an up-to-date and relatively full account of the subject, together with a detailed bibliography. I refer to this book for detailed documentation for this article in which only the most important and representative references will be cited.
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39. J. Treistman [*Science* **160**, 853–56 (1968)], in an attempt to show that "China, at 1000 B.C., was an area of great diversity," tries to refute the whole Lungshanoid concept. She believes that the "so-called prehistoric Lungshanoid is conceived in terms of dynastic succession and expansion as it spreads its enlightening influence throughout China." By using this North China–derived concept to "cover prehistoric and early historic phenomena occurring outside of the nuclear area," my interpretation of this part at least of Chinese prehistory is thus "a reversion to the technique of writing colonial history." That China, at 1000 B.C., or any other time, was a land of diversity is not open to question, but diverse local cultures are subject to classificatory groupings at various levels of contrast for different interpretive objectives. What Dr. Treistman has apparently failed to grasp is that "Lungshanoid" is merely a classificatory label at a high level of contrast, and that under this label cultural regionalization is not only possible but inevitable. Furthermore, the label "Lungshanoid" is descriptive, or even interpretive, but not evaluative. To say that the Lungshanoid *influenced* some culture is not to say that it "enlightened" it. I wish Dr. Treistman had documented her statement that I conceived of the Lungshanoid concept "in terms of *dynastic* succession and expansion as it spreads its *enlightening* influence throughout China" (Italics mine). Migrations, expansions, and mutual influences of prehistoric cultures and peoples took place —or are thought by archeologists as having taken place—throughout human existence all over the world, and I do not see anything peculiar about their having taken place in and around China. The Lungshanoid interpretation simply represents my current view of what took place, according to the available evidence, expressed in a classificatory taxonomy.

Fluorescence Spectroscopy of Proteins

Fluorescent probes provide insight into the structure, interactions, and dynamics of proteins.

Lubert Stryer

Proteins are directly involved in all known biological processes except for the storage of genetic information. The diversity of functions carried out by proteins is matched by the variety of their three-dimensional structures. A challenging area of inquiry in molecular biology is the relation between protein structure and function. In particular, protein chemists are trying to answer three basic questions with respect to the relation between structure and function: (i) How do proteins fold? That is, how does the simple linear array of 20 kinds of amino acids specify the complex three-dimensional structure crucial for the function of the protein? (ii) How do proteins recognize other molecules? What is the basis of the high degree of selectivity displayed by proteins in their interactions with large and small molecules? (iii) How do proteins carry out particular functions, such as catalysis, transport, and motility?

Detailed structural and kinetic data on proteins and their interactions are needed to answer these questions. X-ray crystallography, one of the most powerful experimental approaches, has revealed the structure of five proteins, thereby contributing toward a deeper understanding of how they function. However, the x-ray crystallographic method is not sufficient in itself. The view it affords, although magnificently detailed, is essentially static. Furthermore, not all interesting biological materials can be crystallized. Clearly, no single experimental approach can encompass the richness and complexity inherent in problems of protein structure and function. A number of quite different physical and chemical techniques must be applied and their results correlated.

I shall now discuss some aspects of fluorescence spectroscopy as used to gain insight into the structure and dy-

The author is associate professor of biochemistry at the Stanford University School of Medicine, Palo Alto, California.

namics of protein molecules. In particular, I will illustrate some ways in which fluorescent probes have been used in my laboratory to (i) establish the degree of polarity of a particular region of a protein; (ii) measure distances between groups in a protein; (iii) determine the extent of flexibility of a protein; and (iv) measure the rate of very rapid conformational transitions.

Excited-State Processes

A molecule excited to an upper electronic state (such as S_2) can return to the ground state (S_0) in a number of ways (Fig. 1). First, the molecule very rapidly goes from S_2 to the lowest excited state (S_1) without emitting a photon. Some of the possible fates of S_1 are (i) fluorescence, a transition of S_0 accompanied by emission of a photon; (ii) internal conversion, a return to S_0 without radiation; and (iii) intersystem crossing, a transition to an excited triplet state (T_1) in which the electron spins are no longer paired as in the singlet states. T_1 may return to the ground state in a radiative process termed phosphorescence, or it may return without emitting radiation. Alternatively, S_1 and T_1 may transfer their excitation energy to other chromophores or participate in photochemical reactions.

The time scale of these processes has important experimental consequences. In the absence of nonradiative processes, S_1 typically has a lifetime of a few nanoseconds (1 nsec = 10^{-9} seconds), whereas T_1 usually has a lifetime between a millisecond and several seconds. The long lifetime of T_1 makes it highly vulnerable to quenching processes, particularly those that are limited by diffusion. The result is that phosphorescence is seldom observed except in rigid media. In contrast, chromophores in fluid solution at room temperature are often fluorescent. The fact that fluorescence measurements can be made under a wider range of conditions than phosphorescence measurements accounts for the more extensive use of fluorescence spectroscopy in studies of proteins.

Role of Fluorescent Probes

There are three types of fluorescent chromophores in proteins—intrinsic, coenzymic, and extrinsic. The intrinsic chromophores are the aromatic side chains of phenylalanine, tyrosine, and tryptophan residues. A small proportion of proteins also contain a fluorescent coenzyme such as reduced nicotinamide-adenine dinucleotide, flavin-adenine dinucleotide, or pyridoxal phosphate. Studies of these intrinsic and coenzymic chromophores have furnished important information on the structure and interactions of a number of proteins (*1*). However, nature does not always provide the right chromophore at the right place in a protein. Frequently, the potential benefits of fluorescence methods can be realized only if the investigator first inserts a suitable chromophore into the protein of interest. This approach, introduced by Weber in 1952 (*2*), will be the focus of this article. Some recent developments in the design, calibration, and application of such extrinsic chromophores will be considered.

