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# Green algal cytochrome $b_6$-$f$ complexes: isolation and characterization from *Dunaliella saline, Chlamydomonas reinhardtii* and *Scenedesmus obliquus*

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**Cytochrome $b_6$-$f$ complexes have been isolated from *Chlamydomonas reinhardtii, Dunaliella saline* and *Scenedesmus obliquus*. Each complex is essentially free of chlorophyll and carotenoids and contains cytochrome $b_6$ and cytochrome *f* hemes in a 2:1 molar ratio. *C. reinhardtii* and *S. obliquus* complexes contain the Rieske iron-sulfur protein (present in approx 1:1 molar ratio to cytochrome *f*) and each catalyzes a DBMIB- and DNP-INT-sensitive electron transfer from duroquinol to spinach plastocyanin. Immunological assays using antibodies to the peptides from the spinach cytochrome complex show varying cross-reactivity patterns except for the complete absence of binding to the Rieske protein in any of the three complexes, suggesting little structural similarity between the Rieske proteins of algae with those from higher plants. One complex (*D. salina*) has been uniformly labeled by growth in $NaH^{14}CO_3$ to determine stoichiometries of constituent polypeptide subunits. Results from these studies indicate that all functionally active cytochrome $b_6$-$f$ complexes contain four subunits which occur in equimolar amounts.**

## Introduction

The cytochrome $b_6$-$f$ complex (plastoquinol: plastocyanin oxidoreductase) has been shown to occur in the energy-transducing membranes of a variety of photosynthetic organisms which are widely separated on the evolutionary scale, such as cyanobacteria and the chloroplasts of algae and higher plants [1–3].

The cytochrome $b_6$-$f$ complex from higher plants mediates the flow of electrons between Photosystem II and to Photosystem I, from plastoquinol to plastocyanin and catalyzes the cyclic electron flow around Photosystem I from reduced ferredoxin to plastocyanin [4–6]. The complex also functions in a redox-linked proton-pumping activity [7,8] by either a proposed Q-cycle mechanism [9] or by the putative b-cycle [10]. Isolation procedures of catalytically active and structurally well-defined cytochrome $b_6$-$f$ complexes from higher plant chloroplasts have greatly aided our understanding of the polypeptide requirements for activity [11–12]. These isolated complexes have been studied in a variety of ways, such as in reconstitution experiments of electron transport and proton transport activity [13,14], quinol oxidation mechanisms [15,16], topography experiments [17,18] and fractionation and reconstitution studies of poly-

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DNP-INT, 2-iodo-2′,4′,4′-trinitro-3-methyl-6-isopropyl diphenyl ether; EPR, electron paramagnetic resonance; RFe-S, Rieske iron-sulfur protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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peptides [19,20]. Even with the plethora of accumulated data, some dispute about the number of functionally required polypeptides remains to be resolved. Two recent studies [21,22] of the purified cytochrome $b_6$-$f$ complex from *Chlamydomonas reinhardtii* indicate the possible involvement of five or six polypeptides in its assembly and function.

In the present study, we have characterized the subunit composition of the detergent-purified cytochrome $b_6$-$f$ complex from *C. reinhardtii, Dunaliella salina* and *Scenedesmus obliquus*. These complexes appear to be similar to higher plant complexes, showing analogous polypeptide compositions, activity, and immunological cross-reactivity.

## Materials and Methods

*Culture conditions*

*D. salina* was grown in an NaCl-enriched medium as described by Pick et al. [23]. *C. reinhardtii* (cc-124) was grown in a Tris-acetate-phosphate medium [24]. *S. obliquus* was grown in the medium described in Ref. 25. All strains were grown at 25°C. *D. salina* was cultured in incandescent light with an intensity of 50 $\mu E/m^2$ per s, while *C. reinhardtii* and *S. obliquus* were cultured in fluorescent light at 300 $\mu E/m^2$ per s. In vivo $^{14}C$-labeling was done as described above with the addition of 1.5 mCi of $NaH^{14}CO_3$ with a specific activity of 57 mCi/mmol (ICN Radiochemicals). Cell cultures were harvested in the late-exponential growth phase.

