Site-Directed Photochemical Coupling of Cytochrome b_6f-Associated Chlorophyll[†,‡]

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ABSTRACT: Cytochrome b_6f complexes contain a molecule of chlorophyll *a* (Chl*a*), which, in *Chlamydomonas reinhardtii*, can be exchanged for extraneous chlorophyll during protracted incubation of the purified complex in detergent solution. The specificity of the site and its location in the complex have been studied by photochemical coupling and circular dichroism spectroscopy. Following substitution of the original chlorophyll with [^{3}H]Chl*a*, the complex was irradiated in the Soret absorption band of Chl*a* to complete bleaching and the amount of radioactivity covalently bound to each b_6f subunit determined. Strong labeling was found to be associated with cytochrome *f*. The labeling originates from [^{3}H]Chl*a* molecules bound to a slowly exchanging site and showing the properties of the endogenous Chl, not from molecules dissolved in the detergent belt surrounding the complex. Chlorophyll *b* (Chl*b*) can compete with Chl*a*, albeit with a lower affinity. Irradiation of [^{3}H]Chl*b* introduced into the slowly exchanging site yielded the same labeling pattern that was observed with [^{3}H]Chl*a*. Proteolytic cleavage showed [^{3}H]-Chl*a* labeling to be strictly restricted to the C-terminal region of cytochrome *f*. Circular dichroism spectra of the native complex revealed a bilobed signal characteristic of excitonic interaction between chlorophylls. The structural and evolutionary implications of these findings are discussed.

In the respiratory and photosynthetic electron transfer chains of eukaryotes, cytochromes bc_1 (Cytbc_1)[1] and b_6f (Cytb_6f) perform similar functions: they transfer electrons from a liposoluble carrier to a water-soluble protein, and they transduce part of the free energy released into a transmembrane proton gradient. Among the three redox-active subunits in Cytbc_1, two, cytochrome *b* (Cyt*b*) and the iron−sulfur (Rieske) protein, have homologues in Cytb_6f, the Cytb_6− subunit IV (suIV) tandem and the chloroplastic Rieske protein, respectively (for reviews, see refs *1*−*4*). The two complexes exist as superdimers both in detergent solution (reviewed in refs *4* and *5*) and in crystals (*6*−*10*). The overall arrangement of the bc_1 and b_6f dimers (*10*) and that of their *b*-type hemes (*11*, *12*) are similar, and in both complexes, the Rieske protein exhibits a functionally important mobility (see refs *7*, *13*, and *14* and references therein). Differences are no less manifest. The two extramembrane "core" subunits of mitochondrial Cytbc_1 have functions unrelated to electron transport and no equivalent in Cytb_6f. Conversely, eukaryotic b_6f complexes feature at least four small, ∼4-kDa subunits, PetG, PetL, PetM (formerly PetX), and PetN, each presumably made up of a single transmembrane helix, which have no detectable homology to bc_1 subunits or regions thereof (see refs *15*−*17* and references therein). Functional differences have also been reported: in vivo studies for instance indicate that Cytb_6f is able to pump nonredox protons, a feature which appears to be specific to this complex (*18*−*20*). Other functional differences (see refs *1*−*4* and *19*−*23*) include a larger redox potential gap between the low-potential (*b* hemes) and high-potential (Rieske protein and c_1/*f* hemes) branches of the electron transfer pathway in the b_6f complex, as well as differences in the steps leading to quinone reduction at the reducing, *n*-side quinone-binding site (Q_i) and in the sensitivity of this site to inhibitors. Finally, in eukaryotes, Cytb_6f is involved in regulating the phosphorylation of light-harvesting complex II (LHCII) antenna proteins and, thereby, their distribution between the two photosystems (see refs *17*, *24*, and *25* and references therein). One of the most striking differences between the bc_1 and b_6f complexes is their use of unrelated membrane-anchored hemoproteins, cytochrome c_1 (Cytc_1) and cytochrome *f* (Cyt*f*), to transfer electrons from the Rieske protein to a soluble acceptor protein. Cytc_1 and Cyt*f* have no sequence similarity (*26*); their *c*-type hemes are bound in different ways (*27*) and differently oriented with respect to the membrane plane (see ref *11* and references therein), and they exhibit totally different folds (*7*, *8*, *27*). The catalytic domain of Cytc_1 is evolutionarily related to the soluble mitochondrial cytochrome *c*, while that of Cyt*f* is not: cytochromes c_1 and *f* most likely are derived from different ancestors, independently recruited in the course of convergent evolution to carry out comparable functions (*4*, *26*, *28*).

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[‡] We dedicate this paper to the memory of Matti Saraste, with whom some of the speculations presented here had been discussed.

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[1] Abbreviations: AP-HP, ammonium phosphate buffer containing HG and egg PC (see Media); β-car, β-carotene; CD, circular dichroism; Chl, chlorophyll; Cyt, cytochrome; HG, Hecameg [6-*O*-(*N*-heptylcarbamoyl)methyl-α-D-glycopyranoside]; HPLC, high-pressure liquid chromatography; LHC, light-harvesting complex; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PS, photosystem; SDS, sodium dodecyl sulfate; TAP, Tris acetate-phosphate; T-HP, Tricine buffer supplemented with HG and egg PC (see Media); TMBZ, 3,3′,5,5′-tetramethylbenzidine; TMK, Tris/magnesium/potassium (see Media); TMK-HP, TMK buffer supplemented with HG and egg PC; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine [*N*-tris-(hydroxymethyl)methylglycine].

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Perhaps the most unexpected—and, to this day, unexplained—difference between the two complexes is the presence of chlorophyll *a* (Chl*a*) in Cytb_6f. In highly purified preparations of the b_6f complex from the cyanobacteria *Synechocystis* sp. PCC 6803 (*29*, *30*) and *Mastigocladus laminosus* (*31*), from spinach (*31*, *32*), and from the unicellular freshwater alga *Chlamydomonas reinhardtii* (*15*, *31*, *33*, *34*), Chl*a* generally is present in an ~1:1 stoichiometry with Cyt*f*. Purification of the complex in the presence of radiolabeled Chl*a* and exchange rate measurements have shown it to be an authentic component of the complex, not a contaminant artifactually bound in the course of purification (*33*, *34*). Vibrational spectroscopy using either Raman resonance (*12*, *34*) or fluorescence line narrowing (*30*) techniques shows that the chlorophyll (Chl) is bound to a specific site in *C. reinhardtii* (*34*), in *Synechocystis* PCC 6803 (*30*), and in spinach (*12*). Consistent with these observations, its Q_Y electronic transition moment features a well-defined orientation with respect to the membrane plane (*11*; see also the experiments quoted in ref *29*). The presence of Chl in the complex requires efficient protection against the risk of photo-oxidation by singlet oxygen generated by energy transfer from the triplet state of excited Chl (^{3}Chl*a**) to O_2. The fluorescence of the b_6f-bound Chl is strongly quenched (*30*, *34*, *35*). In most Chl-containing systems, triplet states are quenched by carotenoids (*36*, *37*). The b_6f complexes from *C. reinhardtii* (*15*, *31*, *34*), *Synechocystis* (*30*, *38*), *M. laminosus* (*31*), and spinach (*12*, *31*) do contain carotenoids, either β-carotene (β-car) (*31*, *34*) or echinenone (*38*). Excitation energy transfer from β-car to Chl*a* (*31*) indicates that they lie close to each other. The rate of Chl bleaching in various b_6f preparations inversely correlates with their β-car content (*31*), consistent with a protective role of the latter. However, no energy transfer from ^{3}Chl*a** to β-car has yet been discovered (*30*, *31*), and quenching of the Chl singlet excited state by amino acid side chains has been proposed as the main protecting device (*30*).