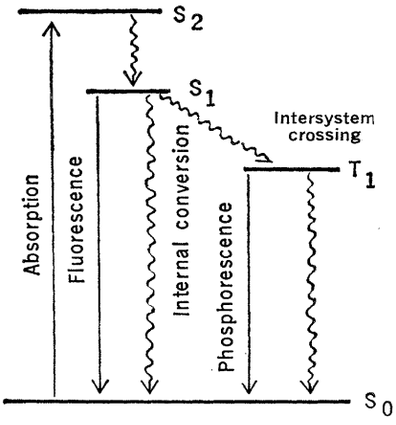


Fig. 1. Excited-state processes. Straight arrows denote processes in which a photon is emitted or absorbed; wavy arrows denote transitions which do not emit radiation.

The first requirement for the optimum use of an extrinsic chromophore is that it be bound or covalently attached to the protein at a unique location, such as the active site. Second, the fluorescence properties of the probe should be sensitive to the structure and dynamics of its environment in ways that are amenable to definitive interpretation. For example, the emission characteristics should reveal whether the probe is in a polar or nonpolar environment and whether it is near or far from another chromophore. Finally, insertion of the probe should not appreciably disturb those features of the protein that are being investigated.

Probes of Polarity

In 1954 Weber and Laurence discovered that a number of polycyclic aromatic compounds which are nonfluorescent in water become highly fluorescent on binding to serum albumin (*3*). One of the chromophores which showed this effect was 1-anilino-8-naphthalene sulfonate (ANS). Subsequent studies have demonstrated that this compound and related chromophores can be used as sensitive probes of the polarity of their environment (Table 1).

Through study of the complex of ANS and apomyoglobin, the factors which influence the emission of the ANS have been clarified (*5*). Apomyoglobin, which is myoglobin minus its heme group, was chosen because it has a highly nonpolar heme-binding site, as shown by high-resolution x-ray studies. For this reason, apomyoglobin was expected to bind ANS. In fact, ANS binds stoichiometrically to a specific site on apomyoglobin with a dissociation constant of the order of $10^{-5}M$.

Furthermore, addition of hemin led to the displacement of ANS from its complex with apomyoglobin, which suggests that ANS and heme bind to the same site or to sites that substantially overlap one another. There was a striking change in the fluorescence of ANS on binding to apomyoglobin. The quantum yield (Q) increased from 0.004 to 0.98, whereas the wavelength of maximum emission (λ_{max}) shifted from 515 to 454 nm. Similar results were obtained for the interactions of ANS with apohemoglobin, which also has a highly nonpolar niche for the heme group.

The inference that the fluorescence

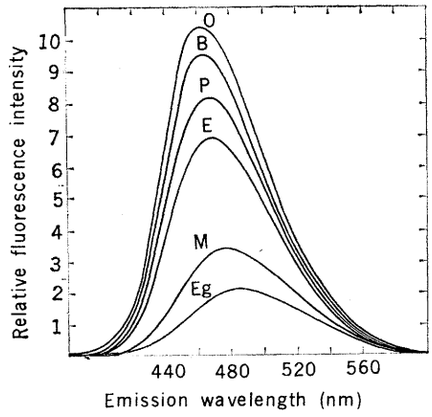


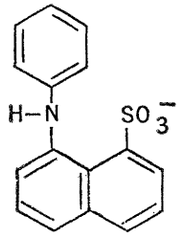
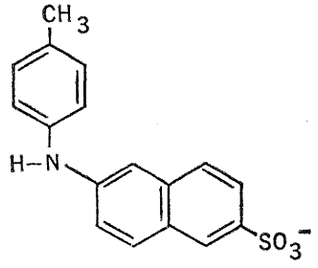
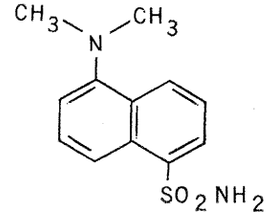
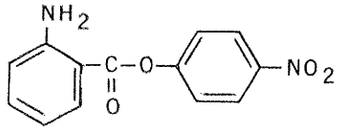
Fig. 2. Fluorescence emission spectra of 1-anilino-8-naphthalene sulfonate (ANS) in alcohols. The quantum yield increases and the emission maximum shifts toward the blue as the solvent polarity decreases in the order: ethylene glycol (*Eg*), methanol (*M*), ethanol (*E*), *n*-propanol (*P*), *n*-butanol (*B*), and *n*-octanol (*O*) (*5*).

properties of the sulfonate depend on the polarity of its environment was further supported by studies of the emission of this compound in various organic solvents (*5*). The emission spectra of ANS in a series of alcohols are shown in Fig. 2. As the polarity of the solvent decreased, Q increased and λ_{max} shifted toward the blue. In ethylene glycol Q was 0.15 and λ_{max} was 484 nm, whereas in *n*-octanol, a much less polar solvent, the corresponding values were 0.63 and 464 nm. A similar effect of solvent polarity on quantum yield and emission maximum was observed for ANS in mixtures of ethanol and water (Fig. 3).

