Purification and Characterization of the Cytochrome \( b_{6}f \) Complex from Chlamydomonas reinhardtii*

(Received for publication, July 21, 1995, and in revised form, September 21, 1995)

Yves Piret, Cécile Breton, David Kramer, and J.-Luc Popot

From the Institut de Biologie Physico-Chimique and Collège de France, CNRS URA 1187, 11 rue Pierre et Marie Curie, F-75005 Paris, France and the Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801

A protocol has been developed for the purification of the cytochrome \( b_{6}f \) complex from the unicellular alga Chlamydomonas reinhardtii. It is based on the use of the neutral detergent Hecameg (6-O-[(N-heptylcarbamoyl)-methyl-\( \alpha\)D-glycopyranoside) and comprises only three steps: selective solubilization from thylakoid membranes, sucrose gradient sedimentation, and hydroxylapatite chromatography. The purified complex contains two \( b \) haems (\( \alpha \) band, 564 nm; \( E_{\text{m,}b} \) = -84 and -158 mV) and one chlorophyll \( a \) (\( \lambda_{\text{max}} \) = 667-668 nm) per cytochrome \( f \) (\( \alpha \) band, 554 nm; \( E_{\text{m,}f} \) = +330 mV). It is highly active in transferring electrons from decylplastoquinone to oxidized plastocyanin (turnover number 250-300 s\(^{-1}\)). The purified complex contains seven subunits, whose identity has been established by \( N \)-terminal sequencing and/or peptide-specific immunolabeling, namely four high molecular weight subunits (cytochrome \( bc_{1} \), Rieske iron-sulfur protein, cytochrome \( b_{6} \), and subunit IV) and three \( 4 \)-kDa miniproteins (PetG, PetL, and PetX). Stoichiometry measurements are consistent with every subunit being present as two copies per \( b_{6}f \) dimer.

The photosynthetic electron transfer chain, the device that provides essentially all of the free energy dissipated by living beings, comprises two membrane protein complexes that are homologous to complexes of the respiratory chain, the \( F_{5}F_{0} \) (respectively, \( F_{0}F_{1} \)) proton ATP-synthase and the \( b_{6}f \) (respectively, \( bc_{1} \)) oxidoreductase. The \( bf/bc \) complexes transfer electrons from a liposoluble quinol to a hydrophilic oxidized protein, plastocyanin or cytochrome \( c \), and couple the resulting electron free energy drop to setting up a transmembrane proton electrochemical potential. Neither the structure of the \( b_{6}f \) nor that of the \( bc_{1} \) complex is known in any detail, and the existence of an unique versus alternative mechanism(s) of electron transfer remains a matter of discussion (see, e.g., Refs. 1-4 and references therein).

Most structural studies of the \( b_{6}f \) complex to date have been carried out either on higher plants or on cyanobacteria (for reviews, see Refs. 1, 2, and 5-8). Unicellular algae, such as Chlorella sorokiniana and Chlamydomonas reinhardtii, have long been recognized as convenient organisms for combined spectroscopic, biochemical and genetic studies of the \( b_{6}f \) complex structure and function (9, 10). In the present article, we describe the purification of the \( b_{6}f \) complex from \( C. \) reinhardtii. From the point of view of the experimentalist, \( C. \) reinhardtii combines a number of advantages: (i) cell suspensions are amenable to in vivo studies of the electron transfer and proton pumping processes using spectroscopic and other biophysical approaches; (ii) \( C. \) reinhardtii being a facultative phototroph, mutant strains with defective photosynthetic complexes can be generated and maintained; (iii) large fractions of its chloroplast genome have been sequenced; (iv) techniques for modification of its chloroplast and nuclear genes are being developed; (vi) it is amenable to in vivo isotopic labeling; (vi) large scale growth, although not currently resorted to, is not an unrealistic objective (for a general review of \( C. \) reinhardtii laboratory use, see Ref. 10).

