Strambini, G. B. (1983) *Biophys. J. 43*, 127–130.
Strambini, G. B., & Galley, W. C. (1975) *J. Chem. Phys. 63*, 3467–3472.
Strambini, G. B., & Galley, W. C. (1976) *Nature 261*, 521–522.
Strambini, G. B., & Gonnelli, M. (1985) *Chem. Phys. Lett. 115*, 196–201.
Strambini, G. B., & Gonnelli, M. (1986) *Biochemistry 25*, 2471–2476.
Strambini, G. B., & Gabellieri, E. (1987) *Biochemistry 26*, 6527–6530.
Strambini, G. B., & Gonnelli, M. (1988) *J. Phys. Chem. 92*, 2850–2853.
Subramanian, S., Ross, J. B. A., Ross, P. D., & Brand, L. (1981) *Biochemistry 20*, 4086–4093.
Theorell, H., & Chance, B. (1951) *Acta Chem. Scand. 5*, 1127–1141.
Theorell, H., & Mckinley-Mckee, J. (1961) *Acta Chem. Scand. 15*, 1811–1865.
Theorell, H., & Yonetani, T. (1963) *Biochem. Z. 338*, 537–553.
Theorell, H., & Yonetani, T. (1964) *Arch. Biochem. Biophys. 106*, 252–258.
Theorell, H., Ehrenberg, A., & deZalenski, C. (1967) *Biochem. Biophys. Res. Commun. 27*, 309–315.
Vekshin, N. L. (1984) *Eur. J. Biochem. 143*, 69–72.
Yonetani, T. (1963) *Acta Chem. Scand. 17*, S96–S101.
Zeppezauer, M. (1986) in *Zinc enzymes* (Bertini, I., Luchinat, C., Maret, W., & Zeppezauer, M., Eds.) pp 416–434, Birkhauser Verlag, Basel.

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

Giovanni B. Strambini* and Margherita Gonnelli
*CNR, Istituto di Biofisica, Via S. Lorenzo 26, 56127 Pisa, Italy*
William C. Galley
*Department of Chemistry, McGill University, Montreal, Quebec, Canada*
*Received March 10, 1989; Revised Manuscript Received June 15, 1989*

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

* To whom correspondence should be addressed.

[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.

0006-2960/90/0429-203$02.50/0 

in the catalytic pathway become available, questions are raised on the validity of the compulsory order model. Moreover, a recent analysis of NADH binding equilibria in terms of this model and of independent subunits fails to account for the experimental data (Bignetti, 1984).

In the preceding paper of this issue the triplet lifetime of Trp-314 was shown to be a sensitive monitor of changes in protein structure that result from binding of the coenzyme to LADH. The aromatic residue is buried in the subunit contact area near the twofold symmetry axis relating the two monomers (Eklund et al., 1976). The possibility that this intrinsic chromophore might report on potential communications between the subunits prompted a detailed study of the room temperature phosphorescence of LADH at partial coenzyme saturation. The results indicate that when the ligand is bound to only one subunit of the macromolecule, the latter attains a remarkably more inflexible structure in the region of the subunit interface. If this finding unveils ligand-induced subunit interactions in LADH, the fraction of singly occupied macromolecules at partial saturation suggests that the interaction does not lead to cooperative phenomena with regard to coenzyme binding.

## Materials and Methods

LADH in a crystalline suspension and highest purity grade NADH and $NAD^+$ were obtained from Boehringer (Mannheim). DMSO and IBA were from Aldrich (Steinheim). ADPR and pyrazole were from Sigma Chemical Co. (St. Louis, MO). Prior to use, IBA and pyrazole were twice recrystallized from water.

The synthesis of 1,4,5,6-tetrahydronicotinamide adenine dinucleotide ($H_2NADH$), the preparation of protein samples for fluorescence and phosphorescence measurements, and the apparatus employed were all described in the preceding paper of this issue. The degree of saturation by the coenzyme was checked by the quenching of the protein fluorescence ($\lambda_{ex}$ = 295 nm; $\lambda_{em}$ = 323 nm) for those complexes involving NADH (Theorell & Tatemoto, 1971) and by the absorbance at 300 nm for the complexes with $NAD^+$ and pyrazole (Theorell & Yonetani, 1963). All experiments were conducted at 20 °C.

