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Room Temperature Phosphorescence and the Dynamic Aspects of Protein Structure

(conformational fluctuations/liver alcohol dehydrogenase)

MARIA L. SAVIOTTI AND WILLIAM C. GALLEY

Department of Chemistry, McGill University, Montreal, Quebec

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ABSTRACT **While the phosphorescence of aromatic chromophores in solution is normally quenched through diffusion of dissolved oxygen and other solvent-mediated processes, the phosphorescence of some proteins in solution is observed at room temperature. The tryptophan phosphorescence arises from residues which are hindered from interaction with oxygen by the folding of the polypeptide chains. Measurements of the phosphorescence lifetime of horse liver alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) as a function of oxygen concentration indicate that internal tryptophan residues are periodically exposed to oxygen. This permits the calculation of rate constants for conformational oscillations in the enzyme. The present article illustrates the feasibility of employing phosphorescence in the study of proteins in solution in general and the utility of such experiments in probing the dynamic aspects of protein structure.**

The properties of the triplet state of aromatic chromophores may be profitably exploited in the study of biomolecular structure and dynamics. Phosphorescence measurements have been used in biochemical systems both to detect the proximity of aromatic groups (1–3) and to monitor long-range interactions (4, 5) in small molecule-biopolymer complexes. In addition tryptophan and tyrosine (6), and in favorable cases individual tryptophan residues (7), can be resolved in protein phosphorescence spectra.

Despite the molecular information which can be derived from triplet-state spectroscopy, phosphorescence has been employed much less frequently than fluorescence as a biochemical tool. This undoubtedly arises from the fact that phosphorescence measurements have been made on biochemical samples in rigid glasses usually formed from glycerol–H_2O or ethylene glycol–H_2O mixtures at liquid nitrogen temperatures. While there is evidence to indicate that biopolymers retain their native structures under these conditions, the inability to directly correlate biological activity with phosphorescence measurements has contributed to its infrequent use.

Rigid media have been employed due to the normally efficient quenching of the triplet state in fluid solution by dissolved oxygen and other solvent-quenching processes. In that aromatic amino acids residues are frequently buried within the globular structure of proteins, it was anticipated that their triplet states would be less susceptible to quenching, the degree of protection afforded a particular aromatic residue being a function of the location of the residue within the protein structure and the flexibility of the protein conformation.

Abbreviation: LADH, liver alcohol dehydrogenase.

Our observations of protein phosphorescence with increasing temperature reveal that the folding of the polypeptide chains in protein molecules does hinder quenching of the triplet states of internal tryptophan residues. Phosphorescence from proteins is observed in fluid solution at temperatures where it cannot be observed from free chromophores, and in the present article preliminary data on the room temperature phosphorescence of horse liver alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) and *Escherichia coli* alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) are presented. The oxygen dependence of the phosphorescence lifetimes observed at room temperature provides a measure of the kinetics of conformational flexibility in proteins. The triplet lifetimes are also found to be sensitive to the binding of coenzymes and the presence of protein stabilizing and destabilizing agents.

We feel the present study not only indicates the usefulness of phosphorescence in probing the dynamic aspects of protein structure, but suggests the feasibility of employing triplet state experiments in general in the study of proteins in solution.

MATERIALS AND METHODS

Horse liver alcohol dehydrogenase (LADH) was obtained from Worthington Biochemical Co., as the 1 × crystallized lyophilized product (lot no. 33E650 in most experiments), and *E. coli* alkaline phosphatase was obtained as an ammonium sulfate suspension from Sigma Chemical Co. NADH was a Calbiochem product, ultra-pure guanidine·HCl was from Schwarz/Mann and urea from the Fisher Chemical Co. Ethylene glycol and glycerol were chromatoquality from Matheson, Coleman, and Bell. Buffers used in the preparation of enzyme solutions were made with double distilled water.

In typical experiments, an enzyme concentration of 10^{-4} M was employed. Experiments with NADH involved the addition of 2-fold excess of coenzyme to assure complete formation of the binary complex.

