Quenching of Room Temperature Protein Phosphorescence by Added Small Molecules†

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ABSTRACT: A number of molecular agents that can efficiently quench the room temperature phosphorescence of tryptophan were identified, and their ability to quench the phosphorescence lifetime of tryptophan in nine proteins was examined. For all quenchers, the quenching efficiency generally follows the same sequence, namely, *N*-acetyltryptophanamide (NATA) > parvalbumin ≈ lactoglobulin ≈ ribonuclease T_1 > liver alcohol dehydrogenase > aldolase > Pronase ≈ edestin > azurin > alkaline phosphatase. Quenching rate constants for O_2 and CO are relatively insensitive to protein differences, while H_2S and CS_2 are somewhat more sensitive. These small molecule agents appear to act by penetrating into the proteins. However, penetration to truly buried tryptophans is less favorable than previously suggested; in five proteins studied, quenching efficiency by O_2 is 20–1000 times lower than for NATA, and up to 10^5 lower for H_2S and CS_2. Larger and more polar quenchers—including organic thiols, conjugated ketones and amides, and anionic species—were also studied. The efficiency of these quenchers does not correlate with quencher size or polarity, the quenching reaction has low energy of activation, and quenching rates are insensitive to solvent viscosity. These results indicate that the larger quenchers do not approach the buried tryptophans by penetrating into the proteins, even on the long phosphorescence time scale, and are also inconsistent with a mechanism in which quencher encounter with the tryptophan occurs in free solution, as in a protein-opening reaction. The results obtained suggest that the quenching process involves a long-range radiationless transfer. In addition, the strong apparent correlation between degree of burial and protection against quenching is consistent with the sharp distance dependence expected for a long-range transfer mechanism. Since the conditions for resonance energy (Forster) transfer do not exist, it seems probable that the quenching reaction occurs by way of an electron transfer or exchange between the buried tryptophan in the excited triplet state and the quencher nearby in solution.

It has recently been recognized that phosphorescence can be observed from most proteins at room temperature when O_2 is removed from the solution [Vanderkooi et al., 1987a; see also Saviotti and Galley (1974), Kai and Imakubo (1979), and Strambini (1987)]. Phosphorescence can be used to study structure and motional processes in proteins in much the same way as fluorescence. This paper reports on the interaction of small molecules with proteins, studied by their ability to quench the phosphorescence of protein tryptophans.

Starting with the work of Lehrer (1971), protein fluorescence quenching has been used to study the ability of added small molecule agents to reach exposed and buried tryptophan indole rings. The work of Lakowicz and Weber (1973) suggested that O_2 might penetrate easily into proteins, and this concept was extended by others to include small organic molecules like acrylamide [reviewed by Eftink and Ghiron, (1981)]. Our more recent studies with a number of proteins and various quenchers now indicate that when measurable quenching of protein fluorescence by added organic quenchers can be demonstrated, tryptophans present are at least partially solvent accessible, so that protein penetration need not be invoked. Further, for several proteins with truly buried tryptophans, fluorescence quenching is not observed (Calhoun et al., 1983b,1986), suggesting that protein penetration does not occur. However, fluorescence quenching experiments are limited to the nanosecond time scale characteristic of fluorescence, and for practical reasons limited to the measurement of rate constants down to about 10^8 M^{-1} s^{-1}. The much longer phosphorescence time scale, in some cases out to seconds, extends the dynamic range of quenching measurements by about 8 orders of magnitude and thus can allow the measurement of reactions that occur much more slowly and provide a more searching test of the ability of various small molecule quenchers to interact with truly buried tryptophans (Calhoun et al., 1983a,b).

As yet, little is known about the kinds of molecules that may influence protein tryptophan phosphorescence. One goal of this study was to identify small molecules that can effectively quench the phosphorescence of tryptophan when it is freely exposed in solution. One can then investigate the ability of such molecules to interact with tryptophans buried within the protein matrix. As in earlier fluorescence work, our experimental approach was to study the patterns of quenching exhibited by a variety of agents on the phosphorescence of a variety of proteins. The quenching efficiency of identified quenching molecules having differing size and charge on the phosphorescence of nine proteins was measured, and the effect of temperature and viscosity was determined. As part of this study, quenching constants for O_2, which has been thought capable of diffusing freely through proteins, were redetermined by use of a novel approach utilizing an internal phosphor to monitor very low solution oxygen concentrations.

The mechanism by which small, large, polar, and nonpolar molecular agents quench the phosphorescence of protein tryptophans is of fundamental interest for the study of protein dynamics. The interaction may reflect some degree of tryptophan exposure to the solvent, a penetration of the quencher into the protein matrix, or some protein unfolding behavior

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that transiently exposes the trytophan. Alternatively, interaction may be achieved without contact by long-range transfer. The results described here bear on these issues.

Materials and Methods

Proteins were obtained from Sigma Chemical Co. as follows: horse liver alcohol dehydrogenase (LADH)[1] as a one-time crystallized product, alkaline phosphatase from *Escherichia coli* as a suspension in 2.5 M $(NH_4)_2SO_4$, bovine liver catalase as a powder, glucose oxidase from *Aspergillus niger* as a powder, Pronase from *Streptomyces griseus*, edestin from hemp seed, aldolase from rabbit muscle, ribonuclease T_1 (RT_1) from *Aspergillus niger*, and β-lactoglobulin from bovine milk. Parvalbumin (with a single tryptophan) was prepared from frozen cod fillets as previously described (Calhoun et al., 1986). Azurin was prepared from *Pseudomonas aeruginosa* (Ambler, 1963; Ambler & Brown, 1967). Sodium dithionite, a gift from Virginia Chemicals Inc. (Portsmouth, VA), was stored under argon at −20 °C. Glycerol, ethanethiol, $NaNO_2$, methyl vinyl ketone, acrylamide, quinaldic acid, cinnamamide, and nicotinamide were obtained from Sigma. Glycerol was stored at 4 °C in the dark.