Previous studies have shown that the \( b_{6}f \) complex from \( C. \) reinhardtii is similar to that of higher plants in its complement of high molecular mass subunits (11, 12). It comprises two heme-bearing subunits (cytochrome \( f \) and cytochrome \( b_{6} \)), a protein carrying a [2Fe-2S] cluster (the so-called Rieske protein), and an integral protein devoid of prosthetic group ("subunit IV") that is homologous to the C-terminal region of mitochondrial cytochrome \( b \) (13). More recent studies have shown that additional, very small subunits (\( \sim 4 \) kDa) are also present, namely: 1) the product of the \( petG \) chloroplast gene, initially identified in higher plants (14, 15) and subsequently shown to be present in \( C. \) reinhardtii \( b_{6}f \) (15, 16); 2) the PetX protein, identified both in \( C. \) reinhardtii (15, 16) and in spinach (15) complexes, which is the product of a nuclear gene (16, 17); and 3) the product of the chloroplast gene \( ycf7 \) (petL),\(^{1} \) a gene that is also present in higher plant chloroplast genomes.

Obtaining large scale preparations of active integral membrane proteins is a major stumbling block in understanding the function of these proteins at the structural level. Two limitations are the amount of protein produced by laboratory organisms and scale limits on purification procedures. Until now, preparations of \( b_{6}f \) complex from \( C. \) reinhardtii (11, 12, 15) have suffered from limited purity, low yields, and low or unknown enzymatic activity. In the present article, we describe a rapid (three-step) protocol for the preparation of highly purified \( C. \) reinhardtii \( b_{6}f \) complex that is enzymatically fully active. The neutral detergent used throughout the purification,6-O-[(N-heptylcarbamoyl)-methyl-\( \alpha\)D-glycopyranoside (Hecameg),\(^{2} \)

---

1 Y. Takahashi, M. Rahire, C. Breton, J.-L. Popot, P. J. oliot, and J.-D. Rochaix, manuscript in preparation.
2 The abbreviations and trivial names used are: Hecameg (HG), 6-O-[(N-heptylcarbamoyl)-methyl-\( \alpha \)-D-glycopyranoside; C\(_{10}\)PQ, decylplastoquinone; C\(_{10}\)PO\(_{4}\), decylplastoquinoid; C\(_{10}\)stigmatellin, tridecylstigmatellin; CMC, critical micellar concentration; HA, hydroxylapitate; LM, laurylmaltoside (dodecyl-\( \beta \)-D-maltoside); OG, octyl-\( \beta \)-D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; PC, \( l \)-phosphatidylcholine; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene

---

* This work was supported by the CNRS, the Collège de France, the Commissariat à l’Energie Atomique, and by grants from the EEC (BIO2-CT93-0076), from the Ministère de la Recherche et de la Technologie (B7C.0385), and from IMABIO (to J.-L. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a National Science Foundation-NATO postdoctoral fellowship.

‡ To whom correspondence should be addressed. Fax: 33-1-40-46-83-31.
Ref. 19, is found to be an interesting substitute to the widely used but much more expensive octyl-\(\beta\)-glucopyranoside (OG). At variance with previous \(b_6\)-f purification protocols, the new procedure does not resort at any step to an electrical charged detergent and it is easily amenable to scaling up: two favorable factors for growing crystals. The purified complex has been characterized with respect to its subunit composition and its spectroscopic and redox properties.

**EXPERIMENTAL PROCEDURES**

**Materials—**Horse heart cytochrome c, decylplastoquinone (C\(_{10}\)-PO), laurylmaltoside (LM), Tricine, egg yolk \(l\)-\(\alpha\)-phosphatidylcholine (PC), Frédéric's adventuar, phenylmethylsulfonyl fluoride (PMSF), \(\epsilon\)-amino-\(\nu\)caproic acid, benzamidine, aprotenin, and sucrose were obtained from Sigma, sodium dodecyl sulfate (SDS) from Pierce, Hexagen (HC) from Vegatec (Villejuif, France), hydroxylapatite (HA) from Bio-Rad, Mycobacterium tuberculosis from Difco, dithiothreitol from Boehringer Mannheim, 3,3,5,5-tetramethylbenzidine (TMBZ) from Fluka, urea from Tébu, immobilization polyvinylidene difluoride (PVDF) membranes from Millipore, \(^{14}\)C-acetate, enhanced chemiluminescence kits, and \(^{32}\)P-labeled polypeptides were detected either by autoradiography of dried gels on Agfa Curix MR4 films or using Phosphorimager plates ( Molecular Dynamics).

**Immunolabeling—**Proteins were electrophoretically transferred onto Immobilon PVDF membranes in a semidyblotting system at 0.8 mA/cm\(^2\) for about 30 min. Immunodetection was carried out either by the enhanced chemiluminescence peroxidase method (Amersham) or by labeling with \(^{125}\)I-protein A, as described previously (33).

