Chemical Modification and Fluorescence Studies of Chloroplast Coupling Factor†

David A. Holowka‡ and Gordon G. Hammes*

ABSTRACT: The reactivity of sulfhydryl groups in the β, γ, and ϵ subunits of latent chloroplast coupling factor with maleimide derivatives is altered by modification of a tyrosine residue in the β subunit with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). The amount of sulfhydryl group in the ϵ subunit labeled with *N*-(3-pyrene)maleimide (MalPy) decreases by a factor of six after modification of the latent coupling factor with NBD-Cl. Circular dichroism and ATPase activity (after heat activation) measurements also suggest that modification of the coupling factor with NBD-Cl is accompanied by a conformational change. This differential labeling of the ϵ subunit and fluorescence resonance energy transfer measurements were used to calculate a minimum distance of 44 Å between MalPy on the sulfhydryl group of the ϵ subunit and NBD on the β subunit of the latent coupling factor (assuming the emission and absorption dipoles are reorienting rapidly relative to the donor fluorescence lifetime). A procedure was developed to remove the ϵ subunit from heat-activated coupling factor. Measurement of the fluorescent properties of MalPy-labeled coupling factor with and without ϵ subunit permitted calculation of the distance between MalPy on the sulfhydryl group of the ϵ subunit and NBD on the β subunit, and between MalPy on the ϵ subunit and 4-dimethylamino-4′-maleimidostilbene on the two sulfhydryl groups of the γ subunit exposed after heat activation. In the former case, the distance is greater than 44 Å while in the latter case the distance is about 39 Å (again assuming rapidly reorienting emission and absorption dipoles). A simple structural model consistent with these results and previous fluorescence resonance energy transfer measurements is presented.

The solubilized coupling factor (CF_1)[1] from spinach chloroplasts is composed of five different polypeptide chains, designated α, β, γ, δ, and ϵ (Nelson, 1976). The smallest subunit, ϵ, acts as an endogenous inhibitor of the ATPase activity of CF_1 (Nelson et al., 1972) and may play a role in the lack of ATP-dependent reversal of energy transfer reactions of intact chloroplasts (Racker, 1976). Amino acid analysis of this subunit has shown the presence of a single sulfhydryl group (Nelson et al., 1972; Binder and Jagendorf, personal communication), and chemical cross-linking studies have shown the ϵ subunit to be adjacent to the α, β, and γ subunits (Baird and Hammes, 1976).

Previous studies have used fluorescence resonance energy transfer measurements to map the spatial relationship between specific sites on CF_1 (Cantley and Hammes, 1975b, 1976a,b). In this present study the location of the ϵ subunit with respect to specific sites on CF_1 has been examined by fluorescence energy transfer measurements. The fluorescent maleimide MalPy was used to label the sulfhydryl group on the ϵ subunit. It is a particularly useful energy donor because of its long fluorescence lifetime (Weltman et al., 1973). The energy acceptors were NBD bound to a tyrosine on the β subunit, probably near the ATPase active site (Deters et al., 1975; Cantley and Hammes, 1975a), and NSM bound to sulfhydryl groups on the γ subunit. Modification of 1 to 2 tyrosines in the β subunits with NBD-Cl markedly alters the reactivity of the sulfhydryl group of the ϵ subunit, although the energy transfer measurements indicate the sulfhydryl group is quite far from the NBD-labeled site ($\geq$45 Å). The sulfhydryl group on the ϵ subunit is about 40 Å from the labeled sulfhydryl groups of the γ subunit.

Experimental Section

Chemicals. The MalPy was purchased from Regis Chemical Co., NBD-Cl was from Pierce Chemical Co., NSM and MalNEt were from Eastman Chemical Co., quinine sulfate was from Aldrich Chemical Co., and β-mercaptoethanol and ATP (Sigma Grade) were from Sigma Chemical Co. The [^{3}H]MalNEt (142 Ci/mol) was obtained from New England Nuclear; the $NaDodSO_4$ employed in the polyacrylamide gel electrophoresis was the electrophoresis purity reagent from Bio-Rad, and the analytical reagent grade glycerol was from Mallinckrodt. All other chemicals were the best commercial grades, and deionized, distilled water was used in all solutions.

CF_1 Preparation. CF_1 was prepared from market spinach by the method of Lien and Racker (1971). The ratio of the fluorescence emission at 305 nm to that at 340 nm (excitation 280 nm with a 290-nm cutoff excitation filter) was greater than 1.5 for the purified enzyme; it was usually necessary to put the DEAE-Sephadex-purified CF_1 through a sucrose gradient (Lien and Racker, 1971) during the winter months (October–March) to achieve this degree of purity. An extinction coefficient of 0.476 mL/(mg cm) (Cantley and Hammes, 1975a) and a molecular weight of 3.2×10^5 (Farron, 1970) were used to obtain the molar concentration of CF_1. The purified latent enzyme was stored as an ammonium sulfate suspension at 4 °C.

The ATPase activity was measured following heat activation (see below) at 23 °C and pH 8.0 using the pH stat technique (Green and Mommerts, 1953) with the following assay concentrations: 10 mM ATP, 10 mM $CaCl_2$, and approximately 10 μg/mL heat-activated CF_1.

Preparation of Latent CF_1 Derivatives. The CF_1 was desalted on an 0.8 cm i.d. × 24 cm column of Sephadex G-25 in

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received June 3, 1977.* This work was supported by a grant from the National Institutes of Health (GM 13292).

‡ National Institutes of Health Postdoctoral Fellow (GM 05413).

[1] Abbreviations used are: CF_1, chloroplast coupling factor; MalPy, *N*-(3-pyrene)maleimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NSM, 4-dimethylamino-4′-maleimidostilbene; MalNEt, *N*-ethylmaleimide; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

50 mM sodium phosphate, 1 mM EDTA, pH 7.1, at room temperature and was incubated at a concentration of 10–25 μM with a twofold molar excess of MalPy for 40–90 min at room temperature in the same buffer with 5 mM ATP. The reaction was quenched with 8 mM dithiothreitol, and the MalPy-modified CF_1 was precipitated with an equal volume of saturated ammonium sulfate. The suspension was then cooled to 4 °C and centrifuged at 18 000*g* for 10 min, and the resulting pellet was dissolved in a small volume of the appropriate buffer and desalted on a column of Sephadex G-25 as before. The effluent was monitored at 280 nm by passing it through a microflow cell (Helma, Inc.) in a Cary 14 spectrophotometer. The MalPy derivative of the NBD-modified enzyme was prepared in a similar manner.

