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1 Cryo-EM structure of the spinach cytochrome 2 *b₆f* complex at 3.6 Å resolution 3

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10 The cytochrome *b₆f* (*cytb₆f*) complex plays a central role in oxygenic photosynthesis, linking electron
11 transfer between photosystems I and II and conserving solar energy as a transmembrane proton gradient
12 for ATP synthesis¹⁻³. Electron transfer within *cytb₆f* occurs via the Q-cycle, which catalyses the oxidation
13 of plastoquinol (PQH₂) and the reduction of both plastocyanin (PC) and plastoquinone (PQ) at two
14 separate sites via electron bifurcation². In higher-plants *cytb₆f* also acts as a redox-sensing hub, pivotal to
15 the regulation of light harvesting and cyclic electron transfer that protect against metabolic and
16 environmental stresses³. Here we present a 3.6 Å resolution cryo-electron microscopy (cryo-EM)
17 structure of the dimeric *cytb₆f* complex from spinach, which reveals the structural basis for operation of
18 the Q-cycle and its redox sensing function. The complex contains up to three natively bound PQ
19 molecules. The first, PQ1, is bound to one *cytb₆f* monomer at the PQ oxidation site (Q_p) adjacent to haem
20 *b_p* and chlorophyll *a*. Two conformations of the chlorophyll *a* phytyl tail were resolved, one that prevents
21 access to the Q_p site and another that permits it, supporting a gating function for the chlorophyll *a*
22 involved in redox sensing. PQ2 straddles the intermonomer cavity, partially obstructing the PQ reduction
23 site (Q_n) on the PQ1 side and committing the electron transfer network to turnover at the occupied Q_n
24 site in the neighbouring monomer. A conformational switch involving the haem *c_n* propionate promotes
25 two-electron, two-proton reduction at the Q_n site and avoids formation of the reactive intermediate
26 semiquinone. The location of a tentatively assigned third PQ molecule is consistent with a transition
27 between the Q_p and Q_n sites in opposite monomers during the Q-cycle. The spinach *cytb₆f* structure
28 therefore provides new insights into how the complex fulfils its catalytic and regulatory roles in
29 photosynthesis.

30 Photosynthesis sustains life on Earth by converting light into chemical energy in the form of ATP and
31 NADPH, producing oxygen as a by-product. Two light-powered electron transfer reactions at photosystems
32 I and II (PSI and PSII) are linked via the *cytb₆f* complex to form the so-called 'Z-scheme' of photosynthetic
33 linear electron transfer (LET)¹. *Cytb₆f* catalyses the rate-limiting step in the LET chain, coupling the oxidation
34 of PQH₂ and reduction of PC and PQ to the generation of a transmembrane proton gradient (Δp), used by
35 ATP synthase to make ATP^{2,3}. The *cytb₆f* complex is analogous to the cytochrome *bc₁* (*cytb_{c1}*) complex
36 found in mitochondria⁴ and anoxygenic photosynthetic bacteria⁵ and both operate via the modified Q-

37 cycle^{2,6}. Both *cytb₆f* and *cytbc₁* are dimeric and have similarly arranged electron transfer co-factors,
38 comprising a 2Fe-2S cluster, two *b*-type haems and a *c*-type haem. However, crystallographic structures of
39 cyanobacterial and algal *cytb₆f* complexes revealed additional co-factors not found in *cytbc₁* complexes,
40 including a chlorophyll *a*, a 9-*cis* β-carotene and an extra *c*-type high-spin haem⁷⁻⁹. The Q-cycle involves
41 bifurcated transfer of the two electrons, derived from oxidising a lipophilic PQH₂ molecule at the Q_p binding
42 site, into the high (2Fe-2S, *cyt_f*) and low (*cytb_p*, *b_n* and *c_n*) redox potential pathways, while the two protons
43 enter the thylakoid lumen^{2,6}. The high potential pathway delivers an electron to a membrane-extrinsic
44 soluble acceptor protein, PC, destined for PSI, while the low potential pathway delivers its electron to a PQ
45 molecule bound at the Q_n site near the stromal side of the membrane. Oxidation of a second PQH₂ at the Q_p
46 site leads to the two-electron reduction of a Q_n site bound PQ, which together with two proton transfers
47 from the stroma, regenerates PQH₂^{2,6}. The Q-cycle thereby doubles the number of protons transferred to
48 the lumen per PQH₂ oxidised. Yet, full understanding of the Q-cycle mechanism is hindered by a lack of
49 information on the binding of the substrate PQ/ PQH₂ molecules within the complex.

50 In addition to its role in LET, *cytb₆f* also plays a key role as a redox sensing hub involved in the regulation of
51 light harvesting and cyclic electron transfer (CET), which optimise photosynthesis in fluctuating light
52 environments^{10,11}. *Cytb₆f* communicates the redox status of the PQ pool to the loosely associated light
53 harvesting complex II (LHCII) kinase, STN7¹²⁻¹⁴. Phosphorylation of LHCII results in a decrease in thylakoid
54 membrane stacking, promoting the exchange of LHCII between PSII and PSI to balance their relative
55 excitation rates¹⁵ and regulate CET¹⁶. CET involves the reinjection of electrons from Ferredoxin (Fd) into the
56 PQ pool, generating Δp, for photoprotective downregulation of PSI and PSII or to augment ATP synthesis,
57 without net formation of NADPH¹¹. The *cytb₆f* complex has been proposed to fulfil the role of the Fd-PQ
58 oxidoreductase (FQR) during CET, with the stromal-facing haem *c_n* suggested to channel electrons from Fd
59 NADP⁺ reductase (FNR) bound Fd to the Q_n site PQ¹⁷. How *cytb₆f* performs these central redox sensing
60 regulatory roles remains unclear. Nonetheless, genetic manipulation of photosynthetic regulation is now
61 recognised as being key to increasing crop yields to feed a global population projected to approach 10
62 billion by 2050¹⁸. Indeed, overproduction of the Rieske iron-sulphur protein (ISP) of *cytb₆f* in *Arabidopsis*
63 *thaliana* led to a 51% increase in yield¹⁹. Further progress in understanding the regulatory roles of *cytb₆f*
64 and potentially manipulating them for crop improvement requires the structure of the higher plant
65 complex. Here, using a gentle purification procedure to obtain a highly active dimeric complex (Extended
66 Data Fig. 1) and single-particle cryo-EM we resolve the *cytb₆f* complex from *Spinacia oleracea* (spinach) at
67 3.6 Å resolution (Extended Data Fig. 2 and Extended Data Table 1).