In order to prepare enzyme solutions at a given concentration of dissolved oxygen, 0.5 ml of buffer was equilibrated with gas mixtures of known composition at 1 atm by bubbling the gas through the buffer for 30 min at a rate of 200 $cm^3 \cdot min^{-1}$. The oxygen content of the gas mixture was controlled by mixing the proper ratios of gases from tanks of known composition of oxygen and nitrogen (Union Carbide Canada Ltd.) in a soap bubble flow meter. The concentration of oxygen in the solution was calculated from the Henry's Law constant for oxygen in water at 25°C (8). After equilibration, the tube containing the buffer was isolated from the gas flow

and the environment. The buffer was poured into a side arm containing a weighed amount of enzyme. The enzyme solution was transferred to a side arm which consisted of a quartz tube (5 mm outside diameter) which served as the emission cell.

Phosphorescence emission spectra were recorded with an apparatus described previously (7). For phosphorescence measurements at room temperature, the intensity was enhanced by removing the excitation monochromator and exciting with the full arc. Phosphorescence lifetimes were recorded with an X-Y recorder for lifetimes longer than 0.1 sec, by photographing the decay of individual phosphoroscope pulses displayed on a Tektronix 546 oscilloscope or, averaging the decay of 10–30 pulses with a C-1024 Varian Time Averaging Computer.

RESULTS AND DISCUSSION

1. Phosphorescence Spectra and Solvent Relaxation. The initial influence of increasing temperature on the phosphorescence spectrum of LADH is depicted in Fig. 1. As has been shown previously (7), the tryptophan phosphorescence spectrum of LADH arises from the independent emission of two tryptophans; the components with peaks at 405 and 410 nm having been assigned as emission from a solvent-exposed residue and an internal residue, respectively. One buried and one exposed tryptophan residue in each of the monomer units of LADH has been observed in the x-ray structure of the enzyme (C. -I. Brändén, personal communication). With decreasing viscosity accompanying the increase in temperature, the tryptophan phosphorescence peak at 405-nm shifts to the red between 100°K and 130°K in 1:1 ethylene glycol–H_2O. At the higher temperature it is hidden beneath the 410-nm peak and can no longer be recognized as a separate component. The temperature range over which the red shift occurs is similar to that observed for indole or tryptophans in small peptides. A red shift in the fluorescence spectrum of tryptophan residues exposed to polar solvents is well known (9). It arises from solvent reorganization about the excited singlet state dipole moment (10). A corresponding, albeit

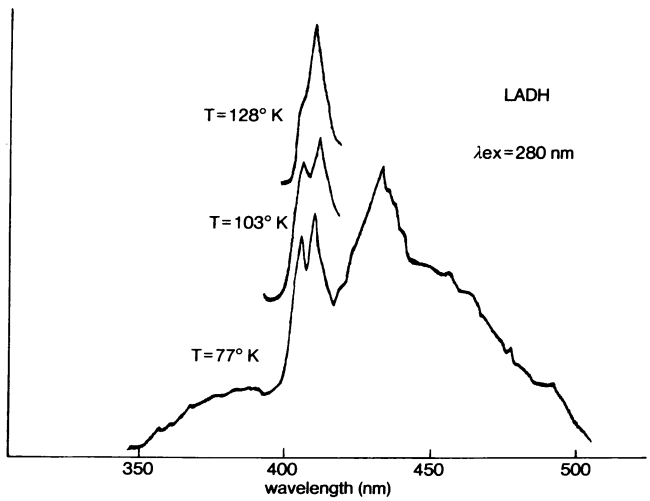

FIG. 1. Phosphorescence spectra of LADH in a 1:1 ethylene glycol-buffer glass as a function of temperature. The spectra illustrate the solvent relaxation red shift of the 405-nm peak as the temperature is increased. λex signifies the exciting wavelength. (The ordinate here and in Fig. 2 represents *intensity* in arbitrary units.)

usually smaller, shift in phosphorescence spectra has been observed in our laboratory for a number of aromatic chromophores (11). The shift in the phosphorescence spectra occurs at low temperatures where the viscosity is such that the relaxation time of the solvent is comparable to the triplet lifetime of the chromophores.

