*TRIPLET-TRIPLET ENERGY TRANSFER IN PROTEINS AS A CRITERION OF PROXIMITY**

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Electronic excitation energy can be transferred between singlet states of chromophores separated by distances of the order of 30 Å.[1] In contrast, energy transfer between triplet states requires a close approach of the donor and acceptor groups, since the transfer occurs by an electron exchange interaction.[2] The different scales of distance dependence of these energy-transfer processes suggest that they can be used in complementary ways to study the structure of biological macromolecules. Singlet-singlet energy transfer can measure distances in the 10- to 60-Å range,[3] while triplet-triplet transfer can show that two groups are less than some shorter distance apart (of the order of 12 Å).[4]

The experimental demonstration of triplet-triplet energy transfer is particularly straightforward when the singlet state of the triplet acceptor is at a higher energy than the singlet state of the triplet donor (Fig. 1).[2] The triplet donor can then be excited by light of a wavelength that is not absorbed by the triplet acceptor. Under these conditions, phosphorescence of the triplet acceptor is observed only if there is triplet-triplet energy transfer. We obtained this arrangement of energy levels in a complex of *m*-acetylbenzenesulfonamide (I) and carbonic anhydrase. In this system, the triplet energy donor is *m*-acetyl-

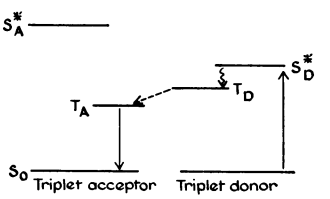
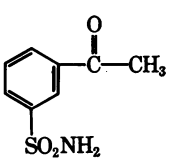


FIG. 1.—Arrangement of energy levels in the enzyme-inhibitor complexes studied. S_D^* and S_A^* are the lowest excited singlet states of the donor and acceptor, and T_D and T_A are the lowest excited triplet states of the donor and acceptor, respectively. S_0 is the ground electronic state. The arrows indicate the path of the excitation energy in the presence of triplet-triplet energy transfer: absorption into S_D^*, intersystem crossing to T_D, triplet-triplet transfer to T_A, followed by the sensitized phosphorescence of the triplet acceptor.



(I)

benzenesulfonamide, an inhibitor that binds to the zinc atom at the active site of the enzyme. The triplet energy acceptor is a tryptophan residue of the protein. Our finding of highly efficient triplet energy transfer between these groups shows that there is a tryptophan residue at or close to the active site of carbonic anhydrase. In contrast, triplet energy transfer was not observed in a

derivative of α-chymotrypsin, suggesting that tryptophan is absent from the portion of the active site that was probed by the potential triplet donor.

Experimental Procedure.—Bovine erythrocyte carbonic anhydrase (Worthington), α-chymotrypsin (Worthington), sodium acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide, Lederle), and *m*-acetylbenzenesulfonyl fluoride (Aldrich) were used. The sample of carbonic anhydrase contained both the B and C forms of the enzyme.[5] *m*-Acetylbenzenesulfonamide (MABS) was synthesized from *m*-acetylbenzenesulfonyl fluoride by addition of ammonia. MABS, twice recrystallized from ethanol-water, had a melting point of 137–139°C (cf. literature[6] value of 135–138°).

The buffers used were 0.05 *M* tris-HCl, pH 9.1, for carbonic anhydrase and 0.1 *M* potassium phosphate, pH 6.8, for α-chymotrypsin. The *m*-acetylbenzenesulfonyl derivative of α-chymotrypsin was prepared by addition of 2.2 μmoles of *m*-acetylbenzenesulfonyl fluoride in 0.1 ml of acetonitrile to 2 μmoles of α-chymotrypsin in 2 ml of buffer. The enzymatic activity of this derivative was assayed with glutaryl-L-phenylalanine-*p*-nitroanilide as substrate.[7] The esterase activity of the reversible complex of MABS and carbonic anhydrase was assayed with *p*-nitrophenylacetate as substrate.[8]

Absorption spectra were obtained at 22° on a Cary 14B recording spectrophotometer. Phosphorescence measurements were carried out at 77°K in 50% sucrose so as to form a rigid glass. The emission spectra were recorded on a spectrophosphorimeter (to be described in detail elsewhere). The exciting and emitted light were mechanically chopped at about 50 cps. The choppers were out of phase so that the photomutltiplier tube detected only the emission that occurred more than 1 msec after excitation. When the excitation wavelength was 330 mμ, a 0.1 *M* solution of indole in ethylene glycol-H_2O (1:1) was placed in the exciting beam to absorb stray light of wavelengths shorter than 315 mμ. The indole filter ensured that the 330-mμ exciting light reaching the sample was free of stray light that could be absorbed by the tryptophan residues of the protein.