**Protein Sequencing—**Polypeptide purification by SDS-PAGE and transfer to PVDF membranes were performed as described previously (33). An amino acid sequence carried out at the Service Central d'Analyse du CNRS (Vernaison, France) by L. Denory.

**Analytical Procedures—**Protein concentrations were determined by the Bradford method (34). Chlorophyll concentrations were determined according to Ref. 35. Critical micellar concentrations (CMC) were estimated either by the pyrene partitioning method (36) or by counting drops from a Hamilton syringe with a non-beveled needle. The accuracy of quoted values for HG is probably approximately \(+1\) mV.

**Spectroscopy—**UV-visible absorbance spectra were recorded either on a home-built spectrophotometer of the Job plot design (37, 38) or on a Kontron Uvikon 930 instrument. Cytochromes were oxidized with ferricyanide and reduced either with ascorbate (cytochrome \(f\)) or with dithionite or borohydride (cytochromes \(b_6\) and \(b_1\)). The spectrum of cytochrome \(c_6\) in the UV-visible region was obtained by recording two spectra, the first one following an air equilibration and the second one after ascorbate reduction, and subtracting from the former the ascorbate-minus-air difference spectrum, scaled so as to cancel the \(\alpha\) peak of cytochrome \(c_6\). Wavelengths of absorbance maxima and isobestic points are rounded off to the nearest nanometer.

**Redox Potentiometry—**Spectroscopic scans on a home-built \(j\) plot spectrophotometer (39). The monochromator was set to a 2.0 nm wavelength resolution. Purified \(b_6\)-f complex was diluted to a concentration of 375 nm in deoxygenated TMK buffer containing 0.3 mV LM and the following redox mediators: anthraquinone-2-sulfonate (10 \(\mu\)m), anthraquinone-1,5-disulfonate (10 \(\mu\)m), 2,5-dimethyl-1,4-benzoquinone (10 \(\mu\)m), pyocyanin (4 \(\mu\)m), 1,4-naphthoquinone (10 \(\mu\)m), phenazine ethosulfate (4 \(\mu\)m), duroquinone (10 \(\mu\)m), N-ethylphenoazonium phenosulfate (4 \(\mu\)m), trimethyl-p-benzoquinone (10 \(\mu\)m), 1,2-naphthoquinone (10 \(\mu\)m), 2,5-dimethyl-p-benzoquinone (10 \(\mu\)m), N,N,N',tetramethyl-p-phenylene diamine (4 \(\mu\)m), and 2,3,5,6-tetra- methyl-p-phenylene diamine (4 \(\mu\)m). Redox potentiometric measurements were made with a platinum electrode, using a standard \(\gamma\) electrode as a reference. The data were corrected to reflect potentials against the standard hydrogen electrode. Redox poising was accomplished by additions of small quantities of concentrated solutions of sodium ferricyanide and sodium hydrosulfite. At least 3 min were allowed for equilibration after each change in ambient redox potential before the spectra were scanned. The pH at the end of the titration was checked to insure that no drift had occurred.

Spectral changes recorded from \(-250\) to \(+450\) mV were analyzed by taking the average of three spectra recorded at \(400 \pm 10\) mV and subtracting the average of four spectra taken at \(230 \pm 30\) mV; the spectrum of cytochrome \(b_6\) was obtained by subtracting from a spectrum recorded at \(-80\) mV a spectrum recorded at 0 mV; the spectrum of cytochrome \(b_1\) was obtained by subtracting from a spectrum taken at \(-250\) mV a spectrum recorded at oxidizing conditions. The ratio of cytochrome \(b_1\) to cytochrome \(b_6\) was estimated by recording two spectro-photometric titration curves, assuming extinction coefficients of 18,000 \(\text{m}^{-1}\cdot\text{cm}^{-1}\) (cytochrome \(f\)) and 20,000 \(\text{m}^{-1}\cdot\text{cm}^{-1}\) (average of the two \(b\)-type cytochromes) (peak absorbance minus absorbance at isobestic point; Ref. 40).

