Temperature dependence of the disulfide perturbation to the triplet state of tryptophan

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ABSTRACT Variability in the temperature dependence of disulfide quenching of the tryptophan phosphorescence of globular proteins in rigid glasses is illustrated with lysozyme and α-bungarotoxin. A laser-pulsed phosphorescence study of this short-range interaction with a model indole-disulfide system is described. The perturbation of secondary dibutyl disulfide on the triplet state of the indole moiety in 2-(3-indolyl)ethyl phenyl ketone in rigid media is found to display a bimodal temperature dependence. The quenching rate constant at contact between chromophore and perturber is observed to be temperature independent below 30 K, but to increase with temperature between 30 and 100 K with an activation energy of ~ 200 cm^{-1}. The results suggest that the underlying quenching interaction involves a photo-induced one-electron transfer from the excited state of indole to the disulfide.

INTRODUCTION

The interaction between internal disulfide linkage and tryptophan side chain is responsible for the anomalous luminescence properties of some disulfide-containing proteins. At the singlet level the interaction manifests itself as an extremely low tryptophan fluorescence quantum yield, although recent direct observations of short-lived components within protein and peptide fluorescence decays have been reported (vandeVen et al., 1987). The interaction at the triplet level is seen as a short-lived component in steady-state protein phosphorescence decays at 77 K (Churchich, 1964, 1966; Longworth, 1971; Li and Galley, 1989). Studies at the triplet level on an indole-disulfide model system have emphasized the short-range nature of the process consistent with the type of wavefunction overlap involved in electron-exchange excitation transfer, or a one-electron transfer process (Li and Galley, 1989).

Maki and co-workers observed that the anomalous tryptophan phosphorescence decay of lysozyme at 77 K becomes lengthened to a decay with a normal phosphorescence lifetime of 6 s at liquid helium temperatures (von Schütz et al., 1974). The demonstration that even in low-temperature rigid media the triplet quenching is a thermally activated process is not consistent with endothermic energy transfer from the triplet state of tryptophan to a putative disulfide triplet state. More recently, the observations with the model system (Li and Galley, 1989) of values for the quenching constant at van der Waals' contact ($\sim 2 \times 10^4$ s^{-1}), which are orders of magnitude lower than anticipated for an exothermic energy transfer ($> 10^{10}$ s^{-1}), support the earlier conclusion. While Maki and co-workers suggested that a barrier-dependent, one-electron transfer was probably involved, excitation transfer involving a disulfide triplet state lying somewhat higher in energy than that of tryptophan could account for the observations.

The ability of disulfide to capture an electron to form the radical anion $(RS\text{-}\dot{S}R)^-$ has been demonstrated in the radiolysis of disulfide compounds both in aqueous solution (Adams et al., 1967) and in rigid glasses (Nelson et al., 1977). Bent and Hayon (1975) found that quenching of the triplet state of tryptophan by disulfide in aqueous solution involved ejection of an electron from such a state to the disulfide. Grossweiner and Usui (1971) had earlier observed in flash photolysis of aqueous lysozyme that the initial photochemical products were photo-oxidized tryptophan residues, hydrated electrons, and the cystine electron adduct. They also found that a portion of the ejected electrons was captured by cystine residues via a short-range intramolecular process having an efficiency of nearly 100%. Thus, while there is evidence in support of electron transfer from indole to disulfide, and evidence that the triplet state of the indole chromophore can be involved, it has not been established that this is indeed the process responsible for the anomalous tryptophan phosphorescence components at 77 K.

In this work the temperature dependence of the disulfide–tryptophan interaction is illustrated with two distinct proteins, and is examined in more quantitative detail with a model system. The characteristics of the temperature dependence obtained in this work favor a barrier-dependent, one-electron transfer process as the source of the triplet quenching.

