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A Spectroscopic Technique for Measuring Slow Rotational Diffusion of Macromolecules. 1: Preparation and Properties of a Triplet Probe†

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ABSTRACT: Slow rotational diffusion may be investigated by measuring the decay of dichroism of flash-induced transient absorption changes of suitable probes. The preparation of the covalent "triplet" probe eosin isothiocyanate is described together with investigations of spectroscopic properties of eosin–protein conjugates. Triplet state lifetimes of air-equilibrated solutions of eosin–protein conjugates are in the order of 10 μs, demonstrating that the probe is protected from oxygen quenching by the protein. Experiments with the sucrase–isomaltase complex from small intestine show that its enzymatic activity is little affected by binding up to 2 mol of eosin/mol of protein.

The method of fluorescence polarization has become a standard technique for investigating the rotational diffusion of macromolecules in aqueous solution (for reviews see Weber, 1973; Yguerabide, 1972). The method is successful because the rotational correlation times of particles of molecular weight in the order of 100 000 are not too much different from typical fluorescence lifetimes, that is, both are in the nanosecond time range. When rotational correlation times are longer than about 10^{-7} s, the fluorescence method is no longer applicable. This will be the case for particles in a relatively viscous environment (e.g., proteins in membranes) and for large assemblies of macromolecules (e.g., virus particles). In order to investigate rotational motion in such systems, it is necessary to develop new techniques.

Triplet states of organic molecules have lifetimes in the order of 10^{-3} s at room temperature. Such long lifetimes may in principle be exploited to extend the measurement of rotational diffusion to the millisecond time range. Radiative transitions (phosphorescence) from the triplet state to the ground state are normally difficult to detect at room temperature in fluid solutions. However, absorption from the lowest triplet state to higher triplet states is readily detected in selected molecules. Thus, measurement of the decay of dichroism of triplet–triplet absorption following excitation by a pulse of polarized light offers a practical way of investigating slow rotational motion.

The feasibility of this proposal has previously been demonstrated using eosin as a probe. Transient dichroism of the eosin triplet–triplet absorption was detected after binding eosin noncovalently to BSA[1] and dissolving the complex in a viscous solution (Razi Naqvi et al., 1973). However, for further progress it is highly advantageous to have a probe that binds covalently to proteins. This is particularly important for investigations with membranes, since it greatly simplifies the task of determining the binding sites.

The lowest singlet–singlet absorption band of eosin is a fully allowed transition. Furthermore, the presence of the four bromine atoms in the structure promotes intersystem crossing from the lowest excited singlet state to the triplet state; in aqueous solution the quantum efficiency of this process is 70% (Bowers and Porter, 1967). For these reasons, eosin is a particularly favorable choice as a triplet probe. Here we describe a simple method of preparing a reactive derivative of eosin, eosin isothiocyanate (Figure 1). This derivative is used to form conjugates between eosin and proteins. We have made measurements of the basic spectroscopic properties of these conjugates that are relevant to our proposed investigations. In the following paper of this issue (part II) we describe experiments that establish that reliable measurements of slow rotations may be made using eosin isothiocyanate as a probe. Some preliminary results of these investigations have been previously reported (Cherry, 1975).

Materials and Methods

Spectroscopic Measurements. Absorption spectra were obtained with a Unicam SP-1700 spectrophotometer. The flash

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[1] Abbreviations used are: BSA, bovine serum albumin; con A, concanavalin A; NMR, nuclear magnetic resonance.

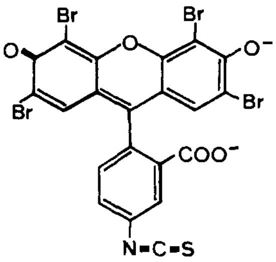

FIGURE 1: Eosin isothiocyanate (isomer I).

photolysis apparatus used for measuring transient absorption signals will be described in detail elsewhere; a brief description is contained in the following paper of this issue (part 2). Samples for flash photolysis were normally bubbled with argon to displace dissolved oxygen. All experiments were made at room temperature. Proton NMR spectra were obtained with a Varian HA-100, 100 MHz spectrometer.

