A Spectroscopic Technique for Measuring Slow Rotational Diffusion of Macromolecules. 2: Determination of Rotational Correlation Times of Proteins in Solution†

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ABSTRACT: Experiments have been performed to test the validity of measurements of slow rotational diffusion using eosin isothiocyanate as a probe. When eosin–protein conjugates are covalently bound to Sepharose 4B, the dichroism of the flash-induced absorption transient does not decay with time. The magnitude of the dichroism is smaller than for eosin in solid solution. These results, together with measurements with highly viscous solutions, demonstrate the existence of a rapid but *restricted* independent motion of the eosin probe. This causes a partial loss of dichroism leaving a residual dichroism whose time dependence is determined by the rotational motion of the protein. Rotational correlation times in the microsecond–millisecond time range have been determined for eosin–protein conjugates dissolved in glycerol–water mixtures of varying viscosity. The measured values are in good agreement with theoretical predictions and show the correct dependence on the viscosity of the medium and the size and state of association of the protein.

In the previous paper of this issue (part 1), the preparation and spectroscopic properties of the "triplet" probe eosin isothiocyanate were described. In principle, the long lifetime of the triplet state permits the investigation of rotational motion that is too slow to be detected by fluorescence polarization.

A principal aim of developing a technique to study slow rotational motion is to investigate the mobility of proteins in cell membranes. However, before applying the method to such complex systems, it is necessary to demonstrate that the technique does indeed provide reliable measurements of rotational correlation times. In the present paper, we report investigations with proteins dissolved in viscous solutions. In such systems the expected rotational correlation time may be calculated from a knowledge of the protein size and the bulk viscosity. In this way, we are able to show that reliable determinations of rotational correlation times in the microsecond–millisecond time range may be obtained from measuring the rate of decay of dichroism of flash-induced transient absorption changes of the eosin probe. The extent of independent motion of the eosin probe when conjugated to protein has also been investigated. Some preliminary results of these investigations were briefly reported previously (Cherry, 1975).

Materials and Methods

Flash Photolysis. A full description of the flash photolysis apparatus used in these experiments will be given elsewhere. Briefly, the exciting source is an Electro Photonics Model 43 dye laser. The dye solution is coumarin 6 (10^{-4} M in ethanol) that has an untuned emission at 540 nm, the light pulse has a half-width of 1–2 μs and an energy of 100–200 mJ. The exciting light is vertically polarized by a Glan–Taylor prism. The sample is contained in a 1-cm fluorimeter cell and the measuring source is a 100 W tungsten-halide lamp. The measuring beam passes through the sample at right angles to the exciting beam. The wavelength of the transient absorption is selected by a Leitz in-line mirror monochromator. After emerging from the monochromator the measuring beam is split into vertically and horizontally polarized components by a Barr and Stroud BC6 polarizing beam splitter. The intensities of the two components are then measured separately by two photomultipliers (EMI 9683 QB) and displayed on a Tektronix 5103 dual-beam oscilloscope. For measurement purposes, it is often convenient to display the difference between the two signals on one trace using the differential input of the oscilloscope amplifier. Low-temperature measurements are made using an Oxford Instruments DN 704 cryostat.

Theory of the Technique. The technique exploits the long lifetime of the triplet state. The lowest triplet state of conjugated molecules is normally populated by intersystem crossing from the lowest excited singlet state following singlet–singlet absorption. Molecules in the triplet state may be detected by the absorption of light of appropriate wavelength that excites transitions from the lowest triplet state to higher triplet states. Alternatively, ground-state depletion signals, that is signals arising from the loss of absorption in the singlet–singlet absorption bands due to removal of molecules to the triplet state, may also be observed.

The principles of measuring rotational diffusion using triplet states are analogous to those employed in the method of fluorescence polarization (e.g., see Yguerabide, 1972) and therefore will not be given in detail. Briefly, excitation with polarized light selectively excites those molecules whose transition moment for absorption lies in or near the direction of polarization, i.e., a partially ordered population of triplet state molecules is photoselected from the randomly oriented population. Transient absorption signals arising from molecules in the triplet state will therefore be dichroic. The dichroism will, however, decay as the triplet molecules again become randomized due to Brownian motion. The anisotropy parameter $r(t)$ is defined by the expression

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

where $A_{\parallel}(t)$, $A_{\perp}(t)$ are the absorbance changes at time t for light polarized parallel and perpendicular to the exciting flash.