The position of the tryptophan phosphorescence maximum at 410 nm in LADH and 415 nm in alkaline phosphatase remains essentially unaltered with increasing temperature. This is consistent with the conclusion that emission maxima in this wavelength region arise from tryptophan residues buried within the polarizable core of the protein (7).

2. Oxygen Quenching of External Residues. At temperatures of the order of 20°C above their respective glass transition temperatures, the ethylene glycol–H_2O and glycerol–H_2O solvent mixtures become sufficiently fluid so that oxygen diffusion in the solution begins to influence triplet state lifetimes. At ambient oxygen concentrations, the lifetime of the triplet state of indole or tryptophan methyl ester markedly decreases and the phosphorescence is quenched at temperatures higher than 183°K with 1:1 ethylene glycol–H_2O. The phosphorescence lifetimes in this temperature region are a function of the oxygen concentration of the solvent.

At temperatures above 173°K (3:2 glycerol–H_2O) with air-saturated solutions, phosphorescence from the external tryptophan residue in LADH is quenched. While at these temperatures due to solvent relaxation, the external residue cannot be distinquished in the phosphorescence spectrum of LADH; quenching is evidenced by the development of a short-lived component in the phosphorescence decay*. With excitation at the red end of the enzyme absorption band, only the internal residue is excited (7). When excitation occurs at 305 nm, the short-lived component in the phosphorescence decay is absent indicating that it arises from the external residue. The short-lived component can no longer be observed in the emission at all exciting wavelengths as the temperature is increased further.

The tyrosine contribution to the phosphorescence spectrum of LADH, which can be observed free of tryptophan at wavelengths below 400 nm at 77°K (compare Fig. 1), is quenched over the same temperature region as the external tryptophan. The quenching of these residues with increasing temperature leaves the internal tryptophan residue in each of the monomer units of LADH as the only phosphorescing center in the molecule. The observation that the tyrosine residues responsible for the phosphorescence are selectively quenched presumably because of their location at the surface of the protein, has also been reported by McGlynn and McCarville (12) who observed the phosphorescence of bovine serum albumin between 20 and 153°K.

3. Room Temperature Protein Phosphorescence. In contrast with the behavior observed with indole or tryptophan methyl ester, the phosphorescence from internal tryptophan residues in proteins is much less affected by increasing temperature or decreasing solvent viscosity. The tryptophan phosphorescence of proteins can be detected at much higher temperatures

* Both the temperature at which external tryptophan residues undergo solvent relaxation and quenching by oxygen appear to depend on the effect of the local environment on solvent structure (J. G. Milton and W. C. Galley, unpublished data).