Oxygen Removal from Samples. Oxygen removal was achieved as described by Englander et al. (1987). In this method, the buffer solution containing 0.3% glucose is initially degassed under an aspirator and then bubbled with argon. The protein is dissolved in the buffer and placed in a cuvette containing a glass-coated micro stir bar, and the air space is filled with argon. A small volume of solution containing glucose oxidase and catalase is added to give a final concentration of 80 nM and 16 nM, respectively. The enzyme system reduces O_2 to H_2O_2 and then to H_2O. After 10 min, 5–10 μL of freshly prepared dithionite is added to give a final concentration of about 0.1 mM. Oxygen-free mineral oil (deoxygenated by stirring under vacuum and shaking vigorously with sodium dithionite solution) is layered on top of the cuvette to a depth of 0.5 cm. The cuvette is then closed with a quartz stopper. Through these operations, air is excluded by a constant flow of argon gas.

Preparation of Solutions of Quenchers. Stock solutions of quenchers were deoxygenated as for the protein solutions, above. Aliquots were added to the phosphor samples by glass λ pipets. The sample was layered with argon during this procedure.

A solution of carbon monoxide at equilibrium with 1 atm of CO was prepared by bubbling CO through the buffer containing glucose, glucose oxidase, and catalase. The CO concentration was taken to be 9×10^{-4} M.

Stock solutions of H_2S were prepared by bubbling with H_2S gas, produced by adding an excess of $MgCl_2$ in solution to solid calcium sulfide. The water was initially treated with the glucose–glucose oxidase–catalase system to remove all traces of oxygen which reacts slowly with H_2S to produce mineral sulfur. The H_2S gas evolved was bubbled for 1–2 h through water with mineral oil layered on the top to yield a saturated solution of H_2S (0.1 M at 25 °C). During this procedure, the pH of the unbuffered water drops to low values since H_2S is a weak acid ($pK = 7.0$). Aliquots of the stock H_2S solutions were introduced to protein samples under flowing argon by λ pipets. This preparation was performed in a fume hood.

Measurement of Oxygen Concentration. A water-soluble oxygen-sensitive phosphor, palladium coproporphyrin (Porphyrin Products, Logan, UT), was used in protein solutions as an internal probe for oxygen concentration. The oxygen concentration present was calculated from the measured lifetime of the probe using a quenching constant of 3×10^9 M^{-1} s^{-1} (Vanderkooi et al., 1987). The measurement is described under Results and in the legends of Figures 1 and 2. Oxygen concentration was varied either by adding aliquots of an oxygen-containing buffer or by allowing the oxygen present to be slowly consumed by the glucose oxidase enzyme system. In the latter mode, sequential lifetime measurements were made, alternating between sample and probe (see Results).

Instrumentation and Analysis. Absorption spectra were obtained with a Perkin-Elmer 200 or a Cary 118 spectrophotometer. Fluorescence and phosphorescence spectra were obtained on a Perkin-Elmer LS-5 luminescence spectrometer. Phosphorescence lifetimes were measured by using the instrument described by Vanderkooi et al. (1987), with an excitation wavelength of 295 nm and an emission wavelength 440 nm. Protein concentrations were typically 2 mg/mL, and the temperature was close to 22 °C except where indicated.

Phosphorescence lifetimes were obtained from decay profiles by analysis for exponential decay using the Asystant program (Macmillan Software Co, New York, NY). The experimental procedure selects for the longest lived component of the decay, and the decay of phosphorescence could generally be fit by a single-exponential function (Figure 2). Quenching was monitored by the decrease in lifetime. Quenching rate constants, k_q, were computed from the equation of Stern and Volmer (1919), modified for lifetimes:

$$(\tau_0/\tau - 1)/\tau = k_q[Q] \quad (1)$$

Here τ_0 represents the lifetime in the absence of quencher and τ is the lifetime in the presence of quencher at concentration [Q].

Results

Phosphorescence Quenching Agents. A survey was performed to identify molecules and ions that can produce significant phophorescence quenching and show other desired properties (solubility and stability). Candidate quenchers were tested against the tryptophan analogue *N*-acetyltryptophanamide (NATA), freely accessible in solution. The phosphorescence lifetime of NATA, about 20 μs [measured by transient absorbance by Evans et al. (1976), Bent and Hayon (1975), and Pepmiller et al. (1983)], is too short to be reliably measured by our instrumentation, so these tests were performed in concentrated glycerol to extend the lifetime, as described by Strambini and Gonelli (1985). In the 85% glycerol used, the NATA lifetime is 0.5 ms.

Table I lists the quenching rate constants measured for a number of efficient quenching agents against NATA (85% glycerol) and a series of proteins. Quenching of NATA phosphorescence in glycerol by O_2 could not be measured due to rapid photolysis of the O_2 present but has been directly measured in water at 3×10^9 M^{-1} s^{-1} (Eftink & Hagaman, 1986).

In order to determine the quenching efficiency of our agents against NATA in water, it is necessary to correct for the effect of the 85% glycerol solvent in which relative viscosity is 100. To a first approximation, the rate constants measured can then be multiplied by 100 to correct for the slowing effect of solvent viscosity, though other problems may enter, including possible solvent effects (Eftink & Ghiron, 1984), long-range transfer, and also the issue of microviscosity, especially for the quenchers that are smaller than the glycerol molecule. In an effort to

[1] Abbreviations: NATA, *N*-acetyltryptophanamide; LADH, liver alcohol dehydrogenase; RT_1 ribonuclease T_1; Tris, tris(hydroxymethyl)-aminomethane; k_q, second-order quenching rate constant.

Table I: Phosphorescence Quenching Constants, log k_q (M^{-1} s^{-1}), at 22 °C

	quencher										
protein	O_2	CO	H_2S[e]	CS_2	NO_2^-	ethanethiol	methyl vinyl ketone	nicotinamide	cinnamamide	quinaldic acid	τ_0 (s)
NATA[a]	(9.5)[f]	6.5	7.2	8.3	7.8	7.5	7.6	7.6	7.6		0.0005
parvalbumin[b]	8.5	5.4	6.4	7.6	6.3	6.5	5.6		7.4	6.8	0.005
lactoglobulin[c]	8.8	4.6		7.8	6.3	5.2	5.7	4.9			0.015
RT_1[c]		4.6	5.0	6.8	5.7	4.3	5.7	4.5	7.6		0.014
LADH[d]	8.0	4.9	6.0	6.5	4.7	4.6	4.3		5.2	5.3	0.3
aldolase[d]	7.6	4.2	3.8	3.8	3.7	2.5	3.2	3.1	4.3	5.4	0.04
Pronase[d]		4.9	4.6	4.7	3.4	2.7	2.8	1.2	4.0		0.8
edestin[d]	7.9	4.6			3.1	2.8	3.8		4.6		0.4
azurin[d]	7.3	4.5	5.3	3.3	2.9	2.1	2.5	0.0	3.4	3.2	0.4
alkaline phosphatase[d]	6.3	4.4	3.9	3.5	1.7	1.0	1.2			3.3	1.5