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MATERIALS AND METHODS

Spectrophotometric grade (Gold Label) secondary dibutyl disulfide was purchased from Aldrich Chemical Co. (Milwaukee, WI). The indole derivative 2-(3-indolyl)ethyl phenyl ketone (IEPK) used as a model compound was obtained from Dr. William E. Lee (Defence Research Establishment Suffield, Ralston, Alberta). The preparation and characterization of this compound have been described by Tamaki (1981) and Lee (1985). The solvent used was a mixture of 70% ethyl alcohol and 30% ethyl ether on a volume basis; both were the highest grade products available from Aldrich Chemical Co. The impurity emission from the solvent did not exceed 1% of the total intensity in any of the experiments.

Chicken egg-white lysozyme and α-bungarotoxin were the highest grade available from Sigma Chemical Co. (St. Louis, MO). The emission samples were prepared by first dissolving the protein powder in potassium phosphate buffer (pH 7.4, 0.2 M) and then mixing with neat glycerol. The glycerol/buffer ratio was rendered 70:30 on a weight basis. The final concentration was 1.0 mM for the lysozyme and 0.5 mM for α-bungarotoxin.

Excitation of the sample was carried out in one of two ways: (*a*) To record emission spectra and follow the decay of the phosphorescence from the steady state, the sample was excited with an Osram HBO 100-W high pressure Hg arc lamp (TJS, Denville, NJ). The excitation wavelength was selected with a 0.25-m Bausch & Lomb monochromator with a resolution of 6 nm/mm. The exciting beam was shuttered with a solenoid shutter and the decay was recorded with an X-Y recorder. (*b*) To emphasize the contribution of "quenched" components in the decay and thereby accurately record their decay times, samples were excited with pulsed excitation. The exciting pulsed light source was a flashlamp-pumped tunable dye laser (model SLL-625A; Candela Laser Corp., Wayland, MA), the specifications of which have been described elsewhere (Li et al., 1989). A frequency doubler was used to produce 296-nm UV pulses from the fundamental rhodamine 6G hydrochloride emission at ~600 nm. The duration of the laser pulse was ~0.5 μs, orders of magnitude shorter than the lifetime of the fastest component in the phosphorescence decays.

The emission cell was a Suprasil quartz tube (i.d. 4 mm) containing the sample. It was sealed with Parafilm, attached to a cell holder, and then mounted inside a CF204 cryostat (Oxford Instrument, Oxford, UK). The cryostat was equipped with two pairs of quartz windows with excitation and emission windows perpendicular to each other. Liquid helium was transferred to the cryostat by a GFS300 transfer tube (Oxford Instrument) and the temperature was regulated by a DTC2 digital temperature controller (Oxford Instrument). Temperature fluctuations were typically controlled to a range of within ±0.1 K.

The emission wavelength was selected by a 0.5-m Bausch & Lomb grating monochromator with a bandwidth of 3 nm/mm. A 325-nm cutoff UV filter was placed in front of the entrance slit of the emission monochromotor to reduce the interference of the exciting pulses when laser excitation was used. The emission signals were detected by an EMI 9635QB photomultiplier tube and amplified by a custom-built DC amplifier. The decay signals were digitized and recorded by an A/D conversion I/O card (model DT2801-A; Data Translation, Marlborough, MA) installed within an IBM PC computer and displayed on the screen. The laser was fired at a pulse rate of only 4–5 min^{-1} to permit the emission signal to decay completely between pulses. The laser pulses and A/D board were triggered simultaneously to ensure that no fast components were missing.

At a given temperature 25 pulsed decays were accumulated. The total decay over 8 s was obtained in "piecemeal" fashion from decays accumulated over different time scales. The experimental decays obtained over the shortest time scale were first normalized to 1 at $t = 0$ by extrapolating the logarithm of the first few points to $t = 0$ in a linear fashion. With a graphics computer program the decays obtained at longer time scale were then matched, in the overlapping time range, to the decays obtained at short times. These were then fitted to the decay function of Inokuti and Hirayama (1965) using a procedure described elsewhere (Li et al., 1989). The parameters K and L were optimized with a χ^2 function minimization computer program.

