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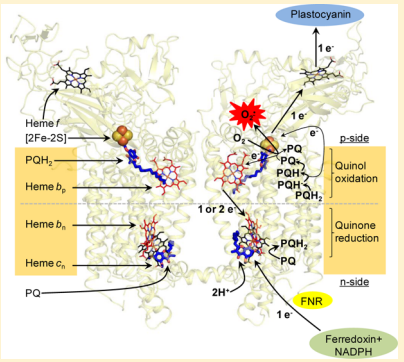
Mechanism of Enhanced Superoxide Production in the Cytochrome b_6f Complex of Oxygenic Photosynthesis

Danas Baniulis,[§,†] S. Saif Hasan,[§] Jason T. Stofleth,[‡] and William A. Cramer*

Department of Biological Sciences, Hockmeyer Hall of Structural Biology, Purdue University, West Lafayette, Indiana 47907, United States

ABSTRACT: The specific rate of superoxide ($O_2^{\bullet-}$) production in the purified active crystallizable cytochrome b_6f complex, normalized to the rate of electron transport, has been found to be more than an order of magnitude greater than that measured in isolated yeast respiratory bc_1 complex. The biochemical and structural basis for the enhanced production of $O_2^{\bullet-}$ in the cytochrome b_6f complex compared to that in the bc_1 complex is discussed. The higher rate of superoxide production in the b_6f complex could be a consequence of an increased residence time of plastosemiquinone/plastoquinol in its binding niche near the Rieske protein iron−sulfur cluster, resulting from (i) occlusion of the quinone portal by the phytyl chain of the unique bound chlorophyll, (ii) an altered environment of the proton-accepting glutamate believed to be a proton acceptor from semiquinone, or (iii) a more negative redox potential of the heme b_p on the electrochemically positive side of the complex. The enhanced rate of superoxide production in the b_6f complex is physiologically significant as the chloroplast-generated reactive oxygen species (ROS) functions in the regulation of excess excitation energy, is a source of oxidative damage inflicted during photosynthetic reactions, and is a major source of ROS in plant cells. Altered levels of ROS production are believed to convey redox signaling from the organelle to the cytosol and nucleus.



The protein subunits and prosthetic groups in the structure from the cyanobacterium *Mastigocladus laminosus*[1] are shown in Figure 1. The role of the cytochrome bc_1 complex in the generation of $O_2^{\bullet-}$ by the mitochondrial respiratory electron transport pathway has been described and reviewed.[2−8] By analogy with the bc_1 complex, the site of production of superoxide in the cytochrome b_6f complex is shown in Figure 2, with an emphasis on the electrochemically positive (p, lumen)-side plastoquinone/plastoquinol binding site that, by analogy with the mechanism proposed for the bc_1 complex, is close to the site of oxygen reduction by plastosemiquinone and resulting superoxide formation. The production of reactive oxygen species (ROS) in chloroplasts not only underlies oxidative damage inflicted in photosynthetic electron transport[9] but also functions in redox signaling from the organelle to the cytosol and nucleus,[10] and in the regulation and dissipation of excess excitation energy.[11] Although the production of $O_2^{\bullet-}$ by the cytochrome b_6f complex of oxygenic photosynthesis has been detected by EPR spectroscopy,[12] details about rates and constraints have not yet been published. This study provides quantitative information about the level of $O_2^{\bullet-}$ generated in electron transport through the b_6f complex that mediates these signaling processes.

$O_2^{\bullet-}$ can be formed through a one-electron reduction of the oxygen molecule, with a midpoint redox potential of −0.14 V in the aqueous phase.[13,14]

$$O_2 + e^- \rightarrow O_2^{\bullet-} \quad \text{(i)}$$

Ubisemiquinone, $UQ_p^{\bullet-}$, formed on the electrochemically positive, p-side, of the complex (reaction iia), is a reductant for oxygen proximal to the bc_1 complex in mitochondrial and photosynthetic bacterial membranes. Because ubisemiquinol is a reductant of low-potential heme b_p in the bc_1 complex,[15] it has been inferred that the ubisemiquinone formed in the bc_1 complex through quinol oxidation by the high-potential segment of the electron transport chain has a sufficiently reducing potential to form superoxide, $O_2^{\bullet-}$ (reaction iia). On the basis of the similarity of the crystal structures of the protein core[16] and of the midpoint redox potentials [E_{m7} values of 80 mV (plastoquinone/quinol)[17] and 60 mV (ubiquinone/quinol)[18]], it is inferred that plastosemiquinone, $PQ_p^{\bullet-}$, can serve as the reductant for the generation of superoxide in the b_6f complex (Figure 2B and reaction iib):

$$UQ_p^{\bullet-} + O_2 \rightarrow UQ(ox) + O_2^{\bullet-} \quad \text{(iia)}$$

$$PQ_p^{\bullet-} + O_2 \rightarrow PQ(ox) + O_2^{\bullet-} \quad \text{(iib)}$$

The p-side semiquinone, $Q_p^{\bullet-}$, is generated through the one-electron oxidation of ubiquinol or plastoquinol, QH_2, by the [FeS] cluster of the high-potential Rieske iron−sulfur protein subunit of the complex:

$$QH_2 + [FeS](ox) \rightarrow QH_p^{\bullet} + [FeS](red) + H^+ \quad \text{(iii)}$$

$QH_p^{\bullet-}$ generated in reaction iii transfers a proton to the p-side aqueous phase through an intraprotein pathway in the

