Penetration of Dioxygen into Proteins Studied by Quenching of Phosphorescence and Fluorescence†

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ABSTRACT: Experiments were done to measure the ability of dioxygen to collisionally quench the phosphorescent and fluorescent tryptophans in alcohol dehydrogenase and alkaline phosphatase. In all cases, luminescence is quenched with rate constants close to 1×10^9 M^{-1} s^{-1}. The rate of reaching the buried tryptophans is little affected by solvent viscosity due to added glycerol. Quenching by dioxygen is not due to a protein-opening reaction. It appears to be rate limited by internal protein diffusion rather than at the entry step. Dioxygen appears to enter the proteins directly, as in liquidlike diffusion, rather than through transiently forming channels that are only present a small fraction of the time. A high-pressure oxygen system is described that considerably facilitates fluorescence quenching experiments.

Work over the last decade on the internal motional behavior of proteins has significantly altered how we think about the behavior of proteins as physical objects and their possible modes of functioning (Englander et al., 1972; Lakowicz & Weber, 1973a,b; Gurd & Rothgeb, 1979; Karplus & McCammon, 1981). One motif in this development concerns the idea that small molecules can penetrate into and move about within proteins more or less as they do in liquid solvents. Indications that this may occur have come especially from observations on the quenching of protein luminescence by several small molecules that exert their quenching effect via direct collision with tryptophan side chains (Eftink & Ghiron, 1981).

Lakowicz & Weber (1973a,b) initially discovered that the fluorescence of apparently buried tryptophan side chains in a variety of proteins could be quenched by oxygen molecules with surprising ease. They found that, in comparison with diffusion-limited quenching rates characteristic of the interaction between oxygen and tryptophan in free solution, rate constants for fluorescence quenching of tryptophans in proteins were reduced by less than 1 decade, consistent with a fairly fluid model for protein structure. But are these protein tryptophans truly totally enclosed by the protein? The criterion used by Lakowicz and Weber was the relative blueness of the fluorescence emission. This parameter is related to hydrophobicity or rigidity of the immediate environment but cannot exclude some exposure to solvent.

Saviotti & Galley (1974) discovered that long-lived phosphorescence of excited-state tryptophans could be measured in certain proteins even at room temperature. The kind of special protection offered these tryptophans by their surrounding protein structure is not yet clear, but long-lived phosphorescence at room temperature presumably provides a good criterion for total internal burial. These tryptophans therefore can provide a test for the reality of oxygen permeability into proteins. In studies of the ability of oxygen to reach the phosphorescent tryptophan in liver alcohol dehydrogenase (LADH),[1] this residue appeared to display some special protection from quenching by oxygen (Saviotti & Galley, 1974; Kishner et al., 1979). This challenges the generality of the oxygen permeation results of Lakowicz and Weber. The results of Galley and his co-workers appeared to require an interpretation in terms of the transient opening of units of protein structure to bring tryptophan side chains out into contact with solvent, rather than the penetration of oxygen molecules into the protein matrix.

In this paper, we compare the ability of oxygen to quench the fluorescence and phosphorescence of the most protected tryptophans in two different proteins, and also the fluorescence of some less protected tryptophans. The results affirm that oxygen can enter proteins and give some indication of the limiting step in this process. A number of other molecules, some considerably larger than oxygen, have been found to quench protein fluorescence, including acrylamide (Eftink & Ghiron, 1977), acetone (Barboy & Feitelson, 1978), iodide anion (Lehrer, 1971), and others (Eftink & Ghiron, 1981). As with oxygen, these agents have been suggested to penetrate into and move about readily within proteins. The following paper (Calhoun et al., 1983) deals with a variety of such quenching agents and their greatly diminished capability for reaching the same tryptophans studied in the present oxygen quenching experiments.

Materials and Methods

Proteins, including horse liver alcohol dehydrogenase (LADH) as a 1 time crystallized product, alkaline phosphatase (type III) from *Escherichia coli* as a suspension in 2.5 M ammonium sulfate solution, and bovine liver catalase, were obtained from Sigma Chemical Co. (St. Louis, MO). A sample of purified LADH was also kindly supplied by Dr. J. D. Shore. Lyophilized glucose oxidase was from Boehringer-Mannheim (Indianapolis, IN). Spectroanalyzed acetone [high-pressure liquid chromatography (HPLC) grade] was from Fisher Scientific Co. (Philadelphia, PA). Other chemicals were reagent grade. Deionized water was used throughout.

Sample Preparation. For phosphorescence studies, enzymes were dissolved in 0.032 M sodium pyrophosphate buffer, pH 8.6, as in the work of Saviotti & Galley (1974) (except where indicated) to a final concentration of $(0.8–2.5) \times 10^{-5}$ M (0.6–2 mg/mL). For fluorescence experiments, the protein concentration was adjusted so that the absorbance at the excitation wavelength was about 0.1 cm^{-1}. The buffer used was 0.01 M sodium phosphate–0.1 M NaCl, pH 7.0. KI solutions were freshly prepared and contained 10^{-4} M sodium thiosulfate

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[1] Abbreviations: LADH, horse liver alcohol dehydrogenase; NATA, *N*-acetyltryptophanamide; AP, alkaline phosphatase.