*Analysis of the Phosphorescence Decay from a Multicomponent System.* In the study of LADH complexes at various degrees of saturation we are faced with the simultaneous presence of two or more conformational states of the protein that may be characterized by distinct tryptophan phosphorescence lifetimes. Interpretation of emission kinetic data can also be complicated by the possibility that during the excited triplet state the macromolecule interconverts between its free and complexed forms. For a two-state model the mathematical formulation that takes into account this process is given below. When an excited chromophore (in our case a triplet state) interconverts between states A and B identified with emission rate constants $k_a$ and $k_b$, respectively

$$\xleftarrow{k_a} A \underset{k_{ba}}{\overset{k_{ab}}{\rightleftharpoons}} B \xrightarrow{k_b} \quad (1)$$

the differential equations describing the time evolution of the concentration of A and B after pulsed excitation are

$$dA/dt = -(k_{ab} + k_a)A + k_{ba}B \quad (2)$$

$$dB/dt = k_{ab}A - (k_{ba} + k_b)B$$

The solution is

$$A(t) = \alpha_{1a}e^{-k_1 t} + \alpha_{2a}e^{-k_2 t} \quad (3)$$

$$B(t) = \alpha_{1b}e^{-k_1 t} + \alpha_{2b}e^{-k_2 t}$$

where

$$k_1, k_2 = \{(k_a + k_{ab}) + (k_b + k_{ba}) \pm \{[(k_a + k_{ab}) - (k_b + k_{ba})]^2 + 4k_{ab}k_{ba}\}^{1/2}\}/2$$

$$\alpha_{1a} = \{A_0(k_a + k_{ab} - k_2) - k_{ba}B_0\}/(k_1 - k_2)$$

$$\alpha_{1b} = \{B_0(k_b + k_{ba} - k_2) - k_{ab}A_0\}/(k_1 - k_2)$$

$$\alpha_{2a} = \{k_{ba}B_0 - A_0(k_a + k_{ab} - k_1)\}/(k_1 - k_2)$$

$$\alpha_{2b} = \{k_{ab}A_0 - B_0(k_b + k_{ba} - k_1)\}/(k_1 - k_2)$$

$A_0$ and $B_0$ are the concentration of A and B at time zero. If the emission spectrum is the same in the two states of the chromophore, then the total emission intensity $I$ decays in time as

$$I_t = A_t + B_t = (\alpha_{1a} + \alpha_{1b})e^{-k_1 t} + (\alpha_{2a} + a_{2b})e^{-k_2 t} \quad (4)$$

i.e., the emission is represented by the sum of two exponential functions. However, when interconversion is rapid, that is, $k_{ab}, k_{ba} > k_a, k_b$, the intensity decays in a monoexponential fashion and the fast exchange limit of the decay rate, $k_f$, is given by

$$k_f = k_a f_a + k_b(1 - f_a) \quad (5)$$

$f_a$ represents the fraction of molecules in state A. For rapid interconversion among $n$ states the general expression is

$$k_f = k_1 f_1 + k_2 f_2 + \ldots + k_n f_n \quad (6)$$

In terms of excited-states lifetimes, $\tau$, the equation becomes

$$\frac{1}{\tau} = f_1/\tau_1 + f_2/\tau_2 + \ldots + f_n/\tau_n \quad (7)$$

This expression is employed to interpret phosphorescence lifetime data at partial saturation with binary complexes with NADH, $H_2NADH$, and ADPR. The dissociation rate constants of these ligands is in general larger than the triplet decay rate. Because the phosphorescence intensity from these samples decays invariably in a monoexponential fashion, we conclude that within the precision of these experiments the magnitude of the dissociation rate constants always satisfies the fast exchange limit.

## Results

*Phosphorescence Decay Kinetics of LADH at Partial Degrees of NADH Saturation in the Ternary Complex with IBA.* In the presence of IBA the reduced coenzyme binds very tightly to LADH and the ligand exchanges with the aqueous phase very slowly ($k_{off}$ = 0.077 $s^{-1}$). In the phosphorescence time scale (~1 s) no interconversion takes place between free and bound states of the protein, and the emission properties of LADH at various degrees of saturation, $Y$, are represented by the sum of individual contributions from unliganded, singly bound, and doubly bound macromolecules.

Coenzyme binding quenches both fluorescence (~78%) and phosphorescence (~86%) intensities, a process accompanied by a small red shift in the fluorescence spectrum but no change in the phosphorescence spectrum. The decay of the phosphorescence intensity at partial degrees of saturation, unlike for unliganded ($E_0$) and doubly bound enzyme molecules ($E_2$), does not follow an exponential law. A long-lived component with a triplet-state lifetime ($\tau_L$) of ~1.3 s, distinct from 0.42 and 0.63 s observed with $E_0$ and $E_2$, respectively, becomes the dominant contribution to the overall intensity at values of $Y$ between 0.6 and 0.9. With limiting amounts of NADH there are three macromolecular species ($E_0$, $E_1$, and $E_2$) and in principle four different protein environments for Trp-314 ($E_{01}$ and $E_{10}$ for the chromophore in the empty and filled subunit, respectively, of $E_1$) that could all display distinct triplet decay

