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Triplet–Singlet Energy Transfer in Proteins*

William C. Galley† and Lubert Stryer‡

ABSTRACT: Triplet–singlet energy transfer was observed in a protein–chromophore complex. The system studied was a complex of proflavin and α-chymotrypsin. The tryptophan residues of chymotrypsin were the triplet energy donors, while proflavin bound at the active site served as the singlet acceptor. The occurrence of triplet–singlet energy transfer was revealed by a delayed fluorescence from bound proflavin and selective quenching of the tryptophan phosphorescence. The over-all transfer efficiency was higher than 80%. The kinetics of the delayed proflavin fluorescence and of the residual tryptophan phosphorescence revealed that there are at least two classes of tryptophan residues and that their rate constants for triplet–singlet transfer are 20 and 2.2 sec^{-1}.

Our observations suggest that triplet–singlet transfer can serve as a useful adjunct to singlet–singlet transfer inducing proximity relationships in the 15–60-Å range in biological macromolecules. Furthermore, triplet–singlet transfer can aid in elucidating processes involving the singlet and triplet excited states of proteins.

The significant finding in this regard is that the major pathway from the excited singlet level of tryptophan residues in chymotrypsin is internal conversion rather than intersystem crossing.

Electronic excitation energy can be transferred between chromophores. Three types of transfer are known: singlet–singlet, triplet–singlet, and triplet–triplet. Singlet–singlet and triplet–singlet transfer can occur over distances of the order of 40 Å (Förster, 1959; Ermolaev and Sveshnikova, 1963), while triplet–triplet transfer requires a much closer approach of the donor and acceptor groups (Ermolaev, 1963). There are many examples of singlet–singlet transfer in proteins (Stryer, 1968). Fluorescence techniques which utilize this process have proven to be particularly useful in studying the binding of ligands to proteins (Velick, 1961; Weber and Daniel, 1966). Recently, triplet–triplet transfer has been demonstrated in an enzyme–inhibitor complex, thereby providing evidence for the presence of a tryptophan residue near the active site (Galley and Stryer, 1968). In this article, we show that triplet–singlet transfer can occur in proteins. This type of energy transfer has previously been observed in DNA–acridine dye complexes (Isenberg *et al.*, 1964).

The system studied was a complex of proflavin and α-chymotrypsin in a rigid glass at 77°K. Proflavin binds specifically to the active site of this enzyme (Glazer, 1965; Bernhard *et al.*, 1966). The tryptophan residues

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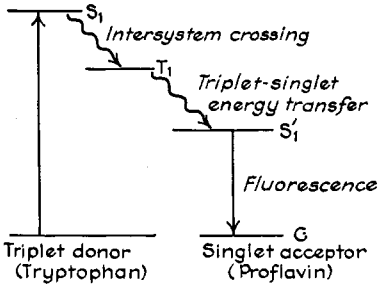

FIGURE 1: Excitation pathway in triplet–singlet energy transfer. S_1 and S_1' denote the first excited singlet states of the donor and acceptor, respectively. T_1 refers to the donor triplet state and G to the ground state. The energy transfer is expressed in terms of a delayed fluorescence of the acceptor.

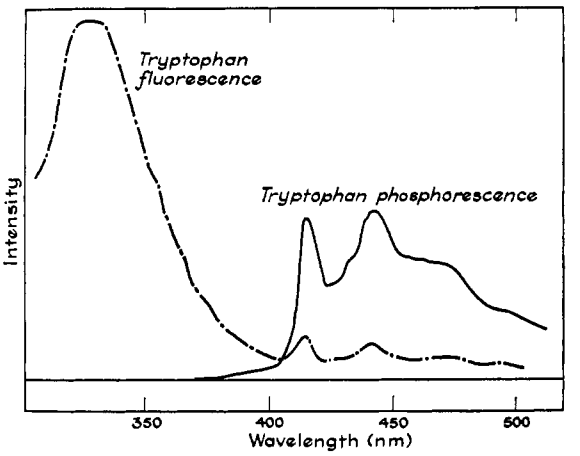

FIGURE 2: Total (—·—) and delayed (———) emission from α-chymotrypsin at 77°K in 2×10^{-3} M pH 6.8 potassium phosphate buffer containing 50% sucrose. The excitation wavelength was 290 nm.

of chymotrypsin were the triplet energy donors, while the bound proflavin served as the singlet acceptor. The path of the excitation energy is shown in Figure 1. *The occurrence of triplet–singlet energy transfer was revealed by a delayed fluorescence from proflavin and a selective quenching of the tryptophan phosphorescence.* The efficiency and kinetics of this transfer process are presented here. Our observations suggest that triplet–singlet transfer can (1) serve as a useful adjunct to singlet–singlet and triplet–triplet transfer in deducing proximity relationships in biological macromolecules, and (2) provide fundamental information concerning processes involving both the singlet and triplet excited states of proteins.