RESULTS

Temperature responses of lysozyme and neurotoxin phosphorescence

In Fig. 1 the nonexponential decay of the tryptophan phosphorescence of hen egg-white lysozyme generated with pulsed excitation at 77 K is compared with the corresponding decay from the steady state. The advantage of pulsed excitation in revealing contributions from short-lived or "quenched" components is apparent from the markedly more rapid decay of the former. This derives from the fact that the initial intensities of the various components in a steady-state decay are a function of their respective excited-state lifetimes, whereas those generated on pulsed excitation are not. While the decay from the steady state can be approximated as essentially the sum of two decay components, the presence of more than the two components is readily revealed with pulsed excitation. The slowest component

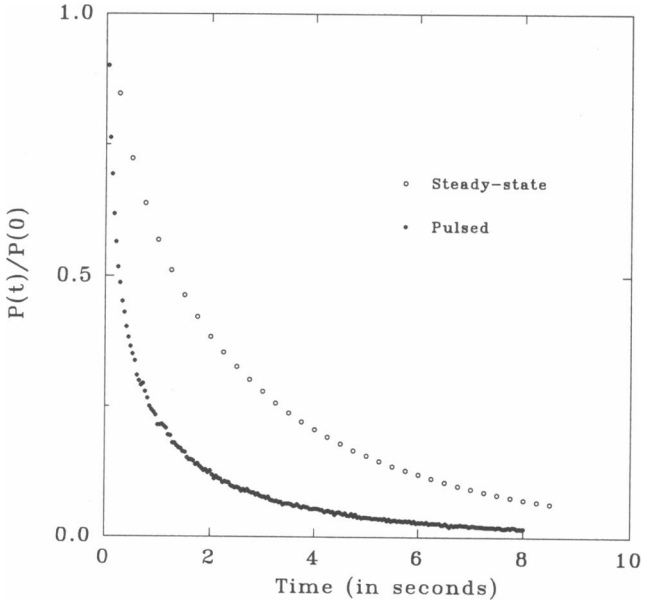

FIGURE 1 Steady-state and pulsed phosphorescence decays of lysozyme in 70:30 glycerol/buffer (pH 7.0) obtained at 77 K. The excitation wavelength was 296 nm for the pulsed decay and 290 nm for the steady state. The emission wavelength was 445 nm.

in the pulsed decay has a lifetime of 3.6 s, somewhat shorter than that of unperturbed tryptophan. The shorter-lived anomalous decay components which vanish on reduction of the disulfide linkages (Churchich, 1964, 1966) derive from the known disulfide–tryptophan proximities in the lysozyme structure (Blake et al., 1967*a*, *b*).

On lowering the temperature from 77 to 4.0 K, the overall decay time of the tryptophan phosphorescence of lysozyme excited with a 0.5-μs pulse is significantly lengthened (Fig. 2). In addition to an increase in the lifetime of the slow component to 6.1 s, a marked reduction in the relative contribution of the shorter-lived components is responsible for the overall slower decay. The observation of this type of temperature dependence for the tryptophan phosphorescence of lysozyme is in agreement with the original observation of Maki and co-workers (von Schütz et al., 1974). While in the original study the presence in the steady-state decay of a long-lived exponential decay at the lower temperature was reported, with pulsed excitation in the present study retention of part of the anomalous short-lived components was detected in the decay at 4 K.

With the neurotoxins a single tryptophan occurs adjacent in the sequence to a cysteine involved in a disulfide bridge in the binding loop region of the protein. Anomalous tryptophan phosphorescence decays have been observed with a number of single-tryptophan proteins and polypeptides that possess disulfides, including α-bungarotoxin (Li, 1990). Given the sensitivity of the interaction to distance, such anomalous behavior is merely a reflection of the distribution of protein conformational states in solution and therefore not unexpected. NMR data have provided evidence for local conformational flexibility of the lone tryptophan side chain within the neurotoxins (Hider et al., 1982), and even in the crystal structure of α-bungarotoxin the relative orientation of the tryptophan side chain to the disulfide in the binding loop differs in the two molecules in the asymmetric unit (Agard and Stroud, 1982). Despite the presence of a single chromophore, the phosphorescence decays nonexponentially with a significant fraction of unperturbed component. The decays of α-bungarotoxin observed at 4.5 and 77 K appear very similar or display a decreased sensitivity to temperature in comparison with lysozyme.

