact, no proton transfer was detected calorimetrically when ADP-ribose or NADH binds to LADH (Subramanian & Ross, 1978, 1979). Since the effects of H^+ and Na^+ are to produce similar spectral changes, it is more likely that a cationic enzyme residue near the adenine group causes the observed spectral changes. It is known (Eklund et al., 1976) that the adenine binding site in LADH is replete with nonpolar residues and that there is an arginine residue (Arg-271) at the entrance of the adenine binding pocket.

Thus, the 260–280-nm spectral features seen in the binding of NAD^+, NADH, or ADP-ribose to LADH can be ascribed largely to the perturbation of the adenine ring by a largely nonpolar milieu in combination with the presence of positively charged groups in the vicinity. We examined the distances of Arg-271 to specific atoms in the adenine ring from the coordinates of the LADH–ADP-ribose complex deposited in the NIH–DCRT computer data bank. The CPK model, generated from the atomic coordinates, shown in Figure 7, indicates the proximity of the guanidinium moiety of Arg-271 to N6 and N1 of adenine. The actual distances of N6 to the two nitrogens of Arg-271 are 2.4 and 1.6 Å and of N1 to the same two nitrogens are 4.5 and 3.3 Å. These are suitable distances for short-range interactions of the positively charged

guanidinium group of Arg-271 with adenine atoms possessing nonbonding electrons. The positively charged Arg-271 and other nonpolar residues in the vicinity of the adenine ring could effectively interact with the nonbonded electrons in the adenine ring to produce the 260–280-nm spectral changes seen when LADH complexes with coenzymes or coenzyme analogues are formed.

The spectral features in the binary and ternary complexes of LADH with ϵNAD^+ further support this conclusion (Figures 3 and 4). The binary and ternary complexes display well-resolved peaks and troughs in the 250–280-nm region. These changes are also seen in solvent perturbation spectra. ϵ-Adenosine and ϵNAD^+ exhibit four maxima (at neutral pH), at 258, 265, 275, and 294 nm (Barrio et al., 1972). We see perturbation of all these bands when ϵNAD^+ binds to LADH similar to the perturbation by NaCl, *tert*-butyl alcohol, or low pH, indicating that the ϵ-adenine ring experiences some kind of a composite environment on the enzyme consisting of nonpolar and cationic residues. Furthermore, there is an intense peak at 240 nm in the difference spectra of LADH–ϵNAD^+ complexes as well as in the solvent perturbation spectra.[2] The intensity of this peak in the ternary complexes with ϵNAD^+ is 10-fold higher than that of the 240-nm peak in LADH–NADH and LADH–NAD^+ ternary complexes with substrate analogues. This argues for the 240-nm peak's origin to be coenzyme perturbation rather than from tyrosine ionization as suggested by Laws & Shore (1979). Adenine has a transition at about 240 nm (Miles & Urry, 1968) (although much less intense than that of the 260-nm band), and perturbation of this transition could manifest in spectral changes in the 240-nm region like those seen in the enzyme–coenzyme complexes or the coenzymes in high concentration of salt (Figure 5B).

Absorption and phosphorescence spectra should, in principle, yield similar information about tyrosine ionization, since the pK of the ground-state singlet of phenols is close to the pK^* of the lowest excited triplet state (Parker, 1968). Using phosphorescence, we were able to detect about 7% total tyrosine ionization in the free enzyme at pH 9.3. The sensitivity of our measurements was sufficient to detect about half of this, that is, about 3% ionization. This estimate of course does not take into consideration a possible contribution to the tyrosinate emission by singlet–singlet energy transfer from the tryptophan residues, nor does it provide for the possibility that those tyrosine residues which are ionized could be quenched by triplet–triplet energy transfer to either Trp-15 or Trp-314. Since there are four tyrosines per subunit, if only one residue is to ionize, as suggested by Laws & Shore (1979), and if it is assumed that the phosphorescence yield of each tyrosine is approximately equal, then tyrosine ionization to the extent of 12% is to be expected, which is well above our detection limit of 3%. However, we find no evidence for tyrosine ionization, at neutral pH, in the ternary complexes LADH–NADH–IBA, LADH–NADH–Me_2SO, LADH–NAD^+–pyrazole, and LADH–NAD^+–TFE. We recognize that the NAD^+ ternary complexes could be slowly converted to NADH ternary complexes in ethylene glycol medium or even in the presence of the supposedly inhibitory TFE. However, in the LADH–NAD^+–Pyr ternary complex, where the extent of quenching is considerably less than that in LADH–NAD^+–TFE or LADH–NADH–IBA complexes, there is still no observable tyrosinate phosphorescence. This tends to rule out the possibility of ionization of tyrosine in ternary complexes of LADH at neutral pH.

