Involvement of plastoquinone and lipids in electron transport reactions mediated by the cytochrome b_6-f complex isolated from spinach

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The isolation of a cytochrome b_6-f complex from spinach, which is depleted of plastoquinone (and lipid), is reported. The depleted complex no longer functions as a plastoquinol-plastocyanin oxidoreductase but can be reconstituted with plastoquinone and exogenous lipids. The lipid classes digalactosyldiacylglycerol, phosphatidylglycerol and phosphatidylcholine were active in reconstitution while monogalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol were not. Neither plastoquinone nor lipid alone fully reconstitutes electron transport in the depleted complex. Saturation of plastoquinol-plastocyanin oxidoreductase activity in the depleted complex occurs at 1 plastoquinone per cytochrome *f*.

Cytochrome b_6-f *complex* *Plastoquinone* *Lipid* *Reconstitution*

1. INTRODUCTION

Evidence has accumulated in support of the view that some of the electron transport complexes of photosynthetic bacteria, plants and mitochondria have special tightly bound quinone molecules. In the photosynthetic bacteria *Rhodopseudomonas sphaeroides* a bound ubiquinone can be extracted from lyophilized chromatophore preparations with organic solvents. Subsequent light-induced electron transport reactions were dependent on readdition of quinone [1]. The isolated cytochrome b_6-f complex from spinach lost the oxidant-induced reduction of cytochrome b_6 upon extraction of the lyophilized complex with acetone. Reconstitution of cytochrome b_6 reduction was dependent on addition of plastoquinone to the depleted complex [2].

Fractionation of mitochondrial succinate cytochrome *c* reductase with cholate and ammonium sulfate results in a quinone- and lipid-depleted preparation with no succinate cytochrome *c* reductase activity. Maximum reductase activity could be restored by the addition of both quinone and lipid to the lipid-depleted reductase [3].

Recent results with isolated oxygen-evolving Photosystem II preparations suggest a lipid requirement for maximum rates of electron transport [4].

This paper describes the isolation of a cytochrome b_6-f complex from spinach which is substantially depleted in plastoquinone (and possibly lipids) without the use of organic solvents. This preparation no longer catalyzes the reduction of plastocyanin by duroquinol. Reconstitution of inhibitor-sensitive electron flow through the depleted complex required addition of both plastoquinone and lipid.

Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; MGDG monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; TLC, thin-layer chromatography; DNP-INT, 2,4-dinitrophenyl ether of iodonitrothymol, PQ, plastoquinone; b_6, cytochrome b_6; f, cytochrome f

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2. EXPERIMENTAL

2.1. *Isolation of the cytochrome* b_6-f *complex*

Plastoquinol-plastocyanin oxidoreductase was prepared from spinach using the method of Hurt and Hauska [5] with the following modifications: (a) omit second NaBr wash; (b) incubate chloroplast thylakoids in octylglucoside mixture at 2.5 mg/ml chlorophyll; (c) omit soybean lecithin from sucrose gradient; (d) centrifuge the final preparation for 16 h at 40000 rpm in a Beckman SW-41 rotor. The greenish-brown complex bands were collected from the gradient at a cytochrome *f* concentration of 25–50 μM.

2.2. *Preparation of a plastoquinone-depleted* b_6-f *complex*

The majority of the plastoquinone in the isolated complex (PQ/*f* = 0.6–1.1) was removed on a second sucrose-density gradient. Complex equivalent to 100–125 nmol cytochrome *f* was dialyzed against 30 mM Tris-succinate (pH 6.5) plus 0.2% Triton X-100 for 1 h to remove the sucrose from the preceding centrifugation. The dialyzed complex was layered on a sucrose-density gradient (10–30%) containing 30 mM Tris-succinate (pH 6.5) plus 0.2% Triton X-100 and centrifuged in a Beckman VTi50 vertical tube rotor at 49000 rpm for 16 h (an alternative method is to use a Beckman SW-41 rotor at 40000 rpm for 40 h). After centrifugation the complex had fractionated into 2–3 bands with about 1/3 of the recovered cytochrome *f* in the bottom band and 2/3 in the upper band(s). The red depleted complex bands were collected from the gradient at a cytochrome *f* concentration of 5–12 μM.

