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Inhibition of electron transfer and the electrogenic reaction in the cytochrome *b/f* complex by 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide (NQNO) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) *

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Key words: Cytochrome *b/f* complex; Electron transport inhibition; Slow electrogenic step; Binding site

We investigated the interaction between the cytochrome *b/f* complex and plastoquinone by determining the effect of the electron transport inhibitors 2-*n*-nonyl-4-hydroxy-quinoline *N*-oxide (NQNO) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) on the flash-induced turnover of cytochromes b_6 and *f*, on the slow phase of the electrogenic reaction (515_s) and on steady-state electron transport. The experiments were performed using thylakoid membranes in the presence of duroquinol, methyl viologen, and diuron, conditions in which electron transport is driven by Photosystem I and includes the cytochrome *b/f* complex. The data from these experiments indicate that NQNO and DBMIB inhibit at two different sites on the cytochrome *b/f* complex. The data can be accommodated by a modified Q-cycle in which the primary site of inhibition by NQNO is the quinone reductase site (Q_c) and the primary site of DBMIB inhibition is the quinol oxidase site (Q_z). NQNO is envisioned to inhibit the oxidation of the cytochrome b_6-heme located nearest the outer aqueous phase, as well as to slow electron transfer between the two *b*-hemes. The effect of NQNO on the 515_s supports the notion that the slow electrogenic reaction is due to electron transfer between the two cytochrome b_6-hemes, followed by a reaction associated with plastoquinone reduction at the Q_c-site (Jones and Whitmarsh (1985) Photobiochem. Photobiophys. 9, 119–127). The results indicate that the Q_z-site and Q_c-site are likely separated by 70% of the dielectrically weighted distance across the

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Abbreviations: C_{50}, the concentration of inhibitor required to give 50% of the maximum inhibitory effect; cytochrome *b/f* complex, plastoquinol-plastocyanin oxidoreductase complex; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DQH_2, duroquinol; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; I_{50}, the concentration of inhibitor required for 50% inhibition of the control signal; NQNO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; P-700$^+$, the oxidized primary electron donor of Photosystem I; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole; Q_c, putative quinone binding site on the cytochrome *b/f* complex located near the outer aqueous phase (stroma); Q_z, putative quinol binding site on the cytochrome *b/f* complex located near the inner aqueous phase (lumen); 515_s, the slow phase of the electrochromic shift (rise time 3–5 ms); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine.

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membrane. At low concentrations of DBMIB the kinetics of the 515_s and cytochrome b_6 reduction are monophasic, indicating that DBMIB moves from one inhibitory site to another within the turnover time of the cytochrome *b/f* complex. This observation suggests that the debinding rate of DBMIB from the Q_z-site is greater than 200 s^{-1}. Lastly, comparing the effect of DBMIB on the extent of cytochrome *f* oxidation and the rate of cytochrome *f* reduction, raises the possibility that DBMIB has a second lower affinity binding site on the cytochrome *b/f* complex, possibly interrupting electron flow between the FeS center and cytochrome *f*.

Introduction

The central element in electron transport between Photosystem II and Photosystem I is the cytochrome *b/f* complex that oxidizes plastoquinol and reduces plastocyanin. The oxidation of quinol involves the removal of two electrons and the release of two protons by the one-electron acceptors of the cytochrome *b/f* complex: the FeS center, cytochrome *f*, and two *b*-cytochromes. (reviewed in Refs. 1–4). Although it can be shown that the electrons from quinol reduce P-700$^+$ and that the protons are deposited into the inner aqueous space, the routes taken by the electrons and protons remain unresolved. Two significant experimental observations associated with quinol oxidation are the occurrence of an electrogenic reaction (e.g., Refs. 5–7) and the transfer of a proton from the outer to the inner aqueous phase by the cytochrome *b/f* complex [8,9]. One mechanism that accounts for these observations is a Q-cycle [10], which, in modified form, has provided the framework for numerous models of quinol oxidation. The Q-cycle is based on a physical model of the cytochrome *b/f* complex that includes two distinct quinone binding sites, a quinol oxidase site (Q_z), and a quinone reductase site (Q_c). The existence of the quinol oxidase site is well established by experimental evidence, whereas that of the Q_c-site remains uncertain and is contentious (e.g., Refs. 1–4 and 11–13). Support for the Q_c-site is based primarily on consistency of the experimental data with electron transport models. For example, the effect of NQNO on the slow electrogenic reaction and cytochrome b_6 reduction is explained by NQNO blocking the oxidation of cytochrome b_6 by binding to the Q_c-site and has led to the proposal that the slow electrogenic step is due to two sequential reactions: electron transfer between the two *b*-hemes and a reaction associated with quinol reduction at the Q_c-site [6]. In contrast to mechanisms of the Q-cycle type, are models of quinol oxidation that exclude a second quinone binding site (discussed in Ref. 2) or suggest that proton transfer associated with redox reactions of the FeS center gives rise to the slow electrochromic shift [14,15].

