**Dimer to Monomer Conversion of the Cytochrome b₆f Complex**

**CAUSES AND CONSEQUENCES**

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The molecular weight of the cytochrome b₆f complex purified from *Chlamydomonas reinhardtii* thylakoid membranes has been determined by combining velocity sedimentation measurements, molecular sieving analyses, and determination of its lipid and detergent content. The complex in its enzymatically active form is a dimer. Upon incubation in detergent solution, it converts irreversibly into an inactive, monomeric form that has lost the Rieske iron-sulfur protein, the b₆f-associated chlorophyll, and, under certain conditions, the small 32-residue subunit PetL. The results are consistent with the view that the dimer is the predominant form of the b₆f in *situ* while the monomer observed in detergent solution is a breakdown product. Indirect observations suggest that subunit PetL plays a role in stabilizing the dimeric state. Delipidation is shown to be a critical factor in detergent-induced monomerization.

In the photosynthetic membrane of plants, algae, and some bacteria, the cytochrome b₆f complex catalyzes reduction by plastoquinol of an acceptor protein, either plastocyanin or a soluble cytochrome. Electron transfer is coupled to proton translocation from the electronegative to the electropositive side of the thylakoid or bacterial membrane (see Refs. 1–4). A homologous complex, cytochrome bc₁, plays a comparable role in mitochondria and in many prokaryotes. The b₆f complex comprises four high *M₄* subunits, cytochromes *f* and *b₆*, the Rieske iron-sulfur protein, and subunit IV (4), and three hydrophobic and very small (3–4 kDa) proteins, PetG, PetL, and PetM (5–12). The seven subunits are present in 1:1 ratio (12). All of them have been shown to be transmembrane except for the Rieske protein, which behaves as an extrinsic protein (see Refs. 9, 10, and 13, and references therein). Prosthetic groups include three hemes, a [2Fe-2S] cluster, and a molecule of chlorophyll *a* (4, 8, 14). The aggregate molecular mass of proteins and prosthetic groups is ~106 kDa per cytochrome *f* (Ref. 12; see Table I).

While membrane proteins frequently are isolated as oligomers, it is often difficult to establish whether oligomerization is purely structural (e.g. as a consequence of the general crowding of the membrane) or whether it is necessary to the function of the protein. Three different examples are photosynthetic reaction centers, bacterial porins, and ligand-gated channels. In reaction centers, the heterodimeric structure is essential to the function, inasmuch as homologous subunits contribute to liganding the special pair of (bacterio)chlorophylls that effects charge separation (15, 16). In trimeric porins, oligomerization is required for insertion of the protein into the bacterial outer membrane, even though each protomer contains its own transmembrane channel (17). The five subunits of the nictinic acetylcholine receptor surround a central transmembrane channel, while pentamers are further associated into supramolecular dimers with no known function (18).

Whether or not the b₆f and bc₁ complexes are functional and/or structural dimers is still a matter of discussion (2–4, 19). The b₆f complex purified from spinach has a Stokes radius corresponding to that of a dimer (20, 21), in keeping with the size of negatively stained particles and with the migration of the complex during electrophoresis under nondenaturing conditions (21). When loaded onto a sucrose gradient containing Triton X-100 (22–24), or upon filtration on a molecular sieve column equilibrated with LM¹ (21), spinach b₆f migrates as a “large” form, prone to conversion into a smaller one. The b₆f complex isolated from *Synechocystis* PCC6803 was concluded to be either a monomer, on the basis of gel filtration sizing and electron microscopy observation of negatively stained particles (25, 26), or a mixture of monomer and dimer, on the basis of electrophoretic analysis of the solubilized thylakoid membranes in a nondenaturing gel system.² The complex from strain PCC6714 had a Stokes radius consistent with a dimer (27).

The minimal size of the functional b₆f complex is unclear. Radiation inactivation (28) and titration with specific inhibitors (29) led to the conclusion that the functional unit is the monomer. On the other hand, Graan and Ort (30) have reported that a single molecule of the inhibitor DBMIB per dimer is sufficient to block the activity. When isolated as a “light”

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¹ The abbreviations and trivial names used are: LM, laurylmaltoside (dodecyl-β-D-maltoside); C₄₋₆-PQ₈, decylplastoquinol; DBMIB, 2,5-di-bromo-6-methyl-3-isopropyl-p-benzoquinone; CMC, critical micellar concentration; EPR, electron paramagnetic resonance; HA, hydroxyapatite; HG, Hecameg (6-O-β-D-galactopyranosyl-α-D-glucopyranosyl)-polyoxyethylene; PAGE, polyacrylamide gel electrophoresis; PC, l-alpha-phosphatidylcholine; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine; MOPS, 3-(N-morpholino)propanesulfonic acid.

