Distance dependence of the tryptoplhan-disulfide interaction at the triplet level from pulsed phosphorescence studies on a model system

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ABSTRACT In the present study the distance dependence of trypotphan-disulfide interaction is examined with a view to both utilizing the interaction as a more quantitative indicator of subtle conformational changes in proteins as well as elucidating the interaction mechanism. To examine pertubations specifically at the indole triplet level 2-(3-indolyl)-ethyl phenyl ketone (IEPK) in which excitation is transferred with high efficiency to the triplet state of the indole moiety was employed. Phosphorescence decays of IEPK excited by a laser pulse in 70/30 (vol/vol) ethanol-ether at 77 K were measured in the presence of various concentrations of simple disulfides. The nonexponential phosphorescence decays arising from a distribution of fixed chromophore-perturber separations and the steady-state quenching of IEPK were accounted for with an exponential dependence of the quenching rate constant with distance. The small effective Bohr radius (0.8 Å) that appears in the exponent emphasizes the localized nature of the interaction. Comparison of the triplet quenching rate constant obtained at quencher contact with IEPK to that estimated in proteins suggests a dependence on the triplet energy of the indole moiety and an endothermic nature for the quenching process. The study predicts that in proteins tryptophan-disulfide interactions are very localized in nature and should give rise to detectable anomalous decays only out to 2 Å beyond van der Waals contact between the interacting partners.

INTRODUCTION

The low-temperature tryptophan phosphorescence decays of some proteins display, in addition to the usual long-lived lifetime, an anomalous short-lived component (Churchich, 1966; Longworth and Hèlène, 1975) that has been attributed to the proximity of tryptophan residues to disulfide linkages. In lysozyme (Blake et al., 1967) and immunoglobulins (Poljak et al., 1973), these structural relationships are apparent from x-ray crystallography. Because disulfides also interact with the singlet states of aromatic amino acids (Cowgill, 1967), marked perturbation of both the fluorescence and phosphorescence will be observed when the distance between the emitter-disulfide pair is sufficiently short.

We have recently provided evidence for ligand-induced conformational changes in the Fab' fragment of the antigalactan murine immunoglobulin J539 and in chicken egg-white lysozyme from changes in the anamolous short-lived tryptophan phosphorescence decay components in these systems (Glaudemans et al., 1987; Li and Galley, 1989). Relating these observed changes in lifetime to changes in the distances between tryptophan side chains and the quenching disulfides in proteins requires knowledge of the dependence of the tryptophan triplet-disulfide quenching constant upon intermolecular separation.

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Disulfide compounds strongly quench both singlet and triplet states of indole-based chromophores. Thus a chromophore with a shorter than normal triplet lifetime has a suppressed fluorescence quantum yield due to singlet quenching and subsequently a suppressed quantum yield for triplet formation. The effects of the perturbation on the singlet and triplet states are not readily separable. In order to simplify the analyses of the distance dependence of the disulfide perturbation we employed the chromophore 2-(3-indoly)ethyl phenyl ketone (IEPK). In the present study the laser-pulsed phosphorescence decays of IEPK in the presence of varying concentration of disulfide in rigid solution were measured. With this chromophore introduced by Tamaki (1981) excitation energy, irrespective of whether it is initially absorbed by the indole or the acetophenone moiety, proceeds very rapidly to the triplet state of the indole chromophore, thereby eliminating singlet quenching phenomena.

As a result of the random but fixed distribution of chromophore-quencher separations that exist in a rigid media very nonexponential phosphorescence decays are observed. In a system of this type in which neither the $S_1 \rightarrow T_1$ intersystem crossing nor the radiative decay constant for the triplet is altered in the presence of the perturber molecule, the phosphorescence decay curves can be described by the model of Inokuti and Hirayama

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(1965). The calculated pulse-induced decay curves were fitted to the experimental ones using a Dexter exponential dependence of the quenching rate constant with distance (Dexter, 1953). The analysis yielded values for the effective Bohr radius L, an exponent which characterizes the distance dependence and K, a rate constant for quenching at van der Waals contact. Comparison of the data with the anomalous lifetimes observed with proteins of known structure suggests that K is a function of the triplet energy of the indole involved. This conclusion coupled with the observation by von Schütz et al. (1974) of a temperature dependence to the anomalous decay in lysozyme emphasizes the endothermic nature of the interaction.