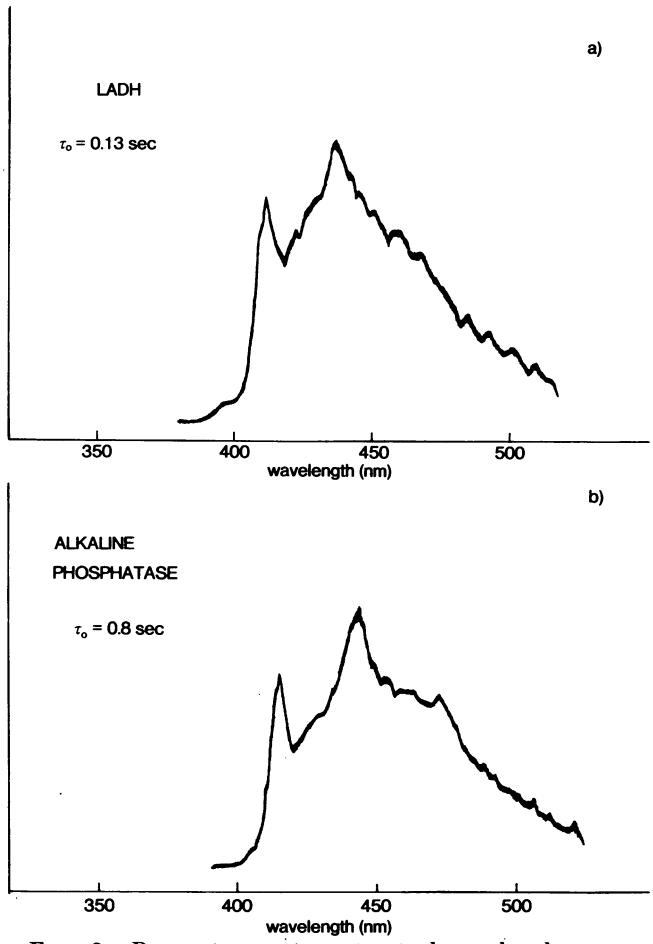

FIG. 2. Room temperature tryptophan phosphorescence spectra of proteins. (a) 10^{-4} M horse liver alcohol dehydrogenase (LADH) in 0.032 M pyrophosphate buffer, pH 8.6; (b) *E. coli* alkaline phosphatase in 0.05 M phosphate buffer, pH 7.5. The oxygen concentration = 3.9×10^{-9} M.

and with some proteins is readily observed at room temperature. The phosphorescence spectra of LADH and *E. coli* alkaline phosphatase in buffer in the absence of glass-forming additives and at room temperature are illustrated in Fig. 2a and 2b. The folding of the polypeptide chains with these two proteins creates an environment for one or more tryptophans in the protein which hinders quenching. As a result, the phosphorescence of a chromophore in a complex structure is observed under conditions where it is normally impossible to observe the phosphorescence of simple molecules.

We have not been successful in observing room temperature phosphorescence from several proteins we have looked at in spite of the fact that they contain tryptophan residues which are buried within the globular protein structure. The phosphorescence of α-chymotrypsin, bovine serum albumin, and bovine carbonic anhydrase was not observed under our present conditions of oxygen removal in ethylene glycol–H_2O mixtures at temperatures above $-30°C$. The solvent viscosity at this point, however, is still many orders of magnitude lower than it is at the temperature at which free tryptophan or indole are quenched by oxygen diffusion. Phosphorescence experiments on proteins in fluid solution, albeit at low temperatures, should also prove to be of interest particularly in view of the recent studies of Douzou and his coworkers on the slow rates of enzymatic activity under these conditions (13).

4. The Dependence of Room Temperature Protein Phosphorescence Lifetimes on Oxygen Concentration. The lifetimes of the room temperature tryptophan phosphorescence of LADH and alkaline phosphatase depicted in Fig. 2 are 0.13 and 0.8 sec, respectively, as compared with 5.5 sec for both proteins in glycerol– or ethylene glycol–H_2O at 77°K. It is not possible to compare the room temperature lifetimes with known oxygen-independent triplet state lifetimes under the same conditions in that data for such comparisons do not exist. While triplet state lifetimes for aromatic hydrocarbons in rigid plastics are only reduced by factors of less than two in going from 77°K to 298°K (14), the triplet states of aromatic residues in proteins may be influenced by environmental fluctuations not present in rigid matrices. The dependence of protein-triplet lifetime on the concentration of dissolved oxygen in the solvent was therefore investigated.

The lifetimes of the tryptophan phosphorescence of both LADH and alkaline phosphatase at room temperature are dependent on the concentration of oxygen in the buffer.

(*i*) *Conformational Fluctuations and O_2-Dependent Triplet Lifetimes.* The phosphorescence lifetime of LADH in pyrophosphate buffer at pH 8.6 tends to a limiting value of 0.13 sec in the absence of oxygen and decreases with increasing O_2 concentration. The triplet lifetime does not go to zero at high oxygen concentration but approaches a limiting finite value. If proteins possessed completely rigid conformations, the triplet lifetimes of buried tryptophan residues would be independent of the oxygen concentration in the solvent. Triplet states of aromatic chromophores in solution are quenched by oxygen at rates which are proportional to the frequency of collisions between the aromatic molecule and the quencher (15), so that tryptophans unavailable to oxygen would not be quenched. While the protein conformation markedly hinders quenching by dissolved oxygen, fluctuations must occur in the structure which periodically expose internal tryptophan residues to perturbation by oxygen.

