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Penetration of Analogues of H_2O and CO_2 in Proteins Studied by Room Temperature Phosphorescence of Tryptophan†

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ABSTRACT: The influence of the protein matrix on the reactivity of external molecules with a species buried within the protein interior is considered in two general ways: (1) there may be structural fluctuations that allow for the diffusive penetration of the small molecules and/or (2) the external molecule may react over a distance. As a means to study the protein matrix, a reactive species within the protein can be formed by exciting tryptophan to the triplet state, and then the reaction of the triplet-state molecule with an external molecule can be monitored by a decrease in phosphorescence. In this work, the quenching ability (i.e., reactivity) was examined for H_2S, CS_2, and NO_2^- acting on tryptophan phosphorescence in parvalbumin, azurin, horse liver alcohol dehydrogenase, and alkaline phosphatase. A comparison of charged versus uncharged quenchers (H_2S vs SH^- and CS_2 vs NO_2^-) reveals that the uncharged molecules are much more effective than charged species in quenching the phosphorescence of fully buried tryptophan, whereas the quenching for exposed tryptophan is relatively independent of the charge of the quencher. This is consistent with the view that uncharged triatomic molecules can penetrate the protein matrix to some extent. The energies of activation of the quenching reaction are low for the charged quenchers and higher for the uncharged CS_2. A model is presented in which the quenchability of a buried tryptophan is inversely related to the distance from the surface when diffusion through the protein is the rate-limiting step. Using this model, upper limit values of the diffusion coefficient in the protein for these molecules can be estimated to be about 7×10^{-11} cm^2 s^{-1} through the protein matrix. Finally, since the quencher molecules H_2S and CS_2 resemble H_2O and CO_2 in size and charge, it is suggested that these molecules would show similar diffusion behavior.

How a molecule that is external to a protein reacts with a species buried within the protein interior will necessarily be influenced by the properties of the protein. As a means to study this process, we have introduced the use of phosphorescence quenching of intrinsic tryptophan by quenchers that are free to diffuse in solution. It is now well recognized that tryptophans in a rigid protein environment exhibit long-lived phosphorescence at room temperature, provided that external quenchers, notably O_2, are removed from the solution [Saviotti & Galley, 1974; Kai & Imakubo, 1979; Strambini, 1987; Vanderkooi et al., 1987; Vanderkooi & Berger, 1989; reviewed by Papp and Vanderkooi (1989)]. Phosphorescence

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can be used to study a reaction in proteins as follows: a reactive species within the protein is formed by exciting the tryptophan to the singlet state, the molecule spontaneously converts to the triplet state by intersystem crossing, and then the reaction of the triplet state with an external molecule is monitored by a decrease in the phosphorescence lifetime.

In previous work, we concluded that tryptophans buried in the core of proteins are not accessible to relatively large molecules (>3 atoms) in solution on the millisecond time scale but can interact with them by a reaction that occurs over distance (Vanderkooi et al., 1990). By examining the distance dependence of quenching, it was concluded that noncolored quenchers react with triplet tryptophan by an electron exchange reaction (Calhoun et al., 1988). It has since been demonstrated by Mersol and co-workers that exchange can also largely account for the quenching of some molecules having absorption in the spectral region of the tryptophan phosphorescence emission (Mersol et al., 1991). A principal indication that the quenching reaction occurred by long-range transfer, as opposed to diffusion of the quencher molecule through the protein matrix, was that the quenching by larger molecules was independent of quencher size and shape. Furthermore, the interaction between tryptophan and larger quencher molecules depended critically on the position of tryptophan in the protein and varied by about 8 orders of magnitude (Vanderkooi et al., 1990).

In contrast, the quenching by small (diatomic) molecules, O_2 and CO, was relatively less dependent on tryptophan position, although the quenching effect of oxygen on tryptophan phosphorescence was reduced somewhat for buried tryptophans. This suggested that there may be some limitation on the access of diatomic molecules to the interior. In a survey of quenching molecules, we saw that triatomic molecules showed intermediate behavior between the two classes of quenchers (Calhoun et al., 1988). In the present paper, we examine the features of triatomic molecules in greater detail by determining the temperature dependence of quenching by the small molecules H_2S and CS_2 and comparing their quenching behavior with the charged species SH^- and NO_2^- for tryptophan that is near the surface (in parvalbumin) or buried (in alkaline phosphatase, azurin, and aldolase).