**Decylplastoquinone Reduction—**A diethylether solution of decylplastoquinone (C\(_{10}\)PO) was removed to decylplastoquinone (C\(_{10}\)PO\(_2\)H\(_2\)) by an aqueous solution of sodium dithionite and sodium borohydride with strong agitation in the dark until the solution became colorless (see Ref. 41). A small volume of chlorhyric acid was then added to eliminate the sodium borohydride in excess. The diethylether phase was collected and evaporated to dryness under N\(_2\). The C\(_{10}\)PO-H\(_2\) was resuspended in 100 mM ethanolic acid with chlorhyric acid, C\(_{10}\)PO-H\(_2\) concentration was estimated from the absorption value at 290 nm using an extinction coefficient \(e_{290} = 3.540 \text{ m}^{-1}\cdot\text{cm}^{-1}\) (42).

**Purification of Cytochrome \(b_6\)—**All steps were performed at 4°C; all
buffers contained 200 μM PMSF, 1 mM benzamidine, and 5 mM ε-amino capric acid as protease inhibitors.

For selective solubilization, thylakoid membranes of wild type cells were resuspended in ice-cold TMK buffer containing 25 mM HG at a chlorophyll concentration of 1.5 μg/ml and incubated at 4 °C for 15 min. with occasional agitation. The suspension was centrifuged either at 160,000 × g (80,000 rpm) for 10 min in the TLA 100.3 rotor of a Beckman TL100 ultracentrifuge, or at 200,000 × g (43,000 rpm) for 30 min in the 70 Ti rotor of a Beckman L8 ultracentrifuge, depending on the volume (6 or 30 ml).

The solubilization supernatant was fractionated by centrifugation in 10–30% (w/v) sucrose density gradients in TMK buffer containing 20 mM HG and 0.1 glit egg PC. The presence of egg PC during this and the following step of purification is necessary to prevent the dissociation of the Rieske protein from the complex (43). The gradients, onto which were layered ~600 μl (Beckman SW41 Ti rotor) or ~3 ml (45 Ti rotor) of supernatant, were centrifuged at 270,000 × g (4,000 rpm) for 24 h (SW41 Ti rotor), or at 180,000 × g (36,000 rpm) for 16 h (45 Ti rotor). The yellow-brown band containing the cytochrome b_{6}f complex was collected with a syringe.

The band collected from the gradient was layered onto a HA column (0.7 cm × 2 cm, or 1 cm × 3 cm) pre-equilibrated with 20 mM Tricine-NaOH, pH 8.0, 20 mM HG, 0.1 glit egg PC. The column was washed with 3 column volumes of 100–200 mM ammonium phosphate (depending on the band to be collected), 20 mM HG and 0.1 glit egg PC. Pure cytochrome b_{6}f complex was eluted with 400 mM ammonium phosphate, pH 8.0, containing 20 mM HG and 0.1 glit egg PC. Typical yields are ~600 μl of pure b_{6}f complex at ~6 μM cytochrome f from one SW41 rotor or ~3 ml at ~10 μM for one 45 Ti rotor, with larger preparations resulting in better overall yields.

Our initial experiments with HG were made somewhat frustrating by irreproducible batch-to-batch results. These appeared to be due to the presence of diheptylurea, a side product from the synthesis. In the presence of traces of diheptylurea, abnormal migration of the complex, resulting in a FACS-like band containing 22,000 M, was observed, and electron transfer activity was inhibited. The presence of diheptylurea is easily detected by leaving a 20 mM solution of HG in water overnight in the cold room. Diheptylurea precipitates as a fine white powder. This problem has been solved by the manufacturer, and recent HG batches (~2001, WK1–20, and CP1–57) have been free from diheptylurea. From a practical point of view, we note that the CMC of HG is similar at 4 °C and at room temperature (see also Refs. 44 and 45), but appears to be sensitive to high salt concentrations, since the CMC at 4 °C dropped from ~19.5 mM to ~15 mM when moving from the low ionic strength TMK buffer to 0.4 M ammonium phosphate (see also Ref. 45).