² R. Barbato, personal communication.
form, the $b_{6}f$ complex from higher plants is generally found to be inactive (21, 22) although an active light form of the spinach complex has been recently reported (24). No activity measurements on cyanobacterial $b_{6}f$ complex preparations are available.

Information concerning the state of association of the homologous $bc_{1}$ complex is abundant but partially contradictory as well. The isolated complex in detergent micelles generally purifies as a heavy form having a Stokes radius or a sedimentation coefficient compatible with its being a dimer and/or crystals as a dimer (Neurospora crassa (31–33), beef heart mitochondrion (34–39), Bacillus PS3 (40)). The active purified $bc_{1}$ complex from the colorless alga Polytomella sp. has a Stokes radius corresponding to a monomer (41). A light form of the beef heart complex, with a Stokes radius corresponding to that of a monomer, was reversibly obtained by varying either the salt or detergent concentrations and was reported to be active (34, 36, 42). Titration of the complex with various inhibitors, on the other hand, led to the conclusion that the functional unit of the $bc_{1}$ complex is a dimer (43–46). It has been proposed that an equilibrium between monomer and dimer plays a role in regulating electron transport in vivo (28, 42, 44).

We have developed a protocol for the purification of cytochrome $b_{6}f$ from the unicellular alga Chlamydomonas reinhardtii (12). The preparations obtained are both highly pure and extremely active in catalyzing electron transport. Chlamydomonas $b_{6}f$ is similar if not identical to the complex of higher plants in its subunit composition and in the spectral and redox properties of its cytochromes (8, 12). In the present work, we have carried out determinations of its $M_{w}$, which show that it is to be a dimer, and of the amount of lipids and detergent associated with it. We have identified conditions under which the dimeric, active complex is stable and conditions that lead to monomerization and inactivation. Delipidation of the complex is a critical factor in these processes. Some of these data have been reported in preliminary form (47).

**EXPERIMENTAL PROCEDURES**

**Materials**—$^{14}C$LM was a kind gift from M. le Maire (Commissariat à l’Energie Atomique, Saclay, France). Sources for other chemicals have been described by Pierre et al. (12).

**Strains and Growth Conditions**, **SDS-Polyacrylamide Gel Electrophoresis**, and **Immunoblotting**—Methods were as described by Pierre et al. (12).

$b_{6}f$ Complex Purification—$b_{6}f$ complex was purified from C. reinhardtii thylakoid membranes as described (12). The protocol comprises the following three steps: specific solubilization of the thylakoid membranes with the neutral detergent Hecameg (HG), fractionation of the supernatant by detergent binding was estimated using $^{[14C]}$LM. Two different procedures were performed after transferring the purified $b_{6}f$ complex into LM.

**Electron Spin Resonance Spectroscopy**—Electron Spin Resonance (ESR) measurements were performed as described previously (48) in a Bruker ER200 spectrometer fitted with an Oxford Instrument helium cryostat and temperature control system. Spectra were recorded at 20 K in 20 mM MOPS-KOH buffer, pH 7.0, containing 5 mM ascorbate and either 20 or 50 mM HG. Instrument settings were as follows: microwave frequency, 9.44 GHz; microwave power, 6.7 milliwatt; and modulation amplitude, 1.6 millitesla. To eliminate the contribution of mitochondrial Rieske protein (49, 50), the spectrum of membrane-bound $b_{6}f$ complex was recorded with membranes prepared from the DUM-1 mutant, which lacks the $bc_{1}$ complex (51).

**Detergent Exchange and Binding**—Molecular mass determinations were performed after transferring the purified $b_{6}f$ complex into LM solution. The $b_{6}f$ complex eluted from the HA column was either (i) concentrated on a Filter 100 membrane (Millipore) supplemented with 1 mM LM, and run through a Sephacryl G-75 column (Pharmacia Biotech Inc.) equilibrated with 20 mM Tricine-NaOH, pH 8.0, 0.2 mM LM; or (ii) supplemented with 1 mM LM and dialyzed for 3 h against 20 mM Tricine-NaOH, pH 8.0, 20 mM HG and then overnight against 20 mM Tricine-NaOH, pH 8.0, 0.2 mM LM.