2.3. *Purification of plastoquinone from spinach*

Plastoquinone was isolated from spinach thylakoids by the method of Redfearn and Friend [6]. The resulting crude extract was further purified by chromatography on an alumina column [7] followed by preparative TLC [7]. The TLC plates were developed using the solvent mixtures chloroform-heptane (80:20) and heptane-benzene (15:85). The plastoquinone A band (as indicated by reference plastoquinone A from Hoffmann-La Roche) was scraped off the plate and stored in petroleum ether at −75°C. Aliquots of the plastoquinone in petroleum ether were dried under N_2 and redissolved in absolute ethanol, assayed spectrophotometrically and used in microliter amounts in the reconstitution experiments. An extinction coefficient of 15 $mM^{-1} \cdot cm^{-1}$ was used to estimate the concentration of plastoquinone from an oxidized minus reduced difference spectrum using 255 nm minus 276 nm.

2.4. *Addition of lipids and plastoquinone to the cytochrome* b_6-f *complex*

Up to 8 μl of purified lipids were added to a dry cylindrical cuvette and the solvent evaporated with N_2. Plastoquinone (1–2 μl) was added on top of the dry lipids followed by either (a) 30 mM Tris-succinate (pH 6.5), 0.2% Triton X-100 in 20% sucrose or (b) 30 mM Tris-succinate (pH 6.5), 30 mM octylglucoside, 0.5% cholate in 20% sucrose. The volume of either complex suspending medium (a) or (b) was determined by the cytochrome *f* concentration of the complex to be added so that the final volume of the lipid, plastoquinone, complex mixture was 50 μl. After a gentle shaking to mix the contents of the cuvette 2.5 μmol $MgCl_2$ were added and the mixture diluted to 4.0–4.5 ml with 20 mM Mes buffer (pH 6.5), 15 mM NaCl and 5 mM $MgCl_2$. Inhibitors and the electron donor/acceptor system were the final addition to the reaction mixture.

2.5. *Other materials and methods*

Spinach plastocyanin was purified by an unpublished method from this laboratory. An extinction coefficient of 4.9 $mM^{-1} \cdot cm^{-1}$ at 600 nm minus 500 nm was used to estimate the concentration of plastocyanin. The reduction of plastocyanin was performed in a stirred cuvette and monitored by an Aminco DW-2a spectrophotometer using a slit width of 3 nm.

Durohydroquinone (ICN Pharmaceuticals) in absolute ethanol under N_2 was completely reduced with a few crystals of sodium borohydride. The excess reductant was driven off as H_2 by the addition of a few microliters of 1.6 N HCl. The plastoquinone content of the cytochrome b_6-*f* complex was determined as in [6]. An extinction coefficient of 18 $mM^{-1}{}' cm^{-1}$ was used to estimate the concentration of cytochrome *f* at 554–540 nm and 20 $mM^{-1}{}' cm^{-1}$ for cytochrome b_6 at 563–575 nm. Lipids were purchased from Sigma except the

SQDG which was the gift of C. Sparace. The Rieske iron-sulfur center was assayed as in [8].

3. RESULTS AND DISCUSSION

When the isolated cytochrome b_6-f complex from spinach [5] was further fractionated on a second Triton X-100 sucrose-density gradient (see section 2.2) several cytochrome-containing bands were present. The lowest band was reconstitutively active in catalyzing the reduction of plastocyanin by duroquinol when supplemented with plastoquinone and lipid. Table 1 shows the requirements for reconstitution of plastoquinol-plastocyanin oxidoreductase activity by the lower band from the second gradient compared to the preparation layered onto the gradient prior to centrifugation. The observation that neither quinone nor lipid alone fully restored the reductase activity was similar to that reported for lipid depleted mitochondrial succinate cytochrome c reductase [3]. The addition of a lipid preparation had previously been observed to increase the oxidoreductase activity of the cytochrome b_6-f complex by a factor of two [5].

