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## 5-Iodoacetamidofluorescein-Labeled Chloroplast Coupling Factor 1: Conformational Dynamics and Labeling-Site Characterization<sup>†</sup>

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**ABSTRACT:** Physical and spectroscopic properties of 5-iodoacetamidofluorescein (5-IAF), a new sulfhydryl-specific fluorescent label, are described. Under certain conditions, 5-IAF labels the chloroplast phosphorylation coupling factor (CF) predominantly on the  $\beta$  subunit. Approximately 88% of the ATPase activity and over 60% of the ability of CF to reconstitute photophosphorylation are retained after labeling. Trypsin activation of the ATPase activity of 5-IAF-labeled CF dramatically alters the fluorescence properties at the labeling site, indicating its involvement in ATPase activation. Studies of the fluorescence emission spectra, fluorescence polarization, and potassium iodide quenching of 5-IAF and 5-IAF-labeled CF demonstrate that the labeling site is in a partially buried hydrophobic region which is partially accessible to potassium

iodide quenching from the solvent phase and which restricts the motion of the fluorescent label. The fluorescence shows little change upon substrate binding. We conclude that the label is located in a cleft region remote from the enzyme active site. Variation of the reaction pH between 6.4 and 8.5 significantly alters the number of attached labels. ATP decreases the extent of labeling over the entire pH range studied. These labeling changes reflect substrate- and pH-induced conformational changes in CF. Certain interdependences observed in these conformational changes suggest that the transmembrane electrochemical gradient may directly induce conformational changes in CF leading to net ATP synthesis. The  $\gamma$  subunit plays a central role in the expression of these intersubunit conformational changes.

The chloroplast coupling factor (CF<sup>1</sup>) is a water-soluble protein associated with the thylakoid membrane surface, where

it catalyzes the terminal step of ATP production during photophosphorylation. It contains five different subunit types ranging in size from 13 000 to 59 000 daltons (Nelson et al., 1973). Two tight-binding sites for ATP or ADP have been characterized (Livne and Racker, 1969; Roy and Moudrianakis, 1971; Cantley and Hammes, 1975a) along with other, weaker sites. These sites have been tentatively assigned to the  $\alpha$  and  $\beta$  subunits (Cantley and Hammes, 1975a). This assignment is verified by the observation that an  $\alpha$  and  $\beta$  subunit complex derived from CF is active as an ATPase (Deters et al., 1975).

In a previous investigation, covalent labeling of tyrosine residues on the  $\beta$  subunit with NBD-Cl led to 80% inhibition of the enzyme activity (Deters et al., 1975). We have used a new sulfhydryl-specific fluorescent label, 5-iodoacetamidofluorescein (5-IAF), to label CF with minimal decrease in the enzyme activity. The label reacts preferentially with the  $\beta$  subunit at pH 7.8 in the presence of ATP. Investigation of the fluorescence emission spectrum, the fluorescence polar-

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<sup>1</sup> Abbreviations used are: CF, chloroplast coupling factor 1; 5-IAF, 5-iodoacetamidofluorescein; TLC, thin-layer chromatography; NEM, *N*-ethylmaleimide; DCCD, *N,N'*-dicyclohexylcarbodiimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; Chl, chlorophyll; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DTT, dithiothreitol; NaDodSO<sub>4</sub>, sodium dodecyl sulfate.

ization, and potassium iodide quenching of the label provides information on the microenvironment at the 5-IAF binding site. The fluorescent label serves as a monitor for the effects of substrate addition and trypsin treatment on the labeled protein.

The fluorescent-labeling reaction itself is used as a probe for structural changes occurring in CF. Both the presence or absence of substrate and small changes in the pH of the labeling mixture cause changes in the extent and subunit localization of the labeling. These labeling changes reflect conformational rearrangements in CF induced by pH changes and by the addition of substrate. The implications of these findings for the conformational theory of energy coupling will be discussed.

### Experimental Section

**5-Iodoacetamidofluorescein.** 5-IAF was synthesized by Dr. Richard Haugland (Hamline University, St. Paul, Minn.) and generously provided to us as a gift. 5-IAF has recently become available from Molecular Probes, Roseville, Minn. It was used without further purification. The material migrates as a major band with several minor components when examined by reverse-phase thin-layer chromatography using Eastman no. 13181 silica gel sheets impregnated with 5% (v/v) Dow Corning 200 silicone fluid (10 cSt viscosity) in anhydrous ethyl ether and run in a solvent of 90% acetone, 10% water saturated with the silicone fluid.

5-IAF exhibits an absorption peak of 491 nm in water solution (40 mM Tricine-NaOH, 2 mM EDTA, pH 8.0) which shifts to 495 nm in methanol and 505 nm in 2-propanol. This corresponds to similar solvent shifts observed for fluorescein (Seybold et al., 1969; Martin and Lindqvist, 1973). Solutions of 5-IAF in certain solvents, such as acetone or dimethyl sulfoxide, are colorless and nonfluorescent. Similar observations for fluorescein have been ascribed to the formation of a nonfluorescent lactone form (Davies and Jones, 1954).

The corrected emission maximum occurs at 518 nm in water solution (40 mM Tricine-NaOH, 2 mM EDTA, pH 8.0) and is shifted to 519 nm in methanol and 525 nm in 2-propanol. Similar results have been obtained for fluorescein (Martin and Lindqvist, 1973). The apparent molar absorptivity for 5-IAF in 40 mM Tricine-NaOH, 2 mM EDTA, pH 8.0 is 60 000 L mol<sup>-1</sup> cm<sup>-1</sup> at 491 nm. The true value may be slightly higher due to the presence of contaminants in our sample.