Detergent binding was estimated using $^{[14C]}$LM. Two different procedures for detergent exchange were compared. In Procedure I, $b_{6}f$ complex in HG was transferred into 0.2 mM LM solution in Tricine-NaOH buffer and molecular sieving. $^{[14C]}$LM was diluted with unlabeled LM to a specific activity of 3.9–4.1 $\times$ 10$^{11}$ cpm/mol (molar fraction of $^{[14C]}$-labeled detergent <3 $\times$ 10$^{-5}$). This solution was used to prepare 2-mL monodisperse <3 $\times$ 10$^{-5}$). This solution was used to prepare 2-mL monodisperse $b_{6}f$ complexes in 20 mM Tricine-NaOH, pH 8.0, 0.2 mM LM. About 15 min before centrifugation, $^{[14C]}$LM was added to the $b_{6}f$ sample to reach the same specific activity as in the gradients. In Procedure II, the $b_{6}f$ complex was transferred into LM solution by dialysis and rate zonal centrifugation. The $b_{6}f$ complex in HG was supplemented with 1 mM unlabeled LM and dialyzed overnight against 0.2 mM Tricine-NaOH buffer, pH 8.0, containing 0.25 mM LM. The following solutions were prepared in 20 mM Tricine-NaOH buffer, pH 8.0, containing protease inhibitors, and containing 0.25 mM LM from a mixture of $^{[14C]}$LM and unlabeled LM with a specific activity of 3.1 $\times$ 10$^{12}$ cpm/mol. Unbound lipids were removed by centrifuging the dialyzed sample on a 5–20% (w/w) sucrose gradient containing 0.25 mM labeled LM. After a second overnight dialysis against 0.25 mM labeled LM, the sample was loaded onto an 15–25% (w/w) sucrose gradient containing 0.25 mM labeled LM. In the two procedures, the gradients were centrifuged for 3.5–4 h at 250,000 $\times$ g (54,000 rpm) in the TLS 55 rotor of a TL100 centrifuge (Beckman Instruments) and collected by 100-ml fractions. The concentration of $b_{6}f$ complex was determined from the peak absorbance of cytochrome $b_{6}$ reduced minus ascorbate-reduced difference spectra ($\epsilon_{564} = 56$) and the absorbance coefficient $\epsilon_{564} = 56$. The concentration of LM was determined by counting 20-$\mu$L aliquots in 5 ml of Aqualuma counting medium (Lumac LSC, Groningen) in a LS180 scintillation counter (Beckman). Two measurements were performed according to Procedure I and one according to Procedure II. Variations of the LM/$b_{6}f$ ratio between experiments were within experimental uncertainty. The most accurate measurement, made using Procedure II, gave a ratio of 130 ± 10 molecules of LM bound per cytochrome $f$.

**Molecular Mass Determination**—The molecular mass ($M_{w}$) of the $b_{6}f$/LM particles (heavy and light forms) was estimated from their specific volume ($\delta^{*}$), diffusion coefficient ($D_{av}$), and sedimentation coefficient ($s_{20,w}$) according to Svedberg’s equation,

$$s_{20,w} = S_{0,0} \frac{M_{w}}{D_{av}} \left( 1 - \delta^{*} \right),$$

where $\rho$ is the density of water at 20°C. In this equation, $M_{w}$ and $\delta^{*}$ include contributions from all constituents of the particle (proteins, pigments, detergents, and lipids) except bound water (see Ref. 52). $\delta^{*}$ was estimated from the particle composition to be 0.797 cm$^{2}$ g$^{-1}$ for the heavy and 0.787 cm$^{2}$ g$^{-1}$ for the light form (see Table I).

Diffusion coefficients were estimated by molecular sieving on a Sephacryl S-300HR column (Pharmacia, 48 × 1 cm; total volume $V_{t} = 37.7$ ml; the void volume $V_{v} = 16$ ml) was measured with dextran blue. The column was calibrated with the following standards (Pharmacia): thyroglobulin ($D_{20,w} = 1.36 \times 10^{-11}$ m$^{2}$ s$^{-1}$), ferritin (3.4), catalase (4.1), aldolase (4.7), bovine serum albumin (6.1), and ovalbumin (7.76; $D_{av}$ values from Refs. 53 and 54). A mixture of standards and $b_{6}f$/LM particles (final volume 0.4 ml) was layered onto the column and eluted with 50 mM ammonium phosphate, pH 8.0, 150 mM NaCl, 0.2 mM LM, 25 mM HG, and 0.1 g/liter PC; 25 mM HG; 0.2 mM LM; 3 mM LM; 3 mM LM; 5 mM LM; 5 mM LM, 0.3 g/liter PC in a 20 mM Tricine-NaOH, pH 8.0, buffer containing protease inhibitors. Before being layered onto a 0.2 mM LM gradient, the purified $b_{6}f$ complex was transferred into 0.2 mM LM by molecular sieving as described above. When layered onto the