In the work presented here, we compare the inhibition of electron transfer and the 515_s by two inhibitors, NQNO and DBMIB, in order to define more clearly the interaction between quinone and the cytochrome *b/f* complex and to characterize the reactions giving rise to the slow electrochromic shift. The experiments were performed using thylakoid membranes in the presence of duroquinol at low flash frequencies (0.1–0.2 Hz). Under these conditions the plastoquinone pool is mainly reduced and cytochrome b_6 is oxidized. The results show that NQNO and DBMIB inhibit electron flow in the cytochrome *b/f* complex at two different sites and are consistent with a modified Q-cycle mechanism of electron transport. In addition, the results support the proposal that the slow electrogenic reaction is due to two sequential steps involving electron transfer between the two *b*-cytochromes and the subsequent oxidation of cytochrome b_6 by plastoquinone [6]. The effect of DBMIB on the flash-induced redox reactions of the 515_s and cytochrome b_6 indicate that DBMIB debinds from the cytochrome *b/f* complex at a rate greater than 200 s^{-1} and raises the possibility that the DBMIB-induced increase in the extent of cytochrome *f* oxidation is due to a second DBMIB binding site on the complex.

Preliminary accounts of this work have been presented previously [16,17].

Materials and Methods

All experiments were performed using thylakoid membranes isolated from market spinach (*Spinacia oleracea*) as described elsewhere [18]. The chlorophyll concentration was determined in acetone/water (4:1, v/v) using the extinction coefficients for chlorophyll *a* and chlorophyll *b* at 664 nm and 647 nm as determined by Ziegler and Egle [19]. The thylakoid membranes were stored on ice in a medium containing 0.2 M sorbitol, 5 mM Hepes-KOH (pH 7.5) and 2 mM $MgCl_2$.

Light-induced absorbance changes were measured using a laboratory-built single-beam spectrophotometer [18]. The half-bandwidth of the measuring beam was 3 nm and the pathlength was 1 cm. To improve the signal-to-noise ratio the flash-induced absorbance changes were signal-averaged at flash frequencies of 0.1–0.2 Hz. Each sample received less than 150 actinic flashes. Short actinic flashes were produced by a xenon flash lamp (FX-193 EG and G, Salem, MA), filtered by a red blocking filter (CS 2-58, Corning Glass Works, Corning, NY) and directed through one of three light guides positioned at right angles to the measuring beam. The half-peak width of the actinic flash was 6 μs, which produced less than 8% double hits as compared to a 0.5 μs duration laser flash. For all measurements shown here the actinic flash was saturating. The photomultiplier tube (EMI 9634 QR, Thorn-EMI, Plainview, NY) was protected from the actinic flash by a blocking filter (CS-4-96, Corning Glass Works, Corning, NY) and a broad-band interference filter (DT-Gruen, Balzers, Rolyn Optics, Arcadia, CA).

For spectrophotometric measurements thylakoids were suspended in 3 cm^3 reaction mixtures (described in the figure legends) that were thermostatted between 16 and 17 °C. All experiments were completed within 4 h of thylakoid isolation except cytochrome b_6 absorbance measurements which were completed within 2 h. Prior to recording flash-induced absorbance changes all samples were pretreated with approx. 10 flashes.

Cytochrome *f* was monitored by the flash-induced absorbance change at 554–540 nm and cytochrome b_6 was monitored by the flash-induced absorbance change at 563–572 nm. The wavelength dependence of the absorbance changes from 540 to 572 nm is shown elsewhere [20,21]. Cytochrome concentrations were determined using a millimolar difference extinction coefficient of 20 $mM^{-1} \cdot cm^{-1}$ for cytochrome *f* at the wavelength pair 554–540 nm, and 15 $mM^{-1} \cdot cm^{-1}$ for cytochrome b_6 at the wavelength pair 563–572 nm. The concentration of the cytochrome *b/f* complex was determined by measuring the total amount of cytochrome *f* present by its chemically-induced reduced minus oxidized spectrum (for further details see Ref. 18). We assumed 1 mol cytochrome *f* per complex.

The slow phase of the electrochromic shift was measured at 515 nm as described elsewhere [6]. Briefly, with DCMU present to prevent the turnover of Photosystem II and DQH_2 present as an electron donor, the flash-induced absorbance change at 515 nm consists of a fast component due to charge separation at Photosystem I and a slow component associated with quinol oxidation by the cytochrome *b/f* complex. Only the slow phase of the electrochromic shift is inhibited by DBMIB and, thus, can be resolved by subtracting the absorbance change at 515 nm measured in the presence of 1 μM DBMIB from the absorbance change measured in its absence. The rate of increase of the slow phase was determined from the initial slope of the absorbance increase.

Steady-state rates of light-driven uncoupled electron transport in thylakoids in the reaction from DQH_2 to methylviologen were measured by oxygen consumption associated with the aerobic oxidation of photoreduced methylviologen in the presence of superoxide dismutase using a Clark-type electrode. Continuous actinic light was provided by an electronically shuttered 250 W Tungsten-Halogen lamp filtered by a Corning CS 2-59 red filter and heat filters. The duration of the actinic light was 30 s and the intensity was saturating. Thylakoids were suspended in a 1.9-cm^3 reaction mixture that is described in the legend to figure 1. The temperature of the sample was between 16 and 17 °C.

The concentrations of NQNO (Research Plus, Bayonne, NJ) and DBMIB (synthesized by Dr. D.R. Ort) were determined gravimetrically. The compounds were suspended in ethanol and stored at −20 °C. Fresh solutions of twice-crystallized NQNO were prepared each week because we found

that older solutions accelerated the decay of the electrochromic shift measured at 515 nm. DQH_2 was prepared from duroquinone as described elsewhere [22]. Gramicidin D (Sigma, St. Louis, MO) and DCMU, when present, were added from ethanolic stock solutions.

