Photosynthetic Electron Transport Chain of Chlamydomonas reinhardi.
V. Purification and Properties of Cytochrome 553 and Ferredoxin

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**Summary.** Cytochrome 553 and ferredoxin were isolated and purified from acetone powders prepared from intact cells of the wild-type strain of *Chlamydomonas reinhardi*. Purification was achieved by ion exchange chromatography on DEAE cellulose and gel filtration on Sephadex G-75.

Cytochrome 553 could be oxidized with potassium ferricyanide and reduced with sodium ascorbate. The absorption maxima of the cytochrome in reduced form were found to be \(\alpha\)-band 552.5 nm, \(\beta\)-band 522.5 nm, and Soret band 416.5 nm. The normal redox potential was +0.37 volt, and the molecular weight was estimated to be 12,000 ± 2000. The cytochrome closely resembled that of the \(c\)-type cytochromes extracted from *Euglena* and *Porphyra*. When cultures were harvested after they had entered stationary phase, the cytochrome could be extracted in soluble form, and in some cases a yield of one cytochrome per 1000 chlorophyll molecules could be obtained.

Ferredoxin showed absorption maxima at 278, 330, 420 and 460 nm. Thus it resembles the ferredoxin extracted from spinach. The molecular weight of the ferredoxin was estimated to be 15,000 ± 2000.

In the previous paper (7) we described a procedure for isolating and purifying plastocyanin from *Chlamydomonas reinhardi*. Both cytochrome 553 and ferredoxin were also isolated and purified by this procedure.

Smillie and Levine (15) observed the presence of a cytochrome similar to cytochrome \(f\) (4) in acetone powders of *C. reinhardi*. This cytochrome from *C. reinhardi*, with its \(\alpha\)-band at 553 nm, is denoted hereafter as cytochrome 553. The investigation by Smillie and Levine (15) failed to yield any significant amount of cytochrome 553 in soluble form. However, in the work reported here it was found that moderate and even relatively large amounts of soluble cytochrome 553 could be obtained from cultures of *C. reinhardi* that were allowed to grow beyond the exponential phase. Accordingly, it became possible to purify the cytochrome and to identify some of its properties.

Ferredoxin from *C. reinhardi*, in soluble form, has been identified (by its activity in NADP photoreduction) in 20,000 to 140,000 \(\times\) cells supernates obtained from preparations of cells that had been disrupted either with the French pressure cell or by ultrasonic disintegration (13). In the work reported here, ferredoxin was isolated and purified from acetone powders and some of its properties were identified.

**Materials and Methods**

**Organisms and Conditions of Growth.** The wild-type strain (137c) of *C. reinhardi* was used and the conditions for its growth were as previously described (7) except that when it was desirable to obtain cytochrome 553 in soluble form cultures were allowed to continue growing for 48 hours after the termination of exponential growth.

**The Extraction and Purification of Soluble Cytochrome 553.** The extraction and purification of soluble cytochrome 553 was carried out by the procedure described for plastocyanin (7). The chromatographic behavior of the cytochrome was found to be very similar to that of plastocyanin, and in fact, plastocyanin was a persistent and troublesome contaminant in the preparations of cytochrome 553. Normally, the cytochrome was separated from the bulk of the plastocyanin in step 2 of the purification procedure (7).

**The Quantitative Determination of Cytochrome 553.** The amount of cytochrome 553 in purified preparations free from plastocyanin was determined by measurement of the reduced-minus-oxidized absorbance difference at 552.5 nm, the \(\alpha\)-band maximum of the isolated cytochrome. The reduction was achieved by the addition of sodium ascorbate.

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and the oxidation by addition of potassium ferri-
cyanide. An extinction coefficient, reduced-minus-
oxidized, of 20 cm²/µmole cytochrome was assumed
(15). This value is very close to the actual extin-
tion coefficients 19.6 and 19.7 cm²/µmole re-
ported for the cytochrome f of parsley (5) and
cytochrome 552 of Euglena (14) respectively.

The Extraction and Purification of Ferredoxin.
Crude ferredoxin was obtained as a byproduct
from step 1 of the plastocyanin purification (7). The
crude ferredoxin was dialyzed for 12 hours at 4°
against 2 liters of 0.01 M phosphate buffer, pH
7.0, and applied to a column (1.3 × 10 cm) of
DEAE cellulose (7). The column was washed
with 0.05 M phosphate buffer, pH 7.0, containing
0.15 KCl until the brown band of ferredoxin had
spread near to the bottom of the column. The
ferredoxin was then eluted with 0.05 M phosphate
buffer, pH 7.0, containing 0.30 M KCl, and the
elution was terminated as soon as 90% of the
ferredoxin had been removed from the column.