The solid 5-IAF was stored at -25 °C in the dark. In solution it is relatively stable when refrigerated in the dark, but it undergoes a slow degradation in the light. Illumination of the parent fluorophore, fluorescein, can lead to free-radical formation (Niizuma et al., 1974) and superoxide production (Balny and Douzou, 1974). For these reasons, the dye and labeled proteins were kept in the dark or under dim illumination, when possible.

**Coupling Factor.** Coupling factor was purified by a method adapted from the pyrophosphate wash procedure of Strotmann et al. (1973). In contrast to their findings, we obtained appreciable protein contamination in the CF samples following this method. However, when the washes were supplemented by subsequent batch-gradient ion-exchange chromatography on DEAE-Sephadex A-50 followed by sucrose gradient centrifugation (Lien and Racker, 1971b), CF was obtained in high yield and purity. We have used this method (Hartig, 1976) to obtain yields comparable to the method of Lien and Racker (1971b). The purity of isolated CF was demonstrated to be greater than 95% by polyacrylamide gel electrophoresis. The fluorescence emission ratio of the purified CF ( $E_{303}/E_{350}$ ) was typically 2.4 or greater when measured using a Perkin-Elmer

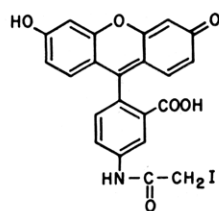
MPF 2A fluorometer with a Hamamatsu R106 photomultiplier: excitation wavelength, 280 nm; emission and excitation slits, 6-nm spectroscopic bandwidth.

**5-IAF Labeling of CF.** We have adopted a labeling technique in which the fluorescent dye is first adsorbed onto Celite (diatomaceous earth) before being introduced into the protein solution (Rinderknecht, 1960, 1962). This technique conveniently provides concentrated 5-IAF solutions for the labeling. Approximately 0.6 g of Johns-Manville acid washed Celite was heated at 350 °C for 45 min and allowed to cool to room temperature. A solution of 0.06 g of 5-IAF in 30 mL of methanol was added to the Celite and the solvent was removed at room temperature on a rotary evaporator. The dried yellow powder was stored in the dark at -25 °C. A suspension of 30 mg of Celite containing adsorbed 5-IAF in 0.2 mL of 40 mM Tricine-NaOH, 2 mM EDTA, pH 8.0, with or without aliquots of 0.1 M ATP was brought to the desired pH by addition of K<sub>3</sub>PO<sub>4</sub> or KH<sub>2</sub>PO<sub>4</sub>. A solution containing 0.5 mg of CF in 1 mL of the Tricine-EDTA buffer was added, and the pH of the reaction mixture was adjusted to the desired value with K<sub>3</sub>PO<sub>4</sub> or KH<sub>2</sub>PO<sub>4</sub>. The mixture was placed in a room-temperature shaker bath and incubated in the dark for approximately 2 h with constant agitation to keep the Celite suspended. Following the incubation, the mixture was centrifuged on a clinical centrifuge and the supernatant was applied to a 0.7 × 15 cm Sephadex G-50 column. The labeled protein was collected in the void column, free from unreacted label. It was stored at 4 °C after precipitation in 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The same results were obtained when Celite was removed from the labeling mixture, leaving behind a millimolar 5-IAF solution for the labeling reaction.

The extent of labeling was determined from the protein concentration and the absorbance of the attached dye at 498 nm. Light scattering, which was observed from the labeled protein, was corrected for by subtracting an interpolated light-scattering curve from the 5-IAF-CF absorption. The scattering curve was drawn between points at 600 and at 400 nm, where the 5-IAF absorption is known to be 0% and 6%, respectively, of the absorption peak of the attached dye at 498 nm. The stability of the covalent labeling was verified by a constancy in labeling ratio following successive desaltings on Sephadex G-50 and precipitations in 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> over several days.

A molar absorptivity of 42 000 at 498 nm was used for 5-IAF covalently attached to CF. This value was derived from the observation that denaturation of singly labeled 5-IAF-CF (labeled at pH 7.8 with ATP) in 7.5 M urea, 0.6 mM DTT at 65 °C induces a 25% increase in the 5-IAF absorption at 498 nm. Heating of free 5-IAF in the same solution induces a 5% absorption decrease at 498 nm. Assuming that the molecular environments of the chromophore will be approximately the same for 5-IAF in both cases, and taking account of a 7% degradation of the dye over the time span of labeling and handling, we calculate an apparent molar absorptivity of 42 000 for the attached label in native 5-IAF-CF.

**Protein Assay.** The protein concentration of CF preparations was determined from a specific absorptivity of 0.54 cm<sup>-1</sup> at 280 nm for a 1 mg/mL solution (Farron, 1970). In the presence of light scattering or interfering absorbance, the protein concentration was determined by a Lowry assay in 40 mM Tricine-NaOH, 2 mM EDTA, pH 8.0 (Lowry et al., 1951). When bovine serum albumin in the same buffer is used as a standard, the value obtained in the Lowry assay for CF must be multiplied by a correction factor of 0.74 to obtain the true dry weight. This is in contrast with the result obtained by Farron and Racker (1970). The buffer used by this group was



5- IODOACETAMIDOFLOURESCIN (5IAF)

FIGURE 1.

TABLE I: 5-IAF Labeling of CF.<sup>a</sup>

Reaction duration (h)	Molar ratio of 5-IAF/CF during reaction	Molar ratio of 5-IAF attached per CF
0.5	100	0.06
1	400	0.26
1	1100	0.82
2	1200	0.80
4	1200	1.04

<sup>a</sup> The labeling reaction occurs at pH 7.8 in 40 mM Tricine-NaOH, 2 mM EDTA, 2 mM ATP. The mixture is incubated at room temperature in the dark.

not specified. Our value was calibrated against both the CF absorption assay and a direct dry-weight measurement of CF. A molecular weight of 325 000 has been used in all calculations (Farron, 1970).