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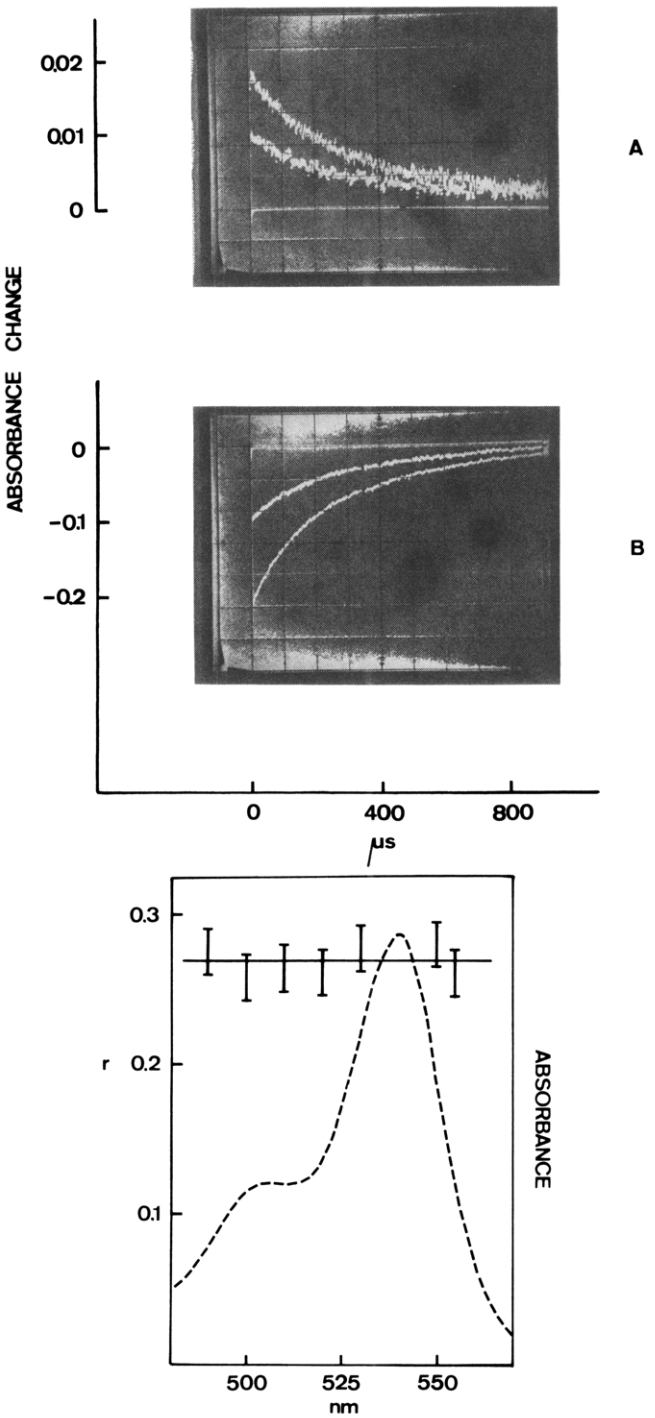

FIGURE 1: Dichroism of eosin transient absorption in poly(methyl methacrylate). Eosin concentration ~1 × 10^{-5} M. (A) Triplet–triplet absorption measured at 650 nm; upper trace parallel, lower trace perpendicular polarization relative to exciting flash. (B) Ground-state depletion measured at 525 nm; lower trace parallel, upper trace perpendicular polarization of measuring beam relative to exciting flash. (C) Dependence of anisotropy parameter on wavelength. Dashed line is S_0–S_1 absorption band of same sample.

For a spherical molecule, $r(t)$ decays exponentially with time according to the expression (Jablonsky, 1961)

$$r(t) = r_0 \exp(-t/\phi) \tag{2}$$

where ϕ is the rotational correlation time and r_0 is the value of r at $t = 0$. For a sphere of radius a immersed in a medium of viscosity η

$$\phi = \frac{4\pi a^3 \eta}{3kT} = \frac{1}{6D} \tag{3}$$

where D is the rotational diffusion coefficient (Einstein, 1906; Perrin, 1926).

For an irregular body the situation is more complicated. In the general case, the right hand side of eq 2 is replaced by the sum of five exponentials (Ehrenberg and Rigler, 1972; Chuang and Eisenthal, 1972). For a body (such as an ellipsoid of revolution) that possesses an axis of symmetry, there are three exponentials. In practice, however, it is unusual with a rigid molecule to observe more than one component of the decay of $r(t)$ unless the molecule is extremely asymmetric (e.g., see Yguerabide, 1972).

