On the Presence and Role of a Molecule of Chlorophyll \( \alpha \) in the Cytochrome \( b_6f \) Complex*

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Highly purified preparations of cytochrome \( b_6f \) complex from the unicellular freshwater alga Chlamydomonas reinhardtii contain about 1 molecule of chlorophyll \( \alpha \) per cytochrome \( f \). Several lines of evidence indicate that the chlorophyll is an authentic component of the complex rather than a contaminant. In particular, (i) the stoichiometry is constant; (ii) the chlorophyll is associated with the complex at a specific binding site, as evidenced by resonance Raman spectroscopy; (iii) it does not originate from free chlorophyll released from thylakoid membranes upon solubilization; and (iv) its rate of exchange with free, radioactive chlorophyll \( \alpha \) is extremely slow. Some of the putative functional roles for a chlorophyll in the \( b_6f \) complex are experimentally ruled out, and its possible evolutionary origin is briefly discussed.

Cytochrome \( b_6f \), the central complex in the photosynthetic electron transfer chain, receives from plastoquinol (PQH\( _2 \)) electrons stripped from water by photosystem II reaction centers and transfers them to plastocyanin, the electron donor to photosystem I. In the process, part of the electron free energy drop is transduced into a proton electrochemical potential gradient (1–5). A homologous complex, cytochrome \( b_c \), carries out an equivalent function in the respiratory chains of mitochondria and many prokaryotes (6, 7). Chlorophyll molecules are part of the two photosystem reaction centers and the associated antenna complexes. In the photosynthetic electron transfer chain, there are 500–1000 chlorophyll (Chl) molecules/\( b_6f \) complex (see Refs. 8 and 9, and Table I). It was no surprise, therefore, that traces of Chl should be found in purified \( b_6f \) preparations (see e.g. Ref. 10), and they have long been regarded as contaminants. In recent years, however, several reports have noted that, in very pure preparations, the amount of Chl tends to be close to 1/cytochrome \( f \) (Cyt\( f \)) (11–14), raising the intriguing possibility that its presence might not be adventitious (12, 14).

In the present article, we present further evidence in support of our earlier conclusion that the native \( b_6f \) complex from Chlamydomonas reinhardtii comprises 1 molecule of chlorophyll \( \alpha \) (Chl\( \alpha \)) per monomer as an authentic component (14). Namely: (i) free \([^{3}H]Chl\alpha\) added to \( C.\) reinhardtii thylakoid membranes at the time of solubilization does not associate with the \( b_6f \) complex; (ii) the rate of exchange of \( b_6f \)-associated Chl\( \alpha \) for free \([^{3}H]Chl\alpha\) is extremely slow; and (iii) Chl\( \alpha \) is bound to the \( b_6f \) complex at a single, specific site. Putative functional roles for a chlorophyll in the \( b_6f \) complex are examined and some of them are experimentally ruled out.

EXPERIMENTAL PROCEDURES

Materials—Decylplastoquinone (C\( _{10} \)-PQ), Tricine, egg yolk 1-\( \alpha \)-phosphatidylcholine (PC), phenylmethylsulfonyl fluoride (PMSF), \( \varepsilon \)-amino- caproic acid, benzamidine, and sucrose were purchased from Sigma; acetone Uvasol was from Merck; sodium dodecyl sulfate (SDS) was from Pierce; Hecameg (HG) was from Vegatec (Villejuif, France); hydroxyapatite (HA) was from Bio-Rad; dithiothreitol was from Boehringer Mannheim; 3,3',5,5' \(-\) tetramethylbenzidine was from Fluka Chemie GmbH; urea was from TEBU; \([^{3}H]\)Acetic acid was from ICN; and Aqualuma was from Packard Instruments.

Media—TMK buffer contained 20 mM Tricine-NaOH, pH 8.0, 3 mM MgCl\( _2 \), 3 mM KCl. TMK-HP buffer contained TMK buffer supplemented with 20 mM HG and 0.1 g/liter egg PC. AP-HP buffer contained 400 mM ammonium phosphate, pH 8.0, 20 mM HG, 0.1 g/liter egg PC, protease inhibitors (200 \( \mu \)M PMSF, 1 mM benzamidine, 5 mM \( \varepsilon \)-amino-caproic acid).

Strains and Growth Conditions—Wild-type strain (WT) and mutant strain LDS (lacking chlorophyll synthesis when grown in the dark) were kindly provided by J. Girard-Bascou and P. Bennoun (CNRS UPR 9072, Institut de Biologie Physico-Chimique). \( C.\) reinhardtii was grown in Trisacetate-phosphate medium (TAP) (15) at 25 °C under illumination of 300–400 lux (WT) or in the dark (LDS) on a rotary shaker until stationary phase (\( 10^8 \) cells/ml). Cells were harvested at 5,000 \( \times \) \( g \) for 10 min. Thylakoid membranes were prepared as described previously (WT, Ref. 16; LDS, Ref. 17), resuspended in 10 mM Tricine-NaOH, pH 8.0, containing protease inhibitors (200 \( \mu \)M PMSF, 1 mM benzamidine, 5 mM \( \varepsilon \)-amino-caproic acid), and stored at –80 °C. The final concentration of WT membranes was adjusted at 3 mg of Chl/ml. The concentration of LDS etioplast membranes was estimated from their optical density at 460 nm.

