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Inhibitor "Double Occupancy" in the Q_o Pocket of the Chloroplast Cytochrome b_6f Complex†

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ABSTRACT: Electron paramagnetic resonance (EPR) spectra of the "Rieske" 2Fe−2S cluster revealed that two molecules of the inhibitor 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) can bind to each monomer of the spinach cytochrome (cyt) b_6f complex, both in isolated form and in intact thylakoid membranes. Binding to the high-affinity site, which accounts for the observed inhibitory effects, caused small shifts in the g_x transition of the 2Fe−2S cluster EPR spectrum, similar to those induced by stigmatellin or 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether (DNP-INT). Occupancy of the low-affinity site was only observed after addition of superstoichiometric amounts of the inhibitor and was accompanied by the appearance of a $g = 1.94$ EPR signal. The shape of the equilibrium binding titration curve, the effects on the 2Fe−2S EPR spectrum, and the ability of the DBMIB binding to displace DNP-INT were consistent with two molecules of DBMIB binding at the Q_o pocket, with the strongly binding species binding close to the 2Fe−2S cluster. Possible implications of these findings for so-called "double-occupancy" models for Q_o site catalysis are discussed.

The cytochrome (cyt)[1] b_6f complex plays a central role in photosynthetic electron transport in cyanobacteria and plant chloroplasts (for reviews, see refs *1* and *2*). It is analogous in both structure and function to the mitochondrial and bacterial cyt bc_1 and *bc*-type complexes (*3*−*9*). The complex transfers electrons from plastoquinol (PQH_2) to a soluble redox carrier, plastocyanin or cyt c_6, which in turn delivers them to photosystem I. The free energy released during the electron-transfer reactions is used to shuttle protons across the membrane from the stroma (*n*-side) to the lumen (*p*-side), contributing to the establishment of proton motive force (*pmf*), which drives the synthesis of ATP via the CF_1-CF_0 ATP synthase.

Electron transfer through the complex occurs through the so-called "Q-cycle", first proposed by Mitchell, and later modified by several groups (*10*−*15*). In the Q-cycle, PQH_2 is oxidized at the quinol oxidase (Q_o) site, which is formed by the interaction of three subunits: the cyt b_6 protein, subunit IV, and the "Rieske" iron−sulfur protein (ISP) (*16*, *17*). Electron transfer from PQH_2 is bifurcated so that the first electron is transferred to the "high-potential chain", consisting of the Rieske 2Fe−2S cluster housed in the ISP

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[1] Abbreviations: cyt, cytochrome; BBB, 2-bromo-5-*tert*-butylbenzoquinone; DIBB, 2,3-diiodo-5-*tert*-butylbenzoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ISP, iron−sulfur protein; *pmf*, protomotive force; ISP_B, iron−sulfur protein (proximal position); ISP_C, iron−sulfur protein (distal position); MOA, (*E*)-*β*-methoxyacrylate; NMR, nuclear magnetic resonance; PQH_2, plastoquinol; Q_i, quinone reductase; Q_o, quinol oxidase; Q_{OS}, strong Q_o binding site; Q_{OW}, weak Q_o binding site; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; UQH_2, ubiquinol.

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and the cyt *f* heme housed in the cyt *f* subunit. The initial one-electron oxidation leaves an unstable semiquinone species at the Q_o site, which is oxidized by the "low-potential chain" consisting of cyt b_L and cyt b_H, which are embedded in the cyt b_6 subunit (*11*, *12*, *18*). The two protons from the quinol oxidized at the Q_o site are released to the *p*-side of the membrane. After two turnovers of the Q_o site, the two electrons sent to the low-potential chain reduce a plastoquinone (PQ) molecule at the quinone reductase (Q_i) site to PQH_2, with an uptake of two protons from the *n*-side of the membrane. As a result, for every electron that is transferred to the high-potential chain, two protons are shuttled to the *p*-side of the membrane. The yield of the bifurcated reaction and proton pumping in the higher-plant cyt b_6f complex is high over a wide range of conditions, both in vitro (*19*) and in vivo (*20*), implying that side reactions which bypass bifurcation are minimized.