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other LM gradients, the complex was diluted and supplemented with detergent and lipids to have the detergent/lipid composition of the gradient. The gradients were centrifuged either at 270,000 × g (40,000 rpm) for 18 to 36 h in the SW41 Ti rotor of a Beckman L8 centrifuge or at 350,000 × g (55,000 rpm) for 3–6 h in the TLS 55 rotor of a Beckman TL100 centrifuge. They were collected by 500- or 100-μl fractions and analyzed by SDS-PAGE. For the analysis of the composition of the two forms, the dimer was obtained after centrifugation of the purified \( b_{6f} \) complex onto a gradient containing either 0.1 g/liter PC + 50 mM HG or 0.2 mM LM; the monomer was obtained after two successive centrifugations, the first one with an excess of detergent (25 mM HG or 5 mM LM), and the second one near the CMC of the detergent (20 mM HG or 0.2 mM LM), to fully separate the complex from PetL, chlorophyll \( a \), and the Rieske protein that have been released from it.

Effect of an Excess of Detergent on Electron Transfer Activity—Purified \( b_{6f} \) complex (6.5 μM \( b_{6f} \), 13 μM PC) was incubated with either 50 or 100 mM HG either with no added lipids or with a molar ratio of egg PC to HG in the micelles of ~1/10 or ~1/5 (3.4 and 6.8 mM PC for 50 mM HG, or 8 and 16 mM PC for 100 mM HG). Electron transfer activity was measured as described by Pierre et al. (12) after incubation in the dark at 4 °C for increasing periods of time.

Reconstitution and Electron Microscopy—Dimeric \( b_{6f} \) complex (eluted from the HA column) and monomeric \( b_{6f} \) (obtained after centrifugation of the purified \( b_{6f} \) complex on a sucrose gradient containing either 25 mM HG or 3 mM LM in a 20 mM Tricine-\( NaOH \), pH 8.0, buffer) were mixed with egg PC (10 μl/giter in 70 mM HG (in this solution, the ratio of PC to micellar detergent is about the same as in a 0.1 g/liter PC, 50 mM HG, or 0.2 mM LM), to fully separate the complex from PetL, chlorophyll \( a \), and the Rieske protein that have been released from it.

Composition of the Heavy and Light Forms of the Complex—The heavy form, collected either from HG or LM gradients, presented every characteristic of the purified \( b_{6f} \) complex (12, 14). All seven subunits, as well as the chlorophyll \( a \) molecule, co-sedimented (Figs. 1 and 2), and the complex was highly active in transferring electrons from \( C_{10} \)-PQH\(_2\) to plastocyanin (not shown). In contrast, the light form had lost the Rieske protein, the chlorophyll \( a \) molecule, and, under some circumstances (see below), subunit PetL (Figs. 1 and 2). As expected from the absence of the Rieske protein, the light form was enzymatically inactive; the spectral properties of its hemes remained unchanged (not shown). The kinetics of dissociation of the complex depended on whether the gradient contained HG or LM. To isolate the light form as a pure species, two sequential centrifugations must be performed (see “Experimental Procedures”); whereas PetL detached from the complex during the first centrifugation in the presence of an excess of LM, it was lost only after the second one in the case of an excess of HG (not shown). On the other hand, the heavy form seems to be more stable in LM than in HG. When layered onto a gradient containing 20 mM HG and 0.1 g/liter PC, the purified \( b_{6f} \) complex sedimented mostly as the heavy form, but the light form was also present, whereas in the presence of 0.2 mM LM and no lipids, the \( b_{6f} \) always remained in the heavy form. With a gradient containing 25 mM pure HG, the heavy form was totally converted into the light one, whereas in 5 mM LM, remnants of the heavy form sometimes were also present (not shown).

Under our experimental conditions, dissociation of the Rieske protein from \( C. \) reinhardtii \( b_{6f} \) complex by exposure to detergent was accompanied by the loss of the iron-sulfur cluster. EPR spectra revealed the presence of the Rieske protein [2Fe-2S] cluster both in \( C. \) reinhardtii membranes and in the purified \( b_{6f} \) complex (Fig. 3, A and B). The derivative-shaped \( g_{xx} \) signal at 1.89 is similar to that of spinach \( b_{6f} \) \( g_{xx} \) (19-20); for example, see Refs. 21 and 48). When HG concentration in the sample was raised from 20 to 50 mM, \( i.e. \) under conditions that induce the transition to the light form and the release of the Rieske protein, the [2Fe-2S]EPR spectrum disappeared, indicating destruction of the cluster (Fig. 3C).