Disulfide–indole perturbation in IEPK

The total emission spectrum of IEPK at 77 K in 2:1 ethanol/diethyl ether is seen in Fig. 3 to consist of indole phosphorescence with no evidence for either indole fluorescence or acetophenone phosphorescence. Singlet-singlet and triplet-triplet energy transfer processes between the aromatic moieties as well as rapid acetophenone intersystem crossing combine to bring the excitation rapidly to the indole triplet state, irrespective of whether it originates in the acetophenone or indole chro-

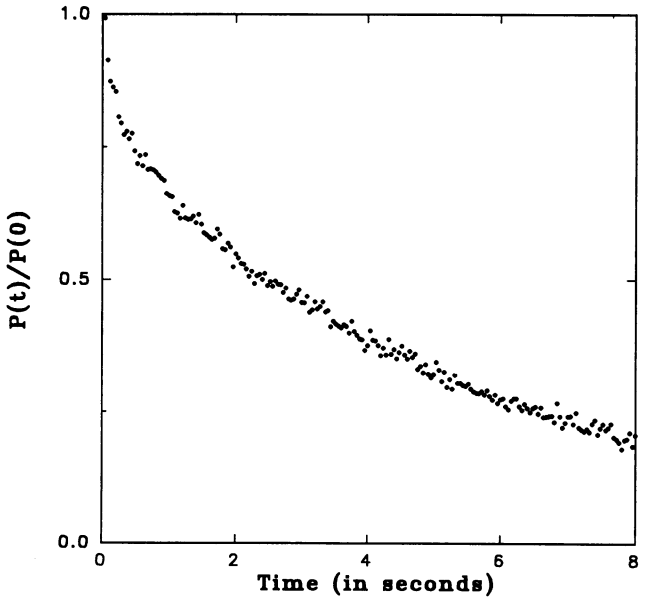

FIGURE 2 Pulsed phosphorescence decay of hen egg-white lysozyme at 4.0 K. Other experimental conditions were the same as in Figure 1.

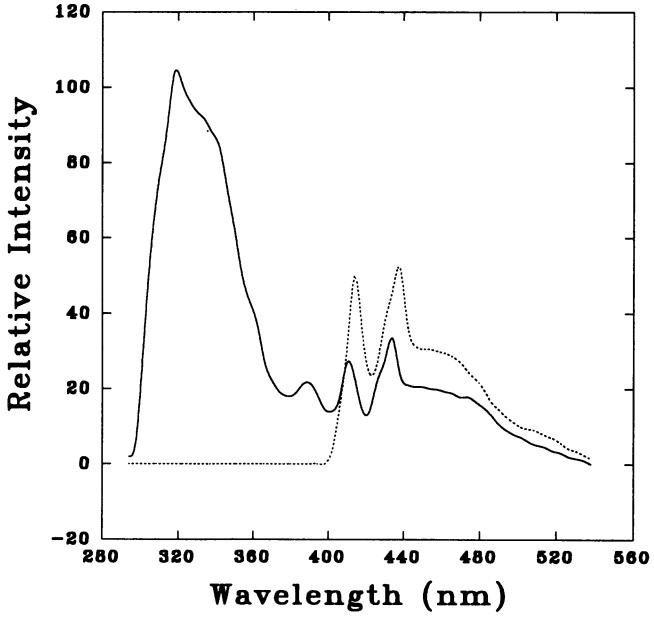

FIGURE 3 Total emission spectra of IEPK (*dashed curve*) and an equimolar mixture (*solid curve*) of indole and acetophenone in 70:30 ethanol/ether at 77 K. The excitation wavelength was 280 nm.

mophores (Tamaki, 1981). As a result, perturbations such as disulfide quenching that would occur at the indole singlet level and thus indirectly influence the triplet population are bypassed with this molecule. The influence of perturbation occurring selectively at the triplet level can be examined (Lee, 1985; Li et al., 1989).

