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# Tryptophan Luminescence from Liver Alcohol Dehydrogenase in Its Complexes with Coenzyme. A Comparative Study of Protein Conformation in Solution

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ABSTRACT: The extent of fluorescence quenching and that of phosphorescence quenching of Trp-15 and Trp-314 in alcohol dehydrogenase from horse liver as well as the intrinsic phosphorescence lifetime of Trp-314 in fluid solution have been utilized as structural probes of the macromolecule in binary and ternary complexes formed with coenzyme, analogous, and various substrate/inhibitors. Luminescence quenching by the coenzyme reveals that (1) while the reduced form quenches Trp emission exclusively from the fluorescent state, the oxidized form is very effective on the phosphorescent state as well and that (2) among the series of NADH binary and ternary complexes known by crystallographic studies to attain the closed form, distinct nicotinamide/indole geometrical arrangements are inferred from a variable degree of fluorescence quenching. Information of the dynamic structure of the coenzyme-binding domain derived from the phosphorescence lifetime of Trp-314 points out that within the series of closed NADH complexes there is considerable conformational heterogeneity. In solution, the variability in dynamical structure among the various protein complexes emphasizes that the closed/open forms identified by crystallographic studies are not two well-defined macrostates of the enzyme.

Alcohol dehydrogenase from horse liver (LADH)[1] is one of the best-characterized enzymes (Eklund & Bränden, 1983, 1985; Eklund et al., 1986). The abundance of information available on kinetic, structural, and physicochemical properties makes it a suitable model system for deeper insight into the chemistry and physics of enzyme catalysis. Refined crystallographic structures for the apo- and holoenzyme show that in general coenzyme binding induces a change in conformation in the macromolecule from an open to a closed form (Eklund, 1986; Eklund & Bränden, 1985; Eklund et al., 1984). The latter is characterized by a rigid body rotation of the catalytic domain relative to the coenzyme-binding domain with closure of the cleft separating the domains. Following these findings, the compulsory binding order mechanism proposed by Theorell and Chance (1951) to describe the kinetics of alcohol oxidation could also be rationalized on a structural basis.

Recent crystallographic investigations with a number of substrate/inhibitor and coenzyme/analogue combinations have pointed out, however, that there is not a single holostructure for LADH, and depending on the ligand combination, ternary complexes do form in which the conformation is either open (similar to that of the apoenzyme) or intermediate between open and closed forms (Cedergren-Zeppezaur, 1986). Evidence that a single holoenzyme structure is inadequate to represent LADH in its complexes with oxidized coenzyme comes also from spectroscopic studies in solution with Co-substituted LADH (Maret & Zeppezauer, 1986; Zeppezauer, 1986).

In this work, through the fluorescence and phosphorescence emission of the two Trp residues, we inquire on the conformation of the LADH molecule in solution in an assortment of binary and ternary complexes for which X-ray data are available. Emission data can provide structural information in two ways: (1) From the extent of fluorescence and phosphorescence quenching emission data can test for possible changes in the geometrical arrangement between tryptophans and the nicotinamide ring of the coenzyme. (2) From the rate of decay of the triplet state of Trp-314, emission data can monitor the dynamical structure of the coenzyme-binding domain (Strambini & Gonnelli, 1985; Strambini & Gabellieri, 1987). The latter approach displays a remarkable sensitivity to changes in protein structure and has been instrumental in highlighting the effects induced, for example, by mild concentrations of guanidine hydrochloride (Strambini & Gonnelli, 1986) or incorporation of the enzyme in reverse micelles

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[1] Abbreviations: LADH, liver alcohol dehydrogenase from horse; NADH, reduced nicotinamide adenine dinucleotide; $NAD^+$, oxidized nicotinamide adenine dinucleotide; MPD, 2-methyl-2,4-pentanediol; DMSO, dimethyl sulfoxide; IBA, isobutyramide; ADPR, adenosine-(5′)diphospho(5)-β-D-ribose; PYR, pyrazole; IMID, imidazole; $H_2NADH$, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide; Trp, tryptophan; EtOH, ethanol; TFE, trifluoroethanol.

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(Strambini & Gonnelli, 1988). Trp-314 is roughly at the center of the macromolecule buried within the large β-sheet in the coenzyme-binding domain (Eklund et al., 1976). The aromatic side chain makes no bonding contact with either coenzyme or substrate. Any change in the phosphorescence lifetime upon ligand binding will signal an altered fluidity of the surrounding protein structure that might arise from bonding rearrangements involving the β-sheet subsequent to the closure of the coenzyme-binding cleft or H-bonding of the nicotinamide moiety to β-strands E and F. In this respect a comparison of the phosphorescence lifetime of complexes having the same crystallographic structure might emphasize differences among them that either are not resolved in the global X-ray analysis or arise from a change in the physical state of the protein (crystal versus dilute solution). The results of the present investigation with reduced and oxidized coenzyme as well as some of its analogues confirm that each complex possesses distinct structural features of its own not correlated to the open/closed crystallographic structures.

## Materials and Methods

Liver alcohol dehydrogenase from horse (LADH), highest purity grade NADH, and $NAD^+$ were obtained from Boehringer (Mannheim). MPD, DMSO, IBA, and acetaldehyde were from Aldrich (Steinheim). ADPR, pyrazole, and imidazole were from Sigma Chemical Co. (St. Louis, MO). Prior to use MPD was distilled under nitrogen atmosphere, whereas IBA and pyrazole were twice recrystallized from water.

The crystalline suspension of the enzyme was dialyzed for at least 24 h (under nitrogen) against 0.1 M sodium phosphate buffer, pH 8, and any remaining insoluble precipitate was removed by centrifugation. Fresh preparations were made weekly, and no loss of activity was found during that time. The activity of LADH preparations, as measured by the method of Dalziel (1957), ranged between 130 and 145%. Active-site concentrations were also determined by the spectrophotometric titration of LADH coenzyme-binding sites with $NAD^+$ in presence of excess pyrazole (Theorell & Yonetani, 1963). On the basis of a molar extinction coefficient of $E_{280} = 3.53 \times 10^4\ M^{-1}\ cm^{-1}$, the coenzyme-binding capacity was typically 95% or better.

