Structural and functional contributions of lipids to the stability and activity of the photosynthetic cytochrome \( b_{6f} \) lipoprotein complex

The photosynthetic cytochrome \( b_{6f} \) complex, a homodimer containing eight distinct subunits and 26 transmembrane helices per monomer, catalyzes proton-coupled electron transfer across the thylakoid membrane. The 2.5-Å resolution structure of the complex from the cyanobacterium \( \text{Nostoc} \) sp. revealed the presence of 23 lipid-binding sites per monomer. Although the crystal structure of the cytochrome \( b_{6f} \) from a plant source has not yet been solved, the identities of the lipids present in a plant \( b_{6f} \) complex have previously been determined, indicating that the predominant lipid species are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PG), and sulfoquinovosyldiacylglycerol (SQDG). Despite the extensive structural analyses of \( b_{6f} \)-lipid interactions, the basis of the stabilization by lipids remains poorly understood. In the present study, we report on the effect of individual lipids on the structural and functional integrity of the \( b_{6f} \) complex, purified from \( \text{Spinacea oleracea} \). It was found that (i) galactolipids (MGDG, DGDG, and SQDG) and phospholipids dilinolenoyl-phosphatidylglycerol (DLPG), 1,2-dioleoylphosphatidylglycerol (DOPG), and 1,2-dioleoyl-\( sn \)-glycerol-3-phosphatidylcholine (DOPC) structurally stabilize the complex to varying degrees; (ii) SQDG has a major role in stabilizing the dimeric complex; (iii) the \( b_{6f} \) complex is stabilized by incorporation into nanodiscs or bicerelles; (iv) removal of bound phospholipid by phospholipase \( A_2 \) inactivates the cytochrome complex; and (v) activity can be restored significantly by the addition of the anionic lipid PG, which is attributed to stabilization of the quinone portal and the hinge region of the iron–sulfur protein.

Four lipids dominate the lipid composition of the plant thylakoid membrane: phosphatidylglycerol (PG)\(^3\) (\(~13\%)\), digalactosyldiacylglycerol (DGDG) (32%), monogalactosyldiacylglycerol (MGDG) (40%), and sulfoquinovosyldiacylglycerol (SQDG) (15%) (1–3). The thylakoid lipids provide a bilayer matrix to support the proteolipid heterooligomeric complexes and, as a result of their hydrophobicity and low dielectric constant, facilitate generation of a transmembrane proton electrochemical gradient coupled to electron transport (3). In addition, the lipids function as essential structural components in the photosynthetic complexes, inferred from high-resolution crystal structures (4–10) and MS analyses (11–16). All four classes of lipids have been implicated in the biogenesis of the thylakoid membrane (3). Although MGDG deficiency has been shown to impair membrane energization and photoprotection (17), lack of DGDG causes dissociation of extrinsic proteins of the photosystem II (PSII) reaction center complex (2). DGDG is also important for the function and stability of PSI (18). The anionic lipid PG has been shown to be critical for electron transport activity as well as the structural integrity of PSII (19, 20). The requirement of SQDG for proper photosynthetic function, however, has been shown to be variable in different photosynthetic environments (3). In \( \text{Arabidopsis} \), it is dispensable under nutrient-rich conditions (21). However, in \( \text{Chlamydomonas} \) green alga and the cyanobacterium \( \text{Synechocystis} \), SQDG is required for maintaining PSII activity (21, 22), although it is not required in the cyanobacterium \( \text{Synechococcus} \) (23). The present study is focused on the effect of the specific lipids on the structural stability and activity of the cytochrome \( b_{6f} \) complex from a plant source.

The \( b_{6f} \) complex from cyanobacteria, algal, and higher plant systems, like \( \text{Spinacea} \), including prosthetic groups, is a \(~250\)-kDa integral membrane-embedded lipoprotein complex that mediates electron transfer between photosystems I and II involved in oxygenic photosynthesis (5, 9, 10, 24, 25). It consists of eight subunits and seven prosthetic groups (Tables 1 and 2). The best-resolved crystal structure of the \( b_{6f} \) complex, solved to 2.5 Å, has been obtained from the cyanobacterium \( \text{Nostoc} \) sp.