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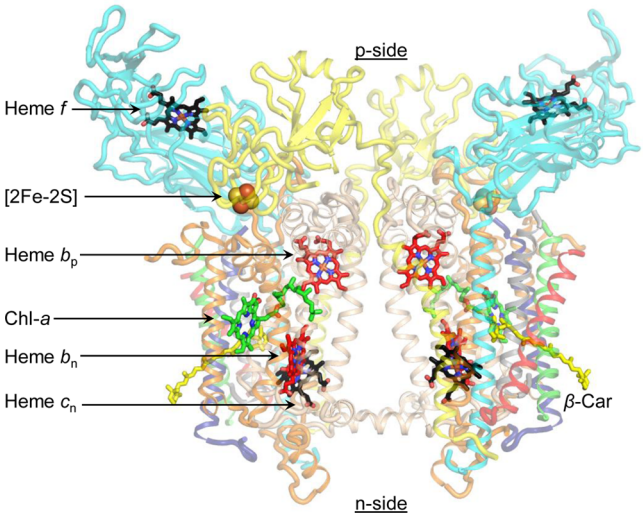

Figure 1. Dimeric cytochrome b_6f complex (PDB entry 4H44). Prosthetic groups in the cytochrome b_6f complex. Hemes b_p (red), b_n (red), and c_n (black) are redox-active prosthetic groups located within the transmembrane domain and constitute the low-potential chain. Heme *f* (black) and the [2Fe-2S] cluster (orange and yellow spheres) form the high-potential chain and are associated with the p-side extrinsic domains of cytochrome *f* and ISP, respectively. A chlorophyll *a* (green) and a β-carotene (yellow) are also associated with the complex. Polypeptides are shown as ribbons. Color code: cytochrome b_6 (cyt b_6), wheat; subunit IV (subIV), orange; cytochrome *f* (cyt *f*), cyan; iron–sulfur protein (ISP), yellow; PetL, red; PetM, green; PetG, blue; PetN, gray.

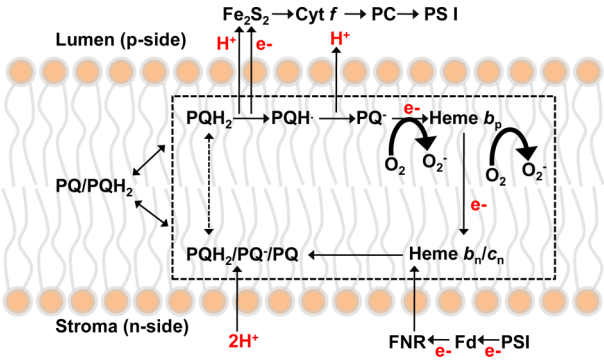

Figure 2. Transmembrane electron transfer and Q-cycle pathway in a schematic of the charge transfer reactions in the cytochrome b_6f complex, indicating the branching of electrons from the anionic semiquinone reductant, $PQ_p^{\bullet-}$, and heme b_p to O_2. The latter reactions are responsible for the formation of superoxide, $O_2^{\bullet-}$.

cytochrome bc_1 complex.[19] A similar p-side proton release pathway, which forms the p-side anionic plastosemiquinone, $PQ_p^{\bullet-}$, has recently been defined by crystallographic analysis of the cytochrome b_6f complex.[20]

In a Q-cycle mechanism,[15,21−24] an electron is transferred from the deprotonated $Q_p^{\bullet-}$ to p-side heme b_p (reaction iv) and then across the hydrophobic domain of the membrane through the n-side heme, b_n, to reduce quinone specifically bound on the n-side of the complex.

$$Q_p^{\bullet-} + b_p(\text{ox}) \rightarrow b_p(\text{red}) + Q_p(\text{ox}) \quad \text{(iv)}$$

Reduced heme b_p may be an alternative source of electrons for the reduction of O_2 to $O_2^{\bullet-}$:[25]

$$b_p(\text{red}) + O_2 \rightarrow b_p(\text{ox}) + O_2^{\bullet-} \quad \text{(v)}$$

It has been inferred[2,8] that superoxide production in the cytochrome bc_1 complex occurs as a bypass of reaction iv above of the Q-cycle by reaction iia, b, or v, the latter reaction having been suggested as being dominant.[4,25] The participation of reactions iib and v in superoxide generation in the cytochrome b_6f complex is shown in the Q-cycle scheme (Figure 2). The n-side ubiquinone reduction in the bc_1 complex is blocked by the quinone analogue inhibitor antimycin A, which occupies the n-side quinone binding site with high affinity.[26] A consequence of this inhibition of the transmembrane electron transfer chain would be accumulation of the p-side semiquinone electron donor, $Q_p^{\bullet-}$ (reaction iv). The probability of electron transfer through the other branch of the $UQ_p^{\bullet-}$ oxidation pathway that forms superoxide (reaction iia) would then be increased. Thus, the specific n-side quinone analogue inhibitor, antimycin A, causes a large increase in the specific rate of formation of $O_2^{\bullet-}$, relative to the electron transport rate, by the bc_1 complex of yeast or bovine mitochondria,[4,7,8,27,28] and the photosynthetic bacterium, *Rhodobacter sphaeroides*.[29] No n-side inhibitor comparable in efficacy to that of antimycin has been found for the b_6f complex, because the unique heme c_n occupies the quinone binding site homologous to that of antimycin,[30] which results in an altered binding interaction of an n-side quinone analogue inhibitor.[31] Although the quinone analogue inhibitor NQNO binds specifically to n-side heme c_n[1] and inhibits the oxidation of heme b_n,[32−34] unlike the inhibition of the respiratory chain resulting from the action of antimycin, the extent of inhibition of linear electron transport, by NQNO, is low.[32]

It has been proposed that $O_2^{\bullet-}$ production occurs in the cytochrome b_6f complex[2] by a mechanism (reaction iib) similar to the alternative pathway for the bc_1 complex (reaction iia).[2,3,5,8] However, experimental details about the rate of $O_2^{\bullet-}$ generation in the b_6f complex have not been described. This study compares the specific rate of $O_2^{\bullet-}$ generation in cytochrome b_6f and bc_1 complexes and proposes that the level of $O_2^{\bullet-}$ production by the b_6f and bc_1 complexes is dependent upon the residence time of reduced quinone species within the Q_p portal.