MATERIALS AND METHODS

Reagent grade s-dibutyl disulfide and dimethyl disulfide were purchased from Aldrich Chemical Company (Milwaukee, WI). The ethanol and ether solvents were Aldrich spectrophotometric grade products. The solvent used in the laser pulsed experiments was a mixture of 70% ethanol and 30% ether by volume. The low-temperature emission from the solvent under excitation at 280–340 nm was found to be negligible. The preparation of IEPK was a modification (Lee, 1985) of the procedure described by Tamaki (1981). This compound was twice recrystallized in absolute ethanol. The total emission spectrum of this compound shows only the characteristic phosphorescence from the indole moiety. The purity of the sample was verified by the absence of either acetophenone phosphorescence or indole fluorescence.

Excitation pulses were provided by a flashlamp-pumped tunable dye laser operating with a CL-500 coaxial flashlamp, model SLL-625A; Candela Corp., Wayland, MA.). Pulses at 296 nm were generated by frequency doubling (model SHG-AT-BF, Candela Corp, Wayland, MA) the fundamental output using rhodamine 6G as the laser dye (Eastman Kodak Co., Rochester, NY). The laser produced 0.5 μs pulses with a uv output of 50 mJ, typically. The pulse-width was a factor of 100< the shortest observable decay components in this work. The laser was fired at low frequences (<0.1 s^{-1}) to allow slow components to completely decay between pulses.

The sample cell was immersed in the liquid nitrogen contained in a Dewar which was equipped with quartz windows. The emission wavelengths were selected by a 0.5 m monochromator (Bauch & Lomb Inc., Rochester, NY). The emission bandwidth was set to 6 nm. The emission was collected at 90° to the excitation beam and detected with an EMI 9635QB photomultiplier whose output was amplified by a custom-built DC amplifier. For steady-state phosphorescence intensity measurements, the signal output was displayed on a X-Y recorder (model F-80A; Varian Associates Inc., Instruments Corp., Palo Alto, CA). The emission cell was made of a pair of quartz plates, whose pathlength was adjustable with a doughnut-shaped spacer of aluminium foil. A 50 μm pathlength was used in all the intensity measurements to efficiently eliminate the competitive absorption by disulfide.

In laser pulsed experiment, a 335 nm cut-off uv filter (Schott Glass Technologies, Inc., Duryea, PA) was placed in the emission pathway to prevent any of the scattered light of the laser excitation pulse from reaching the photomultipliers. The decay signals were digitized and accumulated by an analog to digital I/O conversion board (model DT2801-A; Data Translation Inc., Marlborough, MA) housed in an IBM PC. This system was operated with a graphic display program developed in our laboratory. To obtain the initial intensity, shorter time scale decays were taken and the intensities were extrapolated to zero time. The decays were then normalized and matched with decays at different time scales by using a graphic display subroutine. The data collection intervals were variable 1 to 40 ms per channel. The data fitting task was fulfilled with a conventional least-squares minimization program.

RESULTS

The total emission spectra for IEPK in a 2/1 ethanol/ether glass at 77 K is seen from Fig. 1 to consists solely of an indole phophorescence. The emission is independent of excitation wavelength in agreement with the original observations on this molecule by Tamaki (1981). The absence of either indole fluorescence or acetophenone phophorescence is a consequence of rapid energy transfer to the acetophenone singlet level followed by efficient intersystem crossing to the acetophenone triplet and rapid triplet transfer back to the indole triplet (see Fig. 2). The quenching of the steady-state phosphorescence of IEPK in the presence of methyl disulfide is seen in Fig. 3 to be less than that observed with indole due to the absence of singlet quenching in the former molecule. Triplet quenching is also present with indole as evidenced by both a decrease in the phosphorescence/fluorescence intensity ratio as well as a shortened nonexponential phosphorescence decay. With IEPK the triplet quenching was found to be independent of excitation wavelength, indicating