**ATPase and Photophosphorylation Assays.** The ATPase activity of CF or 5-IAF-CF was determined following trypsin activation of the enzyme by a minor modification (Hartig, 1976) of the procedure of Lien and Racker (1971b). ATP hydrolysis was detected by the inorganic phosphate assay of Martin and Doty (1949) and Lindberg and Ernster (1956). Trypsin activation times of 4 to 12 min were used, and the maximum rate was chosen from this set.

The photophosphorylation assay (Hartig, 1976) was derived from the methods of Shoshan and Shavit (1973) and McCarty (1971). [<sup>32</sup>P]ATP production was monitored by the method of Avron (1960).

**NaDodSO<sub>4</sub> Gel Electrophoresis.** We used the procedure of Laemmli (1970) without a stacking gel for 9% polyacrylamide gels at pH 8.8 with 0.1% NaDodSO<sub>4</sub>. Gels were stained with Coomassie blue using the procedure of Fairbanks et al. (1971). Gel samples contained approximately 0.5 mg of 5-IAF-CF in 1 mL with 10 mg of Pierce NaDodSO<sub>4</sub> and 0.02 mL of 2-mercaptoethanol. Samples were capped and incubated at 37 °C in the dark for 2 h. A few crystals of sucrose were added to each sample before application. Gels were run for approximately 5 h at 1 mA/gel.

No staining or fixation was used on gels that were scanned for 5-IAF fluorescence. Gels were removed from the running buffer immediately after the run with the glass-tube gel casing left intact. The glass tubes were secured to the TLC plate holder of a Model 018-0057 thin-layer chromatography accessory for a Perkin-Elmer MPF 2A spectrofluorometer and scanned mechanically. Excitation wavelength was 480 nm and emission wavelength was 520 nm. A nonreflective background placed behind the gels minimizes scattering artifacts.

**Absorption and Fluorescence Measurements.** We recorded absorption spectra at room temperature using a Cary Model 118 absorption spectrometer, steady-state fluorescence spectra using a Perkin-Elmer MPF 2A spectrofluorometer at ambient temperature, and corrected fluorescence spectra with a Perkin-Elmer MPF 3 spectrofluorometer. Fluorescence polar-

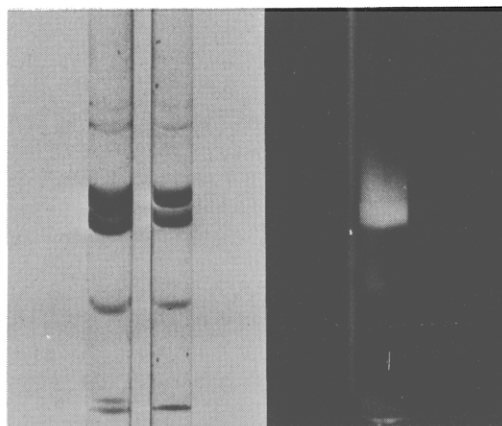


FIGURE 2: NaDodSO<sub>4</sub> gel electrophoresis of 5-IAF-CF labeled at pH 7.8 in the presence of 3.5 mM ATP. From left to right, the gels contain 27, 13, and 110 μg, respectively, of 5-IAF-CF. The photograph of the two gels on the left shows the Coomassie blue staining patterns of the subunits. The two major staining bands in the middle of the gels are the α and β subunits. The γ, δ, and ε subunits are visible toward the bottom of the gels in order of decreasing molecular weight (Nelson et al., 1973; McEvoy and Lynn, 1973). In the middle gel, which was loaded lightly, the δ and ε subunits appear to coalesce into a single band. The photograph of the unstained gel on the right was taken utilizing the green emission from the 5-IAF label as excited by an ultraviolet light source.

ization measurements were obtained on the MPF 2A fluorometer with the standard polarization accessory. A novel single-photon counting fluorescence lifetime system (Hartig et al., 1976; Leskovar et al., 1976) was used for lifetime determinations. Fluorescence polarization, *P*, and the limiting polarization in the absence of molecular motion, *P*<sub>0</sub>, were determined in 40 mM Tricine-NaOH, 2 mM EDTA, pH 8.0. The excitation wavelength was 490 nm and the emission wavelength was 520 nm with an 8-nm band-pass in the excitation and emission slits. Polarization measurements in 95% glycerol at different temperatures were extrapolated to infinite viscosity to determine *P*<sub>0</sub>.

## Results

**5-IAF-Labeled CF.** The structure of 5-iodoacetamidofluorescein (5-IAF) is shown in Figure 1. Labeling of the photophosphorylation coupling factor with 5-IAF at pH 7.8 in the presence of ATP proceeds as shown in Table I. Attachment of the label reaches a plateau with approximately one label bound per coupling factor after 1 h. Labeling under other conditions of pH and substrate proceeds quite differently as discussed later.

The subunit localization of the sites labeled at pH 7.8 in the presence of ATP was determined by separation of the subunits on NaDodSO<sub>4</sub>-polyacrylamide gels. Figure 2 shows a photograph of the Coomassie blue staining pattern and 5-IAF emission from the subunits. The majority of the 5-IAF emission clearly originates in the β subunit, with a small portion distributed among the other subunits and some tailing from the β subunit.