Materials and Sample Preparation. Sucrase–isomaltase was prepared as previously described from rabbit small intestine by either papain treatment (Cogoli et al., 1972) or solubilization with Triton X-100 (Sigrist et al., 1975). Sepharose 4B (cyanogen bromide activated) was obtained from Pharmacia, methyl methacrylate monomer, eosin, and α,α'-azobisisobutyronitrile from Fluka. Glycerol was water-free puriss grade from Fluka; once opened, bottles were tightly stoppered and stored in a desiccator. Preparation of eosin isothiocyanate, labeling of proteins, and all other materials were as described in part 1 (the preceding paper in this issue). Methyl methacrylate was purified by washing with 5% NaOH until the extract remained colorless, then washing three times with 20% NaCl and distilling at 30 Torr and 22 °C under N_2.

In order to obtain a solid solution of eosin, the requisite quantity of dye was dissolved in the minimum possible amount of ethanol and added to a solution containing 1 mg of α,α'-azobisisobutyronitrile/50 ml of methyl methacrylate. After bubbling with argon, the sample was sealed in a glass ampule and polymerized over several days at 120 °C.

Eosin-labeled proteins were conjugated to cyanogen bromide-activated Sepharose 4B in the following manner. Four hundred milligrams of the gel was first swollen and washed with 10^{-3} M HCl. The protein was dissolved in 5 ml of 0.1 M $NaHCO_3$ buffer, pH 8, containing 0.5 M NaCl. The gel was mixed with the protein solution and the reaction was allowed to proceed overnight at 4 °C, the reaction vessel being continuously shaken throughout. Unreacted protein was removed by washing with coupling buffer and the remaining active groups were reacted with 1 M ethanolamine at pH 8 for 1 h. Finally, the sample was washed at pH 4 (0.1 M acetate, 1 M NaCl) followed by washing at pH 8 (0.1 M borate, 1 M NaCl), this cycle being repeated three times.

Solutions of proteins in glycerol–water were normally prepared by first dissolving the protein in 10 mM phosphate buffer, pH 7.4, and then adding the requisite amount of glycerol. The viscosities of the mixture were obtained from published tables (Sheely, 1932). Solutions for triplet–triplet absorption measurement were 10^{-4} M and for ground state depletion 1.5 × 10^{-5} M with respect to eosin unless otherwise stated.

Results and Discussion

Dichroism of Eosin in Solid Solution. Figure 1A,B shows triplet–triplet absorption and ground-state depletion signals for eosin (~1 × 10^{-5} M) in poly(methyl methacrylate). The considerably better signal to noise ratio of the depletion signal is a consequence of the higher extinction coefficient of the S_0–S_1 transition (see part 1). Since there is no rotational motion in this sample (El-Sayed, 1963), the observed dichroism

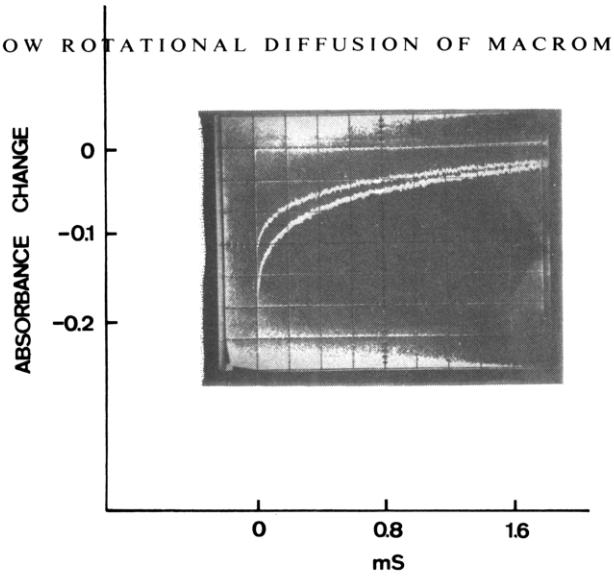

FIGURE 2: Dichroism of ground state depletion signal (λ = 525 nm) of eosin-β-lactoglobulin A conjugated to Sepharose 4B and suspended in 80% glycerol. 3.2×10^{-5} M eosin; eosin-protein mole ratio 0.37. Lower trace parallel, upper trace perpendicular polarization of measuring beam relative to flash.