Results

Inhibition by NQNO

Cytochrome b_6. In thylakoids in the presence of DQH_2, a short actinic flash causes the reduction of a small amount of cytochrome b_6, typically 0.2–0.3 mol cytochrome b_6/mol complex, with a half-time of 1–2 ms (e.g., Ref. 6). In the presence of NQNO, the amount of cytochrome b_6 reduced by the actinic flash increased significantly, while the initial rate of its reduction was unchanged (Fig. 1A). In the presence of 2 μM NQNO the half-time of cytochrome b_6 oxidation was approx. 60 ms (data not shown). The dependence of the extent and rate of cytochrome b_6 reduction on the concentration of NQNO is shown in Fig. 2. NQNO at a concentration of 2 μM caused the extent of cytochrome b_6 reduction to increase from 0.27 mol cytochrome b_6/mol complex in the control to 0.74 mol cytochrome b_6/mol complex. The concentration that resulted in 50% of the maximum

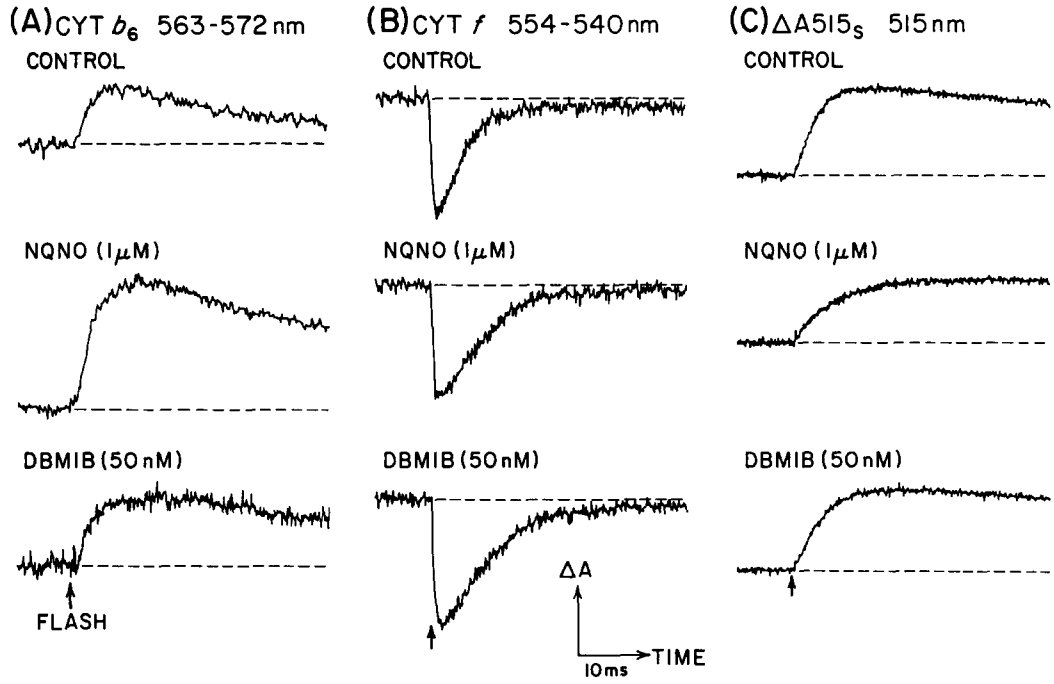

Fig. 1. (A) Kinetics of the flash-induced absorbance change at 563–572 nm in thylakoids due to cytochrome b_6 in the absence of inhibitor (control), in the presence of 1 μM NQNO, and in the presence of 50 nM DBMIB. The electron donor was DQH_2 and the terminal electron acceptor was O_2 mediated by methylviologen. Thylakoid membranes were suspended at a chlorophyll concentration of 40 μM in a reaction medium that contained 100 mM sorbitol, 30 mM Tricine/KOH (pH 8.0), 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM DQH_2, 0.1 mM methylviologen, 10 μM DCMU, 10 $\mu g/cm^3$ gramicidin and inhibitor as indicated. Further details are given in the text. (B) Kinetics of the flash-induced absorbance change at 554–540 nm in thylakoids due to cytochrome f in the absence of inhibitor (control), in the presence of NQNO and in the presence of DBMIB. Experimental conditions were exactly as described in 1A except the chlorophyll concentration was 30 μM. (C) Kinetics of the slow phase of the absorbance change at 515 nm due to the slow electrochromic shift in the absence of inhibitor (control), in the presence of NQNO and in the presence of DBMIB. The fast phase of the absorbance change at 515 nm, measured in the presence of 1 μM DBMIB, has been subtracted (for details see text). Experimental conditions were exactly as those described for A except the chlorophyll concentration was 30 μM and gramicidin was omitted.