The NBD derivative of latent CF_1 was prepared in the same buffer as for modification of CF_1 with MalPy by reaction of 5–15 μM CF_1 with 450 μM NBD-Cl for 1.5–3 h (Cantley and Hammes, 1975a). The NBD was transferred from tyrosine to an amino group on CF_1 for fluorescence identification on $NaDodSO_4$–polyacrylamide gels by incubating NBD-modified CF_1 for 24 h in 40 mM Tris-HCl, 2 mM EDTA, pH 9.0, at room temperature (Cantley and Hammes, 1975b).

Latent CF_1 was modified with [^{3}H]MalNEt as previously described (Baird and Hammes, 1976); the NBD-modified enzyme was further modified with [^{3}H]MalNEt by reacting it at a concentration of 15 μM with 340 μM [^{3}H]MalNEt for 3 h in 40 mM Tris-HCl, 2 mM EDTA, pH 7.1, at room temperature.

The labeling stoichiometries for MalPy and NBD were determined from absorption spectra obtained on a Cary 118 spectrophotometer using the following molar extinction coefficients, ϵ, for the labels: for MalPy, ϵ was taken as 3.75×10^4 M^{-1} cm^{-1} at 343 nm and 1.24×10^4 M^{-1} cm^{-1} at 280 nm, which are the values obtained for the MalPy derivative of β-mercaptoethanol in dioxane; for the NBD–tyrosine derivative of CF_1, ϵ is 1.07×10^4 M^{-1} cm^{-1} at 400 nm and 0.94×10^4 M^{-1} cm^{-1} at 343 nm (Cantley and Hammes, 1975a). Unmodified CF_1 was used to determine the protein light scattering correction at appropriate wavelengths. The amount of [^{3}H]MalNEt incorporated was measured in 10 mL of Aquasol (New England Nuclear) using a Beckman LS-255 liquid scintillation counter.

Preparation of Heat-Activated CF_1 Derivatives. The MalPy-modified CF_1 (0.2–2 mg/mL) in 40 mM Tris-HCl, 2 mM EDTA, pH 8.0, was heat activated at 55–60 °C for 4 min after addition of ATP to 40 mM and dithiothreitol to 6 mM, then cooled immediately to room temperature. The activated enzyme was precipitated with an equal volume of saturated ammonium sulfate in 20 mM Tris-HCl, 2 mM EDTA, pH 7.1, and the centrifuged protein pellet was resuspended in either 40 mM Tris-HCl, 2 mM EDTA, pH 7.1, which leads to a derivative depleted in the ϵ subunit, or in 50% glycerol (v/v), 40 mM Tris-HCl, 2 mM EDTA, pH 7.1, which leads to a derivative in which the ϵ subunit is retained. A quick rinse of the undissolved pellet with buffer before suspension in 50% glycerol buffer aided in the retention of the ϵ subunit. The redissolved enzyme was then desalted in its appropriate buffer on a column of Sephadex G-75 (0.8 cm i.d. × 20 cm).

For modification of heat-activated, MalPy-modified CF_1 with NBD, the heat-activated, MalPy-modified CF_1 (ca. 5 μM) was first reacted with 100 μM MalNEt in the suspending buffer for at least 4 h at room temperature to block the exposed sulfhydryl groups on the γ subunit; it was then precipitated with ammonium sulfate and desalted as above. This enzyme (ca. 5 μM) was reacted with 450 μM NBD-Cl for 3.5–6 h in the 50% glycerol buffer at room temperature. The resulting derivatives, either with the ϵ subunit retained or with it depleted, were again precipitated with ammonium sulfate and desalted in 50% glycerol buffer on Sephadex G-75 or G-25 for subsequent spectroscopic measurements.

To prepare the derivatives in which the γ subunit is labeled with NSM or [^{3}H]MalNEt, the latent MalPy-labeled CF_1 (10 μM) was first reacted with 10 mM MalNEt in 40 mM Tris, 2 mM EDTA, pH 7.1, for 3 h at room temperature; this derivative was heat activated as above and redissolved in the 50% glycerol buffer following ammonium sulfate precipitation. The NSM (50 μM, 8 h) or [^{3}H]MalNEt (350 μM, 4 h) was reacted with the derivative in which the ϵ subunit is retained (9 μM) at room temperature in 50% glycerol buffer. Following ammonium sulfate precipitation and desalting on Sephadex G-75 in the same buffer, the [^{3}H]MalNEt and NSM stoichiometries were determined by scintillation counting of radioactivity and absorption spectrophotometry, respectively. The molar extinction coefficients of the enzyme-bound NSM used were 2.14×10^4 M^{-1} cm^{-1} at 370 nm, 2.11×10^4 M^{-1} cm^{-1} at 344 nm, and 1.03×10^4 M^{-1} cm^{-1} at 280 nm; these extinction coefficients were determined by assuming the extinction coefficient at 350 nm is equal to that of NSM-cysteine, 2.28×10^4 M^{-1} cm^{-1} (Papadakis and Hammes, 1977). Derivatives of these labeled enzymes in which the ϵ subunit is depleted were prepared by ammonium sulfate precipitation, centrifugation, and suspension of the labeled enzyme in 40 mM Tris-HCl, 2 mM EDTA, pH 7.1, at room temperature. Insoluble protein was removed by centrifugation at 18 000*g* for 10 min at 20 °C. The soluble material was then chromatographed on Sephadex G-25 in the 50% glycerol buffer.

Circular Dichroism Measurements. The circular dichroism of latent CF_1 (5.0 μM) and NBD-labeled latent CF_1 (4.3 μM) before and after NBD was displaced with β-mercaptoethanol (2 mM) was measured with a Durrum-Jasco J20A recording spectropolarimeter. The cell path length was 1.00 cm and the molar ellipticity, $[\theta]$, was calculated from the relationship:

$$[\theta] = \theta M / 10cl \tag{1}$$

where M is the molecular weight of CF_1, θ is the observed ellipticity in degrees, c is the concentration of optically active solute in grams per cubic centimeter, and l is the path length in centimeters. A scan speed of 5 nm/min and an instrument time constant of 4 s were employed; all spectra were run in duplicate at room temperature.