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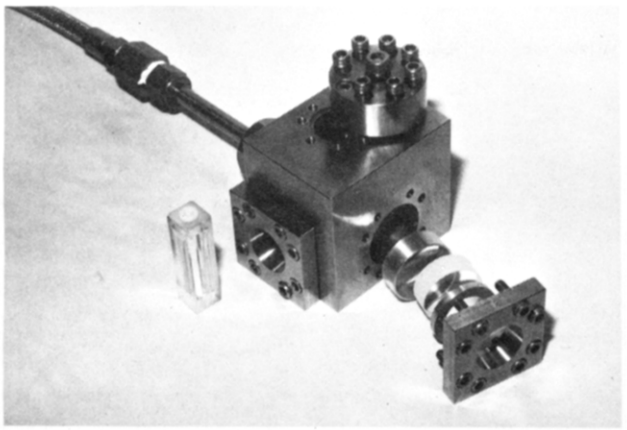

FIGURE 1: High-pressure oxygen cell. The stainless-steel block (5 × 6.5 × 6 cm) has three quartz windows (1 in. in diameter × $^3/_8$ in. thick) and connects to a high-pressure oxygen cylinder via a 36-in. high-pressure flexible cable. A Plexiglass fitting in the chamber holds a 1-cm^2 cuvette. Emission is measured at 90° to the exciting light.

to prevent I_3^- formation (Lehrer, 1971).

For removal of oxygen from buffer diluent, buffer solutions were stirred under vacuum on an aspirator for 10 min, then transferred to a glovebag filled with argon, and bubbled with ultrapure argon (Airco Gas Co., Murray Hill, NJ) for 30 min. Residual oxygen was removed in several ways. For complete deoxygenation, glucose oxidase (0.4 μM), glucose (0.3%), and catalase (0.04 μM) were added. In some experiments, sodium dithionite was then added to a concentration of 1 mM. For experiments in which oxygen concentration was varied, the deoxygenating system was omitted, and the buffer was initially bubbled with argon for 1–2 h. Buffer equilibrated in air (0.25 mM O_2) was suitably diluted into the purged buffer, and aliquots were added to the protein-containing cuvette, which was then stirred and capped with a glass stopper. All these operations were performed in an argon-filled glovebag. In experiments with low oxygen concentrations, it was necessary to limit sample illumination to avoid some photolytic reaction that removes oxygen. This was especially pronounced in the presence of 50% glycerol, which made experiments on phosphorescence quenching by oxygen in glycerol impracticable.

Oxygen Pressure Cell. High-pressure oxygen experiments were carried out in a specially constructed stainless-steel cell (Figure 1) adapted to hold a standard 1-cm fluorescence cuvette. The Teflon cuvette stopper was pierced with a small hole and cut flush with the top of the cuvette. Initial fluorescence intensity was measured. Air spaces in the cuvette and pressure cell were then flushed with oxygen. The cell was closed and placed in the suitably adapted fluorometer sample compartment. Five minutes were allowed for temperature equilibration before the fluorescence intensity was measured. To set solution oxygen concentrations, we increased the oxygen pressure, and the cell was inverted by hand to mix until fluorescence readings remained constant. The cell was connected to the high-pressure gas cylinder via a flexible steel cable, which allowed the cell to be inverted by swinging it through a wide arc. Twenty inversions are sufficient to equilibrate the sample with oxygen. This procedure allowed much more rapid equilibration than heretofore reported (Lakowicz & Weber, 1973a); about 1 h was required to obtain readings up to 1400 psi in increments of 200 psi.

Instrumentation. Steady-state fluorescence spectra and yields were measured either with an SLM-8000 fluorometer interfaced to a Hewlett-Packard 9825 computer or with a Perkin-Elmer 650 fluorometer interfaced to an Apple II computer. The excitation wavelength was 300 nm, and the emission wavelength was 340 nm. Slits were adjusted to give half-maximal bandwidths of 2–4 nm. In both instruments, water baths were used to thermostat the cell compartments at 25 °C.

Commercial steady-state fluorescence instruments were modified to measure phosphorescence. Phosphorescence spectra were obtained with the SLM-8000 fluorometer. The Hewlett-Packard computer triggered a fast mechanical shutter to block the exciting light and concurrently trigger the fluorometer monochromater stepping motors and electronic components of the SLM instrument. Subsequent measurements of emission were on the second time scale since the integration interval of the SLM electronic components allows for a time resolution of 100 ms per point. For measurement of phosphorescence lifetimes, a Perkin-Elmer MPF-2A fluorometer was used. The optical components were used without modification, but the photomultiplier was replaced with an RCA 8850 photomultiplier tube operating at 1800 V. The signal was amplified by a Durrum photometric amplifier, Model D-131, digitized with a Gould-Biomation 2805 transient recorder, and transferred to an Apple II computer for exponential decay analysis by a least-squares method.

Analysis of Data. The apparent rate constant for collisional quenching (k_q) was obtained from measurements of steady-state fluorescence or decay of phosphorescence according to the Stern–Volmer (Stern & Volmer, 1919) equation:

$$\tau_0/\tau = F_0/F = 1 + \tau_0 k_q[Q] \quad (1)$$

Here τ_0 is the luminescence lifetime and F_0 is the luminescence yield in the absence of quencher, and τ and F are the lifetime and yield in the presence of a given quencher at concentration [Q].

A more detailed analysis of this kind of data has been well discussed by Eftink & Ghiron (1981).

Results

Phosphorescence of Tryptophan in Proteins. Luminescence excitation and emission spectra (uncorrected) for alkaline phosphatase at 25 °C are shown in Figure 2. Figure 2A compares the phosphorescence excitation spectrum, using 440 nm as the detection wavelength, with the fluorescence excitation spectrum, using 340 nm for detection. The phosphorescence excitation spectrum appears red shifted. This is expected since a protein tryptophan that displays long-lived phosphorescence at room temperature must be well buried, and the absorption spectrum of tryptophan buried in a hydrophobic environment is known to be relatively red [see also Saviotti & Galley (1974), Eftink & Jameson (1982), Barboy & Feitelson (1978), and Eftink & Ghiron (1981)].