The thin-layer chromatography scanning accessory of the Perkin-Elmer MPF 2A can be adapted to provide spatial scans of the 5-IAF emission from NaDodSO<sub>4</sub> gels (see Experimental Section). Assuming that the emission quantum yield of 5-IAF is the same on each subunit, we can estimate the distribution of the label from the area of the emission peak associated with each subunit. From such scans, we observe that approximately 75% of the label is localized in the α and β subunits. Trailing of the β-subunit emission peak in the vicinity of the α subunit makes estimation of the relative labeling efficiencies between

TABLE II: Photophosphorylation Rates.<sup>a</sup>

Sample	$\mu\text{g}$ of CF added	Photophosphorylation rate	% of control rate	Relative efficiency of 5-IAF-CF/CF (%)
Chloroplasts		705	100	
EDTA chloroplasts		11.8	1.7	
CF + EDTA				
chloroplasts	92	172	24	
(36 $\mu\text{g}$ of Chl)	46	142	20	
5-IAF-CF + EDTA				
chloroplasts	100	116	16	72
(36 $\mu\text{g}$ of Chl)	50	85	12	62

<sup>a</sup> Cyclic photophosphorylation with PMS as the cofactor. The photophosphorylation rate is given as  $\mu\text{mol}$  of ATP produced  $\text{h}^{-1}$  ( $\text{mg}$  of chlorophyll) $^{-1}$ . The 5-IAF-CF efficiency relative to CF is normalized per  $\text{mg}$  of protein. CF was labeled with 5-IAF at pH 7.5 in the presence of 2.5 mM ATP.

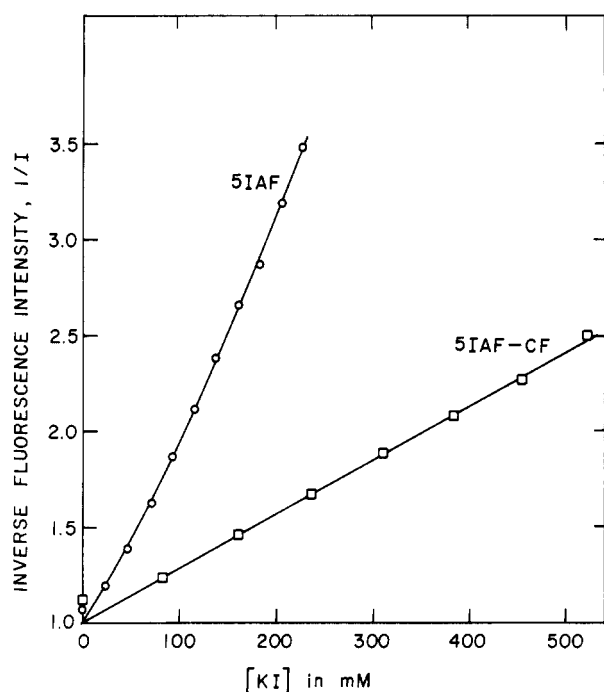


FIGURE 3: Fluorescence quenching by KI of 5-IAF and of 5-IAF-CF labeled at pH 7.8 in the presence of 4 mM ATP. The fluorescence intensity was measured at 520 nm using 490 nm exciting light.

these two subunits more difficult. From the ratio of peak heights, it appears that the ratio of label on the  $\beta$  subunit to label on the  $\alpha$  subunit is at least 4 to 1. Thus, 5-IAF labeling of CF at pH 7.8 in the presence of ATP occurs predominantly on the  $\beta$  subunit. The existence of a distinct plateau in the labeling reaction (Table I) at approximately one label attached per CF suggests that a single fast-reacting site on the  $\beta$  subunit preferentially labels under these conditions.

A calcium-dependent ATPase activity appears in CF following a brief trypsin digestion (Vambutas and Racker, 1965). We observed an average ATPase rate of  $16.5 \mu\text{mol}$  of  $\text{P}_i$  produced  $\text{min}^{-1}$  ( $\text{mg}$  of protein) $^{-1}$  for CF and a rate of 14.5 for 5-IAF-CF labeled at pH 7.9 in the presence of 2 mM ATP. Thus, approximately 88% of the native activity of CF is retained following the labeling reaction. In Table II we observed that labeled CF has the ability to restore photophosphorylation in EDTA-treated chloroplasts. At both the 100 and 50  $\mu\text{g}$  levels we find that the photophosphorylation activity in the reconstitution system using 5-IAF-labeled CF is more than 60% of that obtained using unmodified CF. The ATPase and photo-

phosphorylation data together are suggestive of a minimal perturbation of CF by the presence of the 5-IAF label. The effective retention of native activity in the labeled enzyme provides an indication that the labeled protein will provide an accurate description of processes and properties of native CF.

**Spectroscopy of 5-IAF-CF.** 5-IAF attached to CF at pH 7.8 in the presence of ATP is preferentially localized on the  $\beta$  subunit. Spectroscopic properties of the 5-IAF label provide detailed information on the microenvironment of the attachment sites. The 5-IAF absorption maximum shifts from 491 to 498 nm during labeling at pH 7.8 in the presence of ATP. In addition, the emission maximum shifts approximately 4 nm to 522 nm during labeling. The red shift in the absorption and emission maxima in 5-IAF-CF is consistent with the transfer of the label into a more hydrophobic microenvironment, as noted from solvent effects on 5-IAF absorption and emission.

The quenching of fluorescence emission by heavy ions serves as a probe for the accessibility of the fluorophore to the ionic solution phase (Lehrer, 1971; McGowan et al., 1974). Figure 3 shows a Stern-Volmer plot of the quenching of 5-IAF emission by potassium iodide for the free dye in solution and for the label attached to CF. The free dye deviates slightly from the linear behavior characteristic of pure collisional quenching. The attached label exhibits a significantly shallower slope (decreased Stern-Volmer constant) than the free label. In a control experiment, potassium chloride, a nonquenching salt, had no effect on either 5-IAF or 5-IAF-CF emission. We conclude that 5-IAF in CF labeled at pH 7.8 in the presence of ATP is only partially accessible to quenching by iodide ion from the solvent.