Since bypassing the Q-cycle would be strongly thermodynamically favored (*15*, *19*, *21*), it has been proposed that a catalytic switch gates electron transfer at the Q_o site, to force electron flow down the two pathways (*16*, *22*, *23*). There are at least three readily defined side reactions that could bypass proton pumping, which must be accounted for in Q_o site catalysis models. (1) Quinol at the Q_o site could be oxidized by two sequential electron-transfer reactions from the Q_o site quinol to the high-potential chain, with the release of two protons to the lumen. (2) Given the expected instability of the Q_o site semiquinone, it is thought to be only loosely bound (*11*, *24*), and thus could escape from the site and disproportionate in the membrane. (3) The unstable Q_o site semiquinone could oxidize ferrocytochrome b_L, reforming a quinol.

The recent structural and functional advances in the cyt bc_1 complex offer a good explanation for how the complex can prevent a bypass reaction. Different crystal forms and crystals made in the absence and presence of Q_o site inhibitors showed the hydrophilic "head domain" of the ISP in distinct positions (*3*, *16*, *24*−*27*). One position, termed ISP_B, places the 2Fe−2S cluster close to the Q_o site and cyt b_L; another, termed ISP_C, places the ISP close to cyt c_1 (*3*, *16*, *24*), and intermediate positions have been found (*28*, *29*). Similar conformational changes have also been shown to occur in the chloroplast cyt b_6f complex (*30*, *31*). Although the cyt b_6f complex contained cyt *f* in place of cyt c_1, we retain the term ISP_C to be consistent with the cyt bc_1 complex literature.

The crystal structures of the cyt bc_1 complexes show that in the ISP_B position, the 2Fe−2S cluster is too far from the cyt *c* heme to account for the observed rapid electron-transfer rates (*16*). Likewise, when in the ISP_C position, the 2Fe−2S cluster is too far from the Q_o site to effectively interact with quinol. Therefore, motion of the water-soluble ISP head domain would allow the 2Fe−2S cluster to interact with both ubiquinol at the Q_o site and cyt c_1 (*24*). By restricting the rate of reoxidation of the 2Fe−2S cluster by cyt c_1, the Q_o site semiquinone, which is formed by one-electron reduction of the 2Fe−2S cluster, is prevented from reducing the high-potential chain components and thus is forced to reduce the relatively low-potential cyt b_L, accounting for the high yield of bifurcated electron transfer (*11*, *24*, *26*, *32*). Recent independent evidence also supports the involvement of ISP pivoting in both cyt bc_1 and cyt b_6f catalysis [*30*−*42*; A. G. Roberts, M. K. Bowman, and D. M. Kramer, manuscript to be published (2001)].

In addition to the pivoting ISP mechanism, it has been previously suggested that two quinone−quinol species may bind at the Q_o site during turnover of the enzyme, forming an "electron shuttle" which could act to rapidly dissipate the reactive semiquinone and potentially prevent side reactions 2 and 3 (*43*−*46*). Data supporting this proposal have mostly come from EPR studies (*43*, *45*, *47*) and recently a nuclear magnetic resonance (NMR) study (*48*) of the cyt bc_1 Rieske 2Fe−2S cluster, which is sensitive to the occupancy of the Q_o site. Extraction and reconstitution of native quinones (*43*, *45*, *48*) or inhibitors (*47*, *49*, *50*) suggests the presence of a strong binding site (Q_{OS}) with a K_D of 50 μM and a weak binding site (Q_{OW}) with a K_D of 1 mM, and several kinetic and structural models were proposed to encompass these data (*46*, *48*). Independent spectroscopic data (*51*), as well as the recently determined X-ray structures (*16*), demonstrated that, in the cyt bc_1 complex, different classes of Q_o site inhibitors bind, albeit exclusively, to two distinct binding niches within the Q_o site, the "distal niche" which binds inhibitors such as stigmatellin (*52*) and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) (*53*) and the "proximal niche" which binds (*E*)-β-methoxyacrylate (MOA) inhibitors (see refs *16*, *54*, and *55* and below). Other competing mechanistic models of Q_o site electron transfer suggest that only one quinone species (i.e., quinone, semiquinone, or quinol) molecule occupies the Q_o site during catalysis and can move between the proximal and distal niches of the Q_o site (*56*) or act in a concerted fashion between these niches (*57*).

It has been reported that 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) induces unusually large changes in the EPR spectrum of the cyt b_6f complex 2Fe−2S cluster, including a signal at $g = 1.94$ (*17*, *58*, *59*). This, and related halogenated benzoquinones (*60*, *61*), are the only inhibitors known to induce such large EPR shifts. In this work, we show that these large EPR effects only appear upon binding of two molecules of DBMIB to the Q_o site. Potential implications of this inhibitor double occupancy for the catalytic cycle, as well as the architecture of the Q_o site, are discussed.