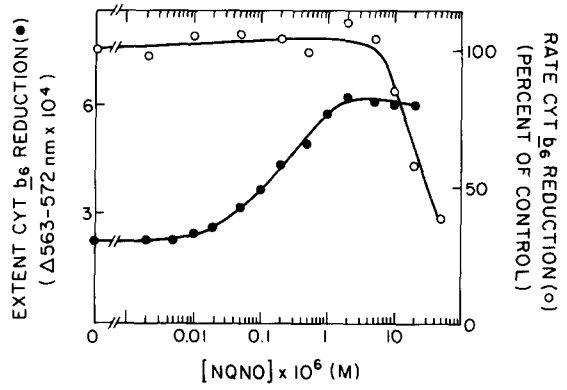

Fig. 2. Effect of NQNO on the extent and rate of the flash-induced reduction of cytochrome b_6 in thylakoid membranes. The reduction of cytochrome b_6 was monitored by the absorbance increase at 563–572 nm. The electron donor was DQH_2 and the terminal electron acceptor was O_2 mediated by methylviologen. The initial rate of cytochrome b_6 reduction observed in the presence of NQNO divided by that observed in its absence is plotted. The initial rate is defined as the maximum slope of the absorbance increase following the flash. The half-time of cytochrome b_6 reduction in the absence of NQNO was 1.2 ms. Thylakoid membranes were suspended at a chlorophyll concentration of 30 uM in a reaction medium that contained 100 mM sorbitol, 30 mM Tricine/KOH (pH 8.0), 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM DQH_2, 0.1 mM methylviologen, 10 μM DCMU and 10 $\mu g/cm^3$ gramicidin. Further details are given in the text.

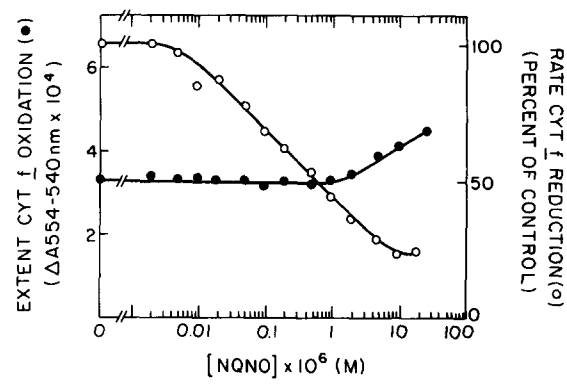

Fig. 3. Effect of NQNO on the extent of cytochrome *f* oxidation and the rate of its rereduction in thylakoid membranes. The redox state of cytochrome *f* was monitored by the absorbance change at 554–540 nm. The electron donor was DQH_2 and the terminal electron acceptor was O_2 mediated by methylviologen. The rate of cytochrome *f* rereduction observed in the presence of NQNO divided by that observed in its absence is plotted. The rate is the reciprocal of the half-time determined from a semi-log plot of the absorbance decrease. In determining the half-time the lag following the flash has been ignored. In the absence of NQNO, the half-time of the decay was 2.0 ms (see text for details). Experimental conditions were exactly as those described in the legend of Fig. 2.

NQNO-induced increase (C_{50}) was approx. 200 nM. The initial rate of cytochrome b_6 reduction was not slowed until the NQNO concentration exceeded 5 μM. Although the initial rate of reduction was the same in the absence and presence of NQNO at concentrations below 5 μM, the observed half-time of the reduction increased from 1–2 ms in the control to 2–3 ms in the presence of NQNO. The longer half-time observed in the presence of NQNO is a consequence of the NQNO-induced increase in the extent of cytochrome b_6 reduction.

Cytochrome f. In a short actinic flash cytochrome *f* is rapidly oxidized ($t_{1/2} = 200$–300 μs), equilibrates with the FeS center and then is subsequently reduced by an electron from quinol [20]. In the presence of DQH_2, the extent of cytochrome *f* oxidation corresponded to 0.30 mol cytochrome *f*/mol complex and the half-time of the rereduction was 2.0 ms (Fig. 1B). The half-time was determined from the semi-log plot of the reduction ignoring the initial lag following the

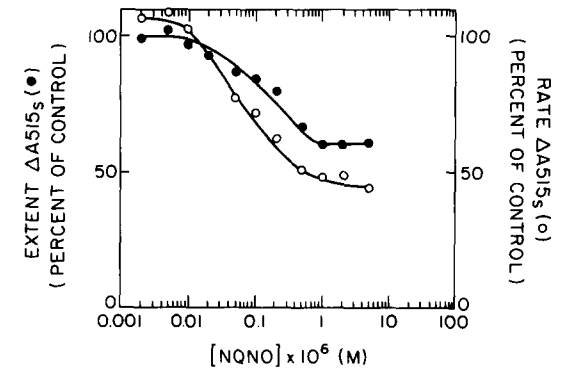

Fig. 4. Effect of NQNO on the extent and the initial rate of the increase of the slow electrochromic shift observed at 515 nm in thylakoids. The electron donor was DQH_2 and the terminal electron acceptor was O_2 mediated by methylviologen. In the absence of NQNO the absorbance increase of the slow component of the electrochromic shift was 0.0011. The rate of the increase of the slow electrochromic shift is defined as the maximum slope of the absorbance in crease at 515 nm immediately following the flash. See text for further details of the measurements. Experimental conditions were exactly as those described in the legend of Fig. 2 except that gramicidin was omitted from the reaction medium.

flash. In the presence of NQNO the rate of cytochrome *f* rereduction was slowed ($I_{50} \approx 600$ nM) and the extent of cytochrome *f* oxidation increased to a maximum value of 1.4-times the control (Fig. 1B, Fig. 3). Note that the rate of cytochrome *f* reduction remains substantial, 25% of the control, even at the highest NQNO concentration used (25 μM).