Experimental Procedures

Materials. Proflavin sulfate (Mann), 9-aminoacridine (Fluka), and three-times-recrystallized α-chymotrypsin (Worthington) were commercial products. Mixtures of proflavin and α-chymotrypsin were made in 2×10^{-3} M potassium phosphate containing 50% sucrose. Samples were equilibrated for at least 6 hr at 4° before they were cooled to 77°K to form rigid glasses. Emission experiments were carried out at liquid nitrogen temperature because the triplet state of the energy donor is rapidly quenched in fluid media at room temperature.

Emission Spectra and Kinetics. Emission spectra were obtained on a recording spectrofluorimeter (described previously by Stryer, 1965) which was modified for phosphorescence measurements at 77°K. A Becquerel-type phosphoroscope was used to isolate the delayed emission. The exciting and emitted light beams were chopped out of phase at about 50 cps. This was accomplished with two slotted wheels which were mechanically coupled and driven by the same motor. The length of the open segment of the excitation disk could be adjusted. Total emission spectra were obtained by setting the excitation and emission wheels in phase. For these spectra, an Ebert–Jarrell Ash monochromator was placed in tandem with the regular excitation monochromator to reduce the level of stray light.

The sample cell consisted of a sheet of aluminum foil (45–50 μ thick) which had a 0.5-cm diameter central hole and two quartz plates (0.1 × 1 × 1 cm). A drop of sample was added to the central depression formed by placing the foil on one of the plates. The other plate was then placed on top of the foil, and the plates were pressed together by a clamp. This thin cell was used to reduce the absorbance of the sample to less than 0.05. In this way, trivial radiative transfer was avoided. The sample cell was immersed in liquid nitrogen in a dewar equipped with quartz windows (Thermal-American Quartz Co.). The sample cell was inclined at an angle of 30° to the incident beam, and the emission was observed from the opposite face at 90° to the direction of excitation.

Emission kinetics were recorded in two ways. For emission lifetimes longer than 100 msec, the exciting light was shuttered with a solenoid switch while the phosphoroscope was rotating at about 50 cps. The emission at a given wavelength was then recorded as a function of time on an XY recorder. Emission kinetics in the 1–100-msec range were recorded on a Tektronix 564 storage oscilloscope with 2A63 and 2B67 plug-in units. Measurements were made while the phosphoroscope was rotating at 1–10 cps. Lifetimes were obtained from the decay of the emission during periods in which light was transmitted by the emission chopping wheel.

Quantum Yields. Emission spectra were plotted on a wave number scale and corrected for the variations with wavelength in the sensitivity of the detection system. The relative sensitivity of the IP28 photomultiplier–grating monochromator system, determined by the method of Weber and Teale (1957), was 3.68, 3.34, 2.97, 2.69, 2.55, 2.29, 1.91, 1.78, 1.59, 1.40, 1.31, 1.20, 1.08, 0.99, 0.89, 0.81, and 0.72 at 10-nm intervals from 340 to 500 nm. The integral of the corrected emission spectrum was compared with one obtained from a standard compound that had the same absorbance at the wavelength of excitation. The quantum yields of fluorescence and phosphorescence of α-chymotrypsin at 77°K were obtained using α-chymotrypsin at 20° as a standard of quantum yield 0.10 (Teale, 1960). 9-aminoacridine served as a standard of quantum yield 0.99 (Weber and Teale, 1957) for the determination of

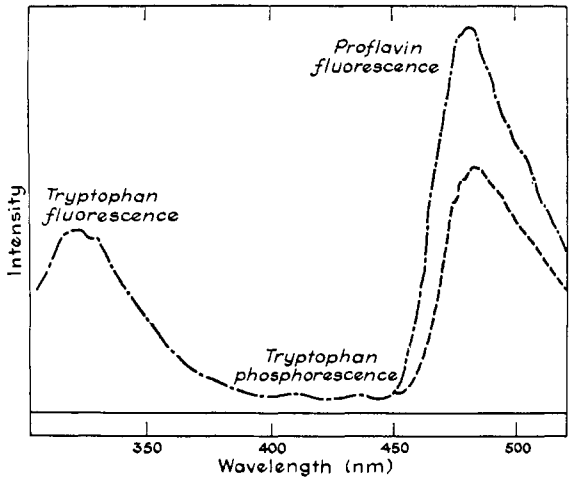

FIGURE 3: Total emission spectra of the α-chymotrypsin–proflavin complex at 77°K excited at 290 nm (—·—) and at 405 nm (– – – –). The concentrations were 4.5×10^{-5} M α-chymotrypsin and 9.0×10^{-4} M proflavin.