The pulsed phosphorescence decays of IEPK in the presence of the 0.3 M disulfide perturber are seen from Fig. 4 to be influenced by the temperature of the rigid glass. While the decays are independent of temperature below 20 K, they become significantly shortened above 30 K. Decay data were obtained up to 100 K, but at temperatures above this the IEPK phosphorescence decays, accompanying the sharp onset of mobility within the glassy solvent, became dramatically shortened. With the appearance of chromophore–perturber diffusion on the timescale of the chromophore triplet lifetime the assumption of a distribution of fixed distances is no longer applicable. The IEPK phosphorescence decay intensities $P(t)$ normalized to the intensity at $t = 0$, $P(0)$, were fitted to a modified form

$$P(t)/P(0) = f(t) \cdot \exp[-\alpha \cdot C \cdot H(t)] \quad (1)$$

of the donor decay function derived by Inokuti and Hirayama (1965). In Eq. 1 C is the concentration of the acceptor at low temperature which was derived from the values at room temperature through multiplication by 1.25 to allow for solvent contraction of the ethanol/ether solvent, α is a proportionality constant depending on the units of C, $H(t)$ is an integral with respect to the chromophore–perturber distance x, and $f(t)$ is the chromophore decay function in the absence of perturber. With an exponential chromophore decay $f(t)$ is given by

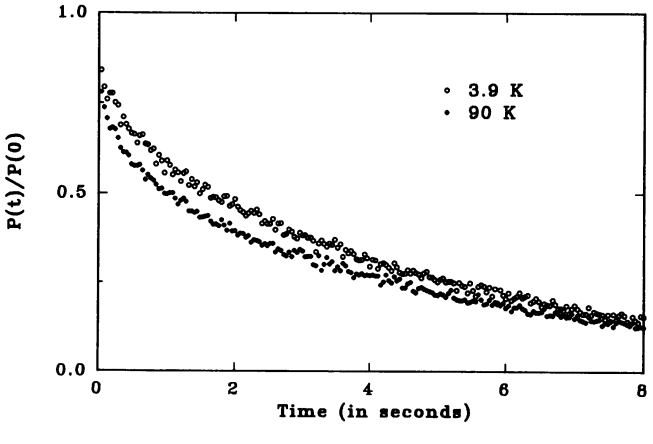

FIGURE 4 Temperature dependence of the phosphorescence decay of IEPK excited by a 296-nm laser pulse. The sample was in 70:30 ethanol/ether solvent.

exp $[-t/\tau]$, and Eq. 1 takes the form given by Inokuti and Hirayama (1965).

At the low end of the temperature range of the measured decays spin-lattice relaxation rates are known to become slower than triplet decay rates. As a result, the emission processes of the triplet sublevels become independent of each other (Maki and Zuclich, 1975) and the overall phosphorescence decays deviate from true exponential behavior even in the absence of quencher. Thus the original IEPK decay functions which were acquired at the same temperature as the IEPK-disulfide decays were substituted into Eq. 1 for $f(t)$.

With an interaction involving overlap of electron clouds the quenching constant can be approximated in the Dexter form $k(x) = K \exp(-2x/L)$, where $k(x)$ represents the quenching constant as a function of chromophore–perturber separation x, K is the quenching constant at van der Waals' contact, i.e., when $x = 0$, and L is the average chromophore–perturber effective Bohr radius. The $H(t)$ integral appearing in Eq. 1 is then given by (Inokuti and Hirayama, 1965)

$$H(t) = 4\pi \int_0^\infty \{1 - \exp[-K \cdot t \cdot \exp(-2x/L)]\} \cdot (x + R)^2 \cdot dx, \quad (2)$$

where R is the sum of van der Waals' radii of the donor and acceptor for which a value of 4 Å was assumed.