The dependence of the λ_{max} of ANS on the polarity of the solvent results from a reorientation of the solvent shell around the chromophore when it is excited. Fluorescent groups which have higher dipole moments in the excited state than in the ground state show this effect (*12*). The more dipolar, excited state of ANS interacts with a polar solvent so as to further align the solvent dipoles, whereas the solvent shell in a nonpolar solvent is less disturbed. A photon of lower energy is emitted by ANS in a polar solvent, since some of the solvation energy of the excited state is lost when the chromophore returns to the ground state. Consequently, the emission is shifted toward the red in a polar solvent. The effect of solvent on the quantum yield is not yet well understood, but the mechanisms involved are distinct from those which influence the wavelength of maximum emission. For ANS, Q, is more sensitive than λ_{max} to the addition of small amounts of a polar solvent to a nonpolar one. (Fig. 3). The wavelength of maximum emission reflects the overall dipolar character of the solvent, whereas Q is sensitive to more localized deactivating processes. This conclusion is further supported by the observation that the quantum yield of ANS is threefold greater in D_2O than in H_2O, whereas λ_{max} is the same in the two solvents (*13*).

Fluorescent probes like ANS have been used to investigate various aspects of the structure and interactions of a number of proteins (*14*) (Table 1). First, the degree of polarity of several active sites has been determined. Apomyoglobin (*5*), apohemoglobin (*5*), and carbonic anhydrase (*10*) are highly nonpolar in this respect. Serum albumin (*4*), antibody to the dimethylaminonaphthalene-5-sulfonyl group (anti-dansyl antibody) (*9*), and alcohol dehydrogenase (*7*) have moderately nonpolar binding sites, whereas chymotrypsin has a highly polar active site (*11*). The degree of polarity of an active site may have important implications for the stability of the prosthetic group, the forces involved in the binding of substrate, or the mechanism of catalysis (*15*). Second, the binding of substrates and coenzymes to proteins can readily be followed if a fluorescent probe is located at or near the active site. The fluorescent probe may be displaced by the substrate, or its fluorescence characteristics may be altered on formation of a ternary complex (*8*). Similarly, conformational changes that influence the catalytic process may be detected (*8*).

Table 1. Fluorescent probes of polarity.

Probe	Protein
1-Anilino-8-naphthalene sulfonate (ANS) 	Serum albumin (*3*, *4*)
	Apomyoglobin and apohemoglobin (*5*)
	Histone (*6*)
	Alcohol dehydrogenase (*7*)
2-*p*-Toluidinyl-6-naphthalene sulfonate (TNS) 	Chymotrypsinogen and chymotrypsin (*8*)
Dansyl sulfonamide 	Anti-dansyl antibody (*9*)
	Carbonic anhydrase (*10*)
p-Nitrophenyl anthranilate (NPA) 	Chymotrypsin (*11*)

Energy Transfer

Still another use of fluorescent probes is based on observations that energy absorbed by a chromophore can be transmitted to another chromophore some distance away. There are three types of energy transfer: singlet-singlet, triplet-singlet, and triplet-triplet (Table 2). In singlet-singlet transfer, the return of the energy donor from S_1 to the ground state is coupled to the excitation of the energy acceptor from the ground state to a higher singlet level. This type of energy transfer occurs over appreciable distances (*16*). Latt, Cheung, and Blout (*17*) observed efficient singlet-singlet transfer between chromophores located at the ends of a rigid steroid molecule 20 Å long, and H. Kuhn and co-workers (*18*) demonstrated efficient transfer between monolayers of chromophores separated by distances of about 50 Å.

Förster proposed that singlet-singlet transfer occurs by a resonance interaction of the dipole pair between the energy donor and acceptor chromophores (*19*). In his quantitative treatment the

Table 2. Types of electronic energy transfer.

Energy transfer	Excited state of donor	State to which acceptor is excited	Transfer mechanism	Effective range (Å)	Experimental criterion
Singlet-singlet	S_1	S_1 or higher	Dipole-dipole interaction	Up to 65	Sensitized fluorescence
Triplet-singlet	T_1	S_1 or higher	Dipole-dipole interaction	Up to 65	Delayed fluorescence
Triplet-triplet	T_1	T_1	Electron exchange	Less than 15	Sensitized phosphorescence

distance R_0 (at which singlet-singlet transfer is 50 percent efficient) is related to spectroscopic and geometric variables by the expression

$$R_0 = 9.79 \times 10^3 (Jn^{-4}K^2Q)^{1/6} \quad (1)$$

in which J is the integral of spectral overlap, n is the refractive index of the medium, K is the orientation factor of the dipole pair, and Q is the quantum yield of the energy donor (*20*). The overlap integral of the extinction coefficient of the acceptor and the emission spectrum of the donor (normalized to unity on a scale of wave number), measures the extent to which the oscillators are in resonance. The medium between the donor and acceptor has no effect on the transfer apart from the refractive index factor (n^{-4}), provided that the medium is transparent at wavelengths at which the energy donor emits. The angular factor K^2 can vary between 0 and 4. For random orientation of the groups, K^2 is ⅔. The quantum yield of the energy donor is a factor in Eq. 1 because internal conversion in the donor competes with energy transfer.