Emission spectra for alkaline phosphatase in the presence and absence of oxygen are given in Figure 2B. In the absence of oxygen, alkaline phosphatase shows, superimposed on the major fluorescence emission, the characteristic emission spectrum of tryptophan phosphorescence in the region between 350 and 550 nm. When the sample was exposed briefly to air and stirred by one inversion of the cuvette, the intensity of emission above 400 nm diminished.

Figure 2C shows emission spectra of anoxic sample recorded at 1-s intervals after the exciting beam was blocked with a mechanical shutter so that the short-lived fluorescence does not appear. The spectrum is characteristic of tryptophan phosphorescence. (A shoulder in the emission spectrum at 390 nm may be due to phosphorescence from tyrosine or some other excited-state species.) Alkaline phosphatase phosphorescence is remarkably long-lived and decays exponentially

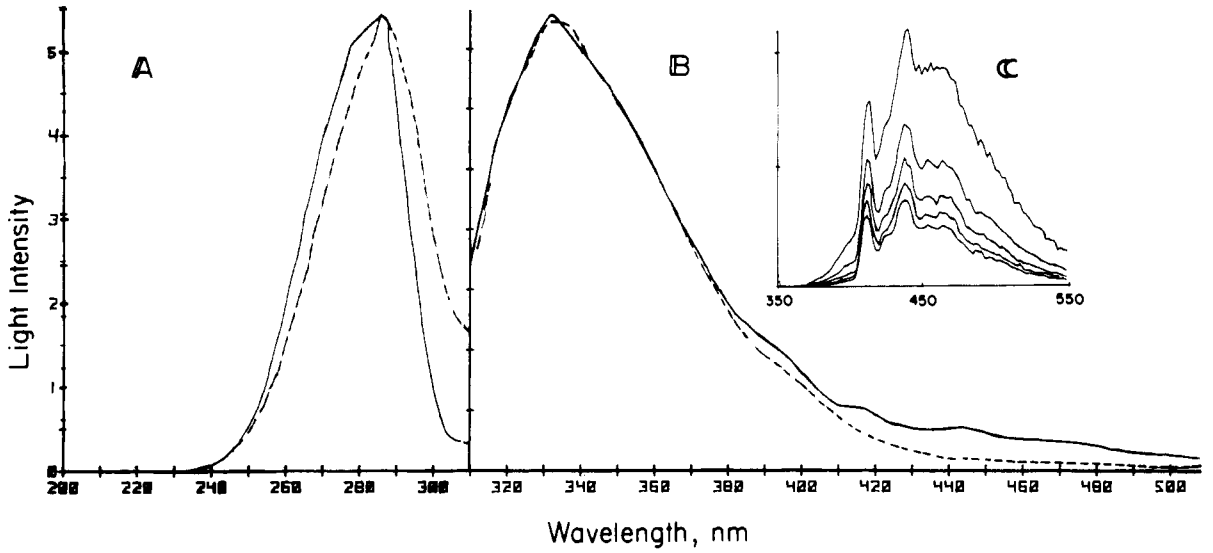

FIGURE 2: Excitation and emission spectra of alkaline phosphatase. (A) Excitation spectra giving rise to phosphorescence emission measured at 440 nm (---) and fluorescence emission at 340 nm (—) of deoxygenated samples of alkaline phosphatase. (B) Steady-state emission spectra of air-equilibrated (---) and deoxygenated (—) samples of alkaline phosphatase. The excitation wavelength was 295 nm. (C) Phosphorescence spectra of deoxygenated alkaline phosphatase. Decreasing spectra represent 1-s intervals after the exciting light was interrupted by closing the shutter. The excitation wavelength was 295 nm.

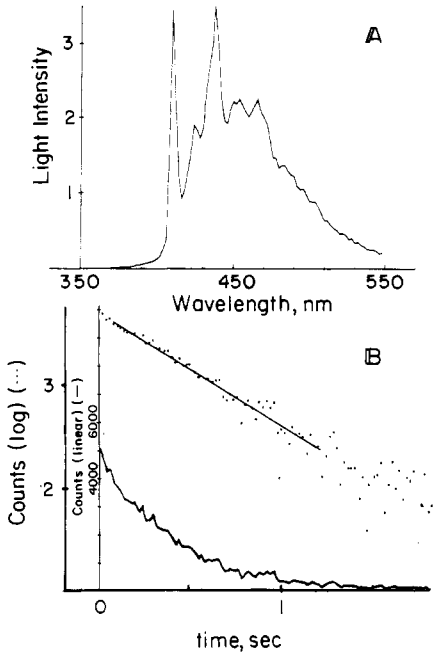

FIGURE 3: Phosphorescence spectrum and decay of alcohol dehydrogenase. (A) Spectrum of a deoxygenated solution of LADH taken in the interval 100–150 ms after the shutter was closed. Excitation was at 295 nm. (B) Decay of LADH phosphorescence. The emission wavelength was 440 nm. The sample was deoxygenated as described under Materials and Methods.

with a lifetime of 1.4 ± 0.1 s in the absence of oxygen or other quenchers (25 °C). Decay is accurately first order, and the emission shows no spectral change in the time interval between 100 ms and 5 s. If there is any structural rearrangement about the excited-state triplet, the altered conformation is obtained before 100 ms.

The phosphorescence emission spectrum from horse liver alcohol dehydrogenase (LADH) at room temperature, shown in Figure 3A, resembles but displays more resolution than that of alkaline phosphatase. The relaxation of LADH phosphorescence is accurately described by a single exponential over 98% of its decay (Figure 3B), consistent with the absence of detectable spectral shifts over the time scale of measurement. The phosphorescence lifetime of fully anoxic LADH was 0.3 ± 0.05 s at 25 °C.