*Synthesis of 1,4,5,6-Tetrahydronicotinamide Adenine Dinucleotide ($H_2NADH$).* Following a modification of the method of Biellman and Jung (1957), NADH (400 mg) was dissolved in 10 mL of doubly distilled water and the pH adjusted to 11 with potassium hydroxide. After the solution was bubbled with hydrogen, 30 mg of dry 10% palladium on charcoal was added to the reaction vessel. The reduction was carried out at room temperature under hydrogen pressure (4 cmHg) and vigorous stirring. As indicated by high-pressure liquid chromatography, the reaction goes to completion in about 4 h. The product was isolated by gel filtration on Sephadex G-25 equilibrated with a mixture of $H_2O$/KOH, pH 9. Its absorption spectrum had characteristic maxima at 287 and 265 nm whose intensity ratio in basic water was 0.89 (287/265 nm).

*Sample Preparation for Phosphorescence Measurements.* To obtain reproducible phosphorescence data in fluid solution, it is of paramount importance to remove thoroughly all dissolved oxygen. Satisfactory deoxygenation was achieved by placing about 0.5 mL of the protein solution in the short arm of an L-shaped quartz cell provided with a small stirrer where gas exchange and equilibration are to take place. The solution is finally transferred to the thin arm (4 mm i.d.) for emission studies. The short arm is connected to a vacuum line for gas exchange by means of a vacuum-tight steel cap (Swagelock Patent D-316) which, upon detachment from the line, avoids air leakage. Satisfactory removal of $O_2$ from the solution was obtained in about 10 min by repeated application of moderate vacuum followed by an inlet of very pure nitrogen (0.1 ppm in $O_2$; SIO, Florence) at a pressure of 3 atm with gentle stirring. A check on the thoroughness of deoxygenation is provided by the dependence of the phosphorescence lifetime on the amount of excitation absorbed by the sample (Strambini, 1983).

*Luminescence Measurements.* Fluorescence spectra and quantum yields were obtained with homemade instruments (Strambini, 1983). The excitation provided by a 100-W high-pressure Hg lamp was selected by a 250-mm grating monochromator (Jarrel-Ash) employing a band-pass ranging from 2 to 10 nm. The emission was dispersed by a 250-mm grating monochromator (Jobin-Yvon H25) and detected with an EMI 9635QB photomultiplier. Phosphorescence decays were monitored at 440 nm by a double-shutter arrangement, permitting the emission to be detected 2 ms after the excitation cutoff. The decaying signal was digitalized by an applescope system (HR-14, RC electronics) and on occurrence averaged on an Apple II computer equipped for exponential decay analysis by a least-squares method.

With relatively weak complexes between LADH and coenzyme large concentrations of coenzyme are needed to saturate over 95% of the binding sites. To correct for the decrease in phosphorescence intensity due to the inner filter effect at 295 nm arising from excess coenzyme, solutions were prepared containing Trp and coenzyme in which their respective absorbances were the same as in the complexes. The correction factor was obtained from the decrease in the fluorescence intensity at 350 nm relative to a coenzyme free solution.

The temperature in these experiments was regulated by the flow of cold nitrogen through a quartz Dewar and a temperature controller (Oxford DTC2), achieving an accuracy of 0.2 K. Enzyme concentrations were typically $15 \times 10^{-6}$ M.

## Results

*Luminescence Quenching in Strong Ternary Complexes between LADH, Coenzyme, and Inhibitors.* The ternary complexes LADH/NADH/IBA, LADH/$NAD^+$/PYR, and LADH/$NAD^+$/TFE have dissociation constants sufficiently strong (nanomolar) that the excess ligand free in solution does not give rise to artifacts from inner filter and trivial energy transfer. Also, they possess small dissociation rate constant ($k_{off}$), so that even at room temperature the coenzyme remains in the bound state for a time longer than the triplet-state lifetime of Trp-314 ($\tau \sim 1$ s). Under these conditions the decay kinetics depend solely on the configuration attained by the macromolecule in the complex and are not influenced by isomerization processes that might accompany coenzyme dissociation.

Binding of NAD(H) leads to substantial quenching of Trp fluorescence. Independent estimates agree that Trp-314, which is nearer the nicotinamide ring than Trp-15, is the residue predominantly affected by it (Table I). Consequently, the room temperature phosphorescence intensity, which is due exclusively to Trp-314 (Saviotti & Galley, 1974), will be reduced to an extent equal to or greater than that of the precursor fluorescent state. Among the three ternary complexes, room temperature phosphorescence was found only with LADH/NADH/IBA and LADH/$NAD^+$/PYR. No phosphorescence could be detected from LADH/$NAD^+$/TFE. As in this complex the fluorescence of Trp-314 is only partially quenched (reference in Table I); the lack of emission from the triplet state implies that its lifetime or quantum yield is below

Table I: Relative Fluorescence ($F/F_0$) and Phosphorescence ($P/P_0$) Quantum Yields of Trp-314 and Trp-15 in Strong Ternary Complexes between LADH and Coenzyme[a]

| | 110 K, $\lambda_{ex}$ = 305 | | 293 K, $\lambda_{ex}$ = 295 | | residual fluorescence (293 K) | | |
|---|---|---|---|---|---|---|---|
| complex | $F/F_0$ | $P/P_0$ | $F/F_0$ | $(P/P_0)(\tau_0/\tau)$ | Tpr-314 | Tpr-15[b] | literature |
| LADH/NADH/IBA | 0.14 ± 0.01 | 0.15 ± 0.01 | 0.24 ± 0.01 | 0.14 ± 0.01 | 0.14 | 0.50 | |
| | 0.30 | 0.30 | | | | | Purkey and Galley (1970) |
| LADH/NAD$^+$/PYR | 0.64 ± 0.02 | 0.69 ± 0.03 | 0.67 ± 0.03 | 0.62 ± 0.04 | 0.62 | 0.80 | |
| | | | | | 0.62 | 0.99 | Ross et al. (1981) |
| | | | | | 0.55 | 0.68 | |
| | | | | | 0.63 | 1.04 | Knutson et al. (1982) |
| LADH/NAD$^+$/TFE | 0.43 ± 0.02 | 0.01 | 0.53 ± 0.1 | | 0.43[c] | 0.79 | |
| | | | | | 0.20 | | Laws and Shore (1978) |
| | | | | | 0.34 | 0.8–0.85 | Eftink and Selvidge (1982) |

