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Coupling Factor for Photophosphorylation Labeled with Eosin Isothiocyanate: Activity, Size, and Shape in Solution[†]

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ABSTRACT: The coupling factor for photophosphorylation (CF1) was covalently labeled with eosin isothiocyanate (ESCN). We found extra binding sites for the label when a protonmotive force existed across the thylakoid membrane during incubation with ESCN. Two out of three such extra sites are located on the γ subunit of CF1. As judged from the (oxygen-dependent) triplet lifetime of bound ESCN, these extra sites are more deeply buried within CF1 than those sites which are already accessible to ESCN in the absence of a protonmotive force. Labeling of the extra sites by only one ESCN per CF1 greatly reduced the activities of the enzyme (more strongly ATP synthesis and Mg^{2+} -dependent hydrolysis of the membrane-bound CF1 than the Ca^{2+} -dependent hydrolysis). On the other hand, a load of up to five ESCN's when bound in the absence of a protonmotive force had hardly

any effect. We isolated and purified active CF1 which was labeled in the absence of a protonmotive force. We studied the rotational diffusion of the isolated enzyme by a photoselection technique aiming at the triplet state of bound eosin. A biphasic decay of the photoinduced linear dichroism of the absorption changes of eosin was observed. The more rapid component was only slightly dependent on the solvent viscosity and therefore attributed to librational motion of the label within the protein. The decay of the slower component was linearly related to the solvent viscosity and therefore attributed to protein rotational diffusion. The theoretical evaluation of the data led us to conclude that isolated CF1 is ellipsoidal rather than spherical in shape, with an axial ratio which is greater than 2.1 if it is prolate and smaller than 1/2.6 if it is oblate.

Synthesis of ATP in green plants is mediated by the coupling factor (CF1).¹ This enzyme is composed from five types of subunits, and it is bound to the thylakoid membrane via its counterpart (CF0), which acts as a proton well [for recent reviews, see Nelson (1976), Kagawa et al. (1979), and Shavit (1980)]. It seems generally accepted that these two together act as a proton-translocating ATP synthase as proposed by Mitchell (1966). In comparison with the ATP synthase complexes of bacteria and of mitochondria, the one of green plants is distinguished by its apparent irreversibility in the

absence of a protonmotive force (Petrack & Lipman, 1961; Kaplan et al., 1967; Mills & Hind, 1979). It is activated under conditions which are also known to induce large conformational changes of CF1 when a protonmotive force is generated by illumination, by an acid-base jump, or by externally applied electric field pulses (Ryrie & Jagendorf, 1971; McCarty & Fagan, 1973; Graeber et al., 1977; Weiss & McCarty, 1977; Kraayenhof & Slater, 1975). The same conditions promote the release (or the exchange) of tightly bound nucleotides

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¹ Abbreviations: CF1, coupling factor for photophosphorylation; ESCN, eosin isothiocyanate; 5-IAF, 5-(iodoacetamido)fluorescein; DTT, dithiothreitol; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; Tricine, N-[tris(hydroxymethyl)methyl]glycine; NaDodSO₄, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; fwhm, full width at half-maximum.

(Harris & Slater, 1975; Boyer et al., 1973; Strotmann & Bickel-Sandkoetter, 1977). The role of conformational changes for energy transduction (from protons to ATP) and/or for the activation of the enzyme is still to be characterized. We searched for a technique to follow conformational changes of the membrane-bound CF1 in real time. For this, we previously tried eosin isothiocyanate (ESCN) as a label for CF1 (Wagner & Junge, 1980). When it is bound to CF1 [as well as to other proteins (Nairn, 1969; Kasche & Lindquist, 1965; Cherry, 1979)], the lifetime of its triplet state is drastically increased [from micro- up to milliseconds (Wagner & Junge, 1980)]. The triplet lifetime is a sensitive measure for the access of dioxygen to the given binding site of the label within the protein. We used it as an indicator for conformational changes of membrane-bound (Wagner & Junge, 1980) and of isolated CF1 (Wagner et al., 1981). The opening of the CF1 structure under the influence of a protonmotive force across the thylakoid membrane became apparent. Another way of looking at conformational changes via ESCN is to monitor the relaxation of the photoinduced linear dichroism of the absorption changes of the label under photoselection. The relatively long triplet lifetime of ESCN makes it superior to fluorescent probes in studies of the rotational diffusion of proteins in biological membranes [for a review, see Cherry (1979)]. We previously reported changes of the rotational diffusion of CF1 in the thylakoid membrane as function of ATP and of a protonmotive force (Wagner & Junge, 1980). Eosin isothiocyanate seems a promising tool for rapid kinetic studies of conformational changes of CF1. On the other hand, careful controls are necessary to place this technique on firm ground. In this paper, we report on different modes of ESCN binding to CF1 and on the influence of bound label on the activities of the isolated as well as of the membrane-bound enzyme. Then we evaluate the size and shape of isolated, ESCN-labeled CF1 as apparent from its rotational diffusion in solution.

Materials and Methods

Chloroplasts were prepared according to standard procedures (Winget et al., 1965) from spinach purchased on the local market.

For photoselection studies, labeled CF1 was suspended in the following viscous medium: a glycerol/water mixture (60:40 w/w) with sucrose (varied between 200 and 800 mM) and buffered at pH 7.6. Glycerol was purchased from Merck (98% pure). The Ca^{2+} -ATPase activity of the labeled CF1 in a mixture of glycerol/water was still 90% of the control in aqueous buffer [$19 \mu\text{M}$ ATP (mg of protein) $^{-1}$ min $^{-1}$].

Eosin isothiocyanate was recently used by Cherry and his co-workers (Cherry, 1979; Cherry et al., 1976) to covalently label proteins for optical studies of their rotational diffusion in biological membranes. It is known that the isothiocyanate group can react with R-NH $_2$ groups of proteins at physiological pH (Nairn, 1969; Means & Feeney, 1971). Following the procedure of Cherry (1979), we prepared eosin isothiocyanate by bromination of fluorescein isothiocyanate (Sigma, grade I). For a stock solution, aliquots were dissolved in the following standard buffer medium: NaCl, 50 mM; MgCl_2 , 5 mM; Tricine (pH 8.25), 50 mM; sucrose, 250 mM at a typical concentration of 4 mg of ESCN/mL. Undissolved ESCN was removed by filtration through Millipore filters (0.45- μm pore size). The concentration of the dye was determined spectrophotometrically by using an extinction coefficient of $8.35 \times 10^4 \text{ M}^{-1} \text{ cm}^{-2}$ for the peak at 518 nm (Cherry, 1979).

Labeling of membrane-bound CF1 with ESCN occurred either under illumination of chloroplasts ("light labeling") or

with the chloroplasts in the dark ("dark labeling"). Chloroplasts were suspended in the above standard buffer medium at a chlorophyll concentration of 50 μM . The suspension was poured into an Erlenmeyer flask which was mounted in a thermostated bath (20 °C), which allowed for illumination from below (white light, 0.7 W cm $^{-2}$). Pyocyanine (3 μM) was added as a cofactor for rapid cyclic electron transport and proton pumping. The mixture was incubated with the lights either on or off. The time protocol was as follows: Before addition of ESCN, the mixture resided for 30 s in the flask under the given light conditions. Then aliquots of the ESCN stock in the same buffer were added to yield the desired final concentration (see figure legends). This was followed by another period of incubation (if not otherwise indicated, 180 s), which was terminated by the addition of glycine to trap unreacted ESCN.

