divided by two to normalize the fact that two donors are absorbing the energy. Using the relation $\sum_{N=0}^{\infty} X^N = 1/(1 - X)$, we finally get eq 10.

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Singlet Energy-Transfer Studies on Associating Protein Systems. Distance Measurements on Trypsin, α-Chymotrypsin, and Their Protein Inhibitors†

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ABSTRACT: Singlet–singlet energy transfer has been measured between fluorescent dyes covalently attached to single proteins and to trypsin–trypsin inhibitor complexes. To maximize the probability of surface labeling, Celite-bound reactive dyes were used. These included fluorescein and Rhodamine B isothiocyanates and dansyl chloride. The Celite technique permits the production of heavily labeled proteins which retain nearly full activity. The experimental energy-transfer results are in excellent agreement with calculations which assume random surface labeling of spherical proteins with known anhydrous radii and a Poisson distribution of the degree of labeling. The experiments on complexes of trypsin with lima and soya bean trypsin inhibitors show that singlet energy transfer is a practical and simple method for determining the distance between specific proteins in a large protein complex. Extrinsic fluorescence labels are capable of providing semiquantitative distance information. Preliminary energy transfer results indicate that trypsin and chymotrypsin form a stable complex at low concentration. The experimental protocol and interpretive framework should be easily generalizable to a variety of complex systems. This rapid and sensitive technique will be of general utility in studies of the size, spatial arrangement, stoichiometry, and kinetics of associating macromolecules.

The use of singlet energy transfer to investigate qualitatively the distances between known sites on a macromolecule (Beardsley and Cantor, 1970) or changes in conformation upon complex formation by proteins (Edelhoch and Steiner, 1965; Millar *et al.*, 1962) is well established. Quantitative measurements of distances by energy transfer have been made only indirectly and only on small molecules (Stryer and Haugland, 1967).

The method of singlet energy transfer seems elegantly suited to the study of the stoichiometry, conformation, and

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kinetics of complex formation in associating protein systems. It should also prove valuable in the investigation of the spatial organization of complicated macromolecular complexes such as ribosomes, membranes, and cell surfaces. One major difficulty is preparing suitable fluorescent derivatives of the components of these systems. Celite-labeling techniques have the remarkable ability to place large numbers of dyes covalently onto proteins without substantial loss of biological activity. However, the dye reactions are not very specific.

Calculations made by Gennis and Cantor (1972) showed that it would be reasonable to attempt to determine semiquantitative distances in Celite-labeled macromolecular systems such as individual and associating proteins. In order to see if the assumptions made for these calculations are at all reasonable, the present experiments were designed for the measurements of radii of single proteins and proximity relationships of protein complexes.

One of the most interesting and best documented systems of associating proteins is trypsin and α-chymotrypsin and their protein inhibitors (Laskowski and Sealock, 1971). From single-sphere transfer experiments, the size (or molecular volume) of individual proteins was determined by measuring the efficiency of energy transfer between two different dyes covalently attached over the protein surface. Double-sphere transfer experiments were performed on binary complexes of trypsin, α-chymotrypsin, Kunitz soya bean inhibitor, and lima bean inhibitor in which one protein of the complex was labeled with only donor chromophores and the other with only acceptor chromophores. Transfer was also observed in the ternary complex of trypsin, α-chymotrypsin, and the double-headed black-eyed pea inhibitor.

The experimentally observed energy transfer is in excellent agreement with expected values calculated from known protein radii. Thus the assumptions needed to perform the calculations appear to be reliable.

Materials and Methods

Proteins, Substrates, and Dyes. Commercial preparations of enzymes and proteins were used without further purification. Salt-free, lyophilized trypsin (mol wt 23,800), α-chymotrypsin (mol wt 24,500), Kunitz soya bean trypsin inhibitor[1] (STI, mol wt 22,000), and lima bean trypsin inhibitor (LTI, mol wt 9000) were purchased from Worthington Biochemical. LTI was also obtained from Pfaltz and Bauer. Bovine serum albumin (mol wt 66,000) was purchased from Kontex and horseradish peroxidase (HRP, mol wt 40,000) from Worthington. Apo-HRP was prepared by the method of Breslow (1964). Black-eyed pea trypsin inhibitor (BEPTI) was isolated from Redbow 98% fat-free, black-eyed peas by the procedure of Ventura and Filho (1966).

Toluenesulfonylarginine methyl ester (TAME) for the assay of trypsin activity and benzoyltyrosine ethyl ester (Bz-L-TyrOEt) for the assay of α-chymotrypsin activity were purchased from P-L Biochemicals and Pfaltz and Bauer, respectively. Celite powder was obtained from Johns-Manville. Fluorescein isothiocyanate (FITC, mol wt 389) and dimethylaminonaphthalenesulfonyl chloride (Dans, mol wt 270) were obtained from Aldrich and Rhodamine B isothiocyanate (RITCB, mol wt 537) from both Sigma and Pfaltz and Bauer.

Pentaalanine was purchased from Sigma. Sephadex gels were obtained from Pharmacia Fine Chemicals and Bio-Gel P2 from Bio-Rad.

Standard solutions of proteases and inhibitors for assays of enzyme activity and determination of protein concentration were prepared in 10^{-3} M HCl at 200 μg/ml. Fresh enzyme standards were prepared at the first sign of loss of activity. Standard solutions of other proteins were prepared in 0.005 M phosphate buffer, pH 7.6, as needed.