68 The colour-coded map (Fig. 1a, b, c) shows the architecture of this dimeric complex surrounded by a
69 disordered density comprising detergent and lipid molecules. The overall organisation of this higher plant
70 *cytb₆f* complex is similar to crystallographic structures of algal and cyanobacterial complexes from
71 *Chlamydomonas reinhardtii*⁷ (PDB ID: 1Q90), *Mastigocladus laminosus*⁸ (PDB ID: 1VF5) and *Nostoc* sp. PCC

72 7120⁹ (PDB ID: 2ZT9) (Extended Data Table 2). Each monomeric unit of the *cytb₆f* complex comprises four
73 large polypeptide subunits that contain redox cofactors (cyt *f*, cyt *b₆*, ISP, subIV), and four small subunits
74 (PetG, L, M, N). Extended Data Fig. 3 shows the density and structural model for each subunit. The extrinsic
75 domains of cyt *f* and the ISP on the luminal face of the complex flank the membrane-integral cyt *b₆*
76 subunits (Fig. 1a, b). The organisation of the transmembrane integral subunits can be seen on the stromal
77 side of the complex (Fig. 1c) with 13 transmembrane helices (TMH) visible within each monomer (Fig 1d, e,
78 f). Peripheral to the core of cyt *b₆* (four TMH) and subIV (three TMH) on the long axis of the complex is the
79 single kinked TMH of the ISP that crosses over to provide the soluble ISP domain of the neighbouring
80 monomer. The single TMH belonging to cyt *f* is sandwiched by the TMH of the four minor subunits PetG, L,
81 M and N, which form a 'picket-fence' at the edge of the complex.

82 Fig. 2a, b show the organisation of the prosthetic groups and lipids, with four *c*-type haems (*f* and *c_n*, dark
83 blue), four *b*-type haems (*b_p* and *b_n*, red), two 2Fe-2S clusters (orange-yellow), two 9-*cis* β-carotenes
84 (orange), two chlorophyll (Chl) *a* molecules (green), three PQ molecules (yellow) and twelve bound lipids
85 (two monogalactosyldiacylglycerol, four phosphatidylglycerol, three sulfoquinovosyldiacylglycerol and
86 three phosphatidylcholine, all shown in white). Extended Data Fig. 4 shows the density map and structural
87 model for each prosthetic group. Fig 2c shows all the bound electron transfer cofactor edge-to-edge
88 distances within the *cytb₆f* complex. Electron transfer from the 2Fe-2S cluster is thought to involve
89 movement of the luminal ISP domain, pivoting between closer association with the Q_p site and the haem
90 *f*²⁰. By comparison with the chicken *cytbc₁* complex where the two conformations of the ISP were resolved,
91 in the spinach *cytb₆f* structure the ISP and bound 2Fe-2S cluster appear to be in the distal position with
92 respect to haem *f*, as in the existing algal and cyanobacterial *cytb₆f* structures (Extended Data Table 2). PQ
93 locations are generally inferred from crystallographic structures containing tightly bound quinone analogue
94 inhibitors²¹⁻²³; here, the spinach cryo-EM structure was obtained with native PQ molecules (Fig. 2d), clearly
95 defined by their respective densities (Extended Data Fig. 4) with their distances from the closest co-factors
96 shown in Fig 2e-g. One PQ molecule (PQ1) is found adjacent to the haem *b_p* and Chl on one side of the
97 dimer (Fig. 2e), and a second (PQ2) binds adjacent to the haem *c_n* - haem *b_n* pair on the opposite monomer
98 to PQ1 (Fig. 2f). A third and less clearly defined PQ (PQ3) lies between the haem *c_n* of one monomer and
99 the haem *b_n* of the other (Fig. 2g). The density map in this region can also be assigned to a phospholipid and
100 Extended Data Figure 5 shows the two possible fits, to a quinone or lipid

101 The 1,4-benzoquinone ring of PQ1 is 16.2 Å from haem *b_p* and 26.4 Å from the 2Fe-2S cluster (Fig. 2e) and
102 distal to the Q_p quinone oxidising site, defined in the *M. lamosus cytb₆f* structure²³ (PDB ID: 4H13) by the
103 inhibitor tridecylstigmatellin (TDS) (Fig. 3a,b). The Q_p site is located within a pocket formed by hydrophobic
104 residues from subIV (Val84, Leu88, Val98, Met101) and cyt *b₆* (Phe81, Val126, Ala129, Val133, Val151,
105 Val154) (Fig. 3c). Since bifurcated electron transfer to the 2Fe-2S cluster and haem *b_p* involves two
106 deprotonation events mediated by the His128 (ISP) and Glu78 (subIV) residues^{2,3}, which are buried inside