Results.—Spectral and binding properties of m-acetylbenzenesulfonamide: MABS, like other sulfonamide compounds,[9] binds specifically to the active site of carbonic anhydrase. The esterase activity of an 8×10^{-7} *M* solution of enzyme was more than 90 per cent inhibited by 5×10^{-5} *M* MABS. The absorption and phosphorescence spectra of MABS make it a highly suitable triplet energy donor. An n-π^* transition in the carbonyl group gives MABS a weak absorption band beyond 300 mμ (Fig. 2). Its extinction coefficient at 330 mμ is 40 M^{-1} cm^{-1}. At this wavelength, the protein has no detectable absorbance. The phosphorescence spectrum of MABS obtained on excitation at 330 mμ is shown in Figure 3*b*. The emission starts at 375 mμ and has its

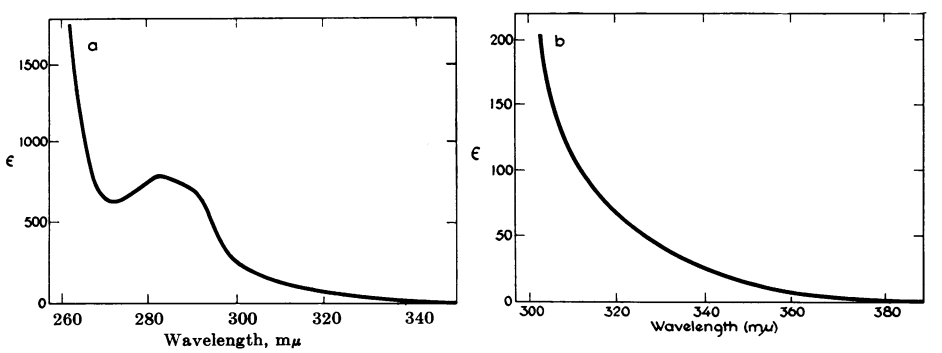


FIG. 2.—Absorption spectrum of *m*-acetylbenzenesulfonamide in 0.05 *M* Tris-HCl, pH 9.1 containing 50% sucrose, at 23°; (*a*) from 260 to 340 mμ and (*b*) from 300 to 380 mμ.

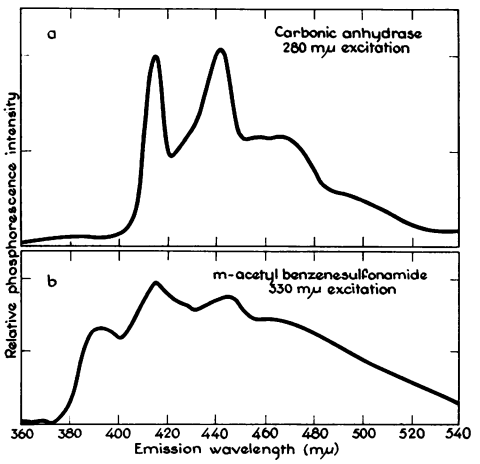


FIG. 3.—Phosphorescence emission spectra of (*a*) carbonic anhydrase (8×10^{-4} *M*), excited at 280 mμ, and (*b*) *m*-acetylbenzenesulfonamide (5×10^{-4} *M*), excited at 330 mμ. The sensitivity of the instrument for spectrum (*b*) was 100-fold greater than for (*a*).

shortest wavelength peak at 391 mμ. The phosphorescence lifetime of MABS was less than 0.1 sec.

Phosphorescence of carbonic anhydrase: The phosphorescence spectrum of carbonic anhydrase obtained on excitation at 280 mμ is shown in Fig. 3*a*. The tryptophan component of the emission starts at 400 mμ. The phosphorescence decayed exponentially with a lifetime of 4.8 sec. The spectrum and lifetime of the phosphorescence identify the emitting species as tryptophan.[10, 11] Excitation of carbonic anhydrase at 330 mμ yielded a low-intensity structureless impurity phosphorescence which had a lifetime of less than 0.1 sec.

Triplet energy transfer in a MABS-carbonic anhydrase complex: The phosphorescence spectrum of a MABS-carbonic anhydrase complex excited at 330 mμ is shown in Figure 4. Though the 330-mμ exciting light was absorbed only by MABS, the observed phosphorescence (Fig. 4) was that of tryptophan, rather than of MABS. Furthermore, the lifetime of this emission excited at 330 mμ was 4.8 seconds, which is characteristic of tryptophan rather than of MABS. It is evident that *the triplet excitation energy of MABS was transferred to a tryptophan*