Förster's theory further predicts that the rate constant for singlet-singlet transfer is proportional to r^{-6}, where r is the distance between the energy donor and acceptor. The efficiency E of singlet-singlet transfer for a donor-acceptor pair at a defined distance and orientation is then

$$E = r^{-6}/(r^{-6} + R_0^{-6}) \quad (2)$$

Stryer and Haugland (*21*) have tested the validity of Förster's theory of singlet-singlet energy transfer by the model system shown in Fig. 4. Oligomers of poly-L-proline served as spacers of defined length to separate an energy donor and acceptor by distances ranging from 12 to 46 Å. The method of solid-phase peptide synthesis was used for the stepwise synthesis of oligomers containing up to 12 prolyl units. The optical rotatory dispersion of these compounds in the ultraviolet region showed that the oligomers containing five or more prolyl residues are in a *trans* helical conformation. Since the atomic coordinates of this helical structure were known, the distances between the energy donor and acceptor in the various oligomers were accurately defined. Two other properties of poly-L-proline were important for this study; in dilute solution intermolecular aggregation did not occur, and the polypeptide did not participate in the transfer process, since the absorption bands of lowest energy of the prolyl units were in the far-ultraviolet region.

The α-naphthyl and dansyl groups were chosen as the donor and acceptor because they meet several spectroscopic criteria. First, their absorption and emission spectra are distinct, which makes it easy to determine how many photons are absorbed and emitted by each group. Furthermore, the emission spectrum of the α-naphthyl group substantially overlaps the absorption spectrum of the dansyl group, so that the magnitude of the integral of spectral overlap (J in Eq. 1) is large. Efficient energy transfer is also favored by the high quantum yield of fluorescence of the α-naphthyl group in the absence of the acceptor. Finally, the dansyl group is highly fluorescent, which simplifies the detection of the energy transfer.

The efficiency of energy transfer in each oligomer was determined from excitation spectra of the dansyl emission (Fig. 5). The excitation spectrum of an isolated chromophore is coincident with its absorption spectrum. However, in the presence of energy transfer, the excitation spectrum shows additional contributions corresponding to absorption by the energy donor. At a given wavelength, the magnitude of the excitation spectrum F is related to the transfer efficiency E and the extinction coefficients of the donor, ε_D, and acceptor, ε_A, by the expression

$$F = \epsilon_A + E\epsilon_D \quad (3)$$

In the present system, the absorption maximum of the acceptor was at 340 nm. The donor exhibited maximum absorption at 290 nm, where the acceptor had an absorption minimum. When one prolyl residue separated the donor and acceptor, the excitation spectrum was identical to the sum of the absorption spectra of the two chromophores, which indicates a transfer efficiency of 100 percent (Fig. 5). As the number of prolyl residues increased, the excitation peak at 290 nm decreased, which demonstrates a reduced transfer efficiency.

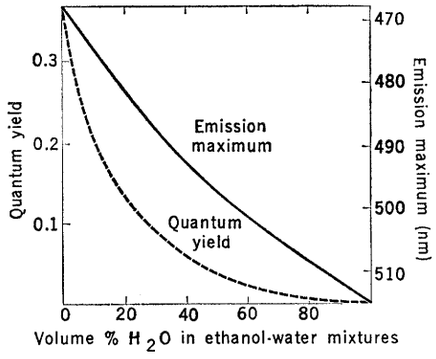


Fig. 3. Dependence of quantum yield and emission maximum of ANS on the percent (by volume) of H_2O in H_2O-ethanol mixtures.

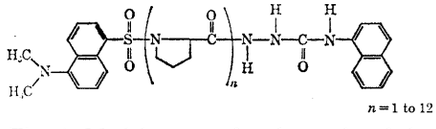


Fig. 4. Model system for the study of the dependence of singlet-singlet energy transfer on distance. The energy donor, a naphthyl group, was separated from the energy acceptor, a dansyl group, by oligomers of poly-L-proline. The number of prolyl residues ranged from 1 to 12 (*21*).

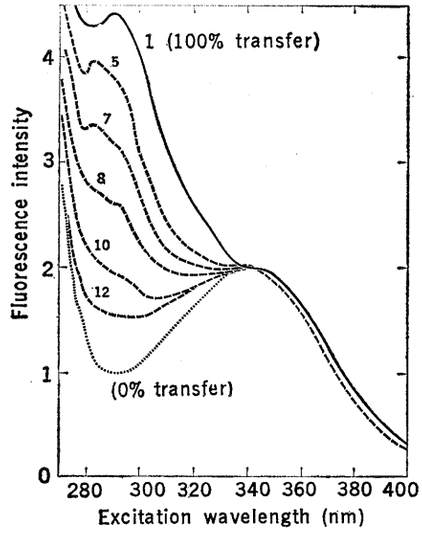


Fig. 5. Excitation spectrum of dansyl-L-prolyl-hydrazide (dotted line, 0 percent transfer); dansyl-L-prolyl-α-naphthyl (unbroken line, 100 percent transfer) and dansyl-(L-prolyl)-α-naphthyl (dashed line, $n = 5, 7, 8, 10, 12$) (*21*).