Conjugation of Dyes to Proteins. For labeling without Celite, approximately 2 mg of protein and a 50 molar excess of dye were dissolved separately in small aliquots of 0.01 M carbonate buffer, pH 9.0, for the inhibitors or 0.01 M borate–0.02 M $CaCl_2$, pH 9.0, for the proteases. For labeling at high pH 0.05 M Tris–0.02 M $CaCl_2$, pH 10.4, was used. A small amount of acetone was necessary to dissolve completely Dans and RITCB. The protein and dye solutions were combined and mixed for 1 hr at room temperature or 2–5 hr at 4°. The reaction mixture was then applied to a small Sephadex G-25 fine column and eluted with 0.01 M Tris-Cl–0.02 M $CaCl_2$, pH 7.0, in order to separate unreacted free dye from the protein–dye conjugate.

Celite–dye powders were prepared by the method of Rinderknecht (1960) except that the dye constituted 20% of the powder. The method of labeling with a Celite–dye was essentially that of Rinderknecht (1962). An amount of Celite–dye to give 50 molar excess dye was suspended in 0.05 M borate–0.02 M $CaCl_2$, pH 9.0, or 0.05 M Tris–0.02 M $CaCl_2$, pH 10.4. A small aliquot containing 2 mg of protein in the same buffer was added and the mixture agitated gently for 4 min at room temperature. The resultant slurry was centrifuged and the supernatant applied to a Sephadex G-25 fine column. Elution was most easily followed by observing the column with a near ultraviolet lamp.

For single-sphere transfer experiments it was necessary to conjugate both the donor and acceptor dyes to the same protein molecule. This was achieved by first labeling a large aliquot of protein with the donor using Dans–Celite. The reaction mixture was centrifuged, and plain Celite was added to half the supernatant to give a control labeled with the donor alone, but subjected to the same treatment as the doubly labeled sample. An equal weight of FITC–Celite or RITCB–Celite was added to the other half to yield molecules with a double label, donors and acceptors. Alternatively, aliquots of the donor-labeled protein were added to plain Celite and a series of weights of FITC–Celite. After centrifugation, all samples were passed through a Sephadex G-25 fine column. The absence of free dye on the column with the donor-labeled control indicated that the second labeling reaction did not cause removal of the first label. The protein was labeled separately with acceptor alone as a control sample. Proteins used for single-sphere experiments were not routinely assayed for residual activity after labeling, since it was not necessary to use fully active molecules for these experiments. However, doubly labeled samples generally retained greater than 70% activity.

For double- and triple-sphere transfer experiments, individual proteins were labeled with a single dye, either donor or acceptor, and only samples which retained full activity (90–100% residual activity) after labeling were used.

Circular dichroism spectra of labeled and unlabeled pro-

[1] Abbreviations used are: STI, Kunitz soya bean trypsin inhibitor; LTI, lima bean trypsin inhibitor; apo-HRP, horseradish apoperoxidase; BEPTI, black-eyed pea trypsin inhibitor; TAME, toluenesulfonylarginine methyl ester; Bz-L-TyrOEt, benzoyltyrosine ethyl ester; FITC, fluorescein isothiocyanate; Dans, dimethylaminonaphthalenesulfonyl chloride; RITCB, Rhodamine B isothiocyanate.

teins were measured from 340 to 200 nm using a Cary 60 spectropolarimeter with the 6001 CD attachment.

Enzyme Activity Assays. Enzyme activity was assayed on a Cary 15 recording spectrophotometer using the synchronous mode. Trypsin samples were assayed with TAME (Hummel, 1959), and their residual activity was expressed as a fraction of the activity of a freshly prepared standard solution of trypsin. It was convenient to use relative activities rather than convert to specific activities expressed in U/mg. Similarly, α-chymotrypsin samples were assayed with Bz-L-TyrOEt (Hummel, 1959). Inhibitor samples were assayed by titrating increasing aliquots of inhibitor with a constant aliquot of protease. The inhibitor–protease complex was allowed to equilibrate for 0.5 hr in the assay buffer before the addition of substrate. The equilibrated samples were then measured for residual activity against TAME or Bz-L-TyrOEt. In the case of the double-headed inhibitor BEPTI, the inhibitor was titrated first with α-chymotrypsin to determine the relative volumes necessary for stoichiometric inhibition. The determined volumes were then allowed to equilibrate 5 min before titration of this binary complex with trypsin. In this way, stoichiometric binding ratios of standard enzyme solutions, labeled enzyme samples, and mixtures of labeled and unlabeled enzymes were determined, in order that transfer experiments could be performed on well-characterized enzyme–complex samples.

Determination of Molar Extinction Coefficients of Dyes. The value for the molar extinction coefficient (ϵ) of protein-bound Dans was determined by Chen (1968) to be 3.4×10^3 using radioactive Dans. The value 4.25×10^4 for ϵ of bound FITC was determined by dialysis by Tengerdy and Chang (1966). No value for ϵ of bound RITCB exists in the literature. A value of 4.7×10^3 was determined by reacting RITCB with pentaalanine overnight at room temperature. The reaction mixture was centrifuged to remove undissolved dye, and the supernatant was applied to a 1×30 cm column of Bio-Gel P2. The RITCB–pentaalanine was separated from unbound RITCB and pentaalanine by elution with 0.005 M phosphate buffer, pH 7.6. The absence of ninhydrin-positive material in the RITCB–pentaalanine eluate indicated that there was no unbound pentaalanine. The molarity of bound pentaalanine was determined by the biuret reaction from a standard curve for free pentaalanine. Since RITCB absorbs at the wavelength for the spectrophotometric determination of peptide bond concentration by the biuret method, it was necessary to prepare blanks containing the appropriate dilution of unbound RITCB. The absorption at 553 nm of the eluate was measured, and ϵ was calculated according to the relation (A_{553} of RITCB–pentaalanine)/(molar concentration of pentaalanine) = ϵ of bound RITCB. This value was corroborated by using a modification of the dialysis method described by Tengerdy and Chang (1966). RITCB was allowed to react with trypsin in pH 10.4 Tris–$CaCl_2$ buffer for 19 hr in the cold. The absorption at 553 nm of aliquots of the reaction mixture was measured before and after the reaction, and after dialysis into 99 volumes of pH 7.6 Tris–$CaCl_2$ buffer. The absorption at 553 nm of the dialysis buffer was 0.01 the concentration of free dye after the reaction. The concentration of free dye before the reaction was determined by comparing the absorption at 553 nm to a standard solution of RITCB. A value of 4.62×10^3 was calculated as ϵ of the bound RITCB.[2]