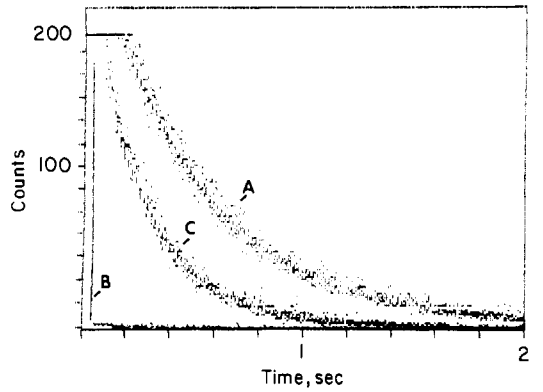

FIGURE 4: Phosphorescence of LADH quenched by oxygen. (A) Solution of LADH deoxygenated with glucose oxidase and catalase as described under Materials and Methods ($\tau \simeq 250$ ms). (B) Air introduced by removing the stopper from the cuvette, fanning the cuvette, replacing the stopper, and inverting the cuvette twice. (C) Subsequent recovery of phosphorescence decay after several minutes in glucose oxidase.

Quenching of Phosphorescence by Oxygen. Lakowicz and Weber have shown that oxygen quenches the fluorescence of a number of proteins with rate constants (k_q) between 2×10^9 and 7×10^9 M^{-1} s^{-1}, compared to the diffusion-limited rate of 1.2×10^{10} M^{-1} s^{-1} found for a number of tryptophan analogoues in free solution. On the other hand, Saviotti & Galley (1974) reported that phosphorescence of tryptophan in LADH appears to resist quenching by oxygen. It was therefore of interest to directly compare the sensitivity of the fluorescence and the phosphorescence of given tryptophans to quenching by oxygen.

The phosphorescence emission of LADH and alkaline phosphatase was extinguished by even brief exposure to atmospheric oxygen. In the experiment displayed in Figure 4, the phosphorescence decay of a deoxygenated sample was initially measured (curve A); then the sample was exposed to air, mixed, and measured again. The phosphorescence was essentially extinguished (curve B; lifetime <1 ms). Over time, the phosphorescence returned (curve C) due to the continuing

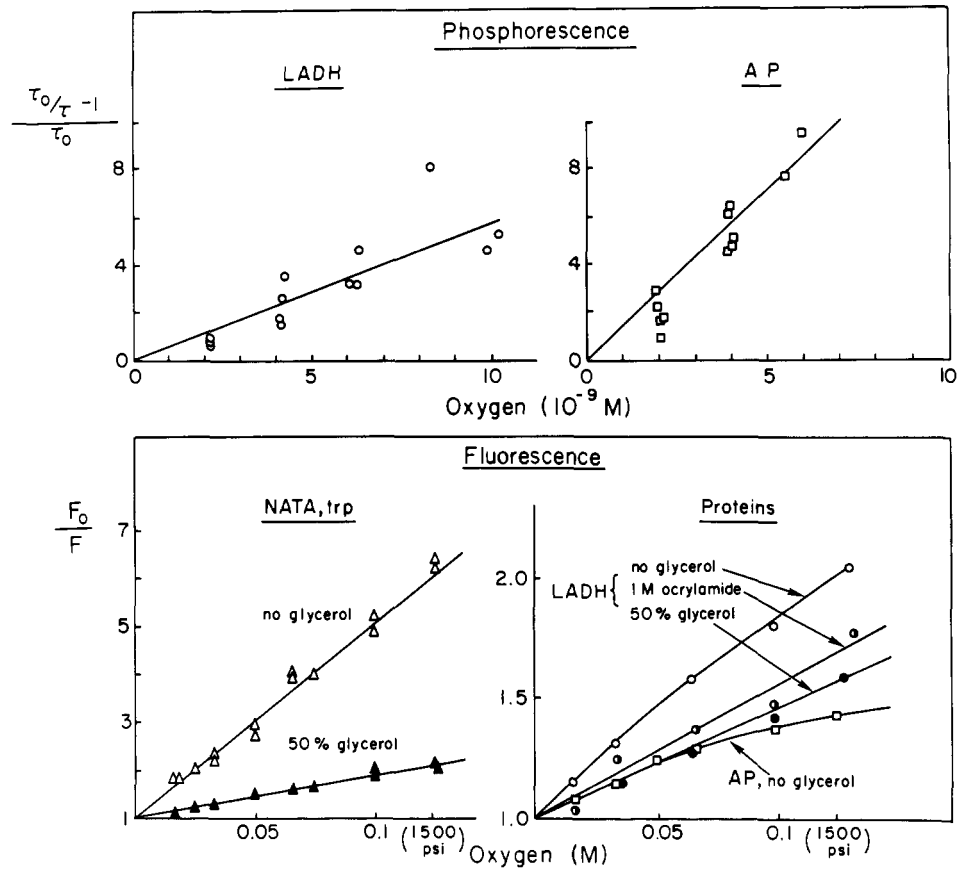

FIGURE 5: Luminescence quenching by oxygen. Phosphorescence quenching data for the single buried tryptophan in LADH and in AP show the decrease in lifetime resulting from addition of small volumes of an oxygen-containing solution. Quenching of the summed fluorescence emission intensity of LADH or AP was measured after LADH or AP was equilibrated with increasing pressures of oxygen. Measurements were made in the presence and absence of 50% glycerol to test the effect of solvent viscosity, and with 1 M acrylamide to remove the contribution of the more exposed tryptophan.

removal of contaminating oxygen by the glucose oxidase catalase system present in the sample. In the absence of the enzyme system, no recovery occurred.