515$_s$. In the absence of NQNO the rise of the 515$_s$ was rapid ($t_{1/2} = 3$–5 ms) (Fig. 1C) and the extent was 0.85–1.0-times that of the fast phase (data not shown). NQNO slowed the rate of the rise of the 515$_s$ ($C_{50} \approx 70$ nM) and decreased the extent ($C_{50} \approx 130$ nM) (Figs. 1C and 4). The effect of NQNO appeared to saturate at approx. 1 μM, where the extent of the 515$_s$ was approx. 60% of the control and the initial rate was slowed to approx. 45% of the control. At concentrations of NQNO above 10 μM, the rate of decay of the 515$_s$ was accelerated making it difficult to analyze the slow phase.

Steady-state electron transport. The effect of NQNO on the rate of steady-state electron transport in the reaction from duroquinol to methylviologen is shown in Fig. 5. NQNO had no effect on the rate of electron transport over a concentration range from 8 nM to 1 μM. At concentrations above 1 μM, steady-state electron transport was partially inhibited. At the highest concentration used (40 μM) the inhibition was approx. 50%.

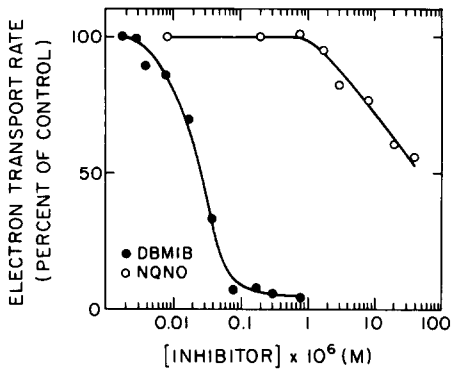

Fig. 5. Effect of NQNO and DBMIB on the rate of steady-state electron transport in thylakoid membranes. Electron transport rates were determined by oxygen consumption using a Clark-type electrode. The electron donor was DQH_2 and the terminal electron acceptor was O_2 mediated by methylviologen. In the absence of inhibitor, the average rate of electron transport was 1410 μmol e^-/mg chlorophyl per h. Thylakoid membranes were suspended at a chlorophyll concentration of 20 μM in a reaction medium that contained 100 mM sorbitol, 30 mM Tricine/KOH (pH 8.0), 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM DQH_2, 0.1 mM methylviologen, 10 μM DCMU, 10 μg/cm^3 gramicidin and 200 units/cm^3 superoxide dismutase. Further details are given in the text.

Inhibition by DBMIB

Cytochrome b_6. The effect of DBMIB on the extent and initial rate of reduction of cytochrome b_6 is shown in Figs. 1C and 6. In the presence of DBMIB, the extent of cytochrome b_6 reduction was decreased ($I_{50} \approx 80$ nM) and the initial rate of reduction was slowed ($I_{50} \approx 40$ nM). At a DBMIB concentration of 200 nM, there was no observable flash-induced turnover of cytochrome b_6.

In order to distinguish between the effects of NQNO and DBMIB on cytochrome b_6, we titrated the effect of DBMIB on the flash-induced turnover of cytochrome b_6 in the presence of 1 μM NQNO (Fig. 7). In these experiments the control was cytochrome b_6 turnover in the presence of 1 μM NQNO. In the presence of DBMIB the extent of cytochrome b_6 reduction was inhibited ($I_{50} \approx 50$ nM) and the initial rate of reduction was slowed ($I_{50} \approx 40$ nM). At a DBMIB concentration of 200 nM, there was no observable flash-induced turnover of cytochrome b_6.

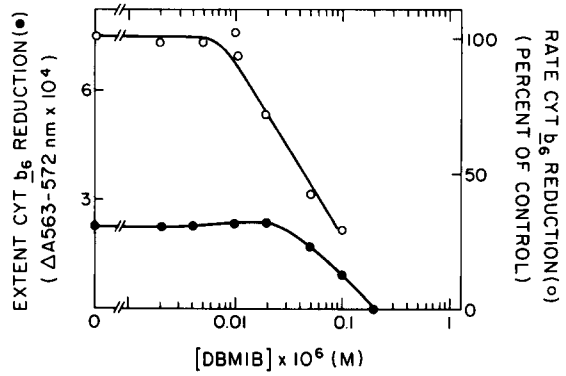

Fig. 6. Effect of DBMIB on the extent and rate of cytochrome b_6 reduction in thylakoid membranes. The reduction of cytochrome b_6 was monitored by the absorbance increase at 563–572 nm. The electron donor was DQH_2 and the terminal electron acceptor was O_2 mediated by methylviologen. Experimental conditions were exactly as those described in the legend of Fig. 2.

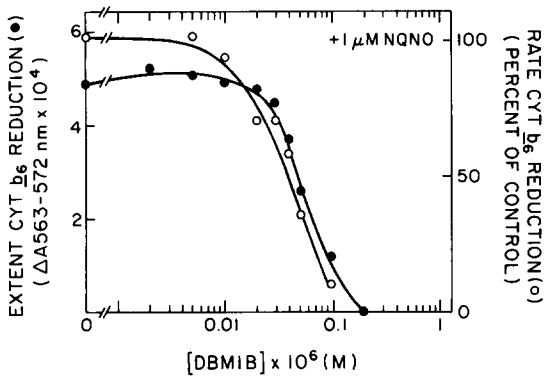

Fig. 7. Effect of DBMIB on the extent and rate of the flash-induced reduction of cytochrome b_6 in thylakoid membranes in the presence of 1 μM NQNO. The reduction of cytochrome b_6 was monitored by the absorbance increase at 563–572 nm. The electron donor was DQH_2 and the terminal electron acceptor was O_2 mediated by methylviologen. Experimental conditions were exactly as described in the legend of Fig. 2 except 1 μM NQNO was present in all samples.