Extraction and Purification of CF1. After incubation, chloroplasts were washed 3 times with the standard buffer medium and centrifuged for 10 min at 10000g. CF1 was then isolated by treatment with chloroform according to Younis et al. (1976). The enzyme was purified in two steps; a Sephadex G-50 (3.0 \times 50 cm) column was followed by a calibrated Sephadex G-200 superfine (2.6 \times 80 cm) column. This purification produced two fractions of CF1, the lighter four-subunit CF1 and a heavier five-subunit CF1 (as apparent from NaDodSO $_4$ gel electrophoresis). Only the latter could be reconstituted into previously depleted thylakoid membranes. In the following, we are dealing only with this fraction. The concentration of CF1 in the eluted buffer was determined according to Lowry et al. (1951) or Bradford (1976). The amount of eosin on CF1 was determined spectrophotometrically. [We observed a bathochromic band shift (518 \rightarrow 531 nm) upon binding of eosin, but we assumed that the peak extinction was unchanged.] To evaluate the proportion of bound to entrapped ESCN, we treated labeled CF1 with sodium dodecyl sulfonate (1%) at 60 °C for 10 min. The sample was then applied to a Sephadex G-50 (2.6 \times 30 cm) column, which was equilibrated with the buffer plus detergent. The protein was eluted in or near to the void volume. We found that between 85% and 95% of the ESCN still resided on the protein.

The ATPase activity of isolated and of membrane-bound CF1 was determined according to standard procedures. The Ca^{2+} -ATPase activity of CF1 in the crude EDTA extract and the one remaining on membranes was activated by trypsin according to Lien & Racker (1971). The rate of ATP hydrolysis was determined from the release of ^{32}P from [γ - ^{32}P]ATP (Lien & Racker, 1971; Sugino & Migoshi, 1964). The Ca^{2+} -ATPase of purified CF1 was activated by heat and DTT (Lien & Racker, 1971). The activation of the membrane-bound Mg^{2+} -ATPase of CF1 was performed as described in Oliver & Jagendorf (1975).

Flash Spectrophotometric Experiments. Labeled chloroplasts or unlabeled depleted chloroplasts which were reconstituted with labeled CF1 were suspended in the standard buffer with 3 μM pyocyanine as the cofactor for cyclic electron transport. The chlorophyll concentration in the absorption cell (1-cm path) was typically 40 μM . The absorption cell was mounted in a rapid kinetic flash photometer (Junge, 1976) where the sample was excited with a short flash of light from a Q-switched Nd-YAG laser (10 mJ/flash at 532 nm, 10-ns duration fwhm). This saturated less than 10% of the eosin chromophores. Absorption changes of eosin at 543 nm (indicating ground-state population) were recorded as previously described (Wagner & Junge, 1980). At this wavelength, the

contribution of the intrinsic electrochromic absorption changes of the chloroplasts was at least 10 times smaller than the one of eosin. For high-time resolution at reasonable signal-to-noise ratios, the electric signals arising from the photodetector were digitized and averaged.

Photoselection Studies. The principle of linear dichroism in photoselection is known from the literature (Weber, 1952; Albrecht, 1961; Cherry, 1979). The sample was excited by a laser pulse impinging on the optical cell at a right angle to the measuring light beam. We used vertical polarization (E vector) of the exciting laser flash. The monitoring beam was linearly polarized (by a rotatable Glan-Thomson polarizer) either parallel or perpendicular to the exciting light. This produced the absorption changes referred to as ΔA_{\parallel} and ΔA_{\perp} , respectively. The beam divergence of the exciting Nd-YAG laser was intrinsically very low; that of the monitoring light (from a tungsten-iodine lamp) was restricted to 5×10^{-2} , which alters the apparent dichroic ratio by a few percent only. Laser stray light and eosin fluorescence caused a flash burst artifact which blindfolded the detection system for less than 1 μ s. Consequently, the time per address setting of the transient recorder was always chosen equal to or longer than this limiting value. ΔA_{\parallel} and ΔA_{\perp} were recorded and averaged in succession. As the photoselection technique requires non-saturating flash energies for excitation, the reproducibility of the laser pulses had to be high. This was achieved by pumping at maximum energy under attenuation of the beam outside of the cavity. The measuring light was gated. It was on during the actual sampling interval plus a short preceding interval (typically 20 ms) to adapt photomultiplier plus amplifiers to the measuring light pulse. The energy of the measuring light pulse was kept below 5% of that of the laser pulse.

Results

Amount of ESCN Binding to CF1 and to Other Chloroplast Proteins. Figure 1 shows the amount of ESCN binding as a function of the ESCN concentration during incubation for 3 min. Figure 1a represents the total amount on chloroplast material and Figure 1b the amount solely on CF1. When it is taken into account that there is approximately 1 CF1 per 1000 chlorophylls (Strotmann & Bickel-Sandkoetter, 1977; Berzborn et al., 1974; Oleszko & Moudrianakis, 1974; Miller & Staehelin, 1976), it can be inferred from a comparison of parts a and b of Figure 1 that ESCN was predominantly taken up by proteins other than CF1. Incubation under light (in the absence of the uncoupler NH_4Cl) greatly enhanced eosin binding. At lower ESCN concentrations, the enhancement was stronger for binding to CF1 than for binding to other proteins. Here the discrimination between ESCN attached to the extra binding sites over ESCN bound to the sites which are also accessible in the dark is better than 2:1 (at 55 μM ESCN in the incubation medium). Addition of the uncoupler NH_4Cl prevented the extra labeling under light. This clearly demonstrated that the extra labeling is brought forth by the protonmotive force across the thylakoid membrane.

At 150 μM ESCN in the incubation medium, the amount of eosin binding to CF1 increased linearly with time for more than 6 min under dark incubation while the extra amount due to light incubation saturated after 3 min at approximately three eosins per CF1 (not shown). In the following, we describe the properties of eosin-labeled CF1 (a) in the labeled membrane, (b) after isolation and purification, and (c) after reconstitution into CF1-depleted, unlabeled membranes.

Triplet Lifetime of Bound ESCN. We monitored the triplet state of bound ESCN via the absorption at 543 nm. Negative-directed absorption changes at this wavelength indicated