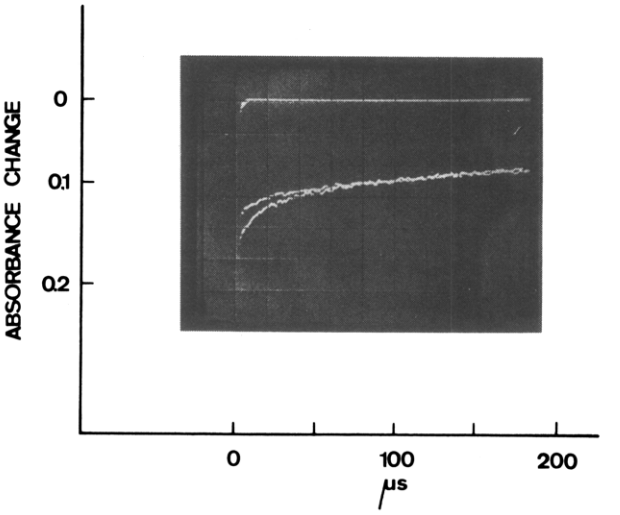

FIGURE 3: Transient dichroism of ground-state depletion signal (λ = 510 nm) of eosin-ovalbumin in 97% glycerol. Eosin-protein mole ratio 0.27 T = 22.5 °C.

represents the maximum obtainable (with our apparatus). As would be expected in an immobilized sample, the dichroism persists for the duration of the signal. The value of r_0 is 0.27 for the depletion signal and 0.25 for absorption.[1]

The value of r_0 depends on the angle γ between the transition moments of excitation and measurement and on instrumental factors. If $\gamma = 0$, the theoretical value of r_0 is 0.4 (Jablonski, 1935). γ must be zero for the depletion signal if the S_0–S_1 band is a pure transition (provided the contribution to the signal from triplet–triplet absorption is negligible; see part 1). This certainly is the case for the related molecule fluorescein, where Wampler and DeSa (1974) have shown that the polarization of fluorescence-excitation spectrum is constant and close to the theoretical value for $\gamma = 0$ over the whole absorption band. We also find that r_0 is constant over the eosin absorption band (Figure 1C). We conclude therefore that our somewhat low value of r_0 is due to instrumental factors and that γ is indeed zero for depletion. We also find that r_0 varies little with wavelength in the range 600–700 nm, indicating that γ is also zero or close to zero for triplet–triplet absorption over this wavelength range (since the magnitude of r_0 is comparable to that found for depletion signals).

Independent Rotation of Eosin in Eosin–Protein Conjugates. Transient absorption signals from eosin report on the motion of the eosin molecule: if eosin were to rotate completely independently of the protein we would learn nothing of the rotation of the protein. The successful use of fluorescence polarization using related probes such as fluorescein suggests that this is unlikely to be the case; however, since we are working in a completely different time range, it is important to investigate this point. This was achieved by conjugating eosin-labeled proteins to Sepharose 4B, hence, immobilizing the protein. Figure 2 shows typical flash photolysis signals obtained with such a sample. A dichroism is observed that persists for the duration of the signal. This is the result that is to be expected for an immobilized sample; similar results were obtained using either absorption or depletion signals for all proteins investigated.

[1] These results were obtained with the laser operating at about one-third maximum power. At higher light intensities the value of r_0 decreases due to saturation effects.

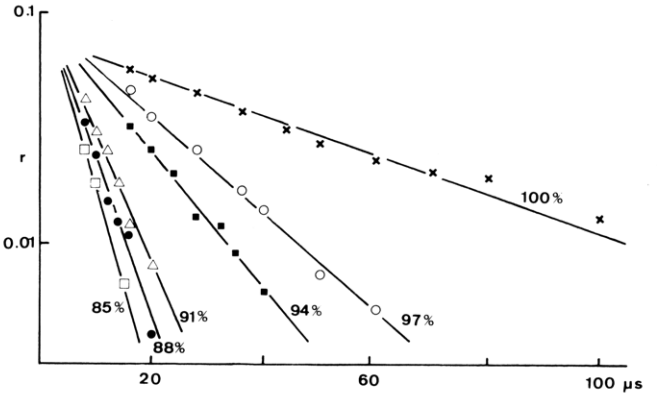

FIGURE 4: Time dependence of r for eosin–ovalbumin complexes in various glycerol–water mixtures; T = 22.5 °C. (The number beside each plot is % glycerol.)