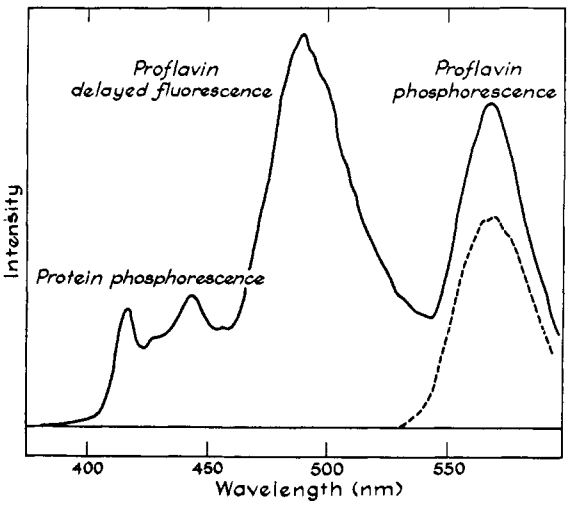

FIGURE 4: Delayed emission spectra of the α-chymotrypsin–proflavin complex at 77°K excited at 290 nm (——) and 405 nm (– – – –). Note that excitation at 290 nm elicited a delayed fluorescence from proflavin with a λ_{max} at 485 nm.

the fluorescence quantum yield of bound proflavin at 77°K. We assume that 9-aminoacridine has the same quantum yield at 77°K as at 293°K, and that the corresponding absorbances are the same. Polarization corrections, which could be as large as 20%, were not applied.

Results

Emission Spectra. The total and delayed emission spectra of chymotrypsin excited at 290 nm are shown in Figure 2. The band centered at 330 nm is tryptophan fluorescence, while the emission between 400 and 500 nm is tryptophan phosphorescence, as initially observed by Freed and Salmre (1958). These bands are also seen in the total emission spectrum of the proflavin–chymotrypsin complex excited at 290 nm (Figure 3). In addition, there is a band at 485 nm, which is proflavin fluorescence. A shoulder at 570 nm due to proflavin phosphorescence also was observed. Excitation of the complex at 404 nm produced only proflavin fluorescence and phosphorescence.

The delayed emission spectra of the proflavin–chymotrypsin complex are shown in Figure 4. Excitation at 404 nm yielded only proflavin phosphorescence. In contrast, excitation of the complex at 290 nm produced tryptophan phosphorescence, proflavin phosphorescence, and a delayed emission peaked at 485 nm. *The spectral distribution of this delayed emission identifies it as proflavin fluorescence.* Delayed proflavin fluorescence was observed only when the excitation wavelength was sufficiently short (less than about 310 nm) so as to also elicit tryptophan phosphorescence. Furthermore, the ratio of the intensity of delayed proflavin fluorescence (at 485 nm) to tryptophan phosphorescence (at 414 nm) was constant over these excitation wavelengths. *This delayed fluorescence at 485 nm arises from triplet–singlet energy transfer in which excitation energy is transferred from the triplet state of tryptophan to the singlet state of proflavin (Figure 1).*

Kinetics of the Delayed Proflavin Fluorescence. The delayed emission at 485 nm of the proflavin–chymotrypsin complex has several kinetic components (Figure 5). About half of the delayed emission decayed too rapidly to be resolved by the XY recorder, *i.e.*, in a time less than 100 msec. The other part is adequately represented (Figure 5) as the sum of two exponentials: about 22% of the delayed emission decays with a lifetime of 450 msec and 22% with a lifetime of 5.2 sec. The 5.2-sec component is tryptophan phosphorescence which overlaps the delayed fluorescence of proflavin. The amount of this long-lived emission deduced from the decay curve (Figure 5) agrees with the intensity of tryptophan phosphorescence expected at 485 nm on the basis of its spectral distribution in the absence of proflavin (Figure 2).

The kinetics of the short-lived portion of the delayed proflavin fluorescence was obtained from single phosphoroscope pulses observed on an oscilloscope. A decay time of about 50 msec was obtained in this way (Figure 6). A faster component was not seen when the speed of rotation of the phosphoroscope was increased, though one would have been detected if its decay time were longer than about 1 msec. *Thus, the delayed proflavin fluorescence consists of two components with lifetimes of 50 and 450 msec.* These values are much shorter than the 5.2-sec lifetime of the tryptophan phosphorescence of chymotrypsin in the absence of transfer, showing that energy transfer did not occur by a trivial radiative process in which proflavin simply reabsorbs phosphorescence already emitted by tryptophan. Rather the 50- and 450-msec lifetimes of the delayed proflavin fluorescence indicate that the triplet-state lifetimes of some tryptophan residues in the complex were markedly shortened as a result of triplet–singlet transfer.