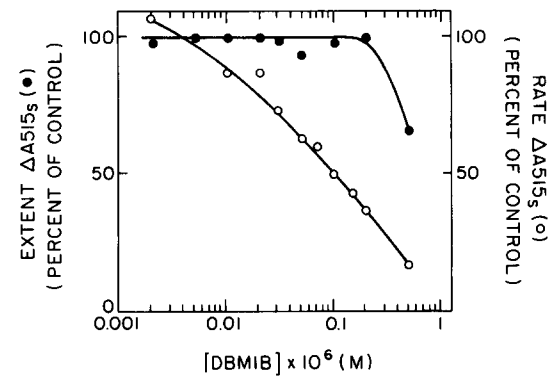

Fig. 9. Effect of DBMIB on the extent and the initial rate of the increase of the slow electrochromic shift observed at 515 nm in thylakoids. The electron donor was DQH_2 and the terminal electron acceptor was O_2 mediated by methylviologen. Measurements were made as described in the legend of Fig. 4. Experimental conditions were exactly as those described in the legend of Fig. 2 except that gramicidin was omitted from the reaction medium.

Cytochrome f. The effect of DBMIB on cytochrome *f* turnover is shown in Figs. 1C and 8. In the presence of DBMIB, the rate of cytochrome *f* rereduction was slowed ($I_{50} \approx 55$ nM). The inhibition was greater than 98% at concentrations above 500 nM. In the presence of DBMIB the extent of cytochrome *f* oxidation increased to a maximum of 1.9-times the control. The C_{50} for the increase was approx. 130 nM.

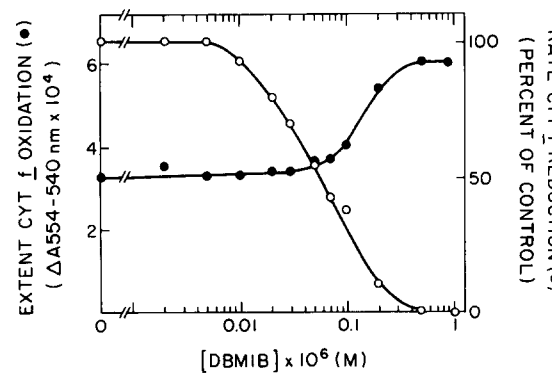

Fig. 8. Effect of DBMIB on the extent of cytochrome *f* oxidation and the rate of its rereduction in thylakoid membranes. The redox state of cytochrome *f* was monitored by the absorbance change at 554–540 nm. The electron donor was DQH_2 and the terminal electron accepter O_2 mediated by methylviologen. Rates of rereduction were measured as described in the legend of Fig. 3. Experimental conditions were exactly as those described in the legend of Fig. 2.

515$_s$. The effect of DBMIB on the slow electrogenic reaction is shown in Figs. 1C and 9. In the presence of DBMIB, the initial rate of the 515$_s$ was slowed, the $I_{50} \approx 100$ nM. The extent of the 515$_s$ was much less sensitive to DBMIB, only at concentrations of DBMIB above 200 nM did the extent begin to lower. At DBMIB concentrations of 1 μM, there was no observable absorbance change due to the 515$_s$.

Steady-state electron transport. The effect of DBMIB on the rate of steady-state electron transport is shown in Fig. 5. DBMIB inhibited the rate 96% at concentrations above 100 nM. The I_{50} for inhibition by DBMIB was approx. 25 nM.

Discussion

In modified versions of the Q-cycle, quinol oxidation is envisioned to occur in two steps, in which one electron goes to the Rieske FeS center and the other electron goes to the *b*-heme located near the inner membrane surface and then to the *b*-heme located near the outer membrane surface. The complete cycle requires a second quinol oxidation reaction to occur, resulting in another

electron passing through the FeS center and another electron going through the cytochrome *b*-hemes. The two electrons passing through the cytochrome *b*-hemes reduce plastoquinone to plastoquinol at the Q_c-site.

Evidence for separate binding sites for NQNO and DBMIB

The results shown here are most simply explained in terms of a modified Q-cycle in which NQNO and DBMIB inhibit electron flow through the cytochrome *b/f* complex at two distinct sites. The data support our earlier proposal that the primary site of NQNO inhibition is the Q_c-site, at which the inhibitor slows the oxidation of the cytochrome b_6 located nearest the outer aqueous phase [6,21]. The primary site of DBMIB inhibition is assumed to be the Q_z-site, at which the inhibitor blocks the oxidation of quinol, as has been proposed previously by Trebst [23]. In the model shown schematically in Fig. 10 we assume that the amount of cytochrome b_6 observed turning over in a short actinic flash is controlled by a competition between the rate of reduction through the Q_z-site and the rate of oxidation through the Q_c-site. Because the two *b*-cytochromes have nearly the same absorption spectra, electron transfer from the inner *b*-heme to the outer *b*-heme is not detectable in these measurements. According to the model, the increase in the extent of cytochrome b_6 reduction is due to NQNO slowing the rate of oxidation of the outer *b*-heme at the Q_c-site without altering the rate of reduction of the inner *b*-heme at the Q_z-site. This mode of inhibition is similar to that proposed for antimycin A in the mitochondrial and photosynthetic bacterial cytochrome b/c_1 complex [1], except that NQNO is not as effective an inhibitor as antimycin A. We have found (personal observation), as have others (e.g., Refs. 1 and 24), that antimycin A does not affect cytochrome b_6 turnover in thylakoids. DBMIB, in contrast to NQNO, slows the reduction of cytochrome b_6 and lowers the extent, providing support for the view that it inhibits quinol oxidation at the Q_z-site.