Quantitative results on the quenching of phosphorescence by oxygen are shown in Figure 5. These data were obtained by deoxygenating protein solutions with argon gas and then adding aliquots of a suitably diluted solution of air-equilibrated buffer (handled in an oxygen-free glovebag). Oxygen at concentrations in the nanomolar range effectively quenches the phosphorescence of the specifically protected tryptophans in both LADH and alkaline phosphatase. Quenching constants are approximately 6×10^8 M^{-1} s^{-1} for LADH and 1×10^9 M^{-1} s^{-1} for alkaline phosphatase (Table I).

Quenching of Fluorescence by Oxygen. The ability of oxygen to quench fluorescence is also indicated in Figure 5 and Table I. In these experiments, we attempted to focus fluorescence measurements selectively on the most protected tryptophan by exciting at the red edge of the tryptophan absorbance spectrum, namely, at 300 nm (see Figure 2A). Emission measurements were at 340 nm. [This tactic was used before by Eftink & Selvidge (1982) and by Barboy & Feitelson (1978)]. Under these conditions, the most protected tryptophan in both these proteins accounts for 70% of the total fluorescence emission [when only the more exposed tryptophans are quenched, Stern–Volmer plots plateau at F_0/F equal to 1.4 [see Calhoun et al. (1983)]]. The quenching by oxygen, however, continues smoothly upward well past this limiting value; the relatively exposed and the well-protected tryptophans are quenched by oxygen at similar rates. The best-fit curve drawn through the data points in Figure 5 for LADH uses values for the quenching rate constant of 1.3×10^9 and 3.5×10^9 M^{-1} s^{-1} for the buried and the exposed tryptophan, respectively, in excellent agreement with the results of Eftink & Jameson (1982). In this computation, we used the lifetimes reported for these residues by Eftink & Jameson (1982) and by Ross et al. (1981) (averaged to 3.7 and 7.0 ns for the buried and exposed residues, respectively). Independent results come from experiments (Figure 5) done in the presence of 1 M acrylamide, which quenches 85% of the exposed tryptophan signal and little of the buried tryptophan [see Calhoun et al. (1983)]. The apparent oxygen quenching constant, dominated by the buried tryptophan, was 1.6×10^9 M^{-1} s^{-1}.

Table I: Luminescence Quenching by Dioxygen[a]

	NATA	LADH	AP
fluorescence	15	3 (exposed) 1 (buried)	0.8 (exposed + buried)
phosphorescence		0.6 (buried)	1 (buried)

[a] Numbers shown are quenching constants in units of 10^9 M^{-1} s^{-1}. For LADH, fluorescence lifetimes used were 3.7 and 7 ns for the buried and more exposed tryptophans, respectively (see text). For AP, 5 ns was used as an average fluorescence lifetime estimate; it was not possible to distinguish between the exposed and buried tryptophans.

The data for alkaline phosphatase in Figure 5 indicate a quenching efficiency somewhat less than that for LADH and qualitatively suggest that the most buried tryptophan is also significantly quenched on the nanosecond time scale.

Since fluorescence and phosphorescence lifetimes differ so greatly, the quenching of these different states occurs on very different time and oxygen concentration scales, as can be seen in the Stern–Volmer plots of Figure 5. However, when the

different lifetimes are taken into account, one finds that oxygen quenches them with comparable rate constants (Table I).

A factor that must be considered is the efficiency of quenching. Does every collision produce quenching? Fluorescence results with tryptophan analogues in free solution (Figure 5) yield a quenching rate constant of 1.5×10^{10} M^{-1} s^{-1}, the expected collisional rate [see also Lakowicz & Weber (1973a)]. Gijzeman et al. (1973) have reported a statistical factor of $^1/_9$ for the quenching of a number of organic triplets with oxygen. On the other hand, Kearns & Stone (1971) point out that the statistical factor can vary from 1 to $^1/_9$, depending upon interaction energies. The present results indicate an apparent statistical factor for tryptophan close to unity. However, for quenching by oxygen inside proteins, it may be necessary to consider the possible role of multiple encounters per effective collision; this would act to enhance the apparent quenching efficiency per collision.

In summary, these results show that oxygen quenches the phosphorescence of the well-protected tryptophans of LADH and alkaline phosphatase, and the fluorescence of these same residues, and also the fluorescence of the more exposed tryptophans with about the same efficiency, about 1–1.5 decades slower than the free solution diffusion-limited rate (Table I).

Effect of Solvent Viscosity. Figure 5 includes some results on the sensitivity of quenching to solvent viscosity. In 50% glycerol (w/w, 25 °C), solvent macroviscosity is increased 5-fold. Fluorescence quenching of NATA by oxygen appears to be decreased 4.5-fold when data are plotted as a function of oxygen pressure (Figure 5). Part of this effect may be due to a 2-fold decrease in the solubility of oxygen (*International Critical Tables*, 1928), leaving another factor of 2 due to a decrease in diffusivity (i.e., a 4.5/2 increase in solution microviscosity). [Note that the equilibrium activity of dissolved oxygen is unaffected by the glycerol, but this fact need not enter the microviscosity calculation since the oxygen–NATA interaction is not an equilibrium one; the quenching reaction, being diffusion limited (Table I), occurs on every collision.]

In the presence of 50% glycerol, the fluorescence quenching of LADH by oxygen appears to be decreased by 2-fold (Figure 5). Again, here the abscissa is in terms of oxygen pressure rather than its corrected concentration in the solvent, which differs in the presence and absence of glycerol. However, it should be appreciated that oxygen concentration *inside* the protein (or bound to the protein surface) is tied to the equilibrating oxygen pressure (activity) rather than to the solution concentration and is therefore expected to be the same in the presence and absence of glycerol (insofar as overall protein structure is not grossly affected).