Materials and Methods

Parvalbumin with a single tryptophan was prepared from frozen cod fillets as previously described (Calhoun et al., 1986). Azurin from *Pseudomonas aeruginosa* was isolated as described in the literature (Ambler, 1963; Ambler & Brown, 1967). Sigma Chemical Co. (St. Louis, MO) supplied *N*-acetyltryptophanamide (NATA),[1] horse liver alcohol dehydrogenase (LADH), alkaline phosphatase from *Escherichia coli* as a suspension in 2.5 M $(NH_4)_2SO_4$, bovine liver catalase as a powder, and glucose oxidase from *Aspergillus niger* as a powder.

Sample Preparation. Oxygen was removed from the buffer solutions by degassing under an aspirator and then bubbling with argon and finally, addition of an enzymatic system to remove oxygen (glucose, glucose oxidase, and catalase) as described in detail by Calhoun et al. (1988).

Stock solutions of H_2S were prepared by bubbling oxygen-depleted water with H_2S gas, produced by adding an excess of $MgCl_2$ in solution to solid calcium sulfide. The water was initially treated to remove oxygen as described above. The H_2S gas evolved was bubbled for 1–2 h through the water held in a long narrow tube and topped with mineral oil; these procedures were necessary to remove all traces of oxygen, which reacts with H_2S to produce mineral sulfur. During this procedure, the pH of the unbuffered water drops to about pH 4. The drop in pH was used to monitor the time course of the equilibration of H_2S with water. At equilibration, a saturated solution of 0.1 M was obtained (Handbook of Chemistry). Aliquots of the stock H_2S were then introduced to the protein samples in cuvettes under flowing argon. The cuvettes, holding 1.4 mL, were filled to the top and capped with sintered glass stoppers. The proteins were buffered, as indicated in the legend of Figure 4, in order to maintain their pH at the desired value. Following measurement of the phosphorescence lifetime after the sequential addition of aliquots of the solution of H_2S, the pH of the solution was measured, to ascertain that the change in pH during the titration was less than 0.2 pH unit.

Instrumentation and Analysis. Absorption spectra were obtained with a Perkin-Elmer 200 spectrophotometer. Fluorescence and phosphorescence spectra were obtained by a Perkin-Elmer LS-5 luminescence spectrometer. The cell compartments of these instruments were thermally regulated by means of a circulting water bath. Phosphorescence lifetimes were measured using the instrument described by Green et al. (1988). The first-order decay constant (k_p) for the phosphorescence is defined as

$$I(t) = I_0 \exp(-tk_p) \quad (1)$$

where I represents the phosphorescence intensity and t is the time. The decay profiles were analyzed for exponential decay using the Asystant program (Macmillan Software Co., New York, NY). The lifetime (τ) is $1/k_p$.

Quenching of phosphorescence was monitored by an increase in the decay rate. The bimolecular quenching rate constant (k_q) was computed from the equation of Stern and Volmer (1919) modified for decay rates rather than for intensities:

$$k = k_0 + k_q[Q] \quad (2)$$

where k_0 is the first-order decay rate in the absence of quencher and k is the observed decay rate (k_p) at quencher concentration [Q]. Least-squares analysis of the data was used to obtain the slope.

The distance of tryptophan from the surface was estimated using protein coordinates from the Brookhaven Protein Data Bank, and protein structure were examined using the Biograf molecular graphics program (BioDesign, Pasadena, CA).

Results

Characterization of Protein Phosphorescence. Phosphorescence from tryptophan was identified by its characteristic excitation and emission spectra and long-lived decay. The emission from aldolase is shown in Figure 1A, and the decay of phosphorescence and Stern–Volmer analysis of quenching for nitrite are shown on Figure 1B,C, respectively.