The observation by Hurt and Hauska that a second sucrose-density gradient centrifugation inactivated the cytochrome b_6-f complex is not inconsistent with the results reported here. The upper band(s) representing 2/3 of the cytochrome complex were inactive even when supplemented with plastoquinone and lipid. When the second centrifugation was for a short time (8 h), one band was present with low oxidoreductase activity (based on cytochrome f) relative to the control complex. Table 2 shows the plastoquinol-plastocyanin oxidoreductase activity of a preparation which fractionated into 3 bands on the second sucrose gradient. (The second band was often a diffuse band between the prominent top and bottom bands and in later preparations was usually combined with band one.)

Under the appropriate conditions Triton X-100 has been demonstrated to selectively solubilize the Rieske iron-sulfur protein from the cytochrome b_6-f complex [9]. Substantial amounts of this iron-

Table 1

The dependence of plastoquinol-plastocyanin oxidoreductase activity on addition of plastoquinone and lipid to a plastoquinone-depleted cytochrome b_6-f complex

Treatment	Plastoquinol-plastocyanin oxidoreductase activity[c]
Control complex[a]	
No addition	18.8
Plus PQ	19.4
Plus lipid	22.9
Plus PQ, lipid	23.5
Plus PQ, lipid, DNP-INT	4.1
Depleted complex[b]	
No addition	5.0
Plus PQ	9.3
Plus lipid	10.8
Plus PQ, lipid	19.4
Plus PQ, lipid, DNP-INT	4.3

In addition, duroquinol (2.5 μM) and plastocyanin (3.5 μM) were added throughout and, as indicated, plastoquinone (200 nM), PG (20 μg/ml) and DNP-INT (2 μM). All activities are corrected for the uncatalyzed rate of plastocyanin reduction by duroquinal (see section 2.4 for additional details)

[a] b_6/f 1.9; PQ/f 0.98; cytochrome f as complex, 50 nM

[b] b_6/f 1.9; PQ/f 0.12; cytochrome f as depleted complex, 48.5 nM

[c] μmol plastocyanin reduced-nmol cyt $f^{-1} \cdot h^{-1}$

Table 2

Partial composition and plastoquinol-plastocyanin oxidoreductase activity of the three bands from the second sucrose-density gradient

Treatment	Rieske center (%)	b_6/f	PQ/f	Plastoquinol-plastocyanin oxidoreductase activity[a]
Depleted complex				
Band 1	40	1.7	0.51	6.3
Band 2	60	1.9	0.24	3.3
Band 3	73	1.9	0.12	19.4

The reaction mixture is described in table 1. The cytochrome f concentration was 37.5 nM (band 1), 44.0 nM (band 2) and 48.5 nM (band 3). The Rieske center percentage was based on the control complex (table 1) as 100%

[a] μmol plastocyanin reduced·nmol cyt $f^{-1} \cdot h^{-1}$

sulfur protein remained with the complex (see band 2) while plastoquinol-plastocyanin oxidoreductase activity was last. No explanation for the loss of reductase activity in the upper band(s) can be offered. It would be tempting to speculate that the upper band(s) have lost an essential component, possibly the small peptides (3–5 kDa) recently found in the cytochrome b_6-f complex [10].

To determine the plastoquinone requirement for reconstitution of electron transport, the plastoquinone-depleted cytochrome b_6-f complex was supplemented with PC and increasing amounts of plastoquinone A as described in section 2.4. As shown in fig. 1 plastoquinol-plastocyanin oxidoreductase activity increased with increasing plastoquinone. Saturation of the reductase activity occurred at about 1 plastoquinone/cytochrome f. The plastoquinone/cytochrome f ratio of the cytochrome b_6-f complex prior to depletion was 0.98 and the depleted complex contained no detectable plastoquinone. These results support the view that a bound plastoquinone molecule in the cytochrome b_6-f complex was required for the reduction of plastocyanin using duroquinol as electron donor. In a highly purified but phosphlipid- and ubiquinone-depleted succinate-cytochrome c reductase preparation, restoration of electron transport required the addition of ubiquinone prior to the addition of phospholipid [3]. Surprisingly the sequence of addition of plastoquinone and lipid in this study had no effect on restoration of activity.