In interpreting these data, one can make the reasonable assumption that the relatively exposed tryptophan is quenched less effectively by the same factor of 4.5 found for NATA in 50% glycerol solution (Figure 5). If so, then curve fitting indicates the buried tryptophan to be hardly affected by the external viscosity; the quenching rate constant is reduced by about 25%, to 1×10^9 M^{-1} s^{-1}. At the other extreme, one might attribute the viscosity-induced slowing equally to the exposed and buried residues. Even here, the apparent quenching rate constant (internal viscosity) is affected only little compared to the 5-fold decrease in external viscosity [0.4 power; see Gavish (1980)]. In making these calculations, we have ignored the small glycerol-induced increases in lifetime noted by Eftink & Ghiron (1977); these would make internal viscosity appear even less sensitive to external solvent viscosity. Our attempts to measure the effect of solvent viscosity on phophorescence quenching failed, owing to some glycerol-enhanced photolytic reaction that removed oxygen rapidly at these low concentrations.

A negligible effect of solution viscosity on the quenching of globin-porphyrin fluorescence by dioxygen has also been noted by Jameson et al. (1981). The possible effect of solvent viscosity on internal protein motions has been discussed by Gavish (1980) and by Beece et al. (1980).

Discussion

Assignment of the Tryptophans Measured. Phosphorescence from tryptophan in proteins is rarely observed at room temperature and is now known to be measurable, in the millisecond to second time range, in only a small number of proteins (Saviotti & Galley, 1974; Domanus et al., 1980; Kai & Imakubo, 1979). It is therefore likely that only one particular tryptophan is responsible for phosphorescence in each protein. This conclusion (Purkey & Galley, 1970) is supported by the sharp emission spectra for room temperature phosphorescence (Figures 2 and 3) and by the simple exponential quality of the phosphorescence decay (e.g., Figure 3B).

LADH is a dimer of two identical monomers and contains two pairs of tryptophans. Trp-314 is known to be rather well buried near the subunit interface (Branden et al., 1975) and presumably accounts for the red-shifted absorption and for LADH phosphorescence. Trp-15 is known to be more exposed to solvent and presumably accounts for the more easily quenchable fraction of LADH fluorescence discussed in the following paper. The same conclusion has been reached by others (Purkey & Galley, 1970; Abdullah et al., 1978; Laws & Shore, 1979; Eftink & Selvidge, 1982). Alkaline phosphatase is also a dimer of two identical subunits. However, it has four pairs of tryptophans, and this complexity precludes the assignment of fluorescence and phosphorescence contributions simply from quenching data.

Phosphorescence Quenching by Oxygen. In their pioneering work on room temperature protein phosphorescence, Saviotti & Galley (1974) noted that the phosphorescence of LADH was quenched rather ineffectively by oxygen, and the rate of quenching appeared to be limited to a maximum of 12 s^{-1}. This result is in conflict with the demonstration of fluorescence quenching by oxygen in many proteins (Lakowicz & Weber, 1973b), even for apparently well-buried tryptophans, at rates in the range of 10 s^{-1}, since both fluoresence and phosphorescence quenching by oxygen undoubtedly involve direct collisional contact with the tryptophan side chain.

The present results resolve this discrepancy; oxygen does reach the most protected tryptophan in both LADH and alkaline phosphatase with rate constants that approach the free solution diffusion-limited rate. This was shown in separate fluorescence and phosphorescence experiments. One cannot be sure why the early results of Galley and co-workers suggested a different conclusion. We noted that prolonged exposure to the exciting beam reduced oxygen levels, and in view of the much higher light levels used in Galley's laboratory (the entire output of a xenon arc), a similar phenomenon might account for the limited quenching they observed.

Mechanism of Oxygen Quenching. It is of great interest to understand the molecular mechanism by which oxygen penetrates into proteins. For most protein tryptophans, some question exists concerning partial solvent exposure (Calhoun et al., 1983), but the phosphorescent tryptophans of LADH and alkaline phosphatase are buried and therefore provide special experimental advantages for such studies.

In analogy with ongoing discussions in the protein hydrogen exchange field (Englander et al., 1980; Woodward & Hilton, 1979), two different points of view concerning the mechanism

of oxygen quenching can be considered. One is that oxygen can reach the buried tryptophan by penetrating into the essentially native protein, either directly through the protein matrix or via transiently formed channels. The other is that the buried tryptophan is repeatedly transiently unmasked in segmental unfolding reactions that expose normally buried residues to the external milieu.

Structural Unfolding Model. The structure opening model can be described as

$$Q + P^*_{cl} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} Q + P^*_{op} \xrightarrow{k_{q,o}} Q{\cdot}P \qquad (2)$$

The subscripts op and cl refer to opening and closing, and the asterisk denotes the excited-state protein. A first-order structural isomerization that exposes the normally buried tryptophan to solvent is followed by a second-order collisional step with quencher. The parameter $k_{q,o}$ is the second-order rate constant for reaction of the relatively exposed form with quencher in solution and is presumably close to 10^{10} M^{-1} s^{-1}. The kinetic expression for this kind of pathway has been much discussed [e.g., see Hvidt & Nielson (1966)] and can be written as in eq 3a.

$$k_q = \frac{k_{op}k_{q,o}[Q]}{k_{cl} + k_{q,o}[Q]} \qquad (3a)$$

Since protein fluorescence quenching is first order in oxygen concentration, eq 3a requires that $k_{cl} > k_{q,o}[Q]$, so that the limiting case in eq 3b is indicated.

$$k_q = K_{op}k_{q,o}[Q] \qquad (3b)$$

Here, the observed quenching rate is equal to the unblocked rate constant (about 10^{10} M^{-1} s^{-1}) multiplied by the fraction of time the structure is open, that is, by the opening equilibrium constant, K_{op}.