Preparation of Eosin Isothiocyanate. Eosin isothiocyanate was prepared by bromination of fluorescein isothiocyanate following a procedure similar to that for converting fluorescein to eosin (Roedig et al., 1960). Fluorescein isothiocyanate (50 mg) (Serva, isomer I) was suspended in 0.5 ml of ethanol. Bromine (164 mg) was added drop by drop, the suspension being thoroughly stirred throughout. A clear solution is obtained on formation of the dibromo product; as the reaction proceeds further, a precipitate of the insoluble tetrabromofluorescein isothiocyanate is formed. After standing for 2 h at room temperature the mixture was filtered and the insoluble material was washed with 5–10 ml of ethanol. The product was solubilized in 10 mM phosphate buffer, pH 7.4, and precipitated at 4 °C with 1 N phosphoric acid. The precipitate was collected by centrifugation and washed three times with double-distilled water. After lyophilization, the product was stored at −20 °C until used.

When examined by thin-layer chromatography (Merck DC Fertigplatten Kieselgel F 254 eluted with benzene–methanol 2:1), the product exhibited a strong main spot together with a very faint second spot. Elemental analysis gave the following results: Anal. Calcd: C, 35.78; H, 1.00; N, 1.99; Br, 45.34; S, 4.54. Found: C, 35.66; H, 1.07; N, 1.96; Br, 45.30; S, 4.65.

Comparison of the proton NMR spectrum of the product with that of fluorescein isothiocyanate (see Corey and Churchill, 1966) demonstrated that the chemical shifts, splittings, and intensities of the resonances are consistent with the replacement of four protons on the xanthene moiety by bromine.

Conjugation of Eosin to Proteins. Isothiocyanates form a covalent linkage to amino and sulphydryl groups according to the reactions:

$$\text{R-N=C=S} + \text{H}_2\text{N-protein} \rightarrow \text{R-NH-CS-protein}$$

$$\text{R-N=C=S} + \text{HS-protein} \rightarrow \text{R-NH-CS-S-protein}$$

Typically, the conjugation of eosin to protein was carried out in the following manner. A solution of eosin isothiocyanate (1.4 mM) in 0.1 M $NaHCO_3$, pH 8, buffer containing 0.5 M NaCl was added to 10 mg of the given protein. The volume of solution added was normally adjusted to give a mole ratio of eosin:protein of unity. Since the amount of eosin bound was typically 50–90%, this procedure yielded conjugates with a mole ratio of eosin:protein between 1:1 and 1:2. The reaction was allowed to proceed for 3 h at room temperature in the dark. The labeled protein was then separated from unreacted dye on a Bio-Gel P10 column. The final product was either lyophilized or, where necessary, concentrated by vacuum dialysis. Protein concentrations were determined by the method of Lowry et al. (1951). Sucrase–isomaltase was prepared by papain solubilization from rabbit small intestine according to the method of Cogoli et al. (1972). Sucrase activity was determined as described elsewhere (Kolinska and Semenza, 1967). Other proteins used in these studies were ovalbumin (two times crystallized, Schwarz/Mann), con A (three times crystallized, Miles), chymotrypsinogen A (six times crystallized, Koch Light), β-lactoglobulin A (Miles), and BSA (fraction V, crystallized, Fluka).

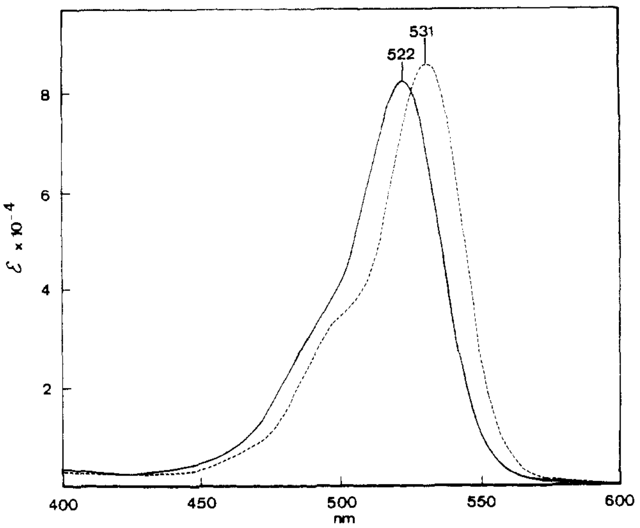

FIGURE 2: Solid line: absorption spectrum of eosin isothiocyanate 12.4 μg/ml in 0.1 M $NaHCO_3$, 0.5 M NaCl buffer, pH 8. Dashed line: same solution on addition of 2 mg/ml of BSA.