The function of b_6f-bound Chl*a* remains a mystery. Bleaching of a large fraction of the Chl associated with *C. reinhardtii* b_6f does not affect the electron transfer activity of the complex in vitro, an observation that does not favor an obligatory role for Chl*a* in electron transfer (*34*). The interpretation of this experiment, however, is complicated by the fact that not all of the b_6f-associated Chl molecules are equally sensitive to bleaching (*35*). On the basis of currently available data, the possibility that Chl*a* is an evolutionary relic with no function in present-day b_6f complexes cannot be excluded (for a discussion, see ref *34*), but a functional role has by no means been definitely ruled out. Identification of the binding site of Chl*a* in the complex would both bring insight into its evolutionary origin and facilitate the design of site-directed mutations aimed at probing its possible role. We have shown previously that, upon extended incubation, the native Chl*a* in Cytb_6f slowly exchanges with an extraneously added one (*34*). Under favorable circumstances, light irradiation can lead to the formation of covalent bonds between a ligand and amino acid residues in its binding site, even in the absence of designed photoactive groups ("site-directed photochemical coupling", reviewed in ref *39*). In the series of experiments presented here, we have photochemically labeled the b_6f complex from *C. reinhardtii* with [^{3}H]Chl*a* introduced into the Chl-binding site. Cyt*f* was found to be the most heavily labeled subunit. The specificity of the site has been probed using [^{3}H]Chl*b* and its location in Cyt*f* examined by limited proteolysis. The possibility that the two Chl*a* molecules in a b_6f dimer interact excitonically has been investigated by circular dichroism (CD) spectroscopy.

EXPERIMENTAL PROCEDURES

Materials. Decylplastoquinone, Tricine, egg yolk L-α-phosphatidylcholine (PC), phenylmethanesulfonyl fluoride, ϵ-aminocaproic acid, benzamidine, papain, sodium ascorbate, potassium ferricyanide, sodium hydrosulfite, and sucrose were purchased from Sigma. Ethanol (analytical grade) and acetone (for UV spectroscopy) were from Prolabo. Benzene (Uvasol) was from Merck. Sodium dodecyl sulfate (SDS) was from Pierce. Hecameg (HG) was from Vegatec (Villejuif, France). Hydroxylapatite was from Bio-Rad. Dithiothreitol was from Boehringer Mannheim. Chl*a*, 3,3′,5,5′-tetramethylbenzidine (TMBZ), and *N*-ethylmaleimide were from Fluka Chemie AG. Urea was from Tebu. Sodium [^{3}H]acetate (~300 MBq/mol) was from ICN or Amersham. Lumasolve and Lipoluma counting media were from Packard Instruments. Acetonitrile and methylene chloride were from SDS.

Media. TMK buffer consisted of Tricine-NaOH (20 mM, pH 8.0), 3 mM $MgCl_2$, and 3 mM KCl. AP-HP buffer consisted of 400 mM ammonium phosphate (pH 8.0), 20 mM HG, 0.1 g/L egg PC, protease inhibitors (200 μM phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 5 mM ϵ-aminocaproic acid). T-HP buffer consisted of 20 mM Tricine-NaOH (pH 8.0), 20 mM HG, and 0.1 g/L egg PC. TAP medium consisted of Tris acetate-phosphate medium (*40*).

Strain and Growth Conditions. Wild-type *C. reinhardtii* (strain WT12$^-$) was grown in 20 L of TAP medium at 25 °C under 300−400 lx illumination until the stationary phase (~10^7 cells/mL), as described in ref *15*. Cells were harvested at 5000*g* for 10 min at 4 °C.

Preparative and Analytical Techniques. Thylakoid membranes were prepared as described in ref *17* and resuspended in 10 mM Tricine-NaOH (pH 8.0) containing protease inhibitors at the same concentrations as in AP-HP buffer (see Media). The Cytb_6f complex was purified as previously described (*15*). UV−visible absorption spectra were recorded on a Hewlett-Packard HP 8453 spectrophotometer. For this article, we have revised the extinction coefficients used in our previous papers for quantification of Cyt*f* and Chl*a* in b_6f preparations in the following manner:

(A) The extinction coefficient of Cyt*f* has been reinvestigated using the pyridine hemochrome method, yielding revised $\Delta\epsilon_{554}$ values (ascorbate-reduced minus ferricyanide-oxidized) of 26 200 and 25 200 M^{-1} cm^{-1} for turnip and spinach Cyt*f*, respectively (*41*). The value for *C. reinhardtii* Cyt*f* is not known. For the sake of comparison with the work of others, we have used here a $\Delta\epsilon_{554}$ of 26 000 M^{-1} cm^{-1}, ΔA_{554} being measured by reference to a baseline joining isosbestic points at 542 and 561 nm (*15*). b_6f complex concentrations are expressed as the concentration of heme *f*.

(B) The Chl*a* concentration was determined as described previously from the absorbance at 667 nm measured by reference to a line joining points at 645 and 700 nm. Instead

Table 1: Synopsis of Photochemical Coupling Experiments

expt no.	label	separation of $b_6 f$ from free [^{3}H]Chl	addition of unlabeled free Chl	total radioactivity in irradiated sample (cpm)	irradiation wavelength (nm)	radioactivity in the acetone pellet: before irradiation (cpm)	radioactivity in the acetone pellet: after irradiation (cpm)	type of gel[a]	analysis of gel[b]	relative labeling: Cyt*f*	Rieske	Cytb_6	suIV	4 kDa
1	[^{3}H]Chl*a*	−	−	630 000	430	∼1000	18000	A	1	1	0.11	0.13	0.17	0.67
2	[^{3}H]Chl*a*	+	−	280 000	430	∼500	14200	A	1	1	0.20	0.27	0.07	0.25
3	[^{3}H]Chl*a*	−	−	236 280	430	nd[c]	15300	A	2	1	0.03	0.15	0.06	0.61
4	[^{3}H]Chl*a*	−	−	118 240	430	nd[c]	nd[c]	A	2	1	0.02[d]	0.17	0.04	0.42
5	[^{3}H]Chl*a*	+	−	53 250	430	nd[c]	5730	B	2	1	0.10	0.24	0.18	nd[c]
6	[^{3}H]Chl*a*	+	+	53 250	430	nd[c]	5380	B	2	1	0.15	0.20	0.15	nd[c]
7	[^{3}H]Chl*a*	+	−	53 250	260	nd[c]	6210	B	2	1	0.18	0.42	0.14	nd[c]
8	[^{3}H]Chl*a*	+	+	53 250	260	nd[c]	5350	B	2	1	0.22	0.39	0.18	nd[c]
9	[^{3}H]Chl*b*	+	−	58 070	455	nd[c]	3990	B	2	1	0.30	0.08	0.19	nd[c]
10	[^{3}H]Chl*b*	+	+	58 070	455	nd[c]	3880	B	2	1	0.17	0.17	0.06	nd[c]
11	[^{3}H]Chl*b*	+	−	58 070	260	nd[c]	4030	B	2	1	0.30	0.33	0.13	nd[c]
12	[^{3}H]Chl*b*	+	+	58 070	260	nd[c]	4350	B	2	1	0.26	0.28	0.11	nd[c]

[a] A, 12−18% polyacrylamide gels containing 8 M urea; B, 15% acrylamide gels. [b] For 1, individual protein bands were excised; for 2, the whole relevant region of the gels was cut into small strips. [c] Not determined. [d] After photoirradiation and incubation of the $b_6 f$ complex with 70 mM HG, the Rieske protein was separated on a hydroxylapatite column.

of an ϵ_{667} of 75 000 M^{-1} cm^{-1}, however, we use here an ϵ_{667} of 77 000 M^{-1} cm^{-1}, which appears to be more appropriate if one combines the absolute extinction coefficient for Chl*a* in acetone reported in ref *42* and the shape of the Chl*a* spectrum (*43*). The extinction coefficient for Chl*b* (ϵ_{647}) was taken to be 47 000 M^{-1} cm^{-1}, without baseline subtraction (*44*).

Electrophoretic fractionation of $b_6 f$ polypeptides was carried out either on 12−18% polyacrylamide gels containing 8 M urea, as described in refs *15* and *45*, or on 15% gels containing no urea as described in ref *45*. Gel staining, using TMBZ for hemes and either Coomassie brilliant blue or silver nitrate for proteins, was performed as described in ref *15*.