Preparative and Analytical Techniques—Cytochrome \( b_6f \) complex was purified and analyzed, and its PQH\( _2 \)-plastoquinoyl oxidoreductase activity was determined as described previously (13). UV-visible absorbance spectra were recorded either on a Kontron Uvikon 930, a Varian Cary 2300, or a Joliot-type homemade spectrophotometer (18), as specified in the legends to Figs. 2, 5, and 6. Cyt\( f \) concentrations were determined from the ascorbate-reduced minus ferricyanide-oxidized
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FIG. 1. Purification of [³H]Chlorophyll a. C. reinhardtii cells were grown on [³H]acetate, thylakoid membranes were purified, and pigments were extracted and separated by thin-layer chromatography. The Chl fraction was further purified by reversed-phase HPLC on a Zorbax-ODS column. The peak eluting at 20.5 min was collected and used for [³H]Chl exchange experiments (see Fig. 5 and Tables III-IV).

Pigment Analysis—Pigments were extracted from thylakoid membranes or from cytochrome b₆f preparations by 10 volumes of ice-cold 100% acetone under vigorous stirring. Precipitated proteins were spun down at 5,000 × g for 10 min. The supernatant was collected, evaporated to dryness in a glass flask under a flow of N₂ and stored at −55 °C. Chl concentrations were determined from the absorption at 668 nm, using εmax = 75,000 M⁻¹ cm⁻¹ (20; we checked that the extinction coefficient of Chl bound to the complex is identical to that in acetone).

Purification of [³H]Chlorophyll a—Wild-type C. reinhardtii cells were grown in TAP medium under standard conditions until stationary phase, diluted 10 times into 200 ml of TAP medium containing 3.7 GBq of [³H]acetate, and further grown under about 1000 lux until stationary phase. Cells were harvested, thylakoid membranes were prepared, and [³H]-labeled pigments were separated as described above (Fig. 1). Their specific activity was determined by liquid scintillation counting in Aqualuma in a LS1801 counter (Beckman) and spectrometry. (Fig. 1). Their specific activity was determined by liquid scintillation counting in Aqualuma in a LS1801 counter (Beckman) and spectrometry.

RESULTS

Highly Purified Preparations of b₆f Complex from C. reinhardtii Contain One Molecule of Chlorophyll a/Cytochrome f—The b₆f complex from C. reinhardtii contains seven subunits in stoichiometric ratio and four identified redox carriers, one c-type heme, two b-type hemes, and a [2Fe-2S] cluster (13). In addition to the three cytochromes, UV-visible spectra of even the most highly purified preparations reveal the presence of carotenoids (absorbance peaks at -460 and 483 nm) and of Chl (peak at 667–668 nm) (13). Within experimental accuracy, the visible spectrum of the Chl does not depend on the redox state of the complex (Ref. 13; see Fig. 3A). Using the in situ extinction coefficient of b₆f-associated Chl (ε₆₆₅ = 75,000 M⁻¹ cm⁻¹; cf. "Experimental Procedures") and an extinction coefficient ε₅₄₄ ~ 18,000 M⁻¹ cm⁻¹ for Cytf (19), the Chls/Cytf ratio was found to be 0.93 ± 0.18 (mean ± S.D. over 26 preparations). Chemical analysis confirmed that b₆f preparations contain essentially pure Chl; Chl, which makes up to -30% of Chl in thylakoid membranes from WT C. reinhardtii, represents less than 10% of Chl in purified b₆f (Table I). Carotenoids are present in substoichiometric ratio with respect to Chl; while other pigments and quinones either are totally absent or are present in trace amounts (Table I).

The approximate 1:1 molar ratio of Chls to Cytf; the excess of Chls over Chl, as compared with thylakoid membranes, and the retention of Chls throughout the purification procedure suggest that there exists, on the b₆f complex, one binding site with high affinity and specificity for Chl. However, the average stoichiometry is somewhat smaller than 1:1, and its variation from preparation to preparation is larger than the uncertainty on the measurements would lead one to expect. Several factors may explain the dispersion of the data. (i) Traces of Chl collected from the sucrose gradient and incompletely washed from the hydroxylapatite column may contaminate some preparations; (ii) the b₆f-associated Chl is easily bleached (see below); and (iii) exposure of the complex to de-
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**TABLE I**

Prosthetic group composition (mol/mol ratios) of thylakoid membranes and cytochrome b₆f complexes purified from either wild-type or LDS C. reinhardtii strains

|          | Membranes | Cyt b₆f |
|----------|-----------|---------|
|          | WT        | LDS     |
| Chla/Cytb₆f | ~900ᵃ | 0.78–1.1 | NDᵇ |
| β-carotene/Cytb₆f | 21–33 | 0.26–0.38 | 0.33 |
| PQ/Cytb₆f | 13 | 0.05–0.07 | NDᵇ |
| β-carotene/Chla | 0.035–0.05 | 0.23–0.48 | NDᵇ |
| Chlb/Chla | 0.37–0.48 | 0.09–0.10 | 1.01 |
| Lutein/Chla | 0.09–0.11 | 0.06–0.08 | 0.35 |
| Neoxanthin/Chla | 0.09–0.11 | 0.01 | 1.10 |
| Violaxanthin/Chla | 0.08–0.09 | 0.02 | 0.36 |

ᵃ Mixture of Chlb and Chla.
ᵇ Concentration of Cyt b not determined.

tergent micelles tends to release the Chl (26).