Assays of Electron Transfer Activity—Cytochrome b_{6}f oxidoreductase activity was measured at room temperature in a Kontron Uvikon 930 spectrophotometer, using 15 μM C_{60},POH2 as an electron donor and either 5 μM oxidized cytochrome c or 5 μM oxidized spinach plastocyanin as an acceptor. The reaction was initiated by adding C_{60},POH2 to a solution containing 20 mM Tricine-NaOH buffer, pH 8.0, 0.3 mM LM, cytochrome b_{6}f (0.5–2 mM), and the electron acceptor. Reduction of horse cytochrome c was monitored during 2 min at room temperature by the absorbance change at 549 nm, assuming an extinction coefficient of 22,000 M−1 cm−1 (22). Reduction of spinach plastocyanin was monitored under the same conditions by the absorbance change at 600 nm, assuming an extinction coefficient of 4,500 M−1 cm−1 (46). We checked that, under the latter conditions, the rate of electron transfer is independent on the concentration of POH2 and linearly related to that of plastocyanin. When measuring the oxidoreductase activity of thylakoid membranes or of fractions containing partially purified b_{6}f complex, using cytochrome c as an acceptor, 1 μM antimycin was added to the solution in order to inhibit the oxidoreductase activity of the contaminating bc_{1} complex (22).

RESULTS

Purification Protocol—Treatment of thylakoid membrane preparations from C. reinhardtii (22, 24) with appropriate concentrations of Hecameg (HG; see “Materials and Methods”) preferentially solubilizes the CF_{2}CF_{1} ATP synthase and the cytochrome b_{6}f complex, resulting in a ~3-fold increase of the b_{6}f/protein ratio (Fig. 1, lane 2, and Table I). Distribution of the b_{6}f complex in this and subsequent fractionation experi-

4 W. Kluge, personal communication.

5 Y. Pierre and J.-L. Popot, unpublished observations.
The concentrations of hemes from the redox titration data were undistinguishable (Fig. 3). The ratio determined from the redox titration data (Fig. 3) and the visible difference spectra (from a different membrane preparation): Em was calculated by subtracting from the spectrum of air-equilibrated ferricyanide-oxidized differences spectra and on direct protein determination (34) and are averages ± S.D. over 3 preparations using SW41 Ti rotors. Larger preparations, using 45 Ti rotors, typically yielded 6–7 mg of purified complex/20-liter growth.

The subunit composition of the cytochrome b₆f complex was studied by SDS-PAGE using either gradient gels in the presence of urea (30) or a three-step gel system (data not shown). The specific activity of electron transfer to cytochrome c was the same whether measured on the HG supernatant, on the b₆f fraction from the sucrose gradient or on purified cytochrome b₆f preparations, indicating that no inactivation occurred during purification (data not shown).

After 2-week storage at 0°C in the dark, purified preparations retained ~75% of their activity and exhibited no obvious change of their polypeptide pattern. They could be frozen without loss of activity.

Polyepitope Composition—The subunit composition of the purified complex was studied by SDS-PAGE using either gradient gels in the presence of urea (29) or a three-step gel system devoid of urea (30). Staining with silver revealed five bands in the first system, with apparent molecular mass values of 39.5, 18.5, 18, 12.5 and 4 kDa (Fig. 1, lane 4). The 4-kDa band often appeared heterogeneous. The five bands comigrated throughout the sucrose gradient and hydroxylapatite purification steps (data not shown). In the discontinuous, three-step gel system, the 4-kDa band was resolved into two, sometimes three or four bands of variable intensity (see Ref. 16). The pattern observed depended on incompletely identified factors that affect the migration and staining of the subunits, and it is not certain that each band represents a distinct polypeptide rather than variable states of denaturation of some of them.

Identification of the bands was based on their apparent Mr, heme content as revealed by TMBZ staining, N-terminal sequencing, and cross-reaction with antipeptide antisera (Table II). These approaches confirmed that the four high Mr bands correspond, in this order, to cytochrome c, the Rieske iron-sulfur protein, cytochrome b₆f, and subunit IV (11, 12). 1) N-terminal sequencing of the TMBZ-stainable 39.5-kDa band yielded a sequence (Table II) that is internal to that of the cytochrome c precursor, as predicted from the sequence of the petA gene (51, 52). The band was labeled by antisera raised against this N-terminal peptide or against a peptide with the predicted C-terminal sequence of cytochrome c (Fig. 1, lane 5, and data not shown). 2) Sequencing of the 18.5-kDa band yielded a sequence (Table II) that is homologous with...
N-terminal sequences of other Rieske proteins. The 18.5-kDa band was labeled by antisera directed against this N-terminal sequence (Fig. 1, lane 6). Oligonucleotides coding for this sequence have been used to clone and sequence *C. reinhardtii* petC nuclear gene (53). 3) The diffuse TMBZ-stainable 18-kDa band did not yield any phenylthiohydantoin derivatives upon amino acid sequencing. It was labeled by antisera directed against peptides corresponding to the predicted N- and C-terminal sequences of cytochrome *b₆* deduced from that of the *petB* gene (52) (Fig. 1, lane 7, and data not shown). 4) The 12.5-kDa band did not yield any N-terminal sequence either. This band was labeled by two antisera (Fig. 1, lane 8, and data not shown), raised respectively against the N-terminal and C-terminal sequences predicted from the *petD* gene, which encodes subunit IV (52).