Preparation of [^{3}H]Chlorophylls a and b. Wild-type *C. reinhardtii* cells were grown in TAP medium under standard conditions until the stationary phase, diluted 10-fold into 200 mL of TAP medium containing 3.7 GBq of sodium [^{3}H]-acetate, and further grown under ∼1000 lx to the stationary phase. Cells were harvested and thylakoid membranes prepared as described above. Radiolabeled thylakoid membranes were collected at 50000*g* for 10 min at 4 °C. The resulting pellet was resuspended in 10 volumes of ice-cold 100% acetone under vigorous stirring. Precipitated proteins were spun down at 5000*g* for 10 min. The supernatant was collected, evaporated to dryness in a rotary evaporator, and stored under argon at −80 °C. For reversed phase HPLC fractionation, ∼1 mg of pigments was redissolved in 100 μL of absolute ethanol. A 20-μL fraction was injected onto a Zorbax-C18 column (Interchim; 4 mm × 250 mm, 5-μm granulometry) equilibrated with an acetonitrile/methylene chloride/methanol mixture (58:12:30, v/v/v). Elution proceeded in two steps at a rate of 1 mL/min. (i) Over the course of 16 min, the solvent mixture used for equilibration was applied to purify chlorophylls *b* and *a*. (ii) Over the course of the next 10 min, 100% methylene chloride was used to elute α- and β-carotenes. Detection of Chl and carotenoids was performed at 450 nm. The fractions containing Chl*a* and Chl*b* were separately pooled, and the purity of the preparations was controlled spectroscopically (not shown) and by HPLC (see ref *34*). The specific activity of the two [^{3}H]Chl preparations used for the experiments described in this article was ∼8×10^{13} cpm/mol. Purified pigments were evaporated to dryness in a rotary evaporator and stored under argon in the dark at −80 °C.

Incubation of Purified $b_6 f$ with [^{3}H]Chlorophylls. A 500-μL sample of purified $b_6 f$ (5 μM in AP-HP buffer, containing ∼2.5 nmol of b_6 *f*-bound Chl*a*) was incubated for 4 weeks at 0 °C in a glass flask in the dark, under argon, with 10 nmol of [^{3}H]Chl*a*. In most photolabeling experiments, the complex was repurified prior to irradiation to eliminate inactivated $b_6 f$ and unbound [^{3}H]Chl*a*: the incubated sample was layered on a 10 to 30% (w/w) sucrose gradient in T-HP buffer and centrifuged at 35 000 rpm (200000*g*) for 15 h in the SW41 Ti rotor of an L8 ultracentrifuge (Beckman). The brown band of the $b_6 f$ dimer was collected with a syringe. Incubation with [^{3}H]Chl*b* was performed in an identical manner, except for using a 5.3-fold excess of [^{3}H]Chl*b*.

Photoinduced Cross-Linking and Radioactivity Measurements. $b_6 f$-bound [^{3}H]Chl*a* was irradiated with monochromatic light at 10 °C at 430 or 260 nm in a quartz cuvette, using the optical irradiation bench described in ref *46* equipped with a 1000 W Osram mercury lamp. [^{3}H]Chl*b* was irradiated at 455 or 260 nm. Irradiation either for ∼20 min in the Soret band or for ∼40 min at 260 nm resulted in 90−95% bleaching of the visible absorbance peak of either Chl. Intact [^{3}H]Chl and those degradation products that had not covalently reacted with the protein were extracted with 5 volumes of ice-cold 80% acetone. Precipitated b_6 *f* was spun down at 5000*g* for 10 min, and the pellet was washed once more with the same volume of acetone. The pellet was resuspended and submitted to SDS−PAGE. After staining with TMBZ and Coomassie brilliant blue had been carried out, either individual protein bands were excised or the whole relevant region of the gels was cut into ∼1 mm × 10 mm strips. Individual bands or pairs of successive strips were incubated overnight at 55 °C in a counting vial containing a mixture of 10% Lumasolve and 90% Lipoluma. The amount of radioactivity was counted in a Beckman type LS 1801 liquid scintillation counter.

In one series of experiments (line 4 in Table 1), the Rieske protein was separated from the rest of the complex prior to SDS−PAGE in the following manner: following irradiation, Cyt$b_6 f$ was supplemented with HG to reach 70 mM and incubated for 30 min on ice, a treatment that causes

dissociation of the Rieske protein and monomerization of the complex (*5*). The preparation was then adsorbed onto a hydroxylapatite column equilibrated with 20 mM Tricine-NaOH buffer (pH 8.0) containing 30 mM HG, and the Rieske protein eluted with 150 mM ammonium phosphate (pH 8.0) containing 30 mM HG. It is likely, although this point was not examined in these particular experiments, that this procedure also removed most if not all of the PetL subunit (see ref *5*). The rest of the complex was eluted with 400 mM ammonium phosphate (pH 8.0) containing 30 mM HG. The two fractions were acetone-precipitated and analyzed by SDS−PAGE and scintillation counting as described above.

Limited Proteolysis. The b_6f complex was purified using the standard protocol (*15*), except for omitting protease inhibitors during hydroxylapatite chromatography. Papain was added at a ratio of 1:130 (w/w) and incubation performed at 37 °C for 10 min. Digestion was stopped with 20 mM *N*-ethylmaleimide. Samples were either kept in an ice bath or frozen at −80 °C before SDS−PAGE analysis was performed on 12−18% polyacrylamide gels containing 8 M urea.

Immunoblotting. Proteins were electrotransferred onto Immobilon NC membranes in a semidry blotting system at 0.8 mA/cm^2 for ∼30 min. An antiserum raised against a peptide mimicking the C-terminus of Cyt*f* (*15*) was used at a 1:5000 dilution and detected using the enhanced chemiluminescence peroxidase method (Amersham).

Circular Dichroism Measurements. The dimeric b_6f complex was separated from the monomeric form and from free Chl*a* by centrifugation on 10 to 30% sucrose gradients in T-HP buffer, as described in ref *5*. CD spectra in the red region of the spectrum were recorded on a Mark V Jobin & Yvon spectropolarimeter, using 2.4 mL samples in 1-cm path length optical glass cuvettes (Hellma, OS 1000 series 181). Samples were analyzed either without treatment or following reduction by solid ascorbate or dithionite. Spectra were scanned between 590 and 710 nm in 0.2-nm steps, using a response time of 1 s and a sensitivity of 1×10^{-6}. The spectra that are shown are the average of four scans minus that of four buffer blanks recorded under the same redox conditions. Control spectra of Chl*a* in an organic solvent or detergent solution were recorded under the same conditions. Extended CD spectra of Chl*a*′ (a kind gift of M. Kobayashi, Tokyo University, Tokyo, Japan), Chl*a*, and whole pigments extracted from purified b_6f by acetone were recorded on a CD6 Jobin & Yvon spectropolarimeter. Samples (0.8 mL) containing 10 μM Chl in benzene were placed in a 0.5-mm path length quartz cuvette (Hellma QS121). Spectra were scanned once from 340 to 710 nm, and the solvent background was subtracted. The CD spectrum of Chl in organic solvents depends on the nature of the solvent, which accounts for the differences between acetonic and benzenic extracts (Figure 5).