The ferredoxin was then dialyzed for 12 hours
against 2 liters of 0.01 M phosphate buffer, pH 7.0
and applied to a small column (0.6 × 5 cm) of
DEAE cellulose. It was eluted with a minimum
volume of 0.05 M phosphate buffer, pH 7.0, con-
taining 0.40 M KCl and then subjected to gel fil-
ltration on Sephadex G-75 as described under step 4
of the procedure for the purification of plasto-
cyanin (7).

In order to concentrate and to store the purified
ferredoxin, it was dialyzed for 12 hours against
0.01 M phosphate buffer, pH 7.0, and applied to a
small column (0.6 × 5 cm) of DEAE cellulose.
It was eluted with a minimum volume of 0.05 M
phosphate buffer, pH 7.0, containing 0.40 M KCl
and dialyzed for 12 hours against 2 liters of 0.005 M
phosphate buffer, pH 7.0. The ferredoxin was
then stored as a frozen solution at −15°.

The Quantitative Determination of Ferredoxin.
The amount of ferredoxin was calculated from the
absorbance at 460 nm using the extinction coeffi-
cient 9.30 cm²/µmole (17).

Results and Discussion

The Isolation and Purification of Cytochrome 553. As shown in figure 1 the presence of plasto-
cyanin in partially purified preparations of cyto-
chrome 553 effectively prevented a direct meas-
urement of its oxidized-minus-reduced absorbance
difference. In such cases the amount of cyto-
chrome 553 was determined by measuring the height
of the α band maximum in the reduced spectrum
above a straight line drawn so as to intersect the
reduced spectrum at 542 and 561 nm. These wave-
lengths approximate the isosbestic points of the
purified cytochrome 553 (fig 3). For pure cyto-
chrome 553 this method gave nearly identical re-

![Fig. 1. The reduced spectrum (A) and oxidized spec-
trum (B) of the cytochrome 553 plus plastocyanin of
wild-type C. reinhardi after purification through step 1.
The preparations differed from those described in the
previous paper (7) only by the fact that the cell culture
had been allowed to continue growing for 48 hours after
the end of the exponential growth phase. Reduction was
achieved with sodium ascorbate and oxidation with po-
tassium ferricyanide. The straight line was drawn so
as to intersect the reduced spectrum at the wavelengths
542 nm and 561 nm in order to illustrate the method
used to measure cytochrome 553 in partially purified
preparations (see text). This spectrum and spectra shown
in succeeding figures were obtained with a Cary Model
14 recording spectrophotometer.

results as the direct measurement of the oxidized-
minus-reduced difference at 552.5 nm.

Figure 2 shows the elution pattern of cytochrome
553 and plastocyanin from DEAE cellulose in step
2 (7) of the purification procedure. The purifi-
cation of cytochrome 553 was found to be strongly
dependent upon the initial yield of soluble cyto-
chrome in the crude extract. The yield, in turn,
was variable and depended upon the age of the
culture from which the cells were harvested. When
cultures were harvested in the exponential phase of

![Fig. 2. The elution pattern of cytochrome 553 and
plastocyanin from DEAE cellulose in step 2 of the puri-
ification procedure. Solid line and circles, total protein
measured as absorbance at 278 nm, broken line and circles
plastocyanin measured as the absorbance at 597 nm after
oxidation with potassium ferricyanide, solid line and trian-
gles cytochrome 553 measured as the absorbance at
553 nm after reduction with sodium ascorbate.

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growth, at a cell density equivalent to 15 to 20 \( \mu g \) 
chlorophyll per ml, the yield of soluble cytochrome 
553 was less than 1 cytochrome per 100,000 
chlorophyll molecules. However, when the cultures 
were harvested after they had been allowed to enter 
the stationary phase, so that the cell density was 
equivalent to 40 to 50 \( \mu g \) chlorophyll per ml, the 
yield was at least 1 cytochrome per 10,000 chloro-
phyll molecules. In several instances a yield of 1 
cytochrome per 1000 chlorophyll molecules was 
obtained.

The soluble cytochrome 553 was extremely sen-
sitive to freezing in aqueous solutions; a single 
freezing and thawing was found to cause it to 
become nearly 100\% destroyed. However, it was 
found to be stable for at least 3 months when 
stored at 4°.

The Properties of Cytochrome 553. The absorp-
tion spectra of the reduced and oxidized forms of 
the purified cytochrome 553 are shown in figure 3. 
Details of the \( \alpha \) and \( \beta \) band regions are shown in 
figure 4, and the positions of the various absorp-
tion maxima are given in table I.

Cytochrome 553 was readily oxidized by potas-
sium ferricyanide and reduced with sodium ascor-
bate. In its reduced form, the cytochrome showed 
no tendency to autoxidize. The normal oxidation-
reduction potential of cytochrome 553 was measured 
spectrophotometrically according to the method 
previously described (7) using a mixture of potassium 
ferri- and ferrocyanide. As shown in figure 4 the 
cytochrome was just 50\% oxidized by a mixture 
having a 10:1 molar ratio of ferrocyanide to ferri-
cyanide. Thus, the normal oxidation-reduction poten-
tial of cytochrome 553 from \( C. \) reinhardi was 
calculated to be +0.37 volts at pH 7.0.