[a] Relative viscosity = 106; 85% glycerol, 0.1 M NaCl, and 0.01 M phosphate, pH 7.0. [b] 0.1 M NaCl, 1 mM $CaCl_2$, and 0.01 M Tris, pH 7.0. [c] 0.1 M NaCl and 0.01 M phosphate, pH 7.0. [d] 0.034 M pyrophosphate, pH 8.6. [e] Same as above at pH 6.5. Values for alkaline phosphatase and LADH correct a misprint in Calhoun et al. (1983b). [f] Measured in water with relative viscosity = 1 (Eftink & Hagaman, 1986).

gain insight here, some quenchers were tested against eosin phosphorescence in water and in 85% glycerol. Phosphorescence quenching constants were all much lower than the diffusion-limited value even in water (range $\sim 10^5$–10^8 M^{-1} s^{-1}). For H_2S and methyl vinyl ketone, the ratios of quenching constants in the two solvents were close to 100 (ratio = 83 and 180, respectively) but not for nitrite (ratio = 7). Though some details remain unclear, we gather from these results that the quenchers listed in Table I can quench the phosphorescence of freely exposed tryptophan with rate constants in the range about 10^9 M^{-1} s^{-1} and that these diffusional encounters respond significantly to solvent viscosity.

The efficient quenching agents used fall into three categories, defined by their protein quenching ability and their molecular characteristics. The small diatomic and triatomic quenchers—O_2, CO, H_2S, and CS_2—tend to maintain relatively high quenching constants even against the most protected tryptophans. An intermediate group—ethanethiol, methyl vinyl ketone, and the small but charged nitrite anion—exhibits lesser quenching ability against the well-protected tryptophans. Even though these agents are very disparate in molecular character, they show similar quenching behavior. Also, nicotinamide (3-pyridineamide) can be placed in this group. The third class consists of the larger molecules, cinnamamide (3-phenylacrylamide) and the bicyclic quinaldic acid (fused phenyl and pyridine rings with a COOH substituent).

Quenching constants due to NaBr, KI, $NaNO_3$, CsCl, acetone, and methyl ethyl ketone were low, less than 10^6 M^{-1} s^{-1} for NATA in 85% glycerol and 100 for LADH (Calhoun et al., 1983b), and were therefore not examined further. The moderately effective quencher acrylamide was not studied since it is sensitive to free radical polymerization under our deoxygenation conditions.

Protein Phosphorescence Quenching by Oxygen. It has proven difficult to determine the quenching of protein phosphorescence by oxygen because of the difficulty of controlling oxygen concentrations in the low nanomolar range required for such studies (Saviotti & Galley, 1974; Calhoun et al., 1983a; Barboy & Feitelson, 1985; Strambini, 1987). The oxygen present may be photochemically destroyed under illumination by the lamp (Calhoun et al., 1983a; Strambini, 1983) or increased by inadvertent contamination from the air.

This experimental difficulty was solved by including in the protein samples the soluble dye palladium coproporphyrin, whose triplet lifetime dependence on oxygen is known (Vanderkooi et al., 1987b). Figure 1 displays phosphorescence excitation and emission spectra of azurin and palladium coproporphyrin. With both phosphors present, tryptophan

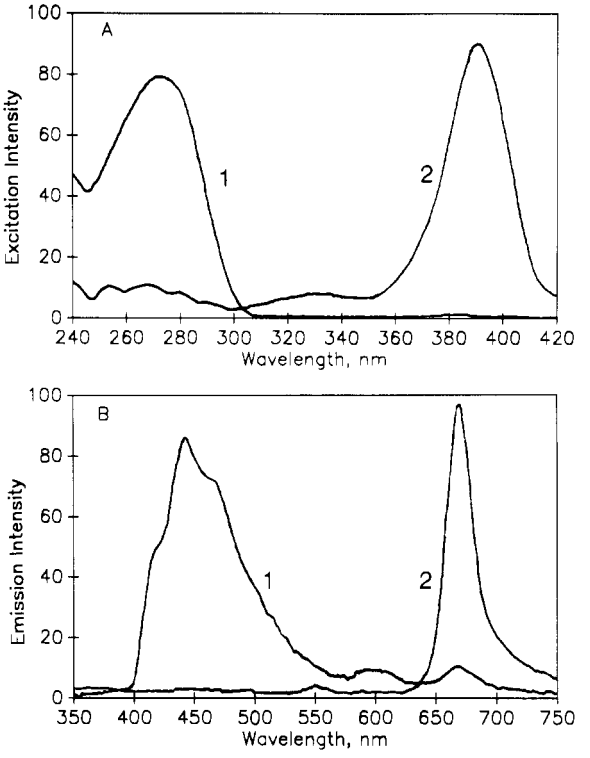

FIGURE 1: Emission spectrum of azurin. The sample contained azurin at 2 mg/mL and 1 nM palladium coproporphyrin in 0.034 M pyrophosphate solution at pH 8.6 with the deoxygenating enzyme system (40 nM glucose oxidase, 16 nM catalase, and 0.3% glucose). (A) Excitation spectra with emission at 445 (1) or 670 nm (2); (B) emission spectra with excitation at 295 (1) or 390 nm (2). Delay time was 0.5 ms and gate time 2 ms. Effective band-pass was 15 nm for excitation and 10 nm for emission. Intensity scales are arbitrary. Gain was 5 times greater for spectra 1 than spectra 2. Temperature was 22 °C.