*Chloroplast membrane isolation*

*D. salina* cells were collected by centrifugation at 5000 × *g* for 5 min. Cells were resuspended in 0.15 M NaCl, which contained the following proteinase inhibitors: 1 mM PMSF, 1 mM benzamidine and 5 mM aminocaproic acid. This suspension was homogenized with a Teflon homogenizer and then centrifuged at 38000 × *g* for 5 min. This step was repeated once. The pellet, which contained the chloroplast membranes, was resuspended in 2 M NaBr in 300 mM sucrose/50 mM Tris-HCl (pH 7.8)/5 mM $MgCl_2$/0.15 M NaCl (buffer 1), to a chlorophyll concentration of 1 mg/ml. The mixture was incubated at 4°C for 30 min, an equal volume of buffer 1 was added, and the suspension was centrifuged at 38000 × *g* for 15 min. The pellet was washed with buffer 1 and centrifuged as before. The pellet was resuspended in buffer 1 to a chlorophyll concentration of 2–2.5 mg/ml. *C. reinhardtii* cells were harvested and resuspended in buffer 1 plus 1 mM EDTA. The cell suspension was passed through a Yeda press as described in Ref. 26 at 1800 lb/in$^2$. Unbroken cells and cell walls were removed by centrifugation at 3000 × *g* for 5 min. The chloroplast membranes were collected by centrifugation at 15000 × *g* for 20 min. The pellet was resuspended in 0.15 M NaCl to a chlorophyll concentration of 1 mg/ml. *S. obliquus* chloroplast membranes were prepared similarly to *D. salina*, except that the cells were initially broken in a glass bead beater (Biospecific Products) for 4 min at 4°C using 1 min on and 1 min off cooling cycles.

*Cytochrome $b_6$-$f$ complex solubilization*

*D. salina* and *S. obliquus* chloroplast membranes were solubilized by adding a mixture of 60 mM octylglucoside and 0.5% sodium cholate in buffer 1 to give final concentrations of 30 mM octylglucoside and 0.25% sodium cholate. *C. reinhardtii* chloroplast membranes were solubilized by the addition of 40 mM octylglucoside and 0.3% sodium cholate to yield a final concentration of 20 mM octylglucoside and 0.15% sodium cholate. The detergent-solubilized material was incubated at 4°C for 30 min and centrifuged at 60000 rpm in a Spinco Ti60 rotor for 1 h. The supernatant was used for further purification of the cytochrome complex. Ammonium sulfate fractionation steps were performed as previously described [11], except for *D. salina*, which utilized a 45–55% second ammonium sulfate fractionation step. The final step of purification was sucrose density gradient centrifugation. Following the sucrose density gradient, the upper reddish-brown band was collected and analyzed for cytochrome content. Samples were stored at −20°C.

Cytochromes were determined by their redox differential absorption spectra utilizing potassium ferricyanide, hydroquinone, sodium ascorbate and sodium dithionite as redox-poising reagents. Molar absorption coefficients ($\epsilon_{553-543}$) of 21 $mM^{-1} \cdot cm^{-1}$ for cytochrome $f$ [27] and ($\epsilon_{563-570}$) 14 $mM^{-1} \cdot cm^{-1}$ for cytochrome $b_6$ [28] were used.

The Rieske iron-sulfur protein was detected in sodium-ascorbate-reduced EPR samples measured at 10 K as well as by chemical analysis of acid-labile sulfide according to Brumby et al. [29]. Plastoquinone content was determined according to Redfearn and Friend [30]. Optical spectra were obtained using a Cary 219 spectrophotometer, while activity assays were obtained using an Aminco DW2A spectrophotometer. EPR spectra were recorded with a Bruker model ER 200 X-band spectrometer equipped with a Nicolet model 535 signal averager.

*Polyacrylamide gel electrophoresis and immunoblotting*

SDS-PAGE was done by the Laemmli system [31] using 10–15% gradient gels. Heme staining was performed according to Thomas et al. [32]. Polypeptides were stained by Coomassie brilliant blue or, in the case of $^{14}C$-labeled complex, by autoradiography of the dried gels using Amersham's Hyperfilm-$\beta$-max without an intensifying screen. Molecular weights of the polypeptides were determined using a low-molecular-weight calibration kit from Bio-Rad.

