Cytochrome b-559 and proton conductance in
oxygenic photosynthesis
(photosystem II/plastoquinol oxidation/uncouplers)

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Contributed by Daniel I. Arnon, September 23, 1988

ABSTRACT Although cytochrome b-559 has long been known as a membrane-bound redox component closely linked to the reaction center of the oxygen-generating photosystem (PSII), its role in photosynthesis has remained obscure. This paper reports evidence and outlines a hypothesis in support of a "b-559 cycle"—i.e., a light-induced, cytochrome b-559-dependent, cyclic electron transport pathway around PSII that promotes translocation of protons from plastoquinol into the aqueous domain (lumen) of photosynthetic membranes (thylakoids). Light-induced proton transport coupled to light-induced electron transport is an essential aspect of energy transduction in photosynthesis because it generates an electrochemical proton gradient that drives ATP synthesis by the process of photosynthetic phosphorylation. The principal carrier of electrons and protons in thylakoids is the plastoquinone/plastoquinol couple. We propose that the b-559 cycle functions as a redox-linked proton pump that may operate jointly with the Rieske iron-sulfur pathway in oxidizing plastoquinol. The overall effect of such concerted oxidation of plastoquinol would be the translocation into the thylakoid lumen of two protons for each electron transferred from water to plastocyanin via plastoquinone.

Light-induced proton transport, coupled to light-induced electron transport, gained prominence in photosynthesis research with the recognition that it produces in photosynthetic membranes (thylakoids) an electrochemical proton gradient (ΔμH+) that drives ATP synthesis (1) in the process of photosynthetic phosphorylation (2). This process consists of cyclic (anoxygenic) photophosphorylation in which ATP is the sole product and noncyclic (oxygenic) photosynthetic phosphorylation in which ATP formation is accompanied by oxygen evolution and the generation of reducing power whose carrier is ferredoxin (2, 3). Oxygen apart, ATP and reduced ferredoxin are the two products of transduction of sunlight's electromagnetic energy into forms of chemical energy that directly or through intermediates, drive the biosynthetic and regulatory reactions of photosynthesis including, but not limited to, CO2 assimilation (4).

In noncyclic, oxygenic photosynthesis, ΔμH+ is generated by protons released from two sources: the photolysis of water (see Discussion) and the oxidation of plastoquinol (PQH2). As for PQH2, the accepted view is that thylakoids can oxidize it only via the Rieske iron-sulfur center pathway that is sensitive to inhibition by dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, DBMIB) (5, 6). However, evidence was recently obtained (7) that PQH2 is also photooxidized by an alternative, cyclic pathway not inhibited by DBMIB. The cyclic pathway was uncovered through the use of uncouplers, specifically proton conductors (photophores). Two chemically diverse photophores, 2,6-di-(t-butyl)-4-(2',2'-dicyano-phenyl)phenol (SF 6847) (8, 9) and carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (10, 11), induced oxidation of PQH2 and dramatically lowered chloroplast fluorescence (7), signifying oxidation of a specialized plastoquinone, QA, when it is reduced to an unprotonated semiquinone anion (Qα) (12).

Uncouplers also induced the oxidation of cytochrome b-559, in either the presence or the absence of DBMIB. Thus, oxidoreductions of cytochrome b-559 appeared to be part of the cyclic pathway for the oxidation of PQH2. Cytochrome b-559 is a thylakoid redox component closely linked to the reaction center of the oxygen-generating photosystem (photosystem II, PSII)—a linkage discovered in 1969 through photooxidation of cytochrome b-559 by PSII at cryogenic temperatures (13) and decisively validated by isolation of a PSII reaction-center complex consisting of the D-1 and D-2 polypeptides and cytochrome b-559 (14).

The preceding paper (7) dealt with evidence pertaining to the oxidation of plastoquinone (PQ) components by the cyclic pathway. We now present evidence pertaining to cytochrome b-559 and propose that the cyclic pathway, which will provisionally call the b-559 cycle, functions as a redox-linked proton pump that operates in concert with the Rieske iron-sulfur pathway in oxidizing PQH2 (see Discussion). The overall effect of such concerted oxidation of PQH2 would be the translocation into the thylakoid lumen of two protons for each electron transferred from PSII via PQ to plastocyanin—i.e., an effect analogous to that resulting from the oxidation of quinol in mitochondria by a Q (15) or b (16) cycle.