Fluorescence Measurements. Steady-state fluorescence measurements were made with a Hitachi-Perkin-Elmer MPF-3 fluorimeter equipped with corrected spectrum and polarization accessories and thermostated to 23 °C. Quantum yield measurements of MalPy on the latent and heat-activated enzyme derivatives were made as previously described (Cantley and Hammes, 1976a), except in the case of the enzyme modified with NBD prior to labeling with MalPy, where the quantum yield was calculated by multiplying the quantum yield of latent MalPy-modified CF_1 by the ratio of the average fluorescence lifetimes of the NBD-modified enzyme to that of the NBD-free enzyme. The quinine sulfate standard in 0.1 N H_2SO_4 was assumed to have a quantum yield of 0.70 (Scott et al., 1970) when excited at 344 nm. The steady-state fluorescence polarization, P, is defined as:

$$P = \frac{F_\parallel - F_\perp}{F_\parallel + F_\perp} \tag{2}$$

where $F_\parallel$ and $F_\perp$ are the respective fluorescence intensities when the emission polarizer is oriented in the parallel and perpendicular directions with respect to the excitation polarizer; P was measured for MalPy with excitation at 343 nm

TABLE I: Subunit Distribution of MalPy and [^{3}H]MalNEt on CF_1.

Derivative	MalPy or MalNEt/CF_1 (mol/mol)	Subunit distribution, mol/mol CF_1 (% of total) $\alpha\beta$	γ	ϵ	
MalPy-CF_1[a]	0.60	0.08 (13)	0.15 (25)	0.37 (62)	
NBD-MalPy-CF_1[b]	0.28	0.12 (42)	0.10 (36)	0.06 (22)	Latent
[^{3}H]MalNEt-CF_1[c]	0.82	0.16 (20)	0.20 (24)	0.46 (56)	Latent
NBD-[^{3}H]MalNEt-CF_1[b]	0.26	0.11 (44)	0.11 (42)	0.04 (14)	Latent
MalPy-CF_1, ϵ retained[d]	0.70	0.07 (10)	0.15 (22)	0.48 (68)	Heat activated
MalPy-CF_1, ϵ depleted[d]	0.23	0.07 (30)	0.12 (52)	0.04 (18)	Heat activated

[a] Average of three determinations: average deviation ±15% for overall MalPy stoichiometry and that of individual subunits. [b] Premodified with NBD-Cl (1.3 mol of NBD/mol of CF_1). [c] From Baird and Hammes, 1976. [d] Prepared from same latent CF_1 (0.74 mol of MalPy/mol of CF_1).

(3-nm slit) and emission at 374 nm (10-nm slit), with appropriate corrections for the effect of the monochromator on polarization (Chen and Bowman, 1965). The light scattering of the protein for all steady-state measurements of MalPy fluorescence was determined by measuring the emission at 360 nm, where no significant MalPy fluorescence emission occurs.

The efficiency of fluorescence resonance energy transfer from MalPy to NBD on CF_1 was measured by adding 1 μL of β-mercaptoethanol (diluted 1:10 in H_2O) to 200 μL of approximately 5 μM of the CF_1 derivative in 40 mM Tris-HCl, 2 mM EDTA, pH 7.1 (latent enzyme), or 50% glycerol buffer (heat-activated enzyme) in a square fluorescence microcell (Perkin-Elmer, 0.3 × 0.3 mm) and monitoring the immediate increase in fluorescence of the energy donor at the wavelength of maximum emission (374 nm). The absorbancy at the exciting wavelength (343 nm) was kept below 0.1 (for a 1-cm path length) to minimize inner filter effects.

Fluorescence lifetime measurements were made using the Ortec 9200 single photon nanosecond fluorescence system, interfaced with a PDP 11/20 computer (Digital Equipment Corp.). Fluorescence microcells were thermostated at 23 °C, and decay spectra for MalPy on CF_1 in the same buffers as used in the steady-state measurements were collected with an excitation interference filter of 340 nm and an emission interference filter of 370 nm (10-nm band-pass, Ditric Corp.). The light scattering due to the protein was corrected for by photon counting a solution of unmodified CF_1 and subtracting the counts from those of the sample. Lamp spectra were collected by scattering light from a solution of Ludox (Dupont) with the emission filter removed. Decay spectra were deconvoluted and analyzed in terms of two fluorescence lifetimes by the method of moments (Isenberg and Dyson, 1969).

$NaDodSO_4$-Polyacrylamide Gel Electrophoresis. One-dimensional rod gels were employed using the general method of Weber and Osborn (1969) and the specific procedure described by Baird and Hammes (1976). The gel concentrations usually utilized were 10% acrylamide and 0.135% *N,N'*-methylenebis(acrylamide). The protein samples were boiled for 2 min in 1% $NaDodSO_4$ and 1% β-mercaptoethanol. To quantitate the fluorescence in individual subunit bands, at least 100 μg of protein was used per gel. After fixation of the electrophoresed gels in 45% methanol, 9% acetic acid, individual fluorescent bands, as visualized with an ultraviolet hand lamp, were excised, homogenized in 1.5 mL of 1% $NaDodSO_4$, 40 mM Tris-HCl, 2 mM EDTA, pH 7, and incubated for at least 1 h at 30 °C with frequent agitation. In most cases a band was excised containing both the α and β subunits since they are normally not well resolved. The samples were then filtered with 0.45 μm Millipore filters, and the relative fluorescence intensity of each sample was measured at the appropriate wavelengths for excitation and emission. Greater than 90% of the fluorophore was solubilized by this procedure. Again, light scattering corrections to the observed MalPy fluorescence were made by determining the emission at 360 nm; for quantitation of NSM-labeled enzyme, the solution was diluted with an equal volume of dioxane to enhance fluorescence.