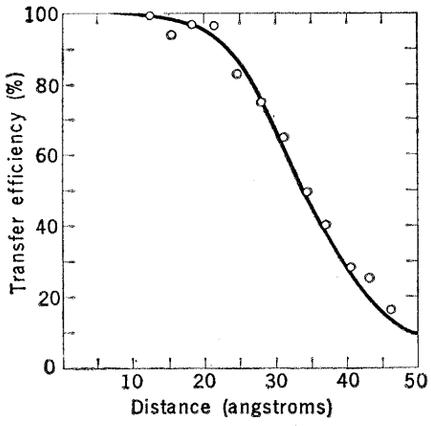


Fig. 6. Efficiency of energy transfer as a function of distance in dansyl-(L-prolyl)$_n$-α-naphthyl. The observed efficiencies of transfer for $n = 1$ to 12 are shown as points. The solid line corresponds to an r^{-6} distance dependence (*21*).

The efficiency of transfer as a function of distance is given in Fig. 6. The exponent j of the distance dependence r^{-j} was determined from the slope of a curve in which log $(E^{-1} - 1)$ is plotted as a function of log r. A linear relation was found with $j = 5.9 \pm 0.3$. Thus, the experimentally observed distance dependence of singlet-singlet energy transfer (*21*) is in excellent agreement with the r^{-6} dependence predicted by Förster.

Spectroscopic Rulers

A particularly attractive prospect suggested by these results is that singlet-singlet energy transfer might be used to reveal proximity relations in the range between 15 and 65 Å in proteins and other biological macromolecules

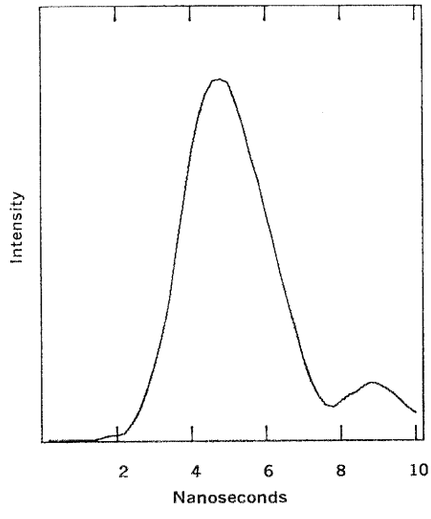


Fig. 7. Nanosecond light pulse (*29*).

(*21*). A number of conditions would have to be met first: (i) there should be a single donor and a single acceptor at specific sites on the macromolecule; (ii) the R_0 value of the donor-acceptor pair should be calibrated, and the value should be comparable to the magnitude of the distance measured; and (iii) information on the relative orientation of the donor-acceptor pair would be needed to determine whether a low transfer efficiency resulted from a large separation of the donor and acceptor or from an unfavorable orientation. Some experimental approaches for meeting these requirements can be suggested. The R_0 value of donor-acceptor pairs could be varied from about 15 to 45 Å by altering the quantum yield of the donor or the absorption spectrum of the acceptor. The orientation ambiguity might be resolved by a series of energy acceptors that have different geometrical modes of attachment to the same side chain of the protein. Alternatively, it might be possible to achieve randomization of the orientation of the donor-acceptor pair, as in the poly-L-proline model system. The requirement for a single donor and acceptor at unique sites in the macromolecule is a particularly challenging one. Techniques for specific attachment of fluorescent groups are being developed; an example is given below.

Triplet-singlet energy transfer (*22*) (Table 2) may also be useful as a spectroscopic ruler. Phosphorescence techniques are used to detect this type of energy transfer since the sensitized fluorescence of the energy acceptor is delayed. The time course of the sensitized emission is determined by the relatively long life of the triplet state of the energy donor. The kinetic studies of Bennett, Schwenker, and Kellogg (*23*) on intermolecular triplet-singlet transfer clearly indicate that its distance dependence is very much like that of singlet-singlet transfer. Galley and Stryer have recently observed triplet-singlet transfer in a complex of chymotrypsin and proflavin in a rigid glass at 77°K (*24*). The tryptophan residues of chymotrypsin were the energy donors, whereas proflavin, specifically bound to the active site, was the energy acceptor. In this system, several rate constants of triplet-singlet transfer corresponding to different distances and orientations of the several tryptophan residues relative to the bound proflavin were found. It seems probable that triplet-singlet transfer will complement singlet-singlet transfer in the study of proximity relations in the range between 15 and 65Å.

Triplet-triplet energy transfer (*25*) is a promising means for showing that chromophores are in close proximity. This process (Table 2) differs from singlet-singlet and triplet-singlet transfer in that it occurs by an electron-exchange interaction. The precise dependence of triplet-triplet transfer on distance is not yet known. However, studies of mixtures of donor and acceptor at high concentration indicate that efficient transfer occurs only when the centers of the groups are less than about 12 Å apart (*25*). Galley and Stryer (*26*) have recently used triplet-triplet energy transfer to determine whether there is a tryptophan residue at the active site of carbonic anhydrase. A potential triplet energy donor, *m*-acetylbenzenesulfonamide (MABS), was specifically bound to the zinc atom at the active site. This sulfonamide was chosen because its excited singlet level is lower than that of tryptophan, whereas its triplet level is higher. Thus it is possible to excite this