107 the Q_p pocket (Fig. 3a,b), it seems unlikely that PQ1 is oxidised in its resolved position, since its -OH group is
108 some ~26 Å from His128, a ligand to the 2Fe-2S cluster (Fig. 3b), and is more likely a snapshot of its
109 approach. It is interesting in this regard that our spinach *cytb₆f* structure resolves two conformations of the
110 Chl phytyl tail, one of which permits access to Q_p site, while the second restricts it (Fig. 3c,d). There is only
111 one position of the phytyl tail for the Chl on the opposing monomer. In this way the bound Chl adjacent to
112 PQ1 may fulfil a gating function at the Q_p pocket, either controlling access of PQH₂ and/or increasing the
113 retention time of the reactive semiplastoquinone (SPQ) intermediate species formed following electron
114 transfer to the 2Fe-2S cluster. Indeed, spin-coupling between the SPQ to the 2Fe-2S cluster has been
115 detected during enzymatic turnover of *cytb₆f* but is absent in *cytbc₁* complexes which lack the Chl
116 molecule²⁴. SPQ in the FeS-bound state does not react with oxygen, which provides a potential mechanism
117 to control release of superoxide from the Q_p site²⁴ and to regulate the activity of the LHCII kinase STN7²⁵,
118 which is proposed to bind to *cytb₆f* between the F and H TMH of subIV²⁶. Another role for Chl in regulating
119 the activity of STN7, could involve PQH₂ displacing the Chl phytyl tail upon binding to the Q_p site and this
120 motion could induce a conformational change in STN7 leading to its activation²⁷.

121 PQ2 binds towards the stromal face of the complex 4.4 Å from the haem *c_n* / *b_n* pair at the Q_n reducing site
122 (Fig. 2f). The *b_n* and *c_n* haems on each monomer are separated by 4.9 Å, with the *b_n* haem coordinated by
123 His202/100 (cyt *b₆*) and the vinyl side-chain of haem *c_n* covalently linked to Cys35 (cyt *b₆*) (Fig. 2f). The
124 dimerisation interface of the *cytb₆f* complex creates a cavity, proposed to promote transfer of quinones
125 between the Q_p and Q_n sites on neighbouring monomers⁸ (Fig. 4a, b). It is significant in this regard that all
126 three resolved PQ molecules inhabit this cavity and that PQ2 assumes a position 'diagonally' opposite to
127 PQ1 (Fig. 4c) on the other monomer, as depicted by Nawrocki et al²⁸. Notably, PQ2 adopts a bowed
128 conformation that straddles the intermonomer cavity and the distal PQ2 tail appears to partially obstruct
129 the Q_n site in the neighbouring monomer (Fig. 4d, e). This arrangement may have functional significance in
130 preventing the simultaneous binding of PQ molecules at both Q_n sites, avoiding competition for electrons
131 and favouring faster turnover of the Q-cycle. Rapid provision of two electrons for PQ2 bound at a particular
132 Q_n site could be facilitated by the 15.3 Å electron-tunnelling distance between *b_p* haems (Fig. 2b), which
133 allows rapid inter-monomer electron transfer via the 'bus-bar' model from the neighbouring low potential
134 chain^{29,30}. Alternatively, the second electron could be provided to the haem *c_n* directly via a FNR-Fd complex
135 bound at the stromal surface via CET^{17,28}. The haem *c_n* propionates on the two halves of the *cytb₆f* dimer
136 adopt different conformations (Fig. 4f, g); in the PQ-vacant site on the opposing monomer the haem *c_n*
137 propionate is more closely associated with Arg207 (Fig. 4f), whereas in the PQ-occupied site the haem *c_n*
138 propionate is twisted towards the 1,4-benzoquinone ring of PQ2 (Fig. 4g). The altered ligation of haem *c_n*
139 upon PQ binding is consistent with the downshift of its redox potential³¹, which would strongly favour PQ
140 reduction. We note that the reduction/oxidation of haem *c_n* is accompanied by the binding/release of one
141 proton³¹ so only one proton is required from the stromal side via the Arg207 and Asp20 residues (Fig 4f, g)
142 and PQ2 reduction could proceed rapidly, avoiding SPQ formation. It is also possible to position an

143 oppositely oriented PQ within the density map, albeit with a less satisfactory fit (Extended Data Fig. 5). A
144 third PQ molecule (PQ3) (Fig. 2g) has been assigned to the density between the Q_p and Q_n binding sites (see
145 Extended Data Fig. 6 for an alternative assignment as phosphatidylcholine) with the 1,4 benzoquinone ring
146 near to the channel that links the two sides of the intermonomer cavity and the isoprenyl tail at the mouth
147 of the Q_p site. This third PQ may therefore capture a snapshot of the molecule transitioning between the Q_p
148 and Q_n sites in opposite monomers.

149 The cryo-EM structure of spinach *cytb₆f* reveals the positions of the natively bound PQ complement and
150 provides new details regarding the conformational switches involved in PQ binding to the Q_n site, Chl gating
151 of the Q_p site and PQ exchange between the sites during Q-cycle operation.

152

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237 AUTHOR CONTRIBUTIONS

238 P.Q, C.N.H, N.R and M.P.J supervised the project. L.A.M., G.E.M, P.Q., C.N.H., R.F.T and M.P.J. designed the
239 experiments. L.A.M. and G.E.M. purified the cytochrome *b₆f* complex, L.A.M., G.E.M., A.H. and
240 D.J.K.S. characterised the cytochrome *b₆f* complex, L.A.M., P.Q., D.A.F. and R.F.T. collected, processed
241 and/or analysed the cryo-EM data. L.A.M., C.N.H. and M.P.J. wrote the manuscript. All authors proofread
242 and approved the manuscript.