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Lipid-dependent properties of cytochrome $b_{6}$ complex

Table 1

| Subunits | Molecular mass (kDa) |
|----------|----------------------|
| Cyt $f$  | 31.9                 |
| Cyt $b_{6}$ | 24.8               |
| ISP      | 18.9                 |
| Subunit IV | 17.3                 |
| PetG     | 4.1                  |
| PetL     | 3.9                  |
| PetM     | 3.4                  |
| PetN     | 3.1                  |

Table 2

| Prosthetic groups | Number per monomer |
|-------------------|--------------------|
| Heme $b$          | 2                  |
| Heme $c_{6}$      | 1                  |
| Heme $f$          | 1                  |
| 2Fe–2S            | 1                  |
| Chlorophyll $a$   | 1                  |
| $\beta$-Carotene  | 1                  |

A distinguishing feature of this structure, compared with previously solved $b_{6}f$ structures, is the documentation of 23 lipid-binding sites per monomer in the dimeric complex (5). Lipids associated with the $b_{6}f$ complex are responsible for regulation of conformation changes associated with the Rieske iron–sulfur protein subunit (26), tethering of the core and peripheral subunits (27), and other heterooligomeric photosynthetic membrane protein complexes like photosystem I (26–28). It is significant to note that a majority of membrane protein structures show evidence of well-ordered lipid headgroups (29, 30). In contrast, the cytochrome $b_{6}f$ crystal structure (PDB code 4OGQ) shows electron density mainly for the hydrophobic fatty acid tails (5). Moreover, the high-resolution crystallographic data (i.e. better than 3.0-Å resolution) of delipidated cytochrome $b_{6}f$ complex was achieved by augmentation of delipidated cytochrome $b_{6}f$ with pure, synthetic lipid, DOPC or DOPG. The fact that high-resolution structural data on the cytochrome $b_{6}f$ complex was obtained in an artificial lipid environment might imply that structural stability and function of the cytochrome $b_{6}f$ complex depends on the lipid environment of the multisubunit membrane protein complex. In the present study, we have used dilinolenoyl-phosphatidylglycerol (DLPG) with an 18:3 carbon side chain that mimics the native lipid found in the thylakoid membrane (31) alongside MGDG, DGDG, SQDG, and the synthetic lipids DOPC and DOPG.

The present study shows that lipid hydrophobic tail groups contribute significantly to the structural stability of the complex. The synthetic lipids DOPC and DOPG and native DLPG and SQDG are approximately equivalent in their ability to stabilize the secondary and tertiary structure of the cytochrome complex. In contrast, the galactolipids MGDG and DGDG do not cause significant stabilization of the complex as seen by far-UV CD. Incorporation of the complex in (a) nanodiscs composed of DOPC and DOPG (3:1) and (b) bicelles (DMPC:CHAPSO = 0.5) stabilized the secondary structure of the $b_{6}f$ complex purified from Spinacea. Although phosphatidylcholine (PC) is not present in the native plant thylakoid membrane, synthetic PC-containing fatty acyl chains mimicking the native hydrophobic environment stabilize the structure of the complex. The native thylakoid membrane sulfolipid SQDG has a significant role in the dimer stabilization of the complex.

Although significant removal of bound phospholipids substantially inactivates the cytochrome complex, subsequent addition of DLPG to the deactivated complex can restore almost complete activity of the complex. In comparison, the 18:1 DOPG can restore the activity of the complex by a factor of 1.6-fold, thus implicating the anionic phospholipid PG as essential for native activity of the $b_{6}f$ complex.

Results

Thermal denaturation/Far-UV CD and differential scanning calorimetry

Effect of lipid in the thermal denaturation of $b_{6}f$ complex—
The secondary structure content of the $b_{6}f$ complex from Nostoc (PDB code 4OGQ) is 45.1% $\alpha$-helix and 12.6% $\beta$-strand (Table 3). The unfolding of the cytochrome complex, as a function of temperature in the presence and absence of lipid, was studied by monitoring the change in molar ellipticity, at a wavelength of 222 nm, of the peak in the far-UV spectrum for $\alpha$-helical secondary structure (32). At this wavelength, the extinction coefficient of the $\beta$-strand structure is approximately a third of that for $\alpha$-helix (33). An overlay of the thermal denaturation spectra of the cytochrome–detergent complex in the presence of DOPG, DOPC, and SQDG (Fig. 1B) reveals an increase in thermal stability of the complex in the presence of lipid. The midpoint of the transition, $T_m$, was determined more precisely from the first derivative of the “melting curve” as a function of temperature (Fig. 1, B, inset, and C). The addition of the native (DLPG and SQDG) and synthetic lipids (DOPG and DOPC) stabilized the structure as indicated by the shift of the $T_m$ to higher temperatures (Table 4). However, the addition of the native galactolipids MGDG and DGDG did not cause any significant stabilization (Fig. 1C and Table 4).