The steady-state fluorescence polarization of fluorescence labels reflects their rotational motion (Weber, 1953). Values of the polarization in aqueous buffer for fluorescein, 5-IAF, and 5-IAF-CF are presented in Table III along with literature polarization values and calculated hard sphere rotational correlation times. As expected, the polarization of emission from fluorescein and from 5-IAF in solution is identical, within experimental error. A polarization of 0.252, which is intermediate between the values for the free and completely immobilized dye, was determined for 5-IAF attached to CF at pH 7.8 in the presence of ATP. The CF protein is known from electron microscopy to be approximately spherical in shape with a radius of 45 Å (Lien and Racker, 1971a; Racker et al., 1972; Howell and Moudrianakis, 1967). This value agrees well with the spherical radius calculated from the known density and molecular weight (Farron, 1970). For a 5-IAF label rigidly

TABLE III: Fluorescence Polarization of Fluorescein, 5-IAF, and 5-IAF-CF.<sup>a</sup>

	Polarization <i>P</i>	Limiting polarization <i>P</i> <sub>0</sub>	Rotational correlation time (ns)
Fluorescein	0.017 <sup>b</sup>	0.44 <sup>c</sup>	
5-IAF-CF	0.252	(0.43) <sup>d</sup>	14
CF			260

<sup>a</sup> The emission polarization was measured at 23 °C in Tricine-EDTA buffer as described in the Experimental Section. Rotational correlation times for free 5-IAF and 5-IAF in 5-IAF-CF were calculated from a rigid sphere model using observed *P* and *P*<sub>0</sub> values in the Perrin equation. The rotational correlation time for CF was calculated from the known spherical radius of 45 Å. The effect of hydration has been ignored. Hydration will slightly increase the indicated correlation times. A measured fluorescence lifetime of 3.8 ns for 5-IAF has been used in correlation time calculations. CF was labeled with 5-IAF at pH 7.8 in the presence of 3 mM ATP. <sup>b</sup> Weber, 1953. <sup>c</sup> Perrin, 1926. <sup>d</sup> Observed polarization in 95% glycerol at 23 °C.

attached to a 45-Å sphere, a fluorescence polarization of 0.42 would be predicted from the Perrin equation (Perrin, 1926). Since the observed polarization for 5-IAF-CF is distinctly lower, we can conclude that considerable independent motion of the label occurs at the labeling site.

Coupling factor displays no catalytic activities when isolated from chloroplast membranes by EDTA treatment. A short trypsin digestion activates a calcium-dependent ATPase activity (Vambutas and Racker, 1965) in both CF and 5-IAF-CF, as described earlier in this paper. Addition of 0.02 mL of 5 mg/mL trypsin in 1 mM H<sub>2</sub>SO<sub>4</sub> to 1 mL of 0.022 mg/mL 5-IAF-CF (labeled at pH 7.8 in the presence of ATP) in 40 mM Tricine, 2 mM EDTA, 2 mM ATP, pH 8.0, induces an increase of approximately 20% in the 5-IAF fluorescence intensity following 1 min of incubation. The 5-IAF fluorescence continues to rise, reaching a final increase of approximately 35% after 10 min of incubation. Addition of H<sub>2</sub>SO<sub>4</sub> alone or prolonged illumination of 5-IAF-CF in the fluorometer causes no significant fluorescence changes. No changes in the position of the excitation or emission peaks are observed during trypsin treatment. The trypsin concentration used corresponds to the normal level for activation of the Ca<sup>2+</sup>-ATPase activity in CF (Lien and Racker 1971b). It is interesting to note that an absorption and fluorescence emission increase of approximately the same magnitude is observed when 5-IAF-CF is denatured by heating at 65 °C in the presence of urea and DTT. We can conclude that trypsin activation of the ATPase activity of CF involves significant changes in the vicinity of the attached chromophore.

We investigated the effect of substrate addition on the fluorescence properties of CF labeled with 5-IAF at pH 7.8 in the presence of ATP. Addition of 2 mM ADP or ATP to the labeled enzyme causes no changes in the 5-IAF emission intensity or peak position. In view of the fact that 5-IAF labeling does not greatly alter the catalytic activities of CF, we can conclude that this labeling, which occurs preferentially on the β subunit, is not at the active site.

**pH and Substrate Effects on Labeling.** Slight variations in the pH of the reaction mixture and the presence or absence of ATP have a profound effect on the extent of the labeling, as shown in Figure 4. The number of attached labels varies between 0.7 and 3.2 for pH values between 6.4 and 8.5 in the presence or absence of 3.5 mM ATP. The extent of labeling reaches a minimum of approximately one label per CF at pH 7.8 in the presence of 3.5 mM ATP and increases dramatically

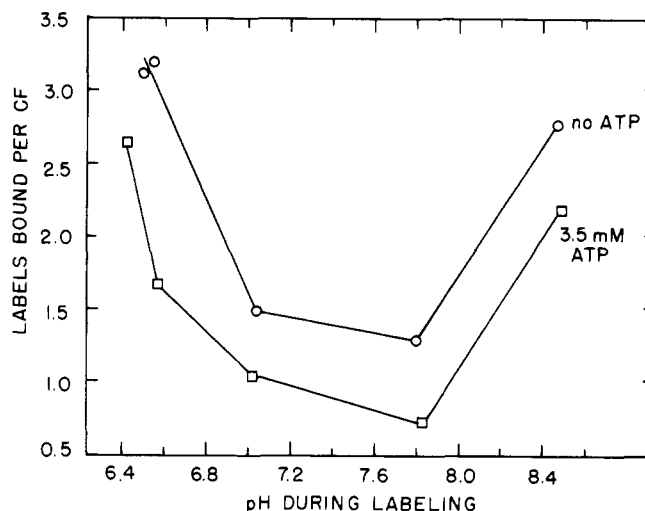


FIGURE 4: pH dependence of the 5-IAF labeling of CF. The conditions for the labeling reaction are described in the text. (○-○) Labeling reactions with no addition; (□-□) labeling reactions with 3.5 mM ATP added during the reaction.

as the pH is either raised or lowered. The effect of ATP addition is to cause a decreased labeling of 0.5 to 1.5 labels per CF over the entire pH range.