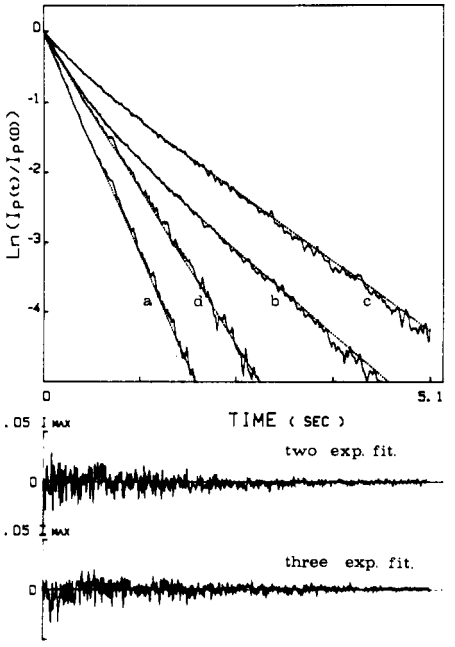


FIGURE 1: Decay of the phosphorescence intensity from LADH (a) at 20 °C in 0.1 M phosphate buffer, pH 8, in the presence of 0.1 M IBA and from its ternary complex with NADH at partial degrees of saturation: 30% (b); 60% (c); 99% (d). The plot of residuals obtained by fitting the decay to two and three exponential components is shown for sample c. The enzyme concentration was 15 $\mu$M.

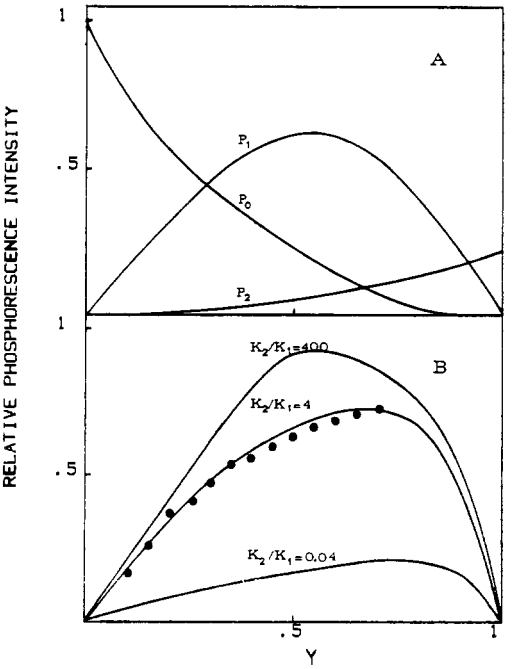


FIGURE 2: (A) Contribution to the total steady-state phosphorescence intensity by unliganded ($P_0$), single-bound ($P_1$), and double-bound ($P_2$) LADH molecules as a function of the degree of saturation with NADH in the ternary complex LADH/NADH/IBA. Intensities were calculated by using relative quantum yields $\phi_1 = 0.42$ and $\phi_2 = 0.14$ and the assumption that binding occurs to independent binding sites. (B) Simulation of $P_1/(P_0 + P_1 + P_2)$ for independent binding sites ($K_2/K_1 = 4$), for positive ($K_2/K_1 = 0.04$) and negative cooperativity ($K_2/K_1 = 400$), where $K_2$ and $K_1$ are the microscopic dissociation constants for the coenzyme in the E/NADH/IBA complex. The experimental points (●) are shown for $Y \leq 0.7$.

kinetics. The decays obtained at various $Y$ were fitted to sums of two and three exponential functions. A sample of such fitting is given in Figure 1. Since there is no improvement on the goodness of the fit, $\chi^2$, with three exponentials as opposed to two, it appears that in general the phosphorescence from two Trp-314 environments is either too weak (as shown in Figure 2, $E_2$ is strongly quenched and will contribute little to the total emission for $Y < 0.7$) or too similar in lifetime to other components to be singled out. At the present signal to noise ratio and precision of decay measurements, a double-exponential decay governed by lifetimes that differed by a factor of 2 would be distinguished from a monoexponential one if the weak component contributed at least 5% to the intensity.

We fitted all decays to a double-exponential function, and the lifetime of the two components together with their steady-state preexponential terms is reported in Table I. Inspection of these data shows that the values of $\tau_s$ and $\tau_L$ are not constant across the saturation range. A sizable variation is noted for $Y$ between 0 and 0.4, a range in which the emission is due almost exclusively to $E_0$ and $E_1$. Within this interval $\tau_s$ decreases slightly from 0.42 s for coenzyme-free LADH to ~0.35 s, whereas $\tau_L$ increases from 1 to 1.3 s. Beyond 90% saturation the nonexponential nature of the decay is less pronounced, and the data from different samples were found also to be less reproducible. If from the rise and fall of preexponential terms there is no ambiguity in assigning $\tau_L$ and $\tau_s$ to molecular species $E_1$ and $E_0$, respectively, differences between $\tau_0$ and $\tau_s$ as well as the variation in $\tau_L$ are not contemplated in a static picture and point therefore to the occurrence of bimolecular quenching processes.