MATERIALS AND METHODS

Materials. ADPH and H_2O_2 were purchased from Anaspec (Fremont, CA), equine heart cytochrome *c*, HRP and SOD enzymes, decyl-ubiquinol, and decyl-plastoquinol from Sigma-Aldrich (St. Louis, MO), DDM and UDM from Anatrace (Maumee, OH), and other reagents from Mallinckrodt/Baker, Inc. (Phillipsburg, NJ).

Preparation of the Yeast Mitochondrial Cytochrome bc_1 Complex. The cytochrome bc_1 complex from yeast was purified in the laboratory of B. L. Trumpower, as previously described.[28]

Preparation of Thylakoid Membranes and Purification of the Cytochrome b_6f Complex. Cyanobacterial thylakoid membranes and the b_6f complex were prepared and purified, respectively, as described previously,[35] as were spinach chloroplast thylakoid membranes and the cytochrome b_6f complex.[35,36] Membranes were resuspended (chlorophyll *a* concentration of 2 mg/mL) in TNE [30 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 0.3 M sucrose, with protease inhibitors benzamidine (2 mM) and ε-aminocaproic acid (2 mM)].

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Purification of Plastocyanin. Plastocyanin from *Nostoc*[36] and spinach[37] was purified as described previously, and for the latter, a modified procedure included size exclusion and anion-exchange chromatography.

Activities for ubiquinol-cytochrome *c* and plasto-quinol-plastocyanin oxidoreductase were assayed with the purified bc_1 and b_6f complexes, respectively. For the bc_1 complex, the assay mixture contained 50 μM equine heart cyt *c*, 50 mM MOPS (pH 6.9), 0.4 mM DDM, 1 mM KCN, and 0.05 mM decyl-ubiquinol. Reduction of cyt *c*, initiated by addition of 5 or 45 nM cytochrome bc_1 for uninhibited or inhibited activity, respectively, was monitored through the change in absorbance at 551 nm, and the activity was based on an extinction coefficient of 2.1×10^4 M^{-1} cm^{-1}.[38] For the plastoquinol-plastocyanin oxidoreductase activity of the b_6f complex or thylakoid membranes, the assay mixture contained plastocyanin (10^{-2} M) from *Nostoc* or spinach, 50 mM Tris-HCl (pH 7.5), 1 mM UDM, and 20 μM decyl-plastoquinol. Changes in absorbance were assayed on a Cary 4000 spectrophotometer. The reaction was initiated by addition of 5 nM cyt b_6f complex or 3 μM Chl *a* equivalent of thylakoid membranes, and activity was monitored as the change in plastocyanin absorbance at 597 nm (ε_{mM} = 4.9 mM^{-1} cm^{-1}).[39] Inhibitors were added as ethanol stocks (<0.5% of the total reaction volume). Initial electron transport rates were determined using the SLOPE function in the support software of a Cary 4000 spectrophotometer and calculated as moles of electrons transferred per mole of *bc* complex per second, or per 10^3 moles of Chl *a* equivalent of thylakoid membranes.

Superoxide Production Assay. The amount of superoxide released from cytochrome *bc* complexes was measured fluorometrically via the formation of H_2O_2. In the presence of saturating levels of SOD, superoxide released from cytochrome *bc* complexes is converted to H_2O_2. ADPH reacts with H_2O_2 in a 1:1 stoichiometry, in the presence of HRP as a catalyst, to produce the fluorescent oxidation product resorufin. The reaction mixture contained for the yeast bc_1 complex 50 mM MOPS (pH 6.9), 0.4 mM DDM, 50 μM ADHP, 1 unit/mL HRP, 50 μM equine heart cyt *c*, and 50 μM decyl-ubiquinol and for the b_6f complex 50 mM MOPS (pH 6.9), 1 mM UDM, 40 μM ADPH, 20 μM decyl-plastoquinol, 1 unit/mL HRP, 300 units/mL SOD, and plastocyanin (10 mM) from *Nostoc* or spinach or 25 μM cyt *c* from equine heart. Inhibitors were added as ethanol stocks (<0.5% of the total reaction volume). The reaction was started by adding the bc_1 complex to a final concentration of 5 or 45 nM for the measurement of uninhibited or inhibited activity, respectively, or the b_6f complex to a final concentration of 5 nM (3 μM Chl *a* equivalent of thylakoid membranes). The fluorescence indicator of superoxide production was measured by excitation and emission at 530 nm (2 nm bandwidth) and 590 nm (1 nm bandwidth), respectively, utilizing a FluoroMax-3 fluorimeter (Horiba Jobin Yvon Inc., Edison, NJ) and calibrated using H_2O_2 standards. The initial rate of superoxide production was estimated using the LINEST function of MS Office Excel and calculated as moles of superoxide generated per second per mole of *bc* complex, or per 10^3 moles of Chl *a* equivalent of thylakoid membranes.