The values of K and L were obtained from the best fits of Eq. 2 to the experimental decay curves at various temperatures. The average effective Bohr radius L which accounts for the fall-off of the perturbation with distance is seen to remain essentially constant, while the quenching constant K at contact is found to increase with temperature. Since the quenching effect is weak at the lower end of the temperature scale, the uncertainties in the values of L and K are relatively larger than the data obtained in a previous study at liquid nitrogen temperature (Li et al., 1989). A value for L of 0.75 Å obtained from the data at 77 K was therefore assumed, and the decays refitted optimizing K alone. The values of K derived in this manner fall into two regions (Fig. 5): a temperature-independent region below 30 K where the quenching constant takes on a value of $\sim 500\ s^{-1}$, and a temperature-dependent one above this temperature. Subtraction of the temperature-independent value of $5 \times 10^2\ s^{-1}$ from the values of K obtained at the higher temperatures produced the values for the temperature-dependent part of the quenching constant depicted in the form of an Arrhenius plot in Fig. 6 The intercept which provides an upper limit for K at infinite temperature is $5 \times 10^5\ s^{-1}$, while a barrier height for the quenching of 190 cm^{-1} is found from the slope.

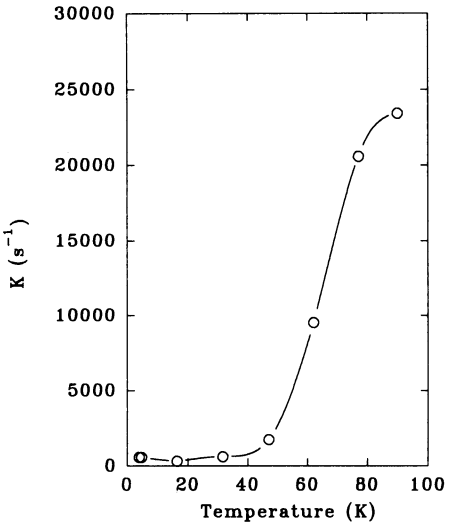

FIGURE 5 Quenching constant K of the IEPK-secondary butyl disulfide as a function of temperature derived from fitting of the decays.

DISCUSSION

After the onset of excitation (ΔI) at $t = 0$ the luminescence intensity of an emitter grows:

$$P(t) = \Delta I \cdot \phi_{ix} \cdot k_p \cdot (1 - e^{-t/\tau})$$

until it reaches a steady-state level ($t \rightarrow \infty$),

$$P(t \rightarrow \infty) = \Delta I \cdot \phi_{ix} \cdot k_p \cdot \tau$$
$$= \Delta I \cdot \phi_{ix} \cdot \phi_p,$$

which is a function of τ. When the emitter–quencher interactions do not involve the heavy atom effects which perturb the intersystem-crossing efficiency ϕ_{ix} and the radiative rate constant k_p, a component of the population with a short lifetime due to nonradiative quenching of the triplet state makes a correspondingly small contribution to the steady-state decay in the presence of unperturbed emitters. However, during the flash time t_f of pulsed excitation ($t_f \ll \tau$) the intensity grows in a linear fashion:

$$P(t_f) = \Delta I \cdot \phi_{ix} \cdot k_p \cdot t_f$$

which is independent of τ, so that in the subsequent decay short-lived components are not selectively suppressed. Discussions on the physical basis of the steady-state and pulsed experiments and the relationships between the two can be found elsewhere (see, for example, Inokuti and Hirayama, 1965 and the references therein). This dependence of nonexponential decays on the mode of excitation is apparent from the comparison of the lysozyme phosphorescence decays in Fig. 1.