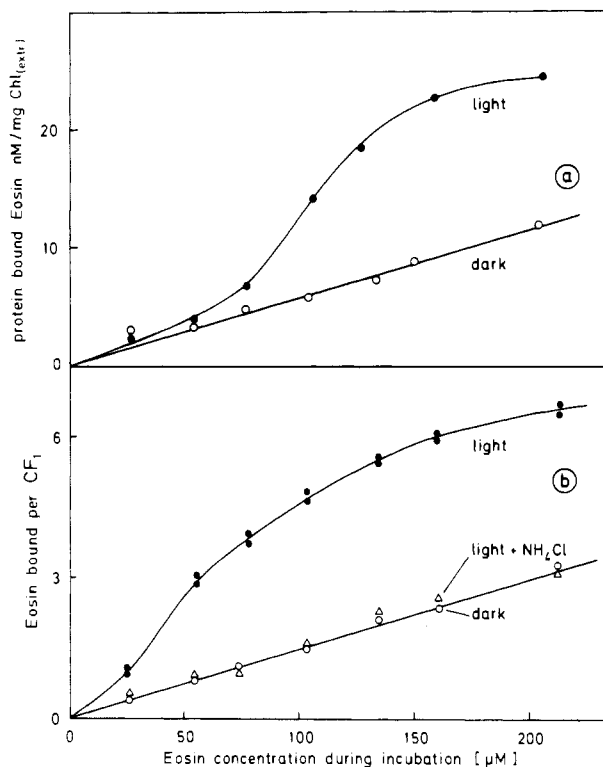


FIGURE 1: Binding of eosin isothiocyanate (a) to all proteins in chloroplasts and (b) solely to CF1 as a function of the ESCN concentration in the incubation medium. The incubation conditions (light, dark, and light plus uncoupler; NH_4Cl , 10 mM) are detailed under Materials and Methods. The amount of eosin binding to CF1 was determined as described under Materials and Methods. The amount of binding to all proteins was determined as follows: Acetone was added (80% v/v), and the sample was centrifuged for 10 min at 10000g. The precipitate was resuspended in Na_2CO_3 , 0.1 mM, and protein and eosin were determined as described under Materials and Methods. The supernatant contained usually less than 5% of the eosin.

ground-state depletion due to the formation of the triplet state. For isolated ESCN-CF1, we observed the following half-decay times of the laser flash induced triplet state: 32 μ s for dark-labeled ESCN-CF1 (at three ESCN's per CF1) and 750 μ s for light-labeled ESCN-CF1 (also at three ESCN's per CF1). In the given aerated buffer, the triplet lifetime of free ESCN was 1.2 μ s. In all samples, the triplet lifetime was dependent on the oxygen concentration in the solution. This indicated that light labeling (i.e., labeling in the presence of a protonmotive force) promoted binding of ESCN to sites which were less accessible to the triple quencher dioxygen, or more deeply buried within CF1, than those which were available for ESCN labeling in the dark. This strong shielding from oxygen was observed, only, if isolated ESCN-CF1 (light labeled) was not activated. We showed elsewhere that the buried extra sites became more exposed after heat activation of isolated CF1 (Wagner et al., 1981) or after induction of a protonmotive force, which acted on membrane-bound CF1 (Wagner & Jung, 1980).

Ca^{2+} ATPase Activity of the Labeled, Isolated CF1. Figure 2 shows the Ca^{2+} activity of the DTT-activated purified CF1 as function of the ESCN load on the coupling factor. It is apparent that the rate of ATP hydrolysis was far more sensitive to ESCN binding under light labeling than under dark labeling. Again light labeling in the presence of the uncoupler NH_4Cl was equivalent to dark labeling. The decrease in the rate of hydrolysis under light labeling did not reflect the saturation of the extra binding sites at three eosins per CF1 which became apparent in Figure 1.

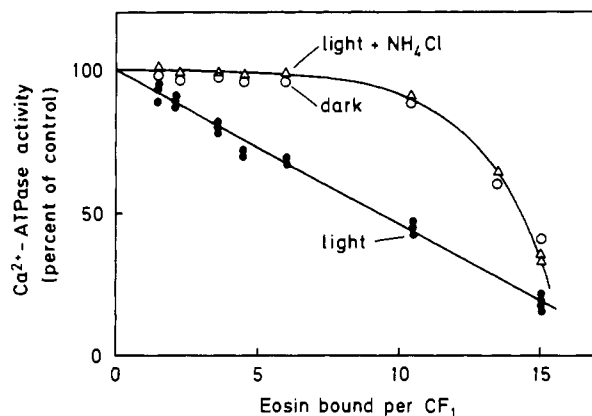


FIGURE 2: Ca^{2+} -ATPase activity of the isolated and purified CF₁ after labeling with ESCN as a function of the eosin load per CF₁. The rate of the control was $28 \mu\text{M}$ ATP hydrolyzed $(\text{mg of protein})^{-1} \text{min}^{-1}$. The incubation conditions were as in Figure 1 and as described under Materials and Methods.

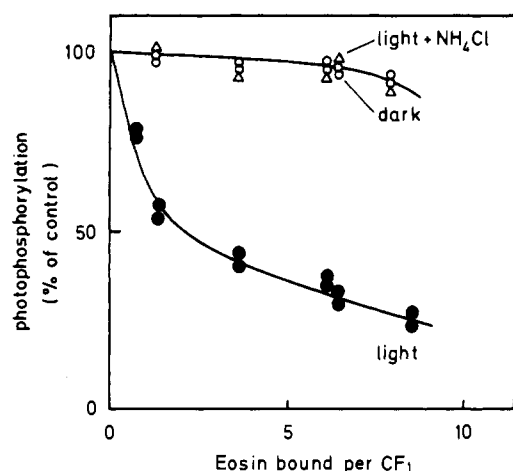


FIGURE 3: Rate of ATP synthesis in chloroplasts incubated with ESCN as a function of the eosin load on CF₁. The rate of the control was $712 \mu\text{M}$ ATP synthesized $(\text{mg of chlorophyll})^{-1} \text{h}^{-1}$. The incubation conditions were as in Figure 1 and as described under Materials and Methods. The sequence of events was as follows: (1) incubation and labeling with ESCN, (2) measurements of the rate of cyclic phosphorylation, and (3) extraction of the CF₁ and analysis for the eosin load (see Materials and Methods).

ATP Synthase Activity of Chloroplasts after Labeling with ESCN. The rate of ATP synthesis under cyclic electron transport (mediated by pyocyanine) was determined as a function of the ESCN concentration during the incubation period (3 min). For these experiments, we used freshly labeled chloroplasts and not depleted and reconstituted ones. The rate of photophosphorylation was dependent on the effect of ESCN via three parameters: the rate of cyclic electron transport, the degree of uncoupling, and the activity of the ATP synthases. While the linear electron-transport chain was very sensitive to ESCN incubation, we found the rate of cyclic electron transport (as apparent via the rate and the steady state of proton uptake) less affected. For example, the steady state of proton uptake of control chloroplasts was 280 nequiv of H^+ per μmol of chlorophyll, while the figures were 266 and 240 nequiv of H^+ per μmol of chlorophyll, respectively, after dark incubation and after light incubation at four ESCN's per CF₁. At higher ESCN concentrations, the steady state of proton uptake decreased; however, there was very little difference between the two incubation modes (data not shown). In conclusion, the impairment of the rate of ATP synthesis, notably after light incubation, seemed mainly caused by the effect of ESCN on CF₁.