Determination of Average Degree of Labeling. The average number of donor–dye molecules per protein (μ_D) and the average number of acceptor–dye molecules per protein (μ_A) were determined by measuring the absorption of the protein–dye sample at the dye λ_{max} and the concentration of protein by the Folin method (Lowry *et al.*, 1951). μ was calculated according to the relations (A_{dye}/ϵ_{dye}) = molar concentration of dye; (molar concentration of dye)/(molar concentration of protein) = μ.

Determination of the Quantum Yield of the Donor. The relative quantum yield ϕ_f of protein-bound Dans used for R_0 calculations was measured by comparing A_{350} of dansylated bovine serum albumin to that of quinine bisulfate in 1 N H_2SO_4 and then measuring the fluorescence of the samples excited at 350 nm on a Perkin-Elmer MPF-2a scanning spectrofluorimeter. The area under the emission curves was compared by numerical integration. The quantum yield of quinine bisulfate in 1 N H_2SO_4 was taken as 0.70 (Scott *et al.*, 1970). ϕ_f of bound Dans was calculated according to the relation

$$\frac{\text{integrated emission of albumin–Dans}}{\text{integrated emission of quinine bisulfate}} = \frac{\phi_f \text{ albumin–Dans}}{0.7} \times \frac{A_{350} \text{ albumin–Dans}}{A_{350} \text{ quinine bisulfate}}$$

to be 0.103, similar to values of ϕ_f for dansylated fibrinogens reported by Mihalyi and Albert (1971b).

Measurement of Singlet Energy Transfer. The spectra of typical donor–acceptor labeled proteins are shown in Figures 1a,1b. Under optimal circumstances one of the most unequivocal ways to demonstrate singlet energy transfer is sensitized emission of the acceptor. Unfortunately in the present work the overlap and relative intensities of the fluorescence of dansyl and fluorescein emission spectra make it very difficult to quantitate the latter in the presence of the former. For this reason, two other possible methods of studying singlet energy transfer have been employed. These techniques have been used successfully in the past to demonstrate energy transfer and must be used where the acceptor is nonfluorescent (Wu and Stryer, 1972; Badley and Teale, 1971). One must be especially careful however to run the proper controls to assure that quenching observed is actually due to energy transfer. See the discussion below. A static fluorescent method involved measurement of the quenching of donor fluorescence on a Perkin-Elmer MPF2a spectrofluorimeter. Samples were excited at 360 nm, and the relative fluorescence intensities were compared at 470 nm for the Dans to FITC pair, or at 325 and 500 nm for the Dans to RITCB pair. A time-dependent method involved measurement of the shortening of the fluorescence lifetime of the donor with a single photon counting apparatus as described by Tao (1969). Exciting light was passed through a Corning CS-754 cut-off filter, and emission was observed at 470 nm for the Dans to FITC pair.

For the single-sphere transfer experiments, samples consisted of the donor-labeled protein, the doubly labeled protein, and the acceptor-labeled protein. The first and third

[2] It is difficult to explain why ϵ of RITCB should be an order of magnitude smaller than ϵ of nonderivitized Rhodamine B considering the similarity in structure to fluorescein and RITC, both of which have $\epsilon \sim 10^4$. It may be that Rhodamine B does not withstand the conditions for conversion to the isothiocyanate as well as does fluorescein. This seems likely since commercial preparations of FITC are relatively pure, while those called RITCB contain only a small fraction of the isothiocyanate, the major portion of the preparation being nonderivitized Rhodamine B.

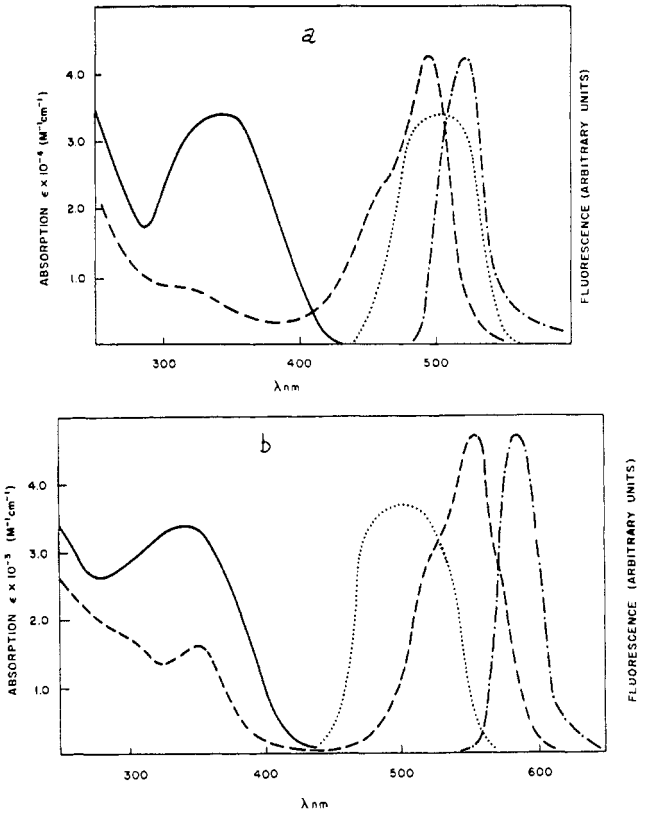

FIGURE 1: Spectral overlap of protein-bound (a) Dans and FITC, (b) Dans and RITCB in 0.01 M Tris-Cl–0.02 M $CaCl_2$, pH 7.6. (——) Absorption spectrum of LTI–Dans (× 10). (· · · · · ·) Emission spectrum of LTI–Dans. (- - - - - - -) Absorption spectrum of trypsin–FITC. (—·—·—·) Emission spectrum of trypsin–FITC. (——) Absorption spectrum of trypsin–Dans. (· · · · · ·) Emission spectrum of trypsin–Dans. (- - - - - - -) Absorption spectrum of LTI–RITCB. (—·—·—·) Emission spectrum of LTI–RITCB.