Antibodies were raised to individual subunits of the spinach cytochrome complex by isolating these proteins from preparative SDS gels. The individual subunits were used for antibody production in rabbits, using standard procedures. Immunoblots were done according to the procedure of Towbin et al. [33]. Protein blocking of the nitrocellulose used 2% non-fat milk in Tris-buffered saline. Detection of antibody binding to polypeptides utilized $^{125}I$-protein A, according to Burnette [34]. Quantitation of antibody binding was determined using a Hoefer scanning densitometer connected to a Hewlett-Packard 3390 A integrator.

*Quantitation of $^{14}C$-labeled proteins in polyacrylamide gels*

Three different procedures were used to determine the amount of $^{14}C$ contained in each subunit of the cytochrome complex purified from *D. salina:* (1) autoradiography of stained and dried slab gels; (2) scintillation counting of single Coomassie blue bands; and (3) scintillation counting of gel slices. After development, the autoradiogram was scanned using a scanning densitometer. The resulting densitometric traces were then digitized on a digitization pad interfaced to a Hewlett-Packard 86B computer. The peak areas were then determined using a program which allowed use of an operator-defined baseline. For scintillation counting, the destained gels were cut into individual lanes with a razor blade. The gel strips were then cut into 130–160 1 mm slices using a Hoefer gel slicer. Each slice was put into a tightly sealable 7 ml plastic scintillation vial containing 0.2 ml ice-cold 70% perchloric acid and 0.4 ml ice-cold 30% $H_2O_2$, then quickly sealed. These vials were then incubated at 65–70 °C overnight, in the dark, with gentle rocking. Amersham PCS scintillation fluid (7 ml) was quickly added to each tube followed by several inversions to assure complete mixing. The tubes were placed in a Beckman LS-1801 scintillation counter and allowed to come to thermal equilibrium in the dark. Counting was performed until each sample reached a 2-sigma level of 5%. No background subtraction was performed. The counts, in cpm, were plotted versus slice number. This plot was then digitized and peak areas were determined as described above. Scintillation counting of single Coomassie blue bands, corresponding to subunits, were excised with a razor blade taking only the visibly blue region. These larger acrylamide slices were treated in an identical fashion to the 1 mm slices above. Each subunit was represented as its total cpm instead of total area.

*Other methods*

Chlorophyll concentrations were measured in 80% acetone using the absorption coefficients of Arnon [35]. Protein was determined according to the method of Smith et al. [36], using bovine serum albumin as a standard.

## Results

Table I summarizes the chemical compositions of the three algal cytochrome $b_6$-$f$ complexes. One criterion for purity of cytochrome $b_6$-$f$ complex preparations from photosynthetic membranes is their separation from pigments. As can be seen from Table I, the chlorophyll/cytochrome $f$ ratios for the three complexes are exceedingly low and

compare favorably to those complexes obtained in similar manner from spinach [11,12]. The cytochrome $b_6$/cytochrome $f$ ratios, as determined from absorption spectra of the isolated complexes, approach a value of 2:1 (see Table I). Since cytochrome $b_6$-$f$ complexes isolated from a variety of sources contain the Rieske ion-sulfur protein [1], the acid-labile sulfide contents of each complex were examined to determine whether these complexes contained the prosthetic groups of the Rieske [37] protein. Table I shows that both *C. reinhardtii* and *S. obliquus* complexes contained sulfide in a 2:1 molar ratio when compared to cytochrome $f$. Since the Rieske protein is known to contain a $Fe_2S_2$ cluster [37], an $S^{-2}$/cytochrome $f$ ratio of 2, as observed for the *C. reinhardtii* and *S. obliquus* complexes, suggests a 1:1 stoichiometry for the Rieske protein and cytochrome $f$. These data are in good agreement with previously characterized cytochrome $b_6$-$f$ complexes which contain equimolar amounts of the $Fe_2S_2$-containing Rieske protein and cytochrome $f$ [1]. In contrast to the *C. reinhardtii* and *S. obliquus* complexes, the *D. saline* complex does not contain any acid-labile sulfide. Direct evidence for the presence of the Rieske iron-sulfur protein in the *S. obliquus* complex came from the EPR spectrum of the ascorbate-reduced cytochrome $b_6$-$f$ complex (data not shown). The spectrum revealed EPR features at $g = 2.03$ and $g = 1.89$ in the reduced state, which are characteristic for the reduced Rieske iron-sulfur protein [37]. The EPR spectrum of the *Dunaliella* complex confirmed the absence of the Rieske center in this preparation. Like other cytochromes $b_6$-$f$ complexes [1], all three complexes were shown to contain co-purifying plastoquinone (see Table I) in similar amounts to complex isolated from spinach [38].