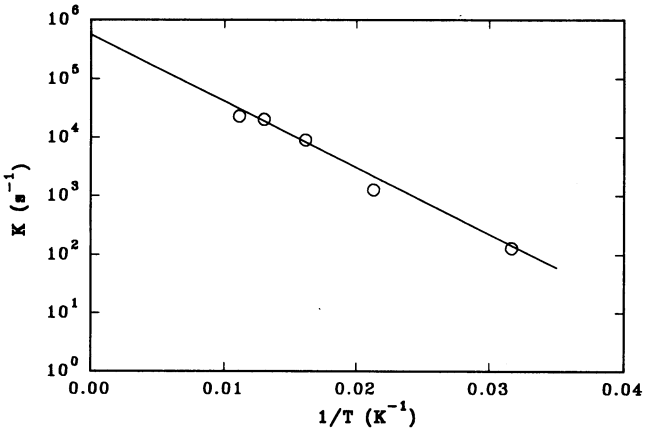

FIGURE 6 Arrhenius plot of the quenching constant K of the IEPK-secondary butyl disulfide (see Results for details).

The observation with hen egg-white lysozyme of a significant temperature dependence of the anomalous short-lived components in the protein phosphorescence decay is consistent with the original observations of Maki and co-workers (von Schütz et al., 1974). The pulse-excited decays observed in this study, while markedly temperature dependent, displayed a residual short-lived component at 4 K not detected in decays from the steady state. Thus, while confirming the endothermic contribution seen earlier, the data also reveal the presence of an additional temperature-independent component in the quenching process.

The surprising observation with α-bungarotoxin of a much more temperature-independent decay emphasizes that the relative temperature-dependent and -independent contributions to the interaction of tryptophan in proximity to disulfides is a function of protein structure. In α-bungarotoxin, cysteine 29, which is involved in a disulfide with cysteine 33, occurs adjacent in the amino acid sequence to the lone tryptophan in the molecule. With lysozyme as well, at least one of the tryptophan–disulfide partners involves an interaction between adjacent residues in the sequence (i.e., Trp 63 with the disulfide involving Cys(64)-Cys(80)). Thus, a "through-bond" transfer might occur and the possibility of its involvement in this case should be considered. If electron transfer were to occur exclusively via the intervening bonds, the observed phosphorescence decay would be a function only of the number of bonds and would be expected to be exponential for single-tryptophan proteins. The existence of both temperature-dependent and -independent quenching processes cannot be related to pathways of "through-bond" and "through-space" elec-

tron transfer in that this behavior is also found with the model system. Thus, it appears that the tryptophan–disulfide interaction at the triplet level is influenced more by the microenvironment in which the interacting pair reside than by the way they are covalently connected.

The temperature dependence of the disulfide interaction with IEPK is similar to that found with lysozyme. The quenching constant at contact falls off with decreasing temperature to ~30 K, below which a temperature-invariant value of 500 s^{-1} is observed. Correction for this value in the temperature-dependent region produced values for the quenching constant which depicted Arrhenius behavior. The results indicated that throughout the temperature region studied, the process was associated with a low activation energy (2.3 kJ · mol^{-1}). The small quenching constant observed, for example at 77 K, derives therefore not from a high energy barrier but rather from the small preexponential factor. Interpreted in terms of transition state theory, the values for A and E_0 imply that it is an unfavorable entropic barrier of −30 J · mol^{-1} · K^{-1} that is primarily responsible for the slow transfer rate.

The involvement in the quenching mechanism of a disulfide excited state lying somewhat higher in energy than the triplet state of tryptophan was considered. This was prompted by the observation of a weak emission (1.2 ms) from a high concentration of secondary dibutyl disulfide samples at 77 K in the absence of IEPK, and with a band origin ~800 cm^{-1} above that of the indole moiety (Li, Z., and W. C. Galley, unpublished data). However, the observed temperature dependence for the triplet quenching does not appear consistent with endothermic excitation transfer to such a putative disulfide excited state. The influence of such a state would be expected to vanish when thermal energies fall much below the difference in excitation energies of the donor and acceptor, rather than to display a temperature-independent region. In addition, an energy gap of 800 cm^{-1} based on the emission data is significantly larger than the 200 cm^{-1} activation energy observed for the quenching process.