Sequence alignment was performed with Clustal-Omega using default parameters.[40,41] The following cytochrome b_6 polypeptide sequences were obtained from the NCBI database: *Synechocystis* sp. PCC 6803, BAA10149.1; *Synechococcus elongatus* PCC 6301, BAD79961.1; *Nostoc* sp. PCC 7120, BAB75120.1; *M. laminosus*, AAR26242.1; *Chlamydomonas reinhardtii*, CAA44690.1; *Arabidopsis thaliana*, NP_051088.1; *Spinacea oleracea*, CAA30128.1.

Panels A and B of Figure 4 were generated with Pymol (http://www.pymol.org), from structural superposition of cytochrome bc_1 complex crystal structures (PDB entries 1NTZ[42] and 3CX5[43]) and cytochrome b_6f complex crystal structures (PDB entries 4H44 and 4H13[20]).

RESULTS

Superoxide Production in the Cytochrome bc_1 Complex. The rate of superoxide ($O_2^{\bullet-}$) production in the yeast mitochondrial bc_1 complex, using decyl-ubiquinol as the electron donor, is 0.1−0.2% of the electron transport rate (Table 1), a value similar to results in the literature obtained with the bc_1 complex from yeast mitochondria.[5,7,8,44] This is approximately the same rate, essentially a background rate, that was obtained in the presence of the p-side quinone analogue inhibitor DBMIB (Table 1), which prevents quinol oxidation by the [2Fe-2S] cluster.[45] Higher rates have been reported for mitochondrial bc_1 complexes, on the basis of a difference in measurements with and without superoxide dismutase to assay $O_2^{\bullet-}$ production.[44] $O_2^{\bullet-}$ generation has also been measured with the bc_1 complex obtained from the purple photosynthetic bacterium *Rb. sphaeroides*,[29,46] for which the rate of $O_2^{\bullet-}$ production relative to the electron transport rate was not reported. In our experiments with the yeast bc_1 complex, the specific rate of $O_2^{\bullet-}$ production, normalized to the electron transport rate, is, however, increased greatly (>40-fold) in the presence of antimycin A (Table 1), in qualitative agreement with data obtained previously for the bc_1 complex.[4,5,8,27−29] The electron transport rate of the isolated b_6f complex or membranes from spinach and *M. laminosus* and the $O_2^{\bullet-}$ generation rate were measured by the rate of reduction of plastocyanin and by the rate of change of fluorescence emission intensity from the resorufin product of ADHP and H_2O_2, respectively (Materials and Methods). Rates of electron transport (Figure 3A) and $O_2^{\bullet-}$ production (Figure 3B) obtained with the yeast bc_1 complex and the b_6f complex from spinach thylakoids and *M. laminosus* are shown, from which electron transport rates were derived for the yeast bc_1 complex (Table 1), the purified b_6f complex isolated from spinach thylakoid membranes (Table 2), the isolated *M. laminosus* cyanobacterial b_6f complex (Table 3), and membranes of *M. laminosus* (Table 4). The rate of production of $O_2^{\bullet-}$ by electron transfer from the decyl-plastoquinol electron donor to the b_6f complex (Table 2) is considerably higher, a factor of 10−20,

Table 1. Production of Superoxide by the Cytochrome bc_1 Complex from Yeast Mitochondria

	electron transfer rate (cyt bc^{-1} s^{-1})	superoxide production rate (cyt bc_1^{-1} s^{-1})	superoxide production (% of electron transfer rate)
control, no inhibitor[a]	151 ± 6.1 (n = 5)	0.21 ± 0.13 (n = 8)	0.14 ± 0.06
antimycin A, 20 μM	1.2 ± 0.1 (n = 4)	0.13 ± 0.03 (n = 6)	10.6 ± 2.3
DBMIB, 2 μM	28.3 ± 3.1 (n = 4)	0.04 ± 0.01 (n = 3)	0.14 ± 0.02

[a]For reactions in the absence and presence of inhibitor, the bc_1 complex was used at concentrations of 5 and 45 nM, respectively, with cytochrome *c* as the electron acceptor. *n* is the number of trials.