The Spectrum of the b₆f-associated Chlorophyll a Is Affected by Its Interactions with the Complex—Exposing the complex to an excess of laurylmaltoside (LM) micelles induced a bathochromic shift of the Chla peak by ~2 nm, from 667–668 to 669–670 nm (Fig. 2A). Similar shifts were observed following denaturing treatments, such as heating the preparation at 50 °C or adding 8 M urea, and occurred whether the b₆f complex was in its detergent-solubilized state or reconstituted into lipid vesicles (Fig. 2B).

The spectrum of Chla in b₆f preparations treated with an excess of detergent resembles that of pure Chla dissolved in LM micelles (Fig. 2A), suggesting that this treatment releases Chl from the complex. Delipidation by detergents indeed induces dissociation of the complex into chlorophyll-free monomers (26). However, closer examination reveals that the spectral shift actually precedes Chl dissociation. We show elsewhere that mild treatment of the complex with detergent first generates a dimeric form that has lost the Rieske protein and retains the Chl, while a harsher treatment is required for the complex to release the Chl and break down into monomers (26). Analysis of the visible spectrum of the Chl bound to purified, Rieske-depleted b₆f dimer revealed a red-shifted (and broadened) absorption peak (Fig. 2C), indicating that the environment of the Chl has been affected even though it is still bound to the complex and co-purifies with it (26).

**Fluorescence Characteristics of b₆f-associated Chlorophyll—**

Interactions of the Chl with its environment in the b₆f complex were further examined by low temperature fluorescence measurements. Cytochrome b₆f exhibits, in the Soret region, several absorption bands due to the Chl, the carotenoids, and cytochromes f and b₆, the latter bands being modulated by the redox potential (Ref. 13 and Fig. 3A). The possible occurrence of energy transfer between hemes and Chla was examined by analyzing the fluorescence characteristics of the Chla under various redox conditions. At 77 K, excitation at 440 nm in the Chlb band of cytochrome b₆f produced an emission of fluorescence with a maximum at 673 nm (Fig. 3B). Excitation spectra of the fluorescence emitted at 673 nm and emission spectra of the fluorescence excited at 440 nm were recorded in the presence or absence of 5 mM ascorbate or ~5 mM sodium dithionite. The effects of these additions on the redox state of the cytochromes were checked by absorption spectroscopy (Fig. 3A). Regardless of the addition, there was no significant change in the fluorescence excitation and emission spectra (Fig. 3C, and data not shown).

Incubation at room temperature for 1 h with 100 mM HgCl₂, which is known to induce partial dissociation of the cytochrome b₆f complex (26), did not modify the shape of the fluorescence spectra, but enhanced fluorescence intensity by a factor of ~2

(Ref. 13 and Fig. 3A). A more limited increase in fluorescence intensity was observed after freezing and thawing the b₆f solution (Table II). A comparison of these fluorescence intensities with that of free Chla in the same buffer is shown in Fig. 3B and Table II. These observations indicate that association of Chla with the b₆f complex results in a redox-independent quenching of its fluorescence (by a factor of ~4), which is partially relieved following detergent treatment.

**Chlorophyll a Is Bound to the b₆f Complex at a Specific Site**—The mode of binding of Chla to the protein was further investigated using resonance Raman spectroscopy. To detect