Results and Discussion

Absorption Spectra. The absorption spectrum of eosin isothiocyanate in pH 8 carbonate buffer is shown in Figure 2. The absorption maximum occurs at 522 nm compared with 517 nm for eosin (e.g., see Kasche and Lindqvist, 1965). The maximum molar extinction coefficient is 8.3×10^4 mol^{-1} cm^{-1}. Upon conjugation to protein, the absorption band shifts to lower energy. The exact position of the absorption maximum varies slightly from protein to protein, usually lying within the range 525–528 nm.

Determination of Eosin–Protein Binding Ratio. The simplest method of determining the amount of bound eosin is based on the assumption that the maximum extinction coefficients of free and bound eosin are identical. The concentration of eosin in a given solution of conjugate is then calculated from the maximum absorbance. However, the assumption that the extinction coefficient is unchanged on binding may be unjustified. In order to check the accuracy of this method, the amount of eosin conjugated to a sample of β-lactoglobulin A was measured in three different ways. First, the bound eosin was determined from the absorption spectrum, as described above, yielding a value of 31.4 ± 0.6 μg of eosin/mg of protein. Second, all the unbound eosin was collected and measured spectrophotometrically, and the amount of bound eosin was then obtained by subtraction from the initial quantity. This method gave a value of 31.6 ± 1.2 μg of eosin/mg of protein. Finally, the bound eosin was determined by measuring the bromine concentration in the sample, and the result was 32.4 ± 0.1 μg of eosin/mg of protein. The good agreement between the different methods demonstrates that any change in extinction coefficient on binding must be small. This conclusion

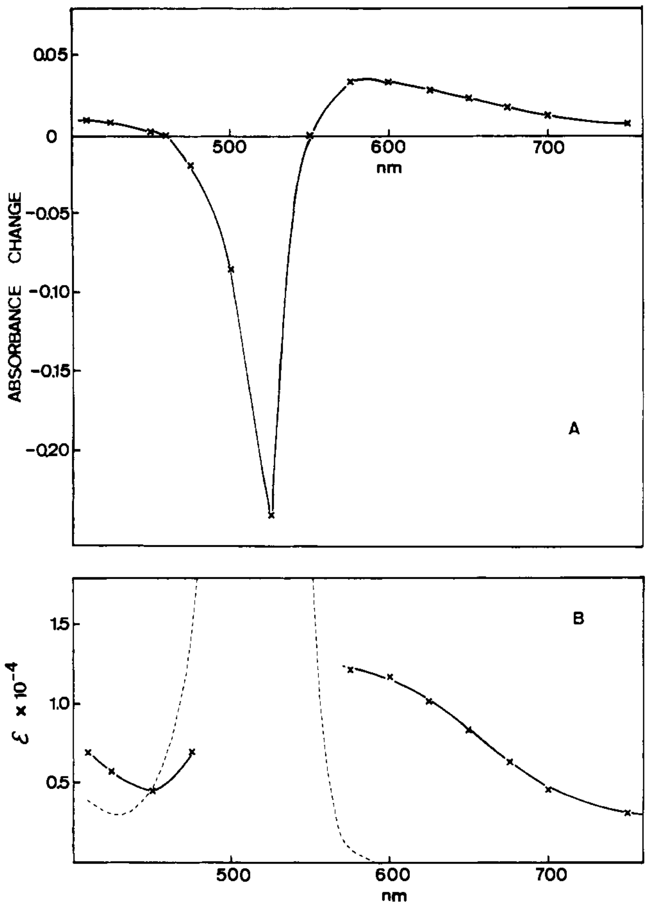

FIGURE 3: (A) Variation of transient absorbance change with wavelength for eosin-ovalbumin conjugate in 10 mM phosphate buffer, pH 7.4 (argon bubbled). Signal measured 20 μs after exciting flash. Eosin-ovalbumin mole ratio 0.78, eosin concentration 1.8 × 10^{-5} M. (B) Solid line: triplet-triplet absorption spectrum calculated as described in text from data in A. The singlet-singlet band (dashed line) is shown for comparison.

was further confirmed by adding excess BSA (2 mg/ml) to eosin isothiocyanate solution (12.4 μg/ml). In this sample there is no unbound eosin, since any unreacted eosin is strongly bound hydrophobically (Youtsey and Grossweiner, 1967). As shown in Figure 2, the absorption maximum shifts to 531 nm on addition of BSA but the maximum extinction coefficient shows an increase of only 3.8%.