Quenching events may be represented by encounters between protein molecules in various states of ligation as well as with the coenzyme or coenzyme-associated impurities. Extensive purification of NADH by chromatographic techniques (Gurr et al., 1972) has had no effect. However, experiments at much higher protein concentrations (up to 10 times) showed an even greater variation in $\tau_s$ and $\tau_L$ and consistently smaller values for the triplet lifetimes over the entire $Y$ range. Our preliminary interpretation of these data is that $\tau_s$ is affected mainly by the concentration of $E_1$ and $\tau_L$ by that of $E_0$ in a process that may involve direct transfer of coenzyme between two macromolecules (Srivastava & Bernard, 1987). At large values of $Y$ a reduction in $\tau_L$ is

Table I: Room Temperature Phosphorescence Lifetimes and Preexponential Terms Obtained for the LADH/NADH/IBA Complex at Partial Degrees of NADH Saturation by Fitting the Decay to Two Exponential Components[a]

| $Y$[b] | $[NADH]_0$ ($10^{-6}$ M) | $\tau_s$ (s) | $\tau_L$ (s) | $\alpha_L$ | $\chi^2$ |
|---|---|---|---|---|---|
| 0 | | 0.42 | | | 1.6 |
| 0.1 | 3.0 | 0.40 | 0.95 | 0.17 | 2.0 |
| 0.15 | 4.5 | 0.38 | 0.97 | 0.27 | 2.1 |
| 0.20 | 6.0 | 0.35 | 1.02 | 0.39 | 2.6 |
| 0.25 | 7.5 | 0.39 | 1.03 | 0.42 | 2.0 |
| 0.30 | 9.0 | 0.37 | 1.07 | 0.48 | 2.0 |
| 0.35 | 10.5 | 0.36 | 1.13 | 0.54 | 2.5 |
| 0.40 | 12.0 | 0.38 | 1.14 | 0.55 | 2.8 |
| 0.45 | 13.5 | 0.36 | 1.20 | 0.59 | 2.7 |
| 0.50 | 15.0 | 0.37 | 1.23 | 0.65 | 3.0 |
| 0.55 | 16.5 | 0.35 | 1.23 | 0.67 | 2.5 |
| 0.60 | 18.0 | 0.36 | 1.23 | 0.70 | 3.6 |
| 0.65 | 19.5 | 0.37 | 1.28 | 0.72 | 2.8 |
| 0.70 | 21.0 | 0.33 | 1.30 | 0.74 | 5.9 |
| 0.80 | 24 | 0.26 | 1.22 | 0.78 | 6.8 |
| 0.90 | 27 | 0.29 | 1.03 | 0.86 | 10.6 |
| 0.95 | 28.6 | | 0.63 | | |

[a] These data are the averages of three independent experiments. All studies were performed at 20 °C in 0.1 M phosphate buffer, pH 8, in the presence of 0.1 M IBA at an enzyme concentration of 15 $\mu$M. [b] $Y$ was calculated by assuming a dissociation constant of $5 \times 10^{-9}$ M equal for both sites (Theorell & McKinley-McKee, 1961).

probably due to the quenching of $E_1$ by excess free coenzyme in solution (Strambini & Gonnelli, 1990). Ideally, to minimize bimolecular quenching by proteins, one should employ very low protein concentrations. However, room temperature phosphorescence is typically a weak emission, and when this is coupled to the large quenching induced by the coenzyme, we find that concentrations much lower than those used in these experiments are not practical in terms of signal to noise ratios (or photodecomposition).

If these quenching reactions introduce a degree of uncertainty in the intrinsic value of $\tau_1$ ($\tau_1 \geq \tau_L \sim 1.3$ s), it is nevertheless possible to determine with accuracy the contribution of this component to the overall phosphorescence intensity. The fortunate circumstance that $E_1$ can be distinguished from $E_0$ and $E_2$ by virtue of its long lifetime allows us to make a reasonable and direct estimate of the fraction of macromolecules in the $E_1$ state as a function of $Y$ and derive from it the microscopic binding affinities to a subunit in $E_0$ and in $E_1$.

The total steady-state phosphorescence intensity $P(Y)$ can be expressed in the form

$$P(Y) = P_0(Y) + P_1(Y) + P_2(Y)$$
$$\propto = f_0(Y)\tau_0 + f_1(Y)\phi_1\tau_1 + f_2(Y)\phi_2\tau_2$$

where $\phi_i$ is the phosphorescence quantum yield relative to uncomplexed LADH and $f_i$ the fraction of protein molecules in the $i$th state of ligation. In this formulation we have assumed a constant absorptivity of Trp-314 upon complexation and also that

$$\tau_{01} = \tau_{10} = \tau_1$$

As stated above the fact that for $Y < 0.5$ the decay is well fitted by the sum of two exponentials leaves us with three possibilities for $\tau_{10}$: $\tau_{10} \approx \tau_0$, $\tau_{10} \approx \tau_{01}$, and $\tau_{10} \ll \tau_0$. Because Trp-314 is at the interface between subunits and the two indole rings are only 6 Å removed from each other (Eklund et al., 1976), it is plausible that the greater rigidity of the protein structure (embodied in $\tau_1 > \tau_0$) is felt by both chromophores. Alternatively, if $\tau_{10} \ll \tau_0$, the contribution from the filled subunit to the steady-state intensity is marginal and may be neglected. If $\tau_{10} \approx \tau_0$, the error induced in the value of $\phi_1$ is not large because as will be shown below the quantum yield of Trp-314 in the filled subunit is much smaller than that in the empty subunit (i.e., $\phi_{01} \geq 4\phi_{10}$) due to preferential singlet energy transfer to nearby NADH.