METHODS

Chloroplasts were isolated from spinach leaves (Spinacia oleracea cv. Marathon) grown in a greenhouse in nutrient solution culture (17) and freshly harvested before each experiment. Published procedures were used for chlorophyll determination (17) and thylakoid preparation (18). The thylakoids consisted of osmotically disrupted chloroplasts that retained the capacity for complete noncyclic electron transport from water to ferredoxin/NADP+ and photosynthetic phosphorylation. Ferredoxin was isolated and purified by R. K. Chain, and photoreduction of NADP+ was measured as described (19). DBMIB was kindly supplied by A. Trebst (Ruhr Universitat, Bochum, F. R. G.), nigericin by Hoffmann-LaRoche, and SF 6847 by Sumitomo Chemical (Osaka, Japan). FCCP and gramicidin were purchased from Sigma.

A dual-wavelength spectrophotometer (Aminco DW-2) was used in the split-beam mode to measure cytochrome

Abbreviations: PS, photosystem; PQ, plastoquinone; PQH2, plastoquinol; QA and QB, specialized membrane-bound forms of PQ; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinione (dibromothymoquinone); SF 6847, 2,6-di-(t-butyl)-4-(2',2'-dicyano-phenyl)phenol; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone.
absorbance changes induced by ferricyanide or uncouplers in the dark and in the dual-wavelength mode to measure light-induced absorbance changes. Samples were illuminated with a red light beam of 663 nm (1.3 × 10⁻³ cm⁻² s⁻¹) isolated from a 150-W tungsten/halogen lamp with a type FCS interference filter (Baird Atomic) while two blue filters (Corning 4-96) were used to shield the phototube.

RESULTS

Oxidation of Cytochrome b-559 Induced by Uncouplers in the Dark. Various redox titrations have demonstrated that there is only one spectral form of cytochrome b-559 in thylakoids but that it exists in different redox states (20, 21). Freshly isolated thylakoids contain a high-potential form, cytochrome b-559HP [λₘₐₓ, 559 nm; midpoint potential at pH 7 (Eₘpₜ) = +330 to +400 mV], and a low-potential form, cytochrome b-559LP (Eₘpₜ ≈ +50 mV) (reviewed in ref. 20). The midpoint potential of cytochrome b-559HP is readily altered; a variety of mild treatments, including aging (22), can lower it considerably without rendering it wholly inactive (20).

Cytochrome b-559HP is reducible by hydroquinone (23). In freshly isolated chloroplasts it is mainly in the reduced state (20). In some investigations, the oxidation of cytochrome b-559 was induced by FCCP (24, 25). Different explanations were advanced to explain these effects, but until recently (25) facilitation of proton translocation was excluded because other uncouplers, such as gramicidin and nigericin, that also facilitate proton translocation were found ineffective (20).

To investigate further whether oxidation of cytochrome b-559 induced by FCCP was related to proton translocation, we compared the effects of FCCP with those of another, chemically different protonophore, SF 6847, which is known as the most potent protonophoric uncoupler of electron transport and ATP formation in mitochondria (8, 9).

Fig. 1 shows the effects of several uncouplers and of ferricyanide. In the dark, 5 μM FCCP, like ferricyanide, induced oxidation of cytochrome b-559 (Fig. 1 Left). Moreover, at 0.1 μM, a concentration at which ferricyanide was no longer effective as a chemical oxidant, FCCP still induced distinct oxidation of cytochrome b-559 (Fig. 1 Right).

SF 6847 induced oxidation of cytochrome b-559 in the dark in a manner similar to that of FCCP. Since protonophores do not readily undergo oxidation–reduction (10) and were effective in our experiments even at very low concentrations at which ferricyanide was no longer an effective oxidant, they must have acted not as electron acceptors but as promoters of the oxidation of cytochrome b-559 by virtue of their special capacity for reversible binding of protons. Gramicidin and nigericin, which do not bind protons but facilitate their translocation by other mechanisms, were ineffective in the dark but were effective in the light (see below).

The identity of the electron acceptor in the oxidation of cytochrome b-559 induced by photonophores is unknown; it might have been the manganese complex of thylakoids. In any case it must have preexisted because the oxidation occurred in the dark. What we deem noteworthy is that even in the dark the oxidation of cytochrome b-559 was induced when proton transport was facilitated by protonophores.

Light-Induced Oxidation of Cytochrome b-559 by Uncouplers. Closer to physiologic conditions were measurements of the redox state of cytochrome b-559 in the light. Tsujimoto and Arnon (26) reported that, under steady-state illumination and in the absence of a terminal electron acceptor, cytochrome b-559 became reduced by PO₂ and that the reduction was not inhibited by DBMIB and the dinitrophenyl ether of iodonitrotymol, inhibitors that block the oxidation of PO₂ by the Rieske iron-sulfur center pathway (5, 6, 27).