During gel filtration, cytochrome 553 was ob-
served to elute from Sephadex G-75 just after 
plastocyanin (7). The distribution coefficient, \( K_b \), 
of the cytochrome was thereby estimated to be 0.51.

Table I. The Properties of Cytochrome 553 of \( C. \) reinhardi and a Comparison with the Photosynthetically Active 
c-type Cytochromes of Other Plants

<table>
<thead>
<tr>
<th>Organism</th>
<th>( C. ) reinhardi</th>
<th>Porphyra tenera (11)</th>
<th>Euglena gracilis (14)</th>
<th>Parsley (4, 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption maxima</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduced form</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha )-band (nm)</td>
<td>552.5</td>
<td>553</td>
<td>552</td>
<td>554.5</td>
</tr>
<tr>
<td>( \beta )-band (nm)</td>
<td>522.5</td>
<td>521</td>
<td>522</td>
<td>524</td>
</tr>
<tr>
<td>Soret band (nm)</td>
<td>416.5</td>
<td>417</td>
<td>416</td>
<td>422</td>
</tr>
<tr>
<td>Oxidized form</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soret band (nm)</td>
<td>411</td>
<td>409</td>
<td>410</td>
<td>411</td>
</tr>
<tr>
<td>Absorbance ratio of reduced form</td>
<td>7.0</td>
<td>6.9</td>
<td>5.6</td>
<td>7.0</td>
</tr>
<tr>
<td>Soret band/( \alpha )-band</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Redox Potential</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( E'_o ) at pH 7.0 (volts)</td>
<td>+0.37</td>
<td>+0.335</td>
<td>+0.37</td>
<td>+0.365</td>
</tr>
<tr>
<td>Molecular wt</td>
<td>12,000</td>
<td>12,000</td>
<td>13,500</td>
<td>250,000</td>
</tr>
</tbody>
</table>
Comparison with the work of Andrews (1) indicated that the cytochrome had a molecular size comparable to that of a globular protein having a molecular weight of 12,000 ± 2000.

The comparison of cytochrome 553 from C. reinhardtii with other photosynthetically active c-type cytochromes (table 1) shows that the cytochrome from C. reinhardtii bears a close resemblance to the cytochromes from Porphyra tenera and Euglena gracilis and that it has a moderately close resemblance to the cytochrome f of higher plants (e.g. parsley).

**The Properties of Ferredoxin.** The absorption spectrum of purified ferredoxin from C. reinhardtii is shown in figure 5. The spectrum had maxima at 278, 330, 420, and 460 nm and resembled closely the absorption spectrum of spinach ferredoxin (9, 10, 12, 16). The purified ferredoxin was found to be active as a cofactor for NADP photoreduction by chloroplast fragments of C. reinhardtii (8).

From the position of the ferredoxin peak following elution from Sephadex G-75, its distribution coefficient, Kd, was found to be 0.44, corresponding in molecular size to a globular protein having a molecular weight of 15,000 ± 2000 (1). By comparison the molecular weight of spinach ferredoxin has been variously reported as 17,000 (2) and 13,000 (3).

**Metalloproteins and Their Relationship to Photosynthetic Electron Transport in Mutant Strains of C. reinhardtii.** We have described in this and in the previous paper (7) the procedure for isolating and purifying plastocyanin, cytochrome 553, and ferredoxin from the wild-type strain of C. reinhardtii. Each of these proteins plays a role in photosynthetic electron transport, and their isolation and purification was undertaken because first, mutant strains of C. reinhardtii have been discovered that lack, or possess in inactive form, either cytochrome 553 or plastocyanin (6, 8), and a mutant strain in which ferredoxin is affected is being sought. Accordingly, it is essential to have a reliable and a well-defined procedure for the isolation, identification, and characterization of these proteins. Second, purified plastocyanin and cytochrome 553 obtained from the wild-type strain were required for the reconstitution experiments in which reactions of the photosynthetic electron chain were investigated in the mutant strains in which one or the other of these proteins was found to be affected (8). Third, purified ferredoxin from the wild-type strain was required for the experiments in which NADP photoreduction was measured (6, 8).

![Absorbance vs Wavelength](image)

**Fig. 4.** Details of the α and β bands of cytochrome 553, and the measurement of its normal oxidation reduction potential. Curve A, reduced by sodium ascorbate at 0.005 M; curve B, in the presence of 0.01 M potassium ferrocyanide plus 0.001 M potassium ferricyanide; curve C, oxidized by potassium ferricyanide at 0.005 M. The spectra were obtained using the Cary Model 14 recording spectrophotometer with the 0 to 0.1 slide wire.

![Absorbance vs Wavelength](image)

**Fig. 5.** The absorption spectrum of the ferredoxin purified from wild-type C. reinhardtii.

**Literature Cited**