The distinguishing characteristic of this model is that the normally protected tryptophan is exposed by a transient structural isomerization that can be considered to behave as a concerted unfolding or collapse of some defined structural segment.

Our results show rate constants for fluorescence quenching of the buried tryptophan reduced by only one-tenth from the diffusion-limited value. This would require the important structural isomerization to be "open" at least one-tenth of the time. This may be inconsistent with the long-lived phosphorescence observed. In addition, the opening rate constant must be faster than observed quenching rates (10^9 s^{-1} at 0.1 M oxygen) and the closing rate faster than this by 10× or more. A structural isomerization, to be so fast, must be very small. An opening pathway further requires that the observed quenching rate in the protein vary in proportion with the free solution rate (eq 3b). In the presence of 50% glycerol, we may then expect the quenching rate to be slowed by a factor of about 4.5, as for free NATA. A maximum factor of 2-fold was observed, and the true factor may be closer to unity. These observations are against the operation of a unitary structural unfolding mechanism for the fast quenching of protein luminescence by oxygen.

[Slower processes, however, such as the quenching of protein phosphorescence by molecules larger than oxygen (Calhoun et al., 1983) and protein hydrogen exchange (Englander et al., 1980), do appear to involve partial protein unfolding reactions.]

Penetration Models. An alternative model visualizes that oxygen reaches buried tryptophans by a penetrational process similar to diffusion through solvent. Here, a large number of rapid small motions of the protein's atoms concert in a statistical sense to allow oxygen to penetrate into and diffuse from point to point within the protein. Equation 4 symbolizes the possibly important kinetic steps along a penetration-based pathway.

$$Q_o + P^* \underset{}{\overset{diff_o}{\rightleftharpoons}} Q_oP \qquad (4a)$$

$$Q_o + P^* \overset{diff_o}{\rightleftharpoons} Q_oP^* \overset{entry}{\rightleftharpoons} Q_iP^* \overset{diff_i}{\rightleftharpoons} Q_iP \qquad (4b)$$

Equation 4a refers to surface, perhaps partially occluded tryptophans. These will probably be quenched largely by external quenchers, with rate constants that are only slightly reduced from the fully exposed free solution value and that vary with solvent viscosity. This is not what we find.

Equation 4b is a minimal reaction scheme for quenching of fully buried tryptophans. The quencher (Q_o) and excited-state protein (P^*) undergo solvent-dependent diffusion ($diff_o$) and collision to form an encounter complex (Q_oP^*). The quencher may then either dissociate or enter the protein (Q_iP^*). Once inside, the quencher may either reexit or accomplish an internal ($diff_i$) quenching collision (Q_iP) with the tryptophan. In addition to the intermediates shown, one can suppose that entry might require the formation of a transient channel, present at some low level in equilibrium with the native protein form (Lumry & Rosenberg, 1975; Richards, 1979). At each of these intermediate points, the balance of outflow (either forward or back) will be determined by the relative size of the forward and reverse rate constants leading away from that intermediate.

The general kinetic expression for such a scheme is complex. For present purposes, we can simplify by considering limiting cases and the observed indifference of quenching rate to solvent viscosity. The latter requires that one of the rate constants to the right of Q_oP^* (eq 4b) is rate limiting. If entry is limiting, Q_oP^* will be at equilibrium with free Q concentration so that its rate of formation, which is viscosity dependent, will not enter the overall kinetic expression. Both Q_oP^* and Q_iP^* will be at equilibrium with solvent Q if internal diffusional collision represents the slow step.

Rate-Limiting Entry. It appears that the entry step is not rate limiting. The conditions of our phosphorescence experiments require that the quenching is due to oxygen molecules that originate in the solvent and not to those that happen to be inside the protein when excitation occurs. In these experiments, oxygen was present at nanomolar concentrations, with an average protein to oxygen distance of 1 μm. If the coefficient for partitioning oxygen between protein and solvent is about unity, only one protein molecule in 10^6 would contain an oxygen at the instant of excitation. Thus, quenching oxygens must come from outside. In contrast, the fluorescence quenching experiments used oxygen concentrations up to 0.1 M; at this condition, the average protein subunit (40 000 daltons) would carry about three embedded oxygen molecules. These oxygens would be already internalized when the exciting photon arrives and thus would escape a limiting entry barrier. Nevertheless, one observes essentially the same quenching rate constant for fluorescence and phosphorescence, suggesting that the same rate-limiting barrier is effective in both cases and that this barrier is not at the entry step.[2]

These results also say something about the mode of entry. In the phosphorescence experiments, oxygen comes from the outside yet quenches the buried tryptophan with a rate constant lower than the diffusion-limited rate by only about 1 decade.

[2] This implies that entry is not slow relative to the phosphorescence time scale. It may be slow on the fluorescence scale; if so, exit must be about equally slowed.

This seems to require that among oxygens that collide with the protein surface, more than10% actually succeed in entering the protein (some reexit; 10% achieve collision with the tryptophan). Entry might be considered to occur in two different ways, either directly into the protein matrix as in liquidlike diffusion or via transient restrictive channels that form predetermined pathways leading inward [see, e.g., Richards (1979)]. In the latter case, where a significant fraction of collision/entry steps are necessarily wasted because they do not lead the oxygen to the tryptophan, a fraction of collisions even larger than 10% must lead to entry. This requirement seems inconsistent with the operation of transient predetermined channels that are only present a small fraction of the time.