[a] The data obtained with selective excitation of Trp-314 at low temperature in a glycerol/phosphate buffer glass (60/40 v/v) are compared to those in buffer at room temperature. [b] The residual fluorescence of Trp-15 was estimated from the total fluorescence intensity of the complex at 323 nm by assuming that the fluorescence of Trp-314 is quenched to the same extent as its phosphorescence and that the contribution of Trp-15 to the fluorescence of unliganded LADH ($\lambda_{ex}$ = 295 nm, $\lambda_{em}$ = 323 nm) is 28% (Eftink & Selvidge, 1982). No correction was attempted for the spectral shift reported by Knutson et al. (1982) for Trp-15 in LADH/NAD$^+$/PYR. [c] With LADH/NAD$^+$/TFE, the quenching of Trp-314 fluorescence was taken to be the low-temperature value.

the detection limit of the apparatus ($\tau < 10^{-3}$ s, $\phi < 10^{-5}$).

Purkey and Galley (1970) have shown that in a glass the individual emissions of Trp-15 and Trp-314 are spectrally resolved, and what is more, Trp-314, unlike at room temperature (Ross et al., 1981), can be selectively excited. Taking advantage of this favorable circumstance, we have examined the low-temperature emission of the ternary complexes in question and determined both the triplet decay rate and the degree of fluorescence and phosphorescence quenching of Trp-314 (relative to the native protein). The phosphorescence spectra obtained in a 60/40 (v/v) glycerol/phosphate buffer glass (110 K) at 280- and 305-nm excitation wavelengths are shown in Figure 1. From the relative intensities of the 405- and 411-nm O–O vibronic bands (corresponding to Trp-15 and Trp-314, respectively), it is evident that both NADH and NAD$^+$ quench preferentially Trp-314. The extent of its quenching was estimated from the decrease in fluorescence and phosphorescence intensity excited at 305 nm and is given in Table I.

For LADH/NAD$^+$/TFE the residual fluorescence of Trp-314 was found to be 43%. No Trp-like phosphorescence, above the solvent impurity, was observed for this complex, setting an upper limit of its eventual contribution to below 1%. As even large quantities of TFE have no influence on the emission of LADH, we conclude that NAD$^+$ in the active site is an efficient quencher of the triplet state of Trp-314. On the other hand, the unchanged phosphorescence lifetime of Trp-15 shows that the perturbation is limited to the nearest Trp residue. Similar conclusions were drawn from the binary LADH/NAD$^+$ complex, although quantitative estimates were precluded by the ease with which NAD$^+$ is converted to NADH even in absence of ethanol (Ross et al., 1981). The mechanism of singlet- and triplet-state quenching by NAD$^+$ is now under investigation and will be dealt with in a separate work. Preliminary results are indicative of electron transfer from the excited states of indole to the nicotinamide ring.

For LADH/NADH/IBA and LADH/NAD$^+$/PYR, the phosphorescence of Trp-314 is quenched to the same extent of its fluorescence, the residual emission being 0.15 ± 0.01 and 0.66 ± 0.03, respectively. In keeping with unaltered phosphorescence to fluorescence intensity ratio, the phosphorescence lifetime is constant throughout, its value being 6.6 ± 0.05 s. Thus, unlike for LADH/NAD$^+$/TFE, in these complexes there is no perturbation of the triplet state of Trp-314 by coenzyme. This is the case with all other complexes with reduced coenzyme/analogues investigated.

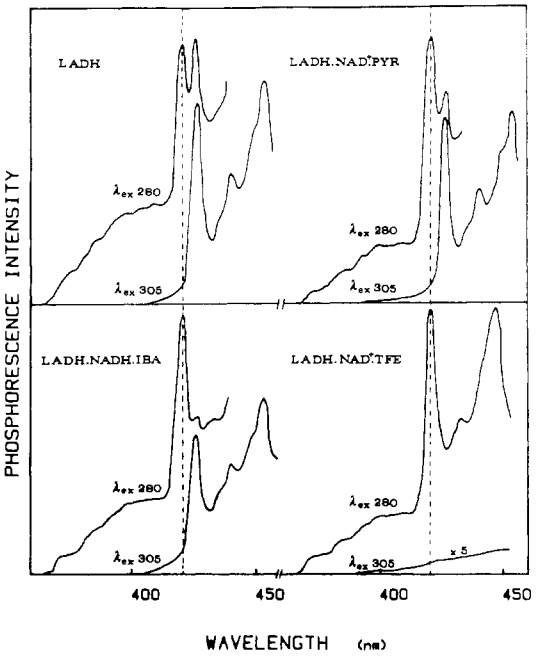


FIGURE 1: Phosphorescence spectra of LADH and the ternary complexes LADH/NADH/IBA, LADH/NAD$^+$/PYR, and LADH/NAD$^+$/TFE in glycerol phosphate buffer (60/40 v/v) at 110 K. The protein concentration was 15 μM.