samples serve as controls for the second. Because static fluorescence measurements are concentration dependent, the Sephadex G-25 eluates were diluted so that the acceptor absorption was the same in both acceptor-labeled samples. For the donor-labeled samples μ was the same as a result of the labeling procedure. Thus relative donor absorptions were given by the relative protein concentrations, since the donor absorptions were always low enough so that corrections for inner filter effects were unnecessary. The sample with acceptor alone served to confirm the absence of FITC emission at 470 nm or of RITCB at 500 nm.

For the double-sphere transfer experiments, samples consisted of donor-labeled inhibitor, donor-labeled inhibitor equilibrated 0.5 hr with acceptor-labeled protease, donor-labeled inhibitor equilibrated for 0.5 hr with nonlabeled protease, and acceptor-labeled protease (or the analogous system with donor-labeled protease and acceptor-labeled inhibitor). For the ternary complex transfer experiment, samples consisted of donor-labeled α-chymotrypsin equilibrated with unlabeled BEPTI and unlabeled trypsin, donor-labeled α-chymotrypsin equilibrated with unlabeled BEPTI and acceptor-labeled trypsin, and donor-labeled α-chymotrypsin equilibrated with acceptor-labeled trypsin in the absence of inhibitor.

The control in which a donor-labeled protein is complexed with an unlabeled protein is necessary since changes in the lifetime or in the fluorescence intensity of the donor may occur upon complex formation in the absence of energy transfer due to conformational changes or due to the transfer from tryptophan to Dans as observed by Edelhoch and Steiner (1965) and Millar *et al.* (1962).

In general, it is best to avoid the necessity of corrections due to bad windows for excitation, inner filter effects, and secondary absorption of donor emission by the acceptor by using very dilute samples with absorptions less than 0.1. However, a high degree of labeling with FITC may result in a larger absorption by FITC than by Dans at the wavelength of excitation, which makes it difficult to measure the relative Dans absorptions needed for the interpretation of static transfer results. In this case, transfer efficiency must be measured as the shortening of the Dans lifetime, since lifetime measurements are not concentration dependent.

Values obtained by static measurements of transfer efficiency are consistently larger than those obtained by time-dependent measurements, since donors which are very close to acceptors are quenched so rapidly that their contributions to the measured donor lifetime are negligible. Thus it is best to use static measurements of transfer in small systems or when there is some reason to suspect adjacent donor–acceptor pairs and to use lifetime methods for large systems such as multiple-sphere complexes.

Calculation of the Average Transfer Efficiency from the Measured Energy Transfer. The average efficiency of singlet energy transfer $\bar{E}$ as described in Gennis and Cantor (1972) is related to the measured quenching of the donor fluorescence or shortening of the donor lifetime as follows:

$$F_D^0 \propto A_D\phi_D^0 = A_D k_f \tau_D^0$$

$$F_D^A \propto A_D\phi_D^A = A_D k_f \tau_D^A$$

$$\frac{F_D^A}{F_D^0} = \frac{\tau_D^A}{\tau_D^0} = 1 - \bar{E}$$

where $\bar{E}$ is the transfer efficiency, k_f the intrinsic rate of fluorescence of the donor; F_D^0, ϕ_D^0, and τ_D^0, the fluorescence intensity, quantum yield, and lifetime of the donor in the absence of the acceptor and F_D^A, ϕ_D^A, and τ_D^A in the presence of the acceptor. A_D is the absorption of the donor at the wavelength of excitation.

Determination of a Distance Corresponding to a Calculated Transfer Efficiency. The average efficiency of transfer may be graphed as a function of the average number of acceptors per sphere μ_A and the ratio R/R_0 of the measured distance R (protein or protein–complex radius) to the R_0 of the particular donor–acceptor pair. Two different sets of curves are generated; one set results from averaging with both dyes on a single sphere (Figure 1, Gennis and Cantor, 1972) and the other from averaging with the donors and acceptors on different proteins (Figure 3, Gennis and Cantor, 1972). Thus the experimental determination of $\bar{E}$ and μ_A allows one to read a distance directly from the appropriate graph. Although a single point is theoretically sufficient, the data are more convincing if the variation of $\bar{E}$ with μ_A can be shown to follow a single curve of a set. Experimentally, this means that a series of samples which differ in their value of μ_A must be prepared, and the $\bar{E}$ corresponding to each value of μ_A is measured.

Results

Conjugation of Dye to Protein without Celite. During the long incubation periods necessary for labeling without Celite,

both trypsin and chymotrypsin were completely inactivated unless the reaction buffer contained a 10^3 molar excess of Ca^{2+} over protein, as expected from the investigations of Sipos and Merkel (1970). At pH 9.0, at least 1 hr at room temperature or 2 hr at 4° were required to achieve 1:1 labeling of trypsin with FITC for 10^{-5} M trypsin and 5×10^{-4} M FITC. After this time the residual activity of a trypsin control in buffer (borate, $CaCl_2$) alone was 61%, and the residual activity of the labeled sample was 41%. At pH 10.4 (Tris–$CaCl_2$) and room temperature, 1 hr was sufficient to achieve 5:1 labeling of trypsin with FITC for 10^{-5} M trypsin and 10^{-3} M FITC. The residual activity of the control was 60%, of the labeled sample 34%. Samples of labeled chicken ovomucoid and lima bean inhibitor retaining 70–80% activity could be obtained by labeling at pH 9.0 and 4°, but not with adequate reproducibility.