Results

The amounts of MalPy and [^{3}H]MalNEt incorporated into latent CF_1 and into NBD-modified latent CF_1 and their distribution in the five subunits are given in Table I. The presence of NBD on CF_1 causes a marked change in both the amount of label incorporated and the subunit distribution. Reaction of a twofold molar excess of MalPy with latent CF_1 at pH 7.1 for 40 min resulted in the labeling of the ϵ subunit with about 62% of the total MalPy incorporated. After modification of latent CF_1 with NBD-Cl (1.3 mol/mol of CF_1), however, only 22% of the total MalPy incorporated is in the ϵ subunit. This corresponds to a sixfold decrease in the absolute amount of MalPy incorporated into the ϵ subunit (Table I). Significant changes in the amount of label incorporated into the α, β, and γ subunits are also seen, but to a lesser extent (<twofold). When $NaDodSO_4$-polyacrylamide gels are run under conditions where the α and β subunits are separated, the MalPy appears in the β but not in the α subunit. The incorporation of [^{3}H]MalNEt shows a similar labeling pattern as MalPy, including the marked decrease in labeling of the ϵ subunit of the NBD derivative (Table I). An absorption maximum at 395 nm for NBD-labeled CF_1 (Figure 2), together with the localization of the fluorescence of the NBD-labeled amino group in the β subunit by $NaDodSO_4$-polyacrylamide gel analysis, is in agreement with the previous assignment of the NBD modification to a tyrosine residue on that subunit (Deters et al., 1975; Cantley and Hammes, 1975a). These results indicate that the reaction of NBD-Cl with the β subunit of latent CF_1 decreases the accessibility of the sulfhydryl moiety on the ϵ subunit. Removal of NBD from CF_1 by addition of β-mercaptoethanol permits labeling of the γ and ϵ subunits with MalPy to about the same levels as before NBD-Cl modification, but the amount of MalPy in the α, β region of the $NaDodSO_4$ gels is significantly higher than before modification with NBD.

The circular dichroism spectrum of NBD-labeled CF_1 in the near-ultraviolet region shows significant changes when compared to that of the unmodified latent enzyme (Figure 1). A broad induced band with a maximum at 400 nm corresponds to the long-wavelength absorption band of the NBD chromophore, and indicates that it binds in an asymmetric environment. A positive change in the region of 280 to 310 nm is also observed for the NBD derivative, as well as a negative change in the region of 250–280 nm. Since one of the NBD–tyrosine

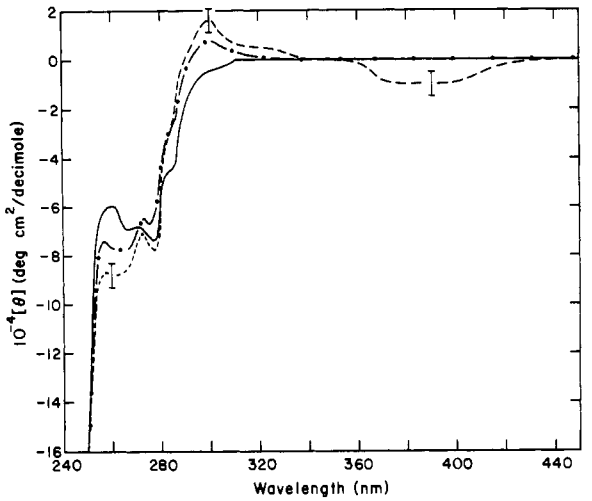

FIGURE 1: Circular dichroism spectra of latent CF_1 (5.0 μM; —) and NBD-modified latent CF_1 (4.3 μM; 1.9 mol of NBD/mol of CF_1) before (- - -) and after (- · - · -) addition of β-mercaptoethanol (2 mM) in 50 mM sodium phosphate, 1 mM EDTA, pH 7.1. The error bars indicate the experimental reproducibility.

absorption transitions has a maximum at about 275 nm (Aboderin et al., 1973), where no significant circular dichroism change is apparent, it is unlikely that all of these observed changes can be attributed to induced circular dichroism of the NBD chromophore alone; therefore, changes in the environments of aromatic amino acid residue(s) due to NBD modification are probable. Addition of β-mercaptoethanol to release the NBD chromophore from the enzyme results in the complete loss of the induced circular dichroism band in the 390-nm region, while changes in the aromatic amino acid residue absorbing region are not fully reversed. The failure of a new circular dichroism band to appear in the 320-nm region, where the NBD-β-mercaptoethanol adduct maximally absorbs, suggests that no significant noncovalent interaction of this adduct with the protein remains, in agreement with the observation that Sephadex G-25 chromatography of this mixture results in removal of any significant NBD absorption in the 300–500-nm region.

Modification of Heat-Activated CF_1. Although the sulfhydryl group of the ϵ subunit could not be selectively labeled, a method was developed by which the heat-activated enzyme could be depleted of ϵ subunit. Heat-activated CF_1 which is precipitated in 50% saturated ammonium sulfate is not entirely soluble in aqueous 40 mM Tris-HCl, 2 mM EDTA (pH 7.1). When the soluble portion of the enzyme is desalted on Sephadex G-75 and examined with $NaDodSO_4$–polyacrylamide gel electrophoresis, very little ϵ subunit is found as judged by the intensity of the protein stain. The yield of this ϵ-deficient enzyme is about 50% of the latent enzyme. Some δ subunit is often lost, but the relative amounts of α, β, and γ subunits appear to be unchanged. This effect can be quantitated by determining the subunit distribution of MalPy in the labeled enzyme after heat activation. The results obtained are included in Table I for heat-activated enzyme in which the ϵ subunit is depleted, and heat-activated enzyme in which the ϵ subunit is retained by dissolving the ammonium sulfate precipitate in buffer containing 50% glycerol. Greater than 90% of the MalPy-labeled ϵ subunit can be removed without loss of MalPy-labeled α and β subunits. This result is somewhat variable: the range of ϵ depletion was 50–100% for different samples.

The heat-activated enzyme was routinely stored in 50% glycerol at room temperature, and the ATPase activity was

TABLE II: Effect of the ϵ Subunit on Ca^{2+}-ATPase Activities.

	Time after activation (h)	ATPase (% control)[a]	Reactivated ATPase (%)[b]
MalPy-CF_1,	0	100	
activation	0.67	80	
mixture (6 mM	3	73	
dithiothreitol,	23	52	
40 mM ATP)	36	46	86
ϵ retained[c]		39	58
ϵ depleted[d]		61	67

[a] Initial activity (100% control) was 6.9 μmol/(min mg) at 22 °C. [b] 60 °C, 4 min with no further additions of dithiothreitol or ATP. [c] ϵ subunit loss less than 10%. [d] Greater than 90% loss of ϵ subunit.

unchanged after 1 month. The ϵ subunit still appears to be a partial inhibitor when bound to heat-activated CF_1 since reactivation at 60 °C for 4 min in 50% glycerol results in a 1.5-fold increase in ATPase activity for the enzyme containing the normal amount of ϵ subunit, but only a 1.1 ($\pm$0.1)-fold increase for the enzyme depleted of ϵ subunit. This reactivation can be compared with the 1.9-fold increase in ATPase activity for heat-activated CF_1 in its original activation buffer after that activity had declined to about half of its initial value. The data demonstrating these phenomena are summarized in Table II.