\[ \text{Visible absorption spectrum of chlorophyll a under various experimental conditions. Panel A, (solid line) visible spectra of purified b₆f in TMK buffer containing either 0.2 or 5 mM LM; (dotted line) visible spectrum of free Chla in TMK buffer + 5 mM LM, using Joliot-type spectrophotometer, [Cyt b₆f] = 0.2 \mu M. Panel B, effects of various perturbations on the spectrum of b₆f-bound Chla; purified b₆f complex (~0.25 \mu M) in TMK buffer containing 0.2 mM LM; (1); same sample ~2 h after [LM] was brought up to 5 mM (2); same sample as in 1 after ~2 h incubation at 50 °C (3); same sample as in 1 after ~2 h incubation at room temperature in the presence of 8 M urea and ~1 mM LM (4); purified b₆f reconstituted into egg PC vesicles according to ref. (26) (5); same sample as in 5 after ~90 min incubation at 50 °C, using Joliot-type spectrophotometer (6). Panel C, spectra of Chla bound to native (dotted line) and Rieske-depleted (solid line) cytochrome b₆f complex in AP-HP buffer (Ref. 26; see "Results"), using Kontron Uvikon 930 spectrophotometer, [Cyt a] = 9.8 \mu M and 6.4 \mu M, respectively. In the three panels, spectra have been arbitrarily displaced vertically.\]
selective contributions from the Chl molecules present in the samples, experiments were performed at 441.6 nm excitation wavelength on oxidized cytochrome b$_{6f}$. This laser line is located on the red side of the Soret electronic transition of Chl$_a$, more than 1500 cm$^{-1}$ away from the Soret band of the oxidized cytochromes (~413 nm). As expected, resonance Raman spectra recorded under these conditions led to barely detectable signals from cytochrome b$_{6f}$. Nevertheless, under these conditions of excitation, intense contributions typical of carotenoid molecules partially masked the middle frequency modes of Chl$_a$ (data not shown). Analysis, therefore, was focused on the high-frequency region of the spectrum (Fig. 4). Below 1600 cm$^{-1}$, resonance Raman spectra of Chl$_a$ molecules typically feature an intense band at ~1550 cm$^{-1}$, which has been attributed to complex vibrational modes of the chlorin ring (27). Between ~1600 and 1710 cm$^{-1}$, two or three bands may be observed: (i) a band between 1595 and 1615 cm$^{-1}$, arising from the stretching modes of the methine bridges of the molecule (27), the frequency of which depends on the conformation of the chlorin ring (28) and is thus sensitive to the coordination state of the central Mg$^{2+}$ ion (27); (ii) a band at ~1620 cm$^{-1}$, arising from the stretching mode of the conjugated vinyl group in position C$_2$ (29), and often just appearing as a weak shoulder on the high frequency side of the methine stretching band (29); and (iii) between 1640 and 1710 cm$^{-1}$, bands arising from the stretching modes of the conjugated 9-keto carbonyl group, the frequency of which is extremely sensitive to the H-bonding state and to the environment of this group (27, 30).

In the resonance Raman spectrum of the b$_{6f}$-bound Chl (Fig. 4), the band arising from the methine bridge stretching modes is observed at 1606 cm$^{-1}$, and is, as expected, asymmetric, because of the presence of the weak contribution of the vinyl stretching modes. At higher frequencies, a single band is observed, at a frequency of 1676 cm$^{-1}$. The full width at half-maximum of these two bands (14 and ~11 cm$^{-1}$, respectively) is similar to that observed in resonance Raman spectra of isolated, monomeric Chl$_a$ molecules (~12 cm$^{-1}$; see Ref. 27). Since the frequency of the 9-keto carbonyl mode is extremely sensitive to the environment of this group, this indicates that the binding sites of all Chl$_a$ molecules share very similar, if not identical physicochemical properties. The frequency of this band is as high as 1696 cm$^{-1}$ when the keto group is free from the binding sites of all Chl$_a$ molecules (data not shown). Analysis, therefore, was focused on the high-frequency region of the spectrum (Fig. 4). Below 1600 cm$^{-1}$, resonance Raman spectra of Chl$_a$ molecules typically feature an intense band at ~1550 cm$^{-1}$, which has been attributed to complex vibrational modes of the chlorin ring (27). Between ~1600 and 1710 cm$^{-1}$, two or three bands may be observed: (i) a band between 1595 and 1615 cm$^{-1}$, arising from the stretching modes of the methine bridges of the molecule (27), the frequency of which depends on the conformation of the chlorin ring (28) and is thus sensitive to the coordination state of the central Mg$^{2+}$ ion (27); (ii) a band at ~1620 cm$^{-1}$, arising from the stretching mode of the conjugated vinyl group in position C$_2$ (29), and often just appearing as a weak shoulder on the high frequency side of the methine stretching band (29); and (iii) between 1640 and 1710 cm$^{-1}$, bands arising from the stretching modes of the conjugated 9-keto carbonyl group, the frequency of which is extremely sensitive to the H-bonding state and to the environment of this group (27, 30).

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The 1606 cm$^{-1}$ frequency observed for the methine bridge stretching mode is somewhat ambiguous with respect to the liganding of the Mg$^{2+}$ ion. It could originate either from a 5-coordinated Chl$_a$ molecule with an unusually planar conjugated system or from a 6-coordinated molecule slightly distorted by its protein environment (28, 31). Imidazole side chains of histidine residues are known to be particularly strong ligands for the central magnesium atom of (bacterio)chlorophyll molecules. In most of the well-documented cases where a magnesium atom interacts with a histidine residue, the methine bridge band is observed at 1612–1615 cm$^{-1}$ at low temperature (see Ref. 31, and references therein). A 1606 cm$^{-1}$ frequency makes it extremely unlikely that the Chl$_a$ of the b$_{6f}$ complex
interacts with such a strong ligand.