Lipid analysis (see “Experimental Procedures”) indicated that \( b_{6f} \) preparations purified according to Pierre et al. (12)
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**FIG. 2.** Subunit and chromophore composition of the dimeric and monomeric forms of the b$_{6f}$ complex. A, SDS-PAGE analysis of purified dimer (D) and monomer (M). The dimer was obtained after centrifugation of the purified b$_{6f}$ complex on a gradient containing 0.2 mM LM in 20 mM Tricine-NaOH, pH 8.0. The monomer was obtained after centrifugation of the purified b$_{6f}$ complex on gradients containing either 3 mM LM (LM) or 25 mM HG (HG) in the same buffer, followed by a centrifugation on gradients containing 20 mM HG or 0.2 mM LM, respectively. B, immunoblot analysis of the dimer and the monomer with antisera against cytochrome f (f), the Rieske protein (FeS), subunit IV (IV), PetG, PetM, and PetL. C, absorbance spectra of the dimer prepared according to Pierre et al. (12) and of the monomer prepared with 25 mM HG as described above. An identical spectrum was obtained when the monomer was prepared with 5 mM LM.

contained no endogenous *C. reinhardtii* lipids, within the detection limit of about 1 molecule of lipid per cytochrome f. Upon further sedimentation of the purified complex in lipid-free sucrose gradients containing either 0.2 mM LM or 25 mM HG, 18 ± 11 molecules of egg PC per cytochrome f were found to comigrate with the heavy form, while the light form contained less than 2. This value can be compared with the 40–50 molecules of PC per cytochrome c$_1$ required for a maximum activity of the bc$_1$ complex (60) and with the 33 molecules of lipids per cytochrome c$_1$ that co-crystallize with the dimeric beef heart enzyme (39). Detergent binding was measured by ultracentrifugation on sucrose gradients containing [14C]LM. The heavy form of the complex was found to bind 130 ± 6 molecules of LM per cytochrome f (not shown; see Ref. 47).

**Determination of the Molecular Mass of the Heavy and Light Forms**—The state of association of the solubilized complex was determined after transfer to LM solutions to make direct measurements of detergent binding feasible. Detergent exchange was achieved either by dialysis and/or by molecular sieving (see “Experimental Procedures”). Sedimentation and diffusion coefficients were determined by ultracentrifugation on sucrose gradients and by molecular sieving, respectively. In dilute LM solutions (0.2 mM), the complex sedimented as the heavy form, with $s_{20,w} = 9.9 ± 0.5$ S and $D_{20,w} = 3.9 × 10^{-11}$ m$^2$·s$^{-1}$ (Table I). The latter value corresponds to a Stokes radius of 5.5 nm for the b$_{6f}$/LM particle. The light form observed in the presence of 5 mM LM migrated with $s_{20,w} = 6.3 ± 0.2$ S and $D_{20,w} = 5.6 × 10^{-11}$ m$^2$·s$^{-1}$ (Stokes radius of 3.8 nm).

The specific volume $\tilde{\nu}$ of the two forms was estimated from their composition (Table I), $D_{20,w}$, $s_{20,w}$, and $\tilde{\nu}$ values were then combined using Svedberg’s equation, yielding molecular mass estimates of $310 ± 46$ kDa for the heavy form and $128 ± 10$ kDa for the light one. Masses were estimated from the chemical composition, assuming the heavy form to comprise two copies of each subunit, 260 ± 20 molecules of LM and 36 ± 22 molecules of egg PC, and the light one to comprise a single copy of each subunit, minus the Rieske protein, PetL, and the chlorophyll, and 130 ± 10 molecules of LM. Estimated masses are 373 ± 28 and 149 ± 14 kDa, respectively, close to but slightly higher than those determined experimentally (Table I). The heavy form thus corresponds to the dimeric form of the b$_{6f}$ complex and the light form to the monomer.

The number of LM molecules bound per dimer, ~260, largely exceeds the aggregation number of LM (~110; see Ref. 61). It is similar to the 215 LM molecules reported to bind to monomeric mitochondrial cytochrome c oxidase (61), which features 28 transmembrane $\alpha$-helices (62) while the b$_{6f}$ dimer is expected to contain 22 (8). Upon isopycnic centrifugation on sucrose gradients in the presence of 0.2 mM LM, the b$_{6f}$ dimer equilibrated at a density of 1.197 g/cm$^3$. We have previously estimated that micellar LM binds 7–8 molecules of water per molecule (63). The density expected for the complex, including LM-bound water, would be 1.228 g/cm$^3$. The difference between measured and calculated densities suggests that, in the presence of 44% sucrose, each b$_{6f}$ dimer additionally binds ~3,000 molecules of water, i.e. ~0.25 g of water/g of protein. This ratio is somewhat lower than that observed for soluble proteins (64),

$^3$ The figures presented in Table I differ slightly from those previously reported (47), mainly due to taking into account the contribution of bound lipids.
as befits a complex whose surface is partially shielded from water.