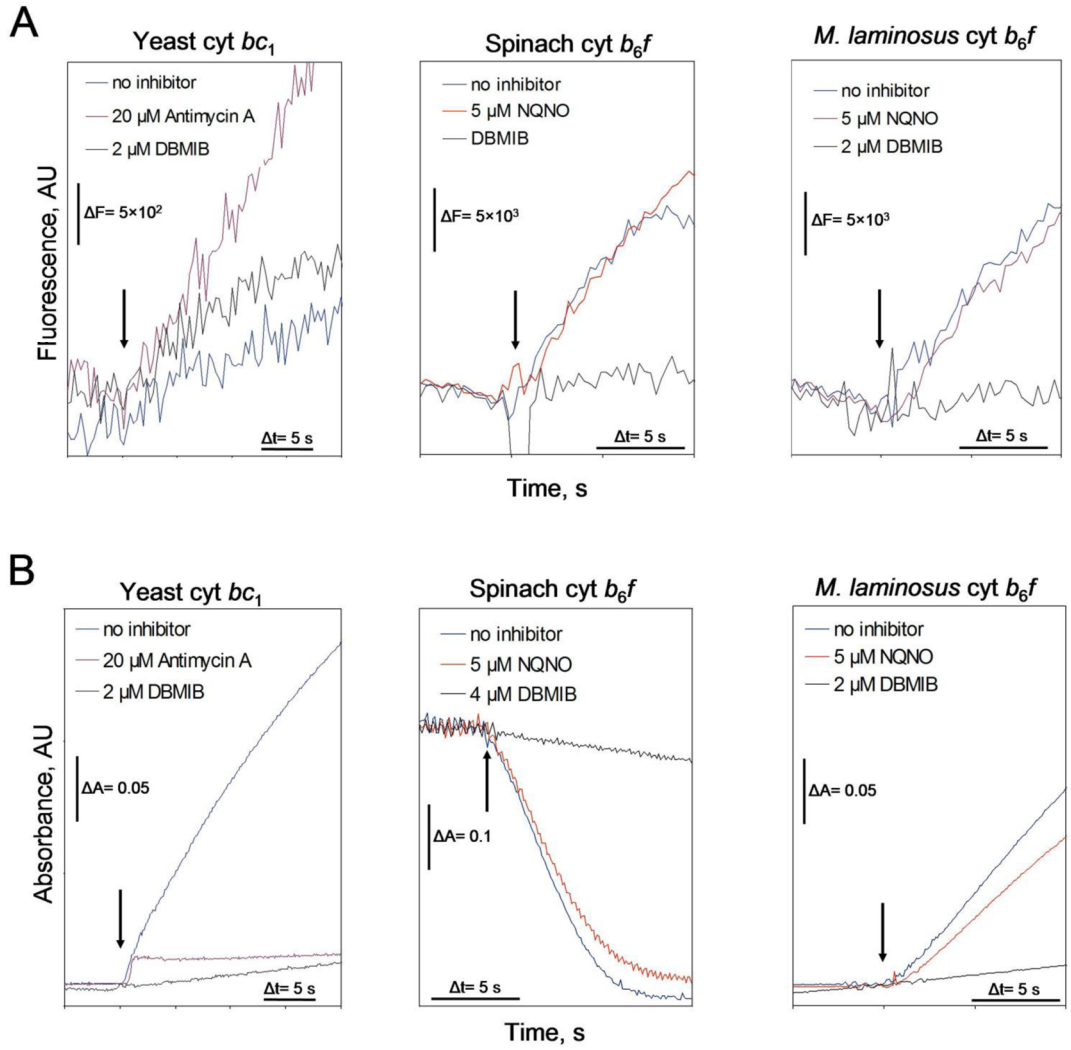

Figure 3. Representative original data traces for determination of rates of (A) superoxide generation and (B) electron transfer in isolated bc_1 and b_6f complexes. The electron transfer and superoxide generation activities were measured as described in Materials and Methods using cytochrome *c* as the electron acceptor for yeast cyt bc_1, cyt b_6f from *M. laminosus*, and cyanobacterial plastocyanin for the b_6f complex from spinach. Traces are averages of two to four measurements. The background fluorescence change measured for the reaction mixture before the addition of the enzyme was subtracted from the superoxide generation activity data (B). The arrow indicates addition of 5 and 45 nM bc_1 complex for reactions in the absence and presence of inhibitor, respectively, and 5 nM b_6f complex.

Table 2. Production of Superoxide by the Isolated Spinach Cytochrome b_6f Complex with Plastocyanin as the Electron Acceptor

	electron transfer rate[a] (cyt b_6f^{-1} s^{-1})	superoxide production rate[a] (cyt b_6f^{-1} s^{-1})	superoxide production (% of electron transfer rate)
control, no inhibitor	245 ± 25 (n = 10)	4.5 ± 0.6 (n = 10)	1.9 ± 0.3
NQNO, 5 μM	196 ± 7 (n = 3)	3.5 ± 0.4 (n = 3)	1.8 ± 0.2
DBMIB, 4 μM	nd (n = 4)	nd (n = 3)	–

[a] n is the number of trials. "nd" indicates that the rate was too low to allow an accurate determination.

Table 3. Production of Superoxide by the Isolated Cytochrome b_6f Complex from the Cyanobacterium *M. laminosus* with Cyanobacterial Plastocyanin as the Electron Acceptor

	electron transfer rate[a] (cyt b_6f^{-1} s^{-1})	superoxide production rate[a] (cyt b_6f^{-1} s^{-1})	superoxide production rate[a] (% of electron transfer rate)
control, no inhibitor	292 (n = 2)	6.3 (n = 2)	2.2 (n = 2)
NQNO, 5 μM	174 (n = 1)	2.3 (n = 1)	2.2 (n = 1)
DBMIB, 2 μM	6.5 ± 1.0 (n = 3)	nd (n = 6)	–
DBMIB, 5 μM	0.5 ± 0.7 (n = 3)	nd (n = 4)	–

[a] n is the number of trials. "nd" indicates not determined.

than that measured for the mitochondrial bc_1 complex (Table 1). Similar results were obtained with the b_6f complex purified from the cyanobacterium *M. laminosus* using cyanobacterial plastocyanin (Table 3). Using intact membranes from *M. laminosus* (Table 4), the specific rate of superoxide production by the b_6f complex is higher than that measured in the isolated bc_1 complex by approximately 1 order of magnitude, but for reasons not understood, this rate is somewhat smaller than that measured with the isolated bc_1 complex (Table 1).

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Table 4. Production of Superoxide by the Cytochrome b_6f Complex in Thylakoid Membranes from the Cyanobacterium *M. laminosus* with Cytochrome *c* as the Electron Acceptor

	electron transfer rate[a] ($\times 10^3$ chl a^{-1} s^{-1})	superoxide production rate[a] ($\times 10^3$ chl a^{-1} s^{-1})	superoxide production rate (% of electron transfer rate)
control, no inhibitor	201 ± 3 (n = 3)	2.6 ± 0.6 (n = 4)	1.3 ± 0.3
NQNO, 5 μM	151 ± 8 (n = 3)	1.5 ± 0.1 (n = 3)	0.9 ± 0.1
DBMIB, 2 μM	11 ± 2 (n = 3)	nd (n = 3)	–
DBMIB, 5 μM	0.2 ± 0.4 (n = 3)	nd (n = 3)	–

[a] n is the number of trials. "nd" indicates not determined.