Evaluation of $f_1(Y)$ requires knowledge of $\phi_1$. This can be obtained from $P_1(Y)/P(Y)$ at low degrees of saturation ($Y \leq 0.2$) for which $f_1$ is, except for large positive cooperativity, practically independent of the type of binding (numerous binding studies reported in the literature rule out this possibility). Fitting $P_1(Y)/P(Y)$ over the 0–0.2 range of the saturation curve, assuming independent binding sites, we find $\phi_1 = 0.42$. Such estimate is in fair agreement with the extent of fluorescence quenching by the first coenzyme molecule. From the fluorescence parameters reported in Table I of the preceding paper and since the reduction in phosphorescence quantum yield is due exclusvely to quenching of the precursor fluorescence state, $\phi_1 = 0.42$ yields a fluorescence quenching of 64.5% ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 323$ nm) relative to 70% estimated by direct titration (Theorell & Tatemoto, 1971).

Finally, from $\phi_1$ and $\phi_2$ the contribution to the phosphorescence emission by the filled and empty subunit of $E_1$ can now be appraised. From the relationships

$$\phi_1 = (\phi_{01} + \phi_{10})/2 = \{k_0/(k_0 + k_2) + k_0/(k_0 + k_1)\}/2 = 0.42$$

$$\phi_2 = k_0/(k_0 + k_1 + k_2) = 0.14$$

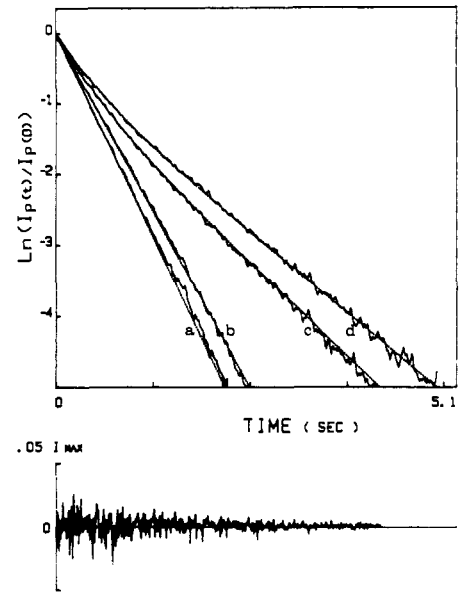


FIGURE 3: Decay of the phosphorescence intensity from the binary complexes LADH/DMSO (a) and LADH/PYR (b) and from the ternary complexes LADH/NADH/DMSO (c) and LADH/$NAD^+$/PYR (d) at partial coenzyme saturation ($Y = 0.4$). Experimental conditions are as in Figure 1 in addition to 10 mM DMSO and 10 mM PYR. The plot of residuals obtained by fitting the decay to two exponential components is shown for sample c.

where $k_0 = 2.6 \times 10^8$ $s^{-1}$ is the singlet decay rate constant of Trp-314 in LADH (Ross et al., 1981), $k_1$ is the rate constant for singlet energy transfer from Trp-314 to NADH in the same subunit, and $k_2$ is the rate constant for singlet energy transfer from Trp-314 to NADH in the adjacent subunit, one finds $k_1 = 1.5 \times 10^9$ $s^{-1}$, $k_2 = 1.15 \times 10^8$ $s^{-1}$, $\phi_{10} = 0.15$, and $\phi_{01} = 0.69$. Thus, assuming $\tau_{10} = \tau_{01}$, we deduce that over 80% of the phosphorescence in $E_1$ originates from Trp-314 in the empty subunit. The alternatives in which $\tau_{10} = \tau_0$ or $\tau_{10} \ll \tau_0$ yield a somewhat larger value for $\phi_{01}$.

The contribution to the overall phosphorescence intensity by $E_0$, $E_1$, and $E_2$ together with $P_1(Y)/P(Y)$ derived for independent binding sites by using $\phi_1 = 0.42$ and $\phi_2 = 0.14$ is shown in Figure 2. Displayed on the same figure is also the experimentally observed fraction of long-lived phosphorescence intensity over a range of $Y$ from 0.1 to 0.7 for which decay data are well fitted and quenching by free coenzyme may be neglected. The line that best fits the experimental points is close to that predicted for independent binding sites. Considering the accuracy of emission data and the assumption involved in deriving $\phi_1$, the small magnitude of positive cooperativity indicated by the best fit ($K_2/K_1 = 1.6$ as opposed to $K_2/K_1 = 4$) is hardly significant.

*Partial Coenzyme Saturation of LADH in Other Strong Ternary Complexes.* To ascertain whether the singular conformation of LADH in the $E_1$ state is exclusive of the LADH/NADH/IBA complex, two other strong complexes, namely, LADH/NADH/DMSO and LADH/$NAD^+$/PYR, were examined at limiting coenzyme concentrations. A sample of their triplet decay kinetics at $Y = 0.4$ is given in Figure 3.