Conjugation of Dye to Protein with Celite. Although the time required for conjugation was minimal, the requirement for Ca^{2+} in the reaction buffer for labeling trypsin and chymotrypsin with Celite–dyes was stringent. After the reaction of 5 mg of protease and 20 mg of FITC–Celite at pH 9.0 or pH 10.4 for 4 min at room temperature and centrifugation for 5 min at 4°, the residual activity was less than 10% in the absence of Ca^{2+}. However, the addition of 10^3 molar excess Ca^{2+} over protease allowed the routine preparation of fully active (90–100% residual activity) labeled protease samples using Celite–dyes.

In order to generate a set of points for the variation of the transfer efficiency with the degree of labeling for a given protein complex, it was necessary to make several samples with different μ_A. (The value of μ_D is unimportant, except that it must be large enough to ensure that most of the donor proteins will be labeled.) Unfortunately, there seemed to be no real way to regulate the degree of labeling. In general, it was necessary to label at pH 9.0 to restrict μ_A to 1.0 or below, at pH 10.4 to achieve μ_A greater than 1.0. To obtain a range of μ_A above 1.0, different proportions of protein and Celite–dye were used. In many cases, samples with the greatest proportion of Celite to protein unexpectedly had the lowest μ_A. In other cases, there was no direct correlation between the proportion of Celite–dye and μ_A. Use of excessive Celite caused loss of protein during centrifugation due to adsorption. This loss was avoided by using a smaller amount of Celite–dye prepared with a greater ratio of dye to Celite (30% w/w) when large proportions of dye to protein were desired.

Characterization of Samples for Double-Sphere Transfer Experiments. In order to ensure maximum complexing of labeled protease and inhibitor for double-sphere transfer experiments, it was originally thought best to label proteins as the complex to avoid interference with the complexing sites by conjugated dyes. Equimolar concentrations of trypsin and STI or LTI were mixed and allowed to equilibrate for 15 min at room temperature in pH 7.6 Tris–$CaCl_2$ buffer. These complexes were then labeled with FITC or Dans by adding the Celite–dye in more concentrated pH 10.4 Tris–$CaCl_2$ buffer. After elution from Sephadex G-25 fine columns, the pH of the samples was carefully lowered from 7.6 to 2.5 to dissociate the complexes. Various standard means of separation of the labeled proteins were attempted including Sephadex G-50, G-75, and G-100 columns with 10^{-3} M HCl, 1 M KCl, pH 2.7 elution buffer; DEAE-cellulose with 6 M urea and a salt gradient; Sephadex G-50 with 0.365% sodium dodecyl sulfate; and SP-Sephadex C25 with a salt gradient. None of these methods proved satisfactory even though all of them have been used successfully for the separation of

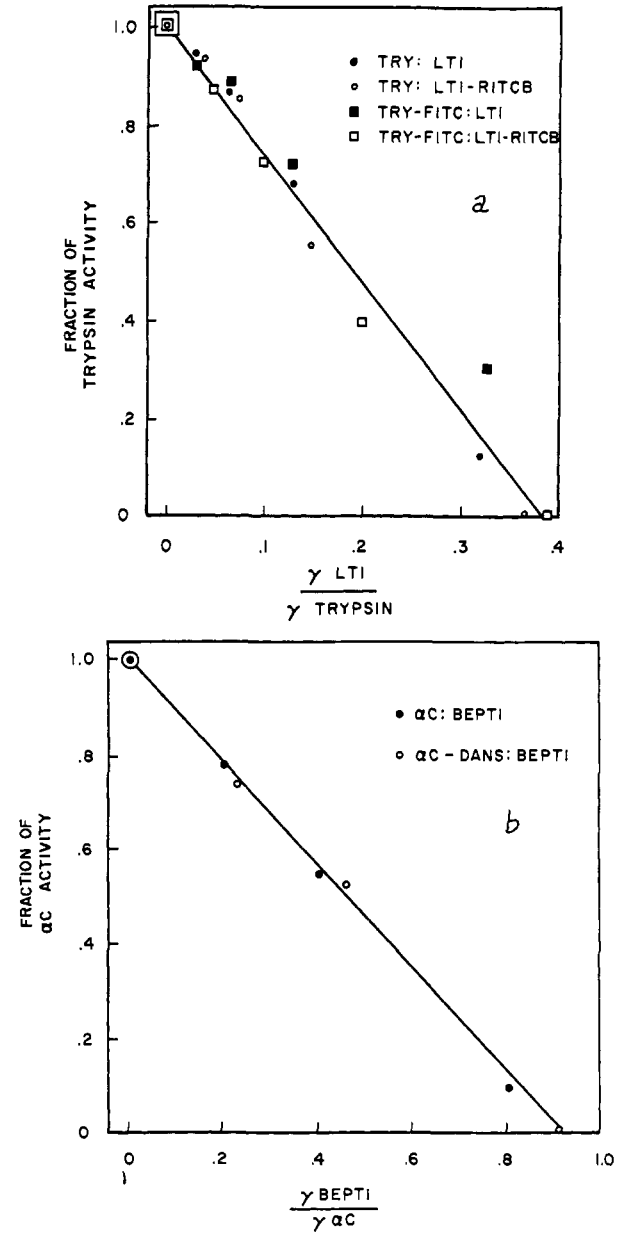

FIGURE 2: Complex formation (a) between labeled and unlabeled trypsin and inhibitor samples prepared for a typical double-sphere transfer experiment. Trypsin–FITC is the donor (μ_D = 1.0) and LTI–RITCB is the acceptor (μ_A = 2.6). (b) Complex formation of unlabeled and Dans-labeled α-chymotrypsin with unlabeled BEPTI used for a ternary complex transfer experiments (μ_D = 1.1).

unlabeled complexes (Laskowski and Sealock, 1971), and Edelhoch and Steiner (1965) were able to separate a dansylated trypsin–pancreatic trypsin inhibitor complex on Sephadex G-100. Fortunately, use of the Celite labeling technique made it unnecessary to label and separate the complex, since it was possible to label the proteins individually while maintaining 90–100% esterolytic activity.