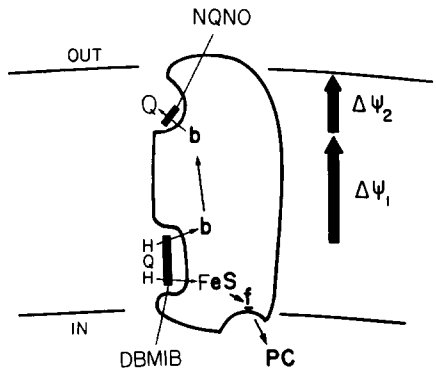

Fig. 10. Model of the cytochrome *b/f* complex showing the pathway of electrons, the electrogenic steps giving rise to the 515_s and the proposed sites of inhibition by NQNO and DBMIB.

Additional evidence that NQNO binds at the Q_c-site is provided by the effect of NQNO on the slow electrogenic reaction. As described earlier, the complicated pattern of inhibition by NQNO can be accounted for by a simple kinetic model based on the assumption that NQNO slows the rate of the rise and lowers the extent of the 515_s [6]. As shown in the model in Fig. 10, the slow phase of the electrochromic shift is envisioned to be due to two consecutive electrogenic reactions: (i) electron transfer from the cytochrome b_6 heme at the inner side the thylakoid membrane to the cytochrome b_6 heme located near the outer membrane surface and (ii) the oxidation of high potential cytochrome b_6 at the quinone reductase site, which could result from electron transfer from cytochrome b_6 to quinone, or proton uptake from the outer aqueous phase associated with quinone reduction, or possibly a combination of the two. Electron transfer between the two *b*-cytochromes contributes approx. 70% of the total electrochromic change, the remaining 30% being associated with cytochrome b_6 oxidation. In the presence of NQNO, the reduction rate of cytochrome b_6 is more rapid than the rise of the 515_s (Fig. 1, A and C), as shown previously [6]. Comparison of the effect of NQNO to that of DBMIB on the initial rate and extent of the 515_s provides further support for this scheme. NQNO slows the initial rate of the slow electrogenic reaction and inhibits the extent to approx. 60% of the maximum. The initial rate slows to approximately half that of the control at an NQNO concentration of 0.5 μM and is then unchanged up to 5 μM. In contrast, DBMIB slows the initial rate of the 515_s to 40% of the

control value without inhibiting the extent, a result consistent with DBMIB blocking at the Q_z-site.

The conclusion that 70% of the 515_s is due to electron transfer between two cytochrome b_6 hemes indicates that the two *b*-hemes are separated by 70% of the dielectrically weighted distance across the membrane [25,26]. It follows that if the Q_z-site is near the inner *b*-heme and the Q_c-site is near the outer *b*-heme, then the Q_z- and Q_c-sites would likewise span 70% of the dielectrically weighted distance across the membrane.

While the effects of NQNO and DBMIB shown here can be accommodated by a modified Q-cycle, other observations are not readily explained within the constraints of a Q-cycle. These observations include the following: (i) NQNO slows the rate of cytochrome *f* rereduction by 50% at a concentration (600 nM) that does not alter cytochrome b_6 reduction nor steady-state electron transport, which indicates that is is unlikely that the site of inhibition is the Q_z-site. It is possible that NQNO bound to the Q_c-site slows electron transfer from the Q_z-site to cytochrome *f*. There is a precedent for this suggestion: antimycin A bound at the Q_c-site slows the rereduction of cytochrome c_1 in the cytochrome b/c_1 complex [27]. Evidence that an inhibitor binding at the Q_c-site may alter the interaction between the two cytochrome *b*-hemes is provided by the observation of Clark and Hind [28] that HQNO raises the mid-point potential of both *b*-cytochromes in the isolated cytochrome *b/f* complex. Davies and Bendall [24] have found that there are 0.77 HQNO binding sites per cytochrome *b/f* complex. Considered together, these observations indicate that an inhibitor bound at the Q_c-site can affect more than one redox center. However, it should be noted that although 600 nM NQNO slows the reduction rate of cytochrome *f* 2-fold, the rate of steady-state electron transport is unchanged. We offer no explanation for the lack of inhibition of steady-state electron transport except the possibility that under continuous light NQNO falls off the Q_c-site (discussed below). There are other examples of an apparent discrepancy between the effect of inhibitors on steady-state and single turnover flash kinetics, one is the I_{50} for DBMIB inhibition of cytochrome *f* reduction following a short flash is 2-fold higher than that for inhibition of steady-state electron transport (Figs. 5 and 8). We are currently investigating the reasons for these differences. (ii) In the presence of NQNO we would expect to reduce both *b*-cytochromes in steady-state light, however, we are able to reduce only one *b*-heme per complex (data not shown). Rich has suggested (personal communication) that when one cytochrome *b* is reduced NQNO may debind from the Q_c-site, which could explain why no one has reported the light-induced reduction of both *b*-cytochromes. However, Joliot and Joliot [15] have shown that NQNO binds when both *b*-cytochromes are reduced in algae. (iii) Girvin and Cramer have challenged the Q-cycle mechanism and our model of the slow electrogenic step based on an experiment which showed that the 515_s was observable using single turnover flashes in conditions under which cytochrome b_6 is reduced and very little cytochrome b_6 turnover occurs [14]. Based in part on these results, they conclude that the cytochromes are not involved in the 515_s. We have confirmed these observations but do not think they are necessarily in conflict with a modified Q-cycle [17]. For example, the semiquinone anion produced at the quinol oxidase site may migrate to the quinone reductase site thereby creating an electrogenic step consisting of Q^- transfer (or a modification thereof, see e.g., Refs. 17, 29, 30). This reaction may be followed by cytochrome b_6 oxidation and contribute further to the electrogeneity of the reaction.