243 COMPETING INTERESTS

244 The authors declare no competing interests

245 FIGURE LEGENDS

246 **Fig. 1 | Cryo-EM structure of the *cytb₆f* complex from spinach.** **a-c**, Views of the colour-coded *cytb₆f*
247 density map showing cytochrome *b₆* (cyt *b₆*, green), cytochrome *f* (cyt *f*, magenta), ISP (yellow), subunit IV
248 (subIV, cyan), PetG (grey), PetM (pale purple), PetN (pale orange), PetL (pink). Detergent and other
249 disordered molecules are shown in semi-transparent light grey. **a**, View in plane of the membrane. The grey
250 stripe indicates the likely position of the thylakoid membrane bilayer. **b**, View perpendicular to the
251 membrane plane from the lumenal (p) side. **c**, View perpendicular to the membrane plane from the stromal
252 (n) side. **d-f**, Modelled subunits of *cytb₆f* shown in a cartoon representation and coloured as in **a-c**.

253 **Fig. 2 | The global arrangement of prosthetic groups, lipids and plastoquinone molecules in the spinach**
254 ***cytb₆f* complex.** **a-b**, The arrangement of molecules in the *cytb₆f* complex viewed in the membrane plane
255 (**a**) and perpendicular to the membrane plane from the stromal side (**b**). **c-d**, Cofactors and edge-to-edge
256 distances (Å) in the dimeric *cytb₆f* complex. **e**, The location of the 1,4-benzoquinone ring of PQ1 adjacent to
257 haem *b_p*, the 2Fe-2S centre and two conformations of the Chl molecule, represented in two shades of
258 green. **f**, Close-up of the 1,4-benzoquinone ring of PQ2 and nearby haem *c_n* and haem *b_n* near the stromal
259 face of the complex. **g**, The 1,4-benzoquinone ring of PQ3, which sits between the haem *c_n* and haem *b_n*
260 from the two *cytb₆f* monomers. The *cytb₆f* complex is coloured as in Fig 1, and shows *c*-type haems (*f*, *c_n*;
261 dark blue), *b*-type haems (*b_p*, *b_n*; red), 9-*cis* β-carotene (*car*; orange), chlorophyll *a* (Chl; green), 2Fe-2S (FeS;
262 orange/yellow), lipids (white) and plastoquinones (PQ1-3; yellow).

263 **Fig. 3 | Conformational alterations in the Chl phytyl chain at the PQH₂-oxidising Q_p site.** **a**, Disposition of
264 the PQ1 in relation to the haem *b_p*, Chl and 2Fe-2S cofactors. The catalytically essential residue E78 is
265 shown, as are coordinating residues of the 2Fe-2S cofactor. TDS is a quinone analogue, superimposed
266 according to its position determined in the cyanobacterial complex (PDB ID: 4H13)²³, and used here to
267 indicate the likely destination for PQ1 in the Q_p pocket. **b**, The same cofactors and residues as in **a**, but now
268 in relation to a surface view of cyt *b₆* (green) and SubIV (cyan). **c**, The Q_p pocket is highlighted with a purple
269 dashed line in relation to the Chl and PQ1 molecules; the hydrophobic residues of subIV (cyan) and cyt *b₆*
270 that line the pocket are shown as sticks and coloured cyan and green respectively. **d**, The two
271 conformations of the Chl tail (represented in dark green and light green) gate (dashed arrow) the entrance
272 to the Q_p pocket.

273 **Fig. 4 | The intermonomer cavity of the spinach *cytb₆f* complex.** **a-b**, Surface representations of the
274 complex, with subunits coloured as in Fig. 1, and cofactors and lipids as in Fig. 2. These two views of the
275 complex are related by a 45° rotation about an axis perpendicular to the membrane, to show two views of

276 the cavity and the locations of PQ molecules. **c**, PQ1-3 are shown in relation to the b_n , c_n and b_p haems in
277 the core of the complex, viewed in the membrane plane. **d**, The complex viewed from the stromal side of
278 the membrane; peripheral helices of cyt b_6 and subIV are shown in cartoon representation for clarity, to
279 show PQ2 straddling the intermonomer cavity and sitting between the two c_n haems. **e**, Close-up of the
280 cavity in panel (**d**). **f-g**, The head and tail regions of PQ2 in relation to the c_n haems on both sides of the
281 cavity, highlighting the different dispositions of the haem c_n propionates, and the Arg207 and Asp20
282 sidechains.

283

284 METHODS

285 **Complex purification**

286 Dimeric cyt b_6f was isolated from dark-adapted market spinach (*Spinacia oleracea*) in a procedure adapted
287 from Dietrich and Kuhlbrandt³².

288 Briefly, spinach leaves were homogenised in Buffer 1 (50 mM Tris-HCl pH 7.5, 200 mM sucrose, 100 mM
289 NaCl). Homogenate was then filtered and centrifuged for 15 min at 4540 RCF, 4°C. Following centrifugation,
290 the supernatant containing cell debris was discarded and the pellet resuspended in Buffer 2 (150 mM NaCl,
291 10 mM Tricine-NaOH pH 8) before centrifugation again for 15 min (4540 RCF, 4°C). The resultant pellet was
292 resuspended in Buffer 3 (2 M NaBr, 10 mM Tricine-NaOH pH 8, 300 mM sucrose) and incubated on ice for
293 15 min before diluting 2-fold with ice cold milliQ H₂O and centrifuging (15 min, 4540 RCF, 4°C). The
294 resultant pellet was resuspended in Buffer 3 and incubated on ice for 15 min before diluting 2-fold with ice
295 cold milliQ H₂O and centrifuging again (15 min, 4540 RCF, 4°C). The pellet was resuspended in Buffer 2 and
296 centrifuged for 15 min, 4540 RCF, 4°C. The final pellet was resuspended in a small volume of Buffer 4 (40
297 mM Tricine pH 8.0, 10 mM MgCl₂, 10 mM KCl). The resultant thylakoid suspension was adjusted to 10 mg
298 ml⁻¹ Chl (Chl concentrations determined as described by Porra et al.³³).