In accordance with the calculations in the accompanying paper, we assumed that the number distribution of label is Poisson (Gennis and Cantor, 1972). Hence a significant proportion of the population of a labeled protein sample will not be labeled at all when μ_A is less than 3. It was feared that complex formation might occur only between unlabeled molecules when labeled inhibitor and protease samples were mixed for double-sphere transfer experiments. That this was

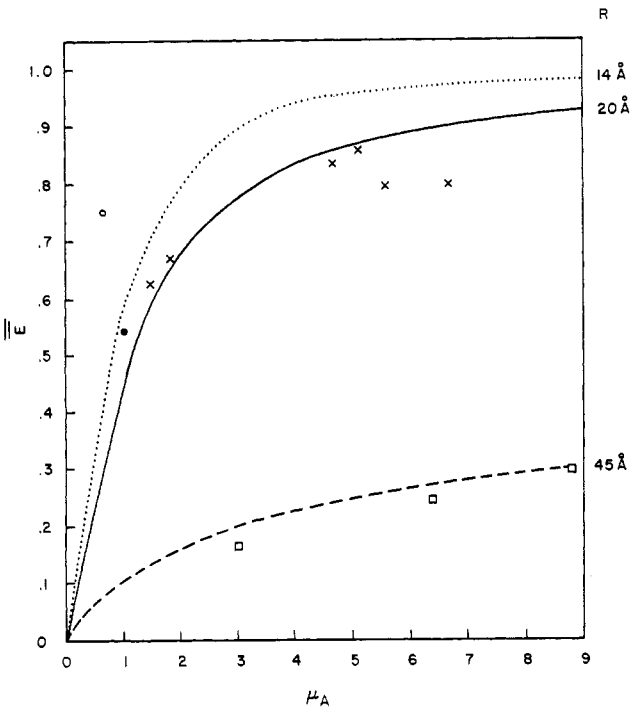

FIGURE 3: Single-sphere energy-transfer experiments. The three curves are expected average energy-transfer efficiency, $\bar{\bar{E}}$, calculated as a function of the ratio of radius, R, characteristic transfer distance R_0, and mean number of acceptors per protein μ_A.(· · · · · ·) Calculated for LTI: anhydrous radius = 14 Å, R_0 (Dans to RITCB) = 24. (——) Calculated for STI and α-chymotrypsin: anhydrous radius = 20 Å, R_0 (Dans to FITC) = 33 Å. (- - - - - -) Calculated for bovine serum albumin: assumed radius = 45 Å, R_0 (Dans to FITC) = 33 Å. Experimental points: (×) α-chymotrypsin–Dans–FITC (static fluorescence); (●) STI–Dans–FITC (static fluorescence); (○) LTI–Dans–RITCB (static fluorescence); (□) bovine serum albumin–Dans–FITC (fluorescence lifetime measurements). For all these samples, protein concentrations were in the range of 100 to 400 μg/ml.

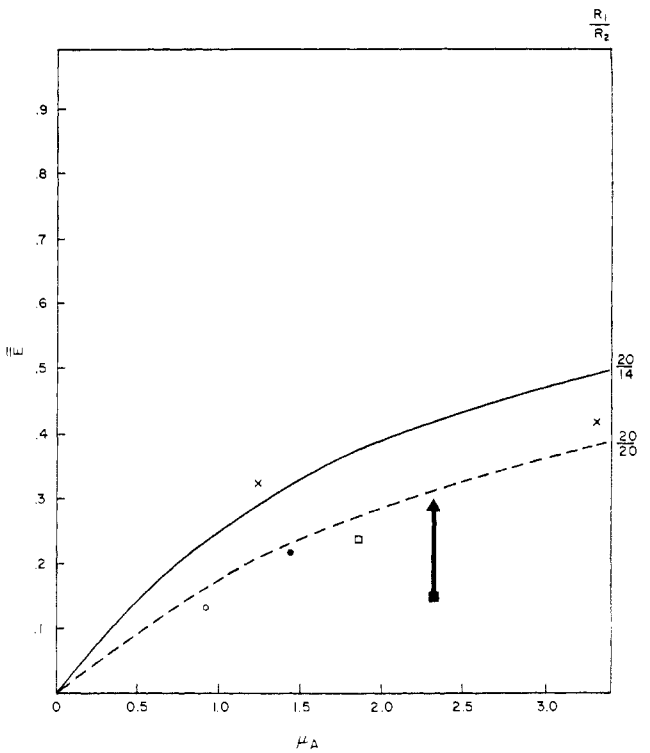

FIGURE 4: Double-sphere energy-transfer experiments. The two curves are expected average energy transfer $\bar{\bar{E}}$ between dyes on two tangential anhydrous protein spheres, one of which is labeled only with donors, the other only with acceptors. (——) Calculated for trypsin, R_1 = 20 Å and LTI, R_2 = 14 Å; R_0 (Dans to FITC) = 33 Å. (- - - - - -) calculated for STI, trypsin, or α-chymotrypsin, $R_1 = R_2$ = 20 Å; R_0 (Dans to FITC) = 33 Å. Experimental points: (×) LTI–Dans–Try–FITC, 1:1 complex (fluorescence lifetime measurements); (○) LTI–FITC–Try–Dans, 1:1 complex (both lifetime and static measurements gave identical results); (●) STI–Dans–Try–FITC, 1:1 complex (fluorescence lifetime measurements); (■) STI–FITC–Try–Dans, 0.5:1 complex, that is, the trypsin activity was only 50% inhibited. The tip of the solid arrow shows the energy transfer that would have been expected for a fully complexed sample (fluorescence lifetime measurements). (□) α-Chymotrypsin–Dans–Try–FITC, 1:1 mixture, apparently a 1:1 complex (static fluorescence). For all of these samples, the individual protein concentrations were in the range of 50 to 300 μg/ml.