The magnitude of the dichroism in Figure 2 is markedly smaller than that of eosin in solid solution. Typical values of r_0 for Sepharose-immobilized proteins are in the range 0.06–0.10. The obvious interpretation of this result is that eosin does have some rapid but *restricted* motion within its binding site. This causes a partial loss of dichroism, *leaving a residual dichroism which reflects the rotational motion of the protein.* Such a result might also have been anticipated from the observation that eosin conjugated to protein is relatively inaccessible to oxygen (see part 1) and hence must to some extent be embedded in the protein. Further evidence for restricted independent motion of the probe is obtained from low-temperature experiments described later.

Measurements of Rotational Correlation Times. Figure 3 is an example of the transient dichroism that is observed when eosin–protein conjugates are dissolved in glycerol–water mixtures. The parameter $r(t)$ is calculated from the experimental data and plotted against time on a semilogarithmic plot. Figure 4 shows a series of such plots for the eosin–ovalbumin conjugate dissolved in various glycerol–water mixtures. Rotational correlation times calculated from the slopes of these plots are plotted against viscosity in Figure 5. As can be seen, there is good agreement between data obtained from ground-state depletion and triplet–triplet absorption measurements;

TABLE I: Results of Rotational Diffusion Measurements of Proteins in 94% Glycerol (22.5 °C).

Protein	Mol Wt	ϕ^a (Expt) (μs)	ϕ^b (Theor) (μs)	$\frac{\phi \text{ (expt)}}{\phi \text{ (theor)}}$	a^a (expt) (Å)	a^b (theor) (Å)
β-Lactoglobulin A[c]	18 400	4.3	2.08	2.08	22.4	17.6
Ovalbumin	43 500	15.1	4.98	3.03	34	23.5
Sucrase-isomaltase	221 000	56	25.0	2.25	52	40.2

[a] ϕ (expt) is the measured value of ϕ, a (expt) is calculated from ϕ (expt) using eq 3. [b] ϕ (theor) and a (theor) are theoretical values calculated assuming the proteins to be spherical and unhydrated. [c] The measurements with β-lactoglobulin A were made under conditions (pH 8.8, 6 $\times 10^{-5}$ M) where the protein is expected to be predominantly monomeric (Georges and Guinand, 1962).

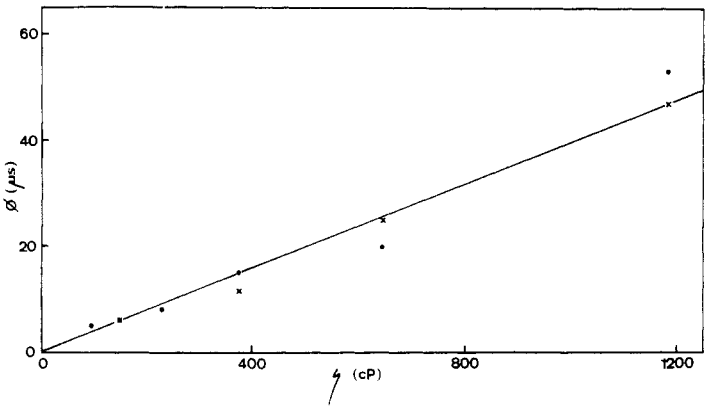

FIGURE 5: Dependence of rotational correlation times calculated from Figure 6 on viscosity. (x) Measured from ground-state depletion signals; (●) measured from triplet-triplet absorption.