The MalPy-labeled, heat-activated enzyme was modified with NBD as described in the Experimental Section. The course of the reaction was monitored by following the absorbance at 400 nm. After 2–3 h the absorbance leveled off with a stoichiometry of 0.7 to 0.9 NBD per mol of CF_1. This value is somewhat less than has been previously reported for heat-activated CF_1 (Cantley and Hammes, 1976b) but may reflect the presence of glycerol in the reaction medium. Removal of NBD from the enzyme with β-mercaptoethanol resulted in restoration of only about 60% of the ATPase activity present before NBD modification.

Labeling of the sulfhydryl groups on the γ subunit of the heat-activated enzyme, which had been previously modified with MalPy and MalNEt, with NSM or [^{3}H]MalNEt as described in the Experimental Section, resulted in 1 to 2 mol of labeled sulfhydryl groups on the γ subunit per mol of CF_1. No significant labeling of other subunits occurred, and the ATPase activity was inhibited less than 40%. Despite the presence of 50% glycerol, some of the ϵ subunit is often lost when labeling the heat-activated enzyme. Therefore, the distribution of MalPy in the subunits was always determined after all labeling reactions by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Energy Transfer Measurements on Latent CF_1. The observed efficiency of fluorescence energy transfer, E, between an energy donor and acceptor is:

$$E = 1 - Q_{D\rightarrow A}/Q_D = 1 - \tau_{D\rightarrow A}/\tau_D \qquad (3)$$

where $Q_{D\rightarrow A}$ and Q_D are the donor quantum yields in the presence and absence of acceptor, and $\tau_{D\rightarrow A}$ and τ_D are the corresponding donor fluorescence lifetimes. For an isolated donor-acceptor pair, the distance at which the energy transfer efficiency is 0.5, R_0, can be calculated from eq 4 (Förster, 1959):

$$R_0 = (9.79 \times 10^3)(J\kappa^2 Q_D n^{-4})^{1/6}\ \text{Å} \qquad (4)$$

where J is the spectral overlap integral of the donor fluorescence emission and acceptor absorption, κ^2 is an orientation

TABLE III: Energy-Transfer Parameters.

	Derivative	mol of NBD or NSM/mol of CF_1	Q_D	C_1	τ_1 (ns)	C_2	τ_2 (ns)	τ_{av} (ns)	E^a	R_0 (Å)	R_{av} (Å)
Latent enzyme	MalPy-CF_1		0.18	0.75	43.3	0.25	120.5	62.6			
	MalPy-NBD-CF_1	1.0		0.68	38.7	0.32	96.5	57.2	0.063[b]	29.7	47[b]
	MalPy-NBD-CF_1 + β-mercaptoethanol			0.70	41.9	0.30	105.6	61.0	0.085[c]		44[c]
	NBD-MalPy-CF_1	1.3		0.60	46.6	0.40	119.8	75.9	0.105[b]	31.6	45[b]
	NBD-MalPy-CF_1 + β-mercaptoethanol		0.26	0.59	55.2	0.41	134.7	87.8	0.123[c]		44[c]
Heat-activated enzyme	MalPy-NBD-CF_1 (ϵ retained)	0.70		0.74	46.8	0.26	129.5	68.3	0.039[b]	30.2	52[b]
	MalPy-NBD-CF_1 (ϵ retained) + β-mercaptoethanol		0.26	0.73	46.7	0.27	133.8	70.2	0.082[c]		45[c]
	MalPy-NBD-CF_1 (ϵ depleted)	0.91		0.76	45.7	0.24	110.0	61.1	0.136[b]	30.2	41[b]
	MalPy-NBD-CF_1 (ϵ depleted) + β-mercaptoethanol		0.26	0.67	48.7	0.33	112.6	69.8	0.208[c]		38[c]
	MalPy-NSM-CF_1 (ϵ retained)	1.9		0.67	31.9	0.33	121.1	61.3	0.107[b]	30.7	44[b]
	MalPy-MalNEt-CF_1 (ϵ retained)		0.22	0.61	40.6	0.39	134.3	76.9			
	MalPy-NSM-CF_1 (ϵ depleted)	1.4		0.70	36.0	0.30	131.9	65.2	0.073[b]	30.7	47[b]
	MalPy-MalNEt-CF_1 (ϵ depleted)		0.22	0.67	39.2	0.33	139.3	72.6			

[a] Efficiency per mole of acceptor; the experimental reproducibility is ±0.02. [b] Calculated from changes in the average fluorescence lifetime. [c] Calculated from changes in the steady-state fluorescence.

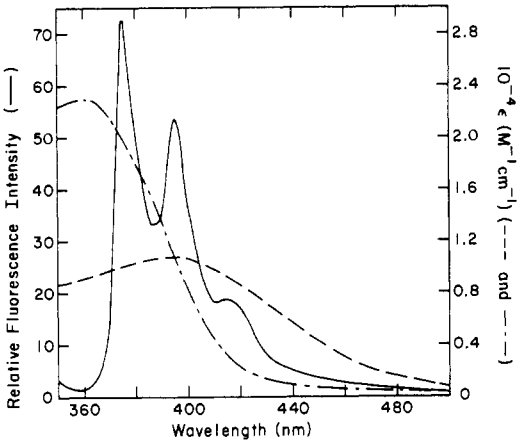

FIGURE 2: Spectral overlap of the MalPy-corrected fluorescence emission spectrum (343-nm excitation) of the MalPy-CF_1 derivative (—) with the difference extinction coefficient (ϵ) of NBD (- - -) and NSM (- · - · -) on CF_1 in 40 mM Tris-HCl, 2 mM EDTA, pH 7.1. The same spectra are obtained when 50% glycerol is present.