Temperature Dependence of the Phosphorescence Lifetime. The phosphorescence lifetimes of tryptophan in proteins are sensitive to temperature. The temperature dependence of the tryptophan lifetime for various proteins is shown in Figure 2 as Arrhenius plots. A simple temperature dependence was observed for aldolase, LADH, and paravalbumin, with the $\Delta E_{act.}$'s being 54, 36, and 38 kJ mol^{-1}, respectively. The phosphorescence lifetime of alkaline phosphatase showed nonlinear behavior in its Arrhenius profile, with 21 kJ mol^{-1} seen in the temperature range of 4–21 °C, and 63 kJ mol^{-1} observed from 21 to 49 °C. Table I summarizes the energy of activation and also gives the phosphorescence lifetimes

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

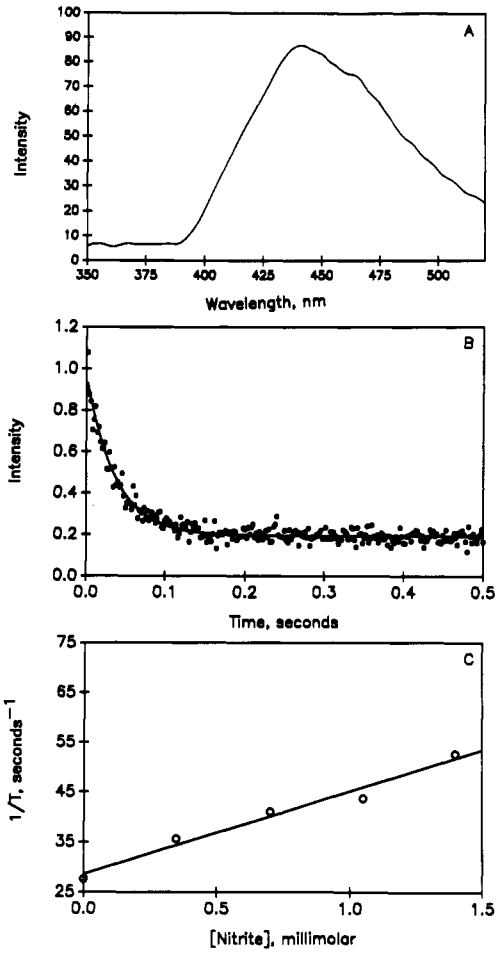

FIGURE 1: Phosphorescence properties of aldolase, 1 mg/mL, in 10 mM phosphate/100 mM NaCl, pH 7.0, 20 °C. (A) Emission spectrum using 278-nm excitation, 0.5-ms delay, and 2-ms gate time; slits of 15 and 20 nm for excitation and emission, respectively. (B) Decay curve, 280-nm excitation, 440-nm emission; the line represents the computer best fit to an exponential function with a decay of 40 ms. (C) Phosphorescence lifetime plotted according to eq 2. Conditions the same as for (B); concentration of $NaNO_2$ indicated on the figure.

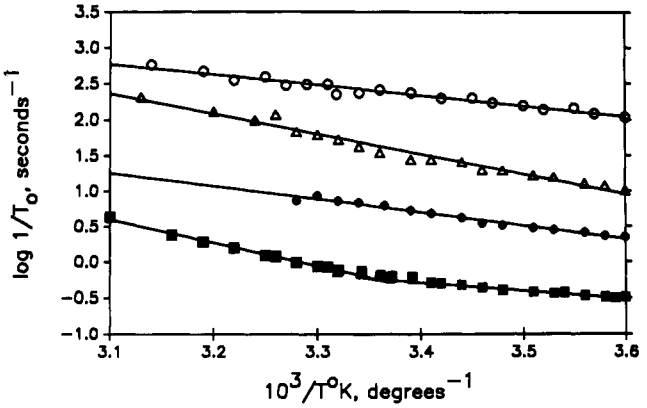

FIGURE 2: Temperature dependence of phosphorescence lifetime in the absence of added quenchers. (○) Paravalbumin, (△) aldolase, and (●) alcohol dehydrogenase were in 0.1 M NaCl/0.01 M Tris, pH 7; (■) alkaline phosphatase was in 0.034 M pyrophosphate, pH 8.6.

$(1/k_p)$ for these proteins at 20 °C in the absence of added quencher molecules. The lifetimes compare favorably with those reported in the literature for proteins at room temperature (Vanderkooi et al., 1987), but are considerably less than the value of ~5–6 s for tryptophan in most proteins at 77 K (Longworth, 1971).

Table I: Parameters of Phosphorescence Decay[a]

protein	ΔE_{act}, kJ mol^{-1}	lifetime, ms at 20 °C
parvalbumin	28	4.5
aldolase	54	45
LADH	36	255
alkaline phosphatase	21, 63	2000

[a] Conditions are given in Figure 2.