MATERIALS AND METHODS

Isolation of Thylakoids. Thylakoid membranes were prepared from market spinach as previously described (*19*) and stored at −80 °C in "resuspension buffer" [30 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.6), 0.5% cholic acid, 0.1% α-lecithin, and 10% $(NH_4)_2SO_4$] at a chlorophyll concentration of ∼8 mg/mL in the presence of 5% dimethyl sulfoxide as a cryoprotectant.

Purification of the Cyt b_6f Complex. The cytochrome b_6f complex was isolated from spinach thylakoids essentially described by Hurt and Hauska (*62*, *63*) with some modifications. Thylakoids (at 3 mg/mL chlorophyll) were solubilized with 40−60 mM *n*-octyl glucoside in a buffer containing 30 mM HEPES, 0.5% cholic acid, 0.1% α-lecithin, and 10% $(NH_4)_2SO_4$ and incubated for 30 min on ice in the dark. Detergent-solubilized suspensions were centrifuged at 141000*g* for 30 min at 4 °C. A buffer containing 30 mM HEPES (pH 7.6), 0.5% cholic acid, 0.1% α-lecithin, and 40% $(NH_4)_2SO_4$ was added to the resulting supernatant to give a final

$(NH_4)_2SO_4$ concentration of 25% and was centrifuged in 250 mL bottles at 15000*g* for 10 min at 4 °C. This was followed by $(NH_4)_2SO_4$ fractionation with 10% $(NH_4)_2SO_4$ fractionation steps, starting with the initial 25% $(NH_4)_2SO_4$. Most of the cyt b_6f complex precipitated in the 45−55% fraction as has been previously described (*63*). The pellet was resuspended in 30 mM *n*-octyl glucoside, 30 mM HEPES, 0.5% cholic acid, and 0.1% α-lecithin and was dialyzed for 1 h against the same buffer, but with 10 mM *n*-octyl glucoside. This was placed onto a sucrose density gradient (from 7 to 30%), which was prepared with a freeze−thaw technique using 30 mM *n*-octyl glucoside, 30 mM HEPES, 0.5% cholic acid, and 0.1% α-lecithin, and was centrifuged at 141000*g* for 14−16 h at 4 °C. After centrifugation, the resulting brown band in the sucrose density gradient was assayed for activity and cyt *f* concentration as previously described (*42*). This was then dialyzed against dialysis buffer (30 mM HEPES, 0.5% cholic acid, 0.1% α-lecithin, and 10 mM *n*-octyl glucoside) for 1 h to remove the sucrose. Detergent was added to the dialyzed protein to a final concentration of 30 mM *n*-octyl glucoside, and this suspension was concentrated to 30 μM cyt *f*, using a 100 kDa cutoff Amicon Centricon concentrator, and then stored with 20% glycerol at −80 °C. The concentration of the cyt b_6f complex was determined by the ferricyanide-oxidized minus ascorbate-reduced absorbance difference spectrum at 554 nm, using the cyt *f* extinction coefficient of 25 mM^{-1} cm^{-1} (*64*). The activity of the protein was determined as described previously (*42*).

EPR Spectroscopy. Electron paramagnetic spectra were obtained using a Bruker 200tt EPR spectrometer (Bruker Instruments, Billerica, MA), using a GFS-300 transfer tube, an ESR-900 helium cryostat, and a model ITC4 temperature controller (Oxford Instruments, Oxford, U.K.). The data were acquired using a CIO-DAS1401/12 digital I/O board (Computer Boards, Mansfield, MA) connected to a Pentium personal computer and software written in Microsoft Visual Basic 6.0 (Microsoft, Redmond, WA). The EPR parameters are given in the figure legends.