Rate-Limiting Internal Diffusion. A physically distinct process that can adequately explain present data involves the equilibration between solvent oxygen concentration and a pool of intraprotein oxygen, with the internal diffusional search and collision process being rate limiting. This kind of process was initially put forward by Lakowicz & Weber (1973b). Here, one visualizes a relatively rapid penetration into proteins of oxygen molecules and a subsequent limitingly slow diffusion of the internalized quencher through the protein until an encounter with the excited-state tryptophan is achieved.

In the limiting case considered here, when internal diffusion and collision are slow, the concentration (activity) of internalized quencher (Q_iP^* in eq 4b) will not vary with solvent viscosity or solvent oxygen solubility as this changes, for example, with added glycerol but will depend directly upon equilibrating oxygen activity (pressure). The internal diffusional process may also be rather insensitive to external solvent viscosity [though the possibility that the rate of protein distortion accompanying internal quencher movement might vary with external viscosity in lesser measure has been discussed (Beece et al., 1980; Gavish, 1980)]. The expected result of rate-limiting internal diffusion then is that the oxygen quenching of fully buried tryptophans should be relatively insensitive to solvent additives like glycerol, even though solvent viscosity is considerably increased and oxygen solubility is decreased. Meanwhile, partially exposed tryptophans should continue to sense solvent viscosity and solvent oxygen concentration (not activity). Our data are most consistent with this expectation.

The penetration model appears to explain available data on oxygen–protein interactions and has achieved a good degree of acceptance among workers in this field. Our results support this model and suggest that the rate-limiting step relates to the internal diffusion process rather to a slow entry step or a requisite unfolding reaction. The most interesting question of how oxygen moves about within proteins is being studied by a number of approaches (Case & Karplus, 1979; Richards, 1979; Beece et al., 1980). The possible penetration into proteins of larger quenching agents is dealt with in the following paper.

Registry No. Oxygen, 7782-44-7; LADH, 9031-72-5; AP, 9001-78-9; tryptophan, 73-22-3.

References

Abdullah, M. A., Biellman, J. F., Wiget, P., Joppich-Kuhn, R., & Luisi, P. L. (1978) *Eur. J. Biochem. 89*, 397–405.

Barboy, N., & Feitelson, J. (1978) *Biochemistry 17*, 4923–4926.

Beece, D., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M. C., Reinisch, L., Reynolds, A. H., Sorenson, L. B., & Yue, K. T. (1980) *Biochemistry 19*, 5147–5157.

Branden, C. I., Jornvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes, 3rd Ed. 11a*, 103–190.

Calhoun, D. B., Vanderkooi, J. M., & Englander, S. W. (1983) *Biochemistry* (following paper in this issue).

Case, D. A., & Karplus, M. (1979) *J. Mol. Biol. 132*, 343–368.

Domanus, J., Strambini, G. B., & Galley, W. C. (1980) *Photochem. Photobiol. 31*, 15–21.

Eftink, M. R., & Ghiron, C. A. (1977) *Biochemistry 16*, 5546–5551.

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.

Eftink, M. R., & Jameson, D. M. (1982) *Biochemistry 21*, 4443–4449.

Eftink, M. R., & Selvidge, L. A. (1982) *Biochemistry 21*, 117–125.

Englander, S. W., Downer, N. W., & Teitlebaum, H. (1972) *Annu. Rev. Biochem. 41*, 903–924.

Englander, S. W., Calhoun, D. B., Englander, J. J., Kallenbach, N. R., Liem, R. K. H., Malin, E., Mandal, C., & Rogero, J. R. (1980) *Biophys. J. 32*, 577–590.

Gavish, B. (1980) *Phys. Rev. Lett. 44*, 1160–1163.

Gijzeman, O. L. J., Kawfman, F., & Porter, G. (1973) *J. Chem. Soc., Faraday Trans. 2 69*, 708–720.

Gurd, F. R. N., & Rothgeb, T. M. (1979) *Adv. Protein Chem. 33*, 74–165.

Hvidt, A., & Nielson, S. O. (1966) *Adv. Protein Chem. 21*, 287–386.

International Critical Tables (1928) Vol. III, p 271, McGraw-Hill, New York.

Jameson, D. M., Coppey, M., Alpert, B., & Weber, G. (1981) *Biophys. J. 33*, 300a.

Kai, Y., & Imakubo, K. (1979) *Photochem. Photobiol. 29*, 261–265.

Karplus, M., & McCammon, J. A. (1981) *CRC Crit. Rev. Biochem. 9*, 293–349.

Kearns, D. R., & Stone, A. J. (1971) *J. Chem. Phys. 55*, 3383–3389.

Kishner, S., Trepman, E., & Galley, W. C. (1979) *Can. J. Biochem. 57*, 1299–1304.

Lakowicz, J. R., & Weber, G. (1973a) *Biochemistry 12*, 4161–4171.

Lakowicz, J. R., & Weber, G. (1973b) *Biochemistry 12*, 4172–4179.

Laws, W. R., & Shore, J. D. (1979) *J. Biol. Chem. 254*, 2582–2584.

Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.

Lumry, R., & Rosenberg, A. (1975) *Colloq. Int. C.N.R.S. No. 246*, 53–61.

Purkey, R. M., & Galley, W. C. (1970) *Biochemistry 9*, 3569–3575.

Richards, F. M. (1979) *Carlsberg Res. Commun. 44*, 47–63.

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

Saviotti, M. L., & Galley, W. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4154–4158.

Stern, O., & Volmer, M. (1919) *Phys. Z. 20*, 183–188.

Woodward, C. K., & Hilton, B. D. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 99–127.