The subunit specificity of the labeling reaction was examined as a function of reaction pH and the presence or absence of ATP. Samples of 5-IAF-labeled CF produced under different reaction conditions were subjected to NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis under dissociating conditions and scanned for 5-IAF fluorescence. Figure 5 shows the fluorescence emission scan for CF labeled with 5-IAF at pH 6.6 in the presence or absence of ATP. The positions of the α, β, and γ subunits of CF (Nelson et al., 1973; McEvoy and Lynn, 1973) on the gels were determined by Coomassie blue staining of other gels in the same batch run under identical conditions. The subunit positions are marked in the figure. We did not investigate the low level of labeling which occurs on the δ and ε subunits due to problems with light scattering and a small amount of free 5-IAF running near the dye front at the bottom of the gels. The sharp spike recorded at the gel origins arises from light scattering at the top surface of the gel. The data indicate that at pH 6.6 a significant decrease in the extent of labeling of the γ subunit occurs when the ATP is added to the reaction mixture. A smaller but reproducible decrease in the labeling of the α subunit is also seen when ATP is added at this pH. Assuming a constant fluorescence quantum yield on all subunits we estimated the total number of labels attached to each subunit from the integrated emission peak areas. The decreased labeling of the γ subunit accounts for the majority of the labeling decrease which occurs when ATP is added at pH 6.6, as seen in Figure 4.

The labeling pattern which occurs at pH 8.5 in the presence or absence of ATP is shown in Figure 6. At this pH, binding of ATP to CF does not induce strong shielding of a specific subunit during the labeling reaction. Since no change is observed in the labeling ratios of specific subunits, we conclude that the decreased labeling seen in Figure 4 at pH 8.5 when ATP is added arises from a generalized decrease in the labeling rate over all three subunits, or from changes in labeling of the δ and ε subunits, which were not examined. The specific increase in labeling of the γ subunit observed in the absence of ATP at pH 6.6 does not occur at pH 8.5. Comparison of the labeling distributions at pH 6.6 and 8.5 (Figures 5 and 6) shows that the higher pH induces a much stronger relative labeling

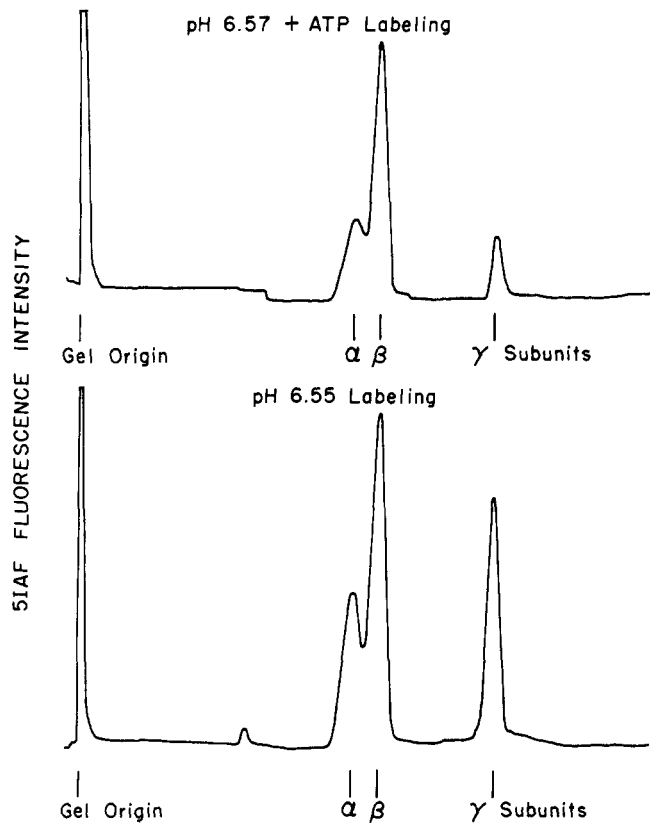


FIGURE 5: Fluorescence emission scans of NaDodSO<sub>4</sub> gels of 5-IAF-CF labeled at pH 6.6. Aliquots of CF labeled with 5-IAF at pH 6.57 in the presence of 3.5 mM ATP and labeled at pH 6.55 without ATP were run on NaDodSO<sub>4</sub>-polyacrylamide gels. The 5-IAF fluorescence was excited at 490 nm and observed at 520 nm as the gels were scanned. The positions of the major CF subunits are marked along the horizontal axis. The sharp spike at the gel origin is a light-scattering artifact.