As for LADH/NADH/IBA, we note a markedly nonexponential decay. The contribution of the long-lived component to the total intensity rises over the initial part of the saturation curve, reaching a maximum around $Y = 0.6$–$0.8$. The lifetime, $\tau_L$, derived from a two-component fitting of the decay, is 1.16 s, constant throughout, for the NAD complex and 0.95 s for LADH/NADH/DMSO. The latter decreases by 16–20% at the lowest degrees of saturation. Thus, in spite of some differences in the value of $\tau_L$ among the three complexes exam-

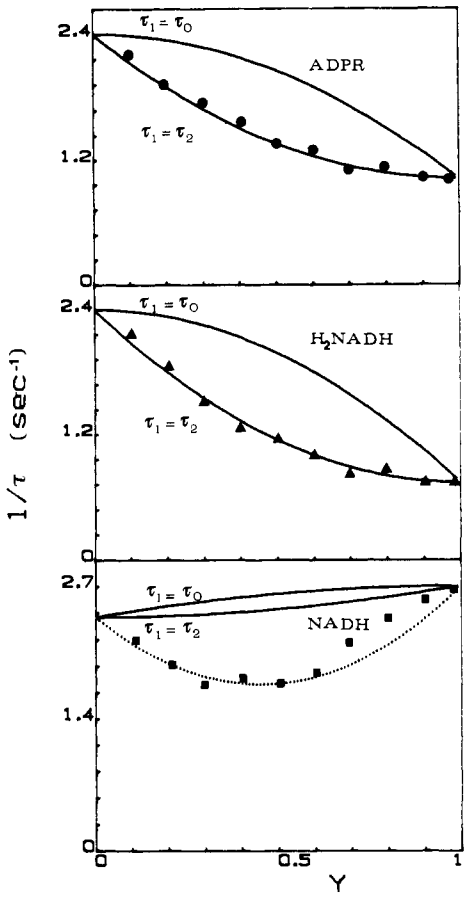


FIGURE 4: Phosphorescence lifetime of LADH as a function of the degree of saturation with NADH and the coenzyme analogues ADPR and $H_2$NADH. Full lines represent simulations of $\tau$ obtained from eq 7 by assuming independent binding sites and $\tau_1 = \tau_0$ or $\tau_1 = \tau_2$. $Y$ was evaluated by using the following dissociation constants: $K_{NADH} = 0.5 \times 10^{-6}$ M (Theorell & McKinley-McKee, 1961); $K_{ADPR} = 21 \times 10^{-6}$ M (Yonetani, 1963); $K_{H_2NADH} = 8 \times 10^{-6}$ M (Dunn et al., 1975). Other experimental conditions are as in Figure 1.

ined, a long lifetime for Trp-314 in the coenzyme-free subunit of $E_1$ is a feature common to all.

An analysis of the total emission in terms of individual components are not carried out with these complexes because the larger phosphorescence yields of $E_2$ ($\phi_2 = 0.20$ and 0.62 for the DMSO and pyrazole complexes, respectively) would involve a greater degree of approximation. By simple inspection of the decay curves, however, it is evident that even for these complexes, extreme forms of cooperativity are ruled out.

*Binary Complexes with Reduced Coenzyme and Analogues.* Phosphorescence lifetimes as a function of $Y$ were measured for the binary complexes LADH/NADH, LADH/$H_2$NADH, and LADH/ADPR. For each ligand and at any degree of saturation the phosphorescence emission decays as a monoexponential function. The value of $\tau(Y)$ derived from the slope of semilog plots is shown in Figure 4. As mentioned under Materials and Methods, these ligands dissociate rapidly from the protein in a phosphorescence time scale, and when the fast exchange limit applies, $\tau(Y)$ is governed by eq 7. Application of eq 7 requires knowledge of coenzyme-binding affinities for $E_0$ and $E_1$. Following the many reports in the literature and also the data with LADH/NADH/IBA, we have assumed throughout that binding occurs to independent subunits.

In Figure 4 together with the experimental points we report simultations of $\tau(Y)$ obtained by letting $\tau_1 = \tau_0$ and $\tau_1 = \tau_2$, namely, for the case in which the half-saturated species has a lifetime identical with either free or doubly bound LADH. Experimental data with NADH clearly depart from either simulation and confirm that at partial saturation there is an additional emission component with $\tau_1 > \tau_0, \tau_2$. Further, it is apparent that eq 7 fails to fit adequately the data at all $Y$ even if a small correction in $\tau$ (less than 12%) was applied to account for external quenching by free NADH (Strambini & Gonnelli, 1990). As pointed out with saturated rapidly exchanging NADH complexes, we suspect that protein isomerization induced by coenzyme binding/release will be a partially quenching event. Were this interpretation correct, then $\tau_0$, $\tau_1$, and $\tau_2$ would not be independent of $Y$. Because the effect will be greatest at large degrees of saturation, we have chosen to fit the experimental data with eq 7 only for the first part of the saturation curve. The best-fit line drawn in Figure 4 corresponds to a value of $\tau_1 = 1.15$ s. If with NADH the information is not sufficient to derive an accurate value for $\tau_1$, we may still conclude that the $E_1$ species, as in ternary complexes, does possess a lifetime quite distinct from $\tau_0$ and $\tau_2$ (the intrinsic value of the latter being also somewhat uncertain under exchange conditions).