Reported in Table I is also the decrease in phosphorescence yield of Trp-314 upon complex formation in buffer at room temperature. When the steady-state phosphorescence intensity is normalized by the change in triplet lifetime, we note that Trp-314 is quenched to the same extent as in the glassy state. If one assumes that the efficiency of intersystem crossing is constant across the temperature range, then we must conclude that, under all conditions, quenching of Trp emission by the coenzyme occurs, with the exception of truly LADH/NAD$^+$ complexes, exclusively at the singlet level.

*Phosphorescence Decay Kinetics in Strong Ternary Complexes.* The kinetics of the phosphorescence emission from LADH/NADH/IBA and LADH/NAD$^+$/PYR at 20 °C in buffer are shown in Figure 2. Above a degree of saturation of 0.98 the intensity decays in a strictly exponential fashion. The value of $\tau$, however, decreases with the excess of free coenzyme in solution. With both NADH and NAD$^+$ a linear dependence was found between $1/\tau$ and the concentration of free coenzyme (Figure 3), a behavior that is characteristic of

Table II: Room Temperature Phosphorescence Yield and Lifetime of Trp-314 in Various Binary and Ternary Complexes of LADH[a]

| complex[b] | [inhibitors/substrates] ($\times 10^3$ M) | [coenzyme] ($\times 10^6$ M) | $(P/P_0)(\tau_0/\tau)$ | $\tau$ (s) | $K_D$ ($\times 10^6$ M) | $K_{off}$ ($s^{-1}$) | $\eta^j$ ($\times 10^{-5}$ P) |
|---|---|---|---|---|---|---|---|
| LADH | | | | 0.420 | | | 1.0 |
| LADH/NADH | | 54 | 0.15 ± 01 | 0.385 | 0.5[c] | 5.8[d] | 0.78 |
| LADH/IBA | 100 | | 1.02 ± 0.02 | 0.440 | | | 1.0 |
| LADH/IBA/NADH | 100 | 30.5 | 0.14 ± 0.01 | 0.635 | 0.005[c] | 0.077[c] | 1.8 |
| LADH/DMSO | 10 | | 0.98 ± 0.03 | 0.476 | | | 1.2 |
| LADH/DMSO/NADH | 10 | 32 | 0.20 ± 0.02 | 0.425 | 0.1 | | 0.95 |
| LADH/EtOH | 500 | | 1.15 ± 0.01 | 0.550 | | | 1.45 |
| LADH/EtOH/NADH | 500 | 36 | 0.15 ± 0.01 | 1.015 | 0.1[c] | 1.4[c] | 4.0 |
| LADH/IMID | 10 | | 1.02 ± 0.02 | 0.502 | | | 1.25 |
| LADH/IMID/NADH | 10 | 70 | 0.13 ± 0.01 | 0.225 | 2.2[c] | 22[e] | 0.3 |
| LADH/MPD | 600 | | 1.01 ± 0.01 | 0.520 | | | 1.3 |
| LADH/MPD/NADH | 600 | 70 | 0.14 ± 0.02 | 0.200 | 2.0 | | 0.27 |
| LADH/PYR | 10 | | 0.99 ± 0.02 | 0.550 | | | 1.45 |
| LADH/PYR/$NAD^+$ | 10 | 30.5 | 0.62 ± 0.02 | 0.693 | 0.01[f] | 0.015[f] | 2.1 |
| LADH/ADPR | | 430 | 0.97 ± 0.05 | 0.990 | 21[g] | 310[h] | 3.8 |
| LADH/ADPR/MPD | 600 | 430 | 1.00 ± 0.01 | 0.985 | | | 3.75 |
| LADH/$H_2$NADH | | 180 | 0.76 ± 0.05 | 1.800 | 8[i] | | 12 |
| LADH/$H_2$NADH/MPD | 600 | 180 | 0.62 ± 0.05 | 1.510 | | | 9.2 |
| LADH/acetaldehyde | 10 | | 1.03 ± 0.03 | 0.390 | | | 0.8 |
| LADH/acetaldehyde/$H_2$NADH | 10 | 368 | 0.50 ± 0.05 | 1.900 | 17[i] | | 14 |

[a] Listed together with the thermodynamic and kinetic coenzyme dissociation constant is the effective protein viscosity ($\eta$) associated with the triplet-state lifetime. [b] All studies were performed at 20 °C in 0.1 M phosphate buffer, pH 8.0, with an excitation wavelength of 295 nm. In all complexes the enzyme concentration was 15 μM. [c] From Theorell and McKinley-McKee (1961). [d] From Kvassman and Pettersson (1979). [e] From Cedergren-Zepperzauer (1983). [f] From Anderson and Dahlquist (1982). [g] From Yonetani (1963). [h] From Theorrel and Yonetani (1964). [i] From Dunn et al. (1975). [j] From Strambini and Gonnelli (1985).

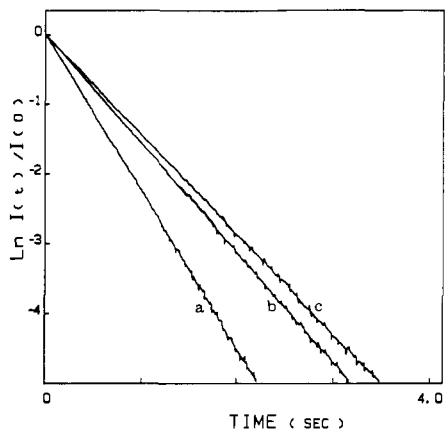


FIGURE 2: Decay of phosphorescence intensity at 20 °C in 0.1 M phosphate buffer, pH 8, of LADH (a) and the ternary complexes LADH/NADH/IBA (b) and LADH/$NAD^+$/PYR (c) ($\lambda_{ex}$ = 295 nm, $\lambda_{em}$ = 440 nm).