The X-ray crystallographic structure of the LADH–ADP-ribose complex (Edlund et al., 1976) reveals that the four tyrosine residues of each subunit are located at the exterior surface of the molecule, and their phenolic hydroxyl groups are fully exposed to the bulk solvent. Thus, there is no reason, a priori, to believe that any of these residues would exhibit "abnormal" tyrosine spectra. This does not rule out the possibility of a strong interaction with a nearest-neighbor residue side chain. However, it is unlikely that such an interaction would take place in an exposed aqueous environment, where there is little thermodynamic driving force for its occurrence. Our phosphorescence and absorption spectral observations provide no evidence for tyrosine ionization. The putative tyrosinate absorption spectrum shown by Laws & Shore (1979) to arise upon ternary complex formation can be accounted for by the spectral changes in the coenzyme itself. The 240-nm peak could arise from adenine perturbation, as demonstrated by us, and the weak 295-nm peak could be caused by nicotinamide interaction with the enzyme.

In summary, the difference spectra produced by binary and ternary complexes of LADH with NAD^+, NADH, or ADP-ribose are very similar to the difference spectra produced by perturbation of the coenzymes by proton, NaCl, or organic solvents. Thus, to a large extent, the difference spectral changes in the LADH–coenzyme complexes are caused by perturbation of the adenine chromophore by cationic and nonpolar residues of the enzyme; any contribution to the difference spectrum from enzyme residues perturbation appears to be minimal. The phosphorescence spectra argue for the absence of any ionized tyrosine as a result of ternary complex formation at neutral pH. These conclusions, taken together, suggest that the mechanism of quenching of tryptophan fluorescence in LADH requires further studies.

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[2] This 240-nm peak is not seen in the low pH vs. neutral pH difference spectrum of either ϵNAD^+ or other ϵ-adenosine derivatives. It is known (Inoue et al., 1980) that there are two sites of protonation in the ϵ-adenine ring in contrast to one site (N1) in adenine and its derivatives. This difference could cause the observed spectral features.

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Use of Resonance Energy Transfer To Monitor Membrane Fusion†

Douglas K. Struck,‡ Dick Hoekstra, and Richard E. Pagano*

ABSTRACT: An assay for vesicle–vesicle fusion involving resonance energy transfer between *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl), the energy donor, and rhodamine, the energy acceptor, has been developed. The two fluorophores are coupled to the free amino group of phosphatidylethanolamine to provide analogues which can be incorporated into a lipid vesicle bilayer. When both fluorescent lipids are in phosphatidylserine vesicles at appropriate surface densities (ratio of fluorescent lipid to total lipid), efficient energy transfer is observed. When such vesicles are fused with a population of pure phosphatidylserine vesicles by the addition of calcium, the two probes mix with the other lipids present to form a new membrane. This mixing reduces the surface density of the energy acceptor resulting in a decreased efficiency of resonance energy transfer which is measured experimentally. These changes in transfer efficiency allow kinetic and quantitative measurements of the fusion process. Using this system, we have studied the ability of phosphatidylcholine, phosphatidylserine, and phosphatidylcholine–phosphatidylserine (1:1) vesicles to fuse with cultured fibroblasts. Under the conditions employed, the majority of the cellular uptake of vesicle lipid could be attributed to the adsorption of intact vesicles to the cell surface regardless of the composition of the vesicle bilayer.

Membrane fusion is involved in a variety of important biological processes, and therefore various attempts have been made to elucidate the mechanism(s) of fusion. In particular, the use of unilamellar lipid vesicles for this purpose has received wide attention (Miller & Racker, 1976; Papahadjopoulos et al., 1976, 1977, 1979; Newton et al., 1978; Pagano & Weinstein, 1978; Portis et al., 1979). Although it is clear that vesicle–vesicle fusion does occur under certain conditions (Maeda & Ohnishi, 1974; Miller & Racker, 1976; Hoekstra et al., 1979; Wilschut & Papahadjopoulos, 1979), the procedures used to detect such fusion suffer from low sensitivity or an experimental design which precludes their use in more complex systems. Consequently, an alternative method(s) by which membrane fusion can be measured which is both sensitive and versatile is needed.

Several recent reports (Keller et al., 1977; Gibson & Loew, 1979; Deamer & Uster, 1980) suggest that membrane fusion in a wide variety of systems might be studied by using resonance energy transfer. This approach relies upon the interactions which occur between two fluorophores if the emission band of one, the energy donor, overlaps with the excitation band of the second, the energy acceptor, and the two probes exist in close physical proximity (Forster, 1949; Fung & Stryer, 1978; Stryer, 1978). When these conditions are met, the energy from a photon absorbed by the energy donor can be transferred to the energy acceptor which will then fluoresce as though it had been excited directly. Since the efficiency of fluorescence energy transfer between two given fluorophores is dependent upon their spatial separation (Fung & Stryer, 1978), this technique provides a means by which lipid mixing

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‡ Present address: Department of Medical Biochemistry, Texas A&M University, College of Medicine, College Station, TX 77843.

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