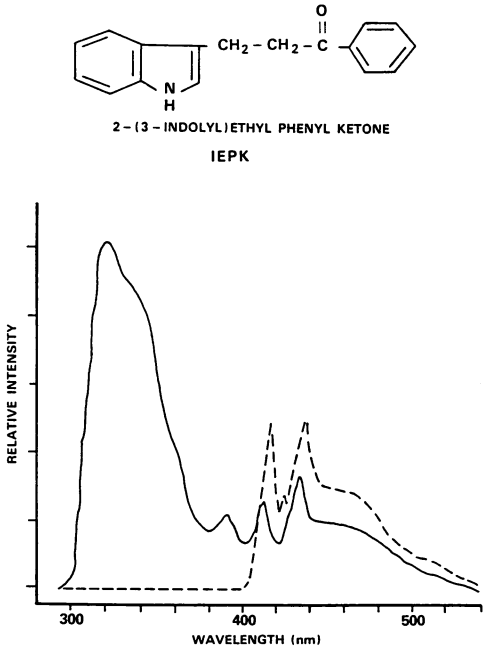

FIGURE 1 The total emission spectra of IEPK (---) and an equimolar mixture (—) of indole and acetophenone in 2:1 (vol/vol) ethanol/ether at 77 K, λ_{ex} = 280 nm.

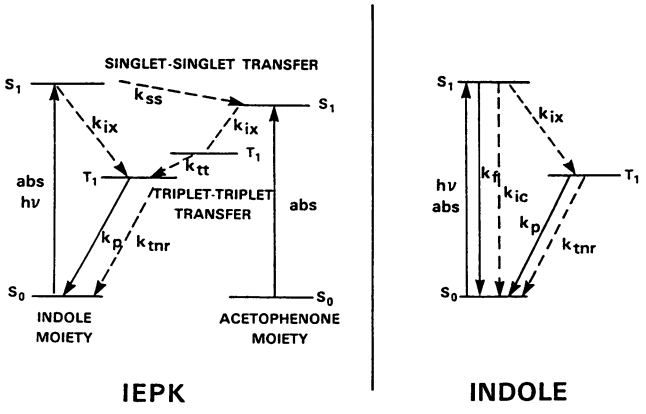

FIGURE 2 The energy levels of the ground state, the first excited singlet and triplet states in indole and IEPK are represented by S_0, S_1, and T_1, respectively. The rate constants for the transitions between states are as following: k_f, fluorescence; k_{ic}, internal conversion; k_{ss}, singlet-singlet energy transfer; k_{ix}, intersystem-crossing; k_{tt}, triplet-triplet energy transfer; k_p, phosphorescence; k_{nr}, nonradiative decay of the triplet state.

that the disulfide perturbation at the singlet level cannot compete with the rapid singlet-singlet transfer from the indole to the acetophenone moiety in this molecule.

After pulse excitation the decays of the indole-like phosphorescence of IEPK in a rigid glass at 77 K in the absence and presence of disulfide are shown in Fig. 4. It is apparent that in the presence of the disulfide perturbation the decay of the triplet emission is nonexponential. The observation that these decays cannot be represented simply as the sum of a perturbed and an unperturbed component indicates that the quenching is not an "all-or-none" effect, the existence of intermediate components in the decay being a reflection of a detectable distance dependence to the interaction. By way of contrast, under steady-state excitation the population of unpertubed or slightly perturbed chromophores are allowed to build up to relatively high levels, and in the subsequent decay tend to overwhelm the "quenched" species. As a result steady-state decays often appear as essentially bi-exponential.