TABLE I

COMPOSITIONS OF THE PURIFIED CYTOCHROME $b_6$-$f$ COMPLEXES FROM *C. REINHARDTII, D. SALINA* AND *S. OBLIQUUS*

All values are expressed as nmol/mg protein.

| | *C. reinhardtii* | *D. salina* | *S. obliquus* |
|---|---|---|---|
| Cytochrome $f$ | 9.1 | 9.8 | 8.5 |
| Cytochrome $b_6$ | 18.4 | 18.5 | 16.5 |
| Acid-labile sulfide | 17.0 | 0 | 16.0 |
| Plastoquinone | 0.7 | 0.5 | 0.7 |
| Chlorophyll | 0.6 | 0.2 | 1.2 |

TABLE II

THE DUROQUINOL : PLASTOCYANIN OXIDOREDUCTASE ACTIVITY OF THE CYTOCHROME $b_6$-$f$ COMPLEXES FROM *C. REINHARDTII* AND *S. OBLIQUUS*

The assay mixture contained 5 μM spinach plastocyanin, about 50 pmol of cytochrome $f$ and 10 mM Tricine (pH 8.0). The reaction was initiated by the addition of durohydroquinone, in an ethanol stock, to give a final concentration of 20 μM. Plastocyanin reduction was monitored at 600–500 nM using 4.9 $mM^{-1} \cdot cm^{-1}$ for the absorption coefficient of plastocyanin. n.d., not determined. The activities shown are corrected for the non-catalyzed rates observed in the absence of cytochrome complex. Activity is expressed as μmol plastocyanin reduced per nmol cytochrome $f$ per h.

| Additions | Activity | |
|---|---|---|
| | *C. reinhardtii* | *S. obliquus* |
| Spinach plastocyanin | 7.5 | 30.5 |
| DBMIB (1 μM) | | |
| + spinach plastocyanin | 0.9 | 1.2 |
| DNP-INT (0.5 μM) | | |
| + spinach plastocyanin | 0.8 | 0.8 |
| Antimycin A (1 μM) | | |
| + spinach plastocyanin | n.d. | 25.2 |

Cytochrome $b_6$-$f$ complexes isolated from a number of sources catalyze electron transfer from a variety of quinols to plastocyanin [1]. As can be seen in Table II, the *C. reinhardtii* and *S. obliquus* cytochrome $b_6$-$f$ complexes possess duroquinol: plastocyanin oxidoreductase activity. The rate for the *S. obliquus* complex is considerably higher than that of the corresponding *C. reinhardtii* complex and approaches the rate observed for the spinach complex. Electron transfer rates of both complexes were markedly inhibited by low concentrations of the known cytochrome complex inhibitors DBMIB and DNP-INT [1]. The *D. salina* complex did not exhibit any detectable duroquinol:plastocyanin oxidoreductase activity, probably due to the absence of the Rieske iron-sulfur protein. Antimycin A, a potent inhibitor of mitochondrial and photosynthetic bacterial $b$-$c_1$ complexes [39,40], shows a slight inhibition of the cytochrome complex from *S. obliquus*. This finding is similar to data previously presented [11] indicating that the cytochrome complex from

chloroplasts was not inhibited by antimycin in either the isolated form or the membrane-bound state undergoing non-cyclic electron transfer [41]. Thus, in terms of activity and inhibitor sensitivity, the *S. obliquus* cytochrome complex would appear to be quite similar to other higher plant cytochrome $b_6$-*f* complexes [1]. It is difficult to ascertain why the *C. reinhardtii* complex is not as active as that from *S. obliquus*. However, since the activity of this complex is fully inhibitor-sensitive and the complex appears to contain all the necessary prosthetic groups for activity, we would argue that it is also similar in composition to higher plant complexes.