Quenching of Tryptophan Phosphorescence on Binding of Proflavin. Binding of proflavin to chymotrypsin was accompanied by a quenching of tryptophan phosphorescence (Figure 7a) and a concomitant increase in the

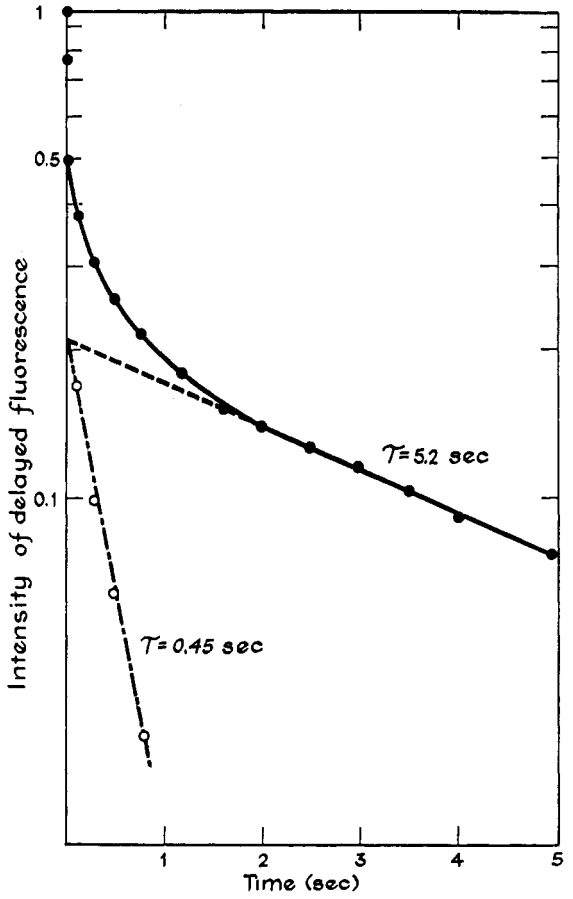

FIGURE 5: Kinetics of the delayed emission of the α-chymotrypsin–proflavin complex at 77°K, measured at 485 nm, the λ_{max} of the proflavin delayed fluorescence. Following an initial very rapid decay, the observed kinetics (●—●) can be represented as the sum of two exponentials with lifetimes of 5.2 sec (– – –) and 0.45 sec (○—○). The excitation wavelength was 290 nm. Concentrations were the same as those in Figure 3.

intensity of delayed proflavin fluorescence (Figure 7b). A plot of $1/\alpha$ *vs.* 1/[proflavin], where α is the ratio of the intensity of delayed proflavin fluorescence to that of tryptophan phosphorescence, gives a binding constant of 1.8×10^{-4} M (Figure 8). The linearity of the plot suggests that proflavin binds to a single site on chymotrypsin at 77°K in much the same way as at room temperature (Bernhard *et al.*, 1966). This suggests that the structure of α-chymotrypsin at 77°K in a rigid glass is not grossly different from that of

TABLE I: Efficiency of Triplet–Singlet Transfer.

Fraction of α-Chymotrypsin Complexed with Proflavin	Fraction of Tryptophan Triplet Quenched by Proflavin
0.75	0.60
0.90	0.72
1.00 (by extrapolation)	0.80

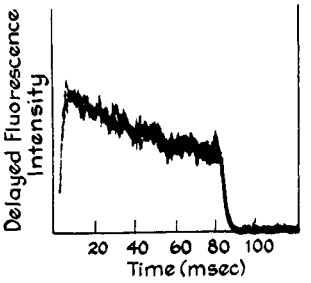

FIGURE 6: Kinetics of the delayed emission of the α-chymotrypsin–proflavin complex at 77°K during individual phosphoroscope pulses. The decay of the short-lived component ($\tau \sim 50$ msec) of the bound proflavin delayed fluorescence is shown here.

the native enzyme in aqueous solution at room temperature. The ratio of proflavin-delayed fluorescence to tryptophan phosphorescence remains constant once this site is saturated, showing that only bound proflavin emits delayed fluorescence (Figure 7b).