For $H_2$NADH and ADPR the experimental values of $\tau(Y)$ are shown in Figure 4 to fall surprisingly well on the curve generated by eq 7 with $\tau_1 = \tau_2$. If the assumption of random binding holds for these coenzyme analogues, the implication of these data is that as far as Trp-314 is concerned all the changes in protein structure induced by the ligands are achieved with the binding to a single subunit of the dimer. Little or no additional perturbation is induced by the second incoming ligand in the adjacent subunit.

## Discussion

Trp-314 is part of the F-strand in the 12-strand $\beta$-sheet forming both coenzyme-binding domains. Its backbone O and N atoms are H-bonded to N and O, respectively, of Trp-314 in the adjacent subunit. The indole ring forms part of the monomer–monomer interface, making contacts with Met-303 and Leu-308 of the opposite subunit (Eklund et al., 1976). This arrangement places the phosphorescence chromophore roughly at the center of the dimeric molecule in a position that may be most suitable for monitoring the transmission of conformational information between the two subunits.

Crystallographic structures of the complexes between LADH and coenzyme show that relative to the apoenzyme large atomic displacements occur mostly in the catalytic domain. In the coenzyme-binding domain the conformational transition is characterized by a large shift in the position of the loop connecting the E- and F-strands and by a sliding of coenzyme-binding domains along each other in a direction opposite to the movement of catalytic domains (Eklund et al., 1984). In the preceding paper we have shown that such structural rearrangements induce considerable changes in flexibility of the polypeptide in the $\beta$-sheet embedding the triplet probe. On the basis of the phosphorescence lifetime the data of binary and ternary complexes at partial degrees of coenzyme saturation provide a first important evidence for the existence of a distinct conformation of LADH when bound to a single coenzyme molecule ($E_1$). The $E_1$ molecular species is characterized by a long phosphorescence lifetime, ranging from 1 to 1.3 s, which, relative to the apoprotein $\tau_0 = 0.42$ s and the ternary complexes with NAD/PYR and NADH/IBA ($\tau_2 \sim 0.65$ s), is 2–3-fold larger. From the relationship between triplet lifetime and microviscosity (Strambini & Gonnelli, 1985) the change in $\tau$ represents a tightening of the protein fold about Trp-314 corresponding to an increase from 2.5 to 6.5 times in local effective viscosity.

A general question with multimeric enzymes is the possible role that the assembly of two or more identical subunits might play in the regulation of catalytic function. As pointed out in the introduction, extensive investigations on both the kinetics and thermodynamics of coenzyme binding have not led to a general consensus regarding allosteric effects with LADH, even if extreme forms of positive and negative cooperativity are now mostly ruled out. If the phosphorescence properties of $E_1$ on one hand imply that the ligand in one subunit affects the structure at the interface, and given the strong connectivity of the $\beta$-sheet, the perturbation presumely extends over large parts of the adjacent coenzyme-binding domain; on the other hand, the concentration of $E_1$ in LADH/NADH/IBA at partial saturation argues for a practically identical affinity for the coenzyme by $E_0$ and $E_1$. Similarly, binding to independent subunits can account also for the distribution of molecular species in binary complexes. Thus, if the emission from Trp-314 emphasizes the occurrence of subunit–subunit interactions, yielding a distinct conformation for $E_1$, it also indicates, in agreement with numerous studies from other laboratories (Anderson et al., 1982; Anderson & Dahlquist, 1982b), that these do not lead to important allosteric effects.

Details on the structure of half-saturated LADH may be difficult to gather by experimental methods. $\tau_1$ reveals a tighter configuration of the polypeptide in the subunit contact region but of course gives no information on the underlying structural changes. At present we can only surmise scenarios that are compatible with the spectroscopic evidence given above as well as the binding and catalytic competence of $E_1$. In terms of gross crystallographic open/closed conformations, one may speculate that (1) as hydride transfer would be inefficient in an aqueous environment, the closed conformation, in which water is expelled from the active site, would be required of the active/coenzyme-bound subunit and that (2) unaltered affinity for the coenzyme by the empty subunit together with unchanged rates of coenzyme exchange with the solvent (in ternary complexes; Anderson & Dahlquist, 1982a; Anderson et al., 1982) presumes an open conformation for the latter. Thus, the $E_1$ species needs to be a conformationally hybrid state of the enzyme. The similarity of $\tau_1$ with the lifetime of complexes with coenzyme analogues ADPR and $H_2$NADH, ligands that form open complexes, suggests that the rigid configuration at the interface, rather than reflecting asymmetry-induced strain in the $\beta$-sheet, represents a perturbed open conformation of the subunit(s). The finding that a single ADPR-like molecule per dimer is sufficient to elicit the change carries the analogy with the $E_1$ state a step closer. In summary, a conformationally hybrid dimer appears a plausible structure for half-saturated LADH in that the large value of $\tau_1$ is consistent with the simultaneous presence of an open subunit and an ADPR fragment bound to the $\beta$-sheet.