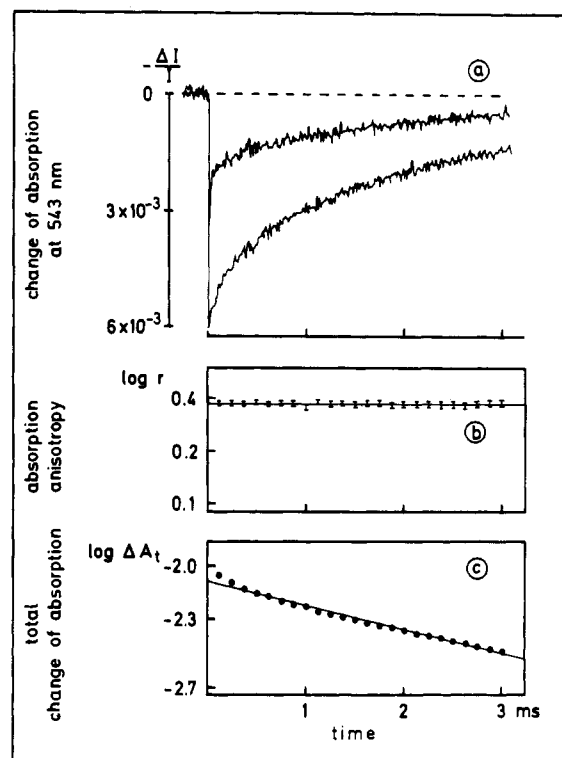


FIGURE 4: Time course of the absorption changes of eosin at 543 nm after excitation with a short laser flash at 532 nm (10-ns duration, $10 \text{ mJ}/\text{cm}^2$). Eosin was embedded in a sucrose glass. (a) Time course of the absorption changes under parallel and perpendicular polarization, respectively, of the measuring and of the exciting light. (b) Calculated time course of the polarization anisotropy from the data in Figure 1a. (c) Calculated time course of the total absorption changes. The eosin candy was prepared as follows: 2 mL of glycerol was heated to 300°C , and a total of 5 g of sucrose was slowly added under stirring. A soft stream of argon was passed over the melt while eosin, dissolved in glycerol, was added until an optical density of 1 was reached in the 1-cm absorption cell. The hot melt was then cooled down to room temperature where it became a glassy substance which was essentially free of cracks or bubbles. The cell was then sealed against oxygen.

Figure 3 shows the effect of ESCN binding on the rate of cyclic photophosphorylation. The ordinate is calibrated in ESCN bound to CF₁. This was determined after termination of the phosphorylation experiment by extraction of CF₁. The salient feature in Figure 3 is the steep decline of the rate of ATP synthesis under light incubation. This decline breaks off at 50% of the control rate from where it becomes much less steep. The steep portion is completed at 1.5 ESCN's per CF₁. Taking Figure 1 into account, a total of 1.5 eosins on CF₁ implies 1 eosin-SCN on the extra binding sites accessible only after "light incubation".

Calibration of Probe and Instrument for Photoselection Studies. To study the properties of the dye and also as a standard for our instruments, we prepared an eosin candy, by carefully dissolving eosin into a heated sucrose solution which became glassy upon cooling. The studied segment of this candy was free of cracks and bubbles. Figure 4 shows the time course of the absorption changes of this sample at two mutually perpendicular polarizations of the measuring light. The virtual absence of any decay of the polarization anisotropy is shown in the middle, and the decay of the total absorption change is shown below, both in half-logarithmic plots. The most important result is the close to ideal dichroic ratio of 2.7 in contrast to an earlier report by Cherry et al. (1976). This demonstrates (1) the close to ideal behavior of this chromophore under the given excitation and observation wavelength and (2) the practical absence of experimental imperfections.

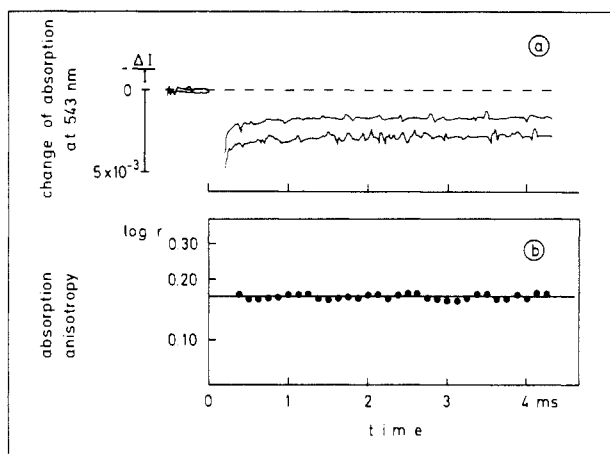


FIGURE 5: Time course of the absorption changes of eosin at 543 nm after excitation with a short laser flash at 532 nm in a sample where eosin isothiocyanate was covalently bound to CF1 (dark incubation) which in turn was isolated from the membrane and attached to DEAE-Sephadex to prevent Brownian rotation of the enzyme. (a) Time course of the absorption changes. (b) Time course of the polarization anisotropy.

It is worth noting that smaller dichroic ratios were obtained under excitation at 532 nm and under observation at other wavelengths, in particular of the triplet-triplet absorption at 600 nm.

Photoselection Experiments on ESCN-Labeled CF1 Immobilized on DEAE-Sephadex. To assess the mobility of ESCN within CF1, we immobilized CF1 on DEAE-Sephadex globules, similar to our previous studies with photosystem I reaction centers (Junge et al., 1977). As documented in Figure 5, the apparent dichroism decreased to $r = 0.16$. The remaining dichroism was stable. We attributed the lowering of the dichroism in Figure 5 as compared to that in Figure 4 to rapid librational motion of covalently bound ESCN within CF1. One might argue that part of the lowering of the dichroism may be accounted for by the slight turbidity of the Sephadex system. This was not so, as apparent from a control experiment with the eosin candy, and with Sephadex cells placed between the candy and the photomultiplier as well as in the exciting beam. It is worth noting that the decay of the triplet state of eosin is furthermore slowed down when CF1 is immobilized on DEAE-Sephadex.

Photoselection Experiments on ESCN-Labeled CF1 in Glycerol/Sucrose/Water Medium. We used highly viscous glycerol/sucrose media for studies on the rotational diffusion of CF1 in solution, mainly to slow down the rotational correlation time, so that it did not fall into the range of the then unavoids flash burst artifact. In the viscous solvents used, the Ca^{2+} -ATPase activity of labeled CF1 was within 90% of that in aqueous buffer solution (even after a 40-min incubation). We measured the viscosity of the mixture with a capillary viscometer (Ubbelohde). The observed linear dependence of the logarithm of the viscosity on the reciprocal temperature was characteristic for an isotropic Newtonian fluid.

The absorption changes of bound ESCN in a glycerol/sucrose medium are documented in Figure 6. As in Figure 4, the middle trace represents the polarization anisotropy (r) and the lower trace the total absorption changes (ΔA_t), both in half-log form. The middle trace is with error bars derived from the peak to peak noise of the two traces in Figure 6a. We have computer fitted the decay of the r parameter with two and three exponentials, respectively. In view of the calculated small amplitude for the intermediate component ($14 \mu\text{s}$), we considered a fit by two exponentials as satisfactory. The respective

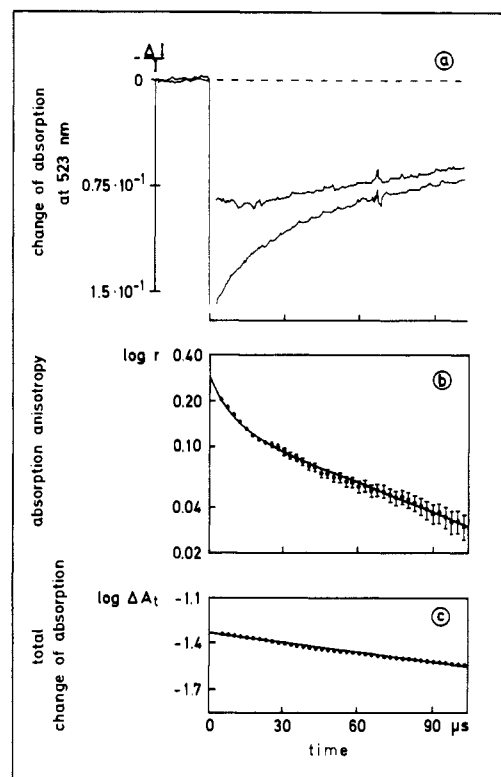


FIGURE 6: Time course of the absorption changes of eosin at 543 nm after excitation with a short laser flash at 532 nm with eosin isothiocyanate bound to CF1 (dark incubation). Labeled CF1 was isolated and purified and then dissolved in glycerol/water (60:40 w/w). (a) Time course of the absorption changes for perpendicular and parallel polarization of the exciting and the measuring light. (b) Time course of the polarization anisotropy. (c) Time course of the total absorption changes. It is apparent that there are at least two exponential decay processes of r .