We have shown previously that the ~4-kDa band(s) represent(s) more than one polypeptide (Ref. 16; see also Ref. 15). The presence of the product of the *petG* gene was inferred from labeling with an antipeptide directed against the C-terminal part of the predicted PetG protein. The presence of a novel subunit, product of an unidentified nuclear gene provisionally named petX, was established by N-terminal sequencing and pulse labeling in the presence of various inhibitors of protein synthesis (Refs. 15 and 16; Table II). Immunolabeling shows that PetX and PetG comigrate during SDS/urea-PAGE and migrate close to one another in three-step gels in the absence of urea (16). The complete sequence of PetX has recently been established by further protein sequencing and by cloning and sequencing of the *petX* gene (17). Finally, the presence of a third ~4-kDa subunit has been recently established by deletion of the chloroplast *ycf7* (*petL*) gene and immunoblot analysis of purified *b₆f* preparations obtained from wild-type and *ycf7*-strains (47).1

Immunoblotting of SDS-PAGE gels shows that all of these seven proteins are absent in thylakoid membranes prepared from mutant FuD4, that lacks the *b₆f* complex (Fig. 1, lanes 5–8, and data not shown).

Posttranslational Modifications—N-terminal sequencing defines the point of cleavage of addressing and signal sequences in the Rieske protein, in cytochrome *f* and in PetX (Table I). Antipeptide antisera indicate that most of the predicted N-terminal and C-terminal sequences are present in mature cytochrome *b₆* and subunit IV. Spinach cytochrome *b₆* has been reported to be a substrate for an endogenous redox-potential-controlled kinase (54). In order to search for phosphorylation sites in the purified *C. reinhardtii* *b₆f* complex, cells were labeled at equilibrium with [³²P]phosphate under the conditions indicated under “Materials and Methods.” None of the *b₆f* polypeptides was found to incorporate a significant amount of radioactivity, under conditions where labeling to an extent of a few percents of each chain or less would have been detected (data...
**Table II**

| Apparent M<sub>b</sub> (SDS-PAGE)<sup>a</sup> | Protein identity | Actual M<sub>b</sub><sup>b</sup> | TMBZ staining | Edman degradation | Immunolabeling<sup>c</sup> | Criteria for identification |
|---------------------------------|-----------------|-----------------|---------------|-----------------|-----------------|-----------------------------|
| kDa                             |                 | kDa             |               |                 |                 |                             |
| 39.5                            | Cytochrome f    | 31.8            | +             | NH$_2$-YPVFAQONYANPREANGRIVXN.<sup>d</sup> | +               |                             |
| 22                              | Rieske          | 18.6            | -             | NH$_2$-AAASEVPMKRN1MNLLAGAXLPM...<sup>d</sup> | +               |                             |
| 18.5                            | Subunit IV      | 17.4            | -             | +               |                 |                             |
| 12.5                            | PetG            | 4.0             | +             | +               |                 |                             |
| 4.5–5.5                         | PetL            | 3.4             | -             | +               |                 |                             |
| ~4                              |                 |                 |               |                 |                 |                             |

**Panel A**

A autoradiogram of purified 14C-labeled b$_6$f complex following urea/SDS-PAGE. B, relative radioactivity associated with each subunit or group of subunits; the radioactivity in each band was quantified using PhosphorImager plates, divided by the number of carbon atoms contained in the subunit(s) present in the band and normalized to the average activity per carbon atom in the complex (see "Materials and Methods"). Panel B shows average ± S.D. of 10 measurements on two different preparations. C, a scan through the middle of the lane shown in panel A; note that because of the variable width and broadness of the bands, peak heights and areas are not proportional to relative activities.