To determine the effects of different hydrophobic tail groups of PC and PG, the effect of dilinolenoyl, dioleyl, and palmitoyl groups on the $T_m$ of the cytochrome complex were compared. DLPG, palmitoyl-oleoyl-PG (POPG), and DOPG increased the $T_m$ of the complex comparably (63.7, 63.8, and 64.4 °C, respectively) (Table 4). However, POPC displayed a larger stabilizing effect on the cytochrome complex than DOPC (Table 4). The increase in structure stabilization by PC, which is not native to the thylakoid membrane, is attributed to its hydrophobic fatty acid acyl chains. The structure stabilization provided by the native lipids DGDG and SQDG, as well as the synthetic lipids DOPG and DOPC, could be reproduced by differential scan-
Lipid-dependent properties of cytochrome b$_{6}$f complex

Figure 1. Lipid dependence of the thermal stability of the cytochrome b$_{6}$f complex helical content. A, representative $\alpha$-helical far-UV CD spectrum of cytochrome b$_{6}$f complex, in the absence of lipids (red) and in the presence of DOPG (blue), DOPC (green), and SQDG (pink), as a function of temperature, measured at 222 nm by far-UV CD. Inset, first derivative of the CD spectra, as a function of temperature, in the absence and presence of DOPC, DOPG, and SQDG. C, first derivative of the CD spectra, as a function of temperature, in the absence (labeled $b_{6}f +$ UDM) and presence of DLPG, MGDG, and DGDG. mdeg, millidegrees.

Table 4
Comparison of the melting temperatures ($T_m$) of the cytochrome b$_{6}$f complex solubilized in UDM, in the absence and presence of lipids, as measured by far-UV circular dichroism at 222 nm

| Cyt b$_{6}$f complex | $T_m$  
|----------------------|--------
| UDM-solubilized      | 62.1 ± 0.2  
| UDM + DLPG           | 63.7 ± 0.2  
| UDM + MGDG           | 63.0 ± 1.2  
| UDM + DGDG           | 61.2 ± 0.4  
| UDM + SQDG           | 65.3 ± 1.0  
| UDM + DOPG           | 64.4 ± 0.3  
| UDM + POPG           | 63.8 ± 2.6  
| UDM + DOPC           | 65.4 ± 1.0  
| UDM + POPC           | 68.4 ± 0.5  

Deconvoluted DSC thermograms showed three distinct transition peaks (Fig. 3), suggesting that the thermal denaturation of the cytochrome complex occurs via three distinct domain unfolding events whose molecular basis is currently not understood. It is evident that the $T_m$ for the three transitions is shifted to higher temperatures in the presence of lipid (Table 6). Removal of internally bound phospholipid which copurifies with the cytochrome b$_{6}$f complex by phospholipase A$_2$ (which cleaves the sn-2 bond of phospholipids) did not affect the $T_m$ of the complex significantly, as determined by the temperature dependence of the amplitude of the CD spectrum at 222 nm (Table 7).

Thermal denaturation of the b$_{6}$f complex in nanodiscs and bicelles—Stabilization of the b$_{6}$f complex by the hydrophobicity of the lipid bilayer was observed when the cytochrome complex was incorporated into more native environments associated with nanodiscs and bicelles. Successful reconstitution of the b$_{6}$f complex in nanodiscs was confirmed by size-exclusion chromatography in which the cytochrome complex in nanodiscs elutes earlier as a consequence of its larger molecular weight (data not shown).

A comparison was made of the thermal melting stability of the cytochrome complex, studied by far-UV CD at 222 nm, incorporated into nanodiscs and phospholipid bicelles. The denaturation profiles of the b$_{6}$f complex in nanodiscs and bicelles reveal a larger increase in the $T_m$ of the b$_{6}$f complex by 7.3 and 5.9 °C, respectively (Fig. 4 and Table 8), when compared with the complex in lipid micelles, implying significant stabilization. The first derivative of the thermal denaturation curve, utilized to more accurately define the temperature associated with the maximum rate of melting for b$_{6}$f in nanodiscs (Fig. 4, blue) shows a residual signal between 20 and 50 °C, caused by the presence of the $\alpha$-helical membrane-spanning protein belt around the nanodisc, which has a broad melting profile in that temperature range (Fig. 4, brown function). These results suggest that the hydrophobic environment of the membrane systems, defined mainly by the nonnative PC lipids, with tail
groups closely mimicking those of the thylakoid lipids in terms of length and degree of unsaturation, is sufficient to increase the structural stability of the b₆f complex.