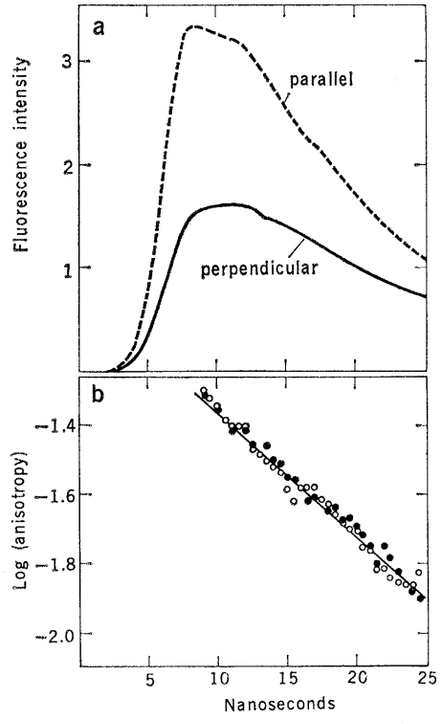


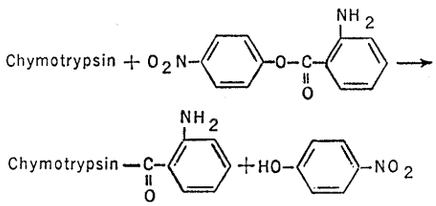
Fig. 8. Nanosecond fluorescence polarization of anthraniloyl chymotrypsin in 0.1*M* phosphate buffer, *p*H 6.8, at 22°C. (a) Intensity of the parallel and perpendicularly polarized components of the fluorescence as a function of time. (b) Logarithm of the emission anisotropy as a function of time. The slope (when corrected for the finite duration of the light pulse) yields a rotational relaxation time of 52 nanoseconds for the anthraniloyl chromophore.

compound at 330 nm, where tryptophan does not absorb. Excitation of the bound sulfonamide compound at 330 nm resulted only in tryptophan phosphorescence. This finding of highly efficient transfer of triplet-triplet energy from the sulfonamide to tryptophan revealed that there is a tryptophan residue at or close to the active site of carbonic anhydrase. The active sites of two other enzymes were probed in this way. A *m*-acetylbenzenesulfonyl group was covalently attached to the serine residue at the active site of chymotrypsin. The absence of triplet energy transfer in that enzyme derivative indicates that none of the eight tryptophan residues is adjacent to the *m*-acetylbenzenesulfonyl group at the active site (*26*). Galley and Stryer also probed the active site of papain with a potential triplet donor. An acetophenone group was covalently attached to the cysteine residue at the active site of papain. Efficient triplet-triplet transfer from the acetophenone group to tryptophan demonstrated that one or more tryptophan residues are close to the active site. These findings are consistent with what is known from x-ray studies about the three-dimensional structures of chymotrypsin and papain (*27*).

Rotational Motions

Very rapid processes in proteins can be directly studied by fluorescence methods that utilize nanosecond light pulses. By this new approach we have investigated the rotational motions of the active sites of several proteins. First, a fluorescent probe with a suitable excited-state lifetime was specifically inserted into an active site. Then the chromophore was excited with a short light pulse, and the polarization of its emission was measured as an explicit function of time in the nanosecond range.

One of the proteins studied in this way was α-chymotrypsin. Haugland and Stryer (*11*) found that *p*-nitrophenyl anthranilate (NPA) reacted specifically with α-chymotrypsin to form a highly fluorescent anthraniloyl derivative:



A number of observations indicated that the anthraniloyl group was attached to the active site of the enzyme, probably at serine 195: (i) only one anthraniloyl group was introduced per molecule of chymotrypsin, even when excess anthranilate was used; (ii) the anthraniloyl derivative of chymotrypsin was devoid of enzymatic activity; (iii) chymotrypsinogen, diisopropylphosphoryl chymotrypsin, and a variety of other proteins were not labeled by the anthranilate compound, which demonstrates the high selectivity of this reagent; (iv) the only other protein tested that reacted with NPA was trypsin, an enzyme similar to chymotrypsin in a number of respects. The anthraniloyl derivative was stable at neutral *p*H and could be crystallized. These crystals have the same unit cell dimensions as those of the native enzyme (*28*). Thus the structure of the protein was not grossly altered by the presence of the fluorescent probe. The absorption and emission properties of the anthraniloyl chromophore also were very favorable. The absorption and emission maxima at 342 and 422 nm, respectively, were well removed from those of the aromatic residues of the protein, making it possible to excite the anthraniloyl group exclusively. The quantum yield of fluorescence (0.53) was conveniently high. Finally, the excited-state lifetime of 7.2 nanoseconds was of suitable duration.

The anthraniloyl chymotrypsin was excited by a nanosecond light pulse (Fig. 7) that was polarized in the *y*-direction. The nanosecond light source and detection system were designed by Hundley, Coburn, Garwin, and Stryer (*29*). The intensities of the fluorescence emitted in directions parallel (y) and perpendicular (x) to the direction of excitation were measured as a function of time (Fig. 8a). The incident light polarized in the *y*-direction produced a population of excited molecules in which the axes of absorption were preferentially oriented in the *y*-direction at the instant of excitation. The process of orienting molecules from an isotropic solution is called photoselection. Shortly after the excitation pulse, the intensity of the parallel component of fluorescence (F_y) was more than twice that of the perpendicular component (F_x) (Fig. 8a). After a few nanoseconds, F_y and F_x were more nearly equal because the protein molecules had undergone rotational Brownian motion. In effect, the assembly of excited molecules seems to forget the direction of excitation with the rate of forgetting dependent on the degree of flexibility of the protein.