Fig. 2 shows that SF 6847 and FCCP promoted the oxidation of cytochrome b-559 in the light. In the absence of protonophores, electrons accumulated in cytochrome b-559, which, in the steady state, became reduced. However, no electrons accumulated in the absence of 1 μM FCCP or 1 μM SF 6847 (Fig. 2 Inset)—i.e., the turnover of cytochrome b-559 was such that the rate of cytochrome b-559 oxidation exceeded its rate of reduction (see ref. 7). Partial accumulation of electrons (i.e., partial steady-state reductions of cytochrome b-559) were observed at concentrations of protonophores between 0 and 1 μM (Fig. 2). Similar results were obtained in the presence of DBMIB (Fig. 3).

Fig. 1. Oxidation of cytochrome b-559 in the dark as induced by ferricyanide, SF 6847, and FCCP. Oxidation was measured in the split beam mode as a difference spectrum: oxidized by ferricyanide (or uncoupler) minus reduced by hydroquinone. The difference spectra included the contribution of cytochrome f, which was not subtracted. Sample cuvettes contained 5 μM (Left) or 0.1 μM (Right) potassium ferricyanide or uncoupler; reference cuvettes contained 400 μM hydroquinone. Common components included spinach thylakoids (equivalent to 100 μg of chlorophyll per ml), 5 mM MgCl₂, and 50 mM Tricine buffer (pH 7.5) for the ferricyanide. SF 6847, and FCCP and pH 8.0 for the nigericin and gramicidin treatments, which also included 50 mM KCl.

Fig. 2. Effect of uncouplers on light-induced redox state of cytochrome b-559. Absorbance changes were measured at 559 nm; reference wave length, 570 nm. Cytochrome b-559 was preoxidized in the dark by adding 10 mM potassium ferricyanide to the reaction mixture, which included thylakoids (equivalent to 100 μg of chlorophyll per ml), 5 mM MgCl₂, and 50 mM Tricine buffer (pH 7.5) for the control, SF 6847, and FCCP and pH 8.0 for the gramicidin and nigericin treatments, which also included 50 mM KCl. (Inset) Tracings of cytochrome b-559 fully photoreduced in the control (no uncoupler added) and fully oxidized in the presence of 1 μM FCCP. Note partial oxidation as a function of uncoupler concentration.
In the light, unlike in the dark (Fig. 1), the redox state of cytochrome $b$-$559$ was also influenced by gramicidin and nigericin, which are not protonophores. Protonophores are weak acids whose lipid-soluble anions reversibly bind protons and facilitate their transport across energy-transducing membranes (8, 11). Gramicidin is a channel-forming ionophore and gramicidin catalyzes an electroneutral exchange of $K^+$ for $H^+$ (11). In the light, gramicidin and nigericin, at somewhat higher concentrations, also promoted the oxidation of cytochrome $b$-$559$ in the absence (Fig. 2) and presence (Fig. 3) of DBMIB.

In sum, under illumination and in the presence of each of the four uncouplers, cytochrome $b$-$559$ became oxidized. The oxidant was most likely $P680^+$, the photooxidized reaction center of PSII (see Discussion). The only feature common to the four chemically diverse uncouplers is that they facilitate proton transport. Thus, these findings are consistent with the view that the reduced form of cytochrome $b$-$559$ was protonated and that uncouplers, by inducing proton dissociation and translocation, favored oxidation.

**Effect of Uncouplers on Redox State of Cytochrome $b$-$559$ in the Presence of Ferredoxin-NADP⁺.** The preceding uncoupler-induced photooxidations of cytochrome $b$-$559$, like those of $Q_A$ and $PQH_2$ (7), occurred in the absence of a terminal electron acceptor. As a consequence there was no photooxidation of water but only a light-induced circulation of intrasytem electrons combined with an uptake of protons needed for the reduction of $PQ$ to $PQH_2$ (7).

For this newly found DBMIB-insensitive, cyclic pathway around PSII to be physiologically significant, it must coexist with the noncyclic (linear) light-induced electron flow from water to ferredoxin-NADP⁺, via $PQH_2$ and the Rieske iron-sulfur center pathway that is sensitive to inhibition by DBMIB. Evidence for the coexistence of the two pathways was obtained by comparing the light-induced redox state of cytochrome $b$-$559$ and the rate of oxygenic NADP⁺ reduction in the presence and absence of uncouplers.