not the case may be seen in Figure 2a,b which give typical titration data comparing the binding of labeled and unlabeled proteins. These results indicated that highly active labeled samples bind as well as unlabeled samples (though the time for the equilibration of the complex may be lengthened). This means that labeled and unlabeled populations of proteins behave similarly as a whole. Further indication that the labeling process did not significantly alter protein conformation was obtained from circular dichroism (CD) measurement. The protein CD of unlabeled and Dans-labeled trypsin, STI, and LTI were identical, where μ_D was ≥ 1 for all samples.

Measurement of Transfer Efficiency in Doubly Labeled Single Proteins. Results of the single-sphere transfer experiments are shown in Figure 3. The donor–acceptor dye pairs of these doubly labeled proteins were Dans and FITC or Dans and RITCB as indicated. The theoretical curves were generated assuming anhydrous spheres as described in Gennis and Cantor (1972) using 33 Å as R_0 for the Dans to FITC pair and 24 Å for the Dans to RITCB. These values were calculated as described by Beardsley and Cantor (1970), the critical assumption being that the orientation factor κ^2 is 0.66. The approximate radii of LTI, α-chymotrypsin and STI, and bovine serum albumin were calculated from partial specific volumes (Tanford, 1961) to be 14, 20, and 27 Å, respectively. Most of the experimental points fall almost exactly on the calculated curves. The slight deviations from expected transfer efficiencies of α-chymotrypsin samples at very high μ_A are probably due to an unfolding of the protein caused by the bulky dyes, so that the increased protein volume is reflected in a decreased transfer efficiency. The albumin data are best fit by a radius of about 45 Å. This is probably the result of two effects. The albumin is sufficiently elongated so that the expected energy transfer will be about 25% less than would occur on a spherical protein. A second possibility is that labeling causes expansion of the protein to a size comparable to what is found for the albumin at acid pH (42–45 Å).

Measurements of Transfer Efficiency in Protease–Inhibitor Complexes. Results of the double-sphere transfer experiments are shown in Figure 4. The theoretical curves were generated assuming a complex of two anhydrous spheres of 14 and 20 Å radii for LTI and trypsin, or both of 20 Å radii for STI and trypsin. The residual esterolytic activity of all labeled protease samples was $\geq 90\%$. The residual protease-inhibiting activity of all labeled inhibitor samples was 100%. Particular attention should be given to the two points representing the STI–trypsin complex (indicated by the closed circle and the closed square), since they indicate

that the measurement of transfer efficiency is a sensitive method for the titration of complex formation. One sample which has the donor protein (trypsin–Dans) only 50% complexed shows only 50% of the expected transfer efficiency (solid arrow). Note that a 1:1 mixture of α-chymotrypsin–Dans and trypsin–FITC shows almost precisely the energy transfer expected if a 1:1 complex has formed. This apparent heterocomplex formed at a total protein concentration of 400 μg/ml.

Energy transfer has been observed in the ternary complex made up of the double-headed BEPTI unlabeled with Dans-labeled α-chymotrypsin and FITC-labeled trypsin, but a quantitative interpretation of the results will require further investigation which will be reported in a future publication.

Measurement of Transfer Efficiency in Samples for Which the Theoretical Assumptions Are Invalid. Although the experimental agreement with the theoretical calculations was impressive for the double-sphere experiments and for the single-sphere experiments involving STI, α-chymotrypsin, and the albumin, it can be seen that the experimental point for the doubly labeled LTI was not in good agreement. Presumably the very small size of LTI (mol wt 9000) considerably increased the experimental errors involved. Best results will be obtained when the protein radius is approximately the same size as R_0 (Gennis and Cantor, 1972).

Spurious results were also encountered in single-sphere transfer experiments using horseradish apoperoxidase doubly labeled with either Dans and FITC or Dans and RITCB. Static measurements of Dans quenching indicated 96–100% transfer efficiency with both dye pairs, while time-dependent measurements detected no shortening of the Dans lifetime. The observation of Dans in the excitation spectrum of the doubly labeled protein, and the measurement of the characteristic Dans lifetime proved that the Dans had not been removed during the FITC-labeling process, since any free dye would have been found on the Sephadex G-25 column. The best explanation for these results is that the free amino groups of apo-HRP are not randomly arranged on the protein surface. Apparently, most of the donor molecules are quite close to the acceptor molecules so that the static measurements indicate total quenching. At the same time, at least one donor molecule must be very distant from any acceptor molecule. This lone donor would not participate in transfer and would give an unshortened characteristic Dans lifetime. The donors which are close to acceptors would transfer so quickly that they would not contribute to the major long exponential of the Dans decay.