Table II: Parameters of the Quenching Reactions[a]

protein	quencher	k_q, M^{-1} s^{-1}, 20 °C	E_{act}, kJ mol^{-1}
parvalbumin	NO_2^-	6.5×10^6	5.4
	CS_2	2.7×10^7	40
aldolase	NO_2^-	1.7×10^4	22
	CS_2	9.7×10^4	49
LADH	NO_2^-	3.8×10^4	7
	CS_2	3.2×10^4	56
alkaline phosphatase	NO_2^-	2.5	46, 110
	CS_2	3.8×10^3	76

[a] Conditions are given in Figures 2 and 3.

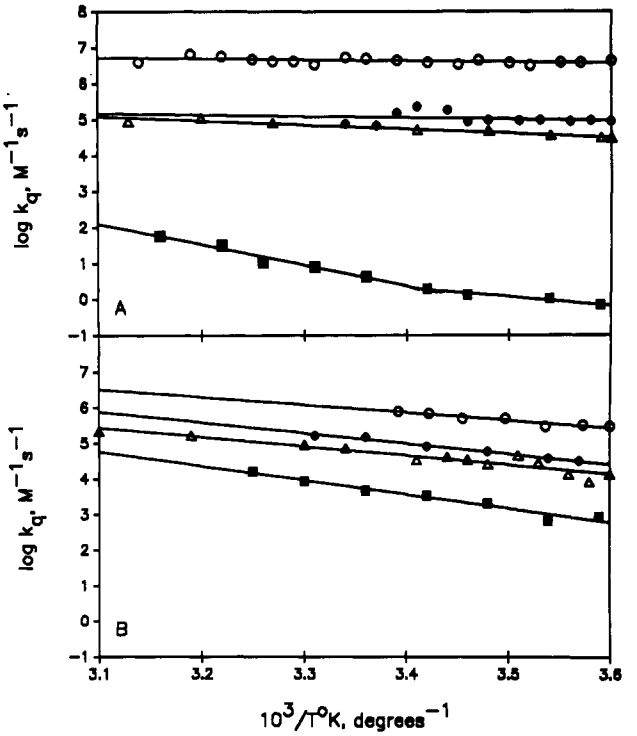

FIGURE 3: Temperature dependence of the bimolecular quenching rate constant, k_q, for phosphorescence quenching. (A) Nitrite; (B) carbon disulfide. (○) Paravalbumin, (●) LADH, and (△) aldolase in 0.1 M NaCl/0.01 M Tris, pH 7; (■) alkaline phosphatase in 0.034 M pyrophosphate, pH 8.6.

Phosphorescence Quenching Constants for CS_2 and Nitrite. We earlier determined that both CS_2 and nitrite quench tryptophan phosphorescence from proteins at room temperature (Calhoun et al., 1988). In this study, we compare the phosphorescence quenching in more detail by determining the quenching constants at different temperatures. The temperature dependences of the quenching constants are shown in Figure 3A for nitrite and in Figure 3B for CS_2. Both molecules showed low energies of activation for all the proteins tested. Nonlinear dependence on the reciprocal temperature was seen for alkaline phosphatase in the case of nitrite quenching; the temperature profiles for other proteins were linear within experimental error. The energies of activation, and the quenching constants at 20 °C, are given in Table II.

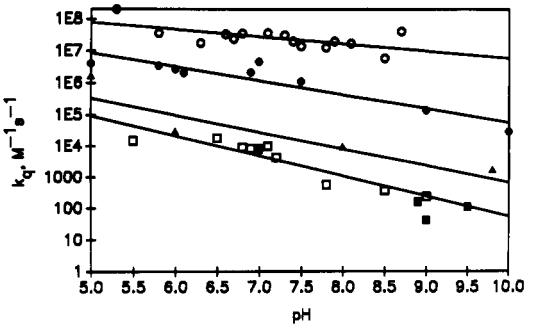

FIGURE 4: pH dependence of the observed bimolecular rate constant, k_q for H_2S quenching. (○) NATA; (●) paravalbumin; (▲) aldolase; (■) azurin; (□) alkaline phosphatase. The media contained 0.3% glucose and 0.1 M NaCl and buffers as follows: pH 5–7.8, 0.01 M PO_4; pH 7.8–9.0, 0.01 M Tris; pH 8.8–9, 0.1–0.15 M serine or 0.1 M L-alanine; pH 9.5, 0.1–0.5 M borate. The sample of NATA also contained 85% glycerol. Temperature: 20 °C.