Two limiting dynamic models may be envisaged which would permit the interaction of oxygen with internal tryptophan residues in proteins. In the first model, small independent structural fluctuations occur in the protein. The motions of amino-acid side chains need only generate sufficient free volume to permit an oxygen molecule to "diffuse" by small random steps to the internal chromophores.

In the second model the structural oscillations tend to occur in a more cooperative fashion. Interaction between an internal tryptophan and oxygen can only occur subsequent to a structural change involving the "simultaneous" rearrangement of a number of intermolecular interactions.

Data on the oxygen dependence of the tryptophan phosphorescence lifetime of LADH are consistent with a model of the latter type in which oscillations occur between two conformational states of the protein. The amplitude of the motion is such that in the more stable state, the tryptophan residues are unavailable to oxygen while in the less stable "open" conformation quenching by oxygen readily occurs. We assume at this point that quenching in this latter state takes place at the rate which would be observed for the free chromophore in solution. This can be represented in a kinetic scheme of the following type:

$$\begin{array}{ccccc} & & k_{op} & & k_q \\ \mathrm{Trp}_{cl} & \rightleftarrows & \mathrm{Trp}_{op} & + \mathrm{O}_2 & \rightarrow \text{quenching} \\ \downarrow & & k_c & \downarrow & \\ 1/\tau_0 & & & 1/\tau_0 & \end{array}$$

in which Trp_{cl} and Trp_{op} represent the triplet states of the tryptophans in the closed and open forms of the protein; k_{op} and k_{cl}, the opening and closing rate constants, τ_0 the tryptophan lifetime, and k_q, the oxygen quenching rate constant for free tryptophan. In that the closed conformation of the protein is the stable one ($k_{cl} \gg k_{op}$), phosphorescence is observed only for tryptophan in the closed form. The rate constant for phosphorescence decay under conditions of steady state illumination is given by:

$$\frac{1}{\tau} = \frac{1}{\tau_0} + k_{op}\left(1 - \frac{k_{cl}}{k_{cl} + k_q[O_2]}\right) \qquad [1]$$

The contribution of the oxygen dependence to the lifetime can be expressed in the form:

$$\tau_{O_2} = \frac{1}{\frac{1}{\tau} - \frac{1}{\tau_0}} = \frac{1}{k_{op}} + \frac{k_{cl}}{k_{op} \cdot k_q} \cdot \frac{1}{[O_2]} \qquad [2]$$

Values of τ_{O_2} are computed from the measured lifetimes and values for τ_0, the oxygen-independent lifetime, are determined at low oxygen concentrations. Eq. [2] predicts that τ_{O_2} should be a linear function of the reciprocal of the oxygen concentration in the solution. As the O_2 concentration goes to infinity, the lifetime is determined by k_{op}, the rate constant for the transition from the "closed" to the "open" conformation. The oxygen dependence is governed by an "effective" quenching constant, $k_q \cdot k_{op}/k_{cl}$, which is the quenching constant for a free chromophore modified by k_{op}/k_{cl}, the ratio of the closing to opening rate constant for the conformational isomerization. A significant feature of the model is that at high oxygen concentration, the triplet lifetime of the internal residue tends to a finite value. Quenching under these conditions is rate-limited by the structural change which exposes the chromophore to oxygen. In a diffusional type quenching, the lifetime of the chromophore would go to zero at infinite O_2 concentration.