Chlorophyll a Does Not Become Artifactually Bound to the Complex during Solubilization—The existence of a single Chla binding site per b$_{6f}$ complex does not in itself exclude the possibility that this site is normally empty, or occupied by another ligand, and that Chla binding occurs artifactually when the thylakoid membrane is disrupted by the detergent. Two indirect arguments militate against this view, but do not strictly rule it out. (i) The presence of Chl in b$_{6f}$ complex preparations has been reported in several species, which implies evolutionary conservation of the site; and (ii) it is not clear where a Chl molecule artifactually picked up by the b$_{6f}$ complex would originate from since very little Chl is actually set free by the solubilization process. Most of the Chl found in the supernatant from HG solubilization indeed is associated to free proteins. Upon fractionating the supernatant on a sucrose gradient, it entered the gradient while free radiolabeled Chl added as a tracer stayed at the top (Fig. 5). Those few Chl molecules that did not enter the gradient, and were presumably free in detergent micelles, represented less than one-tenth of the amount associated with the b$_{6f}$. Should one accept the hypothesis that free Chl becomes artifactually bound to the b$_{6f}$ complex upon solubilization, one would have to contend with the curious coincidence that the solubilization process releases only the amount of Chl that is needed to saturate the complex, and no more.

To more directly rule out this possibility, we have purified C. reinhardtii b$_{6f}$ from membranes solubilized using detergent micelles preloaded with $^3$H-labeled Chl and compared the specific radioactivity of free versus b$_{6f}$-associated Chl. In a first experiment (experiment 1 in Table III), the regular protocol for b$_{6f}$ purification (13) was followed, except for the addition of trace amounts of $^3$HChla to the HG stock solution used to solubilize the membranes (see “Experimental Procedures”). The specific radioactivity of the Chl associated with purified b$_{6f}$ was found to be ~10-fold lower than that of the free Chl collected from the top of the gradient. In a second set of experiments, we checked on the possibility that $^3$HChla initially bound to cytochrome b$_{6f}$ upon solubilization might back-exchange with the unlabeled Chl bound to antenna proteins that comigrate with the complex during the first hours of the centrifugation, thus lowering the final specific activity. Immediately following solubilization, half of the sample was adsorbed onto a HA column, washed, and eluted as described (13). This resulted in the rapid removal (within ~15 min) of most of the antenna proteins. This sample (experiment 3) was then layered on a sucrose gradient along with the other half of the supernatant (experiment 2), and the two samples were purified by ultracentrifugation and HA chromatography. As shown in Table III, early separation of the freshly solubilized b$_{6f}$ from Chl-containing proteins does not increase the specific activity of the Chl molecule in the final purified complex; it actually decreases it, because this protocol prevents the limited exchange of b$_{6f}$-bound for free Chl that can take place at the top of the gradient during the first hours of centrifugation. The different specific activities of b$_{6f}$-bound Chl in experiments 1 and 2 probably stem from a higher level of contamination in experiment 1 by free, radioactive Chl.

Rate of Exchange of Bound and Free Chlorophyll—In keeping with these observations, the rate of exchange between b$_{6f}$-bound Chl and free Chl was found to be very slow. Purified b$_{6f}$ was incubated at 4 °C with $^3$HChla in AP-HP buffer. At intervals, aliquots were removed and free Chl separated from the complex by sucrose gradient centrifugation. The specific radioactivity of b$_{6f}$-bound Chl increased slowly, until after 10 days it reached approximately one-third that of the free Chl recovered from the top of the gradient (Table IV).

Cytochrome b$_{6f}$ Accumulates in a Chlorophyll-poor C. reinhardtii Mutant—Accumulation of the b$_{6f}$ complex was examined in C. reinhardtii LDS mutant. When grown in the dark, cells from this strain etiolate due to the almost complete shutdown of Chl synthesis. The Chl content of thylakoid membranes purified from dark-grown LDS cells was severely depleted, as reflected in the fact that the Chla/$\beta$-carotene ratio

3 P. Bennoun, personal communication.
dropped by a factor of 20–30 (Table I). Such membranes contained almost no photosystem I, photosystem II, and light-harvesting complex proteins (not shown), but they did contain cytochrome b₆f (Fig. 6). No cytochrome b₆f accumulated, on the other hand, in mutant strain C. reinhardtii in which Chl synthesis is totally blocked (119), kindly provided by P. Bennoun, IBPC, Paris) in which Chl synthesis is totally blocked (not shown).

Chlorophyll a Is Not Essential to In Vitro Electron Transfer by Cytochrome b₆f—To examine whether Chl plays a role in the electron-transfer function of cytochrome b₆f, purified samples were exposed to red light, and their enzymatic activity was followed as a function of time, in parallel with the bleaching of Chl. We have shown previously that, under our experimental conditions, the rate of electron transfer from PQH₂ to plastocyanin is limited by the rate of Cyt/f/plastocyanin collisions and is strictly proportional to the concentration of active b₆f complex (13). As shown in Fig. 7, bleaching up to 70% of the Chla in a b₆f preparation had no effect on its PQH₂-plastocyanin oxidoreductase activity.