Properties of Inhibitors. *NQNO.* NQNO is an n-side quinone (Q_n) ligand of heme c_n,[1] and it has been shown to inhibit oxidation of heme b_n.[32−34] Binding of NQNO to the b_6f complex in thylakoid membranes increases the amplitude of the flash-induced heme b_n reduction by a factor of 2−3 without significantly inhibiting linear electron transport or oxygen evolution. In this study, concentrations of NQNO that affect the amplitude of heme b_n reduction only partly inhibit linear electron transport, and NQNO does not increase the rate of $O_2^{\bullet-}$ production as does addition of antimycin A to the bc_1 complex (Tables 2−4 compared to Table 1). The difference in binding of n-side quinone analogue inhibitors in the bc_1 and b_6f complexes resides in the multiple interactions with the amino acid environment that stabilize ligand binding at the Q_n site of the bc_1 complex, whereas in the b_6f complex where heme c_n occludes access to heme b_n, the Q_n site protrudes into the intermonomer cavity, with relatively few stabilizing interactions from coordinating amino acid residues. As a result, binding of a ligand such as NQNO at the Q_n site is expected to be substantially weaker in the cytochrome b_6f complex than in the bc_1 complex.

DBMIB. Halogenated quinone analogues and in particular the dibromo derivative DBMIB[47] have been described as potent inhibitors of the chloroplast cytochrome b_6f complex,[45] and a less efficient inhibitor of the bc_1 complex from mitochondria.[48] DBMIB has been shown to bind at a position distal to the iron−sulfur binding site and also at this site.[45] In this study, 2 μM DBMIB partially inhibits linear electron transport and $O_2^{\bullet-}$ production of bc_1 complex to approximately 20% of the uninhibited rate (Table 1). Efficient inhibition is observed for the cytochrome b_6f complex with rates of electron transport varying from undetectable to a few percent of the control (Tables 2−4), and no detectable $O_2^{\bullet-}$ production above the background signal.

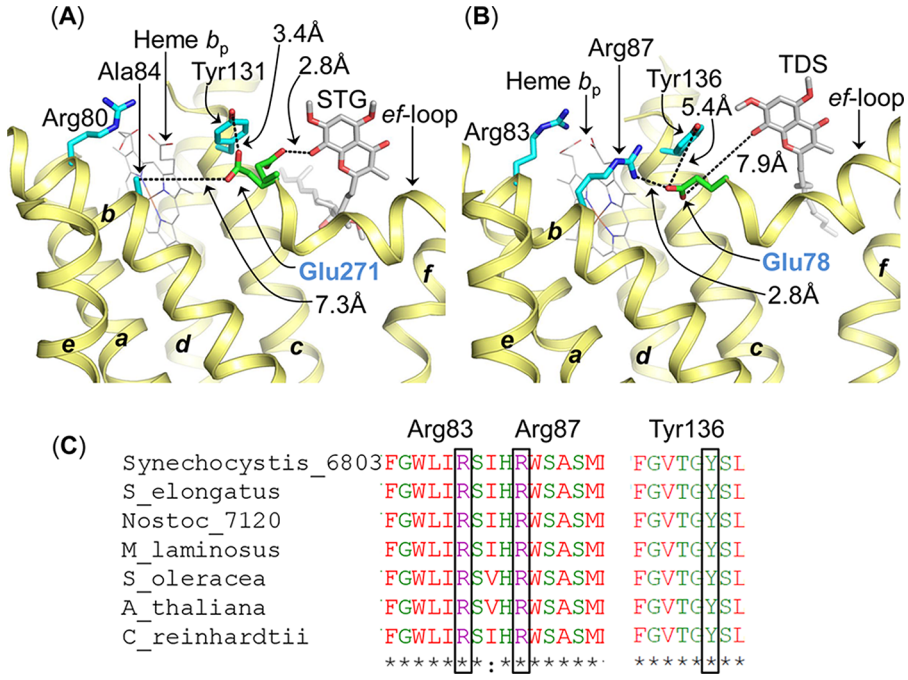

Figure 4. Role of conserved Glu (Pro-Glu-Trp-Tyr) in semiquinone deprotonation in cyochrome *bc* complexes. (A) In the bc_1 complex, the Glu271 residue (green and red sticks, labeled in blue) of the PEWY sequence is found to occupy two distinct locations: quinone-proximal (PDB entry 3CX5) and heme b_p-proximal (PDB entry 1NTZ). In the heme b_p-proximal position, Glu271 inteacts with Tyr131 (cytochrome *b* polypeptide). This structure was generated by superposition of cytochrome bc_1 crystal structures (PDB entries 1NTZ and 3CX5). The STG molecule and the quinone/STG-proximal Glu271 orientation were obtained from PDB entry 3CX5. (B) In the cytochrome b_6f complex crystal structure (PDB entry 4H44), Glu78 (green and red sticks, labeled in blue) of subunit IV, homologous to Glu271 in the bc_1 complex, is located in the heme b_p-proximal orientation. In this position, Glu78 interacts with Arg87 of cytochrome b_6 (b_6f), over a distance of 2.8 Å. Arg87 (b_6f) is replaced with Ala84 (cyan sticks) in the bc_1 complex. Arg83 of cytochrome b_6 (b_6f) and Arg80 of cytochrome *b* (bc_1) are conserved in their locations. The quinone analogue inhibitor TDS has been inserted into the figure from PDB entry 4H13 to mark the Q_p site. Transmembrane helices a−g are labeled. The polypeptides are shown as ribbons. (C) Multiple-sequence alignment of the cytochrome b_6 subunit of the cytochrome b_6f complex from prokaryotic cyanobacteria (*Synechocystis* PCC 6803, *S. elongatus* PCC 6301, *Nostoc* PCC 7120, and *M. laminosus*), a eukaryotic alga (*C. reinhardtii*), and higher plants (*A. thaliana* and *Sp. oleracea*). Arg83, Arg87 (transmembrane helix B), and Tyr136 (transmembrane helix C) are conserved in the cytochrome b_6 polypeptide.