Thus for most practical purposes, the amount of bound eosin may be determined with sufficient accuracy (within 5%) from the absorbance using the extinction coefficient of eosin isothiocyanate in aqueous solution. The result with BSA suggests that for the most accurate value a correction of 3.8% should be applied to account for the change of $\epsilon_{\max}$ on binding.

Triplet-Triplet Absorption Spectrum. Irradiation of eosin-protein conjugates by a 2-μs flash of wavelength 540 nm gives rise to a transient absorption change. In Figure 3A, the absorbance change ΔA (measured 20 μs after the exciting flash) for the eosin-ovalbumin conjugate is plotted against wavelength; similar results were obtained with other proteins. The positive absorbance changes are due to triplet-triplet absorption (e.g., see Kasche and Lindqvist, 1965), while the large negative absorbance change at 525 nm arises from depletion of the ground state. If ϵ_s and ϵ_T are the extinction coefficients of the singlet-singlet and triplet-triplet absorptions at a given wavelength and C_T is the concentration of molecules in the triplet state, then

$$\Delta A = (\epsilon_T - \epsilon_S) C_T L \qquad (1)$$

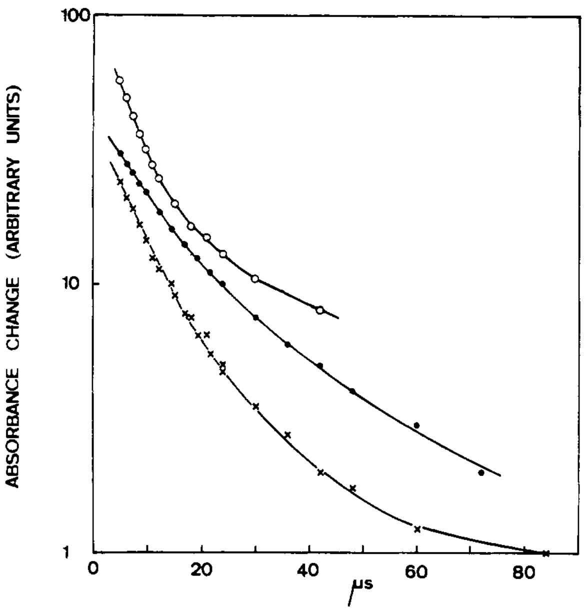

FIGURE 4: Decay of transient absorbance changes measured at 500 nm in air-equilibrated solutions of eosin-protein conjugates. (x) Eosin-ovalbumin; (○) eosin-con A; (●) eosin-BSA. All samples, eosin-protein mole ratio 0.5, eosin concentration 1.4 × 10^{-5} M in 10 mM phosphate buffer, pH 7.4.

where L (= 1 cm) is the path length of the cell. If we assume that $\epsilon_T \ll \epsilon_S$ at the singlet-singlet absorption maximum, we may calculate C_T from the measured absorbance change at 525 nm. The triplet-triplet absorption spectrum may then be calculated from the data of Figure 3A and the known singlet-singlet absorption spectrum; the result is shown in Figure 3B. The triplet-triplet absorption spectrum of the conjugate determined in the above manner is essentially similar to that measured for free eosin by Kasche and Lindqvist (1965). Since there is no reason to expect dramatic changes in ϵ_T on binding, this indicates that the assumption $\epsilon_T \ll \epsilon_S$ at 525 nm is probably justified.