emission can be selectively measured by using excitation at 295 nm and an emission wavelength of 445 nm, while the wavelength pair 390 and 670 nm allows the measurement of palladium coproporphyrin without interference from tryptophan. Variation of palladium coproporphyrin concentration in the range 1–10 nM did not affect the phosphorescence lifetime of tryptophan in the proteins studied. In quenching experiments, oxygen concentration was varied by adding a low concentration of glucose oxidase to protein solutions containing also 0.3% glucose and 16 nM catalase, and the phosphorescence lifetimes of tryptophan and palladium coproporphyrin were measured alternately as the oxygen was slowly consumed. The coproporphyrin measurement served to monitor oxygen

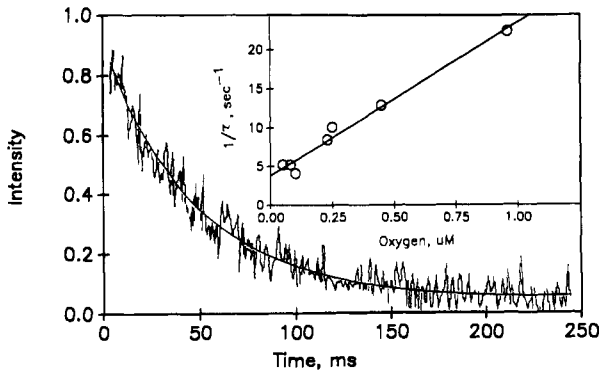

FIGURE 2: Decay profile of azurin. Conditions were as for Figure 1, with excitation at 295 nm and emission at 445 nm. The inset shows an inverse lifetime plot for phosphorescence quenching of azurin by oxygen.

concentration. For each protein studied, phosphorescence decay data like those shown in Figure 2 for azurin were fit by a single-exponential decay function. Lifetimes plotted against oxygen concentration as in eq 1 produced straight-line Stern–Volmer plots from which the second-order quenching rate constants (k_q) in Table I were determined.

Oxygen quenching constants for alkaline phosphatase and liver alcohol dehydrogenase (Table I) are lower than some previously reported values (Calhoun et al., 1983a; Barboy & Feitelson, 1985) but compare favorably with results found by Strambini (1987), 1.2×10^6 and 3.6×10^7 M^{-1} s^{-1}, respectively. The low rate constants reached for these and other proteins with buried tryptophans (Table I), lower than for NATA by 20–10^3-fold, modify the conclusion that O_2 can diffuse easily through proteins in general (Lakowicz & Weber, 1973). Among the 14 proteins studied in the classical O_2 quenching studies of Lakowicz and Weber (1973), only azurin is now known to have a buried tryptophan and no exposed tryptophans.

The oxygen quenching constant measured (Table I) for azurin phosphorescence, 2×10^7 M^{-1} s^{-1}, compares with 1×10^8 M^{-1} s^{-1} determined for fluorescence quenching by Lakowicz and Weber (1973). The difference may be due to a statistical factor for phosphorescence quenching by oxygen between 1/9 (Gijzeman et al., 1973) and 5/9 [reviewed by Saltiel and Atwater (1988)]. A similar effect appears in the oxygen quenching of NATA; reported quenching constants are just over 1×10^{10} M^{-1} s^{-1} for fluorescence quenching (Lakowicz & Weber, 1973; Calhoun et al., 1983a) and 3×10^9 M^{-1} s^{-1} for phosphorescence quenching (Eftink & Hagaman, 1986).

Quenching by Small and Large Molecule Agents. The proteins studied are listed in Table I in order of generally decreasing quenchability. The protein rank order hardly changes from one quencher to another. Figure 3 illustrates these relationships. In comparing quenching efficiencies for proteins and NATA in Table I, it should be remembered that the NATA values listed were measured in 85% glycerol where relative viscosity is 106.

Among the small molecule quenchers (Figure 3A), O_2 maintains the highest quenching efficiency, though this drops off significantly for the well-buried tryptophans in azurin and alkaline phosphatase. CO shows flat behavior and seems less sensitive than oxygen to protein differences, hardly distinguishing one protein from another. Quenching rate constants for the triatomic sulfur-containing agents, H_2S and CS_2, decrease more sharply through the list of proteins. They appear to track each other fairly closely, but note that CS_2 starts with

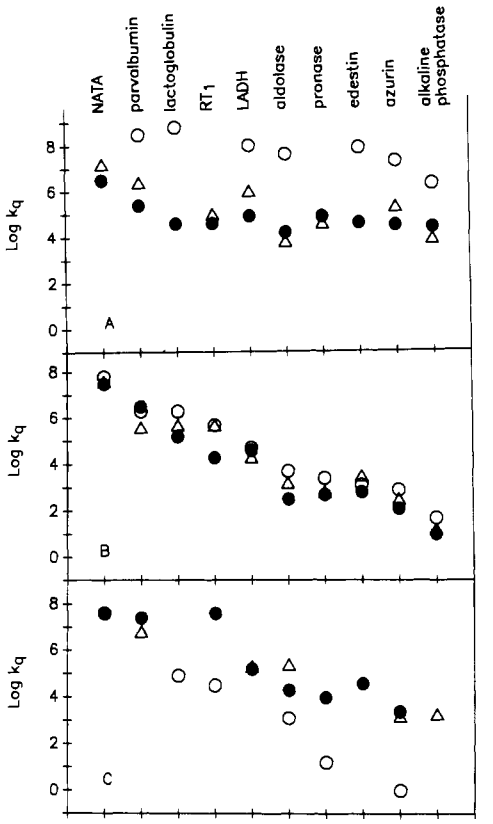

FIGURE 3: Quenching profiles. (A) O_2 (○), CO (●), and H_2S (△); (B) nitrite (○), methyl vinyl ketone (●), and ethanethiol (△); (C) nicotinamide (○), cinnamamide (●), and quinaldic acid (△).

greater efficiency against NATA and reaches a lower level against the most protected tryptophans.

Quenching constants of the larger quenchers change sharply through the different proteins; values of k_q range over 7 orders of magnitude. Through the list of proteins, the quenchers nitrite, ethanethiol, and methyl vinyl ketone, which have quite different molecular character, exhibit regularly decreasing rate constants that are surprisingly similar (Figure 3B). The still larger phenyl ring containing quenchers also decrease in efficiency through the list of proteins, but it is remarkable that, when they are tested against the well-protected proteins, the larger quenchers are more effective than the mid-size ones.