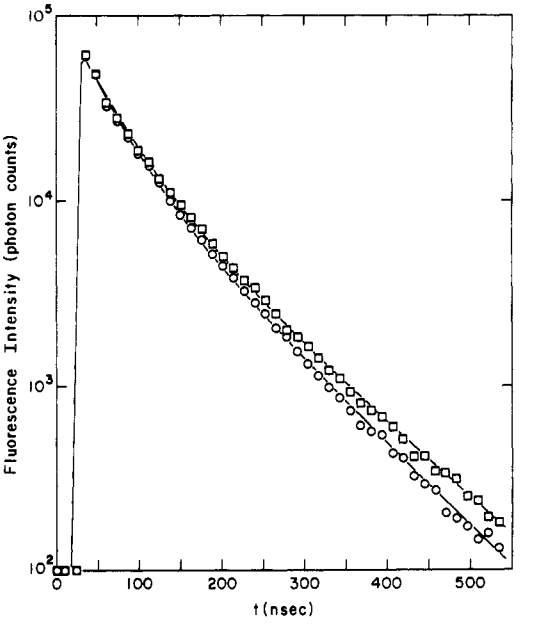

FIGURE 3: Fluorescence emission decay of MalPy on latent CF_1 in 40 mM Tris-HCl, 2 mM EDTA, pH 7.1 (340-nm excitation, 370-nm emission): (○) CF_1 modified with MalPy and NBD (MalPy-NBD-CF_1); (□) after addition of β-mercaptoethanol to remove NBD. The line is the reconvoluted best fit of the data to eq 5; the best fit parameters are given in Table III.

factor characterizing the relative orientation of the donor and acceptor transition dipoles, and n is the refractive index of the medium through which transfer takes place. In the cases to be considered here, the donor is probably heterogeneous in its fluorescence properties since it binds to several different subunits. Therefore, we can determine only an efficiency of energy transfer averaged over all environments and we can calculate an R value which is some averaged measure of energy transfer. The average quantum yields of MalPy on latent CF_1, prepared by reaction of MalPy before and after NBD modification, and with the NBD displaced by β-mercaptoethanol are given in Table III. The overlap integrals were calculated as previously described (Cantley and Hammes, 1975b) from the spectral data shown in Figure 2; κ^2 was assumed to be $\frac{2}{3}$, which is the value for the case where the donor and acceptor rotate rapidly relative to the donor fluorescence lifetime, and n was taken as 1.4 in the Tris-EDTA buffer, and 1.5 in the 50% glycerol buffer. The values of R_0 calculated for the two modified latent CF_1 species are given in Table III.

The energy transfer from MalPy to NBD on CF_1 was measured by observing the change in fluorescence and the change in fluorescence lifetimes when NBD is displaced by β-mercaptoethanol. Since NBD did not alter the shape of the wavelength dependence of the MalPy emission, the change in fluorescence is proportional to the change in quantum yield. The fluorescence decay for MalPy-labeled CF_1 is multiexponential as might be expected when at least three different sites are labeled. As shown in Figure 3, a good fit of the fluorescence decay, $F(t)$, is obtained with the two-exponential equation:

$$F(t) = C_1 e^{-t/\tau_1} + C_2 e^{-t/\tau_2} \quad (5)$$

where C_1 and C_2 are constants which will be arbitrarily normalized to $C_1 + C_2 = 1$, and τ_1 and τ_2 are fluorescence life-

times. In view of the complexity of the labeling stoichiometry, the lifetimes cannot be attributed to MalPy in any one particular environment; the data are most conveniently summarized by an average lifetime, τ_{av}, defined as:

$$\tau_{av} = C_1\tau_1 + C_2\tau_2 \quad (6)$$

This average lifetime is the normalized area under the decay curve and, therefore, represents a relative quantum yield. To obtain meaningful comparisons, it is important to collect and analyze donor decay data in the presence and absence of acceptor under identical experimental conditions (i.e., concentrations, instrument settings, etc.). The fluorescence decay parameters for the two derivatives MalPy-NBD-CF_1 and NBD-MalPy-CF_1, where the order of labels indicates the order in which they are reacted with the enzyme, in the presence and absence of β-mercaptoethanol are given in Table III. The energy-transfer efficiencies determined from both the change in the average fluorescence lifetime and from the change in steady-state fluorescence are also presented in Table III.

In view of the complexity of the system, the agreement between the nanosecond and steady-state measurements of energy-transfer efficiency is satisfactory although the consistently greater efficiencies obtained from the quantum yield determinations could be due to a static quenching component which is independent of the energy-transfer process (Schiller, 1975). In both cases, the species in which very little of the donor is on the ϵ subunit has a higher efficiency of energy transfer indicating the donor on the ϵ subunit is farther away from NBD than one or more of the donors on the other subunits. Nevertheless, the change is rather small suggesting that some energy transfer between MalPy on the ϵ subunit and NBD occurs. In fact, the apparent change in energy-transfer efficiency is primarily due to the increased quantum yield of the derivative in which very little label is on the ϵ subunit (NBD-MalPy-CF_1). For an isolated donor-acceptor pair, the distance between the donor and acceptor, R, is related to the efficiency of energy transfer by:

$$E = \frac{1}{1 + (R/R_0)^6} \quad (7)$$

The values of R calculated from the data and the above equation are also in Table III. Obviously a molecular interpretation of the measured distance is not possible, but since a slightly shorter distance is obtained for the derivative in which only a small amount of label is on the ϵ subunit, the distance between MalPy on ϵ and NBD must be greater than the calculated value of R, 44 Å.

The fluorescence polarizations of all of the derivatives were essentially the same, 0.235 ± 0.004.

Energy-Transfer Studies on Heat-Activated CF_1. Energy-transfer experiments with the heat-activated enzyme were performed in 50% glycerol to stabilize the interaction of the ϵ subunit with the enzyme. Since the NBD is slowly transferred from tyrosine to an amino group (Ferguson et al., 1975; Cantley and Hammes, 1975a), measurements with the NBD-labeled enzyme were made as soon as possible after labeling. Under the conditions used, <0.1 mol of NBD per mol of CF_1 was transferred to the amino group. It was also noted that NBD slowly dissociates with a half-time of 50–70 h.