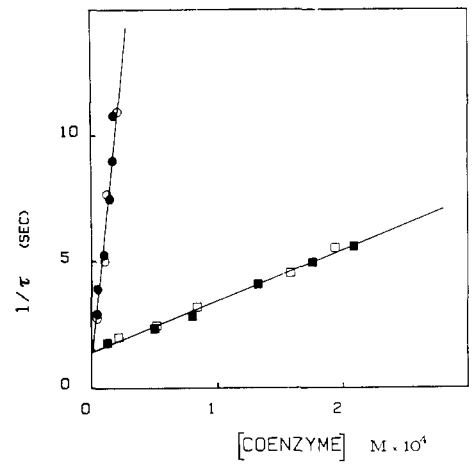


FIGURE 3: Dependence of the phosphorescence lifetime of LADH/NADH/IBA (●, ■) and LADH/$NAD^+$/PYR (○, □) on the concentration of free coenzyme. The protein concentration was 30 μM, and the degree of saturation in coenzyme cross experiment, when the coenzyme bound is different from that added in excess, was 0.98.

a bimolecular dynamic quenching process. The bimolecular rate constants derived from the slopes of the Stern–Volmer plot are (4 ± 1) × $10^5$ and (2.5 ± 0.3) × $10^4$ $M^{-1}$ $s^{-1}$ for $NAD^+$ and NADH, respectively. As evidenced in Figure 3, these rates are the same with both ternary complexes. Bimolecular quenching constants in the range $10^4$–$10^5$ $M^{-1}$ $s^{-1}$ were previously reported for a number of aromatic chromophores (Calhoun et al., 1983) that, in common with NADH, have triplet energy levels suitable for triplet–triplet energy transfer from Trp. With $NAD^+$, the observation that several nonchromophoric oxidizing agents have comparable quenching constants (Gonnelli and Strambini, unpublished data) would suggest again a mechanism of quenching by electron transfer.

In Table II we report the phosphorescence lifetimes of LADH complexes corrected for the external/bimolecular quenching of free NAD(H) according to the above-mentioned rate constants. With LADH/NADH/IBA and LADH/$NAD^+$/PYR the intrinsic lifetimes are 0.64 ± 0.01 and 0.69 ± 0.015 s, respectively, similar between the two complexes but distinct from the 0.42 ± 0.01 s of the apoprotein or its binary complexes with inhibitor pyrazole ($\tau$ = 0.55 s) and IBA ($\tau$ = 0.45 s). From the lengthening of $\tau$ and the dependence of the triplet lifetime on viscosity (Strambini & Gonnelli, 1985), we deduce that the protein in the ternary complexes has a more rigid structure about the triplet probe. Quantitatively, the net effect of coenzyme binding is a 2-fold increase in local viscosity.

*Weaker Binary and Ternary Complexes with NADH and Analogous.* Table II summarizes the results of phosphorescence yields and lifetime measurements on Trp-314 at room temperature in a number of well-characterized binary and ternary complexes with NADH, the analogue 1,4,5,6-tetrahydronicotinamide adenine dinucleotide ($H_2$NADH), and ADPR. Among the NADH complexes with inhibitors (imidazole) and abortive substrates (ethanol and MPD), the phosphorescence yield is roughly the same as for LADH/NADH/IBA and about 14–15% of the apoenzyme. One exception is the complex LADH/NADH/DMSO, for which it is 20%. Once again, from the constancy of the phosphorescence to fluorescence ratio and the triplet lifetime of Trp-314 in the glassy state, we conclude that the room tem-

perature emission is quenched entirely at the fluorescence level. Thus, the greater yield observed with DMSO ought to be interpreted in terms of a less efficient energy transfer between Trp-314 and the nicotinamide ring due to a less favorable distance/orientation.

As implicit in the lifetimes reported in Table II, the phosphorescence decays monoexponentially with every LADH complex examined. The binary complexes with inhibitors/substrates possess a lifetime that is either the same as that of native protein (IBA, TFE, acetaldehyde) or slightly larger (DMSO, MPD, 0.5 M EtOH, IMID). If in the cases of ethanol and MPD the large concentration employed may exert a bulk solvent effect on protein flexibility, and therefore on $\tau$, the results with pyrazole, imidazole, and DMSO are suggestive of structural adjustments in the coenzyme-binding domain following coordination of the inhibitor to the catalytic zinc ion or simply occupation of the substrate channel.

Among NADH complexes the intrinsic lifetime displays a remarkable variability, $\tau$ ranging from 1.03 s with EtOH down to 0.22 s with MPD and IMID. In terms of effective viscosity the variation in $\tau$ represents a change by a factor of 10. In view of the analogous crystallographic structure (closed) for the EtOH, DMSO, and MPD complexes, this difference in dynamical structure comes somewhat unexpectedly. However, since in all these complexes the coenzyme dissociates during the excited-state lifetime, the value of $\tau$ cannot be analyzed simply in terms of the protein configuration in the holoconformer. Indeed, the possibility exists that during the isomerization between apo and holo forms transient configurations of the macromolecule are achieved that enjoy considerable flexibility about the chromophore. In this event the apparent value of $\tau$ will be shorter and will be determined by, among other things, the frequency of exchange. To test this hypothesis we have compared $1/\tau$ with the coenzyme dissociation rate constant ($k_{off}$) within a series of closed complexes. For DMSO and MPD the value of $k_{off}$ is not known. However, since with other complexes $k_{on} = k_{off}/K_D$ is roughly constant (1.0–1.4 $\times 10^6$ $M^{-1}$ $s^{-1}$; references cited in Table II), we may estimate $k_{off}$ from the equilibrium dissociation constant. Using the fluorescence enhancement of bound NADH ($\lambda_{ex}$ = 340 nm) (Theorell & Chance, 1951), we find that in 0.1 M phosphate buffer, pH 8, 20 °C, $K_D$(LADH/MPD/NADH) is (2 ± 0.2) $\times 10^{-6}$ M and $K_D$(LADH/DMSO/NADH) is less than $10^{-7}$ M. Adopting $k_{on} = 1.2 \times 10^6$, we estimate $k_{off}$ to be less than 1.2 and 24 $s^{-1}$ with DMSO and MPD, respectively. Within the complexes investigated then, $k_{off}$ follows the order DMSO (<1.2 $s^{-1}$) ≤ EtOH (1.4 $s^{-1}$) < $H_2O$ (6 $s^{-1}$) < MPD (24 $s^{-1}$). Except for the DMSO complex, this order parallels also that of the decay rate ($1/\tau$). Namely, EtOH (1 $s^{-1}$) < DMSO (2.35 $s^{-1}$) < $H_2O$ (2.6 $s^{-1}$) < MPD (4.5 $s^{-1}$).