RESULTS

Chlorophyll Exchange within Native Cytochrome b_6f. Chl*a* is firmly bound to native Cytb_6f (*34*). However, upon extended incubation in mixed lipid/detergent micelles in the presence of an excess of [^{3}H]Chl*a*, it slowly exchanges for the radioactive Chl (*34*). The extent of Chl*a* exchange in these experiments was monitored by separating aliquots of b_6f from free Chl*a* on a sucrose gradient (*34*) and determining the specific activity of Chl*a* bound to the b_6f dimer. The molar ratio of Chl*a* to Cyt *f* in these aliquots varied from 0.9 to 1.25, and their enzymatic activity was similar to that of native preparations. After 4 weeks, equilibration was nearly complete. Kinetics were close to what should be expected if the native b_6f-bound Chl*a* exchanges with [^{3}H]-Chl*a* with a half-time of ∼10 days, although there was some indication of a fast phase involving a small fraction of the sites (not shown; cf. ref *34*). Incubation in the presence of detergent is deleterious to the b_6f complex from *C. reinhardtii*, which first loses the Rieske subunit (and thereby its electron transfer enzymatic activity), then releases its Chl, monomerizes, and finally loses the PetL subunit (*5*). Consistent with previous observations (*47*), a 4-week incubation of *C. reinhardtii* b_6f in the presence of mixed micelles containing a 4-fold excess of [^{3}H]Chl*a* resulted in the inactivation and monomerization of approximately half of the enzymatically active, dimeric complexes present in the original preparations (not shown). The remaining dimers, however, once isolated by sucrose gradient ultracentrifugation, featured the same enzymatic activity and Chl*a*:Cyt*f* molar ratio as fresh preparations. The specific radioactivity of b_6f-bound Chl*a* was close to that of free Chl*a*, indicating that the exchange had essentially reached equilibrium and had affected most of the Chl-binding sites. The position of the Chl Q_Y absorption band, 667−668 nm, was characteristic of Chl*a* bound to unaltered b_6f sites (*34*). Raman resonance spectra recorded on parallel samples incubated with identical concentrations of unlabeled Chl*a* under the same conditions and washed free of unbound Chl by sucrose gradient fractionation revealed no difference in the liganding of the Chl*a* molecules as compared to that in freshly purified complexes (B. Robert, personal communication; see ref *34*).

Photochemical Coupling of [^{3}H]Chlorophyll a to Cytochrome b_6f Subunits. Following Chl exchange, Cytb_6f was irradiated with 430-nm light. After ∼20 min, 90−95% of the Chl was bleached. Unbound [^{3}H]Chl*a* degradation products were removed by two acetone washes. Five to twelve percent of the radioactivity initially noncovalently associated with the complex precipitated with the acetone pellet following irradiation, against <0.2% in the absence thereof (Table 1), depending on the experiment. The b_6f subunits were then separated by SDS−PAGE. Following silver staining, the electrophoretic pattern of irradiated b_6f showed no significant alteration as compared to that of the native complex (Figure 1). Protein bands were excised from the gels (rows 1 and 2 of Table 1), or the whole relevant region of the gels was cut into thin strips (rows 3−12 of Table 1 and Figures 2 and 4). The amount of radioactivity associated with each subunit was determined by liquid scintillation counting. Tritium was found to be principally present in the band containing Cyt*f* and in that containing the four low-M_r subunits (Figure 2A and rows 1 and 2 of Table 1). No significant level of radioactivity was associated with any band if the irradiation step was omitted (Figure 2B).

To rule out the possibility that Cyt*f* would preferentially but nonspecifically react with any light-activated molecules of Chl*a* present in the detergent belt surrounding the transmembrane region of the complex, control samples were

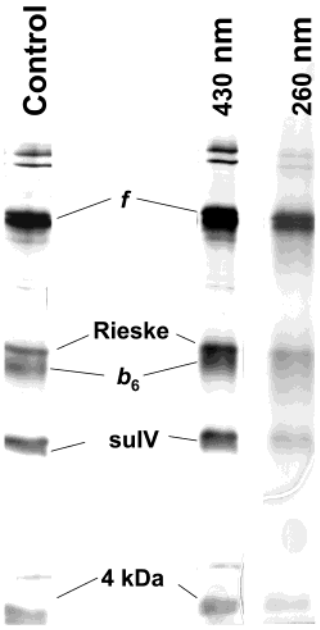

FIGURE 1: Urea/SDS−PAGE analysis of the purified $b_6 f$ complex before (left) and after (right) a 20 min irradiation at 430 nm or a 40-min irradiation at 260 nm. Heme staining followed by silver staining.

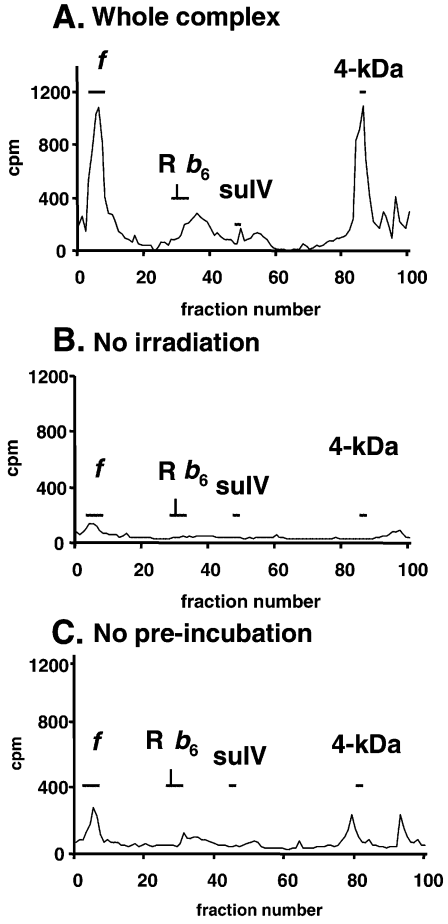

FIGURE 2: Labeling pattern of the $b_6 f$ complex with [^{3}H]Chl*a* and control experiments. (A) Cyt$b_6 f$ was incubated for 4 weeks with [^{3}H]Chl*a*. The complex was not separated from excess [^{3}H]Chl*a* before a 20-min irradiation at 430 nm. Following irradiation, the preparation was extracted with acetone and the pellet analyzed by SDS−PAGE. The gel was stained with TMBZ and silver nitrate and the relevant region cut into ~200 strips (1-mm wide), the amount of radioactivity of which was determined by liquid scintillation counting; one "fraction" corresponds to the pool of two successive strips. Data that were summed to estimate the amount of radioactivity associated with each subunit (row 3 of Table 1), located by TMBZ and silver staining, are indicated by horizontal bars. (B) Control without irradiation. The sample was treated and analyzed as described for panel A, except that the irradiation step was omitted. (C) Control without preincubation. The sample was treated and analyzed as described for panel A, except that [^{3}H]-Chl*a* was added immediately before irradiation rather than 4 weeks before.

irradiated immediately after addition of [^{3}H]Chl*a*, before extensive exchange with the native, unlabeled Chl*a* could take place. These samples showed a pattern akin to that obtained after protracted incubation, but with a much reduced intensity (Figure 2C). This observation indicates that most of the covalent labeling originates from slowly exchangeable Chl*a*. At least part of the limited amount of labeling observed in this experiment is likely to be due to a fraction of the sites exchanging during the irradiation (cf. Experimental Procedures). Removing free [^{3}H]Chl*a* from the $b_6 f$'s environment by repurifying the complex on a sucrose gradient prior to irradiation had no effect on the prevalence of Cyt*f* labeling (row 1 vs 2, Table 1), further supporting the conclusion that this subunit reacts with Chl bound to the slowly exchanging site. It did, on the other hand, decrease the amount of radioactivity comigrating with the low-M_r subunits (row 1 vs 2, Table 1).

When the environment of the $b_6 f$ complex was supplemented, after incubation and removal of free [^{3}H]Chl*a*, with a 5-fold excess of unlabeled Chl*a*, neither the extent nor the pattern of photolabeling was affected either (row 5 vs 6, Table 1). This observation further confirmed that most of the labeling originates from molecules that do not exchange rapidly with their environment, since rapid exchange should have displaced them to the detergent phase, from which, as shown above, labeling is inefficient. The absence of any change in labeling intensity indicates that the excess unlabeled Chl, although it was also excited and bleached, did not compete with excited [^{3}H]Chl*a* for binding to the protein.