These results can be readily interpreted in terms of a theory derived by Perrin and further developed by Jablonski (*30*). The important parameters are $A(t)$, the emission anisotropy, and ρ, the rotational relaxation time. The anisotropy, defined as

$$A(t) = [F_y(t) - F_x(t)]/[F_y(t) + 2F_x(t)] \quad (4)$$

depends on time according to

$$A(t) = A_0 e^{-3t/\rho} \quad (5)$$

where A_0 is the anisotropy at the instant of excitation. In fact, the emission anisotropy of anthraniloyl chymotrypsin obeys this relationship. The plot of log A as a function of time is linear (Fig. 8b). The slope of this line is equal to $-3/\rho$. The application of a convolution analysis (*29*), which takes into account the finite duration of the exciting light pulse, gives a rotational relaxation time of 52 nanoseconds for the anthraniloyl chromophore attached to chymotrypsin.

One can interpret this value of ρ by considering two very different models. In the first, the active site of the enzyme is taken to be highly flexible during the nanosecond range so that the anthraniloyl chromophore rotates as though it were independent of the rest of the molecule. In the second model, the enzyme is taken to be a rigid sphere, and the anthraniloyl chromophore is assumed to rotate in common with the entire protein molecule. The value of ρ can be estimated for these two models in terms of the expression for the rotational relaxation time ρ_0 of an unhydrated, rigid sphere (*30*)

$$\rho_0 = 3\eta V/kT \quad (6)$$

where V is the volume of the sphere, η is the viscosity of the solution, k is the Boltzmann constant, and T is the absolute temperature. The value ρ for model 1 is estimated to be less than 1 nanosecond, whereas for model 2, ρ is calculated to be 22 nanoseconds. Clearly, the observed ρ of 52 nanoseconds is incompatible with the model of a flexible active site. In fact, the observed ρ is even greater than ρ_0 calculated for an anhydrous, rigid sphere of the size of α-chymotrypsin. This result would be expected if the chymotrypsin molecule were hydrated or had a nonspherical shape, as is probably the case. These nanosecond polarization data indicate that the active site of anthraniloyl

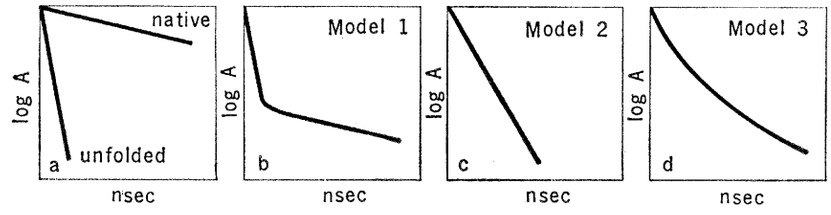


Fig. 9. Predicted dependence of the emission anisotropy on time for a protein undergoing a reversible transition between a rigid, native form and a flexible, unfolded form, according to (b) model 1, in which the two forms do not interconvert in times of the order of nanoseconds; (c) model 2, in which the two forms readily interconvert in times of the order of nanoseconds; and (d) model 3, in which there is a range of partially unfolded molecules in slow equilibrium. Log A plotted as a function of time for the fully native and fully unfolded forms is shown in (a).

chymotrypsin is rigid in the nanosecond range. The linearity of the plot of log A as a function of time emphasizes that the chromophore has no rotational mobility independent of the motion of the whole protein molecule.

This nanosecond approach (*31*) complements steady-state polarization methods that have been fruitfully used in studies of proteins since their introduction by Weber (*32*). It is of interest to compare the two approaches. The steady-state method is less direct, since ρ cannot be determined from a single experiment. The quantity ρ is related to $\bar{A}$, the average anisotropy observed in the steady-state mode, by the expression

$$\rho = 3\tau\bar{A}/(A_o - \bar{A}) \qquad (7)$$

where τ is the lifetime of the excited state. The difficulty with the steady-state approach is that ρ is specified only if A_o is known, yet A_o cannot be directly measured. Rather, it is obtained by measuring $\bar{A}$ in solutions of increasing viscosity and extrapolating to infinite viscosity. The value for ρ obtained in this way for anthraniloyl chymotrypsin (*11*) agrees well with the value determined by the nanosecond approach described above. However, the extrapolation procedure is by no means universally applicable; addition of sucrose or glycerol to increase the viscosity of the solution frequently alters the structure of the protein or the spectroscopic properties of the probe. This problem is entirely obviated by the nanosecond pulse method, which directly reveals ρ in a single experiment. A second advantage of the nanosecond approach is that the presence of more than one rotational relaxation time can be more easily detected by it than by the steady-state method. For example, anisotropic macromolecules such as DNA are expected to have two rotational relaxation times (*30*).

Nanosecond fluorescence measurements can also reveal whether a conformation transition occurs in a very short time interval. Our studies of the rotational motions of anthraniloyl chymotrypsin in solutions of urea illustrate this application of the nanosecond pulse technique. Chymotrypsin undergoes a reversible unfolding in solutions that contain a high concentration of urea (*33*). The steady-state emission anisotropy of the unfolded form of anthraniloyl chymotrypsin ($\bar{A}_u$) is much lower than that of the native, folded form ($\bar{A}_f$). The concentration of urea can be chosen so that the observed emission anisotropy is intermediate between $\bar{A}_f$ and $\bar{A}_u$. This intermediate value of the steady-state anisotropy can be interpreted in terms of several models. In model 1 half the molecules are native, the other half are completely unfolded, and the two forms do not interconvert in times of the order of nanoseconds. In model 2 some of the molecules are native, the others are completely unfolded, and the two forms interconvert readily in the time range of nanoseconds. In model 3 there is a spectrum of forms, such that the extent of folding of many molecules is intermediate between the completely native and completely unfolded states. Furthermore, it is assumed that these partially unfolded species do not interconvert in the nanosecond range.