The decrease in the ratio of tryptophan phosphorescence to tryptophan fluorescence on binding of proflavin (Figure 7a) demonstrates that the triplet level of tryptophan serves as the energy donor. The efficiency of transfer, E, is given by

$$E = 1 - \frac{(p/f)_s}{(p/f)_0} \qquad (1)$$

where $(p/f)_0$ is the phosphorescence to fluorescence ratio of tryptophan in the absence of proflavin, and $(p/f)_s$ is the ratio deduced by extrapolation for a saturating amount of proflavin. The triplet–singlet transfer efficiency determined in this way is 80% (Table I). This value taken by itself has two possible interpretations: (a) all tryptophan residues transfer some of their triplet energy, so that the average transfer efficiency is 80%; or (b) some of the tryptophan residues do not transfer at all, whereas others transfer their energy with an efficiency close to 100%. The kinetics of tryptophan phosphorescence and of delayed proflavin fluorescence support the latter alternative. Most of the residual tryptophan phosphorescence of the proflavin–chymotrypsin complex has a lifetime of 5.2 sec, which is the same as in chymotrypsin alone. Clearly, one or more tryptophan residues do not transfer any of their triplet energy to proflavin. These tryptophan residues might be located on chymotrypsin molecules which are not complexed with proflavin, in which case the transfer efficiency in the proflavin–chymotrypsin complex would be greater than 80%. Furthermore, the 50- and 450-msec lifetimes of delayed proflavin fluorescence indicate that there are tryptophan residues which transfer 99 and 90%, respectively, of their triplet energy. *Thus, the efficiency and kinetics of triplet–singlet transfer reveal that there are at least two distinct classes of tryptophan residues.*

Efficiency of Excited-State Processes of Tryptophan. The efficiency of intersystem crossing (ϕ_{is}) to the triplet level of tryptophan (S_1 to T_1, in Figure 1) and the radiative effiiciency (ϕ_p) of the triplet state can be determined

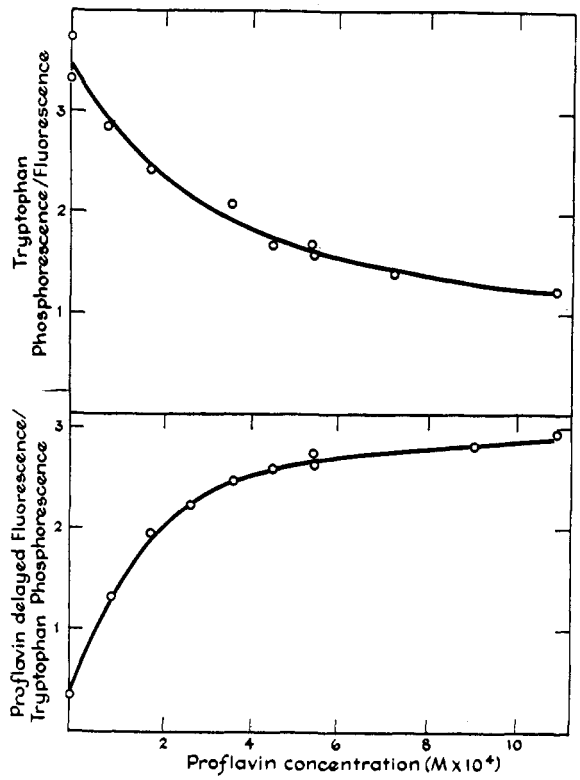

FIGURE 7: Dependence of the donor phosphorescence and acceptor delayed fluorescence on proflavin concentration. The α-chymotrypsin concentration was 4.5×10^{-5} M. Upper: The ordinate is the (phosphorescence)/(fluorescence) ratio of the tryptophan emission. Lower: The ordinate is the (delayed fluorescence))/(tryptophan phosphorescence) ratio. The excitation wavelength was 290 nm. Tryptophan phosphorescence was measured at 414 nm and proflavin delayed fluorescence at 485 nm. The nonzero value of the ratio of proflavin delayed fluorescence to tryptophan phosphorescence in the absence of proflavin arises from tryptophan phosphorescence which occurs at the emission peak of the delayed fluorescence.

as a result of the occurrence of triplet–singlet energy transfer. These parameters are not usually experimentally accessible. Rather, what is usually observed is the quantum yield of phosphorescence, Q_p, which is related to these parameters by

$$Q_p = \phi_{ic}\phi_p \qquad (2)$$

The quantum yield of phosphorescence is defined as the ratio of the number of photons emitted as phosphorescence to the number of photons absorbed at the singlet level. The individual parameters can be determined by using the following relationships. The number of photons, n_p, emitted as tryptophan phosphorescence in the presence of proflavin is given by

$$n_p = n_t\phi_p(1 - E) \qquad (3)$$

where n_t is the number of tryptophan molecules which reach the triplet state per unit of time, ϕ_p is the radiative efficiency of the triplet state, and E is the efficiency of triplet–singlet transfer (given by eq 1). The number of