A summary of the energy-transfer measurements between MalPy and NBD on heat-activated CF_1 with the ϵ subunit depleted and with it retained is given in Table III. The changes in quantum yield and in fluorescence lifetimes were determined and used to calculate the efficiency of energy transfer as with the latent enzyme. The fluorescence decay parameters and quantum yield parameters are included in Table III. The value of the average distance between donor and acceptor was calculated as for the latent enzyme. Although the energy-transfer efficiencies measured by fluorescence lifetimes and steady-state fluorescence differ somewhat as with the latent enzyme, the observed energy transfer is much less for the ϵ-retained derivative than for the ϵ-depleted derivative. Assuming that the only difference between the ϵ-depleted and ϵ-retained species is the amount of ϵ subunit present, the fluorescence lifetime for the MalPy label on the ϵ subunit, τ_ϵ, can be estimated from the measured average lifetimes and the known amount of label on each subunit as determined by Na-DodSO_4-polyacrylamide gel electrophoresis. The average lifetime can be approximated as:

$$\tau_{av} = X_\epsilon\tau_\epsilon + X_{\beta\gamma}\tau_{\beta\gamma} \quad (8)$$

where X_ϵ and $X_{\beta\gamma}$ are the fractions of fluorescence donor on the ϵ and the β and γ subunits, and $\tau_{\beta\gamma}$ is the average lifetime of the donor on the β and γ subunits. Since τ_{av} is known for the ϵ-depleted and ϵ-enriched enzyme, the two simultaneous equations can be solved to give τ_ϵ and $\tau_{\beta\gamma}$. The results obtained are $\tau_\epsilon = 76.4$ ns and $\tau_{\beta\gamma} = 57.0$ ns for the NBD-labeled enzyme and $\tau_\epsilon = 70.7$ ns and $\tau_{\beta\gamma} = 69.6$ ns for the enzyme in which NBD has been displaced by β-mercaptoethanol. These changes in lifetime can be used to calculate energy-transfer efficiencies. Essentially no energy transfer occurs between MalPy on the ϵ subunit to NBD (the apparent efficiency is actually somewhat negative) while the efficiency of transfer from MalPy on the β and γ subunits to NBD is 0.18. This analysis of the data is quite crude, but both it and qualitative consideration of the results obtained for the latent and heat-activated enzyme indicate the MalPy on the ϵ subunit is greater than 45 Å from the NBD label on the β subunit.

Energy-transfer measurements also were carried out using MalPy as the energy donor and NSM, which was used to specifically label γ-sulfhydryl groups following heat activation, as the energy acceptor. Since the NSM cannot be removed from the enzyme, steady-state fluorescence determinations of transfer efficiency are unreliable due to the relatively large uncertainties in correction factors for nonsensitized emission and in normalization factors for absorption of the exciting radiation. The average fluorescence lifetime for the NSM-labeled enzyme was compared with that for a derivative in which MalNEt was substituted for NSM for determination of the energy-transfer efficiency. This should effectively eliminate any difficulties in the determination of the energy-transfer efficiency due to possible conformational changes caused by the labeling of the γ-sulfhydryl groups. The results obtained for the ϵ-retained and ϵ-depleted heat-activated CF_1 are included in Table III. The energy-transfer efficiencies are normalized to 1 mol of NSM/mol of CF_1 assuming a linear extrapolation.

The apparent energy-transfer efficiency from MalPy is greater on the ϵ-enriched CF_1 than on the ϵ-depleted enzyme. This indicates the distance from MalPy on the ϵ subunit to NSM on a sulfhydryl group of the γ subunit is closer than the average distance of 44 Å given in Table III. The fluorescence lifetime for MalPy on the ϵ subunit and the average lifetime for MalPy on the β and γ subunits can be calculated as above. The values obtained are $\tau_\epsilon = 71.6$ ns and $\tau_{\beta\gamma} = 65.0$ ns for the enzyme modified with NSM, and $\tau_\epsilon = 88.2$ ns and $\tau_{\beta\gamma} = 61.3$ ns for the enzyme not containing NSM. This leads to an energy-transfer efficiency of about 0.19 with MalPy on ϵ as the energy donor. This in turn implies a distance between the MalPy on the ϵ subunit and NSM on the γ subunit of about

39 Å. The energy transfer from MalPy on the β and γ subunits is zero within experimental uncertainty, indicating that on the average they are greater than 45 Å from the NSM.

No significant differences between the fluorescence polarization of MalPy in the presence and absence of acceptor were seen; the average value is 0.278 ± 0.013.

Discussion

The results obtained indicate that NBD modification of the β subunits causes major conformational changes in the chloroplast coupling factor. The reactivity of the sulfhydryl group of the ϵ subunit, which is greater than 44 Å from the NBD sites, is markedly diminished in the NBD-modified protein. The circular dichroism results show that NBD modification of CF_1 results in significant changes in the environments of some aromatic amino acid residues, and that these changes are not fully reversed when the NBD moiety is removed. Moreover, the induced circular dichroism of the NBD chromophore indicates a strong interaction with the protein. The inability to completely reverse the intrinsic circular dichroism changes caused by NBD is consistent with the facts that the ATPase activity of heat-activated CF_1 modified with NBD is restored to only about 60% of its value before modification by addition of β-mercaptoethanol and that less MalPy is incorporated into the α and β subunits of CF_1 before modification with NBD than after displacement of NBD with β-mercaptoethanol. The NBD modification also alters the binding of [^{3}H]ADP and 1,N^6-etheno[^{3}H]ADP to the tight sites of latent CF_1 (Cantley and Hammes, 1975a). In light of these results, the possibility that NBD may be inhibiting the ATPase activity via a conformational effect should be considered.

The fluorescence energy-transfer measurements between MalPy on the ϵ subunit and NBD are complicated by a number of considerations; of most concern is the possibility that the release of NBD by β-mercaptoethanol could cause a conformational change that alters the fluorescence lifetime of MalPy in its various environments on the enzyme. Although the steady-state fluorescence polarization of MalPy and the shape or position of its emission spectrum were not altered by NBD release in either the latent or heat-activated derivatives, an environmental effect cannot be ruled out. Nevertheless, the basic conclusion that the ϵ subunit is far away (>44 Å) from the NBD site appears to be justified, as it was arrived at by studying derivatives of both the latent and heat-activated enzymes which were prepared by different methods. The fluorescence lifetime data indicate that the environments for MalPy on the latent and heat-activated derivatives are quite different, but it would be necessary to assume that NBD modification enhances the fluorescence of MalPy on the ϵ subunit to the same extent on both derivatives to account for the data by a strictly conformational effect. Although the efficiency measurements have been interpreted assuming only one ϵ subunit and that the two NBD sites are close to each other (Cantley and Hammes, 1975a), the presence of a second ϵ subunit (Baird and Hammes, 1976) would not alter the interpretation provided that the ϵ subunits are symmetrically arranged with respect to the NBD sites.