The inactive coenzyme analogue $H_2$NADH forms ternary complexes in which LADH adopts the closed structure with the aldehyde substrate DACA and the open structure with MPD (Cedergren-Zeppezauer, 1986). Unfortunately, the chromophoric aldehyde is not suitable for emission work because the phosphorescence is considerably quenched by this substrate. We have replaced DACA with acetaldehyde and shall assume that the conformation of LADH is not affected by the substitution. The drop in phosphorescence yield by complexation with $H_2$NADH is substantially smaller than with NADH, the residual emission being 0.76 in the binary complex and 0.62 and 0.50 in the ternary complexes with MPD and acetaldehyde, respectively. As with NADH, this analogue quenches Trp emission by singlet–singlet radiationless energy transfer, the smaller quenching efficiency relative to NADH arising probably from the reduced emission/absorption overlap integral. Accordingly, the difference in quantum yield among the three complexes studied is indicative of distinct Trp-314/nicotinamide ring separations/orientations. While we have no structural information on the binary complex, X-ray data on the two ternary complexes are consistent with a lower quenching efficiency with MPD. In this case the nicotinamide ring lies at the entrance of the coenzyme channel and is further removed from Trp-314 (Cedergren-Zeppezaur, 1986).

The phosphorescence lifetime of $H_2$NADH complexes shows the most dramatic increases relative to the apoenzyme. In spite of X-ray data that report only minor differences between LADH and LADH/$H_2$NADH/MPD, the change in $\tau$ (from 0.42 to 1.51 s) attests to a remarkable reduction in the flexibility of the coenzyme-binding domain quantifiable as a 10-fold increase in local viscosity. In the presumably closed complex with acetaldehyde we observe an even greater tightening of the structure about the probe ($\tau$ = 1.9 s).

The association of ADPR to LADH has always been considered to have no influence on the native fold of the enzyme. We studied the emission of LADH bound to ADPR both in buffer and, to compare our data with crystallographic studies, in the presence of MPD. In keeping with previous fluorescence work, we find no change in fluorescence and phosphorescence spectra as well as quantum yields. We do observe, however, a remarkable increase in the phosphorescence lifetime ($\tau$ = 0.9 s), the change being exactly the same in buffer as in MPD.

## Discussion

*Quenching of Trp Emission by Bound Coenzyme.* NAD(H) binding to dehydrogenases in general leads to quenching of Trp emission. Since the coenzyme analogue ADPR is uninfluential, it is widely accepted that the process is due to the interaction between nicotinamide moiety and the aromatic residues. In the closed conformer of LADH the nicotinamide ring is 17 and 22 Å from Trp-314 in the same and in the adjacent subunit, respectively (Eklund et al., 1984). As these separations are within the range of singlet–singlet radiationless energy transfer, fluorescence quenching by NADH (and NADH-like adducts such as $NAD^+$–pyrazole) which has a suitable overlap integral is well accounted for by energy migration. The closer proximity of NMN to Trp-314 explains also the preferential quenching of this residue over the distal (27 Å) Trp-15. The mechanism of quenching by $NAD^+$, on the other hand, is still debated, and although several proposals have been advanced in the literature, none is really satisfactory (Subramanian et al., 1981; Eftink & Selvidge, 1982; Vekshin, 1984).

If, on one hand, knowledge of the relative quenching of Trp-15 and Trp-314 by coenzyme may provide useful indications as to the mechanism involved, on the other, dissimilar quenching patterns in LADH complexes formed with the same coenzyme reveal distinct Trp–NMN geometries. In the present investigation advantage is taken of the blue shift that occurs to the absorption spectrum of Trp-15 at low temperature to selectively excite Trp-314 and obtain quantitative quenching data on strong ternary complexes. The results lead to the following conclusions: (1) NADH does not affect the triplet state of Trp-314, and, as reported by Purkey and Galley (1970), quenching occurs exclusively at the fluorescent level. (2) $NAD^+$, in LADH/$NAD^+$/TFE, quenches less effectively the fluorescence of Trp-314 (57% vs 85% of NADH), but unlike the reduced coenzyme it quenches entirely its phosphorescence. Energy migration to $NAD^+$ is not a plausible mechanism. If singlet–singlet energy transfer is precluded by an unfavorable overlap integral (Abdalla et al., 1978; Ross

et al., 1980), triplet–triplet energy transfer is improbable owing to the large separation between chromophores (Strambini & Galley, 1975, 1976). Both singlet and triplet quenching by $NAD^+$ can apparently be accounted for instead by electron transfer from the excited states of Trp to the oxidizing center (Miller et al., 1982; Petrich et al., 1987), the greater efficiency with the triplet state reflecting its long lifetime. The behavior of $NAD^+$ in LADH/$NAD^+$/PYR is anomalous because the fluorescence is quenched to a lesser extent than in LADH/$NAD^+$/TFE and the phosphorescence is unaffected. In this respect the covalent adduct formed between $NAD^+$ and pyrazole (Chen et al., 1987; Eklund et al., 1982) is NADH-like. Indeed, similarly to NADH, quenching of the fluorescence is well accounted for by Förster-type energy transfer (Ross et al., 1981).