Effect of Solvent Viscosity. Quenching constants were determined for representative proteins and quenchers as a function of solvent viscosity, adjusted by glycerol concentration. Results are given in Table II. Relative viscosity is 4 in 45% glycerol, 6 in 50% glycerol, 22 in 70% glycerol, and 106 in 85% glycerol. The presence of glycerol had little effect on protein phosphorescence lifetimes. The protein quenching constants show only small effects of solvent viscosity, much smaller than expected for a reaction limited by diffusion through the solvent, and found in the tests with eosin described above. This is so even for parvalbumin (Table II), the tryptophan of which apparently abuts the protein surface (Calhoun et al., 1986).

LADH is the only protein that consistently exhibits a measurable glycerol effect, and this is generally small. Since LADH phosphorescence is due to a tryptophan enclosed at the intersubunit interface (Saviotti & Galley, 1974), some contribution due to a transient glycerol-sensitive subunit separation (Gekko & Timasheff, 1981) that opens the way for in-diffusing quencher may be indicated.

Energy of Activation. The temperature dependence for quenching by nitrite and methyl vinyl ketone was determined for both aldolase and parvalbumin. Results are shown in

Table II: Viscosity Dependence of Quenching Constants, k_q (M^{-1} s^{-1})[a]

protein	quencher	glycerol (%) 0	45	50	70	85
parvalbumin	$NaNO_2$	2×10^6		2×10^6		4×10^5
	methyl vinyl ketone	4×10^5		4×10^5		6×10^5
LADH	CO	9×10^4			1×10^5	
	H_2S	1×10^6			6×10^5	
	CS_2	3×10^6			4×10^5	
	ethanethiol	4×10^4			1×10^4	
	$NaNO_2$	5×10^4	2×10^4			
Pronase	$NaNO_2$	2×10^3			2×10^3	
azurin	$NaNO_2$	7×10^2			4×10^2	
alkaline phosphatase	CO	3×10^4			2×10^4	
	H_2S	8×10^3			5×10^3	
	CS_2	3×10^3	2×10^3		5×10^2	
	ethanethiol	11			9	
	$NaNO_2$	50			45	
	methyl vinyl ketone	16			20	

[a] Medium conditions are given in Table I. Relative viscosities at 22 °C are 4 in 40% glycerol, 6 in 50% glycerol, 22 in 70% glycerol, and 106 in 85% glycerol.

Figure 4. The energy of activation found for both proteins is low, 3 kcal/mol for nitrite and 6 kcal/mol for methyl vinyl ketone.

DISCUSSION

This paper reports an initial survey of the characteristics of protein phosphorescence quenching, using nine proteins and a range of molecular quenchers. One wants to use the data available to consider the mechanisms at work.

Tryptophan Accessibility, Rigidity, and Burial. The proteins used in this study, chosen from a list of 29 proteins that show easily measurable phosphorescence in oxygen-free solution at room temperature (Vanderkooi et al., 1987), are listed in Table I in order of decreasing quenchability. The high quenchability of the three proteins at the top of Table I appears to reflect their closeness to the protein surface. In an earlier *fluorescence* quenching study, the single tryptophan in parvalbumin and that in ribonuclease T_1 were judged to be partially solvent exposed, since they were rapidly reached and quenched by added quenchers that are unable to penetrate into proteins on the nanosecond fluorescence time scale (Calhoun et al., 1986). Indeed, the RT_1 tryptophan has been found by X-ray crystallography to have one edge marginally accessible at the protein surface (W. Saenger, personal communication). In the same fluorescence study, these partially exposed protein tryptophans and also the well-protected tryptophans in LADH and alkaline phosphatase were found to be quenched effectively by NO_2^- and methyl vinyl ketone acting as Forster transfer agents with a small characteristic transfer distance, R_0, of about 10 Å. Thus, even the most protected tryptophans of the proteins in Table I can be rather close to the protein surface. This can be seen as a simple consequence of the geometry of protein molecules, which does not allow for very deep burial (Chothia, 1976).

The results in Table I display a strong correlation between phosphorescence lifetime and protection against quenching ($\tau_{ph} \sim 1/k_q$). We earlier observed (Vanderkooi et al., 1987a) that protein phosphorescence lifetime tends to correlate with tryptophan burial, judged by the blue shift in fluorescence emission ($\tau_{ph} \sim$ burial). It appears that tryptophan burial produces both long phosphorescence lifetime and protection against quenching.

The detailed factors that determine the great range of protein phosphorescence lifetimes [about 6 orders of magnitude; see Vanderkooi et al., (1987a)] are not known. Site rigidity seems to be a major factor. Proteins in general exhibit

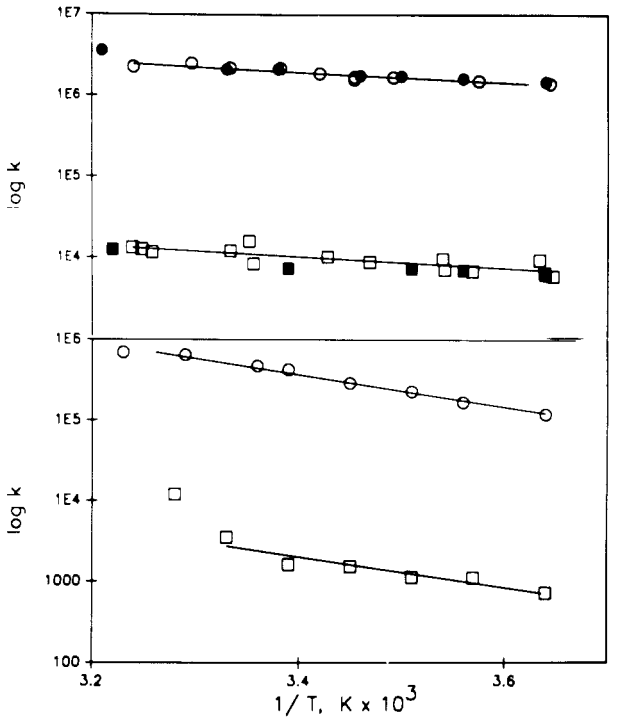

FIGURE 4: Temperature dependence for phosphorescence quenching of parvalbumin and aldolase. The temperature dependence of quenching by nitrite (top) and methyl vinyl ketone (bottom) is shown. The samples contained parvalbumin (○, ●) at 2 mg/mL in pH 7, 0.1 M tris, 0.1 M NaCl, and 2 mM $CaCl_2$ or aldolase (□, ■) at 2 mg/mL in pH 7, 0.1 M phosphate, and 0.1 M NaCl. The enzymic deoxygenating system was also present.

phosphorescence lifetimes of ≥5 s when frozen into rigid glasses. That this is not simply a low-temperature phenomenon is suggested, for example, by the behavior of alkaline phosphatase, which approaches this same lifetime in solution at room temperature. Further, Strambini and Gonelli (1985) showed that the phosphorescence lifetime of free tryptophan increases regularly with solvent viscosity, which can kinetically inhibit out-of-plane distortions that produce radiationless decay.