In case of s-dibutyl disulfide, the phosphorescence decays of IEPK at higher disulfide concentrations were always accompanied by a spike with a lifetime of ~1.2 ms, which undoubtedly came from the disulfide in the sample and masked the initial intensities of the IEPK phosphorescence decays. Subtraction of the corresponding disulfide emission from the decays of IEPK in presence of disulfide yielded a negative spike at all concentrations. The initial pulsed intensities of IEPK were obtained by linearly extrapolating the logarithm of the decay starting from 10 ms to zero time.

Disulfides do not posses excited singlet states lower in energy than the indole triplet state, so that the triplet quenching interaction cannot be dipolar in nature. Rather the interaction must occur as a result of a one-electron transfer or two-electron exchange requiring the overlap of electron orbitals. With a Dexter-type distance dependence for the triplet quenching constant the predicted intensity-normalized pulsed decay function $P(t)/P(0)$ takes the form (Bazhin, 1980):

$$\frac{P(t)}{P(0)} = \exp(-t/\tau_0)\exp[-6.023 \times 10^{-4} \cdot C \cdot H(t)], \quad (1)$$

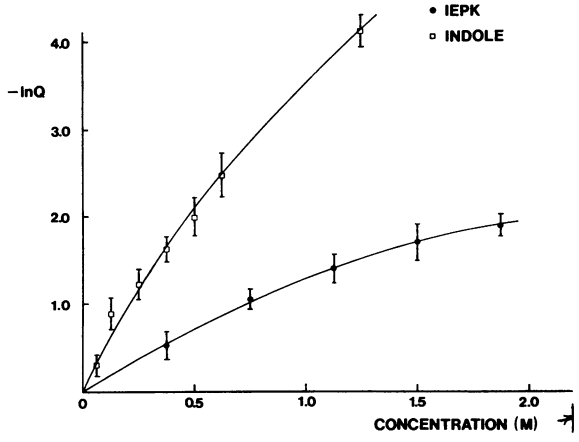

FIGURE 3 The steady-state phosphorescence quenching curves of IEPK (λ_{ex} = 337 nm) and indole (λ_{ex} = 296 nm) at 77 K as a function of dimethyl disulfide concentration. The solvent used was a mixture of 70/30 ethanol/ether (vol/vol). The concentrations of IEPK (1.25×10^{-3} M), indole (1.25×10^{-3} M) and disulfide have been corrected by a factor of 1.25 for glass contraction at 77 K.

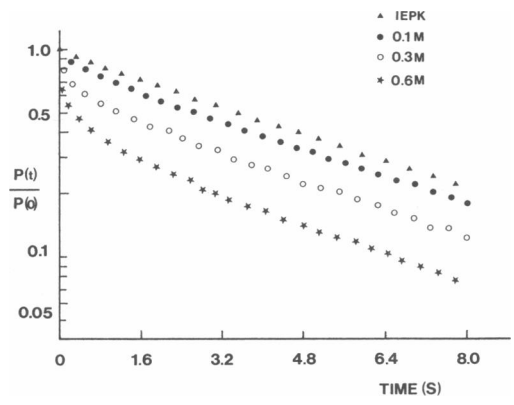

FIGURE 4 The laser pulsed phosphorescence decays (λ_{ex} = 296 nm) of IEPK in the absence (*top curve*) and presence of sec-dibutyl disulfide at 77 K. The intensities of the decays are presented on a natural logarithmic scale and the initial intensities were normalized to unity. The numbers appeared on the diagram stand for the uncorrected disulfide concentrations. Other experimental conditions were as in Fig. 3.

where $\exp(-t/\tau_0)$ is the unperturbed decay, C is the molar concentration of the quencher and $H(t)$ is an integral of the form:

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

where χ is the separation beyond the van der Waals contact distance; R the center-to-center distance between the chromophore and the quencher at $\chi = 0$; K the rate constant at van der Waals contact; L the average effective Bohr radius for the ground and excited states involved. From Eq. 2, $H(t)$ is clearly independent of quencher concentration. Rewriting Eq. 1 in term of $H(t)$ after taking logarithms yields:

$$H(t) = \frac{1}{6.023 \times 10^{-4} C} \ln\left[\frac{\exp(-t/\tau_0)}{P(t)/P(0)}\right]. \quad (3)$$

If the data are consistent with the model, calculation of the right hand side of Eq. 3 from the experimental decays obtained in the presence and absence of quencher should yield values that are independent of quencher concentration and that can be equated to $H(t)$. In Fig. 5 values for $H(t)$ obtained in this way are seen to be independent of disulfide concentration as predicted. As values of $H(t)$ approach a constant at long times the experimental decay curves become essentially parallel to the unperturbed decay.