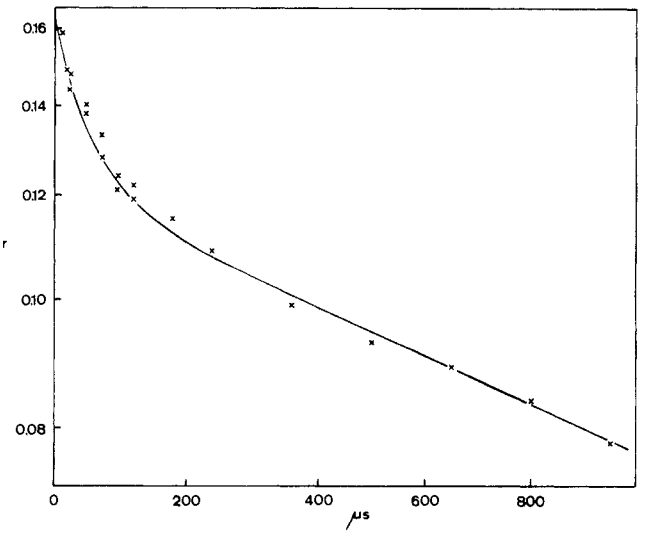

FIGURE 6: Time dependence of r for eosin-ovalbumin complex in 100% glycerol; $T = -16$ °C. (x) Experimental points; solid line calculated from expression $r(t) = 1.37 \exp(-t/(1.9 \times 10^{-3})) + 0.50 \exp(-t/(51 \times 10^{-6}))$.

further, ϕ varies linearly with viscosity as is to be expected from eq 3.

In Table I, results obtained with three different proteins in 94% glycerol are compared. The theoretical values of ϕ and a are calculated assuming the proteins are spherical and unhydrated. In each case the experimental value of ϕ is markedly larger than the theoretical value. This is in part due to hydration effects, since ϕ depends on a^3 (eq 3) a small increase in size has a marked effect on rotation. It may be estimated that hydration could account for about 30% of the difference between ϕ_{expt} and ϕ_{theor}; the remaining difference is probably due to deviation from a spherical shape. Although, in principle, bodies of lower symmetry should exhibit multiple components of rotation (Ehrenberg and Rigler, 1972; Chuang and Eisenthal, 1972), the observed effect is usually a single value of ϕ that is longer than that expected for a sphere of equivalent mol wt. Thus, ϕ_{expt}/ϕ_{theor} values in the order of 2 are typically found using fluorescence polarization techniques (e.g., see Yguerabide, 1972). Thus, the experimental values of ϕ in Table I are reasonable; furthermore, ϕ responds in the expected manner to variation in the size of the protein.

Further investigations of the validity of the method were made with the eosin-ovalbumin conjugate that was found to be soluble in 100% glycerol. By cooling a glycerol solution of the protein it was feasible to obtain data from solutions varying in viscosity by more than two orders of magnitude. At temperatures above about −10 °C it was possible to fit the $r(t)$ vs. t curves to a single exponential decay. However, below −10 °C marked deviations were detected at short times. This is illustrated by the result obtained at −16 °C, which is plotted in Figure 6. The solid line in Figure 6 shows that the experimental points may be fitted by the sum of two exponentials of time constants 1.9 ms and 51 μs. Further experiments with free eosin in glycerol revealed that transient dichroism became detectable below −10 °C and had a time constant of decay of 19 μs at −16 °C. In the ovalbumin experiment, we therefore identify the 1.9-ms component as resulting from rotation of the protein and the 51-μs component as resulting from independent motion of the probe that is only 2–3 times slower than that of free eosin at the same temperature. These results further confirm the conclusions concerning the independent motion of the probe reached from studies with Sepharose-bound proteins.

The values of ϕ for ovalbumin (i.e., the component assigned to rotation of the protein) obtained with glycerol solutions at various temperatures are plotted against viscosity on a log–log plot in Figure 7. The data fit reasonably well to a straight line of slope unity as predicted by eq 3. Thus, the measured rotation times vary in the expected manner when the viscosity is varied either by changing the temperature or adding water.

Effects of Multiple Binding. Most of the experiments reported here were carried out with eosin–protein mole ratios of below 1:1 to obviate the risk of depolarization by energy transfer between eosins on the same protein. That depolarization by energy transfer can occur was clearly demonstrated by preparing eosin–ovalbumin complexes of varying mole ratio. A noticeable decrease in r_0 was detected when more than 2 mol of eosin/mol of protein was bound. At a mole ratio of 3.4:1 the measured value of r_0 was only 50% of that found at low mole ratio, while the intensity of the absorbance change was little altered.