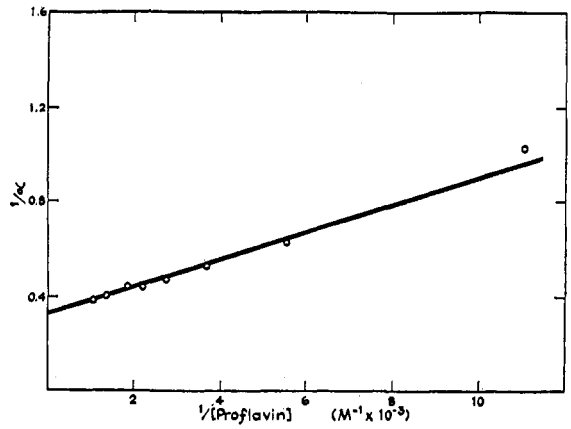

FIGURE 8: Plot of $1/\alpha$ *vs.* 1/[proflavin], where α is the ratio of the intensity of proflavin delayed fluorescence to that of tryptophan phosphorescence. The dissociation constant obtained from the slope and intercept is 1.8×10^{-4} M.

photons, n_{df}, emitted as delayed fluorescence by proflavin is given by

$$d_{df} = n_t E Q_{acc} \qquad (4)$$

where Q_{acc} is the quantum yield of fluorescence of bound proflavin at 77°K (the energy acceptor). Thus, as previously derived by Kellogg and Bennett (1964), the radiative efficiency of the triplet state is given by

$$\phi_p = \frac{n_p}{n_{df}} Q_{acc} \frac{E}{(1 - E)} \qquad (5)$$

Then, the efficiency of intersystem crossing can be calculated from eq 2, if the quantum yield of phosphorescence is known.

The observed values for the proflavin–chymotrypsin system are 0.80 for E, 0.35 for n_{df}, 0.32 for Q_{acc}, and 0.021 for Q_p. From these values, it follows that for the tryptophan residues of chymotrypsin at 77°K, the radiative efficiency of the triplet state is 0.23 and the efficiency of intersystem crossing is 0.09. Since the quantum yield of tryptophan fluorescence under these conditions is 0.18, the efficiency of internal conversion from the excited singlet level is 0.73. *Thus, the major pathway from the excited singlet level of tryptophan residues is internal conversion.* The eightfold difference in the efficiencies of internal conversion and intersystem crossing makes this conclusion a firm one, beyond the uncertainties of experimental errors which may arise from difficulties in measuring absolute intensities in glasses.

Discussion

The occurrence of triplet–singlet energy transfer in the proflavin–chymotrypsin complex is unequivocally demonstrated by the following experimental findings. (1) *The tryptophan phosphorescence to fluorescence (p/f) ratio is reduced on binding of proflavin* (Figure 7a), *showing that the triplet level of tryptophan is selectively depopulated.* It should be noted that the informative parameter in this regard is the *p/f* ratio, rather than the

phosphorescence intensity. Singlet-singlet transfer, in the absence of triplet-singlet transfer, would reduce the phosphorescence intensity by lowering the population of the precursor excited singlet state; however, the *p/f* ratio would remain the same. The triplet level of a chromophore is demonstrated to be an energy donor only if its phosphorescence is quenched to a greater extent than its fluorescence. (2) *The bound proflavin exhibits a sensitized delayed fluorescence, showing that the triplet excitation energy is transferred to an excited singlet state of proflavin* (Figure 4). Delayed fluorescence is not in itself proof of triplet-singlet transfer, since delayed fluorescence may also result from triplet-triplet annihilation (Parker and Hatchard, 1962; Sternlicht *et al.* 1963), thermal activation of an excited triplet to an excited singlet state (Lewis *et al.*, 1941), or recombination following photoionization (Lim and Swenson, 1962). An important finding in support of triplet-singlet transfer here is that the delayed fluorescence of proflavin could not be elicited by direct excitation of bound proflavin; excitation of tryptophan was required (Figure 4). (3) The occurrence of triplet-singlet transfer introduces an additional pathway for the decay of the donor triplet so that its lifetime, and *therefore the lifetime of the delayed fluorescence, must be shorter than the phosphorescence lifetime of the donor in the absence of transfer*. This requirement is met here. The lifetimes of the delayed fluorescence of proflavin are 50 and 450 msec, whereas the phosphorescence lifetime of tryptophan in the absence of transfer is 5.2 sec (Figure 5).