In the absence of uncouplers, cytochrome $b$-$559$ became, in the steady state, predominantly reduced (Fig. 4 Inset). In the presence of uncouplers at about 1 $\mu$M, cytochrome $b$-$559$ was predominantly in the oxidized state. Decreased amplitudes of cytochrome $b$-$559$ reduction were observed at uncoupler concentrations between 0 and 1 $\mu$M (Fig. 4). In short, the effect of uncouplers on the redox state of cytochrome $b$-$559$ was the same in the presence (Fig. 4) as in the absence of a terminal electron acceptor (ferredoxin-NADP⁺) (Fig. 2).

Fig. 5 shows that the uncoupler-induced shift in the steady-state level of cytochrome $b$-$559$ from reduced to oxidized did not diminish the rate of NADP⁺ reduction. Since effects of uncouplers on photosynthetic electron flow depend on external pH, each uncoupler was tested at a predetermined external pH at which the uncoupler had little or no effect on the rate of electron transport in the controls (18). In sum, the oxygenic, noncyclic electron transport from water to ferredoxin-NADP⁺, which is sensitive to DBMIB, was concurrent and compatible with the DBMIB-resistant, light-induced oxidoreductions of the $b$-$559$ cycle.

**DISCUSSION**

Mitochondrial investigations have linked oxidoreductions of $b$ cytochromes with proton uptake and release (28-32). Among the divergent and in some cases mutually exclusive roles proposed for cytochrome $b$-$559$ (reviewed in refs. 20 and 21) are hypotheses from the laboratories of Butler (33, 34) and Losada (25, 35) depicting cytochrome $b$-$559$ as both an electron and a proton carrier in noncyclic electron transport. These hypotheses share certain premises. They envision that cytochrome $b$-$559$ binds protons and exists in a high-potential or low-potential state depending on whether it is protonated. As the low-potential unprotonated form is re-
duced by PQH$_2$, it binds a proton. The binding by the reduced form is stronger than the binding by the oxidized form ($pK_{\text{red}} > pK_{\text{ox}}$), and thermodynamic considerations (36) dictate that the reduced protonated form of cytochrome b-559 must have a higher midpoint potential than the unprotonated form. Both groups postulated that the reduced, high-potential protonated form of cytochrome b-559 in turn reduces cytochrome f and, upon oxidation, releases its proton into the thylakoid lumen. Cytochrome b-559 was thus viewed as a link between PSII and PSI. The Osada group (35) depicted all electrons originating from PSII as passing through cytochrome b-559, whereas the Butler group considered that only 25–50% of the electron transport originating from PSII needs to pass through cytochrome b-559 (34). Furthermore, Butler’s, but not Osada’s, group considered that the proton bound by cytochrome b-559 is the proton released by photooxidation of water.

We envision similar but not identical roles for low- and high-potential forms of cytochrome b-559 in electron and proton transport of the b-559 cycle, which, based on previous (7) and present evidence, provides a DBMIB-resistant pathway for the oxidation of PQH$_2$. We do not regard cytochrome b-559 as a link in electron transport between PSII and PSI but limit its activity to a cyclic electron flow around PSII. Further, we ascribe the oxidation of cytochrome b-559$_{\text{Hp}}$ to P680. As stated, this relation has long been known at cryogenic temperatures (13). It was not adequately exploited because it could not be detected at physiological temperatures without experimentally suppressing the photooxidation of water (13). However, as originally suggested (13), no photooxidation of cytochrome b-559 would be detected at physiological temperatures if it were immediately balanced by a reduction—a sequence of events that would, however, be expected from the operation of the proposed b-559 cycle.

The nature of proton release from water is germane to the assessment of proton conductance by the b-559 cycle. In chloroplasts, $\Delta \mu_{\text{H}+}$ is an electrochemical proton gradient between two aqueous phases, stroma and lumen, separated by a hydrophobic barrier that is poorly permeable to protons (1). Thus, protons released in the hydrophobic region would require the mediation of a lipophilic carrier to reach the lumen.

It is now accepted that PQ is the lipophilic carrier that shuttles protons from stroma into the lumen, but no role has been assigned to PQ in the conductance of protons liberated by the photooxidation of water (5). That reaction is often represented as occurring in the lumen with no need for proton carriers (Fig. 6A).