299 For selective solubilisation of cyt b_6f , the thylakoid suspension (10 mg ml⁻¹ Chl) was diluted with Membrane
300 Extraction Buffer (40 mM Tricine pH 8.0, 10 mM MgCl₂, 10 mM KCl, 1.25% (w/v) Hecameg) to a final
301 concentration of 2 mg ml⁻¹ Chl, 1% (w/v) Hecameg. The resultant solution was mixed thoroughly then
302 incubated for 2 mins at room temperature before dilution to 0.75% (w/v) Hecameg with Buffer 4.
303 Unsolubilised material was removed by ultracentrifugation at 244,000 RCF at 4°C for 30 min in a Beckman
304 Ti50.2 rotor.

305 The solubilisation supernatant was concentrated using a Centriprep 100K centrifugal filter (Merck Millipore
306 Ltd.) before loading onto a 10-40% (w/v) continuous sucrose gradient containing 40 mM Tricine pH 8, 10
307 mM MgCl₂, 10 mM KCl, 0.8% (w/v) Hecameg, 0.1 mg ml⁻¹ egg yolk L- α -phosphatidylcholine (Sigma). This
308 was ultracentrifuged at 174,587 RCF at 4°C for 16 h in a Beckman SW32 rotor.

309 A brown-ish band containing cyt *b₆f* was harvested from a region of the gradient corresponding to ~16%
310 sucrose. This band was concentrated and loaded onto a ceramic hydroxyapatite column (CHT) (Type I, Bio-
311 Rad) pre-equilibrated in 20 mM Hecameg, 0.1 mg ml⁻¹ Phosphatidylcholine, 20 mM Tricine pH 8. The
312 column was washed with 5 column volumes of CHT Wash Buffer (20 mM Hecameg, 0.1 mg ml⁻¹
313 Phosphatidylcholine, 100 mM ammonium phosphate pH 8) before bound material was eluted with CHT
314 Elution Buffer (20 mM Hecameg, 0.1 mg ml⁻¹ Phosphatidylcholine, 400 mM ammonium phosphate pH 8).

315

316 **Detergent exchange and gel filtration**

317 Concentrated CHT eluate was loaded onto a 10-35% (w/v) continuous sucrose gradient containing 50 mM
318 HEPES pH 8, 20 mM NaCl, 0.3 mM tPCCαM and ultracentrifuged at 175,117 RCF at 4°C for 16 h in a
319 Beckman SW41 rotor.

320 A single brown band containing cyt *b₆f* was harvested from a region of the gradient corresponding to ~22%
321 sucrose. This band was concentrated and loaded onto HiLoad 16/600 Superdex 200 pg gel filtration column
322 (GE Healthcare) connected to an ÄKTA prime plus purification system (GE Healthcare). The column was run
323 at a rate of 0.2 ml min⁻¹ with 145 ml with Gel Filtration Buffer (50 mM HEPES pH 8, 20 mM NaCl, 0.3 mM
324 tPCCαM). Eluted fractions comprising dimeric cyt *b₆f* were pooled and concentrated.

325

326 **SDS-PAGE and BN-PAGE analysis**

327 Samples collected from each purification step were analysed by SDS-PAGE and BN-PAGE. For SDS-PAGE,
328 precast NuPAGE 12% Bis-Tris gels (Invitrogen) were run for 60 min at 180 V before staining with Coomassie
329 Blue. For BN-PAGE, precast NativePAGE 3-12% Bis-Tris gels (Invitrogen) were run for 120 min at 160 V
330 before staining with Coomassie Blue. Gels were imaged using an Amersham 600 imager (GE Healthcare).

331

332 **Quantification of purified dimeric cytochrome *b₆f* using redox difference spectra**

333 Absorbance spectra were recorded at room temperature on a Cary60 spectrophotometer (Agilent). For
334 redox difference spectra cytochromes were first fully oxidised with a few grains of potassium ferricyanide
335 followed by reduction with a few grains of sodium ascorbate (cyt *f*) then sodium dithionite (cyts *f* and *b₆*).
336 At each stage the sample was mixed thoroughly and incubated for ~1 min before recording spectra. Redox
337 difference spectra (ascorbate-reduced minus ferricyanide-oxidised and dithionite-reduced minus
338 ascorbate-reduced) were calculated and used to determine the concentrations of cyt *f* and the two *b*-type
339 cyts using extinction coefficients of 25 mM cm⁻¹ (*f*) and 21 mM cm⁻¹ (*b₆*)³⁴.

340

341 **Reduction of decylplastoquinone**

342 Approximately 0.1 mg decylplastoquinone (Merck, UK) was dissolved in 100 μl ethanol, mixed with a few
343 grains of sodium dithionite dissolved in 100 μl milliQ H_2O and vortexed until the solution became
344 colourless. Decylplastoquinol was extracted by mixing with 0.5 ml hexane, vortexing and centrifuging at
345 16,000 RCF for 2 mins. The hexane layer was carefully removed ensuring none of the aqueous phase was
346 collected. Hexane extraction was repeated on the aqueous phase twice more, then the hexane solutions
347 were pooled and dried in a rotary evaporator at 30 $^\circ\text{C}$ for 1 hour prior to re-dissolving in ~ 100 μl DMSO.
348 Decylplastoquinol concentration was determined by dilution of 10 μl of the DMSO solution into 795 μl
349 ethanol, recording the absorbance spectrum between 250 and 350 nm and using an extinction coefficient
350 of $3540 \text{ M}^{-1} \text{ cm}^{-1}$ at 290 nm³⁵.