Fig. 1 shows the peptide composition of the isolated cytochrome $b_6$-*f* complexes, as determined by staining the protein subunits following SDS-PAGE. Spinach, *S. obliquus* and *C. reinhardtii* $b_6$-*f* complexes each contain four major peptides. In some preparations we noted minor contaminations in the 45–60 kDa range which were presumed to be ATPase subunit contaminants. The

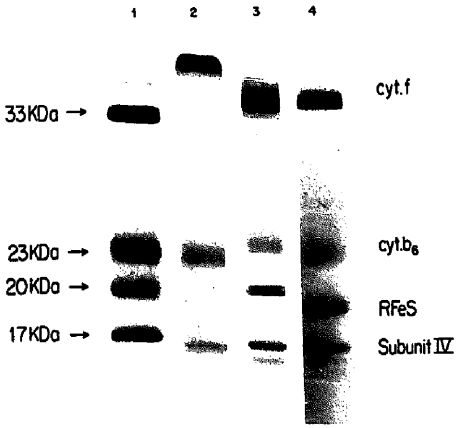


Fig. 1. Polypeptide composition of cytochrome $b_6$-*f* complexes. Gel was stained for protein with Coomassie brilliant blue: Lane 1, spinach complex equivalent to 2 nmol cytochrome *f*; Lane 2, *D. salina* complex equivalent to 0.5 nmol cytochrome *f*, Lane 3, *S. obliquus* complex equivalent to 0.9 nmol cytochrome *f*; Lane 4, *C. reinhardtii* complex equivalent to 1.0 nmol cytochrome *f*. Molecular weights were determined for the spinach complex using low-molecular-mass standards from Bio-Rad: phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa.

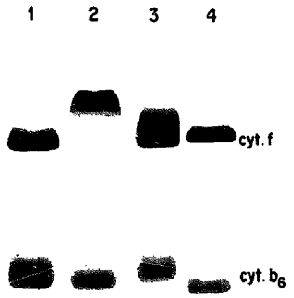


Fig. 2. Heme composition of cytochrome $b_6$-*f* complexes. Gel was stained for heme with tetramethylbenzidine + $H_2O_2$ (as described in Materials and Methods): Lane 1, spinach complex equivalent to 2.0 nmol cytochrome *f*; Lane 2, *D. salina* complex equivalent to 0.5 nmol cytochrome *f*; Lane 3, *S. obliquus* complex equivalent to 1.5 nmol cytochrome *f*; Lane 4, *C. reinhardtii* complex equivalent to 1.0 nmol cytochrome *f*.

apparent molecular masses of the spinach subunits are 32, 23, 20 and 17 kDa; for *C. reinhardtii*, 35, 23, 19 and 16 kDa; and for *S. obliquus*, 35, 23, 20 and 16 kDa. The *D. salina* complex shows only three prominent peptides of 37, 23 and 16 kDa. The *Scenedesmus* complex shows an additional band at 15 kDa which is absent from all other complexes. As shown in Fig. 2, staining the gels for heme [32] to identify cytochrome *f* and cytochrome $b_6$ [1] showed that only two peptides are heme-stained in each complex.

Figs. 3A and B show antibody binding data for each of the three complexes, compared to the spinach cytochrome $b_6$-*f* complex. As can be seen for the spinach complex, all four major peptides show good cross-reactivity with their corresponding antibodies. Each antibody was specific only to its corresponding polypeptide. Both *D. salina* and *S. obliquus* show three peptides which cross-react to antibodies made from the spinach complex. The only antibody which does not show cross-reactivity with a peptide in these complexes was the Rieske iron-sulfur protein (Fig. 3B). Since previous data (activity assays, chemical analysis and peptide composition) have revealed the absence of

the Rieske iron-sulfur protein in the *D. salina* cytochrome $b_6$-$f$ complex, it is not surprising that there is no cross-reactivity with the Rieske antibody. However, convincing data argue for the presence of the Rieske iron-sulfur protein in the *S. obliquus* $b_6$-$f$ complex. The fact that no cross-reactivity is observed with the Rieske antibody leads us to conclude that this protein, in *S. obliquus*, is not antigenically related to the corresponding protein from spinach. Only one prominent peptide from *C. reinhardtii* (cytochrome $f$) cross-reacts in this cytochrome complex (Fig. 3A), even though this complex has activity and a similar polypeptide composition when compared to the spinach cytochrome $b_6$-$f$ complex. Table III indicates the quantitation of antibody binding to each of the subunits in the respective cytochrome complex in comparison to the spinach complex. This numerical representation was obtained from densitometric integrated scans. This data indicates that the Rieske iron-sulfur proteins from these chlorophyta species share little similarity with those proteins from higher plant complexes (Fig. 3B).