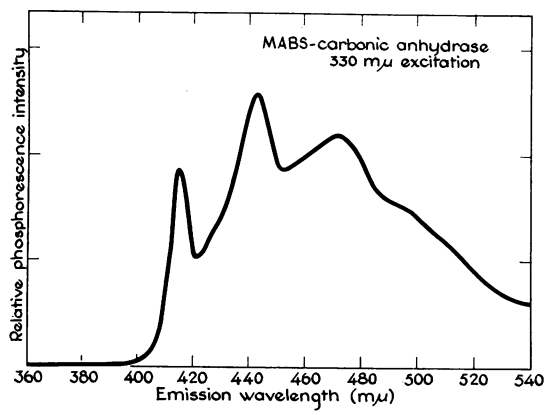


FIG. 4. — Phosphorescence emission spectrum of the complex of *m*-acetylbenzenesulfonamide (5×10^{-4} *M*) and carbonic anhydrase (8×10^{-4} *M*), excited at 330 mμ. The sensitivity of the instrument was the same as in Fig. 3*b*. This phosphorescence spectrum is characteristic of tryptophan, rather than MABS, showing that highly efficient triplet transfer has occurred.

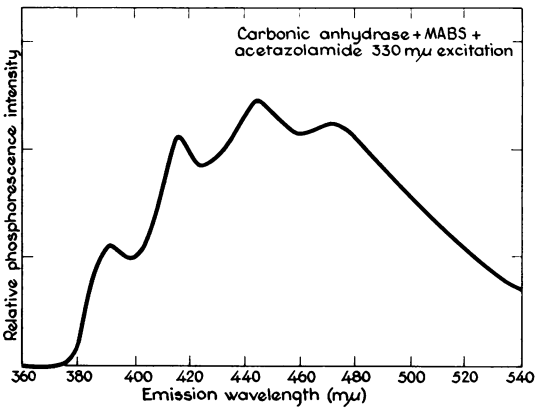


FIG. 5. — Phosphorescence emission spectrum of a mixture of *m*-acetylbenzenesulfonamide (5×10^{-4} *M*), carbonic anhydrase (8×10^{-4} *M*), and acetazolamide (10^{-3} *M*), excited at 330 mμ. The sensitivity of the instrument was the same as in Fig. 3*b*. This phosphorescence spectrum is very nearly that of MABS, rather than tryptophan, showing that triplet energy transfer was lost on displacement of MABS from the active site of the enzyme.

residue in the enzyme-inhibitor complex. The absence of any detectable phosphorescence in the 380- to 400-mμ region (Fig. 4), where unbound MABS has its shortest wavelength phosphorescence peak (Fig. 3*b*), suggests that *the efficiency of triplet energy transfer in the complex was close to 100 per cent.* The slight distortion of the sensitized tryptophan spectrum at longer wavelengths resulted from a short-lived impurity emission.

Loss of triplet energy transfer on displacement of MABS: Two equivalents of acetazolamide were added to a solution of MABS-carbonic anhydrase to displace MABS from the active site of the enzyme. Acetazolamide, a sulfonamide inhibitor of carbonic anhydrase, is known to bind with high affinity to the zinc atom at the active site.[9] Acetazolamide does not absorb or phosphoresce in the spectral region of interest here. The phosphorescence spectrum of a mixture of MABS, acetazolamide, and carbonic anhydrase is shown in Figure 5. The phosphorescence observed was that of MABS. The tryptophan emission seen in the absence of acetazolamide has almost completely disappeared. The lifetime of the emission shown in Figure 5 was less than 0.1 second, indicative of phosphorescence from MABS. It is evident that displacement of MABS from the active site of the enzyme resulted in a loss of triplet energy transfer.

Absence of triplet transfer at the active site of α-chymotrypsin: A potential triplet energy donor was covalently attached to the active site of α-chymotrypsin to determine whether a tryptophan residue is in proximity. *m*-Acetylbenzenesulfonyl fluoride reacted specifically and stoichiometrically with α-chymotrypsin. Addition of 1.1 equivalents of the sulfonyl fluoride to α-chymotrypsin resulted in a complete loss of enzymatic activity. It is likely that the *m*-acetylbenzenesulfonyl group was attached to serine 195 at the active site, since other sulfonyl fluorides have been shown to react with this residue.[12] The phosphorescence spectrum of *m*-acetylbenzenesulfonyl-α-chymotrypsin excited at 330 mμ was that of the *m*-acetylbenzenesulfonyl group. The lifetime of the emission was shorter than 0.1 second. There was no evidence of sensitized tryptophan phosphorescence. Thus, none of the eight tryptophan residues of α-chymotrypsin was in sufficiently close proximity to the *m*-acetylbenzenesulfonyl group to serve as a triplet energy acceptor.