Table I^a

sample	η (cP)	τ_1 (μs)	a_1	τ_2 (μs)	a_2	$\sum_{i=1}^2 a_i$
60/40 (w/w)	66	8	0.150	31	0.155	0.305
glycerol/	57	7	0.145	28	0.150	0.295
water plus 100	52	7	0.145	25	0.160	0.305
mM CaCl_2	44	6	0.155	20	0.155	0.310
	38	6	0.150	17	0.155	0.305
60/40 (w/w)	72	8	0.150	33	0.15	0.300
glycerol/	56	7	0.145	28	0.15	0.295
water	53	7	0.145	25	0.16	0.305
	44	6	0.140	20	0.16	0.300
	38	6	0.150	17	0.155	0.305

^a The table shows the rotational correlation times for eosin isothiocyanate on CF1 in glycerol/water mixtures at different temperatures. Experimental data as documented in Figure 6 were evaluated for two exponentials in the decay of the polarization anisotropy. The decay times τ_1 and τ_2 plus their respective amplitudes a_1 and a_2 are given.

decay times of a two-component fit (9 and 72 μs) are 1 order of magnitude apart from each other. The amplitude of the slower relaxation process was 0.145, which was close to the amplitude of the steady dichroism under conditions where CF1 was immobilized (see Figure 5). Similar data as in Figure 6 and similar analyses for two exponentials were carried out at different viscosities. The result is given in Table I. The following features are apparent: (1) The total amplitude of the r parameter is independent of the viscosity, $a_1 + a_2 \approx 0.3$ (as well as the ratio of the two partial amplitudes, $a_1/a_2 \approx 1$). (2) The rapid relaxation time is only slightly dependent

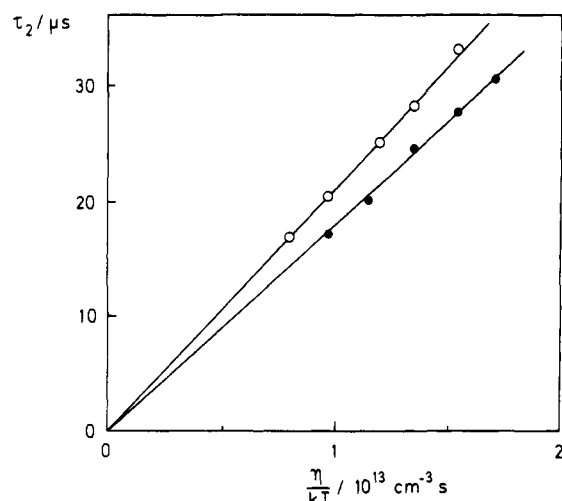


FIGURE 7: Slower rotational relaxation time of ESCN on CF1 (in glycerol/water) as a function of the reduced viscosity. The two lines refer to two different media: the closed circles are for 60/40 (w/w) glycerol/water and the open ones for this medium in the presence of 100 mM CaCl_2 . For each of these media, the viscosity was varied by variation of the temperature.

on the solvent viscosity while the slower relaxation time increases approximately linearly with the solvent viscosity. The most probable conclusion is that the rapid component reflects the internal motion of the chromophore within the protein and the slower component the rotation of the protein in the solvent. This is also consistent with the result from Figure 5. (3) Within the slower relaxation of r , only one phase is discernible. A closer analysis revealed that the given signal-to-noise ratio would not have justified discrimination between two relaxation processes with their relaxation times differing by less than a factor of two.

Interpretation of the Rotational Diffusion of CF1 in Viscous Solvent. Figure 7 summarizes the experimental results. The slow rotational relaxation time was plotted over the viscosity divided by kT . According to Perrin's formula [$\tau = \eta v / (kT)$], which describes the dependence of the rotational relaxation time (τ) of a spherical body on the viscosity (η) (Perrin, 1934), one expects the relaxation time to vary linearly with the ratio of viscosity over temperature times the Boltzmann constant. The slope is then equal to the volume of the sphere in cubic centimeters, provided that the viscosity is in poise ($\text{g cm}^{-1} \text{s}^{-1}$) and kT is in ergs. The plot which is shown in Figure 7 proves a good approximation of this linearity, the nonexperimental zero included. The respective sphere volumes are 2.1×10^{-18} and $1.7 \times 10^{-18} \text{ cm}^3$, depending on the solvent.

How does this compare with the solvent-free volume of the protein, which can be calculated according to eq 1? In eq

$$v = M_r \bar{v} / N \quad (1)$$

1, \bar{v} is anhydrous specific volume, M_r is the molecular weight, and N is Avogadro's number. For CF1, a molecular weight of 325 000 and an anhydrous specific volume of 0.73–0.745 mL/s (Farron, 1970; Paradies et al., 1978) have been determined. [Whether the true molecular weight is higher, if loss of subunits is prevented, is under debate (Kagawa et al., 1979).] Hence, one calculates a dry volume of $4 \times 10^{-19} \text{ cm}^3$. The ratio between the apparent volume (which we calculated according to Perrin's equation for a sphere on the basis of the observed relaxation time) and the dry volume of the protein is the form factor, F (Tanford, 1961; Hiemenz, 1977). The observed relaxation time exceeds the expected one for two reasons: (1) The real volume of the protein is greater because of solvation, and (2) the shape of the protein deviates from

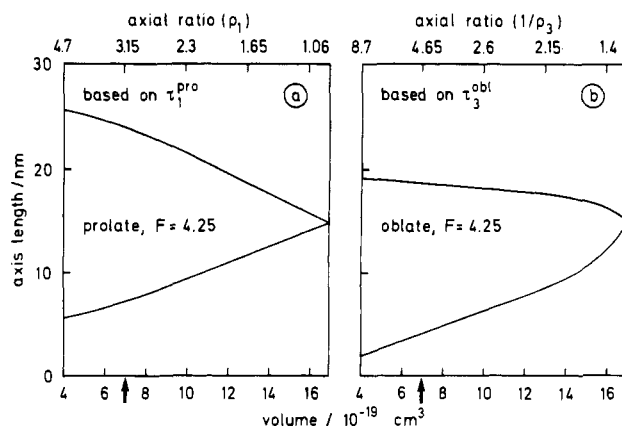


FIGURE 8: Full length of the axes of ellipsoids of revolution as a function of the volume under the assumption that the rotational correlation time coincides with the experimentally determined one for CF1. The graphs were calculated according to eq 3 and 4 and by using Figure 9 under the assumption that τ_1 was dominating for the prolate ellipsoid (a) and that τ_2 was dominating for the oblate one (b).