A plot of τ_{O_2}, obtained from the tryptophan triplet lifetime of LADH in pyrophosphate buffer at 298°K, as a function of the reciprocal of the oxygen concentration appears in Fig. 3. A linear relationship is observed and the lifetime extrapolates to a finite value as the oxygen concentration goes to infinity. The intercept at infinite O_2 concentration results in a value of $k_{op} = 12\ sec^{-1}$, and the slope leads to an effective quenching constant of $6.5 \times 10^4\ l\ mole^{-1}\ sec^{-1}$. The oxygen triplet quenching constant for tryptophan in water at room temperature has not been determined to date. Employing an average value of $10^9\ l\ mole^{-1}\ sec^{-1}$ [obtained from the data of Gijzeman, Kaufman, and Porter (15) who have recently determined quenching constants for aromatic chromophores in solution at room temperature] results in a k_{cl} of $1.8 \times 10^5\ sec^{-1}$. The ratio of k_{op}/k_{cl} is 6.7×10^{-5} indicating that the equilibrium lies far in the direction of the closed conformation, so that the protein exists for only a small fraction of the time in the more open structure. The calculation of k_{cl} is based on the assumption that the tryptophan in the more "open" conformation is quenched at the rate observed for aromatic chromophores in solution. Quenching in the conformational state in which the tryptophan can interact with oxygen will depend on its extent of exposure in this state. Quenching at the diffusion-controlled rate represents an upper limit and thus the calculated value for k_{cl} must be regarded as a maximum.

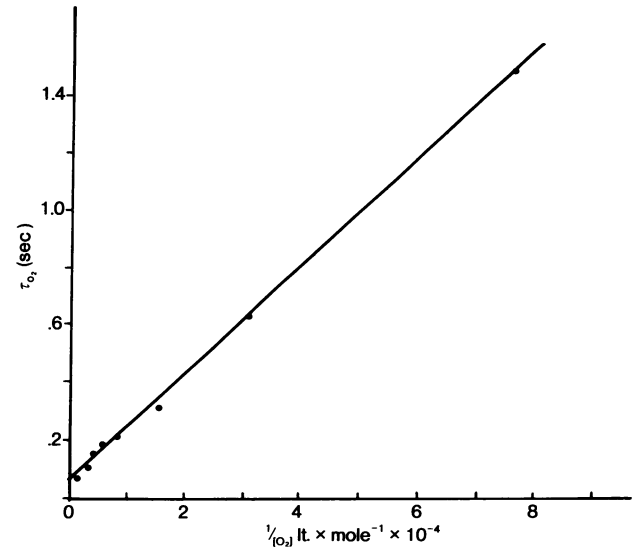

FIG. 3. The contribution of dissolved oxygen to the room-temperature phosphorescence lifetime of LADH at pH 8.6 as a function of [oxygen]. $\tau_{O_2}^{-1} \cdot \tau_0 = (1/\tau - 1/\tau_0)^{-1}$. In terms of the proposed model, the finite lifetime observed as $[O_2] \rightarrow \infty$ provides a measure of k_{op} and the slope $= k_{cl}/k_{op} \cdot k_q$.

The general conclusion from our data that conformational fluctuations must occur in proteins in solution is consistent with recent interpretations of tritium exchange data (16), and with the conclusions of Lakowicz and Weber (17) who observed the influence of high concentrations of dissolved oxygen on the fluorescence of aromatic residues in proteins. In their studies, Lakowicz and Weber found that quenching of even internal tryptophan residues in a number of proteins occurred with a quenching constant close to the diffusion-controlled one. Furthermore, the fluorescence lifetimes in their experiments tend toward zero with increasing oxygen concentration. The oxygen dependence of the triplet lifetime for LADH in the present work results in an effective oxygen quenching constant which is four orders of magnitude less than the triplet quenching rate constants of $10^9 - 3 \times 10^9\ l\ mole^{-1}\ sec^{-1}$ observed for aromatic molecules in solution (15), and the lifetimes do not extrapolate to zero. Comparison between our data with LADH and alkaline phosphatase and the fluorescence results of Lakowicz and Weber for a number of other proteins, suggests that globular proteins differ markedly in the rate at which they undergo conformational fluctuations. In order to clearly resolve this question, experiments in which fluorescence and phosphorescence quenching by oxygen are measured in the same proteins will be needed since singlet and triplet quenching appear to occur through different mechanisms and with different rate constants (15).