In the SDS−PAGE system used for the experiments of Figure 2, the Rieske protein and Cytb_6 are not well resolved one from another (Figure 1). Subsequent experiments using a different gel system, which does not resolve the 4-kDa subunits from the migration front but cleanly separates the Rieske protein from Cytb_6, indicated the presence of a comparable, relatively small amount of radioactivity at positions corresponding to Cytb_6, the Rieske protein, and suIV (rows 5 and 6 of Table 1). To further ascertain whether the Rieske protein was labeled, it was dissociated from the photolabeled complex by treatment with an excess of detergent (*5*) and separated from the Rieske-depleted complex (see Experimental Procedures). No radioactivity was found to elute with it (row 4 of Table 1). A significant fraction of the radioactivity that migrated in the region of the 4 kDa subunits in the untreated samples appeared to be either displaced or lost following detergent treatment (row 3 vs 4, Table 1). While this treatment is known to remove at least one of the small subunits, PetL (*5*), it is also possible that part of the radioactivity in this region of the gels represents lipid labeling.

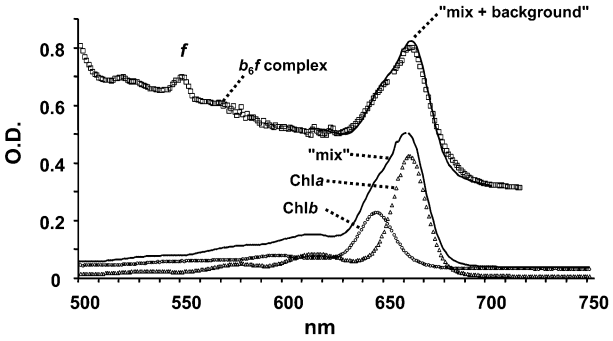

FIGURE 3: Absorption spectrum of a preparation of the b_6 *f* complex incubated with [^{3}H]Chl*b*. The purified b_6 *f* complex was incubated for 4 weeks under argon in the dark at 0 °C with a 5.3-fold excess of [^{3}H]Chl*b*; the complex was separated from free chlorophylls by ultracentrifugation on a sucrose gradient and its visible spectrum recorded; it is shown here after being shifted by 2 nm to the blue (□). Absorption spectra of Chl*a* (△) and Chl*b* (○) in acetone were summed in a 57:43 molar ratio ("mix", solid line, bottom) and superimposed on a linear background to fit the blue-shifted experimental spectrum (solid line, top). The concentration of Cyt*f* was determined from a separate spectrum after reduction of the sample with ascorbate.

Irradiation at 260 nm, while also exciting the chlorin ring, makes it in principle possible for excited amino acid side chains to react with any region of the Chl molecule, including the phytol chain. Excitation at this shorter wavelength yielded photolabeling patterns similar to those obtained following illumination at 430 nm, except for some increase in the amount of radioactivity found at the position of Cytb_6 (rows 7 and 8 of Table 1). Weakening and blurring of the protein bands were apparent upon SDS−PAGE, indicative of radiation damage to the proteins (Figure 1).

Photolabeling with Tritiated Chlorophyll b. The low Chl*b*:Chl*a* ratio of purified b_6 *f* complex preparations (∼1:10, as compared to ∼1:2 in *Chlamydomonas* thylakoid membranes) suggests that the b_6 *f* binding site is specific for Chl*a* (*34*). Assuming that the ∼1:10 ratio reflects the occupancy of the site (certainly an overestimate given the probability of some contamination) would imply a binding constant ratio of ∼5:1 in favor of Chl*a*. Incubation of Cytb_6 *f* with a 5:1 Chl*b*:Chl*a* ratio therefore ought to result in approximately equivalent occupancies of the site. Following a 4-week incubation with a 5.3-fold excess of [^{3}H]Chl*b* over Chl*a*, the complex was separated from free Chl by sucrose gradient centrifugation. Decomposition of the visible absorption spectrum (Figure 3) indicated that the Chl comigrating with the complex (1.17 molecules/Cyt*f*) was comprised of Chl*b* and Chl*a* in an ∼43:57 molar ratio. Depending on whether the excess Chl (∼0.17 mol/mol of Cyt*f*) is assumed to be pure Chl*b* or to have the same composition as that in the binding site, this indicates that between ∼33 and ∼43% of the sites were occupied by Chl*b* (corresponding to dissociation constant ratios in the 20−40:1 range).

Irradiation of the repurified complex at 455 nm (the absorption maximum of Chl*b* in the Soret region) resulted in the same labeling pattern that was observed with [^{3}H]-Chl*a*, independent of whether an excess of unlabeled Chl*b* was added prior to irradiation (rows 9 and 10 of Table 1). Labeling, therefore, again occurred at a slowly exchanging Chl-binding site, most likely the same as that labeled by [^{3}H]-Chl*a*. Irradiation at 260 nm resulted in a pattern similar to that obtained with [^{3}H]Chl*a* under the same conditions (rows 11 and 12 of Table 1).

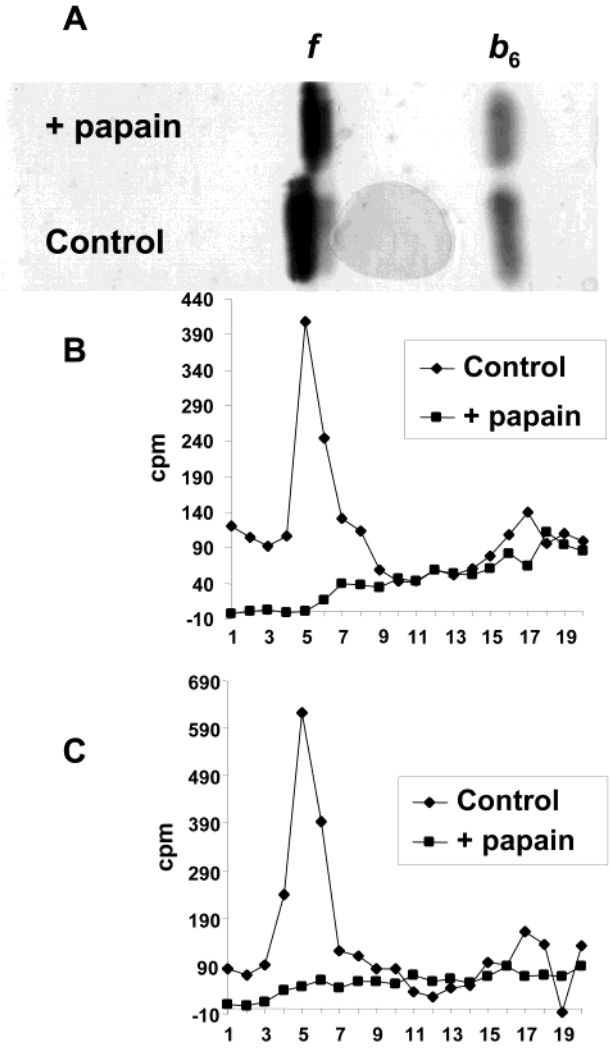

FIGURE 4: Papain digestion of the b_6 *f* complex photocoupled to [^{3}H]Chl*a*. (A) Urea/SDS−PAGE analysis of the photolabeled b_6 *f* complex before and after proteolysis with papain (see Experimental Procedures); the gel was stained with TMBZ. (B) The lanes shown in panel A were cut into thin strips, the amount of radioactivity of which was determined by scintillation counting as described in the legend of Figure 2: (◆) before proteolysis and (■) after proteolysis. The dimensions of the graph were adjusted so that fraction numbers match band positions in the gel above it. Panel C is a repeat of panel B.

Proteolytic Digestion of Photolabeled Cytochrome f. Following limited proteolysis of Cytb_6 *f* with papain, the apparent M_r of Cyt*f* diminished slightly (Figure 4A). TMBZ staining indicated that the large Cyt*f* fragment still contained the heme (which is covalently bound to cysteine residues 21 and 24) (Figure 4A). Edman degradation yielded the Tyr-Pro-Val-Phe-Ala sequence, i.e., the N-terminal sequence of mature *C. reinhardtii* Cyt*f* (*15*). An anti-C-terminal antiserum raised against a synthetic peptide corresponding to the 16 C-terminal residues of Cyt*f* (*15*), on the other hand, labeled the intact protein, but not the papain fragment (not shown). Papain cleavage therefore removes a short C-terminal fragment of Cyt*f*. The apparent M_r of the N-terminal fragment in SDS−PAGE was slightly higher than that of a soluble fragment of Cyt*f* obtained by inserting a stop codon after that encoding Ile252 (*48*), suggesting that papain cleavage

occurs downstream of this residue (not shown).