The thermodynamic driving force of electron transfer process is provided by the ionization potential and electron affinity of the donor and acceptor. However, unlike most electron transfer reactions that normally take place at the ground state, the rate of quenching process is a function of excited state ionization potential rather than the ground state IP of the electron donor. The excited state IP is given by the ground state IP minus energy of the excited state involved. The protein environment may affect electron transfer process by varying the excited triplet energy. We have found that tryptophans in disulfide-containing proteins and peptides with increasing triplet energy of the O-O vibronic bands from 405 to 417 nm have increasing phosphorescence lifetimes of the perturbed components (Li, 1990).

The demonstration of both temperature-dependent and -independent contributions to the disulfide quenching appears more consistent with a mechanism involving a one-electron transfer from the triplet state of the indole chromophore to disulfide, as suggested by Maki and co-workers (von Schütz et al., 1974). This type of temperature behavior has been observed for one-electron transfer processes with a variety of systems, including proteins and biomolecular complexes (DeVault and Chance, 1966; McElroy et al., 1974; Moore et al., 1982). An example involving the triplet state as the electron donor is found in the recent work of Zang and Maki (1990) on the temperature dependence of the phosphorescence quenching of Zn-substituted cytochrome c by covalently attached $Ru^{III}(NH_3)_5$. The temperature-dependent and -independent behavior have been examined from a theoretical point of view (DeVault, 1980; Marcus and Sutin, 1985) and are generally agreed to involve a combination of electron tunneling through a barrier as well as activation energy over it. From the small activation energy involved, the triplet tryptophan–disulfide interaction must be considered to lie near the "barrier-free" limit of Marcus' theory (Marcus and Sutin, 1985).

The distinguishing feature of the kinetics of the present donor–acceptor process lies in the small values for the electron transfer rate constants close to the barrier-free limit. However, the conditions under which the present process occurs differ from those normally present. (*a*) The precess is observed in rigid media where permanent dipolar relaxation about either the excited state or charge separated species does not contribute to stabilizing such species. (*b*) In that the photoexcited donor is a triplet state, the rate of spin interconversion might play a significant role in determining the kinetics of the quenching process. The charge-separated species formed immediately after electron transfer would possess "triplet character," i.e., $S = 1$. Depending on the energy of such a putative state it could exist as a long-lived state or quickly revert back to the triplet indole, neutral disulfide, state. In the latter case further relaxation involving this biradical species could be limited by spin-lattice relaxation that would convert the "triplet" biradical species to that of a "singlet" preventing back transfer and promoting return to the original ground-state species. Based on this model the low preexponential factor associated with the quenching process would derive from slow spin-interconversion rates.

Tryptophan–disulfide proximities are an integral part of protein structure and markedly influence both the

singlet and triplet emission properties of some proteins. The perturbations at both levels are very sensitive to distance (Cowgill, 1970, Longworth and Hélène, 1975; Li et al., 1989). The observations of an essentially unperturbed phosphorescence decay for the lone tryptophan in a number of globular proteins, including erabutoxin (Ménez et al., 1980), phospholipase A_2, and serum albumins (Li, Z., and W. C. Galley, unpublished data), despite the presence of many disulfide linkages in these systems, emphasize that fact. The observation of anomalous phosphorescence decays, the distribution of components within these, as well as the temperature dependence of the quenching are all functions of protein structure and thereby potentially useful intrinsic probes. Observations on the influence of temperature on disulfide quenching of protein phosphorescence to date have been confined to rigid media at low temperature. However, this intrinsic interaction in proteins is potentially a significant factor in determining the tryptophan phosphorescence intensities and lifetimes that are observed for proteins at room temperature (Saviotti and Galley, 1974). This needs to be evaluated from temperature-dependent phosphorescence experiments that extend upward into the solution region.

With increasing evidence pointing to electron transfer as the origin of disulfide quenching of the triplet state of tryptophan in proteins, and given the speculations at present in attempting to model this type of quenching, more direct evidence for the existence of charge-separated species as well as knowledge of their lifetimes are required to generate a more complete picture of this intrinsic perturbation.

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