The unknown orientation of the donor and acceptor dipoles must also be considered. Limits on the value of κ^2 can be assessed from the amount of fluorescence depolarization of the donor fluorophore occurring independently of that due to rotation of the macromolecule (Dale and Eisinger, 1974a). The reported steady-state polarization values and rotational correlation times of 220 ns for latent CF_1 and 3300 ns for heat-activated enzyme in 50% glycerol (Baird and Holowka, unpublished results) were used to calculate the half-angle of restricted rotation on the surface of a cone (Dale and Eisinger, 1974a,b). A probability density distribution of distances subject to the calculated half-angle of restricted rotation for the donor and with the acceptor fixed in space was then generated (Hillel and Wu, 1976). This probability density distribution also includes the experimental uncertainty in the efficiency, ±0.04 for energy transfer between MalPy and NBD on latent CF_1 and ±0.08 for energy transfer between MalPy on the ϵ subunit and NSM on the γ subunit on heat-activated CF_1. For the energy transfer between MalPy and NBD, the half-angle is 23° and the most probable value of the minimum value of R is 44 Å (as is obtained with $\kappa^2 = 2/3$). The probability of R being within 4 Å of this most probable value is 44%, and a minimum distance of 35 Å has greater than 90% probability. For the energy transfer between MalPy and NSM, the half-angle is 25°. The most probable value of R is 37 Å (R = 39 Å when $\kappa^2 = 2/3$), and the probability R lies between 30 and 48 Å is 90%. If the possibility that NBD and NSM also have some rotational mobility is considered, the probability density distribution of distances would be even more sharply peaked. Also, the very irregular nature of the emission polarization spectrum of the pyrene fluorophore (Hudson, 1970) makes it probable that emission occurs from more than one discretely oriented dipole, further reducing the probability of an extreme value for κ^2. These calculations indicate that the uncertainties in the experimental measurements and the assumption that $\kappa^2 = 2/3$ are unlikely to lead to a major error in the calculated distances.

The problem of environmental effects for the case of energy transfer between MalPy and NSM on the γ subunit of heat-activated CF_1 has been minimized by comparing the NSM derivative to one in which the γ sulfhydryl groups have been labeled with MalNEt, a derivative for which no spectral overlap with MalPy fluorescence occurs. The accuracy of the energy-transfer measurements in this case is somewhat lessened by the need to compare τ_{av} of two different derivatives, but the finding that the transfer efficiency is greater for the ϵ-retained derivative than for the ϵ-depleted one is in contrast to the results obtained with NBD as the energy acceptor. The two sulfhydryl groups on the γ subunit which react with NSM are assumed to be close together relative to the distance from the ϵ sulfhydryl group(s), but the validity of this assumption is unknown (Cantley and Hammes, 1976b). Despite the many uncertainties, a distance of about 37–39 Å between the ϵ sulfhydryl groups of the γ subunits suggested by these studies is consistent with previously measured distances (Cantley and Hammes, 1976b) and chemical cross-linking studies (Baird and Hammes, 1976). The distances measured are also consistent with the estimated radii of the subunits: 16, 23, and 25 Å for the ϵ, γ, and β subunits, respectively.[2]

The model shown in Figure 4 incorporates the results presented here with those obtained in previous studies from this laboratory (Cantley and Hammes, 1976b; Baird and Hammes, 1976) to give a plausible structure of CF_1. The diagram depicts the view from above the plane of the chloroplast membrane on which CF_1 is attached in vivo; the δ subunit mediates this attachment (Nelson and Karny, 1976; Younis et al., 1977), and is beneath the plane of the diagram. The tight adenine nucleotide binding sites have been localized arbitrarily on the β subunits. The previous assignment of these sites to the α subunit (Cantley and Hammes, 1975b), which is an alternative possibility, was based solely on the relatively long distance

[2] This assumes the subunits are spherical and that the radius is equal to $(3\bar{v}M/4\pi N)^{1/3}$ where $\bar{v}$ is the partial specific volume (0.735), M is the molecular weight, and N is Avogadro's number.

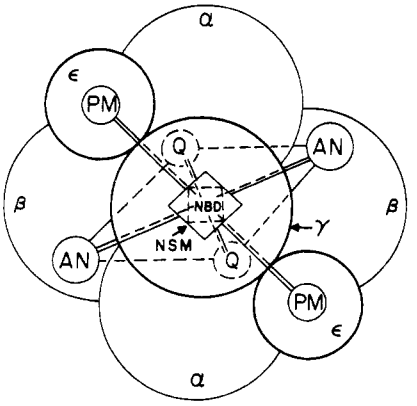

FIGURE 4: Pictorial representation of the chloroplast coupling factor. In this diagram AN represents the tight adenine nucleotide binding sites and Q the quercetin binding sites. The quercetin binding sites are located on either the α or β subunits (Cantley and Hammes, 1976a). The NBD site between the β subunits is located beneath the γ subunit in this diagram, and the δ subunit is beneath the plane of the paper. The lines represent distances which have been measured by fluorescence energy transfer; dotted lines are beneath the γ subunit.

(~40 Å) between the tight nucleotide sites and the NBD site on the β subunit; the measured distance does not preclude the possible location of the tight nucleotide sites on the β subunits. If the NBD is assumed to be near the ATPase active site, then present energy-transfer results indicate that the inhibitory action of the ϵ subunit does not involve a direct interaction with the active site. In this regard, the inhibition of ATPase activity by the ϵ subunit has been shown to be kinetically noncompetitive for the *Escherichia coli* coupling factor (Smith et al., 1976). Both the energy-transfer measurements and the chemical cross-linking studies suggest that the ϵ subunit does not change its location appreciably following heat activation, so that more subtle conformational interactions must be involved in the inhibition of ATPase activity.

Fluorescence energy-transfer measurements have given considerable insight into the structure of the solubilized coupling factor; future measurements will be aimed at extending this approach to the membrane-bound system.

Acknowledgments

We wish to thank Dr. R. E. Cathou for the use of the Durrum-Jasco J20A recording spectropolarimeter and Dr. C.-W. Wu for the computer program used to calculate the probability density distributions of R.

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