**DISCUSSION**

Our conclusion that a molecule of chlorophyll a is a genuine component of the cytochrome b₆f complex is based on a central observation and backed by a number of corroborative experiments. The most direct observation is the lack of radioactivity in b₆f complex preparations that have been solubilized and purified in the presence of [³H]Chl(a). Depending on the exact way the experiment was performed, the radioactivity of the Chl associated with the complex varied between −10 and <1% that of the free Chl present in the supernatant, demonstrating that b₆f-bound Chl does not originate from free Chl artificially picked up by the complex. Our data rule out the (far-fetched) possibility that free, radioactive Chla becomes initially bound upon solubilization but is replaced in the course of purification by back-exchange with the non-radioactive Chla present on the small fraction of antenna proteins co-solubilized with the b₆f complex.

More circumstantial evidence runs as follows: (i) the mole ratio of Chla to Cyt(f) in highly purified preparations is always close to 1:1 (0.93 ± 0.18 over 26 preparations); (ii) very little Chl is actually set free by the solubilization process (<10% of that recovered in the b₆f complex); (iii) spectral changes upon mild treatment of the complex with detergents show that the Chl, while still bound to the complex, is sensitive to its conformational state and/or to the presence of the Rieske subunit; (iv) resonance Raman data indicate that all Chl molecules in a b₆f preparation experience an identical, specific environment; and (v) there is spectroscopic evidence for the presence of Chl in the b₆f complex in vivo (32; see below).

While these observations leave little doubt that the native b₆f dimer contains two molecules of Chl(a), they raise a number of questions. One of them is how the complex is protected from oxidation by the triplet state of Chl(a) generated upon illumination. From this point of view, it is probably significant that purified b₆f preparations also contain carotenoids, albeit at a substoichiometric level. Experiments are in progress to determine their origin and the extent of protection they confer against photooxidation.

Another question is the localization of the chlorophyll a molecule in the complex and the identity of the subunit(s) it interacts with. Spectroscopic evidence indicates that a molecule of Chl(a) is located close to the Qₐ site in vivo in both C. reinhardtii and Chlorella sorokiniana (32). This conclusion is based on the observation of a Chl spectral shift that correlates with proton release at this site. Our observations indicate that, while the Chla molecule remains bound to cytochrome b₆f dimer depleted of the Rieske protein (26), its spectrum shifts to the red (this work). The latter effect is compatible with a localization of the Chl close to Qₑ, where it would become exposed to a more polar environment upon removal of the Rieske protein. It cannot be excluded that part of the red shift of the Chl spectrum is a consequence of the delipidation that is used to trigger the dissociation of the Rieske protein (26), but it is notable that a similar shift also occurred following heat treatment of purified b₆f reconstituted into lipid vesicles. Also compatible with a localization close to Qₑ is the recent observation that chlorophyll comigrates with cytochrome b₆ upon SDS-polyacrylamide gel electrophoresis analysis of Synechocystis PCC 6803 thylakoid membranes.⁴ We have not been able, unfortunately, to corroborate this experiment using purified C. reinhardtii b₆f. Resonance Raman data indicate that the 9-keto group of the Chl is, in the native complex, involved in a medium strength hydrogen bond. They are more ambiguous in regard to the number of ligands to the Mg²⁺ ion, but they do not support support 5-fold liganding with a strong electron donor such as

⁴ R. Barbato, personal communication.
FIG. 6. Visible absorption spectra of $b_{6f}$ complexes purified from wild-type and LDS C. reinhardtii strains. Cytochrome $b_{6f}$ was purified from WT and LDS strains as described under “Experimental Procedures,” and the preparations were analyzed for Cyt$f$ (left), following addition of ascorbate, and for Chl (right). Spectra were recorded on a Kontron Uvikon 930 spectrophotometer normalized to the same absorbance at 554 nm and vertically displaced.

histidine as the fifth ligand. Experiments are in progress to determine the orientation of the chlorin ring with respect to the membrane plane and the accessibility of the Chl from the lipid and aqueous phases.

More fundamentally, the presence of Chl in the $b_{6f}$ complex raises the question of its eventual function. The plastoquinol-plastocyanin oxidoreductase activity of the $b_{6f}$ complex is not driven by the energy of light, and the redox potentials of either ground state or excited state Chl are too different from those of either of the redox carriers in the $b_{6f}$ for electron transfer to the Chl to be contemplated. The spectrum of the $b_{6f}$-associated Chl, indeed, does not depend on the oxidized or reduced state of the cytochromes. The homologous ubiquinol-cytochrome $c$ oxidoreductase, the cytochrome $bc_1$ complex, does not require any Chl for its function. We have examined a number of possibilities—none of which, admittedly, is very compelling—and tried to rule out some of them.

Light Protection—A role of the Chl in deactivating the hemes appears a priori unlikely, given the high efficiency of quenching of heme fluorescence by the iron atom. Indeed, fluorescence energy transfer measurements show no change of the excitation spectrum of the Chl upon heme reduction.

Facilitation of Electron Transfer—This also is not a priori very likely given that conjugated double bond systems such as that of the chlorin ring are thought to hamper rather than facilitate electron tunneling (34). Bleaching of the $b_{6f}$ Chl with red light resulted in no loss of the PQH$_2$-plastocyanin oxidoreductase activity. It should be noted, however, that this experiment rules out a complete shut-down of electron transfer upon Chl bleaching, but not a slowing down, since electron tunneling is not the rate-limiting step under our experimental conditions. This point is under more detailed examination.