351

352 **Activity assays**

353 Reduction of PC by cyt *b₆f* was monitored by stopped-flow absorbance spectroscopy using an Olis RSM
354 1000 rapid-scanning spectrophotometer equipped with a USA-SF stopped flow cell at 20 $^\circ\text{C}$. Solution A
355 (231.25 nM cyt *b₆f* and 62.5 μM PC in 50 mM HEPES pH 8, 20 mM NaCl, 0.3 mM tPCC α M) and solution B
356 (1.25 mM decylplastoquinol in the same buffer) were prepared and the reaction was initiated by mixing the
357 solutions in a 4:1 volumetric ratio (final concentrations: 185 nM cyt *b₆f*, 50 μM PC, 250 μM
358 decylplastoquinol). PC reduction was monitored by recording absorbance spectra between 420-750 nm at a
359 rate of 62 scans sec^{-1} and plotting the change in absorbance at 597 nm³⁶. In a control reaction cyt *b₆f* was
360 omitted to record the uncatalysed reduction of PC by decylplastoquinol. Fitting of the initial reaction rates
361 was performed in Origin. All measurements were carried out in triplicate.

362

363 **Purification of plastocyanin**

364 Plastocyanin was purified in its oxidised form from market spinach. Briefly, spinach leaves were
365 homogenised in buffer containing 50 mM sodium phosphate pH 7.4, 5 mM MgCl_2 , 300 mM sucrose.
366 Homogenate was then filtered and centrifuged for 15 min at 4000 RCF. Following centrifugation, the
367 supernatant containing cell debris was discarded and the pellet resuspended in buffer containing 10 mM
368 Tricine pH 7.4, 5 mM MgCl_2 . The solution was incubated on ice for 1 min before diluting 2-fold with buffer
369 containing 10 mM Tricine pH 7.4, 5 mM MgCl_2 , 400 mM sucrose and centrifuging for 15 mins at 4000 RCF.
370 Following centrifugation, the pellet was resuspended to a chlorophyll concentration of 2 mg ml^{-1} in buffer
371 containing 10 mM HEPES pH 7.6, 5 mM NaCl, 5 mM EDTA, and sonicated for 10 min, at 30 sec intervals. The
372 solution was centrifuged at 200,000 RCF for 1 h to pellet any large unbroken material. The supernatant was
373 applied to 4 x 5 ml GE Healthcare Hi-TRAP Q FF anion exchange columns chained together, equilibrated in

374 HEPES pH 8, 5 mM NaCl. A gradient of 0.005-1 M NaCl was used for elution, with PC eluting at around 200
375 mM. PC-containing fractions were identified by the blue colour upon addition of potassium ferricyanide.
376 These fractions were pooled, concentrated in a Vivaspin 3 kDa molecular-weight cut-off spin concentrator
377 and loaded onto a Superdex™ 200 16/600 FPLC column, equilibrated with 20 mM HEPES pH 8, 20 mM NaCl.
378 The resulting PC fractions were pooled, concentrated, and frozen at -80°C until use.

379

380 **CryoEM specimen preparation and data acquisition**

381 3 μ l of purified cyt *b₆f* (~17 μ M) was applied to freshly glow discharged holey carbon grids (Quantifoil
382 R1.2/1.3, 400 mesh Cu). The grids were blotted for 2 sec at 8°C then plunge frozen into liquid ethane using
383 a Leica EM GP at 90% relative humidity. Data acquisition was carried out on a Titan Krios microscope
384 operated at 300 kV (Thermo Fisher) equipped with an energy filtered (slit width 20 eV) K2 summit direct
385 electron detector. A total of 6,035 movies were collected in counting mode at a nominal magnification of
386 130,000 X (pixel size of 1.065 Å) and a dose of 4.6 e⁻ Å⁻² s⁻¹ (see Extended Data Table 1). An exposure time of
387 12 sec was used and the resulting movies were dose-fractionated into 48 fractions. A defocus range of -1.5
388 to -2.5 μ m was used.

389

390 **Image processing and 3D reconstruction**

391 Beam-induced motion correction and dose-fractionation were carried out using MotionCor2. Contrast
392 transfer function (CTF) parameters of the dose-weighted motion corrected images were then estimated
393 using GCTF³⁷. All subsequent processing steps were performed using RELION 2.1³⁸ or 3.0³⁹ unless otherwise
394 stated.

395 In total, 422,660 particles were manually picked from 6,035 micrographs. These particles were extracted
396 using a box size of 220 x 220 pixels and subjected to reference-free 2D classification. A typical micrograph
397 showing picked particles is shown in Extended Data Fig. 2a,b. Particles that categorised into poorly defined
398 classes were rejected, while the remaining 292,242 (69.2%) particles were used for further processing. A
399 subset of 30,000 particles was used to generate a *de novo* initial model using the '3D initial model'
400 subroutine. The initial model low pass filtered to 20 Å was used as a reference map for subsequent 3D
401 classification into 10 3D classes. One stable 3D class at a resolution of 5.38 Å was selected for high
402 resolution 3D auto-refinement; this class accounted for a subset of 108,560 particles (25.6%). This subset of
403 refined particles was then re-extracted and re-centred before another round of 3D auto refinement was
404 carried out. The resultant 4.85 Å density map was corrected for the modulation transfer function (MTF) of
405 the Gatan K2 summit camera then further sharpened using the post-processing procedure to 4.02 Å. Per-
406 particle CTF-refinement was carried out and a soft mask was created which included the detergent shell.

407 The final global resolution estimate of 3.58 Å was based on the gold-standard Fourier shell correlation (FSC)
408 cut off of 0.143.

409 Local resolution was determined using one of two unfiltered half-maps as an input, a calibrated pixel size of
410 1.065 and a B-factor of -103. The output local resolution map is shown in Extended Data Fig 2d,e.