**DISCUSSION**

Purification Protocol—Our protocol for purification of the b$_6$f complex from C. reinhardtii thylakoid-rich membranes is derived from the classical Hauska-Cramer procedures used to purify higher plant b$_6$f complexes (49, 50, 55, 56). It presents a number of advantages over current methods (see e.g. Refs. 15 and 49, and references therein). As regards the C. reinhardtii complex, it is much simpler than the 6-step protocol of Schmidt and Malkin (15), and it yields a preparation that is free of polypeptide contaminants and that exhibits a 20-fold higher, native-like electron transfer activity. The greater purity probably reflects the fact that the Schmidt-Malkin protocol has been optimized for spinach rather than for C. reinhardtii b$_6$f.

The better preservation of the activity is presumably linked to limiting delipidation. As regards the b$_6$f complex in general, our protocol yields preparations whose purity and activity are comparable to or better than those reported for the best higher plant or cyanobacterial preparations (see e.g. Refs. 15, 48–50, 56–58, and references therein). It is nonetheless simpler than the usual spinach protocol (49, 50), it does not resort to any heterogeneous detergent mixture, and it presents the added advantage of making use of a facultative phototrophic organism. Finally, this protocol is easily amenable to scaling up. Current limitations on mass production are the size of C. reinhardtii growths (20-liter flasks, yielding 5–7 mg of purified b$_6$f) not shown).

Subunit Stoichiometry—In order to examine subunit stoichiometry, b$_6$f complex was purified from C. reinhardtii cells grown on [14C]acetate and the radioactivity associated with polypeptide bands on SDS-PAGE gels quantified using PhosphorImager plates (Fig. 5). The ratio of 14C activity in the high M<sub>b</sub> bands was very close to stoichiometric, when normalized to the number of carbon atoms per protein (Fig. 5B). The relative amount of radioactivity in the five bands suggests that the 4-kDa band contains ~3 polypeptides/cytochrome f. These data are compatible with all seven subunits being present in equimolar amount. The protein content of the purified preparations (112 ± 9 mg/μmol of cytochrome f) is close to that expected assuming this stoichiometry (103 mg/μmol). However, the dimeric nature of the complex (43), the uncertainty in the determinations, and the small size of the ~4-kDa subunits combine to make it difficult to rule out two possible cases of figure, namely (i) the existence of still other unidentified 4-kDa subunits, and/or (ii) a substoichiometric complement of one or more of the 4-kDa subunits, that would be present as one copy per b$_6$f dimer rather than one per monomer. A direct, reliable quantitation of each of the 4-kDa subunits has not been possible yet. As noted above, these subunits are not well resolved under our usual SDS/urea-PAGE conditions (Fig. 1, lane 4). Three-step gels (30), which give a better but variable resolution, were found to be unreliable for this purpose, presumably because of the incomplete denaturation of some subunits in the absence of urea.

**Fig. 5. Subunit stoichiometry in 14C-labeled b$_6$f complex.** A, autoradiogram of purified 14C-labeled b$_6$f complex following urea/SDS-PAGE. B, relative radioactivity associated with each subunit or group of subunits; the radioactivity in each band was quantified using PhosphorImager plates, divided by the number of carbon atoms contained in the subunit(s) present in the band and normalized to the average activity per carbon atom in the complex (see "Materials and Methods"). Panel B shows average ± S.D. of 10 measurements on two different preparations. C, a scan through the middle of the lane shown in panel A; note that because of the variable width and broadness of the bands, peak heights and areas are not proportional to relative activities.
and the cost of detergent.

In this latter respect, Hecameg (HG) appears as an economically interesting substitute to the much more expensive octyl-
β-D-glucopyranoside (OG) for membrane protein purification. HG has been developed by Plusquellec and co-workers (19). Its chemical structure, molecular mass, aggregation number, and CMC are similar to those of OG (19, 44, 45), and it is likely to be able to substitute for it under many circumstances. HG has been hitherto applied to the purification of mycoplasma surface antigens (19, 59), to the solubilization of the sarcoplasmic Ca2+-ATPase (45), and to the crystallization of beef heart cytochrome b3 (60). Proteins that are sensitive to OG are not good candidates for purification with HG, as indicated by preliminary experiments with Ca2+-ATPase (45) and with the nicotinic acetylcholine receptor. C. reinhardtii b,f complex, on the other hand, is solubilized by HG somewhat more specifically than by OG, and purified preparations in HG are stable for weeks provided lipids are also present.