Discussion

Choice of Dye Pairs. In order to maximize the random spatial distribution of dyes on the protein surface, it is desirable to use dye derivatives with functional groups such as isothiocyanate or sulfonyl chloride which attack all free α- and ε-amino groups, rather than dye derivatives which specifically modify the side chains of infrequently occurring amino acids. Thus the commercial dye preparations Dans, FITC, and RITCB have been used. Aside from the convenience of using commercial samples, these dyes have some theoretical advantages. Lysine attachment will help ensure flexible dye orientation and thus make κ^2 approach 0.66. The huge Stokes shift of Dans causes the R_0 for self-transfer between Dans labels to be zero. Thus corrections due to donor–donor interactions are unnecessary. It is desirable to have a number of dye pairs with a range of R_0 values, since transfer experiments are most sensitive when the distance to be determined is approximately the same as R_0. The fluorescence lifetime of Dans bound to protein (13–17 nsec) is much longer than that of bound FITC or RITCB (less than 7 nsec), so that lifetime data are easily interpreted. On the other hand, these dye pairs suffer the disadvantage of having bad windows for donor excitation (see Figures 1a and 1b). The best window for Dans excitation in the Dans to FITC pair is at 360 nm, while that for the Dans to RITCB pair is about 325 nm. The difficulty in measuring sensitized fluorescence in the Dans to FITC pair has been mentioned. New isothiocyanate derivaives of fluorescent dyes are being explored for future use.

Choice of pH for Labeling. To label randomly as many surface amino groups as possible with the acceptor FITC or RITCB, it is necessary to buffer the labeling reaction mixture at pH 10.4, above the pK_a of the ε-amino group of lysine, since Maeda *et al.* (1969) have shown that only α-amino groups are labeled by FITC below pH 9.5. For trypsin, at least, maximum labeling of the ε-amino groups of lysine preserves the activity of the trypsin sample by preventing autolysis, as described by Labouesse and Gervais (1967) for acetylated trypsin. Maximum labeling is also advantageous since the dependence of transfer efficiency on R/R_0 is most sensitive in the region of large μ_A (Gennis and Cantor, 1972). In this respect, transfer results from samples with μ_A much less than 1.0 as probably used by Edelhoch and Steiner (1965; see Steiner and Edelhoch, 1963) in their investigation of the trypsin complex with basic bovine pancreas trypsin inhibitor could not be interpreted quantitatively. Moreover, the Poisson distribution for such low values of μ_A indicates that most of the protein molecules would be unlabeled.

In some cases, a suitable degree of labeling can be obtained at pH 9 or below. Important examples can be seen in the Dans labeling of fibrinogen by Mihalyi and Albert (1971a) and in the labeling of the 30S ribosome with FITC (Huang and Cantor, 1972).

Advantages of Labeling with Celite. The use of Celite–dye powders in the labeling of proteins allows the use of water-insoluble dyes in completely aqueous buffers. Thus the denaturation of protein due to small additions of acetone for Dans labeling can be avoided. Dyes which hydrolyze rapidly in solution are extremely stable when adsorbed to Celite (Mihalyi and Albert, 1971a). Protein denaturation is minimized because of the very short reaction time (4 min). When α-chymotrypsin is labeled with Dans by slow diffusion of the dye into the protein during long reaction times in the cold, Dans combines 1:1 stoichiometrically in the active site, resulting in 100% inactivation of the enzyme (Hartley and Massey, 1956). However, labeling of α-chymotrypsin with Celite–Dans in 4 min produces surface-labeled samples with large μ_A which retain 100% esterolytic activity. Thus the assumption of surface labeling used in the calculations of efficiency is reasonable. Surface labeling also ensures the homogeneity of local environment, and consequently the uniformity of the quantum efficiency and fluorescence lifetime, of the attached dyes, since they are all in contact with the aqueous buffer.

Conclusions

The excellent agreement of experimentally determined protein and protein complex radii with values calculated for anhydrous spheres is highly gratifying. It appears that the method of singlet energy transfer is capable of providing quantitative distance information in macromolecular systems,

even where the donors and acceptors are not located in unique sites.

Earlier studies on singlet–singlet energy transfer have been plagued by two difficulties. One is uncertainty about what value to use for κ^2, the dipole–dipole orientation factor. The second is that realizable values of R_0 for single chromophore pairs are often too small to measure the distances of interest. The present experimental protocol appears to surmount these two difficulties. By using random dye labeling, a choice of 0.66 for κ^2 becomes more easy to rationalize and is certainly likely to be sufficiently accurate for most cases. By using multiple labeling, the effective R_0 of a dye pair can be increased. The excellent experimental agreement also indicates that the additional assumptions used for calculation of transfer efficiency (Gennis and Cantor, 1972), anhydrous spheres, random spatial distribution of dyes, and Poisson distribution of number of dyes per protein are probably adequate.

Since the method can be used to measure an effective anhydrous protein radius, it will be a valuable means of investigating the swelling which accompanies protein denaturation. Such studies are under way in our laboratory. The method of singlet energy transfer seems especially applicable to the investigation of associating systems. It is applicable at greater dilutions than other physical techniques. It is capable, in principle, of monitoring even very rapidly associating and dissociating complexes. Energy transfer between extrinsic probes should be an extremely useful and sensitive tool for the investigation of the protein protease inhibitors. The apparent heterocomplex of trypsin and α-chymotrypsin (Figure 4) could not have been detected by changes in the esterolytic activity of either protease, since the sample containing the heterocomplexes showed 100% activity for both.

It should also be possible to use energy transfer to ascertain whether the protein inhibitors bind to inactive proteases, a question central to the "acyl-enzyme mechanism" of inhibitor–protease interaction proposed by Finkenstadt and Laskowski (1965) and later contradicted (Foster and Ryan, 1965; Feinstein and Feeney, 1966).

Finally, singlet energy transfer should prove invaluable in the investigation of multimacromolecular associates such as the ribosome. By reassociating ribosomal particles from proteins and RNA which have been Celite-labeled pair by pair, the detailed spatial organization of the ribosome might be revealed. Some success has already been had in preparing active Celite-labeled ribosomes (Huang and Cantor, 1972).

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