Table III: Calculated Quenching Constants for H_2S and SH^- for NATA and tryptophan in proteins[a]

protein	k_q, M^{-1} s^{-1}	
	H_2S	SH^-
NATA	4×10^7	6×10^6
parvalbumin	3×10^6	2.5×10^4
LADH	2×10^6	$0 \geq k_q < 1 \times 10^3$
aldolase	2×10^4	2×10^3
azurin	1.8×10^4	<100
alkaline phosphatase	1.3×10^4	50

[a] The conditions are given in Figure 4. [b] The dissociation constant for H_2S was taken to be 10^{-7}.

Phosphorescence Quenching by H_2S. H_2S, previously observed to be a quencher of tryptophan phosphorescence, resembles H_2O in size and shape, and so its quenching behavior may give some insight about the interaction of water with proteins. The p*K* of H_2S is 7, and therefore at neutral pH the quenching may be due to H_2S, SH^-, or both. pH studies were undertaken to evaluate the relative contribution of each species. In Figure 4, the observed quenching constants are given as a function of pH for the four proteins and for NATA in a glycerol medium. It can be seen that as the pH increases, the quenching constant decreases for all the proteins studied.

A dependence of the quenching on pH may arise from several factors. The efficiency of quenching of tryptophan in proteins may be pH-dependent. That this is unlikely is supported by the observation that quenching of tryptophan phosphorescence of alkaline phosphatase by nitrite was independent of pH from 7 to 9 (data not shown). It may be that the quenching efficiencies of H_2S and SH^- differ or that the indole triplet state is affected by pH. Both of these effects were tested by monitoring the phosphorescence quenching of NATA by H_2S. The quenching was only weakly dependent upon the pH (Figure 4), suggesting that both forms can quench. Finally, we are left with the explanation that the accessibilities of H_2S and SH^- to the tryptophan may be different and that this can account for the observed quenching. Taking the p*K* to be 7, we can separate the relative contribution of each species to the quenching. The quenching constants are given in Table III.

Discussion

At a given temperature, phosphorescence lifetimes vary dramatically from protein to protein (Vanderkooi et al., 1987). The determinants of long lifetime in proteins are not known, although it is generally accepted that rigidity of the site contributes to a long lifetime (Strambini & Gonnelli, 1985). This idea is consistent with the observation that tryptophans with long lifetimes are in structured regions. For example, the emitting tryptophan of LADH, Trp-314 (Saviotti & Galley, 1974), is in a β-sheet (Eklund et al., 1976). The single tryptophan of azurin, Trp-48, is within a β-barrel (Admen et al., 1978), and the likely emitting tryptophan of alkaline phosphatase, Trp-109, is on an α-helix that is connected to a β-sheet region (Sowadski et al., 1985). Presumably, a rigid site leads to a long lifetime by protecting against interaction with reacting moieties within the protein, which can include free amino groups and disulfides (Bent & Hayon, 1975) or proton and electron donors (Colucci et al., 1990).

Although the magnitude and specificity of quenching by neighboring amino acid moieties are unknown, temperature changes would cause displacement of the atoms, and since quenching is skewed toward close distance, an increase in temperature would decrease the phosphorescence lifetime, whatever the quenching species and mechanism. The phosphorescence decay of aldolase, paravalbumin, and LADH was observed to show a simple temperature dependence with low energy of activation in the temperature range of 5–50 °C, whereas the temperature dependence of alkaline phosphatase phosphorescence showed a nonlinear Arrhenius behavior (Figure 2) and the $\Delta E_{act.}$ was 21 kJ mol^{-1} in the lower temperature range and 63 kJ mol^{-1} in the higher temperature range (Table I).

Since the phosphorescence decay in the absence of external quenchers and the quenchability of phosphorescence are both likely to be influenced by structural changes in the protein, the first-order decay constant and the second-order quenching constant may show similar temperature dependence. The experimental findings are shown in Figures 2 and 3. In the case of alkaline phosphatase, a discontinuity in the temperature profile of quenching by nitrite was observed, and this occurred in the same temperature range that the phosphorescence lifetime showed a discontinuity in the absence of added quencher (Figure 3). This suggests that a single structural change with temperature could have altered the phosphorescence lifetime and have made the tryptophan more susceptible to quenching by nitrite. In the case of quenching by large molecules, a temperature-dependent quenching of a buried phosphorescent probe has been suggested to arise from domain structural flexibility (Koloczek & Vanderkooi, 1987), and perhaps a similar phenomenon can explain the temperature dependence of alkaline phosphatase phosphorescence.