**Thermal denaturation/native PAGE**

The effect of different lipids on the thermal stability of the b₆f dimeric complex was determined by heating the protein complex in the presence and absence of lipids as described earlier and subsequently displaying the complex on 4–12% clear native PAGE (Fig. 5A). The monomer-to-dimer ratio for each sample on the native gel was calculated using densitometry analyses using ImageJ software (34, 35). The monomer-to-dimer ratio was plotted against temperature for the b₆f complex in UDM and in the presence of DOPC, DOLPG, DLPG, MGDG, DGDG, and SQDG (Fig. 5, B and C). All the lipids stabilize the dimeric form, except MGDG, relative to the complex without lipids. SQDG shows a 3-fold stabilization of the dimeric complex, which is greater than the effects seen in the presence of any of the other lipids.

**Electron transfer activity**

Removal of phospholipids by phospholipase A₂ totally inactivated the cytochrome complex (measured as the rate of electron transfer between plastoquinone (PQH₂) and plastocyanin) within an hour of incubation at room temperature. However, the activity could be restored completely upon incubation with the native DLPG within an hour. DOPG could also restore the activity but only ~65% (Fig. 6), whereas DOPC and SQDG were unable to restore the activity to the complex.

**Discussion**

The effect of lipids on the structural integrity and the function of the photosynthetic cytochrome b₆f lipoprotein complex was investigated in the present study. Both the native (DLPG and SQDG) and synthetic lipids (DOPC and DOPG) stabilize the secondary structure of the protein as shown by the change in molar ellipticity at 222 nm monitored by far-UV CD (Fig. 1, B and C, and Table 3). The effect of neutral galactolipids on the secondary structure stability was tested with the addition of MGDG and DGDG. Addition of either lipid did not affect the Tₘ of the complex, implying no additional structural stabilization (Fig. 1C and Table 4). Varying the lipid hydrophobic tails, keeping the headgroups constant as either PC or PG (Table 4), stabilized the complex. This result suggests that for the phosphocholines, the stabilization is provided by both the head- and tail groups, whereas in the case of the anionic lipid phosphatidylglycerol, stabilization is provided mainly by an electrostatic interaction associated with the headgroup.

Interestingly, upon removal of bound phospholipids by phospholipase A₂, there is no statistically significant decrease in the Tₘ of the complex (Table 7). This is in contrast to the effect of phospholipase on other membrane protein complexes such as cytochrome c oxidase (36).

The stabilization effect of the native thylakoid (DGDG and SQDG) and synthetic (DOPC and DOPG) lipids was further demonstrated by differential scanning calorimetry. The addition of the phospholipids and SQDG increased the tertiary structure stability of the complex (Fig. 2A and Table 5). Deconvolution of the DSC data reveals three distinct domain transitions, which are shifted to higher temperatures in the presence of lipids (Fig. 3 and Table 6) but whose structural basis is not known. The enthalpy change (ΔH) and the melting temperature of the protein (Tₘ) are increased by incorporated lipids (Tables 5 and 6). Upon addition of DGDG, there was no increase in Tₘ of the complex, but there was a substantial increase in the enthalpy of unfolding of the cytochrome complex (Fig. 2B and Table 6).

Nanodiscs are model detergent-free membrane systems, which enable biophysical and biochemical characterization of membrane proteins in a physiological lipid bilayer environment (37, 38). Incorporation of integral membrane proteins into nanodiscs formed by various combinations of lipids has been shown to provide a more native membrane environment than detergent micelles (38). Similarly, phospholipid bicelles can preserve the integrity of integral membrane proteins by providing a native-like membrane environment (39–41). Nanodiscs and bicelles comprising synthetic lipids stabilize the secondary