The in vivo $^{14}$C-labeled cytochrome $b_6$-$f$ complex from *D. salina* had a specific activity of 230000 cpm per nmol cytochrome $f$. Fig. 4 summarizes the results of the three different methods used to quantitate the peptides in the complex.

TABLE III

QUANTITATION OF ANTIBODY CROSS-REACTIVITY TO SUBUNIT PEPTIDES OF CYTOCHROME $b_6$-$f$ COMPLEXES PURIFIED FROM *C. REINHARDTII, D. SALINA* AND *S. OBLIQUUS*

Values were obtained from densitometric scans and integration of the results of Fig. 2 and are represented in terms of percentages when compared to the spinach $b_6$-$f$ complex.

| | Relative cross-reactivity | | | |
|---|---|---|---|---|
| | α-Cyt. $f$ | α-Cyt. $b_6$ | α-RFe-S | α-IV |
| Spinach | 100 | 100 | 100 | 100 |
| *C. reinhardtii* | 34 | 5 | 0 | 5 |
| *D. salina* | 27 | 24 | 0 | 30 |
| *S. obliquus* | 62 | 95 | 0 | 70 |

The actual peak area or the total cpm measured for each protein band has been divided by the molecular mass of each subunit present in the complex. This result yields the amount of label per subunit with units of area or total cpm per kDa protein. To determine the number of subunits per complex, we have defined the cytochrome $f$ subunit to be present is one copy per complex. Therefore, the other polypeptides are present with a copy number relative to this value. As can be seen from this figure, the three procedures used in the analysis of the in vivo labeled complex agree well

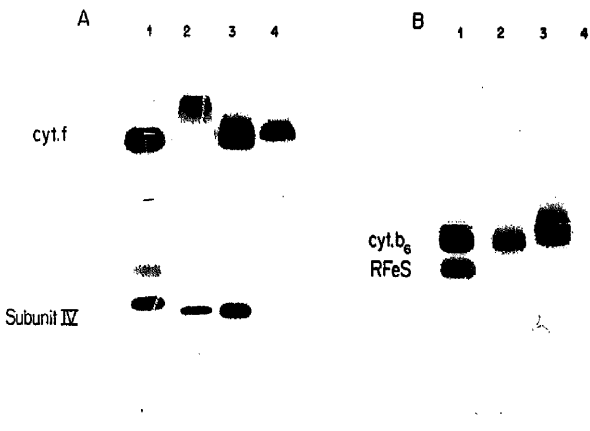


Fig. 3. Immunoblot analysis of cytochrome $b_6$-$f$ complexes. Cytochrome $f$ concentration was equivalent to 1.0 nmol cytochrome $f$ for each complex. Lanes 1–4 were the same as in Fig. 1. (A) Probed with antibodies to cytochrome $f$ and subunit IV. (B) Probed with antibodies to cytochrome $b_6$ and the RFe-S.

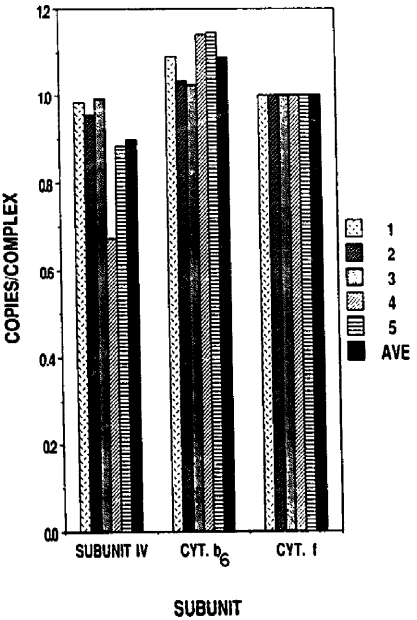


Fig. 4. Subunit stoichiometry of the *D. salina* cytochrome $b_6$-$f$ complex. Subunit quantitation was performed by the three methods described in Materials and Methods. The amount of $^{14}C$ per gram equivalent of each subunit was determined by normalization with the following molecular masses: cytochrome $f$, 33 kDa; cytochrome $b_6$, 23.5 kDa and subunit IV, 17 kDa. The subunit number per complex was determined by assuming one copy of cytochrome $f$ per complex. Data from No. 1 and No. 2 were obtained from autoradiography, No. 3 and No. 4 from single band counting and No. 5 from slicing of the whole gel (see Materials and Methods). AVE refers to average of 1–5.

with one another. Therefore, we conclude that each subunit peptide is present in equimolar amounts.