In relating the temperature dependence and quenching profiles to interaction processes between external molecules and buried tryptophans, we use the experimental data to distinguish between cases which appear to work via a long-range interaction such as electron exchange and cases in which the quenching molecule is thought to approach the lumiphore by diffusive penetration of the protein surrounding the tryptophan residue. Previously, it has been argued that quenching by larger molecules proceeds by long-range transfer and does not involve binding to, or penetration of, the protein. This argument was based in part on two observations for systems in which (1) the tryptophan was sufficiently buried in the protein, and (2) quenchers were sufficiently large, that the quenching rate was dependent only on the rate of reaction with quenchers which were at the distance of closest approach. The first was that the rate-limiting step in these systems was the quenching reaction itself, since the overall rate did not depend upon the diffusion coefficient of the quencher in the aqueous phase (Calhoun et al., 1983). The second was that quenching by these molecules was relatively independent of the size and shape of the quencher (Calhoun et al., 1988). Finally, a

characteristic of the long-range transfer mechanism will be a sensitivity to the distance, falling off exponentially in the case of electron exchange (Marcus & Sutin, 1985). This was seen experimentally for large quenchers and sufficient depth of burial (Vanderkooi et al., 1990).

For the triatomic molecules H_2S and CS_2, however, different patterns of quenching constants and temperature dependence are seen, which suggest that charge appears to play a role in preventing the penetration of NO_2^- into the protein. Whereas nitrite quenching varied strongly from protein to protein, the quenching constants of CS_2 for the proteins alkaline phosphatase, aldolase, and alcohol dehydrogenase were all about 10^4 M^{-1} s^{-1}, in spite of the fact that the tryptophan burial depth ranged from ~7 to 15 Å. The quenching reaction of alkaline phosphorescence by CS_2 showed simple temperature dependence, and unlike the reaction with NO_2^-, CS_2 did not show a break in the temperature profile, suggesting that different processes limit the reaction rates for the two quenchers (Figure 3). For the other proteins, the energies of activation for the first-order quenching, derived from phosphorescence decay in the absence of added quencher (Table I), and for second-order quenching (Table II) are low, but different. Furthermore, the energies of activation differ for the two quenchers nitrite and CS_2 for a given protein. Finally, the charged species, nitrite, showed lower energy of activation and lower quenching constants than did CS_2.

An exception was noted, however, to the pattern that the quenching by charged species depended dramatically upon the location of tryptophan in the protein. The quenching constant of LADH by H_2S was ~10^6 M^{-1} s^{-1}, whereas for both CS_2 and NO_2^- the value was ~10^4 M^{-1} s^{-1}. The emitting tryptophan of LADH is in a β-sheet in the coenzyme binding domain near the interface between each dimer. It may be that H_2S may be small enough to be in contact with the tryptophan, whereas CS_2 and NO_2^- are not, but that the tryptophan is close enough to the surface so that there is no distinction between the charged and uncharged quencher.

The role of charge in preventing penetration of the charged species can be invoked to explain the pH dependence of quenching by H_2S. It was observed that H_2S and SH^- showed comparable quenching of NATA, but differed significantly in their quenching of proteins (Figure 4). The quenching constants for aldolase, azurin, and alkaline phosphatase were all about 10^4 M^{-1} s^{-1}, whereas the quenching constant for parvalbumin was 10^6 M^{-1} s^{-1}. On the basis of the pH dependence, the quenching for SH^- is much less, and its quenching ability is especially reduced for the highly buried tryptophans. Again, we consider that these observations are consistent with diffusion of H_2S and CS_2 through the protein as contributing to the quenching reaction. We need to point out that there is no fundamental difference in the quenching mechanism per se between reactions that depend upon long-range transfer or penetration. When the rate of penetration is faster than long-range transfer, the long-range effects will be negligible.