Lipid-dependent properties of cytochrome b₆f complex

**Table 5**

Comparison of the enthalpies and melting temperatures (Tₘ) of the delipidated cytochrome b₆f complex and in the presence of DOPG, DOPC, DGDG, and SQDG

| Cyt b₆f | Tₘ (°C) | Enthalpy, ΔH (kJ/mol) |
|---------|---------|----------------------|
| UDM     | 62.8    | 115.3                |
| UDM + DOPC | 63.1    | 220.0                |
| UDM + DOPG | 64.5    | 170.6                |
| UDM + SQDG | 64.6    | 218.2                |
| UDM     | 61.4    | 271.3                |
| UDM + DGDG | 61.4    | 340                  |

- Figure 2. DSC spectra of cytochrome b₆f complex solubilized in UDM in the absence of lipids (red) and in the presence of DOPC (magenta), DOPG (cyan), and SQDG (green) (A) and in the absence (green) and presence (magenta) of DGDG (B).

- Table 5. The effect of different lipids on the thermal stability of the cytochrome b₆f complex and on the thermal stability of the complex in the presence or absence of lipids as described earlier and subsequently displaying the complex on 4–12% clear native PAGE (Fig. 5A). The monomer-to-dimer ratio for each sample on the native gel was calculated using densitometry analyses using ImageJ software (34, 35). The monomer-to-dimer ratio was plotted against temperature for the cytochrome b₆f complex in UDM and in the presence of DOPC, DOLPG, DLPG, MGDG, DGDG, and SQDG (Fig. 5, B and C). All the lipids stabilize the dimeric form, except MGDG, relative to the complex without lipids. SQDG shows a 3-fold stabilization of the dimeric complex, which is greater than the effects seen in the presence of any of the other lipids.
Lipid-dependent properties of cytochrome b₆f complex

Figure 3. DSC thermograms of the deconvoluted cytochrome b₆f complex in the presence of UDM (A), DGDG (B), SQDG (C), DOPG (D), and DOPC (E) reveal three distinct transitions.

Table 6
Comparison of the Tₘ of the three transitions (1, 2, and 3) obtained from deconvolution of the DSC thermograms for the cytochrome b₆f complex in the presence of DOPG, DOPC, and SQDG

|           | Delipidated | Delipidated + DOPG | Delipidated + DOPC | Delipidated + SQDG | Delipidated + DGDG |
|-----------|-------------|--------------------|--------------------|--------------------|--------------------|
| Tm        | °C          | kJ/mol             | °C                 | kJ/mol             | °C                 | kJ/mol             |
| Transition 1 (magenta) | 60.8 ± 16    | 62.9 ± 60          | 61.1 ± 53          | 63.6 ± 129         | 59.1 ± 14          |
| Transition 2 (blue)      | 62.6 ± 73    | 64.8 ± 84          | 62.9 ± 121         | 65.4 ± 61          | 61.5 ± 159         |
| Transition 3 (green)     | 64.3 ± 32    | 66.5 ± 31          | 64.7 ± 106         | 66.9 ± 28          | 63.4 ± 48          |

Table 7
Comparison of the melting temperatures (Tₘ) of the delipidated cytochrome b₆f complex before and after addition of phospholipase A₂ measured by far-UV CD

| Cyt b₆f | Tm | °C |
|---------|----|----|
| UDM-solubilized | 62.1 ± 0.25 |
| Phospholipase A₂ | 61.6 ± 0.4 |

structure of the complex significantly (Fig. 4 and Table 8), which implies that the hydrophobic environment, irrespective of the polar headgroups, is sufficient to stabilize the integrity of the cytochrome complex. SQDG and DGDG show the greatest dimeric stabilization when compared with the other lipids (Fig. 5, B and C). A structural basis for the involvement of SQDG in dimer stabilization of the complex, based on the 2.5-Å structure (5), may be due to its unique, conserved binding site (Fig. 7). The SQDG is within hydrogen-bonding distance to the highly conserved asparagine residue (Asn-20) on the Rieske iron–sulfur subunit and a lysine residue (Lys-275) on the cytochrome f subunit (5, 42). Deletion of the homologous conserved asparagine, Asn-17, residue in Chlamydomonas has been shown to be deleterious for the assembly of the cytochrome b₆f complex (42). The unique domain-swapped Rieske subunit of the cytochrome complex is considered essential for dimerization of the b₆f complex (43). Thus, by stabilizing the essential domain-swapped Rieske subunit, SQDG contributes to the dimeric stability of the complex.