In correlating these parameters, one can infer that phosphorescence lifetime tends to increase with degree of burial because of the concomitant increase in site rigidity. Depth of burial in itself will determine quenchability when the quenching mechanism involves long-range transfer as suggested below. That is, the cause–effect relationship may be as follows:

Table III: Characteristics of Quenching Mechanisms

	quencher size effect	viscosity effect	ΔE^*_{app} (kcal)
surface contact	no	yes	3
surface insertion	yes	yes	~3
penetration	yes	no	≫3
opening	no	yes	3 + 0–10
long range	no	no	low

$1/k_q \leftarrow$ burial $\rightarrow$ rigidity $\rightarrow \tau_{ph}$.

Experimental Recognition of Different Quenching Mechanisms. Does the quenching process involve collison of the quenchers with tryptophan at the protein *surface*, a *penetration* of quencher into the protein, a protein-*opening reaction* that transiently exposes the normally buried tryptophan, or a *through-space transfer*? One appreciates that in the different cases observed here various mechanisms may play a role. Formal rate expressions and energy relationships characteristic of *surface contact*, *penetration*, and *opening* mechanisms can be found in Englander and Kallenbach (1984). We consider here the defining characteristics of these different possible quenching mechanisms so that they can be compared with the experimentally evaluated parameters, namely, the effect of quencher size and polarity (Table I and Figure 3), solvent viscosity (Table II), activation energy (Figure 4), and the lifetime–quenchability relationship (Table I). Expectations for the different quenching mechanisms are summarized in Table III.

The possible penetration of small molecules into proteins has been addressed by many authors in relation to protein hydrogen exchange behavior [reviewed by Englander and Kallenbach (1984)], in relation to fluorescence and phosphorescence quenching [Lakowicz & Weber, 1973; Coppey et al., 1981; see review by Eftink and Ghiron (1981); Gratton et al., 1984; Calhoun et al., 1983a,b], and by computer simulations (Richards, 1973; Case & Karplus, 1979). The term penetration might be considered to include a shallow *insertion* reaction past the most exposed surface groups. As for free surface collisions, insertion of the active substituent of a quencher molecule into crevices of the protein surface can be expected to depend kinetically on solvent viscosity and in this way to be operationally identified as an exposed *surface contact*.

We will consider *penetration* to imply actual entry of the small molecule quencher into a nonaqueous region of the protein accompanied by some (hindered) permeation through the protein matrix, so that the rate-limiting diffusive step leading to quencher–phosphor encounter occurs away from the solvent. Experimentally, one can expect penetration-dependent quenching to be largely insensitive to solvent viscosity (van Gunsteren & Karplus, 1982) but very sensitive to quencher size (Richards, 1973) and polarity. It can further be expected that penetration-dependent quenching will show sizable activation energy, i.e., significantly above the ~3 kcal characteristic of aqueous diffusion (Eftink & Ghiron, 1975; Beece et al., 1980).

The quenching reactions may be mediated by gated *opening or unfolding* reactions that expose normally masked sites to direct contact with solvent species. Here, as for normally surface-exposed groups, the quencher–phosphor encounter occurs in the aqueous solvent, so that sensitivity to solvent viscosity can be expected. Concentrated glycerol can further slow opening-dependent encounters by its inhibitory effect on the protein-opening reaction itself (Gekko & Timasheff, 1981), as has been shown in hydrogen-exchange experiments (Calhoun & Englander, 1985). If the phosphor is freely exposed to solvent at the instant of encounter, the quenching reaction will be insensitive to quencher size. However, if the dominant quenching reaction still involves some insertion behavior, then quencher size and shape may still be a factor. For gated exposure, the apparent activation energy will include the activation energy characteristic of encounters in the freely exposed state plus a contribution from the structural gating reaction. Since reclosing of the structural gate (nanoseconds to milliseconds) is certainly much faster than the phosphorescence quenching time scale (milliseconds to seconds), gating enters the kinetic expression as a prior equilibrium step, and it is the equilibrium free energy of the gating reaction that enters the apparent activation energy. Experience with hydrogen-exchange reactions suggests that segmental opening energies are in the range 0–10 kcal (Englander & Kallenbach, 1984).

Finally, one can consider long-range virtual photon or electron transfer between quencher and phosphor as a possible quenching mechanism. The activation energy for long-range transfer is small. The rate of transfer reactions depends strongly on distance, so that the quenching rate will decrease sharply with depth of burial. Again, quencher size and polarity will be important if the quencher approaches the phosphor by a through-protein penetration, but not if the quencher remains in the solvent. In either case, solvent viscosity will generally not be a factor. Even if the quencher remains in the solvent, the quenching reaction will be viscosity independent, since a long-lived phosphorescing system will be in the so-called rapid-diffusion limit (Thomas et al., 1978). For example, in 1 ms, short on the phosphorescence time scale, quencher molecules in free solution diffuse about 1 μm on average, much further than the average distance between quencher molecules (~100 Å at 1 mM). Thus, quenching rate will respond to the time-averaged concentration of quencher molecules and not to their diffusional velocity.