a sphere. Following the literature (Tanford, 1961; Hiemenz, 1977), we consider the form factor F as a product of two factors (for details, see Appendix):

$$F = f(v)g(\rho) \quad (2)$$

The first factor is the ratio of the real volume (solvated volume) over the dry volume, and the second term, Perrin's function, corrects for the deviation of the solvated molecule from spherical shape. Both factors are given in the Appendix. From our experimental data, we find form factors of 4.25 and 5.25, respectively (in the glycerol/water/sucrose media). This is at the upper limit for globular proteins. The form factor for bovine serum albumin, for comparison, is 2.22 (Squire & Himmel, 1979). Higher figures suggest ellipsoidal or irregular geometry. We also determined the intrinsic viscosity of CF1 in the glycerol/water medium. This again is a measure for the eccentricity of a macromolecule in a viscous solvent. We obtained an intrinsic viscosity of 4.6 mL/g for CF1 in the above medium (data not shown). This is well above the theoretical figure for a sphere (2.5) and also above the figure for bovine serum albumin in water [3.9; see Younis et al. (1976)]. If eccentricity were the only reason for this, the axial ratio of the ellipsoid of revolution had to exceed 3 [e.g., Figure 7.2 in Van Holde (1971)]. The interpretation of the form factor in terms of the size and shape of the protein is ambiguous for two reasons: (1) The form factor is a function of two variables, volume and axial ratio, and (2) the Perrin function $g(\rho)$ is 6-fold degenerate (as shown in Figure 9 in the Appendix). We postpone the appraisal of this ambiguity, and we start to interpret the data under the assumption that the transition moments of the eosin labels were parallel to the symmetry axis of a prolate ellipsoidal protein; i.e., we had observed the relaxation time τ_1^{pro} .

The measured form factor (4.25) in the glycerol/water medium defines a line in the volume over axial ratio space. For each chosen volume, v , it follows a unique value for ρ . This pair of variables leads us to the (full) length of the axes x_3 and $x_1 = x_2$, which we name $2b$ and $2a$ to indicate that they are diameters and not radii.

$$2b = \rho 2a \quad (3)$$

$$2a = 2[3v / (4\pi\rho)]^{1/3} \quad (4)$$

For the above figure of the form factor (4.25), we calculated (by eq 3 and 4 with the use of Figure 9) the full length of the

axes as a function of the solvated volume of CF1. This is plotted in Figure 8a, which refers to a prolate ellipsoid where τ_1^{pro} was observed. The equivalent for an oblate ellipsoid is shown in Figure 8b (based on τ_3^{obl} from Figure 9). Previously, we mentioned the ambiguity. Here we note that Figure 9 (Appendix) shows that the observation of τ_2^{pro} of a prolate ellipsoid, or of τ_2^{obl} and τ_1^{pro} of an oblate one, would have led to even higher eccentricities. Hence, Figure 8 describes the situation with the minimal eccentricity for CF1. The arrows pointing to the abscissa show the volume which was determined by Paradies et al. (1978) by X-ray small-angle scattering. With this volume, we obtain axes 7.52 and 23.7 nm long ($\rho = 3.15$) for the prolate case and 18.5 and 3.9 nm ($\rho^{-1} = 4.8$) for an oblate geometry. The given volume was attributed to a normal degree of hydration (0.59 g of water/g of protein). Higher solvation degrees have been observed for other proteins. The largest figure inferred from hydrodynamic measurement was 1.04 for phosphoglycerate kinase (yeast) [see Squire & Himmel (1979) for a review]. If, for example, we assume that solvation in the denser glycerol/water medium is even higher, e.g., 1.2, we calculate a volume of $1.04 \times 10^{-18} \text{ cm}^3$, which corresponds to the following lengths of the full axes of CF1: in the prolate case, 10 and 21 nm ($\rho = 2.1$); in the oblate case, 18 and 7 nm ($\rho^{-1} = 2.6$). Thus, under any assumption, CF1 behaves hydrodynamically as a rather eccentric molecule. However, we cannot yet discriminate between the prolate and the oblate shape. One might argue that the relatively large form factor that we observed was due to dimerization of CF1 in the glycerol/water medium. We first note that this would not abolish the eccentric shape for the following reason. If dimeric, the volume of CF1 would be at least twice as big as that of a monomer ($7 \times 10^{-19} \text{ cm}^3$). When the volume approaches $1.7 \times 10^{-18} \text{ cm}^3$, the rotational diffusion corresponds to that of a spherical body (see Figure 9). Each half of a sphere, however, is similar to an oblate ellipsoid. Besides this argument, we put the possibility for dimerization on two experimental tests. (1) We ran CF1 over a calibrated Sephadex column, which was equilibrated with the glycerol/water medium, and found it to have a molecular weight on the order of 300 000. (2) We observed the same relaxation times as shown in the table independent of the dilution of CF1 in the viscous solvent (variation by a factor of 10). This strongly argued against dimerization.

Discussion

Mode of ESCN Binding and the Activities of Labeled CF1.

The above experiments have demonstrated the following: (1) ESCN binding to chloroplast proteins (including the coupling factor for photophosphorylation, CF1) is stronger when chloroplasts are incubated with the label under illumination than when incubated in the dark. (2) Light enhanced binding via the generated protonmotive force. (This was apparent through the reversal of the light effect by an uncoupler.) (3) There were two classes of binding sites for ESCN on CF1: those already accessible under dark incubation and others which were more available in the presence of a protonmotive force. (4) The number of extra binding sites on CF1 is limited (three per CF1). (5) Two of these extra sites are on the γ subunit of CF1. (6) The triplet lifetime of ESCN on CF1 depends on the nature of the site ("dark site" or "light site") to which it is attached. For the dark sites, it is 25 times longer than for ESCN in solution, and it is again 25 times longer for the light sites (in the absence of a protonmotive force). This can be interpreted as follows: Under dark incubation, ESCN binds to sites which are nearer to the outer surface of CF1. Binding sites inside CF1 are inaccessible to the label because

CF1 is in a "closed" conformation in the absence of a protonmotive force. Under illumination and in the absence of uncoupler, i.e., under the influence of a protonmotive force, CF1 opens up, and it exposes the extra binding sites. The structure closes again when the protonmotive force has decayed. The closed structure also prevails when CF1 is isolated at temperatures below 30 °C. We showed elsewhere (Wagner et al., 1981) that heating of CF1 above a transition temperature of 30 °C exposed (reversibly) previously shielded binding sites for ESCN. The exposure of internal sites of membrane-bound CF1 under the influence of a protonmotive force is not new. It was also apparent in previous studies of the exchange of protons for tritium by Ryrie & Jagendorf (1971), of the binding of *N*-ethylmaleimide to the γ subunit by McCarty & Fagan (1973), and of the binding of fluorescein (Ellenson & Pheasant, 1978; Prochaska & Dilley, 1978). It is difficult to compare the binding sites for ESCN with those of labels which were used by other authors. The finding of two binding sites for ESCN on the γ subunit could be related to the two SH groups which are known to exist in the γ subunit (McCarty & Fagan, 1973; Cantley & Hammes, 1976). However, it is unknown whether the dark-accessible sites for ESCN are identical with the ones on the β subunit which are reached by 5-(iodoacetamido)fluorescein (Hartig et al., 1977) or by NBD-Cl (Holowka & Hammes, 1977). (7) Neither of the known activities of the isolated or of the membrane-bound CF1 (ATP synthesis and hydrolysis) is greatly impaired if a few ESCN molecules are bound to sites at the "outer surface" (dark labeling). (8) In contrast to this, even then light labeling inhibits the ATP synthase activity (and the Mg^{2+} ATPase activity) of the membrane-bound enzyme down to the 50% level if only one ESCN is bound to one of the three internal sites. (9) The Ca^{2+} ATPase activity of the isolated CF1 is also more sensitive to ESCN under light labeling than under dark labeling. However, the dependence is not as sharp as for ATP synthesis. These results corroborate earlier suggestions (Shoshan & Selman, 1980) that the catalytic sites for the Ca^{2+} ATPase activity of CF1 may be different from those of the ATP synthase (and the Mg^{2+} ATPase) of the membrane-bound enzyme.