Discussion.—The observation of efficient triplet energy transfer from MABS to tryptophan in bovine carbonic anhydrase indicates that at least one tryptophan residue in the enzyme is close to the active site. It is evident that triplet energy transfer can be used to reveal the proximity of groups in proteins, as shown by Galley and Davidson for nucleic acids.[4] Triplet-triplet energy transfer, first observed by Terenin and Ermolaev[2] in mixtures of small molecules in rigid media, requires a very close approach of the donor and acceptor chromophores.[13] Theoretical and experimental studies have shown that the transfer does not occur by dipole-dipole coupling.[13] Instead, triplet transfer occurs through an electron exchange interaction that depends on the overlap of the donor and acceptor electronic wave functions.[14] Furthermore, the efficiency of triplet transfer has been shown to be independent of the nature of the solvent.[15]

The precise dependence on distance of the kinetics and efficiency of triplet energy transfer is not yet known. Studies of mixtures of donor and acceptor at high concentration indicate that the centers of these groups must be less than 12 Å apart for highly efficient triplet transfer.[13] Clearly, in such a mixture, the peripheral portions of the donor and acceptor are even closer. Model systems of defined structure are needed to solve this problem.[16] Also, measurements of the kinetics of triplet transfer may aid in determining whether the groups are, say, 4 or 10 Å apart. Rate constants for triplet-triplet transfer can be as high as 10^{10} sec^{-1} if the groups are in van der Waals contact.[17, 18] Our observation of highly efficient triplet transfer indicates only that the triplet transfer rate constant in the carbonic anhydrase complex is higher than 10^2 sec^{-1}.

A maximal distance between the tryptophan triplet acceptor and the zinc atom at the active site of carbonic anhydrase can be estimated for the case that the triplet donor and acceptor are virtually in van der Waals contact. Model building shows that the carbonyl group of MABS is not further than 8 Å from the zinc atom, if the nitrogen atom of MABS is directly bonded to zinc, as suggested by studies of other sulfonamide inhibitors.[19–21] The indole moiety of a tryptophan residue must then be within 12 Å of the zinc atom to be in van der Waals contact with MABS. Furthermore, this tryptophan residue must form part of the binding site of the aromatic sulfonamide inhibitor. In contrast, the absence of triplet energy transfer in *m*-acetylbenzenesulfonyl-α-chymotrypsin suggests that none of the eight tryptophan residues[22] of the enzyme is located in the portion of the active site contiguous to the *m*-acetylbenzenesulfonyl group. The validity of these conclusions based on triplet energy transfer can be rigorously assessed once the high-resolution X-ray crystallographic studies of α-chymotrypsin[12] and carbonic anhydrase[20] are complete. A particularly attractive possibility is that the precise mode of interaction of MABS with the tryptophan residue at the active site of carbonic anhydrase can be determined by the difference Fourier method.

The triplet energy transfer method should be applicable to a variety of proteins. A number of chromophores which have appropriate spectroscopic properties can be covalently attached to selected sites on proteins. For example, α-bromoacetophenone is a suitable alkylating reagent, since the acetophenone moiety has absorption and emission properties like MABS. The arrangement of

energy levels shown in Figure 1 is not essential for triplet energy transfer studies, though it makes the experimental demonstration of triplet transfer particularly straightforward. There are many more potential triplet donor-acceptor pairs in which the lowest excited singlet state of the triplet acceptor is below that of the triplet donor.[23] A present limitation of the triplet energy transfer method in studies of biological macromolecules is the requirement that the sample be a rigid glass at low temperature, since phosphorescence is typically quenched in fluid media. The possibility that the structure of a protein may be altered on cooling to 77°K must always be considered.

This study shows that triplet energy transfer can provide definitive information about protein structure. The observation of efficient triplet transfer has an unambiguous interpretation, namely, that the triplet donor and acceptor are in proximity. A high degree of spatial resolution is inherent in the method, since the interaction responsible for triplet transfer is a short-range one. Triplet energy transfer can be expected to reveal the presence of certain chromophoric residues at active sites and thereby contribute to an understanding of enzymatic function. Furthermore, triplet transfer may be useful in demonstrating the proximity of groups far apart in the linear amino acid sequence. Proximity relationships of this kind would provide insight into the three-dimensional structures of proteins.

Summary.—Triplet-triplet energy transfer was observed in a complex of bovine carbonic anhydrase and a chromophoric inhibitor. The triplet energy donor was *m*-acetylbenzenesulfonamide, an inhibitor that binds specifically to the active site, while the triplet acceptor was a tryptophan residue of the protein. The finding of highly efficient triplet-triplet transfer between these groups shows that there is a tryptophan residue at the active site of bovine carbonic anhydrase. In contrast, triplet energy transfer was not observed in *m*-acetylbenzenesulfonyl-α-chymotrypsin, suggesting that tryptophan is absent from the portion of the active site probed by the potential triplet donor. These studies show that triplet energy transfer can be used to reveal the proximity of chromophores in protein molecules.

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