Larger Quenchers. Let us compare the mechanism-related characteristics listed in Table III with the quenching behavior found for the mid-sized quenchers, ethanethiol, methyl vinyl ketone, and nitrite. The possibility of *surface contact* can be rejected. Little viscosity effect is seen (Table II), and the phosphorescent tryptophans of LADH, azurin, and alkaline phosphatase are known to be buried. A *penetration* mechanism can also be ruled out, since these quenchers, even though they differ greatly in size and polarity, exhibit impressively similar rate constants against each of the proteins through the whole range tested. Further, the activation energies measured (3–6 kcal; Figure 4) are too small to support a penetration mechanism. In earlier work with a more limited data set, we noted the similarity in phosphorescence quenching rate constants for disparate quenchers seen for LADH and suggested that this might reflect an *opening-dependent* pathway. Indeed, the data in Table II do show a persistent albeit small viscosity dependence for LADH, consistent with an opening pathway, but this is not seen for the other proteins. Therefore, with the possible exception of LADH, the data available when considered in light of the criteria listed in Table III are against surface contact, penetration, and opening mechanisms. The data do appear to be consistent with a long-range transfer mechanism.

The same reasoning applies to the larger, cyclic quenchers in Table II. Quenching cannot represent surface contact since many of the tryptophans are not surface exposed. Penetration is unlikely, since quenching does not decrease with increasing quencher size. In fact, a reverse size dependence is seen; cinnamamide and quinaldic acid, though they are larger than

the mid-size quenchers, exhibit a higher quenching constant against the more protected tryptophans. An opening pathway also seems improbable. Any opening reaction that would produce the quenching constants found for the larger agents should have been available also to the smaller ones. These considerations again point to a long-range transfer mechanism.

If quenching is to be explained by long-range transfer, why is it that some of the larger quenchers exhibit higher quenching rate constants than the smaller ones? One possibility is that the conjugated cyclic quenchers have a more favorable redox potential. Alternatively, these agents may have significant binding affinity to the proteins. Long-range transfer exhibits strong distance dependence, and binding even at a low level can greatly reduce the time-averaged transfer distance. It is interesting that the phenyl-containing quenchers show this behavior while nicotinamide does not. The general ability of phenyl derivatives to bind to proteins has been noted before (Behe & Englander, 1979).

Smaller Quenchers. That small molecules might diffuse rather freely through proteins was first suggested by Lakowicz and Weber (1973) on the basis of the ability of O_2 to quench the *fluorescence* of a number of proteins with high quenching rate constant. Over the years, there has been some uncertainty about the generality of this result. *Phosphorescence* quenching experiments can provide a good test of this thesis since the measurement selectively detects the most buried tryptophans. To measure the low concentrations of O_2 that have previously made this kind of experiment so difficult, the present work used an internal O_2-sensitive phosphorescent probe. Table I shows that O_2 does indeed maintain fairly high quenching ability against even the most protected tryptophans, though somewhat less than has been expected. Between NATA and the partially exposed tryptophan of parvalbumin, the O_2 quenching constant decreases by a factor of 10, then by another factor of 10 for the well-protected tryptophan of azurin, and then another 10-fold for alkaline phosphatase. The behavior of CO shows some similarities and some differences. CO appears to have lower quenching efficiency than O_2. It similarly decreases by about 10-fold between parvalbumin and azurin; then, however, it appears to maintain this quenching level against alkaline phosphatase.

The behavior seen for O_2 and CO is suggestive of a penetration mechanism. Since the tryptophans are not very deeply buried, however, one can consider for these smallest quenchers the possibility of a near-surface insertion reaction that might reach the tryptophan without the need for significant internal diffusion through the protein matrix. This kind of behavior should presumably be marked by sensitivity to solvent viscosity (Table III). In general, O_2 and CO exhibit non-Stokes behavior with respect to solvent viscosity; diffusional rate constants in glycerol–water mixtures vary inversely only with the square root of viscosity [e.g., see Hasinoff and Chisti (1983)]. The results in Table II, however, show that the quenching of LADH and alkaline phosphatase by CO has no significant viscosity dependence, even when viscosity is increased by 20-fold. Apparently, the rate-limiting diffusional approach and encounter with the buried tryptophans occurs away from the influence of solvent, i.e., within the protein.

Through the list of increasingly protected tryptophans, quenching constants for the triatomic quencher H_2S decrease somewhat more sharply than for O_2 and CO but much less sharply than for the mid-sized quenchers. One suspects that H_2S is also able to penetrate the proteins, albeit less readily than O_2 and CO, as might have been expected since H_2S is more polar. The comparison between ethanethiol and the small thiol H–SH is indicative; the much smaller H_2S analogue shows significantly greater quenching ability, as expected qualitatively for a penetration mechanism. However, if both thiols acted by a penetration mechanism, one might expect a much sharper distinction between these agents, with ethanethiol quenching falling off more drastically. The behavior observed can be explained by the conclusion reached before; ethanethiol, though it cannot significantly penetrate the proteins, still maintains a good degree of quenching ability by virtue of an alternative pathway, namely, a long-range transfer mechanism. H_2S quenches more effectively because it can approach the buried tryptophans more closely.

CS_2 is larger than H_2S and, as expected for a penetration mechanism, exhibits a generally steeper decrease in quenching ability than H_2S through the proteins studied (Table I). However, Table II shows that CS_2 maintains some sensitivity to solvent viscosity, more than for any of the other quenchers tested. It also seems significant that CS_2 possesses unusually high quenching ability against the model NATA and the partially exposed protein tryptophans. A possible conclusion is that quenching by CS_2 has a significant component due to long-range transfer but because of its good transfer capability is not yet fully in the fast diffusion limit, so that some viscosity dependence is still seen. The alternative source for viscosity dependence, an opening reaction, seems unlikely since the defining characteristics of an opening pathway do not appear for the other quenchers. Opening with some residual insertion or penetration requirement can still be considered.

Luminescence Quenching by Long-Range Transfer. The quenching of excited states by radiationless transfer can occur either through a dipolar (Forster, 1948) or through an electron-exchange (Dexter, 1953) or -transfer (Marcus, 1956) mechanism. The rate of dipolar transfer is proportional to the sixth power of distance and the spectral overlap between donor emission and acceptor absorption. For an electron-transfer mechanism, rate decreases exponentially with distance (R), as in

$$k_q = k_0 \exp[-(R - R_0)/L] \quad (2)$$

Here, k_0 is the quenching constant at van der Waals contact where $R = R_0$ and L is a distance scaling factor.