Cyt$b_6 f$ was photolabeled with [^{3}H]Chl*a*, treated with papain, and acetone-extracted, and the intact and proteolyzed complexes were analyzed by SDS−PAGE. TMBZ staining revealed, in addition to the large N-terminal Cyt*f* fragment, Cytb_6, with a reduced intensity, and a number of heme-containing fragments that can originate from either cytochrome (Figure 4A). The gels were cut into thin strips, the amount of radioactivity of which was measured. The untreated complex presented the usual photolabeling pattern, with strong labeling comigrating with Cyt*f*. Following papain treatment, radioactivity, on the contrary, was totally absent from the band containing the large N-terminal Cyt*f* fragment (Figure 4B,C). The amount of radioactivity present in the low-M_r region of the gel increased, yielding a complex pattern that made identification and recovery of the clipped fragment(s) problematical (not shown).

Evidence for Excitonic Interaction between the Two Molecules of Chla in a $b_6 f$ Dimer. The CD spectrum of $b_6 f$-associated Chl was examined with the aim of establishing constraints on the distance separating the two Chl*a* molecules within a $b_6 f$ dimer. The extended CD spectrum in benzene of whole pigments extracted from the purified complex showed that the $b_6 f$-associated Chl indeed is Chl*a*, not Chl*a*′ (Figure 5A). This identification was confirmed by HPLC analysis according to ref *49*: under our experimental conditions, Chl*a*′ eluted after 4.97 min and Chl*a* and the $b_6 f$-associated Chl after 7.12 min (not shown; we verified that Chl*a*′ does not epimerize to Chl*a* when added to the purified $b_6 f$ complex prior to Chl extraction). When the intact $b_6 f$ complex in HG/lipid mixed micelles was examined in the red region of the spectrum, a bilobed signal centered at ∼673 nm was recorded, characteristic of excitonic coupling between two molecules of Chl*a* (Figure 5C). Monolobed, negative signals were observed with pure Chl*a* dissolved in either detergent, ethanol, or acetone (Figure 5B). The bilobed signal from intact $b_6 f$ is asymmetrical, and clearly results from the superposition of a symmetrical, conservative excitonic component and a negative signal similar to that of free Chl.[2] We have reported previously that the absorbance of the $b_6 f$-associated Chl*a* is insensitive to the redox state of the hemes (*15*). Similarly, the excitonic Chl*a* signal was not affected by the addition of either ascorbate or dithionite (*35*). When free Chl*a* was added to the complex, the excitonic signal did not change; it simply became superimposed onto the larger monolobed signal of free Chl (*35*). These data are consistent with the excitonic component of the signal originating from specifically bound Chl*a* molecules.

Further investigations, however, revealed a complex situation. Namely, upon illumination of Chl*a* in situ with red light (λ > 635 nm), the CD signal proved to be more resistant to bleaching than the Chl*a* visible absorbance (*35*). This observation implies that only a subpopulation of the $b_6 f$-bound Chl contributes to the CD signal. This point will be analyzed in more detail elsewhere.

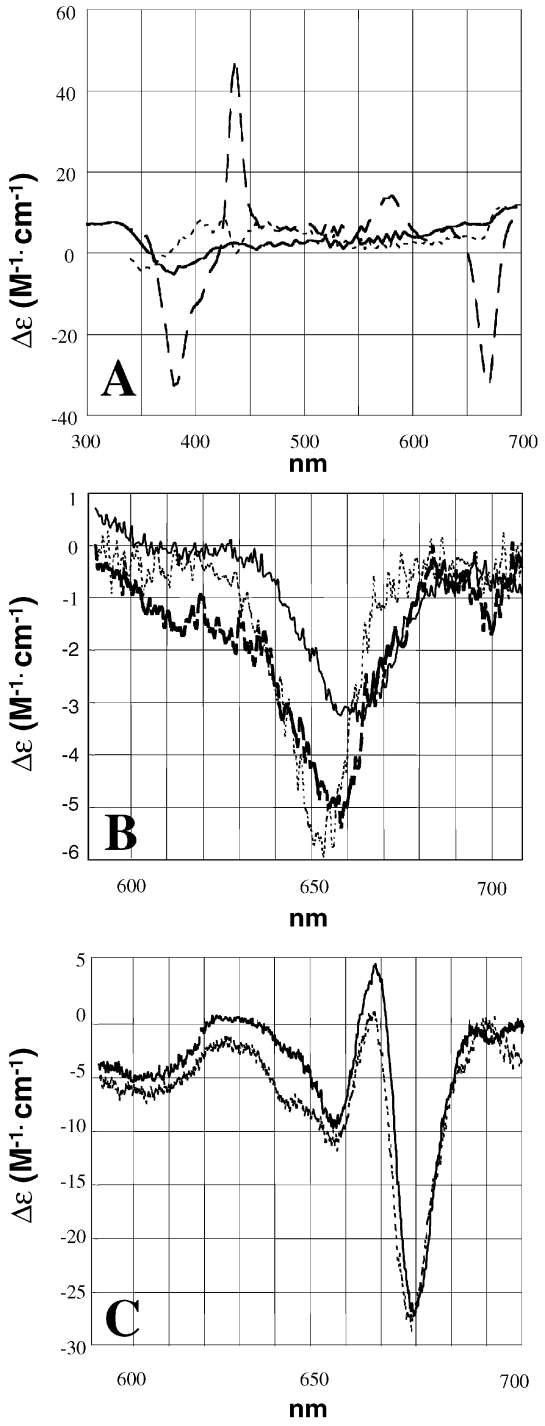

FIGURE 5: Circular dichroism spectra of (A) Chl*a* (···), Chl*a*′ (− − −), and the acetonic extract from purified $b_6 f$ (—) adjusted to 10 μM Chl in benzene, (B) Chl*a* (∼1 μM) in 20 mM HG (—), in absolute ethanol (− − −), or in acetone (···), and (C) the purified dimeric $b_6 f$ complex in AP-HP buffer. The superimposed data are from two distinct preparations.

DISCUSSION

Cytochrome f Forms Part of the Binding Site of Chlorophyll a. Following the exchange of the $b_6 f$-bound Chl*a* molecule for [^{3}H]Chl*a* and irradiation at 430 nm, covalently bound radioactivity was found to be associated predominantly with Cyt*f*. The low yield of labeling (0.1−2.7% of the amount of [^{3}H]Chl*a* introduced into the site) is typical for site-directed photocoupling (*39*). Some radioactivity, the specificity of which is more difficult to ascertain, also comigrated with the small, ∼4-kDa subunits. Labeling of Cytb_6, the Rieske protein, and suIV was weak. That the binding to Cyt*f* is a consequence of a light-induced reaction between this subunit and Chl*a* specifically bound to a slowly exchanging site is supported by the following observations:

(i) [^{3}H]Chl*a*, even if present in a 4-fold molar excess over the complex, reacts only weakly with it in the absence of protracted incubation (4 weeks) that allows it to displace the native, unlabeled Chl.

(ii) Conversely, preferential labeling of Cyt*f* is not affected if, after incubation and prior to irradiation, unbound [^{3}H]-Chl is separated from the complex.

(iii) Neither the intensity nor the pattern of photolabeling is affected by the further addition of a large excess of free, unlabeled Chl*a* prior to irradiation.