Regulation of $b_{6f}$ Assembly—In this hypothesis, binding of Chl would regulate the stability of the complex, preventing accumulation of $b_{6f}$ in the absence of light-energy transduction. This idea holds little appeal given that the complex accumulates in cells of mutant LDS grown in the dark, even though their low content of Chl prevents accumulation of both reaction centers and light-harvesting complexes. The substoichiometric ratio of Chl to cytochrome $f$ in $b_{6f}$ preparations purified from etioplasts of this mutant suggests that, while the complex may bind Chl even under these conditions, a full complement of 2 Chl/$b_{6f}$ dimer is not necessary to its accumulation. We have not, on the other hand, observed any accumulation of $b_{6f}$ in mutant ∆Gid, which does not synthesize any Chl at all. Whether this is a direct or an indirect consequence of the absence of Chl is not known.

A Structural Role—There are precedents for prosthetic groups that serve no catalytic function or that are used in atypical manners. The inactive electron transfer branch in purple bacteria reaction centers appears to fulfill a primarily or purely structural role (see e.g. Refs. 35 and 36). Pyridoxal 5’-phosphate, whose role in enzymatic catalysis usually depends on Schiff base formation between its aldehyde function and amino acid amino groups, is used by glycogen phosphorylase in a totally different way. Its 5’-phosphate group serves as a proton donor-acceptor shuttle while the Schiff base that associates it to the protein can be reduced without loss of activity (see Refs. 37 and 38). Chl being freely available in thylakoid membranes could have been recruited by the $b_{6f}$ either as a mere building block in the assembly of the complex or in a catalytic function not necessarily related to its usual roles as a light harvester and an exciton or electron carrier. The hypothesis of a purely structural role, of course, is very difficult to rule out. In the present case, it is made particularly unappealing by the fact that incorporation of Chl implies the simultaneous development of photoprotecting devices, such as the additional recruitment of carotenoids.

An Evolutionary Relic—One possible view would be that some of the small subunits of the $b_{6f}$ complex, which have no equivalent in $bc_1$ complexes, originated from Chl-binding proteins that were recruited by the complex at a late stage in
evolution and that have lost most, but not all, of their Chl-biding sites. There is indeed a very low level of sequence identity between $b_6f$ subunits PetG and PetM and the alpha unit of purple bacteria light-harvesting proteins. There are several arguments against this hypothesis, among which are the following. (i) Chloroplasts originate from cyanobacteria with quite different light-harvesting systems (however, see Ref. 39); (ii) in Synechocystis PCC 6803, Chl has been observed to be associated with apocytochrome $b_6$, even though (iii) the genome of Synechocystis PCC6803 contains genes homologous to petG and petM (40).

An alternative view would hold that Chl, similar to the inactive electron transfer branch in reaction centers, is a relic from an earlier evolutionary stage of the $b_6f$ complex. This could be understood, for instance, if quinol oxidoreductases and reaction centers shared a common, photochemically active ancestor. Such a hypothesis is relatively straightforward if, as is often thought, photosynthesis predate respiration (see e.g. Refs. 36 and 41). It becomes more involved if, as proposed by some recent evolutionary schemes, reaction centers evolved in the context of electron transfer chains where $b$-type cytochromes already operated (7, 39, 42, 43). Further studies of the $b_6f$ Chl-binding site and of the arrangement of the two Chl in the $b_6f$ dimer, examination of the distribution of $b_6f$-associ-ated (bacterio)chlorophylls among phyla, and comparison of the upcoming three-dimensional structure of mitochondrial $b_6$ complex with those of reaction centers should contribute to shedding light on this puzzling question.

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REFERENCES

1. Malkin, R. (1992) Photosynth. Res. 33, 121–136
2. Hepe, A. B. (1995) Biochim. Biophys. Acta 1143, 1–22
3. Cramer, W. A., Martinez, S. E., Furbacher, P. N., Huang, D., and Smith, J. L. (1994) Curr. Opin. Struct. Biol. 4, 536–544
4. Cramer, W. A., Soriano, G. M., Ponomarev, M., Huang, D., Zhang, H., Martinez, S. E., and Smith, J. L. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 477–508
5. Hauska, G., Schütz, M., and Büttner, M. (1996) in Photosynthesis: The Light Reactions (Ört, D. B., and Yocum, C. F., eds), pp. 377–398, Kluwer Academic Publishers, Dordrecht
6. Trumpower, B. L., and Gennis, R. B. (1994) Annu. Rev. Biochem. 63, 675–716
7. Nitschke, W., Mühlenhoff, U., and Liebl, U. (1997) in Photosynthesis: A Comprehensive Treatise (Raghavendra, A. S., ed) Cambridge University Press, Cambridge, in press

J.-L. Popot, unpublished observations.