At room temperature the phosphorescence is due to Trp-314 alone. The correspondence between the extent of phosphorescence quenching in the glassy state and in fluid solution shows that, even at room temperature, Trp-314 in NADH-like complexes is perturbed at the singlet level only. This finding is useful because from the reduction in phosphorescence yield one has a selective measure of the change in fluorescence yield of Trp-314, a parameter that with weak complexes is not accessible from low-temperature studies. When this information is combined with the overall change in fluorescence yield, one may also assess the extent to which Trp-15 is quenched by bound coenzyme.

The outcome of this analysis with the three strong ternary complexes is given in Table I, and the results are compared to those available in the literature from fluorescence spectral and lifetime resolution. For LADH/$NAD^+$/PYR, the agreement between our data and those of Ross et al. (1981) and Knutson et al. (1982) on the extent of Trp-314 quenching is excellent. On Trp-15 we find a 20% quenching, whereas these authors find practically none. The discrepancy may, in part, be due to the red shift in the spectrum of Trp-15 (Knutson et al., 1982) not corrected for in our analysis. In the complex formed with $NAD^+$ and TFE the residual fluorescence of Trp-314 is 43% as compared to 20% reported by Laws and shore (1978) and 34% reported by Eftink and Selvidge (1982). Again, Trp-15, in agreement with Eftink and Selvidge (1982), is only marginally affected, retaining about 80% of its emission. The relative fluorescence yield with LADH/NADH/IBA, determined for the first time on an NADH complex at room temperature, reveals the strongest quenching of Trp-314 (86%). Contrary to the conclusion reached by Purkey and Galley (1970) at low temperature, and in keeping with less efficient energy migration over large distances, the emission from Trp-15 is quenched by at most 36%.

Among the series of weaker NADH complexes listed in Table II we note that at room temperature the phosphorescence yield is roughly 14% constant throughout. The exception is LADH/NADH/DMSO, for which it is 20%. If a fixed Trp-314–NMN geometry is a requisite for a constant quenching efficiency, then the larger yield with DMSO points out that the mutual arrangement between chromophores in this complex is different from the others and less favorable for energy transfer. This finding contrasts with the unaltered geometrical arrangement observed in the crystallographic structures of these complexes (Eklund et al., 1981, 1984). On the other hand, a different orientation between Trp-314 and NMN, not evidenced in the quenching pattern, is instead contemplated for the complex with imidazole (Cedergren-Zeppezaur, 1983) where the protein in the crystal maintains the open structure. Only a comparison of quenching parameters between solution and crystalline states might resolve whether the discrepancies pointed out here are due to the stabilization of a different average holoenzyme conformation by crystal lattice forces.

*Phosphorescence Lifetimes and Flexibility of the Coenzyme-Binding Domain.* In the crystallographic closed configuration of LADH the nicotinamide ring is totally buried near the catalytic Zn ion, the carboxamide apparently H-bonded to Val-292 ($\beta$E), Ala-317 ($\beta$F), and Phe-319 in the central region of the $\beta$-sheet. The adenine moiety binds next to $\beta$A- and $\beta$D-strands of this sheet in a pocket that is largely hydrophobic but with the possibility to H-bond to polar groups Arg-223 and Arg-271 (Eklund et al., 1984). The nicotinamide moiety is believed to be indispensable for the open/closed transition to occur, an isomerization of protein structure that most likely represents the rate-limiting step in catalysis (Hardman, 1981; Plapp et al., 1986).

Trp-314 is buried within the 12-strand $\beta$-sheet (F-strand) that overlaps the two subunits of the protein and constitutes the coenzyme-binding domain. Any conformational change in this domain that alters the existing H-bonding pattern (increasing or decreasing the number and strength of H-bonds) or any extension of this highly connected bonding network by ligands is bound to have important consequences on the dynamical structure of the region embedding the triplet probe.

Both coordination of the substrate (inhibitor) to catalytic Zn and closure of the coenzyme channel lead to the expulsion of water molecules from the active site and to a more intimate contact between catalytic and coenzyme-binding domains. On this ground one might anticipate a large increase in internal viscosity associated with the closed holoconformer of LADH. The lengthening of the triplet lifetime in strong ternary complexes does indeed confirm a 2-fold increase in local viscosity about the chromophore. This tendency, however, is not generalized. There are closed conformers (such as in LADH/NADH/MPD) in which the environment of Trp-314 is even more flexible than in the apoenzyme and, conversely, open configurations of LADH (as in LADH/$H_2$NADH/MPD), which are far more rigid than strong ternary complexes. Thus, in solution every complex is characterized by dynamical features of its own, and a classification in terms of the crystallographic open and closed structures, on the basis of the flexibility of the coenzyme-binding domain, is not possible. While we find no unifying element to rationalize the flexibility changes in LADH complexes, some general remarks can nevertheless be made:

(a) Binary complex formation with substrates and inhibitors has at best a modest effect. When changes do occur, as with MPD, pyrazole, imidazole, DMSO, and EtOH, they always indicate an increased rigidity. Hence, coordination to Zn and/or the extrusion of water molecules from the substrate crevice would seem to increase the connectedness between the core region of LADH and the rest of the subunit. To our knowledge there is no evidence that these ligands induce changes in the native fold of the enzyme. However, as they do exert important cooperative effects on coenzyme binding, it is not unreasonable that they should influence protein structure.

(b) Crystallographic closed complexes are in general more rigid, although the evidence for an additional tightening of the structure subsequent to closure of the coenzyme-binding cleft is not compelling. The only open complexes studied are LADH/NADH/IMID and LADH/$H_2$NADH/MPD, and both are the most flexible of their respective series.

(c) Within the series of closed and labile NADH complexes the weaker ones appear also to be more flexible. Since the rate of coenzyme release in these complexes is larger than the phosphorescence decay rate, the possibility arises, as mentioned under Results, that during the isomerization between open and closed conformers transient flexible configuration may be visited by the macromolecule. The triplet lifetime will then be reduced by the ongoing exchange process, and a correlation should exist between $\tau$ and the dissociation rate constant. The situation envisaged here is somewhat analogous to the protein quakes observed by Ansari et al. (1985) upon CO binding to myoglobin. Except for LADH/NADH/DMSO, for which the distinct NMN–Trp-314 geometry suggests also an unusual binding configuration, $1/\tau$ was found indeed to increase with $k_{off}$.