These observations imply that Chl bound to a slowly exchanging site is responsible for the labeling, and exclude the possibility that it reacts with Cyt*f* after leaving the site in an excited state and diffusing in the detergent layer. It is more difficult to rule out experimentally the possibility that some of the labeling occurs at an intermediate location, namely, after the excited Chl leaves the site and before it reaches the surface of the complex. This is a concern in all photolabeling experiments, except those involving extremely short-lived excited intermediates. If previous experience is any guide, however, photolabeling is a generally reliable technique: in what is probably the most documented case, that of the nicotinic acetylcholine receptor, an excellent correlation has been found between those residues that were identified by photochemical coupling experiments similar to those reported here, those that reacted with photoactive probes, and those later found by X-ray crystallography to actually line the acetylcholine binding sites (see refs *39* and *50*). It seems safe to conclude that [^{3}H]Chl*a* reacts with amino acid residues that line a slowly exchanging Chl-binding site, and that the most strongly labeled subunit, Cyt*f*, must form part of this site. That this site indeed is the native, unaltered Chl*a* binding site is supported by (i) its stoichiometry (one per monomer), (ii) its resistance to exchange (swamping the sample with unlabeled Chl prior to irradiation affects neither the intensity nor the pattern of labeling), (iii) its specificity (it discriminates against Chl*b*; see below), and (iv) the Raman and visible spectra of the bound Chl, which are identical after the 4-week exchange to those of the original b_6 *f*-bound Chl*a*.

Labeling of Other Subunits. The amount of radioactivity found at the levels of Cytb_6, the Rieske protein, and suIV was consistently much weaker than that comigrating with Cyt*f*. The Rieske protein was found to be unlabeled once extracted from the labeled complex by detergent. The origin of the radioactivity that comigrates with the small subunits, close to the dye front, is ambiguous. Part of the radioactivity found in this region was removed upon washing the complex with detergent. It may therefore result from incomplete extraction of some side products, e.g., lipid conjugates, and/or of the labeling of subunit PetL, the association of which with the complex is detergent-sensitive (*5*). Because covalent coupling to Chl*a* (M_r = 893.5 Da) or photolytic fragments from it may significantly affect the SDS−PAGE behavior of 4-kDa subunits, which may not comigrate with their unlabeled forms, their identification would call for methods other than those used in the work presented here. The recovery of smaller amounts of radioactivity in the low-M_r region when free [^{3}H]Chl was removed prior to irradiation is consistent with at least some of it being due to spurious labeling from the detergent belt.

At 430 nm, the wavelength at which most irradiations were performed, only the conjugated double bond system of the Chl chlorin ring is excited. Attempts to change the photolabeling pattern by irradiating at a shorter wavelength (260 nm), at which amino acid side chains can be directly excited and react with any region of the Chl molecule, yielded very similar results, with, however, a somewhat larger amount of radioactivity comigrating with Cytb_6.

Comparison with Previous Data. Previous information about the location of Chl*a* in the b_6 *f* complex is scarce. We have shown previously that the dimer of *C. reinhardtii* b_6 *f* retains its Chl following removal of the Rieske protein (*5*). Such is also the case for the b_6 *f* monomer from *Synechocystis* (*30*). The Rieske protein therefore is excluded as a main contributor to forming the binding site, in agreement with the results presented here. Following SDS−PAGE (*51*) or ion exchange chromatography (*38*, *52*, *53*) analyses of *Synechocystis* PCC 6803 Cytb_6 *f* under mildly denaturing conditions, Chl*a* was observed to comigrate with dissociated Cytb_6. The association of Chl*a* with the b_6 *f* complex from *C. reinhardtii* is highly sensitive to exposure to "nondenaturing" detergents (*5*). It would not be expected to resist exposure to dodecyl sulfate, and indeed, we have not been able to observe any comigration of dissociated Cytb_6 and Chl*a*, even under particularly mild lithium dodecyl sulfate−PAGE conditions (unpublished data). Care should be exercised, however, when comparing experiments performed on complexes originating from different organisms. The data available for preparations from *Synechocystis* PCC 6803 (*29*, *30*, *38*), *C. reinhardtii* (*5*, *31*, *34*), spinach (*31*), and *M. laminosus* (*31*) clearly indicate that Chl*a* and its associated carotenoid exhibit different degrees of resilience to detergent treatment from one organism to the next. Our experiments do not rule out the possibility that Chl*a* interacts, in situ, with both heme-carrying subunits, but preferentially reacts with Cyt*f* upon light irradiation.

Specificity of the Binding Site for Chlorophyll a. The current belief that the b_6 *f*-associated Chl is mostly Chl*a* is based on the following analytical data, obtained on purified preparations: (i) the position of the Chl's Q_Y absorption band, as observed in *Synechocystis* (*29*), spinach (*32*), *C. reinhardtii* (*15*), and *M. laminosus* (*31*) b_6 *f* preparations; (ii) its migration upon reverse phase HPLC analysis of acetonic extracts from *C. reinhardtii* (*34*), spinach (*31*), *M. laminosus* (*31*), and *Synechocystis* (*53*) b_6 *f*, as compared to Chl*a* standards; and (iii) mass spectrometry data on a spinach preparation (*31*). None of these approaches (barring, perhaps, HPLC, but no appropriate controls have been reported) would have distinguished between Chl*a* and its epimer Chl*a*′, a molecule of which has been shown to be part of the special pair in PSI reaction centers (*54*, *55*). We have checked, on the basis of CD and HPLC measurements, that most of the Chl molecules associated with the b_6 *f* complex of *C. reinhardtii* indeed are Chl*a*, not Chl*a*′. This is consistent with the stoichiometry of ∼1 Chl*a*′ per PSI reaction center observed on whole-cell extracts from higher plants and cyanobacteria (*54*).

Our b_6 *f* preparations, on the other hand, contain a small proportion (∼10%) of Chl*b* (*34*). The present exchange experiments show that, when given in large (∼5-fold) excess, some Chl*b* does associate with purified b_6 *f*. Part of this binding most likely occurs at the Chl*a* binding site, given

that (i) the remaining amount of Chl*a* in such preparations is substoichiometric, consistent with part of it having been displaced, (ii) photolabeling with [^{3}H]Chl*b* yields a pattern similar to that obtained with [^{3}H]Chl*a*, indicating a similar protein environment, and (iii) labeling, as for [^{3}H]Chl*a*, is done from a site that is in slow equilibrium with free Chl. Both the composition of purified b_6f (*34*) and exchange experiments (this work) nevertheless indicate that the binding site presents more affinity for Chl*a* than for Chl*b*. This suggests that position 7 of the chlorin ring, where a methyl group in Chl*a* is replaced with a bulkier and more hydrophilic formyl group in Chl*b*, is either deeply buried in the lipids or in contact with the protein. The results of substitution experiments using a series of Chl and bacteriochlorophyll homologues tend to favor the latter interpretation (unpublished data in collaboration with W. Rüdiger and H. Scheer).

Which Region of Cytochrome f Is in Contact with Chlorophyll a? In vivo observations on suspensions of green algae under conditions where the transmembrane potential is collapsed have shown that cycling of Cytb_6f is accompanied by the bathochromic shift of the visible absorption spectrum of a Chl*a* molecule (*56*). Like the absorption spectrum of the b_6f-associated Chl studied in vitro (*15*), the difference spectrum recorded in vivo centers on an unusually short wavelength (~669 nm). There is little doubt that in vivo and in vitro observations relate to the same Chl*a* molecule. The bathochromic shift increases upon repeated turnover of the b_6f complex, pointing to an electrochromic effect linked to proton movements (*56*). Whether those occur on the luminal or stromal side of the membrane is unclear.

It seems unlikely that the binding site of Chl*a* is located within the luminal, catalytic domain of Cyt*f*. The absence of Chl (*27*) in the heme-bearing domain from turnip Cyt*f*, proteolytically cleaved from its transmembrane anchor after residue 252 (homologous to *C. reinhardtii* residue 253), does not by itself rule out such a hypothesis: because the purification protocol involves acetone extraction (*57*), Chl would be removed if initially present. An extramembrane, luminal location is not, however, supported by the results of our proteolysis experiments. SDS−PAGE, heme-specific staining, and Edman degradation indicate that limited papain treatment of the purified b_6f complex releases a large Cyt*f* fragment that contains the heme and covers at least residues 1−252. When the same treatment is applied to a preparation of Cytb_6f that has been photolabeled with [^{3}H]Chl*a*, this N-terminal fragment is found to contain no radioactivity whatsoever. All of the labeling therefore must occur downstream of residue 252. Our current data do not rule out the possibility that [^{3}H]Chl*a* labels the extramembrane C-terminus of Cyt*f*, in the stromal space. Alternatively, and more consistent with in vivo observations and with the usual location of Chl in photosynthetic membranes, the contribution of Cyt*f* to forming the binding site of Chl*a* may involve its transmembrane helix, and/or regions very close to it.