Characterization of Redox Components—Knowledge of the spectral and redox properties of the C. reinhardtii b,f complex will be essential for conducting experiments on its function. The absorbance spectra of the cytochromes of the b,f complex were found to be similar to those of higher plants and of other algae (61–63). The only minor differences were the absorbance peaks in the α band regions of the hemes; those for cytochrome f, cytochrome b3, and cytochrome b2 were found to be 554, 564, and 564 nm, respectively. Within the noise level the peaks from the two cytochrome b2 hemes were indistinguishable. In contrast, spinach cytochrome b3 was found to have an α peak shifted by 1–2 nm to the red (64, 65), whereas, in C. sorokiniana, cytochrome b3 is shifted by approximately 1 nm to the blue (18). It seems from these data that the cytochrome b3 α peaks can vary by a few nm from species to species, even though their respective function and redox properties are conserved. The absorbance spectrum of cytochrome f in the purified b,f complex is nearly identical to that in intact cells of C. reinhardtii.

Anaerobic redox titrations were performed on isolated cytochrome b,f complex in order to determine the redox midpoint potential of the components. Cytochrome b3 and cytochrome b2 were found to have E° values of −158 and −80 mV, respectively, similar to those found in spinach cytochrome b2,f in thylakoids at pH 8.3 (65). Cytochrome f was found to have a slightly lower E° (approximately +340 mV) than reported for other systems (+350 to −370 mV; Refs. 1 and 65).

Polypeptide Composition—Earlier work on C. reinhardtii b,f complex has led to the identification of four subunits, cytochromes f and b3, the Rieske protein, and subunit IV (11, 12). More recent studies have revealed the additional presence of a number of other proteins that are not highly pure and highly active in electron transfer. It establishes the polypeptide composition of the complex and some of its physico-chemical properties. These data further assert the great similarity of the b,f complex from C. reinhardtii to that from higher plants and, therefore, its usefulness as an experimental model. The purification protocol, as well as a number of other experimental tools established in the course of this work, such as a library of subunit-specific antisera, should be of great use in future studies of the complex. Examination of its non-protein components and crystallogenesis experiments are currently in progress (47).

Acknowledgments—We are particularly grateful to J.-L. Girard-Bascou and R. Matagne for making available the DUM-1/BF4 and DUM-1 C. reinhardtii strains, respectively, to L. Denory (Service Central d’Analyse du CNRS, Vernaison) for amino acid sequencing, to D. Drapier for collaboration in early phases of this work, to Y. Takahashi and J.-D. Rochaix for a gift of anti-PetL antisera, to P. Rich for a gift of C6-Sephalapur, to H. Couratier for the photographic work, and to A. Alonso, P. Bennoun, W. A. Cramer, L. Dutton, J.-L. Eiselle, P. J. Oliot, W. Klagba, M. le Maire, W. Nitschke, D. Pictet, D. Plusquellec, F. Reiss-Huison, P. Rich, B. Schoepf, A. Verméglio, C. de Vitry, F.-A. Wollman, and H. Wrzblewski for useful discussions and/or for sharing materials or unpublished information. Work with Hecameg was facilitated by the purchase of batch WK-120 by the CNRS Groupement de Recherche 1082 “Systèmes Colloïdes Mixtes” and by information exchanged among members of this network.

REFERENCES
1. Hope, A. B. (1993) Biochim. Biophys. Acta 1143, 1–22
2. Trumpower, B. L., and Gennis, R. B. (1994) Annu. Rev. Biochem. 63, 675–716
3. J. Oliot, P. J. Oliot, and J. Oliot, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1030–1038
4. Kramer, D. M., and Crofts, A. R. (1994) Biochim. Biophys. Acta 1184, 193–201
5. M. de Vitry, C. de Vitry, and J.-L. Popot, unpublished observations.
6. C. de Vitry, C. Breyton, Y. Pierre, and J.-L. Popot, submitted for publication.
Purification and Characterization of the Cytochrome $b_{6}f$ Complex from *Chlamydomonas reinhardtii*

Yves Pierre, Cécile Breyton, David Kramer and Jean-Luc Popot

*J. Biol. Chem.* 1995, 270:29342-29349.
doi: 10.1074/jbc.270.49.29342

Access the most updated version of this article at [http://www.jbc.org/content/270/49/29342](http://www.jbc.org/content/270/49/29342)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 10 of which can be accessed free at [http://www.jbc.org/content/270/49/29342.full.html#ref-list-1](http://www.jbc.org/content/270/49/29342.full.html#ref-list-1)