One requirement of the modified Q-cycle discussed here is that a monomeric cytochrome *b/f* complex must turn over twice to reduce quinone to quinol. Another possibility, originally suggested for the mitochondrial cytochrome b/c_1 complex [31], is that cytochrome *b/f* exists as a dimer that catalyzes the concerted oxidation of two quinol molecules to quinone and the net reduction of one quinone to quinol. Evidence for a cytochrome *b/f* dimer has been provided by freeze-fracture electron micrograph studies of thylakoid membranes suggesting a multimeric complex [32] and by DBMIB-binding studies indicating a functional dimer [33]. Recently, Nugent and Bendall have determined the functional size of the cytochrome *b/f* complex by radiation inactivation [34]. The results indicate that in thylakoid membranes the

complex is a functional monomer for plastoquinol-1 · plastocyanin oxidoreductase activity, although the possibility of a slight increase in activity by dimerization could not be excluded. In contrast, radiation inactivation studies of Pan et al. on the reaction from H_2O to methylviologen in thylakoid membranes indicate the complex is a functional dimer [35].

Evidence for possible secondary binding sites for NQNO and DBMIB

NQNO concentrations above 2 μM inhibit steady-state electron transfer and the rate of reduction by cytochrome b_6, indicating that NQNO has a secondary site of inhibition on the cytochrome b/f complex at the Q_Z-site. The I_{50} for inhibition of electron transport is approx. 50 μM and the I_{50} for inhibition of the rate of cytochrome b_6 reduction is 20 μM.

The results shown here for NQNO are similar to those found with HQNO [7,9,12,21,36] except that NQNO is effective at a lower concentration. A significant advantage of NQNO is that concentrations that inhibit the 515_s do not accelerate the decay of the electrochromic shift [6]. In addition to inhibiting at the cytochrome b/f complex, both NQNO and HQNO (e.g., Ref. 37) inhibit photosystem II, presumably at the Q_B-site.

The effect of DBMIB on cytochrome b_6, cytochrome f, the 515_s and steady-state electron transport can be explained by DBMIB binding at the Q_z-site, with the following exception. The I_{50} for the DBMIB-induced increase in the extent of cytochrome f oxidation is over 2-fold higher than the I_{50} for the rereduction of cytochrome f. Note that at 55 nM DBMIB the rate of cytochrome f rereduction is slowed 2-fold and the extent of the oxidation is the same as the control. DBMIB [38] as well as UHDBT [20] increase the extent of cytochrome f oxidation in a short flash. These observations lead to the suggestion that these inhibitors bind to the Q_z-site, blocking electron donation to the FeS center, as well as preventing electron donation from the FeS center to cytochrome f [20,38]. As a consequence, the removal of a single electron from the high potential donor pool to P-700 that excludes the FeS center, i.e., cytochrome f and plastocyanin, results in a larger oxidation of cytochrome f. This notion is supported by the work of Graan and Ort who found that DBMIB removed one equivalent from the high potential donor pool [39]. The fact that we observe that DBMIB inhibits the rate of cytochrome f reduction without increasing the extent of its oxidation raises the possibility that the latter effect is due to a second, lower affinity binding site, distinct from the Q_z-site. The putative second binding site would inhibit electron flow from the FeS center to cytochrome f.

Evidence that the debinding rate of DBMIB is higher than 200 s^{-1}

Under the experimental conditions described here, the rise of the 515_s is monophasic, even in the presence of low concentrations of DBMIB, and the extent is the same as in the control (Fig. 1C). The observation of monophasic kinetics at low concentrations of DBMIB supports the notion that DBMIB moves from one binding site to another within the turnover time of the cytochrome b/f complex (approx. 5 ms). If DBMIB were not mobile, then the kinetics would be biphasic at low concentrations, where distinct populations of DBMIB-bound and DBMIB-free complexes with different turnover rates would coexist. The result would be that the observed 515_s would rise with the same rate constant as the control but would reach a smaller extent. The notion that DBMIB is mobile was first suggested by Velthuys [40] based on the observation that in the presence of DBMIB the reduction of cytochrome b_6 was monophasic. We have confirmed his observation measuring cytochrome b_6 reduction in the presence of 1 μM NQNO. Based on the turnover rate of the cytochrome b/f complex the debinding rate of DBMIB would have to be at least 200 s^{-1}.

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