411

412 **Model building**

413 Initially, a homology-based approach was performed using the crystallographic structure of *Nostoc* sp. PCC
414 7120 cyt *b₆f* (PDB: 4OGQ)⁴⁰ as a template. Sequence alignments of the 8 polypeptide subunits of cyt *b₆f*
415 were carried out using Clustal Omega (Extended Data Fig. 7 and 8). The model was rigid-body docked into
416 the density using the 'fit in map' tool in Chimera⁴¹. This was then followed by manual adjustment and real-
417 space refinement using COOT⁴². Sequence assignment and fitting was guided by bulky residues such as Arg,
418 Trp, Tyr and Phe. After fitting of the polypeptide chains and cofactors in one half of the dimeric complex,
419 the other half of the complex was then independently fitted into the C1 density map. Once both halves of
420 the complex were fitted, cofactors, lipids and plastoquinone-9 molecules were fitted into regions of
421 unassigned density. The final model underwent global refinement and minimisation using the real space
422 refinement tool in PHENIX⁴³. The final refinement statistics are summarised in Extended Data Table 1.

423

424 **Pigment analysis by reversed-phase HPLC**

425 Pigments were extracted from purified cyt*b₆f* with 7:2 acetone/methanol (v/v) and clarified extracts were
426 separated by reversed-phase HPLC at a flow rate of 1 ml min⁻¹ at 40°C using a Supelco Discovery® HS C18
427 column (5 µm particle size, 120 Å pore size, 25 cm × 4.6 mm) on an Agilent 1200 HPLC system. The column
428 was equilibrated in acetonitrile: water: trimethylamine (9:1:0.01 v/v/v) and pigments were eluted by
429 applying a linear gradient of 0-100% ethyl acetate over 15 min followed by isocratic elution with 100% ethyl
430 acetate for a further 5 min. Elution of carotenoid and chlorophyll species was monitored by absorbance at
431 400, 450, 490 and 665 nm. Chlorophyll *a* was identified by its absorption spectra and known retention
432 time⁴⁴. The major carotenoid species was confirmed as 9-*cis* beta-carotene using a standard obtained from
433 Sigma-Aldrich (Product number: 52824).

434

435 **DATA AVAILABILITY STATEMENT**

436 All relevant data are available from the authors and/or are included with the manuscript or Supplementary
437 Information. Atomic coordinates and the cryo-EM density map have been deposited in the Protein Data

438 Bank (PDB) under accession number 6RQF and the Electron Microscopy Data Bank (EMDB) under accession
439 number EMD-4981.

440

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469

470 **Extended Data Table 1. Cryo-EM data collection, refinement and validation statistics.** *Peter B Rosenthal
471 and Richard Henderson (2003) Optimal determination of particle orientation, absolute hand and contrast
472 loss in single particle electron cryomicroscopy. *J. Mol. Biol.*, 333(4):721-745.

473 **Extended Data Table 2. A comparison of edge-to-edge cofactor distances (Å) in each half of the b_6f dimer**
474 **from different species (6RQF, 1Q90, 2E74, 4OGQ) and the bc_1 dimer from *G. gallus* with the Rieske ISP in**
475 **its distal (1BCC) and proximal (3BCC) positions.** * Inhibitors are indicated by the abbreviations TDS
476 (tridecylstigmatellin), STG (stigmatellin), AMY (antimycin).

477 **Extended Data Figure 1. Purification of cytochrome b_6f from spinach.** **a**, Absorption spectrum of
478 ascorbate-reduced purified b_6f complex. The peak at 421nm corresponds to the Soret band of bound
479 pigments (chlorophyll *a* and haems). The peaks at 554 and 668 nm correspond to *c*-type haem of
480 cytochrome *f* and chlorophyll *a* respectively. The inset panel shows a redox difference spectra of ascorbate-
481 reduced minus ferricyanide-oxidised b_6f (dashed line) and dithionite-reduced minus ascorbate-reduced
482 (dotted line) $cytb_6f$. Redox difference spectra show cytochrome *f* absorption peaks at 523 and 554 nm as
483 well as absorption peaks at 534 and 563nm corresponding to the *b*-type cytochromes of cytochrome b_6 .
484 The calculated ratio of cytochrome b_6 to cytochrome *f* was ~ 2 using extinction coefficients of 25 mM cm^{-1}
485 (*f*) and 21 mM cm^{-1} (b_6) (Cramer and Whitmarsh, 1977). The spectra exhibit the absorption properties
486 characteristic of intact cytochrome b_6f . Spectra were recorded at room temperature. **b**, SDS-PAGE analysis
487 of purified cytochrome b_6f indicates the sample is highly pure with the four large subunits of the complex
488 (*cyt f*, *cyt b₆*, the Rieske ISP, subunit IV) running at ~ 31 kDa, ~ 24 kDa, ~ 20 kDa and ~ 17 kDa respectively and
489 the 4 small subunits (PetG, L, M, and N) running at around 4 kDa (not shown). **c-d**, Negative stain and BN-
490 PAGE analysis of purified cytochrome b_6f demonstrates the sample is dimeric and highly homogenous, with
491 a single band corresponding to dimeric cytochrome b_6f shown in lane 1. Lane 2 is a sample which has been
492 deliberately monomerised following incubation with 1% Triton-X-100 for 1 hour. For gel source data see
493 Supplementary Information Figure 1. **e**, The catalytic rate of plastocyanin reduction by the purified dimeric
494 $cytb_6f$ complex as determined by stopped-flow absorbance spectroscopy. A rate of $200 \text{ e}^- \text{ s}^{-1}$ was
495 determined by taking the initial linear region from the enzyme-catalysed reaction (solid line) and deducting
496 the background rate measured in the absence of enzyme (long-dashed line). Plastocyanin reduction was
497 not observed in the absence of decylplastoquinol (short dashed line). Reactions were initiated upon
498 addition of decylplastoquinol to the solution containing plastocyanin and b_6f whilst monitoring the loss of
499 absorbance at 597nm. Final concentrations were 50 μM plastocyanin, 185 nM b_6f and 250 μM
500 decylplastoquinol. All experiments were performed in triplicate and controls were performed in the
501 absence of b_6f or decylplastoquinol.