Protein–Protein Interactions. Rotational diffusion measurements are a sensitive method of detecting protein associ-

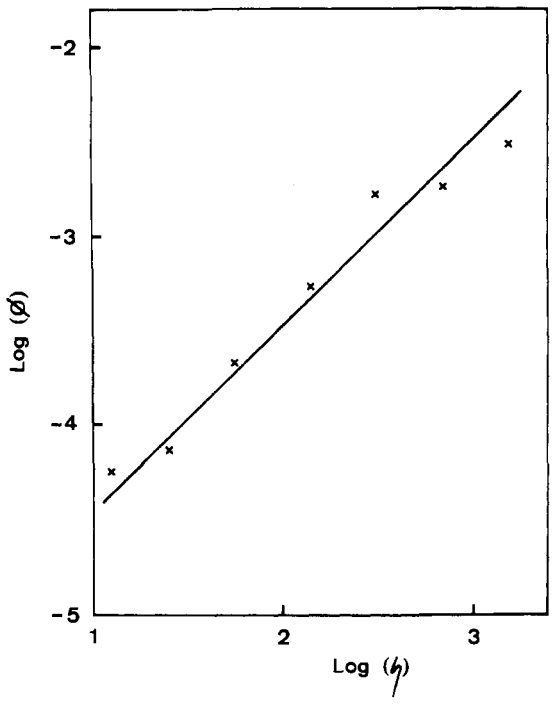

FIGURE 7: Variation of rotational correlation time of protein with viscosity for eosin-ovalbumin in 100% glycerol. The viscosity was varied by varying the temperature over the range +22 to −21 °C.

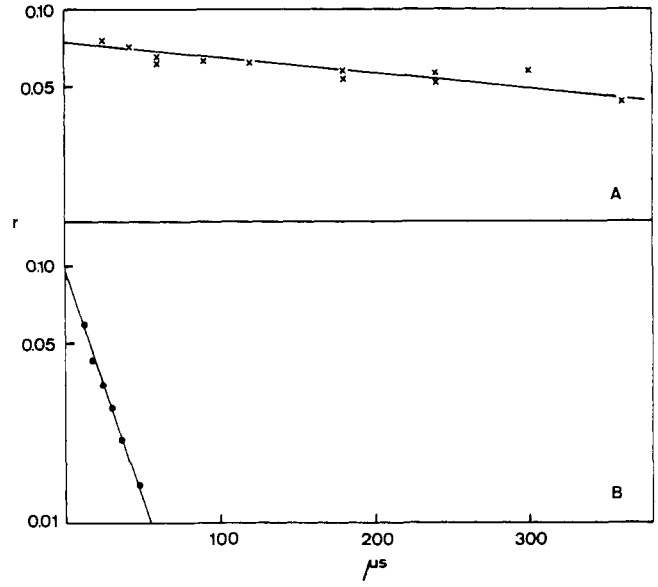

FIGURE 8: Time dependence of r for sucrase-isomaltase in 89% glycerol. (A) Enzyme prepared by extraction with Triton X-100. Eosin-protein mole ratio 0.72. (B) Enzyme prepared by papain solubilization. Eosin-protein mole ratio 2.3.

ations, because ϕ depends on the cube of the particle diameter. For example, Wahl et al. (1969) were able to clearly distinguish monomeric, dimeric, and tetrameric forms of β-lactoglobulin A by fluorescence polarization measurements. (Our determination of the radius of the monomeric form (22.4 Å) is in good agreement with their value (21 Å).)

We have investigated protein aggregation of the sucrase-isomaltase complex. Figure 8 shows $\log r(t)$ vs. t plots for papain- and Triton-solubilized enzyme, both in 89% glycerol. As discussed in a previous section, the rotational diffusion of papain-sucrase-isomaltase is consistent with the molecular weight of the monomer and corresponds to a diameter of 104 Å. In the case of Triton-sucrase-isomaltase, however, the value of ϕ (800 μs) corresponds to a diameter in the order of 300 Å, clearly demonstrating the formation of aggregates. This finding is in harmony with the results of Sigrist et al. (1976) who directly detected such aggregates (in the absence of glycerol) using negative-staining techniques. In their case, a somewhat lower value (240 Å) for the particle diameter was obtained.

Conclusions

The experiments described here clearly demonstrate that rotational correlation times of proteins in the microsecond-millisecond time range may be determined using eosin isothiocyanate as a probe. Eosin bound to proteins exhibits a rapid but restricted independent motion that causes a partial loss of dichroism. The rate of decay of residual dichroism is determined by rotation of the protein. Measured rotational correlation times correlate with the viscosity of the medium and the size and state of association of the protein.

These experiments thus establish the validity of the technique in preparation for investigations with proteins in membranes and other complex systems. Some preliminary observations with erythrocyte membranes have already been reported (Cherry, 1975). Recently, we have established that eosin isothiocyanate when added to intact human erythrocytes binds almost exclusively to band 3 proteins. Investigation of the rotational mobility of these proteins is currently in progress.

Acknowledgments

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