In the introduction, we have referred to our purpose to use ESCN-labeled CF1 as a probe for conformational changes of membrane-bound CF1 and also for determinations of the size and shape of CF1 in solution. With respect to these goals, the present work has demonstrated the following: dark-labeled CF1 is hardly affected in any of its activities provided that the load of ESCN per CF1 is not too high (less than six). Photosynthesis experiments aiming at its rotational diffusion both in the membrane (Wagner & Junge, 1980) and in solution are probably dealing with the enzyme in a native conformation, if restricted to dark-labeled material. Light-labeled CF1 has been previously used to monitor the "opening" of membrane-bound CF1 via the triplet lifetime of inside-located ESCN (Wagner & Junge, 1980). The results presented in this paper show that CF1 was unable to phosphorylate ATP at high rates under these conditions. The conformational changes, which were apparent under the influence of a protonmotive force, were therefore attributable to a functionally impaired enzyme.

Size and Shape of Isolated CF1. The structure of CF1 is still in contention. The widely believed molecular weight (325 000; Farron, 1970; Paradies et al., 1978) was challenged for loss of subunits [M_r 390 000 (Yoshida et al., 1979)]. The standing subunit composition [2:2:1:1:1 (Nelson, 1976) or 2:2:1:1:2 (Baird & Hammes, 1976)] of CF1 seems debatable

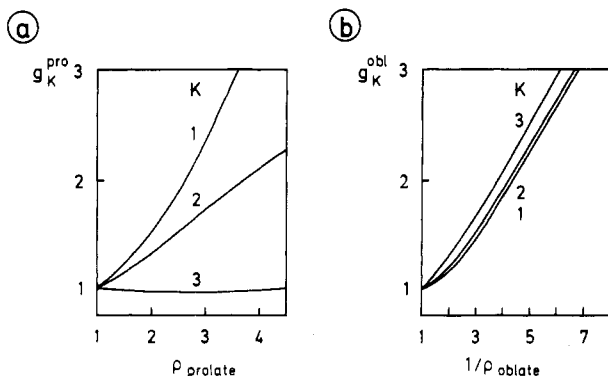


FIGURE 9: Perrin function $g(\rho)$ for the rotational diffusion as a function of the axial ratio of ellipsoids of revolution. Calculated according to eq A1 and A3–A5.

in light of the subunit stoichiometry which was reported for the related coupling factors in thermophilic bacterium and in mitochondria [3:3:1:1:1; for a review, see Kagawa et al. (1979)]. Controversy also exists concerning the volume and shape of CF1. Electron microscopy at different types of staining revealed a spherical molecule which protruded outward from the thylakoid membrane by 10 nm (Howell & Moudrianakis, 1969; Oleszko & Moudrianakis, 1974; Miller & Staehelin, 1976). The isolated enzyme was prepared at a diameter of 9 nm under negative staining and at 15 nm in freeze etching (Garber & Steponkis, 1974). Chemical cross-linking and energy-transfer studies were compatible with distances below 10 nm [see Baird & Hammes (1979) for a review]. X-ray small-angle scattering studies by Paradies et al. (1978) led these authors to conclude that CF1 is a (hollow) sphere with a diameter of 11.8 nm. However, in their impressive paper, Paradies et al. (1978) have also reported on hydrodynamic studies, which led them to conclude that CF1 is eccentric at an axial ratio of 1.5–2 (see their Table I), which is at variance from the result of their own X-ray work. Also in another X-ray study, Suess et al. (1978) came to the conclusion that CF1 is by more than 50% larger in volume and also oblate in shape with axes 8.4 and 16.8 nm long. Our own hydrodynamic work suggested a relatively high eccentricity of CF1; the axial ratios are even larger than 2 as concluded by Suess et al. (1978). We could not contribute to a resolution of the apparent discrepancies between the X-ray work in itself, or between hydrodynamic and X-ray work. However, the present work shows a relatively handy way to monitor the rotational diffusion of CF1 in solution. Together with the possibility to monitor conformational changes of CF1 via the triplet lifetime of bound eosin isothiocyanate [see Wagner & Junge (1980) and Wagner et al. (1981)], this technique will hopefully lead to a better understanding of the variability of CF1 structure as a function of preparation, treatment, and chemical modifications.

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Appendix

Decay of the Polarization Anisotropy for Ellipsoids of Revolution. The time course of the fluorescence depolarization in photoselection experiments with macromolecules, which are idealized as ellipsoids of revolution, was treated by Tao (1969)

on the basis of Perrin's pioneering work (Perrin, 1934, 1936). In the coordinate system of the macromolecule, x_3 is the symmetry axis and x_1 and x_2 are the axes which are perpendicular to it. With D_i ($i = 1-3$) denoting the diffusion coefficients around the respective axes, the following relaxation times for the polarization anisotropy are expected (note that $D_1 = D_2$):

$$\tau_1 = (6D_1)^{-1} \quad (\text{A1a})$$

$$\tau_2 = (5D_1 + D_3)^{-1} \quad (\text{A1b})$$

$$\tau_3 = (2D_1 + 4D_3)^{-1} \quad (\text{A1c})$$

and the amplitudes of the three exponentials are

$$a_1(\theta) = (\frac{3}{2} \cos^2 \theta - \frac{1}{2})^2 \quad (\text{A2a})$$

$$a_2(\theta) = 3 \cos^2 \theta \sin^2 \theta \quad (\text{A2b})$$

$$a_3(\theta) = \frac{3}{4} \sin^4 \theta \quad (\text{A2c})$$

where θ is the angle of the transition moment with the symmetry axis of the ellipsoid of revolution. The respective diffusion constants D_3 and D_1 for prolate and oblate ellipsoids are given in eq 53 of Wegener et al. (1979) as a function of the axial ratio of the ellipsoid (ρ), which is >1 for prolates and <1 for oblates. With D being the isotropic diffusion constant of an equivalent sphere (same volume v) in the same medium (same viscosity η and temperature T)

$$D = \frac{kT}{6\eta v} \quad (\text{A3})$$

the diffusion constants of a prolate ellipsoid become

$$D_3^{\text{pro}} = \frac{3}{2} \frac{\rho}{\rho^2 - 1} \left\{ \rho - \frac{\ln [\rho + (\rho^2 + 1)^{1/2}]}{(\rho^2 - 1)^{1/2}} \right\} D \quad (\text{A4a})$$

$$D_1^{\text{pro}} = \frac{3}{2} \frac{\rho}{\rho^4 - 1} \left\{ \frac{2\rho^2 - 1}{(\rho^2 - 1)^{1/2}} \ln [\rho + (\rho^2 - 1)^{1/2}] - 1 \right\} D \quad (\text{A4b})$$