8. Kok, B. (1956) Biochim. Biophys. Acta 22, 399–400
9. Joliot, P., and Joliot, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1034–1038
10. Hutt, C., and Hauska, G. (1981) Eur. J. Biochem. 117, 593–599
11. Bald, D., Kraup, J., Boekema, E. J., and Rögner, M. (1992) in Research in Photosynthesis. Proceedings of the IXth International Congress on Photosynthesis (Murata, N., ed), pp. 629–632, Kluwer Academic Publishers, Dordrecht
12. Huang, D., Everly, R. M., Cheng, R. H., Heymann, J. B., Schagger, H., Sied, V., Ohnishi, T., Baker, T. S., and Cramer, W. A. (1994) Biochemistry 33, 4401–4409
13. Pierre, Y., Breyton, C., Kramer, D., and Popot, J.-L. (1995) J. Biol. Chem. 270, 29342–29349
14. Popot, J.-L., Pierre, Y., Breyton, C., Lemoine, Y., Takahashi, Y., and Rocheaix, J.-D. (1995) in Photosynthesis: From Light to Biosphere. Proceedings of the Xth International Congress on Photosynthesis (Mathis, P., ed), pp. 507–512, Kluwer Academic Publishers, Montpellier
15. Germain, D. S., and Levine, R. P. (1965) Proc. Natl. Acad. Sci. U. S. A. 54, 1665–1669
16. Attea, A., de Vitry, C., Pierre, Y., and Popot, J.-L. (1992) J. Biol. Chem. 267, 226–234
17. Bennoun, N., Pierre, Y., and Delomme, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10202–10206
18. Joliot, P., and Joliot, A. (1984) Biochim. Biophys. Acta 765, 210–218
19. Rich, P. R., Heathcote, P., and Moss, D. A. (1967) Biochim. Biophys. Acta 982, 138–151
20. Dawson, R. M. C., Elliot, D. C., Elliot, W. H., and Jones, K. M. (1986) Data for Biochemical Research, pp. 232–233, Clarendon Press, Oxford
21. Eichenberger, W., and Groh, E. C. (1962) Helv. Chim. Acta 45, 974–981
22. Lichtenthaler, H. K. (1987) Methods Enzymol. 148, 350–382
23. Britton, G. (1985) Methods Enzymol. 148, 113–149
24. Breyton, C., and Crane, F. L. (1973) Methods Enzymol. 23, 372–408
25. Ajlani, G., Vernotte, C., DiMaggio, L., and Haselkorn, R. (1995) Biochim. Biophys. Acta 1231, 189–196
26. Breyton, C., Tribet, C., Olive, J., Dubaq, J.-P., and Popot, J.-L. (1997) J. Biol. Chem. 272, 21892–21900
27. Lutz, M., and Mantele, W. (1991) in The Chlorophylls (Scheer, H., ed), pp. 865–902, CRC Press Inc., Boca Raton, FL
28. Fujiwara, M., and Tasumi, M. (1986) J. Phys. Chem. 90, 5405–5450
29. Feiler, U., Mattiodi, T. A., Kathered, L., Scheer, H., Lutz, M., and Robert, B. (1994) J. Raman Spectrosc. 25, 365–370
30. Luth, M., and Robert, B. (1988) in Biological Applications of Raman Spectroscopy (Spirito, G. T., ed), pp. 137–411, John Wiley and Sons, New York
31. Naveka, A., Lapouge, K., Sturgis, J. N., Hartwich, G., Simonin, I., Scheer, H., and Robert, B. (1997) J. Raman Spectrosc., in press
32. Joliot, P., and Joliot, A. (1995) in Photosynthesis: From Light to Biosphere. Proceedings of the IXth International Congress on Photosynthesis (Mathis, P., ed), pp. 615–618, Kluwer Academic Publishers, Montpellier
33. Watanabe, T., and Kobayashi, M. (1991) in Chlorophylls (Scheer, H., ed), pp. 287–315, CRC Press Inc., Boca Raton, FL
34. Marcus, R. A. (1993) Angew. Chem. Int. Ed. Engl. 32, 1111–1121
35. Woodbury, N. W., and Allen, J. F. (1995) in Anoxygenic Photosynthetic Bacteria (Blankenship, R. E., Madigan, M. T., and Bauer, C. E., eds), pp. 527–557, Kluwer Academic Publishers, Dordrecht
36. Blankenship, R. E. (1992) Photosynth. Res. 33, 91–111
37. Palm, D., Klein, H. W., Gschwain, R., Buehler, M., and Helmreich, J. M. (1990) Biochemistry 29, 1099–1107
38. Holm, L., and Sander, C. (1995) EMBO J. 14, 1287–1293
39. Meyer, T. E. (1994) BioSystems 33, 167–175
40. Kaneko, T., Sato, S., Kogami, H., Tanaka, A., Asanuma, E., Nakamura, Y., Miyajima, N., Hirohashi, M., Sugita, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Maruki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) DNA Res. 3, 109–136
41. Olson, J. M., and Pierson, B. K. (1987) Organisms of Life 17, 419–430
42. Castresana, J., Lubben, M., and Saraste, M. (1995) J. Mol. Biol. 250, 202–210
43. Castresana, J., and Saraste, M. (1995) Trends Biochem. Sci. 20, 443–448