Excited-State Processes in Tryptophan Residues. Triplet-singlet energy transfer can reveal the efficiencies of internal conversion and intersystem crossing processes. Insight into these radiationless processes would undoubtedly contribute toward a more effective and comprehensive use of fluorescence and phosphorescence techniques in studies of the conformation of biological macromolecules. Interpretation of the quantum yields of fluorescence of most compounds is uncertain at present. It is not even generally known whether internal conversion or intersystem crossing is the major dissipative pathway. The relative importance of these has previously been determined for only a few chromophores (Lamola and Hammond, 1965). The present study appears to provide the first determination of this kind for the macromolecule. Our results demonstrate that rapid intersystem crossing to the triplet state is not responsible for the low fluorescence yield of the tryptophan residues of α-chymotrypsin. *The major pathway from the excited singlet level of these tryptophan residues is internal conversion.* The possibility that photochemical events at the excited singlet level also contribute to the decay of that state cannot be excluded at this time.

The high efficiency of internal conversion observed for the tryptophan residues of α-chymotrypsin contrasts with predictions based on current theoretical treatments of radiationless transitions. Efficient internal conversion is not expected for chromophores like tryptophan which have a large energy difference between the ground and the first excited singlet state (Robinson and Frosch, 1963; Siebrand, 1967). The theory, which emphasizes the importance of vibrational changes in internal conversion, has been successful in accounting for the excited-state properties of aromatic hydrocarbons (Kellogg and Convers Wyeth, 1966; Powell, 1967). However, the theory has failed to account for the high internal conversion efficiency observed in purines and pyrimidines (Guéron *et al.*, 1967), as well as for tryptophan.

The nonexponential decay of the proflavin delayed fluorescence in the complex with α-chymotrypsin indicates that all of the tryptophan residues in the enzyme do not transfer to the bound proflavin at the same rate. The eight tryptophan residues in the protein can be divided into different classes on the basis of the rate at which they undergo triplet-singlet transfer to proflavin. The resolution of at least two exponential components in the delayed fluorescence indicates that there are at least two classes which transfer with rate constants of 20 and 2.2 sec^{-1}.

It is apparent that the triplet state of a number of tryptophans is populated in the complex and that intersystem crossing does not occur at a single tryptophan residue. It may also be concluded that the tryptophans in a α-chymotrypsin are not strongly coupled at the triplet level by triplet-triplet energy transfer. In that case, triplet-singlet transfer to proflavin would occur at a single rate. Our conclusion is in agreement with that of Konev and Bobrovich (1965) based on observations of the polarization of tryptophan fluorescence and phosphorescence in chymotrypsinogen.

Triplet-Singlet Transfer as a Spectroscopic Ruler. The efficiency and kinetics of triplet-singlet energy transfer are markedly dependent upon the distance between the donor and acceptor (see Appendix). This suggests that triplet-singlet transfer can be used to reveal distance relationships in proteins and other macromolecules, as proposed for singlet-singlet transfer (Latt *et al.*, 1965; Stryer and Haugland, 1967) and triplet-triplet transfer (Galley and Davidson, 1966; Galley, 1968; Galley and Stryer, 1968). The dependence of the transfer efficiency upon distance is the same for singlet-singlet and triplet-singlet transfer. Since both can occur over distances of the order of 40 Å, it is of interest to consider their relative merits as spectroscopic rulers. The major advantage of singlet-singlet transfer is that it can be observed in fluid media at room temperature. In contrast, triplet-singlet transfer is readily detected only in rigid media at low temperature, because the triplet state of the donor is usually rapidly quenched under other conditions. An important compensating advantage of triplet-singlet transfer is that it is much slower than singlet-singlet transfer, and so the kinetics of the transfer can be measured readily. The time course of efficient singlet-singlet transfer is typically in the subnanosecond time range, whereas for triplet-singlet transfer it is in the microsecond or millisecond time range. The present study demonstrates that the kinetics of the transfer process can be highly informative. The different tryptophan residues that serve as triplet donors here are clearly distinguished by the 50 and 450 msec delayed fluorescence lifetimes (Figures 5 and 6). A greater range of distances can be measured by a particular donor-acceptor

pair if kinetic measurements are made. For example, it would be difficult to distinguish between a transfer efficiency of 99 and 99.9%; however, a tenfold reduction in the lifetime of the delayed fluorescence would be easily detected. Thus, distances shorter than R_0 (see Appendix) should be more readily measured from the kinetics than from the efficiency of transfer.