The diffusion constants of an oblate ellipsoid are

$$D_1^{\text{obl}} = \frac{3}{2} \frac{\rho}{\rho^2 - 1} \left\{ \rho - \frac{\tan^{-1} [(1 - \rho^2)^{1/2} / \rho]}{(1 - \rho^2)^{1/2}} \right\} D \quad (\text{A5a})$$

$$D_3^{\text{obl}} = \frac{3}{2} \frac{\rho}{\rho^4 - 1} \left\{ \frac{2\rho^2 - 1}{(1 - \rho^2)^{1/2}} \tan^{-1} [(1 - \rho^2)^{1/2} / \rho] - \rho \right\} D \quad (\text{A5b})$$

Belford et al. (1972) have pointed out that Tao's derivation holds only for the case where there is one and the same transition moment involved in excitation and observation. According to the result shown in Figure 1, this is fairly well approximated under our conditions. Also Wegener et al. (1979) have warned that the ellipsoidal approximation does not cope with the rotational properties of any irregularly shaped molecule. We consider this objection of minor importance for the interpretation of our data (which revealed only one relaxation time). We extracted a single relaxation time from our experiments. In a first approximation, we interpreted the relaxation of the polarization anisotropy in terms of the rotational diffusion of a spherical body under Discussion. The ratio of the apparent sphere volume v and the dry volume of CF1 is the form factor F (see eq A6). From eq A1 and

$$F = f(v)g_k^{\text{ell}}(\rho) \quad (\text{A6})$$

A3–A5, we see that the magnitude of F is a composite of two factors, the volume ratio f and Perrin's function g . The first factor $[f(v)]$ is the ratio of the real volume (v) and the dry volume of the macromolecule:

$$f(v) = \frac{v_{\text{real}}}{v_{\text{dry}}} \quad (\text{A7})$$

and the second factor $[g_k^{\text{ell}}(\rho)]$ is a complicated function of the axial ratio (ρ) which is implicit in eq A1 and A3–A5. The subscript k of $g_k^{\text{ell}}(\rho)$ refers to the particular relaxation time (τ_k , $k = 1-3$), and the superscript to the type of ellipsoid (prolate or oblate). We do not explicitly write out $g_k^{\text{ell}}(\rho)$ because of its complexity but, instead, present the functional dependence as graphs in Figure 9. It is not a priori evident which of the six (or practically five) possible graphs for $g_k^{\text{ell}}(\rho)$ is appropriate for our experimental situation. The right choice would require information on the angle the chromophore makes with the symmetry axis of the presumably ellipsoidal protein (see eq A2).

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Retinoid Affinity Label for the Binding Site of Retinol-Binding Protein†

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ABSTRACT: Three radioactive retinoid bromoacetates were synthesized as potential retinoid affinity labels for the retinol binding site of human plasma retinol-binding protein (RBP). The compounds synthesized were β -[9-³H]ionyl bromoacetate (IBA), β -[11-³H]ionylideneethyl bromoacetate (IEBA), and [15-³H]retinyl bromoacetate (RBA). When excess ligand was incubated with RBP for 5 h at 37 °C, IBA and IEBA formed nearly 1:1 molar complexes with RBP, whereas RBA bound only approximately one-third as well. Subsequent addition of retinol to the retinoid-RBP complex resulted in complete displacement of IBA and RBA from the protein, whereas a large proportion (37%) of the [³H]IEBA remained bound to the retinol binding site of RBP. For maximization of covalent bonding of IEBA to RBP, IEBA was incubated with RBP for

varying lengths of time, followed, in each instance, by addition of retinol to displace noncovalently bound IEBA. The amount of IEBA remaining bound to RBP increased with increasing incubation time, reaching a maximum of about 0.66 mol/mol of RBP at 18 h. Moreover, at each time point, the binding of retinol to RBP was inhibited to an extent that was equivalent to the amount of [³H]IEBA that was not displaced from RBP by retinol. Only a very small proportion of the bound [³H]-IEBA that was not displaced with retinol could be extracted from the protein with chloroform-methanol. Taken together, these several lines of evidence strongly suggest that the IEBA was bound in the retinol binding site of RBP and was attached to the protein in a covalent manner. Thus, IEBA appears to be an effective affinity label for the retinol binding site of RBP.

Retinol (vitamin A) circulates in plasma bound to a specific transport protein, retinol-binding protein (RBP) (Kanai et al., 1968). RBP is a single polypeptide chain with a molecular weight of approximately 20 000 [see Goodman (1980) and Smith & Goodman (1979) for recent reviews]. The primary structure of human plasma RBP is now known (Rask et al., 1979, 1981; Kanda & Goodman, 1979). RBP serves to mobilize vitamin A from its stores in the liver and to deliver it to peripheral target tissues. RBP has a single binding site for one molecule of retinol and circulates mainly as the retinol-RBP complex. RBP also interacts strongly with plasma prealbumin and normally circulates as a 1:1 molar RBP-prealbumin complex.

The binding of *all-trans*-retinol to RBP is reversible and fairly specific. A variety of retinoids can bind to apo-RBP with varying degrees of effectiveness [see Goodman (1980) for review and references]. Some of these (e.g., *all-trans*-retinoic acid) (Cogan et al., 1976) bind to RBP with an affinity similar to that of retinol. Other retinyl derivatives have also been shown to bind well to RBP, including compounds with shortened side chains such as β -ionone and β -ionylideneacetic acid (Hase et al., 1976). It appears from these studies that some degree of structural similarity to retinol, particularly in the area of the cyclohexene ring, is necessary for binding to RBP.

These studies with retinoids have provided some information about the structural requirements of the retinol binding site on RBP. No information is, however, available about the amino acid residues in RBP that are involved in the binding

site. Acetylation of lysine residues of RBP did not affect its binding of retinol (Heller & Horwitz, 1975). Modification of one of eight tyrosine residues and two of four tryptophan residues of RBP also had no effect on the retinol-RBP interaction (Heller & Horwitz, 1975; Horwitz & Heller, 1974b). The binding site was, however, disrupted by reduction and alkylation of disulfide bonds (Raz et al., 1970).

This paper reports the results of the first phase of a retinoid affinity labeling study of the retinol binding site of RBP. The goal of the work reported here was to synthesize a retinoid with a reactive functional group that would bind reversibly to the retinol binding site of RBP and would then react and form a covalent bond with proximate amino acid residues. The reactive retinoid would thus serve as a label of the binding site. Subsequently, proteolytic cleavage and peptide mapping of the labeled RBP should yield one or more peptides containing the label, thus identifying them as having some involvement in the retinol binding site.

We now report the synthesis of three retinoid bromoacetates that comprise a homologous series of compounds with varying chain lengths. We also report studies of the binding of these three retinoids to RBP, together with data which demonstrate the covalent binding of one of the compounds to the retinol binding site of RBP.

Materials and Methods

Synthesis of Retinoid Bromoacetates. Radioactive compounds that were structurally similar to retinol and contained the bromoacetyl functional group were prepared and tested for their ability to bind to RBP. The three compounds synthesized and studied were retinyl bromoacetate (Figure 1, RBA), the bromoacetyl analogue of retinol, and two compounds with shorter side chains, β -ionyl bromoacetate (Figure 1, IBA), and β -ionylideneethyl bromoacetate (Figure 1, IEBA). All three analogues were prepared from the respective

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