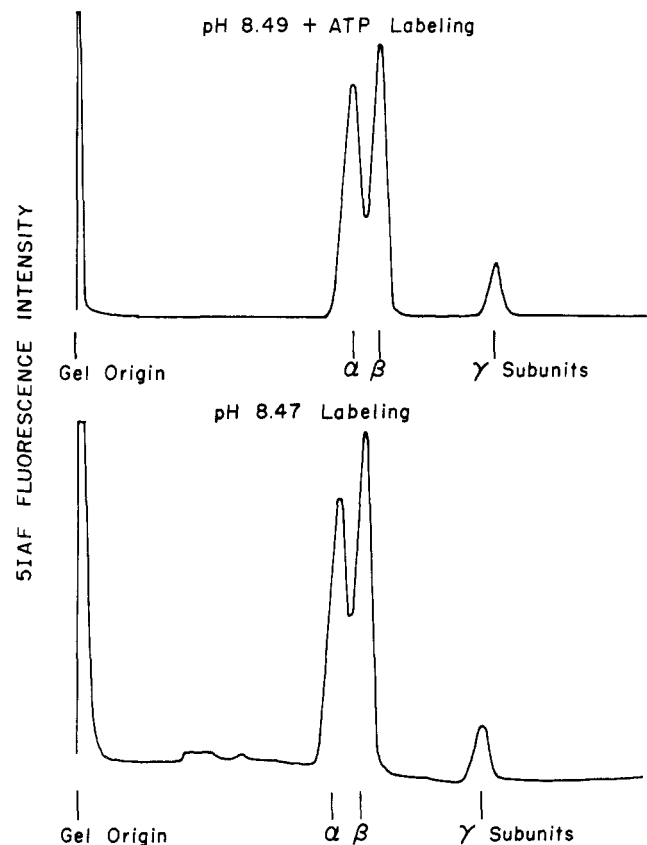


FIGURE 6: Fluorescence emission scans of NaDodSO<sub>4</sub> gels of 5-IAF-CF labeled at pH 8.5. Samples of 5-IAF-CF labeled at pH 8.49 with 3.5 mM ATP and at pH 8.47 without ATP were run on NaDodSO<sub>4</sub> gels, and the fluorescence emission was analyzed as described in the caption to Figure 5.

of the  $\alpha$  subunit in both the presence and absence of ATP. Thus, the effect of high pH is expressed primarily as an increase in the extent of labeling of the  $\alpha$  subunit.

At pH values between 6.6 and 8.5 the labeling pattern can be generally described as intermediate between the two extremes present in Figures 5 and 6. For example, labeling at pH 7.0 produces a fluorescence emission peak height ratio ( $\gamma/\beta$ ) of 0.80 in the absence of ATP and only 0.40 in the presence of ATP. Thus, at pH 7.0, as well as at pH 6.6, addition of ATP induces a selective decrease in labeling on the  $\gamma$  subunit. Labeling reactions which occur near pH 7.8 in the presence of ATP produce a low level of labeling of all subunits except the  $\beta$  subunit. Labeling reactions at pH values above 7.5 produce increased labeling of the  $\alpha$  subunit.

#### Discussion

The unusually high absorptivity and fluorescence efficiency of the fluorescein function makes 5-IAF a sensitive fluorescent probe molecule, useful at low concentrations. It can be covalently attached to proteins by the iodoacetamido moiety, which exhibits a high degree of selectivity for SH groups under certain conditions (Means and Feeney, 1971). 5-IAF possesses long-wavelength emission and excitation bands that provide freedom from interfering light scattering and protein emission artifacts. These same properties also make 5-IAF desirable as a donor or acceptor for long-range excitation transfer studies (Zukin et al., 1977).

Attachment of 5-IAF to CF at pH 7.8 in the presence of ATP results in a red shift in both the emission and excitation

maxima of the dye. This red shift is consistent with transfer of the dye to a more hydrophobic environment. Potassium iodide quenching studies on 5-IAF and 5-IAF-CF indicate that the label on the protein is partially accessible to quenching by iodide, but much less accessible than the free dye in solution. The steady-state polarization measurements show that the label demonstrates motion that is independent of the tumbling of the whole protein, but with a much longer correlation time than for the free dye. Taken together, these three independent findings suggest that 5-IAF, which is preferentially attached to the  $\beta$  subunit of CF, is in a cleft region of the protein. This region is partially hydrophobic and partially accessible to bulk solvent, and it tends to restrict the rotational motion of the label. We can further conclude that the label is remote from the enzyme active site, because the catalytic activities are only slightly affected by the presence of the label and the binding of substrate has no detectable effect on the probe's fluorescence properties. In another investigation (Deters et al., 1975), attachment of 2 equiv of NBD-Cl to tyrosine residues of the  $\beta$  subunit led to a loss of 80% of the ATPase activity of CF. We conclude that these two fluorescent probes bind to different regions of the  $\beta$  subunit, because their effects on the catalytic activities are so different.

The polarization data for 5-IAF-CF could also be interpreted in terms of a model in which the label is rigidly immobilized on the protein backbone, but the local protein chain exhibits considerable independent motion from the protein as a whole on the short time scale. The greater than tenfold difference in rotational correlation times for a rigid-sphere CF and the attached 5-IAF label seems large for local chain mo-

tion alone, but little information exists to quantitate such motions. In either case, the protein region containing 5-IAF experiences rapid local motion. Also, the spectral shift in the label may arise partially from changes in solvent shell motion or other factors in addition to solvent dielectric constant (Radda and Vanderkooi, 1972). Again, such factors are difficult to quantitate.

Photophosphorylation reconstitution studies also present certain ambiguities. DCCD can cause a significant restoration of photophosphorylation in EDTA-treated chloroplasts that have been depleted of bound CF (McCarty and Racker, 1967). This effect presumably arises from the ability of DCCD to decrease the proton permeability of the membrane and activate ATP production in the residual CF left on the membrane following EDTA treatment (McCarty and Racker, 1967; Uribe, 1972). Thus, stimulation of photophosphorylation on addition of CF does not prove that the added enzyme is active. Added CF may bind to the membrane, decrease proton permeability, and activate ATP production in residual CF without itself catalyzing ATP production. As a minimum conclusion, 5-IAF-CF does retain the ability to bind to the membrane and stimulate photophosphorylation.