The presence of the phospholipids DLPG, DOPG, and DOPC in the complex shows a significant reduction in the rate of monomerization of the cytochrome b₆f complex as a function of temperature (Fig. 5, B and C). In comparison, the native MGDG does not show appreciative dimer stabilization (Fig. 5C)

Anionic lipids like PG and cardiolipin have been found to be essential for the activity of many membrane protein complexes such as photosystem II (44–46), respiratory complex I (47), and respiratory complex III (48). PG has been shown to be required for the photosynthetic activity of the PSII complex in cyanobacteria (44, 45) and plants (46).

The present study shows that the removal of bound phospholipids completely deactivates the cytochrome b₆f complex (Fig. 6). Subsequent addition of the native anionic lipid DLPG could restore at 4 the activity within 1 h of incubation at 4 °C (temperature). In comparison, synthetic DOPG restores ~2/5 of the activity of the complex in 2 h. The native anionic lipid SQDG and the neutral synthetic lipid DOPC, however, are unable to reactivate the cytochrome complex, as is the neutral lipid DOPC (Fig. 6). From this observation, it is inferred that the anionic phospholipid headgroup is essential for the activity of the b₆f complex. This result is consistent with the observation that in the homologous cytochrome bc₃ complex, the anionic lipid cardiolipin restored most of the activity in phospholipase-treated complex (48, 49). Furthermore, (i) the oxygen-evolving photosynthetic activity of the cyanobacterium Synechocystis sp. PCC 6803 has been found to be dependent on the hydrophobic chain length of the PG lipid moieties (50) and (ii) the native unsaturated di-oleoyl-PG restored the photosynthetic activity in photoinhibited cyanobacterium much more efficiently than the saturated counterparts (dimyristoyl-PG, dipalmitoyl-PG, and distearoyl-PG) (50). In the present study, it was
found that POPG could not restore the activity of the \( b_{6f} \) complex (data not shown).

A structure-based explanation for the effect of PG on the activity of the \( b_{6f} \) complex can be inferred based on putative PG-binding sites, as obtained from the 2.5-Å crystal structure (5)( Fig. 8A; PDB code 4OGQ). (i) There are two phospholipid-binding sites, PG1 and PG2 (Fig. 8B), near the quinone portal lined by the C- and F-helices, which connect the intermonomer cavity and the heme \( b_p \) (52). By stabilizing the portal, the PG could facilitate the passage of plastoquinone for reduction of the heme \( b_p \). (ii) A third PG-binding site, PG3 (Fig. 8C), stabilizes the hinge region of the Rieske iron–sulfur protein (ISP). In the homologous cytochrome \( b_{c1} \) complex, the presence of a similar phospholipid-binding niche to support the large-scale movement of the soluble domain of ISP for electron transport (53, 54) has been documented. Although the sulfolipid SQDG increases the dimeric stability of the \( b_{6f} \) complex, PG is required for activity. The consequences of endogenous lipid on the directionality of electron transfer through the low-potential hemes in the \( b_{6f} \) complex have been discussed previously (56, 57). Thus, the relationship between the structure and function of a hetero-oligomeric lipoprotein complex such as the cytochrome \( b_{6f} \) complex must take into account not only the protein but also the internal lipid environment.

**Materials and methods**

**Purification of cytochrome \( b_{6f} \) complex from spinach**

Cytochrome \( b_{6f} \) complex was isolated from leaves of commercially purchased spinach as described previously (58). Dimeric \( b_{6f} \) complex was separated from monomer fractions by sucrose density gradient and size-exclusion chromatography. All further analysis was performed in 30 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, 10% sucrose (TNES) and 0.04% UDM. For experiments with the cytochrome complex supplemented with additional lipids, 1 mM solutions of individual lipids were prepared from stock solutions of 25 mg/ml concentration.

**Circular dichroism**

Far-UV CD spectra were measured in the wavelength range of 200–250 nm using a spectropolarimeter (Chirascan, Applied Photophysics Ltd., UK) with a cell path length of 1 cm and protein concentration of 0.5 \( \mu \)M. For thermal denaturation studies, the wavelength was fixed at 222 nm, and simultaneous OD and CD scans were measured from 30 to 70 °C at a scan rate of 1 °C/min. Data fitting and analyses were performed using SigmaPlot software.
Differential scanning calorimetry

DSC analyses were carried out on a Nano-DSC instrument (TA Instruments) equipped with two 0.3-ml platinum capillary cells. Sample solutions, at a concentration of 3 mM, were degassed for 10 min at 20 °C before loading in the capillary cells. DSC scans were conducted from 25 to 90 °C at a scan rate of 1 °C/min. Experiments were monitored using DSCRun software, and data analyses were performed using NanoAnalyze software. A two-state model was used to determine whether the transition is two-state or multistate based on the values of $\Delta H$ measured by DSC.