Optimal use is made of triplet–singlet transfer as a spectroscopic ruler when there is only one energy donor and one energy acceptor. The energy donor should have a high efficiency of intersystem crossing to the triplet level. If distances of the order of 40 Å are to be measured, the radiative efficiency of the triplet state of the donor should also be high. Acetophenone derivatives appear to be very suitable triplet donors. Bromacetophenone, for example, can be used as a labeling reagent for this purpose. The energy acceptor has to absorb light of wavelengths longer than about 400 nm. Also, the acceptor should have a high quantum yield of fluorescence. Fluorescein is one chromophore that meets these criteria for an energy acceptor, and also it can be covalently linked to proteins.

A problem that will be encountered in the use of singlet–singlet or triplet–singlet energy transfer to measure distance is that the relative orientation of the donor and acceptor are also important (Appendix, eq 7). A low transfer rate or efficiency may result from a large separation or an unfavorable orientation of the donor–acceptor pair. This ambiguity may be resolved in part by utilizing a series of energy acceptors that have different geometrical modes of attachment to the same point in the macromolecule. Another approach is to use donor–acceptor pairs which have spectroscopic properties that allow both triplet–singlet and singlet–singlet energy transfer. The orientation factor, K^2, will be different for these two types of transfer because the transition moment for phosphorescence has a direction that is usually different from that for fluorescence. The likelihood that K^2 will be unfavorable for both transfer processes is rather low. Thus, triplet–singlet and singlet–singlet transfer can complement each other in studies of biological macromolecules.

Appendix

Efficiency and Kinetics of Triplet–Singlet Transfer. The dependence of triplet–singlet transfer upon distance has not yet been calibrated in a model system of defined length. However, there is strong evidence that Förster's theory of dipole–dipole coupling applies to triplet–singlet transfer as well as to singlet–singlet transfer (Ermolaev and Sveshnikova, 1963; Bennett *et al.*, 1964; Kellogg, 1967). Singlet–singlet transfer has been shown to depend upon the inverse sixth power of the distance between the donor and acceptor (Stryer and Haugland, 1967).

According to Förster's theory, the rate constant, k_{ts}, for triplet–singlet transfer is given by

$$k_{ts} = k_p r^{-6} K^2 J n^{-4} \times 8.71 \times 10^{23} \qquad (6)$$

where k_p is the rate constant of phosphorescence of the triplet donor, r is the distance (in ångströms) between the donor and acceptor, K^2 is the orientation factor for dipole–dipole coupling, n is the refractive index of the medium, and J is the spectral overlap integral. The orientation factor is given by

$$K^2 = (\cos\gamma - 3\cos\alpha\cos\beta)^2 \qquad (7)$$

where γ is the angle between the donor and acceptor transition moments; α is the angle between the transition moment of the donor and the line joining the centers of the two groups; and β is the angle between the transition moment of the acceptor and this line. J, a measure of the overlap of the phophorescence spectrum of the donor and the absorption spectrum of the acceptor, is defined as

$$J = \frac{\int P(\lambda)\epsilon(\lambda)\lambda^4 d\lambda}{\int P(\lambda) d\lambda} \qquad (8)$$

where $P(\lambda)$ is the phosphorescence intensity at wavelength λ (in cm) and $\epsilon(\lambda)$ is the extinction coefficient (in cm^2/mmole) of the energy acceptor. The units of J are cm^6/mmole.

The efficiency of triplet–singlet transfer is given by

$$E = \frac{r^{-6}}{r^{-6} + R_0^{-6}} \qquad (9)$$

where R_0 is the distance at which the transfer efficiency is 50%. From eq 6, and noting that

$$E = \frac{k_{TS}}{k_{TS} + k_x + k_p} \qquad (10)$$

where k_x is the rate constant for radiationless processes (other than triplet–singlet transfer) at the triplet level of the donor, it follows that R_0 (in ångströms) is given by

$$R_0 = (K^2 J n^{-4} \phi_p)^{1/6} \times 9.79 \times 10^3 \qquad (11)$$

ϕ_p, the radiative efficiency of the triplet state, is defined as $k_p/(k_p + k_x)$.

It should be noted that the expressions for the efficiency and kinetics of singlet–singlet transfer are analogous to the ones given above. The rate constant k_F for fluorescence of the donor substitutes for k_p in eq 6. The fluorescence spectrum of the donor, rather than the phosphorescence spectrum, enters into eq 8. The fluorescence quantum yield replaces ϕ_p in eq 11. The essential difference between triplet–singlet and singlet–singlet transfer is that the former process is much slower, since k_p is typically 10^8-fold less than k_F. However, the efficiencies of the two types of transfer are comparable, because the competing processes at the triplet level are also much slower than at the singlet level.

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