**Dimer to Monomer Transition**—Conditions under which the *C. reinhardtii* $b_{6f}$ dimer converts into the monomer were further investigated. In our regular purification protocol (12), sucrose gradient sedimentation and HA chromatography are performed near the CMC of HG (20 mM) and in the presence of lipids (0.1 g/liter egg PC). Under these conditions, the complex appeared as relatively homogeneous particles with an average diameter of 10–11 nm for the dimer and 8 nm for the monomer (whether obtained by incubation with HG or with LM, i.e. having retained or not subunit PetL). This Rieske-less dimer retained the chlorophyll molecule whose spectroscopic properties were similar to those of the wild-type complex, but it lost the Rieske protein (47) (50). The density of the dimeric state of the complex at 20 molecules of bound detergent and lipids is in fair agreement with that calculated for a dimer (373 kDa), assuming it comprises two copies of each prosthetic group, assuming the heavy form to comprise 2 copies of each subunit (PetL). In the present work, we have determined the molecular mass of the purified $b_{6f}$ complex in LM solution with an accuracy of approximately ±15%, by measuring its sedimentation and diffusion coefficients and determining the amounts of bound detergent and lipids. The measured value, 310 kDa, is in fair agreement with that calculated for a dimer (373 ± 28 kDa), assuming it comprises two copies of each subunit (12), 36 ± 22 lipids (this work), 260 ± 20 molecules of detergent (47 and this work), and 2 molecules of chlorophyll (8, 12, 14). In agreement with this finding, the $b_{6f}$ complex from *C. reinhardtii* is a dimer (65). The dimeric state of the $b_{6f}$ complex from *C. reinhardtii* evidenced here is consistent
with lipids.

with high concentrations of detergent supplemented or not

b

the spinach

centrily, Chain and Malkin have described an

was reported to retain the molecule of chlorophyll (21). Re-

C. reinhardtii b

form of the spinach complex obtained by incubation in 3 mM LM

✴

100 mM HG containing either no added lipids (21).

Purified b

f complex was incubated with either 50 mM or

3 m M) LM (21).

f

6

f

6

b

Purified b

f complex was incubated with either 50 mM or

100 mM HG containing either no added lipids (x) or a molar ratio of egg

PC to micellar detergent of 1:5 (++) or 1:10 (■).

with previous conclusions based on analyzing the migration of

the spinach enzyme either during sucrose gradient sedimenta-

tion in the presence of 0.1% Triton X-100 (22) or upon molecu-

lar sieving in the presence of either 0.2% Triton X-100 (20) or

0.15% (~3 mta) LM (21).

Monomerization of the Complex and Loss of the Rieske Pro-

tin—A lighter, inactive form is produced upon exposure of the

C. reinhardtii b

f dimer to an excess of detergent. Its molecular

mass is that expected for a monomer (molecular mass = 128 ±

10 kDa; calculated molecular mass = 149 ± 14 kDa; cf. Table I).

The monomer has lost the molecule of chlorophyll, the Rieske

protein, and, under certain conditions, the small subunit PetL.

Light, inactive forms of spinach b

f complex, depleted of

Rieske protein, have similarly been observed following incuba-

tion with detergent (0.2% Triton X-100 (22, 23), or 3 mta LM

(21)). Unlike the C. reinhardtii b

f monomer, the inactive light

form of the spinach complex obtained by incubation in 3 mta LM

was reported to retain the molecule of chlorophyll (21). Re-

cently, Chain and Malkin have described an active light form of

the spinach b

f complex, obtained by treatment with 0.2%

Triton X-100, which retains the Rieske protein and seems
depleted of chlorophyll (24). In our hands, when treated with

0.2% Triton X-100 under similar conditions, the purified com-

plex from C. reinhardtii monomerized and lost both the Rieske

protein and the chlorophyll. Active light forms of the bc

complex from beef heart mitochondria (36, 42) and from the color-

less alga Polytomella sp. (41) have also been reported. These

observations suggest that, despite the intimate association into
dimers revealed by x-ray data on beef heart bc

(39), bc

1 and

b

f complexes can be resolved into monomers without (com-

plete) inactivation. In the case of C. reinhardtii b

f, however, this possibility remains to be demonstrated.