The temperature dependences of the two types of reaction (i.e., one in which the limiting step is diffusion through the protein or one determined by long-range interactions) have different interpretations. On the basis of crystallographic structures, it is recognized that in order for molecules to diffuse through a protein there must be displacement of the polypeptide chain (Case & Karplus, 1979; Frauenfelder et al., 1979). The energy of activation for this process has been reported in the example of CO through myoglobin and related monomeric heme proteins to range from 22 to 35 kJ mol^{-1} at 300 K (Ansari et al., 1986). Similarly, low energies of activation have also been observed for quenching by diatomic molecules in alkaline phosphatase where the energies of activation of O_2 and NO were 7.4 and 7.9 kcal mol^{-1} or 30.9 and 33.0 kJ mol^{-1}, respectively (Strambini, 1987). For O_2, the triplet quenching activation energy was found to be about the same for both buried and exposed tryptophan (Ghiron et al., 1988).

For the larger molecule CS_2, we observed the energy of activation to be 40–70 kJ mol^{-1} (Table II), significantly higher than observed for diatomic molecules. The diffusion of the larger CS_2 would require larger displacements. One treatment for such a case is given by Kramers (1940), where the diffusing molecule is thought to experience barriers of both heights. A higher energy of activation would be a reflection of the higher barriers experienced by the larger molecule.

The temperature dependence for the electron exchange/transfer mechanism is expected to be low, although some temperature dependence could arise from several factors. Since long-range reaction is nonlinearly dependent upon distance and orientation, increased fluctuations in the polypeptide chain will increase the quenching rate because the reaction rate for a quencher transiently closer to the excited-state tryptophan will be highly enhanced. In addition, temperature may affect the reaction rate by changing the reorganization energy and redox potential (Marcus & Siders, 1982), although we may suppose that these effects could be smaller for the case considered here since there are no detectable temperature-dependent spectral shifts. This supposition is also supported by the observation that for electron transfer between heme-like molecules in proteins, the primary determinant of rate is the distance between redox pairs (Moser et al., 1992).

An important feature of diffusive penetration of uncharged quenchers in the protein is that quenching will be relatively independent of tryptophan location. It can be theoretically shown (see Appendix) that the application of the Smoluchowski treatment of diffusion-dependent quenching to this system leads to a prediction that the observed rate of quenching will be sensitive to the effective diffusion constant (D_s) for motion of the quencher through the protein "shell" separating the tryptophan from the buffer but only weakly dependent on the thickness of that shell. In this treatment of the problem, the D_s dependence gives rise to the temperature dependence of the quenching effects, and D_s is assumed to be essentially zero for charged (nonpenetrating) quenchers and to have approximately the same nonzero value for most uncharged triatomic quenchers.

The value of the effective diffusion constant D_s for triatomic molecules penetrating a protein by diffusion can be estimated from the treatment presented in the Appendix. Taking a value of 2×10^4 M^{-1} s^{-1} as the quenching rate constant for the diffusive penetration process, eq A6 of the Appendix shows that this corresponds to a value of approximately 7×10^{-1} cm^2 s^{-1} for D_s. If we introduce a correction for the fact that the experimental case is not spherically symmetric with a full 4π solid angle of access for the quenchers to the protein, then the calculated value for D_s will be correspondingly greater. If the valid angles of approach for diffusion pathways through the shell correspond to only about $^1/_3$ to $^1/_5$ of a full sphere, then the observed quenching rate constant would correspond to a diffusion constant within the protein of $D \sim (2\text{–}4) \times 10^{-10}$ cm^2 s^{-1}. This calculation ignores all particular features of the protein but provides a simple model which can explain the similar values that were found for the three different proteins aldolase, azurin, and alkaline phosphatase, in which the dis-

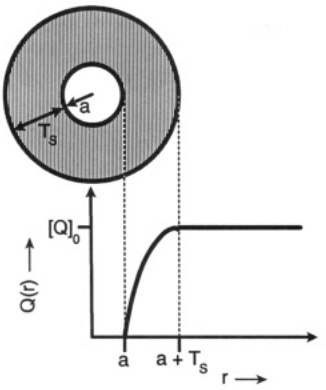

FIGURE 5: Spherical model for protein. The tryptophan has a radius a, and the protein shell of thickness is T_s. The shaded area indicates the shell of protein around the tryptophan. Bottom: Quencher concentration relative to the radius of the protein.

tances of tryptophan from the surface were greatest.