These alternatives can be distinguished by fluorescence polarization measurements in the nanosecond range, since the three models would yield distinctly different curves of log $A(t)$ plotted as a function of time. For model 1 two distinct slopes which correspond to fully native and fully unfolded molecules would be observed (Fig. 9b). For model 2 a single slope intermediate in magnitude between those of the fully native and fully unfolded forms is expected (Fig. 9c). For model 3 the dependence of log $A(t)$ on time would not be linear, since the spectrum of partially unfolded molecules would give a wide range of rotational relaxation times (Fig. 9d). The experimental finding is that the nanosecond emission anisotropy of anthraniloyl chymotrypsin in urea corresponds to model 1. Thus, the unfolding of anthraniloyl chymotrypsin is an all-or-none process. Furthermore, the interconversion between the fully folded and fully unfolded states takes longer than a few nanoseconds.

Thus fluorescence pulse methods can be used to explore the conformational motility of proteins. It is important to know whether conformational transitions in proteins occur in the nanosecond range. The rates of the most rapid structural rearrangements in proteins set an upper limit on the number of conformations that a newly synthesized polypeptide chain could try out before folding into its characteristic form. If conformational transitions occur in nanoseconds, then fluorescence pulse methods will be invaluable for their study, because few other techniques extend to such short times.

Summary

Fluorescent probes which have readily interpretable emission properties can be specifically inserted into proteins to reveal facets of their structure and dynamics. The degree of polarity of sites on proteins can be determined from the emission characteristics of probes such as 1-anilino-8-naphthalene sulfonate. In a highly polar environment, they exhibit a very weak, green emission, whereas in a nonpolar environment, they show an intense, blue fluorescence. Distances in proteins can be deduced from the efficiency of energy transfer between chromophores, expressed in terms of sensitized fluorescence or sensitized phosphorescence. Singlet-singlet and triplet-singlet energy transfer are suitable spectroscopic rulers in the range between 15 and 65 Å, whereas triplet-triplet transfer can be used to show that two groups are less than 12 Å apart. Rotational motions of proteins and the degree of flexibility of their active sites can be determined by measurements of fluorescence polarization as a function of time, after an exciting light pulse of nanosecond duration. Nanosecond fluorescence spectroscopy can also be used to ascertain the rates of very rapid conformational transitions.

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Primate Color Vision

The macaque and squirrel monkey differ in their color vision and in the physiology of their visual systems.

Russell L. De Valois and Gerald H. Jacobs

Visual perception depends upon certain basic information: the intensity and wavelength of light coming from each point in a scene. Every animal with vision makes use of intensity information, and various animals appear to do so in much the same way. The situation is different, however, for wavelength information. Some animals have no color vision at all, others apparently discriminate wavelengths to some extent, and still others have excellent color vision. A number of years ago, Walls (*1*) argued that color vision has evolved independently in different classes of animals, human color vision having probably developed within the primate order. One basis for this view is the fact that many other mammals seem to have no more than rudimentary color vision, and that, in those lower animals which do have excellent color vision, the physiological means by which it is achieved may differ. Man, for example, has receptors containing different photopigments which provide the essential first step in differentiating wavelengths; some reptiles and birds, on the other hand, appear to have just a single photopigment in combination with oil droplets of various colors which serve to differentially filter the light reaching the pigment in different receptors.

Although evidence that the color vision of mammals developed independently from that of reptiles and birds is fairly strong, the course of that evolution within the mammals, or the extent to which color vision developed within the primates, is by no means so clear. Although no mammal other than the higher primates has been found with color vision approximating that of normal man, ground squirrels (*2*) appear to have some color vision, and, as Polson (*3*) has recently shown, the tree shrew (*Tupaia*) has color vision. This latter animal, which is intermediate between the insectivores and the primates, is highly diurnal, has an all-cone retina, and color vision of the deuteranopic variety.

Many tests have been made of color vision in primates, mostly in various Old World species. Many of the early studies were essentially anecdotal, but, in carefully controlled experiments, Grether (*4*) and Trendelenberg and Schmidt (*5*) showed that macaques and several other Old World primates have excellent color vision similar to that of normal human trichromats. These experiments included neutral-point tests, hue discrimination, and anomaloscope measurements.

The results of tests of New World monkeys have been quite different. Grether (*4*) found that *Cebus* monkeys had deviant color vision on all of his tests, and concluded that they were protanopic dichromats. The squirrel monkey (*6*) was also found to be deviant in hue discrimination, but the hue-discrimination test alone does not allow diagnosis of the type of defect. On the other hand, one spider monkey studied by Grether was found to have normal color vision and hue discrimination (the fact that this animal was a female whereas all of Grether's *Cebus* were males may be significant).

Unfortunately, in regard to the question of the evolution of primate color vision, no recent studies have been

Dr. De Valois is professor of psychology at Indiana University, Bloomington; Dr. Jacobs is assistant professor of psychology at the University of Texas, Austin.