## Acknowledgments

We are grateful to L. Favati and A. Puntoni for their excellent technical assistance and to C. Neri and to M. A. Baldocchi for typing the manuscript.

**Registry No.** LADH, 9031-72-5; Trp, 73-22-3.

## References

Anderson, D. C., & Dahlquist, F. W. (1982a) *Biochemistry 21*, 3569–3578.

Anderson, D. C., & Dahlquist, F. W. (1982b) *Biochemistry 21*, 3578–3584.

Anderson, D. C., Wilson, M. L., & Dahlquist, F. W. (1982) *Biochemistry 21*, 4664–4670.

Andersson, L., & Mosbach, K. (1979) *Eur. J. Biochem. 94*, 565–570.

Andersson, P., & Pettersson, G. (1982) *Eur. J. Biochem. 122*, 559–568.

Baici, A., & Luisi, P. L. (1977) *J. Mol. Biol. 114*, 267–279.

Bernard, S. A., Dunn, M. F., Luisi, P. L., & Shack, P. (1970) *Biochemistry 9*, 185–192.

Bignetti, E. (1984) *Physiol. Chem. Phys. Med. NMR 16*, 21–27.

Dunn, M. F., & Bernard, S. A. (1971) *Biochemistry 10*, 4569–4575.

Dunn, M. F., Biellman, J. F., & Branlant, G. (1975) *Biochemistry 14*, 3176–3182.

Dunn, M. F., Bernard, S. A., Anderson, D., Copeland, A., Morris, R. G., & Roque, J. P. (1979) *Biochemistry 18*, 2346–2354.

Eklund, H., Nordstrom, B., Zeppezauer, E., Soderlund, G., Ohlsson, I., Boiwe, T., Soderberg, B.-O., Tapia, O., Bränden, C.-I., & Akenson, A. (1976) *J. Mol. Biol. 102*, 27–59.

Eklund, H., Samama, J. P., & Jones, T. A. (1984) *Biochemistry 23*, 5982–5996.

Gurr, P. A., Bronskill, P. M., Hanes, C. S., & Wong, J. T. (1972) *Can. J. Biochem. 50*, 1376–1384.

Hadorn, M., John, V. A., Meier, F. K., & Dutler, H. (1975) *Eur. J. Biochem. 54*, 65–73.

Koerberg, S. C., & Dunn, M. F. (1981) *Biochimie 163*, 97–102.

Kordal, R. J., & Parsons, S. M. (1979) *Arch. Biochem. Biophys. 194*, 439–443.

Kvassman, J., & Petterson, G. (1976) *Eur. J. Biochem. 69*, 279–287.

Lindman, B., Zeppezaner, M., & Akeson, A. (1972) *Biochim. Biophys. Acta 259*, 173–177.

Luisi, P. L., & Favilla, R. (1972) *Biochemistry 11*, 1486–1493.

Luisi, P. L., & Bignetti, E. (1974) *J. Mol. Biol. 88*, 653–670.

McFarland, J. T., & Bernard, S. A. (1972) *Biochemistry 11*, 1486–1493.

McFarland, J. T., Chen, J., Wnuk, M., De Traglia, M. C., Li, A. Y., Peterson, R., Jacobs, J. W., Schmidt, J. L., Feinberg, B., & Watters, K. L. (1977) *J. Mol. Biol. 115*, 355–369.

Pettersson, G. (1976) *Eur. J. Biochem. 69*, 273–278.

Ross, J. B. A., Schmidt, C. J., & Brand, L. (1981) *Biochemistry 20*, 4369–4377.

Shore, J. D. (1969) *Biochemistry 8*, 1588–1590.

Srivastava, D. K., & Bernard, S. A. (1987) *Annu. Rev. Biophys. Chem. 16*, 175–204.

Strambini, G. B., & Gonnelli, M. (1985) *Chem. Phys. Lett. 115*, 196–201.

Strambini, G. B., & Gonnelli, M. (1990) *Biochemistry* (preceding paper in this issue).

Tatemoto, K. (1975) *Arch. Biochem. Biophys. 166*, 16–24.

Theorell, H., & McKinley-McKee, J. (1961) *Acta Chem. Scand. 15*, 1811–1865.

Theorell, H., & Yonetani, T. (1963) *Biochem. Z. 338*, 537–553.

Theorell, H., & Tatemoto, K. (1971) *Arch. Biochem. Biophys. 142*, 69–82.

Yonetani, T. (1963) *Acta Chem. Scand. 17*, S96–S101.