It was possible to find optimum values for L and K in Eq. 2 that would produce values for $H(t)$ in agreement with those obtained experimentally (Fig. 5). However, we found it more useful to fit the original experimental pulsed phosphorescence decay curves. This was done using both a nonlinear least-square minimization program Parafit (Ruckdeschel, 1981) and a program written in BASIC in which values for the parameters L and K

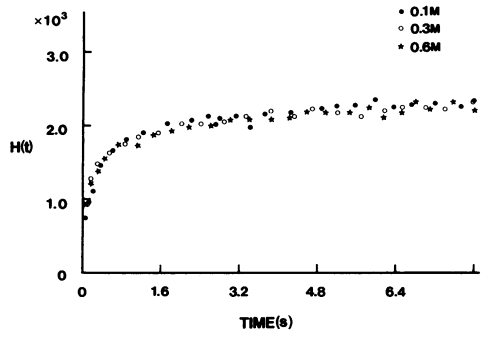

FIGURE 5 The calculated $H(t)$ functions obtained from decays of IEPK in the presence of 0.1 M, 0.3 M, and 0.6 M s-dibutyl disulfide are shown to be independent of disulfide concentration. $H(t)$ tends to approach a limiting value at longer decay time.

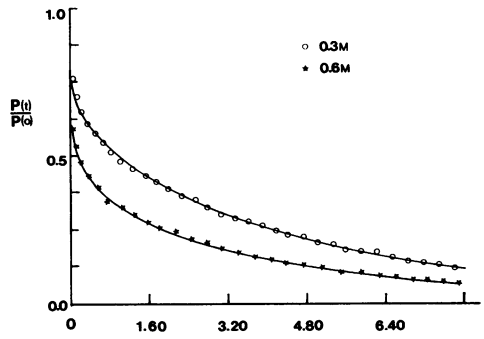

FIGURE 6 The normalized laser pulsed phosphorescence decays (λ_{ex} = 296 nm) of IEPK in the presence of 0.3 M and 0.6 M s-dibutyl disulfide appear as the data points. The solid curves represent the calculated decays using L = 0.75 Å and $K = 2.2 \times 10^4$ s^{-1}.

were systematically varied and agreement with the experimental decay curves evaluated. The latter approach was more rapid in finding reasonable values of the parameter that could then be refined with Parafit. Using Parafit alone if initial pairs of input-parameter values were not chosen sufficiently close to the final values, then the convergence was very slow or false minima were produced. Using a value for R of 4 Å the best values obtained were L = 0.75 Å, $K = 2.2 \times 10^4$ s^{-1} for secondary butyl disulfide, and L = 0.77 Å, $K = 1.9 \times 10^4$ s^{-1} for methyl disulfide. In Fig. 6 the solid lines through the data points represent curves calculated with the parameters above. The steady-state phosphorescence intensity (Q) of IEPK as a function of disulfide concentration can be calculated using the values of L and K according to the method of Inokuti and Hirayama (1965):

$$Q = \frac{P}{P^0} = \frac{\int_0^\infty P(t) \cdot dt}{\int_0^\infty P(t)_{un} \cdot dt}, \quad (4)$$

where the numerator and the demoninator represent the total area under the IEPK decay curves in the presence and absence of disulfide, respectively. In Fig. 7 the calculated phosphorescence intensity of IEPK as a function of s-dibutyl disulfide concentration is compared with the experimental data, the agreement providing additional support for the reasonable nature of the parameters derived from the fit to the pulse decays.