The stability of the isolated Rieske protein appears to vary

depending on species and experimental conditions. In our

hands, the C. reinhardtii protein lost the characteristic EPR

signal of the [2Fe-2S] cluster upon dissociating from the com-

plex. However, isolation of a native b

f Rieske protein from

spinach (66) and its reconstitution into an active complex (67)

have been described, as is also the case with the bc

1 Rieske

protein (68–70). A native-like catalytic domain from the Rieske

protein of spinach b

f has recently been obtained by proteolytic

cleavage, purified, and crystallized (71).

Following reconstitution into lipid vesicles, freeze-fractur-

ing, and electron microscopy examination of metal-shadowed

replicas, the dimeric and monomeric forms of C. reinhardtii b

f complex appeared as homogeneous particles with diameters

10–11 nm and ~8 nm, respectively. These dimensions are

consistent with those of the dimer (~8.8 × 5.3 nm) and mono-

mer (~5.3 nm diameter) observed in the projection map ob-

tained from negatively stained two-dimensional crystals (65),

assuming shadowing to increase apparent diameters by 3–4

nm. Mörschel and Staehelin have reported a diameter of 8.5 nm

for spinach b

f particles, which they interpreted as dimers

(72). Such a large size discrepancy between C. reinhardtii and

spinach b

f dimers would be surprising, given the high simi-

larity of the two complexes. Unless it originates from differ-

ences in the shadowing protocol and/or the measurement pro-

cedures, it seems more likely that these authors actually

observed a monomeric form of the spinach complex.

Involvement of PetL, the Rieske Protein, and Lipids in Sta-

bilizing the b

f Dimer—Several observations indicate that both

the presence of subunit PetL and the composition of the micel-

lar phase surrounding the solubilized complex are important

factors for the stability of the b

f dimer. The involvement of

PetL is suggested by two lines of indirect evidence, (i) the

monomer purified in 5 mta LM has lost PetL (this work), and (ii)

the purified b

f complex from a PetL

mutant migrates as a

monomer under non-delipidating conditions (9). There is no

absolute correlation between the presence of PetL and the

oligomeric state of the b

f complex, however, since (i) PetL

co-migrates with the monomer purified in 25 mta HG (this

work), and (ii) the PetL

complex is a dimer when initially

solubilized from thylakoid membranes. It monomerizes only

during the second step of purification (9). These data suggest

that the formation of the b

f dimer stabilizes the association of

PetL with the other subunits in the complex and vice versa. A

speculative interpretation of these observations would be that

PetL is located at the periphery of the complex and interacts

with subunits belonging to the two monomers. More indirect

effects, of course, cannot be excluded.

The destabilizing effect of raising the detergent concen-

tration can receive a priori two types of interpretation. A direct

effect could originate from the displacement of a dimer ↔

monomer equilibrium due to the increased number of micelles

in the solution. In our hands, monomerization is irreversible;

however, some of our data would be compatible with a two-step

process in which dilution into detergent micelles generates an

unstable monomer (Fig. 6, intermediate ◊) that loses rapidly

Fig. 4. Kinetics of inactivation of the b

f complex incubated

with high concentrations of detergent supplemented or not

with lipids. Purified b

f complex was incubated with either 50 mta or

100 mta HG containing either no added lipids (x) or a molar ratio of egg

PC to micellar detergent of 1:5 (++) or 1:10 (■).
An indirect effect of raising the detergent concentration might originate from the loss of a subunit or cofactor by dilution into the micellar phase, resulting in the destabilization of the dimer. From this point of view, the number of candidates as stabilizing factors is relatively limited. The only subunit whose loss is generally correlated with monomerization is the Rieske protein. However, this correlation is not absolute since (i) mild treatment with HG produces a Rieske-depleted form of the wild-type $b_6f$ dimer (47 and this work) and (ii) PetL-less $b_6f$ can transiently form monomers that retain the Rieske protein (9). Furthermore, we have previously shown that the Rieske protein released by detergent treatment does not bind to detergent micelles (13). Loss of the Rieske protein, therefore, appears more likely to be a consequence of a detergent-induced modification of the $b_6f$ complex rather than its cause. As far as cofactors are concerned, plastoquinol and carotenoids are present in substoichiometric amounts (14) and, therefore, could hardly stabilize all of the complexes. Chlorophyll $a$ is present stoichiometrically (8, 12), but it is released much too slowly (weeks; cf. Refs. 8 and 14) for its loss to be the primary event that initiates the fast inactivation induced by detergents (minutes; cf. Fig. 4). Furthermore, the Rieske-depleted dimeric complex obtained by mild treatment with HG still retains the chlorophyll. Finally, a protecting effect of endogenous lipids appears unlikely in purified preparations since their concentration is undetectably low (1/cytochrome $f$); it could, however, explain why high concentrations of HG are less destabilizing when applied to partially rather than to totally purified complexes.