(d) The effect of ADPR binding on LADH is most remarkable in that the considerable loss in protein mobility was not predictable on the basis of available crystallographic and spectroscopic evidence. Although ADPR forms strong bonds with the protein (Chen et al., 1987), perturbations of the native fold have never been reported. It is widely accepted that protein isomerization is triggered by the nicotinamide moiety and that the rest of the coenzyme serves merely the role of site recognition (Theorel et al., 1967; Shore, 1969; Eklund et al., 1984). Given the large perturbation induced by ADPR, one may speculate that besides target recognition this fragment of the coenzyme might play also an important structural role. It is possible, for example, that the distortion of the native fold following ADPR binding poses the macromolecule for the insertion of the nicotinamide ring. Subsequent interactions of the latter within the active-site pocket will then trigger protein isomerization, a readjustment of protein structure that partly releases the stress imposed by ADPR. In this perspective, the puckering of the nicotinamide ring in $H_2$NADH might not allow its perfect accommodation in the binding site, and although closure can be achieved in the aldehyde complex, the coenzyme-binding domain does not relax, as implied by the large value of $\tau$, to the normal configuration. In LADH/$H_2$NADH/MPD the protein remains in the open conformation, with the nicotinamide ring outside the binding pocket, and, indeed, the lifetime of this complex is not much dissimilar from that with ADPR. Whatever the actual role of ADPR is, the perturbed state of LADH revealed by the phosphorescence probe represents in all likelihood a transient conformation of the macromolecule in the pathway of coenzyme binding and release.

In summary, the dynamical features reported for LADH in various binary and ternary complexes emphasize that the classical open and closed conformers are at best two gross macrostates of the protein and that, as suggested by spectroscopic, kinetic, thermodynamic, and recent crystallographic data, the panorama in solution comprises an assortment of configurations, each peculiar of a given ligand combination. The experimental approach adopted here based on a natural probe reporting on the flexibility of a *limited region* of the macromolecule, while providing no details of the molecular architecture, represents perhaps the ultimate in sensitivity toward changes in intramolecular bonding patterns. In this respect its main usefulness will be in pointing out the detail to which structural data must reach to provide a satisfactory global understanding of structure–function relationships.

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# Room Temperature Phosphorescence of Trp-314 as a Monitor of Subunit Communications in Alcohol Dehydrogenase from Horse Liver

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ABSTRACT: The phosphorescence properties of liver alcohol dehydrogenase from horse were characterized at limiting concentrations of coenzyme and coenzyme analogues. The emission decay kinetics of Trp-314 in strong, slowly exchanging, ternary complexes with NADH/isobutyramide, NAD/pyrazole, and NADH/dimethyl sulfoxide displays a markedly nonexponential character. The analysis of decay components over the saturation curve reveals that the phosphorescence from singly bound protein molecules has a lifetime from 1 to 1.3 s, which is 2–3 times larger than observed with fully bound and unliganded enzyme. The remarkably tighter configuration reported by the triplet probe for the coenzyme-binding domain in half-saturated macromolecules is not exclusive of strongly inhibited ternary complexes. Measurements on binary complexes with NADH, ADPR, and the inactive coenzyme analogue 1,4,5,6-tetrahydronicotinamide adenine dinucleotide confirm that binding of the ligand to one subunit has qualitatively the same influence on protein structure. If the lifetime of Trp-314 provides clear evidence for an appreciable change in conformation at half-binding that is apparently triggered by the ADPR fragment of the coenzyme, such communication between subunits does not lead to allosteric phenomena in coenzyme binding.

Equine liver alcohol dehydrogenase (LADH)[1] is one of the best-characterized dimeric enzymes. In spite of a considerable number of equilibrium and rapid kinetic studies, some aspects of the catalytic mechanism are not fully elucidated.

One of the most controversial points has concerned the existence or degree of ligand-mediated subunit interactions during catalysis. Although in a number of laboratories no evidence was found for allosteric phenomena (Shore, 1969; Tatemoto, 1975; Hadorn et al., 1975), numerous studies have implied half-of-the-sites reactivity (Bernard et al., 1970; Dunn & Bernard, 1971; McFarland & Bernard, 1972; Luisi & Favilla, 1972; Luisi & Bignetti, 1974; Baici & Luisi, 1977; McFarland et al., 1977) or subunit interactions (Lindman et al., 1972; Dunn et al., 1979; Koerber & Dunn, 1981).

Most of the data purporting to support the existence of subunit interactions stem from the initial observation of Bernard et al. (1970), who noted that the reaction with various aromatic aldehydes above pH 8 and under single-turnover conditions is associated to biphasic absorbance changes. A subsequent theoretical analysis presented by Petterson (1976) challenged this interpretation and showed that biphasic kinetics can be predicted for a two-substrate reaction proceeding by an ordered ternary complex mechanism. Later experimental work (Kvassman & Petterson, 1976; Andersson & Petterson, 1982) lent further support to this view, and investigations designed to characterize one subunit of the dimer pointed out that biphasic transient kinetics appear to be a property of a single site (Kordal & Parsons, 1979; Andersson & Mosbach, 1979; Anderson & Dahlquist, 1982a). The matter is not settled, however. As more details of the possible steps involved

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[1] Abbreviations: LADH, liver alcohol dehydrogenase from horse; NADH, reduced nicotinamide adenine dinucleotide; $NAD^+$, oxidized nicotinamide adenine dinucleotide; DMSO, dimethyl sulfoxide; IBA, isobutyramide; ADPR, adenosine(5′)diphospho(5)-β-D-ribose; PYR, pyrazole; $H_2$NADH, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide; Trp, tryptophan.

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