Since H_2S resembles H_2O in size and can substitute for water in some enzymatic reactions (Sivaraja et al., 1988), it can be taken as an analogue of H_2O. Likewise, on the basis of size and shape, CS_2 can be considered as an analogue of CO_2. Then we would conclude from the quenching constants and temperature dependence that structural changes occur which allow for the penetration of these species also and that their diffusion would be on the order of magnitude of the sulfur-containing analogues. The actual value for the oxygen-containing derivative would be larger based upon size, but for H_2O would be countered by its hydrogen-bonding properties.

Appendix

Consider the case of lumiphores (fluor or phosphor) which have a radius a and are shielded from the buffer by a protein shell of thickness T_s. The depth, T_s, can range from zero (for location at the protein–buffer surface) to >10 Å. If the molecules are optically excited at time zero, there is an intrinsic lifetime for the observed luminescence (fluorescence or phosphorescence) which depends in some complex manner upon the environment of the lumiphore. If a quenching molecule is added to the buffer, additional loss of signal will be observed due to the interaction of quenching molecules (Q) with the excited donor lumiphores. In general, the time dependence of the quenching process arises from (1) the intrinsic rate constant $k(r)$ for interaction between donor and quencher molecules separated by a distance r, (2) how close the quenchers are allowed to get to the donor (the depth T_s), and (3) the rate of diffusion of quenchers toward those donors (since, in the random distribution at time zero, some donors will happen not to have nearby quenchers).

The theory of quenching is usually treated by an approximation in which one transforms the problem into a single donor lumiphore at the origin which is surrounded by quenchers which can diffuse up to the interaction distance and react. In the limit of rapid interaction times, quenching is completely diffusion-limited, and the problem is the well-known one originally discussed by Smoluchowski (1918). Conversely, in the limit of rapid diffusion, the rate of quenching depends on the rate constant $k(r)$ but is independent of the diffusion constant (Thomas et al., 1978). In both cases, quenching also depends on the overall quencher concentration Q_0.

In order to estimate the quenching rate for a lumiphore embedded in a protein, we adopt a spherical approximation as shown in Figure 5 (Owen & Vanderkooi, 1991). The diffusion constant for quencher molecules through the protein is given by D_s. The diffusion equation which the quencher concentration $Q(r,t)$ must satisfy in the shell region is the same as that solved by Smoluchowski (1918). Because diffusion within the protein shell will be what limits the rate of quenching, one can use the Smoluchowski boundary conditions of instantaneous reaction at the distance of closest approach ($r = a$). The diffusive shell problem differs from that of Smoluchowski only in the choice of the second spatial boundary condition. Since diffusion in the buffer outside the protein is rapid in comparison with the rate at which quenchers enter the protein, the concentration in the buffer at distances $r \geq a + T_s$ should be constant and equal to the average quencher concentration, Q_0. The usual Smoluchowski problem is just the limiting case of this more general problem when the thickness T_s approaches infinity.

The diffusion equation is

$$D_s\nabla^2 Q(r,t) = \partial Q(r,t)/\partial t \quad \text{(A1)}$$

which is subject to the boundary conditions:

$$Q(a,t) = 0 \quad \text{(A2)}$$

$$Q(a + T_s,t) = Q_0 \quad \text{(A3)}$$

$$Q(r,0) = Q_0 \text{ for } a < r < a + T_s \quad \text{(A4)}$$

These are solved by the usual approach (Carslaw & Jaeger, 1947) and yield the complete time-dependent solution for lumiphores activated at time zero, from which one obtains the time-dependent rate of quenching after excitation at time zero:

$$\Phi(t) = 4\pi a D_s Q_0[1 + a/T_s + a(\pi D_s t)^{-1/2}] \quad \text{(A5)}$$

For the long times of interest in these experiments, the transient term can be ignored. Dividing by the concentration Q_0 in order to obtain a steady-state rate constant, one finds

$$k_q = 4\pi D_s(a + T_s)a/T_s \quad \text{(A6)}$$

A salient feature of quenching in this model is that the rate is only weakly dependent on the thickness of the protein shell, since a and T_s are of comparable size, but it is directly proportional to the diffusion constant D_s. In this model, the temperature dependence of quenching by molecules which show little dependence on the distance from the Trp to free buffer could arise through the temperature dependence of D_s.

Registry No. ADH, 9031-72-5; Trp, 73-22-3; H_2S, 7783-06-4; CS_2, 75-15-0; NO_2, 14797-65-0; SH^-, 15035-72-0; alkaline phosphatase, 9001-78-9; aldolase, 9024-52-6.

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