Secondary structure calculation

The content of secondary structure in the cytochrome $b_{6f}$ complex was measured using the crystal structure data (PDB ID 4OGQ) and the software STRIDE.

Figure 5. Monomerization of the cytochrome $b_{6f}$ complex as a function of temperature (38–44 °C). A, representative clear native gel image showing dimer (−240 kDa) and monomer bands (−120 kDa) of cytochrome $b_{6f}$ complex. The rate of monomerization of the cytochrome complex was measured in the absence of lipids (red) and in the presence of DOPG, DOPC, and SQDG (B) and MGDG, DGDG, DLPG, and SQDG (C).

Figure 6. Kinetics of the electron transfer activity of the cytochrome $b_{6f}$ complex after incubation with phospholipase $A_2$ (dotted line) and after addition of DLPG (red), DOPG (green), SQDG (pink), and DOPC (blue) to the deactivated complex. Error bars represent S.D.

Thermal denaturation and native PAGE

Cytochrome $b_{6f}$ complex (5 μM) in TNES-UDM buffer (pH 8.0), in the presence and absence of lipids, was heated at the indicated temperatures for 5 min and immediately transferred to ice. The samples were run on 4–12% gradient native gels to study the dependence of the monomerization process on temperature. Densitometry analyses were done using ImageJ software (34, 35).

Encapsulation of cytochrome $b_{6f}$ complex into nanodiscs

Cytochrome $b_{6f}$ (5 μM) was incubated overnight with mixed micelles of DOPC:DOPC at a molar ratio of 1:1000, buffer (30 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.03% UDM), and membrane scaffold protein MSP2N2 (provided by A. Kossiakoff) with the $b_{6f}$ complex at a molar ratio of 1:10, and Biobeads (Sigma). The Biobeads were removed from the nanodisc complex, which was concentrated and checked for successful reconstitution by size-exclusion chromatography and SDS-PAGE.

Encapsulation of cytochrome $b_{6f}$ complex into bicelles

A mixture of DMPC:CHAPSO at a molar ratio of 0.5 was prepared by measuring the appropriate amounts and dissolving in deionized water. The final bicelle concentration was kept at 12% (v/v). 5 μM cytochrome $b_{6f}$ was added to the bicelle suspension, mixed by pipetting, and incubated for 1 h on ice.

Removal of phospholipids by phospholipase $A_2$

15 μM bee venom phospholipase $A_2$ (Sigma) was mixed with 15 μM cytochrome $b_{6f}$ complex in 20 mM MOPS (pH 7.2), 20% glycerol, 50 mM CaCl$_2$, 0.04% UDM and incubated at room temperature for 2 h. The reaction was stopped by addition of 50 mM EDTA.
**Lipid-dependent properties of cytochrome b₆f complex**

**Lipids: sources and fatty acid composition**

Lipids were purchased from Avanti Polar Lipids Inc. The major fatty acid compositions of the lipids, as listed in the provided product data sheets, are: MGDG, 16:3–18:3 (66.8%), 18:3–18:3 (14.1%), and 16:3–18:2 (12.9%); DGDG, 18:3–18:3 (44.5%), 16:3–18:3 (21.1%), 18:2–18:3 (10.7%), and 16:0–18:3 (9.7%); DLPG, 18:3 (99.5%); SQDG, 16:0 (45%) and 18:3 (50%); DOPG, 18:1 (99%); and DOPC, 18:1 (99%).

**Activity assay**

The ability of the cytochrome b₆f complex, purified from spinach thylakoid membranes, to conduct electrons from donor decylplastoquinol (dPQH₂) to acceptor plastocyanin was determined. dPQH₂ at a concentration of 10 μM was assayed in Tris-HCl (pH 6.0), 0.04% UDM, and a baseline was recorded. To initiate the electron transfer reaction, 5 μM cytochrome b₆f and plastocyanin were added to the reaction mixture, and absorbance kinetics was studied by the absorbance change at 597 nm (51), characteristic of the reduction of plastocyanin.

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