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DISCUSSION

Origin of the Elevated Rate of Superoxide Production in the Cytochrome b_6f Complex. *More Favorable Redox Potential of Heme b_p in the b_6f Complex.* In the literature, there are large variations in midpoint redox potentials for heme b_p in the isolated complex: (i) −90 mV at pH 7,[49] (ii) −172 mV at pH 6.5,[50] and (iii) −80 mV at pH 6[51] and −158 mV at pH 8.0.[52] For the bc_1 complex, reported midpoint potentials of heme b_p are approximately −30 mV in yeast and mammals,[53,54] −90 to 50 mV in purple bacteria,[55,56] and 0 mV.[57] It is possible, but not determined by existing data, that a difference in redox potentials, e.g., a more negative potential of heme b_p in the b_6f complex, could contribute to the difference in the efficiency of superoxide formation. However, the spread of data in the literature on the heme b_p redox potentials is presently too great to allow this explanation.

Longer Residence Time. The higher rate of superoxide production in the b_6f complex than in the bc_1 complex could arise from a longer residence time of the semiquinone in its binding niche ("Q_p pocket") proximal to the iron−sulfur cluster of the Rieske iron−sulfur protein. The 10−20-fold higher rate of $O_2^{\bullet-}$ production by the cytochrome b_6f complex compared to that of the bc_1 complex (Tables 2−4 vs Table 1) implies that the rate constant in the b_6f complex for reactions iib, iii, and v is increased relative to that for reaction iv. The source of this difference can be the fact that the $PQ_p^{\bullet-}$ generated in the b_6f complex having a lifetime in the Q_p pocket longer than that of $UQ_p^{\bullet-}$ in the bc_1 complex. An identifiable structure-based cause of this longer residence time could be the partial occlusion of the Q_p portal for quinone entry and exit by the phytyl chain of the unique chlorophyll *a* molecule embedded in each monomer of the complex.[31] This occlusion does not affect the overall rate of electron transfer through the b_6f complex, as the isolated b_6f complex supports an electron transfer rate of ∼200 electrons monomer^{-1} s^{-1} at room temperature,[58−60] which is comparable to the activity of the bc_1 complex measured in ref 61 and larger that that measured in these experiments, compared to the activity of isolated bc_1 complex (Tables 2 and 3 vs Table 1). Therefore, the passage of quinol through the chlorophyll-obstructed portal is not the rate-limiting step in the photosynthetic linear electron transport chain.

Role of the Glutamate in the Conserved PEWY Sequence in Semiquinone Formation. The neutral semiquinone species bound within the Q_p site of cytochrome *bc* complexes undergoes deprotonation to form an anionic semiquinone (reaction iii), which can be a proton donor to the low-potential chain. It has been suggested that the Glu residue (Glu78 in subunit IV of the cytochrome b_6f complex and Glu271 or Glu272 in the cytochrome *b* polypeptide in the bc_1 complex) of the conserved Pro-Glu-Trp-Tyr (PEWY) motif located on the p-side *ef* loop is involved in deprotonation of the neutral semiquinone to the anionic semiquinone[62] (Figure 4A,B), which acts as the electron donor to heme b_p (reaction iib). The motion of the Glu271 residue in the cytochrome bc_1 complex during proton transfer has been demonstrated.[19,63−66] The Glu residue undergoes a rotation from a proton-extracting Q_p niche-proximal position to a proton-releasing heme b_p-proximal position, where it interacts with the Tyr131 residue of cytochrome *b* (Figure 4A). Hence, the motion of the Glu residue constitutes an important step in the transfer of semiquinone from the Q_p site. The kinetics of these reactions have recently been described.[67]