In Table 1 the quenching constant calculated from the parameters given above appears as a function of the separation beyond the van der Waals contact of the interacting disulfide and indole chromophore. The actual center-to-center distance between the interacting partners is the sum of χ and R, for which a value of 4.0 Å was used. It is apparent from the sharp fall of $k(\chi)$ with distance (Table 1) that the disulfide perturbation with

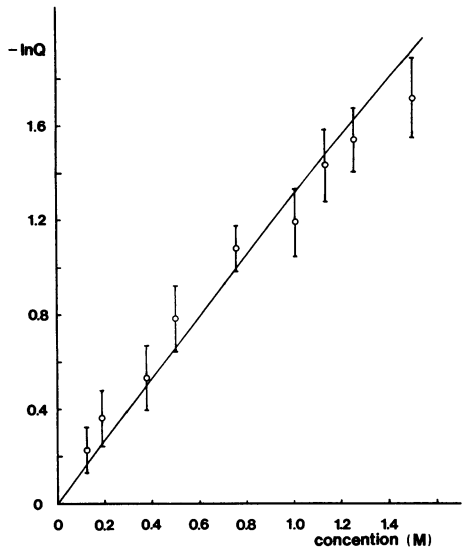

FIGURE 7 The steady-state phosphorescence intensity at 77 K (*open circles*) as the function of s-dibutyl disulfide concentration is compared with the calculated quenching curve (*solid curve*), using $L = 0.75$ Å and $K = 2.2 \times 10^4\ s^{-1}$. The excitation and emission wavelengths were 337 nm and 438 nm, respectively. Other experimental conditions were as described in Fig. 3.

IEPK essentially vanishes for separation >4 Å beyond van der Waals contact.

DISCUSSION

From the value for the average effective Bohr radius (L) found for the exchange interaction between the indole chromophore and the disulfides here it is possible to quantitatively interpret the ligand-induced conformational changes that we have recently observed in proteins (Glaudemans et al., 1987; Li and Galley, accompanying article). For example, on addition of a galactan antigen to the antigen-binding fragment of the J539 antibody, an increase in the tryptophan triplet lifetime from 0.7 to 1.4 s was detected. This increase in lifetime corresponds to a decrease in the quenching rate constant from 1.08 to 0.54 s^{-1}. According to Table 1 this translates into a change of only 0.4 Å between the interacting groups in the protein in response to antigen binding. The low L value for the interaction under investigation results in a very sharp dependence of quenching rate constant on distance so that very subtle structural changes in proteins can be revealed as significant lifetime changes. The quenching rate constant changes by a factor of 10 to 20 for each angstrom of separation.

TABLE 1 Quenching constant k of indole in IEPK by s-dibutyl disulfide as a function of the separation χ beyond van der Waals contact

χ	k
Å	s^{-1}
0.5	5.8×10^3
1.0	1.5×10^3
2.0	1.1×10^2
3.0	7.4
4.0	0.51
5.0	3.6×10^{-2}

The values for L and K are 0.75 Å and $2.2 \times 10^4\ s^{-1}$, respectively.

The L value found here is smaller than that found for triplet-triplet energy transfer between the π-electron orbitals of some aromatic chromophores (Kobashi et al., 1973; Strambini and Galley, 1975) or for one-electron transfers (Alexandrov et al., 1978, and the references therein). Following Marcus' nomenclature (Marcus and Sutin, 1984) for one-electron transfers and writing the exponential distance dependence in Eq. 1 as $k(\chi) = K \cdot \exp(-\beta\chi)$, an L value of 0.75 Å corresponds to a β of 26.7 nm^{-1} that is larger than most values found in the literature (Marcus and Sutin, 1984). The restricted nature of the delocalization is somewhat surprising in that the extension of electron clouds, seen in terms of the exponential part of Slater atomic-orbitals, increases with n the principal quantum number. Orbital overlap in an indole-disulfide interaction will occur between $2_{p\text{-}\pi}$ orbitals on indole with 3_s or 3_p orbitals on sulfur. We suspect that there are two compensating factors which result in low L value actually found in this work. Firstly the more localized nature of the σ^* orbitals that likely act as an acceptors for either the excitation or an electron are a contributing factor, and in addition the interactions probed in these experiments tend to be at short range where wavefunctions drop off more quickly with distance. An L-value of 0.76 Å, essentially the same as found here, was observed for triplet energy transfer from acetophenone to indole by Strambini and Galley (1976, and the references therein). In the latter study a short-lived energy donor was specifically used to examine the distance dependence at shorter interaction separations and a more steep dependence was found than when a long-live donor (Strambini and Galley, 1975) was involved. Because of the small rate constants that are found for the disulfide-tryptophan perturbation at van der Waals contact, the data in this case also, of necessity, derive from interaction that are very short range in nature where one expects the distance dependence to be steep.