Constraints from Circular Dichroism Data. CD measurements were undertaken to examine whether there exists any excitonic coupling between the two Chl*a* molecules in a b_6f dimer and, if so, whether the intensity of the signal could be used to determine the distance that separates them. Given the *C*2 symmetry of the dimer (*9*, *10*), this information would severely constrain possible locations for the Chl*a* binding site in the structural map. An excitonic signal was indeed observed, which is specific of the b_6f-bound Chl. It is moderately strong, ~$^1/_{10}$ of that resulting from the coupling between the two pairs of b_L and b_H hemes in the same complex (*11*). Interpreting it in a quantitative manner, however, turned out to be impossible. Detailed analysis indeed shows that the Chl*a* molecules bound to purified *C. reinhardtii* b_6f are not homogeneous in terms of their fluorescence lifetime, their resistance to bleaching, or their contribution to the CD signal (*35*), a heterogeneity that may well be related to the substoichiometric complement of β-car found in these preparations (*31*, *34*). Heterogeneity and/or partial release from the site may possibly account for the absence or near absence of a Chl excitonic signal in the CD spectrum of the spinach b_6f complex published in ref *31*. The resistance to bleaching of the bilobed CD signal of *C. reinhardtii* b_6f (*35*) suggests that it is primarily due to β-car-associated Chl, which is better protected against photooxidation (*31*). This observation is consistent with the excitonic interaction being a genuine feature of intact Cytb_6f.[2] At the same time, it makes it more difficult to derive precise distance constraints from the intensity of the signal.

However, the very fact that an excitonic signal is observed suggests that the two Chls present in a b_6f dimer cannot lie very far away from its *C*2 axis of symmetry. The projection maps of the transmembrane regions of the b_6f and bc_1 complexes show many similarities (*10*). In the bc_1 dimer, the axis of the single transmembrane helix of Cytc_1 lies, on most of its length, ~3.5 nm from the *C*2 axis (closest approach, at its luminal end, being ~3 nm). The ease with which Chl molecules can be removed from the monomer of the *C. reinhardtii* b_6f complex (*5*) suggests that they are unlikely to be deeply buried within the monomer. If it is assumed that the transmembrane helix of Cyt*f* occupies a position equivalent to that of the Cytc_1 anchor in the bc_1 complex, Chl*a* molecules located toward the protein−lipid interface and in contact with this helix would lie 7−8 nm apart, which seems impossibly far for a detectable excitonic effect to occur (see ref *58*). On the other side of the helix, toward the *C*2 axis, two Chl*a* chlorin rings in van der Waals contact with the Cyt*f* helix would be no less than ~3 nm edge to edge from each other. The latter is still very long a distance for direct excitonic interactions. A location closer to the *C*2 axis would imply one of the two following situations: either (i) the Chl interacts with Cyt*f* residues that do not belong to the transmembrane helix, or (ii) this helix does not occupy, in the b_6f structure, a position equivalent to that of the Cytc_1 helix within the bc_1 complex. It may be worth recalling, in this context, that Chl release upon mild detergent treatment of the *C. reinhardtii* complex is concomitant with monomerization, which would be consistent with (but does not prove) a location close to the monomer−monomer interface (*5*). Identification of the Cyt*f* residues that interact with Chl*a* should help define its most likely location in the complex.

[2] Note added in proof: In keeping with the view that the bilobed signal originates from excitonic interaction between the two Chls in a dimer (but not proving it), the CD spectrum of Chl*a* bound to the monomeric b_6f complex from *Synechocystis* sp. PCC 6803 shows a monolobed, negative signal peaking at 674 nm, comparable in shape and molar amplitude to that shown in Figure 5B for free Chl*a* (A. Tietjens and M. Rögner, personal communication).

Evolutionary Implications. These findings have important bearings regarding the evolutionary origin of the b_6f-associated Chl. In an earlier discussion of this matter (*34*), we considered two alternative hypotheses. The first one postulated that b_6f complexes had incorporated Chl*a* at a late stage in evolution, after the separation of the b_6f and bc_1 lineages. According to the second one, Chl on the contrary would have been present in an early ancestor common to both complexes. Our observation that Cyt*f* forms at least part of the binding site of Chl*a* clearly favors the first proposal, in keeping with recent evolutionary schemes that postulate that cytochrome *b*-type oxidoreductases predate photosynthesis (see refs *28* and *59* and references therein) and with the apparent absence of bacteriochlorophyll in bacterial photosynthetic bc_1 complexes (*60*).

Why Chl came to be associated with the complex remains open to speculation. One can perhaps hypothesize that, as a soluble precursor of the catalytic domain of Cyt*f* became involved in relaying electrons from the Rieske protein to PSI electron donors (*28*), selection pressure favored a variant of it the C-terminus of which had become fused to a transmembrane, Chl-bearing protein. Subsequent evolution would have altered the sequence of this acquired anchor beyond recognition and eliminated all but one of its Chl-binding sites. Fusion or fission of transmembrane regions, and loss or acquisition of transmembrane helices, are very common events in the evolution of membrane proteins (see refs *28*, *61*, and *62* and references therein), and indeed, such a process must account for the homology between soluble cytochrome *c* and its membrane-anchored relative Cytc_1 (cf. ref *4*). Factors that would contribute to blurring any evolutionary relationship between current-day Cyt*f* anchors and their postulated antenna protein ancestor include (i) evolutionary distance, since this hypothetical event would have to predate the radiation of cyanobacteria, 1−2 billion years ago (*28*), (ii) the restricted set of amino acid residues commonly encountered in transmembrane segments, and (iii) functional and structural constraints. Evolutionary pressure indeed would be expected to favor (a) the loss of residues involved in forming most of the original Chl-binding sites and any antenna protein oligomerization interfaces, (b) the development of mechanisms for efficiently quenching the excited states of the one remaining chlorophyll, and (c) the optimization of interactions with other subunits in the b_6f complex. Not surprisingly, sequence comparisons give few clues. Chl-binding transmembrane helices whose sequences are similar (∼35% identity score) to that of the putative transmembrane helix of Cyt*f* are encountered in LHCI and in several subunits of the PSI and PSII reaction centers, but the significance of these similarities is far from obvious. It is interesting to note, however, that while present-day cyanobacteria rely essentially on extramembrane light-collecting devices (phycobilisomes), evidence has been presented that the common ancestor of cyanobacteria and chloroplasts may have featured both phycobilisomes and transmembrane, LHC-like complexes (*63*), making the postulated fusion event not unrealistic. Also worth noting is the fact that, while most proteins of the LHC family feature three (or four) transmembrane helices, current-day cyanobacteria harbor genes encoding proteins of the same family that are predicted to feature a single transmembrane helix per subunit (*64*).

The hypothesis of a fusion event attaching a soluble ancestor of Cyt*f* to a transmembrane antenna protein may perhaps help in the understanding of the origin of the b_6f-associated Chl. It is fair to say, however, that it sheds no particular light on the reason any molecule of Chl should have been retained at all. The presence of Chl*a* in all b_6f complexes that have been examined thus far, despite the risks of photo-oxidation it exposes the oxidoreductase to, suggests that it fulfills either a currently unidentified function or a structural role that cannot be easily substituted.

CONCLUSION

In conclusion, these data establish that the Chl*a* molecule present in each cytochrome b_6f monomer is in contact with the C-terminal region, presumably the transmembrane anchor, of cytochrome *f*, and that the two chlorophylls in a b_6f dimer are in excitonic interaction with each other. These observations place constraints about the localization of the Chl molecules and the cytochrome *f* anchor with respect to the *C*2 axis of symmetry of the complex, strongly suggest that recruitment of the Chl is posterior to the divergence of the bc_1 and b_6f lineages, and provide a basis for genetic experiments aimed at examining the functional role of the Chl in the complex.

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