In the cytochrome b_6f complex, electron density for the Glu78 residue side chain, homologous to the Glu271 residue in the bc_1 complex, was assigned recently in the 2.7 Å resolution crystal structure (PDB entry 4H44).[20] The Glu78 residue side chain is found in a heme b_p-proximal position, in which it interacts with a conserved Arg87 of the cytochrome b_6 polypeptide over a short distance of 2.8 Å (Figure 4B). In this position, the Glu78 side chain does not interact with the quinone analogue inhibitor TDS (inserted into the Q_p site from PDB entry 4H13). The side chain of the conserved residue Tyr136 [cytochrome b_6 in the b_6f complex, transmembrane helix C (Figure 4B,C)], which replaces Tyr131 of cytochrome *b* [bc_1 complex (Figure 4A)], does not interact with Glu78 in the heme b_p-proximal position. It has previously been reported that the conserved Arg87 of transmembrane helix B of the cytochrome b_6 polypeptide [b_6f complex (Figure 4C)] is replaced by a small uncharged residue, such as Ala84 (or Ala83), in cytochrome *b* (bc_1 complex, transmembrane helix B).[68] An analysis of the amino acid environment of Glu271 of cytochrome bc_1 shows that the Glu271 side chain is separated from the Ala84 residue by a distance of 7.3 Å (Figure 4A). Hence, unlike the Glu78 residue in the b_6f complex that is inferred to participate in a relatively strong interaction with Arg87 in the heme b_p-proximal position, Glu271 in the bc_1 complex does not interact with a basic residue from transmembrane helix B in the heme b_p-proximal position. Thus, Glu271 may be relatively free to undergo motion in the cytochrome bc_1 complex, from a Q_p niche-proximal position to a heme b_p-proximal position, making proton extraction and translocation from the semiquinone a relatively efficient process. On the other hand, in the b_6f complex, the motion of Glu78 is expected to be comparatively restricted because of the interaction with Arg87, thereby making proton translocation a less efficient process. As a consequence, the lifetime of the neutral semiquinone is expected to be larger within the Q_p site of the cytochrome b_6f complex.

Further Consequences of the Increased Semiquinone Retention Time. Photosynthetic state transitions depend on the redox poise of the thylakoid membrane quinone pool.[69−71] The presence of reduced plastoquinone in the Q_p site activates an LHCII kinase bound to the b_6f complex.[72,73] With regard to the application of this signaling process to cyanobacteria, although the latter possess state transitions that involve the mobility of the phycobilisomes, this does not involve kinase activation but is dependent on redox equilibria involving plastoquinone.[11]

Chloroplasts as a Major Source of ROS in Plant Cells. High irradiance and other stress conditions that affect the photosynthetic electron transport rate and create hyperoxic conditions result in a transient increase in the level of ROS in chloroplasts.[9,74] The major sources of ROS generated in chloroplasts are PSII (singlet oxygen) and PSI ($O_2^{\bullet-}$). The production of $O_2^{\bullet-}$ by the cytochrome b_6f complex, as described in this study, has been detected by EPR spectroscopy.[12] Accumulated $O_2^{\bullet-}$ can also be metabolized to another more stable form of ROS, hydrogen peroxide.[11,74]

The production of ROS in chloroplasts underlies oxidative damage inflicted during photosynthetic reactions,[75] and the redox state of photosynthetic electron transport components conveys information about environmental light conditions. Thus, chloroplasts can function as mediators of environmental signals.[76] Light- and stress-induced generation of ROS contributes to redox signaling inside the chloroplast and from

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the organelle to the cytosol and nucleus.[10,77,78] ROS function in chloroplast signaling was demonstrated in relation to the regulation of dissipation and avoidance of excess excitation energy mechanisms.[11] The ROS generated in chloroplasts act as a retrograde signal to the nucleus for the regulation of plant responses to environmental stress and pathogen defense responses.[79,80] Increases in H_2O_2 concentrations have been shown to be important for the induction of the ascorbate peroxidase gene, APX2, and for the expression of a number of genes involved in plant development and stress responses.[81] There is evidence that in plant disease resistance responses, ROS produced in chloroplasts interact with the signal from other intracellular and extracellular ROS sources in the modulation of the pathogen-induced hypersensitive response.[82,83]

AUTHOR INFORMATION

Corresponding Author

*Department of Biological Sciences, Hockmeyer Hall of Structural Biology, Purdue University, West Lafayette, IN 47907. E-mail: waclab@purdue.edu. Telephone: (765) 494-4956.

Present Addresses

[†]D.B.: Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry, Babtai, Kaunas reg. Lithuania, LT 54333, Lithuania.

[‡]J.T.S.: Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA 92037.

Author Contributions

D.B. designed superoxide experiments, purified the cytochrome b_6f complex, performed enzymatic assays, analyzed results, and contributed to the manuscript. S.S.H. applied results to critical aspects of the crystal structure and wrote the manuscript. J.T.S. purified the cytochrome b_6f complex, performed enzyme assays, and contributed to the analysis of the results. W.A.C. conceived the original idea, designed the experiments, analyzed the results, and wrote the manuscript.

Author Contributions

[§]D.B. and S.S.H. contributed equally to this work.

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Notes

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ABBREVIATIONS

ADPH, 10-acetyl-3,7-dihydroxyphenoxazine; b_p and b_n, b hemes on electrochemically positive and negative sides of the b_6f complex, respectively; Chl a, chlorophyll a; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DDM and UDM, n-dodecyl and undecyl β-D-maltopyranoside, respectively; $\Delta\tilde{\mu}_{H^+}$, transmembrane proton electrochemical potential gradient; E_{m7}, midpoint oxidation−reduction potential at pH 7; ISP, iron−sulfur protein; HRP, horseradish peroxidase; MOPS, 3-(N-morpholino)propanesulfonic acid; NQNO, 2-n-nonyl-4-hydroxyquinoline N-oxide; p-side and n-side, electrochemically positive and negative sides of the membrane and cytochrome bc complexes, respectively; PC, plastocyanin; PSI and PSII, photosystems I and II, respectively; Q, quinone; Q_p, p-side quinone binding site; PDB, Protein Data Bank; PQ and PQH_2, plastoquinone and plastoquinol, respectively; $PQ_p^{\bullet-}$, plastosemiquinone; ROS, reactive oxygen species; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; STG, stigmatellin; TDS, tridecylstigmatellin; UQ and UQH_2, ubiquinone and ubiquinol, respectively; $UQ^{\bullet-}$, ubisemiquinone.

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