We do not as yet have sufficient data to attempt to correlate the rate constants for conformational oscillations in LADH observed in these studies with enzyme activity. It is of interest that isomerization of the enzyme-coenzyme complex has been proposed (18) to occur in LADH with a rate constant ($11\ sec^{-1}$) essentially equal to the opening rate constant in our studies. More detailed studies in the presence of coenzyme and substrate will be required to ascertain whether the structural motions monitored by this technique are involved in the mechanism of enzyme catalysis.

TABLE 1. *Phosphorescence lifetimes of LADH under various experimental conditions*

Enzyme concentration = 10^{-4} M, $[O_2] < 10^{-6}$ M (Lot no 33 E 650) T = 25°C	
Experimental conditions	Phosphorescence lifetimes (msec)
pH 8.6*	134
pH 7.5, 0.05 M phosphate buffer	90
NADH:enzyme (3:1), pH 8.6	70
6 M Urea, pH 8.6	97
1 M Guanidine·HCl, pH 8.6	60
Ethylene glycol:buffer (1:1)	260
Glycerol:H_2O (3:2)	240

* Unless otherwise stated the buffer used in the experiments was 0.032 M pyrophosphate buffer at pH = 8.6

While the present data indicate that a periodic loosening of the protein structure must occur in the region of the internal tryptophan in LADH, they do not establish the amplitude of the oscillations nor the extent to which these are delocalized over the protein. Triplet quenchers that have a variety of molecular diameters may help to resolve the former question, but the charge, chemical nature, and hydration must also be considered in accessing their effectiveness as quenchers.

Phosphorescence studies of the dynamic aspects of the structure of LADH will become more meaningful when examined in the light of the x-ray structure of the enzyme in the crystalline form (19, 20). The room temperature phosphorescence observed in these studies is undoubtedly from Trp 314 in the sequence (21) which from the x-ray data of C. -I. Brändén *et al.* (personal communication) is buried in a hydrophobic environment close to the contact region between the two subunits in the enzyme. Trp 15 which is partially exposed to solvent correlates well with the residue whose phosphorescence properties reflect an external environment. Analysis of the x-ray data particularly in the region of Trp 314 should aid in elucidating the structural basis of the conformational fluctuations which we appear to be monitoring in these studies.

(*ii*) *Oxygen Independent Triplet Lifetimes.* The O_2-independent lifetime for the tryptophan residue in LADH of 0.13 sec is markedly shorter than the 5.5 sec lifetime typically observed from the phosphorescence at 77°K. It is also considerably shorter than the 0.8 sec O_2-independent tryptophan phosphorescence lifetime observed for alkaline phosphatase. At very low O_2 concentration, the triplet lifetime for tryptophan residues in a protein is clearly influenced by the particular environment in which the chromophore is located as well as by the temperature. The O_2-independent triplet lifetime of LADH was observed to be extremely sensitive to perturbations to the protein structure. The lifetime measured at room temperature was reproducible from day to day with a given lot of enzyme, but both the enzyme activity and the lifetime varied between 80 and 200 msec from one lot number to the next; the enzyme batches with the shorter lifetimes appearing to be the most active. With a given lot of enzyme, the tryptophan triplet lifetime of LADH at room temperature is influenced by changes in solvent, pH, and the addition of coenzyme and denaturants. The influence of variations in the experimental conditions of the O_2-independent phosphorescence lifetime of LADH are summarized in Table 1. Our knowledge of the factors affecting radiationless transitions in aromatic chromophores is inadequate at the present to interpret these lifetimes in terms of particular environmental perturbations. It is likely that time-dependent perturbations arising from fluctuations in the protein structure and/or periodic exposure to a mobile polar solvent may play a role in determining the lifetime. More detailed studies of this type which include comparisons with x-ray structural data should help to establish the factors which influence triplet-singlet radiationless transitions at ambient temperatures, not only for chromophores in proteins but for molecules in general. In order to determine in a more definitive way the influence of these various experimental conditions on the rate of conformational fluctuations in the protein a study of the oxygen dependence in each case will be required.

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