On the other hand, a stabilizing effect of exogenous lipids is clearly indicated. For a given concentration of detergent micelles, the rate of dissociation and inactivation of the $b_6f$ complex is reduced in direct relationship to their lipid content. The hypothesis that delipidation leads to monomerization is further corroborated by the observation that dimeric $b_6f$ retains sev-
eral dozens of lipid molecules, while the monomeric form does not. A destabilizing effect of delipidation is consistent with a number of earlier observations, both on spinach b$_{6}f$ (22–24, 73, 74) and on the bc$_{1}$ complex (36, 60, 75), and is very commonly encountered with other membrane proteins (see e.g. Refs. 76 and 77, and references therein). Its mechanism will deserve further examination. Occupancy by lipids of certain critical sites on the protein could stabilize the protein, e.g. by promoting folding or interaction of certain regions. Alternatively, lipids could exert their protective effect by competing for sites where the binding of detergent would favor transition toward inactive protein conformations, e.g. because of the detergent’s greater ability to intrude into the protein structure (cf. Ref. 78). 

**Steps in b$_{6}f$ Dissociation**—Whatever the mechanism, much of our data is consistent with delipidation first inducing a change of the b$_{6}f$ structure that weakens both monomer/monomer and Rieske/b$_{6}f$ interactions (Fig. 6). Dissociation and denaturation of the Rieske protein, currently an irreversible step with the complex from C. reinhardtii, may either precede or follow monomerization depending on the detergent used; delipidation by LM leads directly to the most dissociated form (Fig. 6, form ⑤) while denaturation by HG is less easily controlled but more progressive: loss of the Rieske protein precedes monomerization, which occurs concomitantly with the loss of the chlorophyll $a$ molecule, the last step being the dissociation of the PetL subunit. Forms ①, ②, ③, and ④ of Fig. 6 have been observed in the present work. 

As argued above in the case of PetL, the limited stability of the Rieske-depleted dimer suggests that the Rieske protein may interact with subunits belonging to both monomers. This hypothesis is consistent with electron microscopy data that suggest the Rieske protein lies close to the monomer/monomer interface (65) and might explain why its loss and monomerization of the complex generally occur concomitantly. 

The pathway described above (Fig. 6, bottom) does not account for the fact that, for a given lipid-to-detergent ratio in mixed micelles, inactivation is more rapid when the concentration of micelles is raised. Indeed, the degree of occupancy of lipid-binding sites, considered as the factor controlling the rate of dissociation of the complex, ought to depend on the composition of the micelles but not on their concentration. We must envision, therefore, an alternative route (Fig. 6, top) where formation of an unstable monomer precedes the loss of the Rieske protein and is driven by dilution of the dimer in a large pool of micelles rather than by delipidation. The postulated intermediate ③, a monomeric form retaining all seven subunits and the chlorophyll, has not been isolated in our experiments with wild-type C. reinhardtii b$_{6}f$. The possibility of its existence, however, is suggested both by the transient presence of a monomeric form containing the Rieske protein during purification of PetL b$_{6}f$ (9) and by the recent report of an enzymatically active light form of spinach b$_{6}f$ (24). Micelle composition and concentration, and possibly species differences, would determine the rate of formation and dissociation of this intermediate, which may explain some seemingly inconsistent observations reported in the literature regarding the composition and activity of light forms of the b$_{6}f$ complex. 

**Conclusion**—In the present work, we have precisely measured the $M_{f}$ of the purified b$_{6}f$ complex from C. reinhardtii, showing it to be a dimer, and undertaken the identification of conditions under which the complex is stable, of conditions under which it loses the Rieske protein and dissociates into inactive monomers, and of the steps in this process. Our results reinforce the general belief that the dimer is the predominant form of the b$_{6}f$ in situ while the monomer observed in detergent solution is a breakdown product. Indirect observations suggest that the small 32-residue subunit PetL plays a role in stabilizing the dimeric state. Delipidation is shown to be a major factor in detergent-induced inactivation. Efficient control of the stability and monodispersity of the preparations is a prerequisite to further progress in studying the structure and function of the b$_{6}f$ complex in vitro. The work described in the present article has formed the basis for setting up conditions favoring the growth of well ordered two-dimensional crystals of C. reinhardtii b$_{6}f$, which have yielded an 8 Å-resolution projection map of the negatively stained complex (65).
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