Values of K of $2 \times 10^4\ s^{-1}$ provide a measure of the lower limit of the triplet lifetimes for a chromophore in van der Waals contact with disulfide. These values are orders of magnitude smaller than the values of $10^{10} - 10^{12}\ s^{-1}$ that would be anticipated for either a normal exothermic T-T energy transfer process (Nieman and Robin-

son, 1962; Anderson et al., 1974) or a one-electron transfer (Alexandrov et al., 1978 [and the references therein]; Marcus and Sutin, 1984). On the other hand the values we observe are much larger than those responsible for the anomalous tryptophan phosphorescence lifetimes that have been measured in globular proteins to date. Emission data with secondary butyl disulfide suggests the presence of a disulfide excited state that displays an emission with a band origin at ~380 nm (Li and Galley, unpublished), so that the quenching investigated here could arise from an endothermic energy transfer through this state that lies ~1,000 cm^{-1} above the triplet state of indole. At 77 K one would expect the transfer rate constant to be reduced, assuming an Arrhenius temperature dependence, by a factor of $\sim 10^8$ by virtue of the endothermic nature of the process, and thus a typical transfer rate constant of 10^{12} at room temperature would be reduced to 10^4 due to the height of the energy barrier and thereby fall within the range that is observed in this work. A tryptophan phosphorescence lifetime of 0.8 s such as one finds with J539 corresponds to a quenching constant of only 1.0 s^{-1}. Examination of the immunoglobulin structure (Suh et al. 1986) from the coordinates provided by the Brookhaven Protein Data Bank (Berstein et al., 1977) reveals that the relevant tryptophan-disulfide pairs are 0.6 A out of van der Waals contact. Assuming an L value of 0.75 Å this still only predicts a rate constant at contact of 10 s^{-1}, two orders of magnitude smaller than the value predicted here. The perturbed tryptophans in J539, however, have a 0-0 transition at 417 nm whereas the indole chromophore in IEPK shows the corresponding transition at 411 nm. The lower triplet energy of the indole chromophore in the protein would add 400 cm^{-1} to the energy barrier; further reducing the observed rate constant at 77 K to ~10 s^{-1}.

The mechanism proposed above for the disulfide-triplet indole perturbation is consistent with the observations of von Schütz et al. (1974) that the short-lived anomalous tryptophan phosphorescence lifetimes observed for lysozyme vanish at 4.2 X; the decay under these conditions consisting entirely of the usual 6 s lifetime. Von Schütz et al. (1974) have speculated that the disulfide perturbation is the result of a barrier-dependent one-electron transfer process. Convincing evidence has been presented for the occurrence of a one-electron transfer from tryptophan to disulfide in aqueous solution, and that the triplet state of the chromophore is capable of participating in that process (Bent and Hayon, 1974). It is still not clearly established at present that the shortened triplet lifetime observed at 77 K is, in fact, the result of this one-electron transfer process or whether it arises from an endothermic two-electron exchange.

In steady-state experiment, because the more strongly perturbed chromophores contribute correspondingly less to the overall intensity, the range over which the interaction can be detected is probably 2 to 4 Å. The perturbation revealed by the short components observed from the decays of lysozyme and immunoglobulins falls within this range.

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