502 **Extended Data Figure 2. Cryo-EM micrographs of the spinach cytochrome b_6f complex and calculation of**
503 **the cryo-EM map global and local resolution.** **a**, Cytochrome b_6f particles covered by a thin layer of
504 vitreous ice on a supported carbon film. **b**, Examples of dimeric cytochrome b_6f particles are circled in
505 green. 6,035 cryo-EM movies were recorded, from which 422,660 particles were picked manually for
506 reference-free 2D classification. 108,560 particles were used for calculation of the final density map. **c**, Gold
507 standard refinement was used for estimation of the final map resolution (solid black line). The global

508 resolution of 3.58 Å was calculated using a Fourier shell correlation (FSC) cut-off at 0.143. A model-to-map
509 FSC curve (solid grey line) was also calculated. **d-e**, A C1 density map of the cytochrome *b₆f* complex both
510 with **(d)** and without **(e)** the detergent shell. The map is coloured according to local resolution estimated by
511 RELION and viewed from within the plane of the membrane. The colour key on the right shows the local
512 structural resolution in Angstroms (Å).

513 **Extended Data Figure 3. Cryo-EM densities and structural models of polypeptides in the cytochrome *b₆f***
514 **complex.** Polypeptides are coloured as in Fig. 1. The contour levels of the density maps were adjusted to
515 0.0144

516 **Extended Data Figure 4. Cryo-EM densities and structural models of prosthetic groups, lipids and**
517 **plastoquinone molecules in the cytochrome *b₆f* complex.** *c*-type haems (*f*, *c_n*; dark blue), *b*-type haems (*b_p*,
518 *b_n*; red), 9-*cis* β-carotene (orange), chlorophyll *a* (major conformation, dark green) (minor conformation,
519 light green), 2Fe-2S (orange/yellow), plastoquinones (yellow), monogalactosyl diacylglycerol (light pink),
520 phosphatidylcholine (light cyan), sulfoquinovosyl diacylglycerol (light green), phosphatidylglycerol (light
521 purple). The contour levels of the density maps were adjusted to 0.0068.

522 **Extended Data Figure 5. Alternative interpretation of the region assigned as PQ2.** The density map
523 showing two possible alternative conformations for PQ2. **a)** The major conformation of PQ2 and **b)** the
524 alternative conformation of PQ2. Cofactors are coloured as in Extended Data Figure 4 with *b*-type haems
525 (*b_p*, *b_n*) coloured red, *c*-type haems (*c_n*) coloured dark blue, chlorophyll *a* (major conformation) coloured
526 dark green, plastoquinones coloured yellow and the cytochrome *b₆* subunit coloured light green. The
527 contour level of the density map was adjusted to 0.0089.

528 **Extended Data Figure 6. Alternative interpretations of the density map in the region assigned as PQ3.** The
529 density map modelled with **(a)** a plastoquinone molecule and **(b)** a phosphatidylcholine molecule. Upper
530 panels show the protein-free density map and the lower panels include cyt *b₆* (green). The 2.9 Å distance
531 indicates a close contact between the PQ3 head group and the conserved Lys208. Cofactors are coloured as
532 in Extended Data Figure 4 with *b*-type haems (*b_p*, *b_n*) coloured red, chlorophyll *a* (major conformation)
533 coloured dark green, plastoquinones coloured yellow, phosphatidylcholine coloured light cyan,
534 sulfoquinovosyl diacylglycerol coloured mint green, and the cytochrome *b₆* subunit coloured light green.
535 The contour level of the density map was adjusted to 0.0127.

536 **Extended Data Figure 7. Multiple sequence alignment of cytochrome *b₆f* subunits cytochrome *f*,**
537 **cytochrome *b₆*.** Sequences of cytochrome *f* **(a)**, cytochrome *b₆* **(b)** from cyanobacterial (*Mastigocladus*
538 *laminosus*, *Nostoc* sp. PCC7120), algal (*Chlamydomonas reinhardtii*) and plant (*Spinacia oleracea*) subunits
539 were aligned in Clustal Omega v 1.2.4. Conserved identities are indicated by asterisks (*), and similarities by
540 double (:), then single dots (.). Polar residues are coloured in green, positively charged residues are

541 coloured pink, hydrophobic residues are coloured red and negatively charged residues are coloured blue.
542 The sequences omit signal peptides.

543 **Extended Data Figure 8. Multiple sequence alignment of the Rieske ISP, Subunit IV, PetG, PetL, PetM and**
544 **PetN.** Sequences of Rieske ISP **(a)**, Subunit IV **(b)**, PetG **(c)**, PetL **(d)**, PetM **(e)** and PetN **(f)** from
545 cyanobacterial (*Mastigocladus laminosus*, *Nostoc* sp. PCC7120), algal (*Chlamydomonas reinhardtii*) and
546 plant (*Spinacia oleracea*) subunits were aligned in Clustal Omega v 1.2.4. Conserved identities are indicated
547 by asterisks (*), and similarities by double (:), then single dots (.). Polar residues are coloured in green,
548 positively charged residues are coloured pink, hydrophobic residues are coloured red and negatively
549 charged residues are coloured blue. The sequences omit signal peptides.

550

551