Oxygen Quenching in Eosin-Protein Conjugates. No transient absorption signal is detected with our apparatus in air-saturated aqueous solutions of free eosin. Presumably, oxygen quenching reduces the triplet state lifetime below 2 μs, the shortest lifetime which is measurable with the present instrumentation. However, in air-saturated solutions of eosin-protein conjugates, a transient absorption of lifetime in the order of 10 μs is detected in all cases examined (ovalbumin, BSA, chymotrypsinogen B, con A, and sucrase-isomaltase). Thus, covalent binding of eosin to protein provides some protection from oxygen quenching, demonstrating that the dye is at least partially embedded in the protein.

Some typical decay curves for the transient depletion signal are shown in Figure 4. The decay of the triplet-triplet absorption measured at 610 nm follows the same time course as the decay of the ground state depletion signal for a given sample. The semilogarithmic plots in Figure 4 are all nonlinear, demonstrating that in each case there is more than one component of the decay. Probably this is because the binding sites are heterogeneous.

It is of interest to see whether the triplet lifetime is sensitive to the conformation of the protein. Decay curves were measured for the eosin-ovalbumin complex in the presence and absence of 8 M urea; the slopes at short times gave time constants of 5.7 and 10.2 μs, respectively. As might be anticipated, the triplet lifetime is shorter in the denatured protein due to

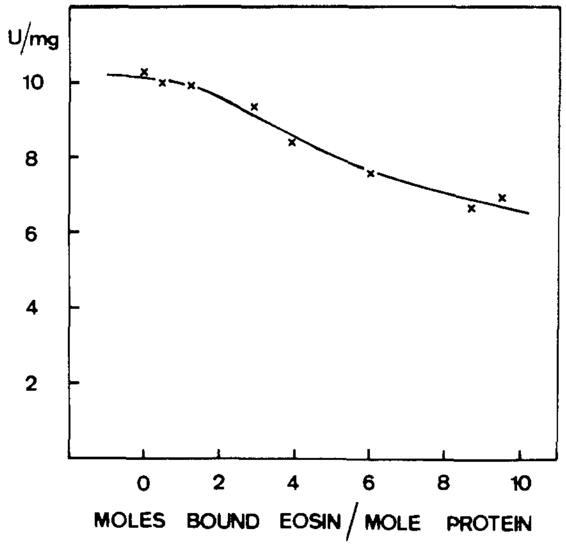

FIGURE 5: Sucrase activity of sucrase-isomaltase complex as a function of moles of bound eosin.

increased exposure to oxygen in the aqueous solution. However, since a transient absorption change is still observable, there is some protection from oxygen even in the denatured state. Previously, Geacintov et al. (1975) demonstrated that triplet lifetimes of aromatic hydrocarbons bound hydrophobically to BSA are sensitive to conformation. Razi Naqvi and Gonzales-Rodriguez (1973) found that the triplet-triplet absorption of tryptophans in fibrinogen could be used to monitor denaturation, while room temperature tryptophan phosphorescence has recently been reported from horse liver alcohol dehydrogenase and *E. coli* alkaline phosphatase (Saviotti and Galley, 1974) and from wool keratin (Pailthorpe, 1975). These various observations indicate that oxygen quenching of the triplet state of intrinsic or extrinsic chromophores is a useful method of monitoring protein conformational changes.

Effect of Labeling on Enzymatic Activity. An ever present danger of probe techniques is that the probe may perturb the system under investigation. It is of course not possible to provide a general answer to the question of what perturbation occurs with the labeling techniques described here; each system must be individually investigated. For present purposes, we have examined the effect of eosin labeling on one system that is of interest to us, namely, the sucrase-isomaltase complex. The dependence of enzymatic activity on the amount of eosin bound is shown in Figure 5. The point for zero eosin is obtained by subjecting the enzyme to the standard labeling procedure in the absence of eosin. Since the original activity of the enzyme was 11.6 U/mg, the labeling conditions alone produce a small loss of activity. However, Figure 5 shows that at least 2 mol of eosin/mol of protein can be bound without further loss of activity. Thereafter, there is a gradual loss of activity with increasing amounts of eosin bound, but even at a mole ratio of 9:1 over 60% of the activity is retained. These results are in good agreement with those of Braun et al. (1976) who found that sucrase-isomaltase is not inactivated by amino group reagents (unless they bind in the active site). Sucrase-isomaltase does not have free SH groups and is not inactivated by SH reagents (Cogoli et al., 1972).

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