We consider such direct release improbable because the lumen’s aqueous phase would favor random recombination of negative and positive charges derived from the photooxidation of water and preclude their directional separation as required for photosynthetic energy transduction. In our view, photooxidation of water must take place in the hydrophobic regions of the membrane that permit directional uptake of released electrons and protons by appropriate acceptors embedded in the lipophilic membrane. Specifically, we envision a role for PQ as such an acceptor (18, 37).

A schematic overview of this concept is given in Fig. 6B. On accepting two electrons from the PSII reaction center, PQ takes up two protons, one from stroma and one from water, and is reduced to PQH$_2$. The oxidation of PQH$_2$ releases two protons into the lumen but transfers only one electron to plastocyanin.

A more detailed description of a scheme that would accomplish this is diagrammed in Fig. 7. The photoreduction of the PQ pool is represented in accord with known pathways (5). Two electrons are sequentially transferred from P680, the photoactive pigment in the PSII reaction center, to QA, the single-electron-carrying, bound PQ, and thence to QB, the bound secondary PQ, which accepts two electrons and becomes protonated to form PQH$_2$. As Q$_B$ is in exchange equilibrium with PQ in the pool, the reduction and protonation of Q$_B$ results in the reduction of the pool PQ to PQH$_2$ (5).

As stated, it is generally assumed that the two protons needed for the formation of PQH$_2$ come from the stroma (5). However, we assume that only one proton comes from the stroma and that the second proton is derived from the photooxidation of water (Fig. 7). Thus, we postulate that Q$_B$ and not cytochrome b-559 (33, 34) provides the binding site for protons released by the oxidation of water.

Turning to the oxidation of PQH$_2$, we propose, by analogy with the Q (15) and b (16) cycles, two sequential one-electron oxidations along two distinct pathways that operate with different electron carriers and with semiquinone (PQH$^-$) as an intermediate. In the first one-electron oxidation, PQH$_2$ is oxidized to PQH$^-$ by the Rieske iron-sulfur center. The electron in the iron-sulfur center is transferred to cytochrome f and plastocyanin while one proton is released into the lumen (Fig. 7). This "noncyclic" electron, which reaches plastocyanin and eventually ferredoxin, is ultimately replaced by an electron from water.

In the second one-electron oxidation (PQH$^-$ $\rightarrow$ PQ), the electron cycles by returning to P680 via cytochrome b-559 while a second proton is released into the lumen when cytochrome b-559$_{\text{Hp}}$ is oxidized (Fig. 7). In sum, the concerted oxidation of PQH$_2$ by the Rieske iron-sulfur pathway and the b-559 cycle would provide for translocation into the lumen of two protons for each electron transferred by noncyclic electron transport from water to plastocyanin.

Unlike the Q cycle (15), which has been implicated in noncyclic electron transport, largely from considerations of comparative biochemistry (27), the scheme in Fig. 7 includes no steps that are sensitive to antimycin inhibition, in agreement with the long-known resistance of noncyclic electron transport to antimycin inhibition (38).

Economy of hypothesis dictates the exclusion of cytochrome $b_6$ from our scheme. A previous paper (38) associated cytochrome $b_6$ with cyclic phosphorylation and attributed the antimycin sensitivity of that process to inhibition of cytochrome $b_6$ turnover. From this perspective, the inclusion in our scheme of components of the cytochrome $b_6f$ complex...
(Rieske iron-sulfur center and cytochrome f) need not imply the involvement of the entire complex (27); the existence of a subcomplex comprising these two components would suffice. Recent evidence supports the existence of such a subcomplex. A binary subcomplex consisting of the Rieske iron-sulfur protein and cytochrome f was recently isolated from spinach chloroplasts (39), and evidence was obtained that in the cyanobacterium *Nostoc* PCC 7906 the genes encoding the Rieske iron-sulfur and cytochrome f polypeptides are cotranscribed as one 2.0-kilobase message (40).

The operation of the b–559 cycle reinforces and clarifies previous findings that uncouplers significantly relieve the inhibition by DBMIB of noncyclic electron transport from water to NADP⁺ (18, 37). The present findings and those previously reported (7) suggest that uncouplers restore NADP⁺ reduction because they activate the DBMIB-resistant pathway for PQH₂ oxidation via the b–559 cycle. Other proposals for a cytochrome b–559 cycle around PSI have been made previously. These include "a side pathway connected to the reaction center of PSI" (41), a cyclic pathway around PSIII that saturates at low light intensities and "is therefore probably not physiologically significant" (42), and a cyclic pathway around PSI at high light intensities when "oxidation of cytochrome b–559 would induce cyclic electron flow preventing photooxidative reactions of the strong oxidant generated by PSI" (24).