One problem in the study of electron transport through the isolated cytochrome b_6-f complex was that quinones had to be used as substrate electron donors. This fact complicated the investigation of the plastoquinone requirement for electron transport. Duroquinol was found to donate electrons to the cytochrome b_6-f complex in spinach thylakoids [11] but fortunately proved to be ineffective in substituting for the bound plastoquinone in the depleted isolated cytochrome b_6-f complex.

The lipid requirement for reconstitution of electron transport activity in the plastoquinone depleted cytochrome b_6-f complex is presented in fig. 2. (One other lipid class, SQDG, was found to be ineffective in restoring activity.) Although MGDG represents nearly 50% of the total thylakoid lipid [4] and has been reported to be present in the isolated b_6-f complex [5], there was no apparent role for this lipid class in the depleted cytochrome b_6-f complex's electron transport reac-

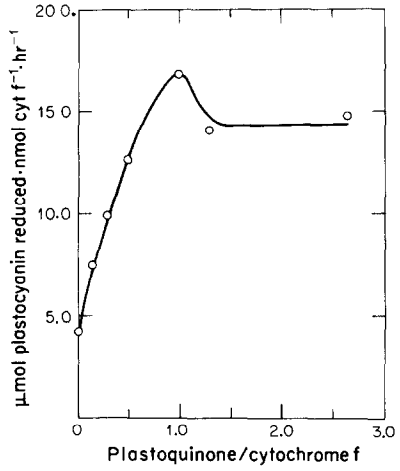

Fig. 1. Plastoquinone requirement for reconstitution of plastoquinol-plastocyanin oxidoreductase activity. The reaction mixture was as described in table 1 except PC (2.5 μg/ml) was added throughout and plastoquinone as indicated. The cytochrome f concentration, added as depleted complex was 41.9 nM. There was no detectable plastoquinone in the depleted complex.

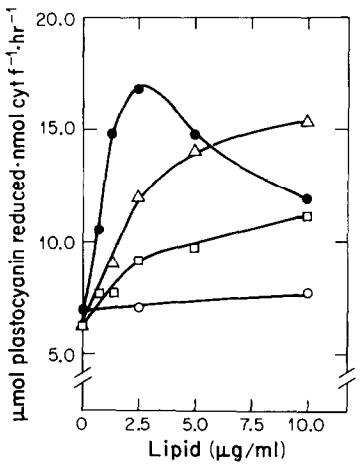

Fig. 2. Lipid requirement for reconstitution of plastoquinol-plastocyanin oxidoreductase activity. The reaction mixture was as described in fig. 1 except plastoquinone (140 nM) was added throughout and PG (□—□), PC (•—•), DGDG (△—△) and MGDG (○—○) added as indicated.

tions. It has been proposed that MGDG may be involved in packaging super-molecular complexes in planar bilayer regions of photosynthetic membranes [12]. This would be an attractive idea to explain both the occurrence of MGDG in the isolated complex and its being inactive in restoring electron transport in the depleted cytochrome b_6-f complex.

In Photosystem II preparations, isolated by Triton X-100 fractionation of spinach thylakoids DGDG and PC were found to stimulate oxygen evolution (electron transport) while MGDG caused a slight inhibition [4]. These results with Photosystem II are similar to those reported here where DGDG and PC produced the greatest stimulation of electron transport.

Unfortunately this study cannot explain why DGDG, PG and PC are required for plastoquinol-plastocyanin oxidoreductase activity in the plastoquinone-depleted cytochrome b_6-f complex. (Limited experiments with mixtures of these 3 lipids did not demonstrate enhanced activity.) Current studies of the redox reactions of the cytochromes in the depleted complex as a function of added plastoquinone and lipid may yield some insight into the mechanism of the lipid stimulation of electron transport.

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