Addition of ATP to CF at pH 6.6 causes a substantial decrease in the accessibility of the  $\gamma$  subunit to labeling by 5-IAF. Direct shielding of the  $\gamma$ -subunit labeling sites by local ATP binding is ruled out because ATP binds only to the  $\alpha$  and  $\beta$  subunits (Deters et al., 1975; Cantley and Hammes, 1975b). Another possibility is that the  $\alpha$  and  $\beta$  subunits shield the  $\gamma$  subunit and that the binding of ATP changes the degree of shielding. However, it is likely that the  $\gamma$  subunit is directly accessible to 5-IAF because antibody-binding studies (Nelson et al., 1973) have shown that the  $\gamma$  subunit is directly accessible from the solvent phase. In addition, energy-transfer measurements demonstrate that the tight-binding sites for ADP on the  $\beta$  subunit are approximately 47 Å from labeled cysteines on the  $\gamma$  subunit (Cantley and Hammes, 1976). Thus, direct shielding of the  $\gamma$  subunit by contact with the  $\beta$  subunit is unlikely. We conclude that a substrate-induced conformational change occurs in the  $\alpha$  or  $\beta$  subunit upon ATP binding and that this change is transmitted via intersubunit forces to induce a conformational change in the  $\gamma$  subunit. This conformational change decreases the extent of  $\gamma$  subunit labeling by 5-IAF.

pH-induced conformational changes are also revealed by changes in the labeling pattern. 5-IAF labeling at pH 7.8 most likely occurs at cysteine sulfhydryl sites, since the other potentially reactive nucleophilic amino acids are significantly less reactive at this pH (Means and Feeney, 1971). Cysteine sulfhydryl groups normally exhibit a  $pK_a$  of approximately 9 (Means and Feeney, 1971). The increased labeling that we observe between pH 8.0 and 8.5 may be due to the increase in unprotonated sulfhydryl groups that appears in this range. However, the decreased reactivity of CF observed as the pH is raised from 6.4 to 8.0 cannot be assigned to any direct effect of pH on the side-chain reactivity.

The effect of pH on the reactivity of CF to 5-IAF is also not an artifact of denaturation. The  $Ca^{2+}$ -ATPase activity of soluble CF is stable at room temperature at pH 8.0 and 6.5 (McCarty and Racker, 1966). Between pH 6.7 and 8.7 there is no pH dependence to the  $Mg^{2+}$ -ATPase rate of light-activated chloroplasts in Tricine buffer (McCarty and Racker, 1968). These data indicate that CF is stable over the physiological pH range used in this study. We conclude that the variations observed in the extent and distribution of the labeling as a function of pH can be assigned to a pH-induced conformational change. These conformational changes may be of physiological significance, since they occur over a narrow range

of pH which corresponds to the pH range thought to exist across the illuminated chloroplast membrane (Uribe and Jagendorf, 1967; Rumberg and Siggel, 1969; Heldt et al., 1973; Portis and McCarty, 1974).

The pH- and substrate-induced conformational changes revealed in these experiments are interdependent. For example, at pH 6.6 we observe that ATP addition induces conformational changes specific for the  $\gamma$  subunit. At pH 8.5, addition or deletion of ATP does not induce the same effect. A similar interdependence has been observed in conformational changes induced by substrate binding and illumination of chloroplasts (McCarty and Fagan, 1973; Ryrie and Jagendorf, 1972). The existence of these interdependent ATP-induced and pH- or illumination-induced conformational changes suggests that the active-site conformation may be sensitive to the bathing pH in solubilized CF or to the illumination-induced transmembrane proton potential in membrane-bound CF. This evidence provides support for the conformational coupling model of Boyer (1975) in which conformational forces serve to couple ATP production to the transmembrane potential. The current study suggests that pH changes which occur across the chloroplast membrane upon illumination may serve directly to induce conformational changes at the active site of CF leading to net ATP synthesis.

The binding of ATP to remote subunits ( $\alpha$  and  $\beta$ ) is sensed by the  $\gamma$  subunit via conformational changes. This conformational sensing is dependent on both substrate binding and pH. The  $\gamma$  subunit also appears to play an active role in phosphorylation coupling. Presence of the  $\gamma$  subunit is essential for photophosphorylation (Deters et al., 1975), it exhibits conformational changes on the membrane during illumination (McCarty and Fagan, 1973), and it is accessible from the solvent phase but apparently oriented toward the membrane face (Nelson et al., 1973) where it may have direct access to the transmembrane pH differential. These data suggest that the  $\gamma$  subunit may serve as an essential allosteric mediator which transmits conformational forces derived from the transmembrane proton potential to the active site for use in ATP synthesis. Alternatively, the  $\gamma$  subunit conformation may provide a passive monitor of these induced conformational changes.

5-IAF preferentially attaches to the  $\beta$  subunit of CF at pH 7.8 in the presence of ATP. The labeled enzyme has been characterized biophysically and the labeling site identified as remote from the active site, probably in a cleft region. Changes in the vicinity of the labeling site are involved in trypsin activation of the ATPase activity of CF. The ATPase activity and the ability to reconstitute photophosphorylation are retained by the labeled enzyme.

Changes in labeling efficiency under varying reaction conditions demonstrate the presence of complex pH- and substrate-induced conformational changes in CF. Conformational changes induced by substrate binding and by pH changes in solution exhibit certain interdependences. These observations on solubilized CF suggest that CF on the membrane may experience conformational changes by direct action of the transmembrane pH gradient. These conformational forces may be directed to the active site for use in net ATP synthesis. The  $\gamma$  subunit of CF appears to play a central role in the expression of these conformational changes.

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