Evidence obtained in solid media indicates that the rate of transfer for an electron-exchange reaction decreases by a factor of 10 for every 1.5–2 Å (Turro, 1978). Li et al. (1988) have found this same factor for isobutyl disulfide quenching of tryptophyl phosphorescence. This sets the value of L in eq 2 at ~0.8 Å. Evidence for long-range electron transfer involving indole and other aromatic hydrocarbons comes from the observation of nonexponential phosphorescence decay in vitrified glasses due to quenching by potassium iodide or propyl bromide (Lessard & Durocher, 1979; Birks et al., 1976; Najbar et al., 1977). The quenching of porphyrin phosphorescence has also been interpreted in terms of electron transfer or exchange occurring over long distances during the extended excited-state lifetime (Zemel & Hoffman, 1981; McLendon et al., 1985; Peterson-Kennedy et al., 1986; Koloczek et al., 1987).

The quenchers used in the present study show no absorption in the spectral region where tryptophan phosphoresces that could fulfill the requirement for resonance energy transfer. Three of the quenchers used here contain sulfur (H_2S, CS_2, and ethanethiol), and the larger ones contain conjugated bond systems or are amides. These molecules are potential electron donors and acceptors. Thus, the interesting suggestion emerges that the quenching of protein phosphorescence observed here may represent long-range electron transfer or exchange. If

so, it is noteworthy that the study of phosphorescence quenching in solution may provide an easily manipulatable system for the study of the dependence of electron transfer on distance, the character of the intervening medium, and the like.

Radiationless Transfer in Fluorescence Quenching. In earlier work on the fluorescence quenching of a number of proteins by acrylamide, Eftink and Ghiron (1975, 1977) and Hagaman and Eftink (1984) showed that, for proteins with progressively lower k_q values, the quenching reaction becomes less and less sensitive to solvent viscosity. These authors considered the possibility that this behavior indicates acrylamide penetration through the proteins. More recent work on fluorescence (Calhoun et al., 1986) and phosphorescence (1983b) quenching and the work described here indicate that molecules like acrylamide cannot rapidly penetrate into proteins. We have previously suggested that the acrylamide quenching observed against various proteins represents surface contact, since the protein tryptophans that exhibit measurable fluorescence quenching are now known to be partially exposed to solvent. However, surface contact does not account for the interesting viscosity effect found by Eftink and Ghiron for the least exposed tryptophans.

An alternative possibility can be considered in view of the present results. In the case of the more protected tryptophans studied by Eftink and Ghiron, the acrylamide fluorescence quenching reaction may be limited by a low radiationless transfer rate between the tryptophan and near-surface quencher molecules. In the fluorescence experiments, very high concentrations of quenchers, in the 1 M range, are used due to the short fluorescence lifetime. Here, the average separation distance between quencher molecules is about 10 Å, a distance that can be traversed by quencher diffusion in less than 1 ns. Thus, insofar as a long-range transfer may contribute to quenching of the more protected tryptophans, the system can approach the fast diffusion limit and exhibit little viscosity dependence, as was observed. Whether the *fluorescence* quenching mechanism represents dipolar or electron transfer remains to be seen.

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The Linkage with Apolipoprotein (a) in Lipoprotein (a) Modifies the Immunochemical and Functional Properties of Apolipoprotein B†

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ABSTRACT: Lipoprotein (a) [Lp(a)] was isolated from several donors and its apolipoprotein (a) [apo(a)] dissociated by a reductive treatment, generating the apo(a)-free form of Lp(a) [Lp(a-)] that contains apolipoprotein B (apo B) as its sole protein. Using anti-apo B monoclonal antibodies, the properties of apo B in Lp(a), Lp(a-), and autologous low-density lipoprotein (LDL) were compared. Marked differences in apo B immunoreactivity were found between these lipoproteins, due to the presence of apo(a) in Lp(a). Apo(a) enhanced the expression of two epitopes in the amino-terminal part of apo B while it diminished the immunoreactivity of three other epitopes in the LDL receptor binding domain. Accordingly, the binding of the lipoproteins to the LDL receptor was also decreased in the presence of apo(a). In a different experimental system, the incubation of antibodies that react with 27 distinct epitopes distributed along the whole length of apo B sequence with plastic-bound Lp(a) and Lp(a-) failed to reveal any epitope of apo B that is sterically hindered by the presence of apo(a). Our results demonstrate that the presence of apo(a) modified the organization and function of apo B in Lp(a) particles. The data presented indicate that most likely the modification is not due to a steric hindrance but that some more profound conformational changes are involved. We suggest that the formation of the disulfide bridge between apo B and apo(a) in Lp(a) alters the system of disulfide bonds present in apo B and thereby modifies apo B structure.

Discovered by Berg in 1963, Lp(a)[1] was long considered to be a genetic variant of LDL, but it was later found to be present in most human subjects (Albers et al., 1977; Albers & Hazzard, 1974). Recently the Lp(a) apolipoprotein was shown to be protein of about 1.2 million daltons which upon reduction yielded two subunits with molecular weights of about 645 000 and 490 000 that have been respectively identified as apo(a) and apo B (Gaubatz et al., 1983). The apo B component was identified on the basis of its apparent molecular weight and its cross-reactivity with an antiserum to LDL (Mondola & Reichl, 1982; Utermann & Weber, 1982; Fless et al., 1984). Subsequently, it was also demonstrated that Lp(a) can show both inter- and intraindividual heterogeneity with respect to particle size, and this heterogeneity was found to be related at least in part to the size of apo(a) (Seman & Breckenridge, 1986; Utermann et al., 1987).

Several methods have been reported recently that allow the selective removal of apo(a) from Lp(a) (Fless et al., 1985; Armstrong et al., 1985; Seman & Breckenridge, 1986) and which demonstrate that apo(a) is linked to Lp(a) apo B via disulfide bonds and that it has little avidity for the lipids. Particles remaining after the selective removal of apo(a) from Lp(a) [apo(a)-free Lp(a), Lp(a-)] have been found to be

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[1] Abbreviations: Lp(a) lipoprotein (a); apo(a), apolipoprotein (a); Lp(a-), apo(a)-free Lp(a); apo B, apolipoprotein B; LDL, low-density lipoprotein; HDL, high-density lipoprotein; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Na_2EDTA, sodium salt of ethylene diaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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