## Discussion

Cytochrome $b_6$-$f$ complexes with characteristic 2:1 cytochrome $b_6$/cytochrome $f$ stoichiometry, similar to that found in other cytochrome $b_6$-$f$ complexes [1], have been isolated from the photosynthetic green algae *C. reinhardtii, D. salina* and *S. obliquus*. This report represents, to our knowledge, the first such isolation from *D. salina* and *S. obliquus*. In addition to the cytochromes, these complexes also contain plastoquinone. Chemical analysis for acid-labile sulfide suggest that both *C. reinhardtii* and *S. obliquus* contain equimolar amounts of the Rieske iron-sulfur protein and cytochrome $f$. The fact that *S. obliquus* has been shown to contain a Rieske EPR signal is further proof of the presence of a Rieske center. Oxidoreductase activity measurements (duroquinol: plastocyanin) from *C. reinhardtii* and *S. obliquus* indicate that the basic characteristics of electron flow through the native complexes have survived the isolation procedure. Inhibition of electron flow by low concentrations of DBMIB or DNP-INT indicate some similarity to higher plant cytochrome $b_6$-$f$ complexes [1]. The marginal effect of antimycin A on electron transfer activity in *S. obliquus* is also characteristic of cytochrome complexes from oxygenic photosynthetic systems [1]. The relative differences between the activities of the complexes from *C. reinhardtii* and *S. obliquus* may reflect some differences in the acceptors themselves, or may indicate that the former complex is more unstable and loses activity during purification.

In contrast to the previous report [21] describing the cytochrome $b_6$-$f$ complex in *C. reinhardtii*, we have detected only four major polypeptides per complex in *C. reinhardtii* as well as *S. obliquus*. Based on heme staining and antibody cross-reactivity, we have assigned the bands between 34 and 36 kDa to cytochrome $f$ and those between 22 and 23 kDa to cytochrome $b_6$. The 17 kDa protein has been identified to be antigenically similar to the 17 kDa from the spinach cytochrome complex. Additional antigenic binding to cytochrome $f$ and cytochrome $b_6$ from spinach was found for subunits from *D. salina* and *S. obliquus*. While cytochrome $f$ from *C. reinhardtii* bears some antigenic similarity to cytochrome $f$ from spinach, no other polypeptides show cross-reaction in this complex when using antibodies raised against subunits from the higher plant cytochrome $b_6$-$f$ complex. One additional absence of cross-reactivity relates to the Rieske protein of *S. obliquus*. The basis of these differences is not clear at this time and will require isolation and characterization of the Rieske protein from these algae as well as a comparison of properties with the spinach protein.

Due to the convenience of radiolabeling, *D. salina* was uniformly $^{14}$C-labeled for accurate determination of subunit stoichiometry. The three methods employed in this study yielded similar values for the stoichiometry. We therefore conclude that the stoichiometry of the *D. saline* complex is 1:1:1 (cytochrome *f*/cytochrome $b_6$/subunit IV). A previous report on the subunit stoichiometry of the higher plant cytochrome complex [42] utilized analyses of stained SDS gels, but this technique is not considered to be as reliable as $^{14}$C-labeling because of differential reactivities of different stains with different protein subunits. Several attempts were made to isolate a complex from *D. saline* which retained the Rieske protein, but these efforts were unsuccessful. The Rieske protein can be found in the detergent-solubilized extract from *D. salina*, but it is apparently released from the complex more readily than from other complexes.

In summary, the present results indicate photosynthetic cytochrome $b_6$-*f* complexes from oxygenic organisms have a conserved composition comprised of four polypeptide subunits present in an equimolar ratio: cytochromes *f* and $b_6$, subunit IV and the Rieske iron-sulfur protein. These complexes are sufficient for inhibitor-sensitive electron transport from reduced quinones to plastocyanin.

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