RESULTS AND DISCUSSION

Figure 1 shows the effects of a range of DBMIB concentrations on the EPR spectrum of the 2Fe−2S cluster from the isolated cyt b_6f complex. In the absence of inhibitors (Figure 1A), the spectrum exhibited g_z, g_y, and g_x transitions at *g* values of ∼2.03, ∼1.89, and ∼1.75, respectively, as previously observed in refs *62* and *65*−*68*. The addition of 1 equiv (20 μM) of DBMIB (Figure 1B) caused small but reproducible shifts, particularly in the g_x transition, which shifted from a *g* value of 1.75 to ∼1.745. In addition, a small signal appeared at $g = 1.94$, which we interpreted as a small fraction of complexes with two DBMIB molecules bound at the Q_o site (see below). It may be important to note that previous EPR results obtained under similar conditions showed a somewhat larger $g = 1.94$ signal (*60*). We interpreted this as reflecting the addition of a slight excess of DBMIB to the cyt b_6f complex, probably the result of previously used, inaccurate cyt *f* extinction coefficients [i.e., 18 mM^{-1} cm^{-1} (*62*)]. When 2 equiv of DBMIB (Figure 1C) was added, large transitions at $g = 1.94$ and 1.99 appeared, while the amplitude of the $g \sim 1.745$ and $g = 1.89$ signals decreased, consistent with previous observations (*17*, *59*, *60*).

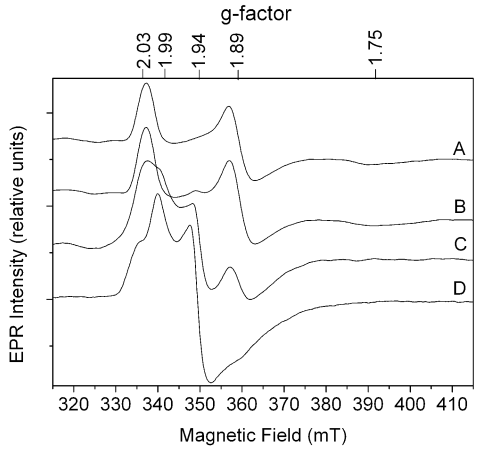

FIGURE 1: Effects of different concentrations of DBMIB on the Rieske 2Fe−2S EPR spectrum. Suspensions of the isolated spinach cyt b_6f complex (20 μM) were prepared in 30 mM Hepes (pH 7.6), 0.5% cholic acid, 0.1% α-lecithin, and 30 mM *n*-octyl glucoside in the presence of no inhibitors (A) or 20 (B), 40 (C), and 100 μM (D) DBMIB. EPR spectrometer settings were as follows: microwave frequency, 9.429 GHz; microwave power, 6.32 mW; time constant, 1000 ms; modulation amplitude, 1.6 mT; center field, 370 mT; sweep width, 200 mT; sweep time, 2 min; averages, 32; and temperature, 20 K. Traces were normalized to receiver gain.

The extent of the $g = 1.94$ shift continued to increase with increasing concentrations of DBMIB, saturating at ∼100 μM (see Figure 1D and below), leading to the disappearance of the $g = 1.89$ and $g \sim 1.745$ signals. This, and the relatively large amplitude of the $g = 1.94$ transition, gives the appearance that the spectrum became nearly axial, but with relatively large *g* strain at these high concentrations of DBMIB (see reviews in refs *69*−*72*), although the spectrum could also be explained by the overlap of the g_y and g_x transitions. The DBMIB-shifted spectrum is also strikingly similar to that of a two-point-mutated 2Fe−2S human ferredoxin (C46S/C95A) (*73*). Residue C46 is a ligand to the 2Fe−2S cluster, while C95 is located quite distant from the cluster as determined from sequence alignments and the structures of putidaredoxin and *Anabaena* 7120 ferredoxin (*73*−*78*). Single mutations to either residue, C46S and C95A, did not change the 2Fe−2S EPR spectrum, whereas together they induced quite dramatic shifts and broadening. The long distance between the cluster and C95 suggested that the effect on the 2Fe−2S geometry was conformational. We thus suggest that DBMIB induces similar changes in the conformation of the 2Fe−2S cluster of the cyt b_6f complex, which is in general agreement with the conclusions of Britt and co-workers (*79*).

Figure 2 shows a more detailed view of the effects of DBMIB on the $g \sim 1.75$ region, shown to be sensitive to the occupancy of the Q_o site (*66*). Stoichiometric amounts of DBMIB (Figure 2C) had effects comparable to those induced by two other Q_o site inhibitors, stigmatellin (*66*) (Figure 2D) and 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether (DNP-INT) (*60*) (Figure 2B). Such shifts in the g_x transition of the 2Fe−2S cluster EPR spectrum have been interpreted as reflecting a close interaction between the Q_o site inhibitor and the 2Fe−2S cluster (*66*), most likely via hydrogen bonding to one of the cluster's histidine ligands, as revealed by the X-ray structures in the presence of stigmatellin (*16*).

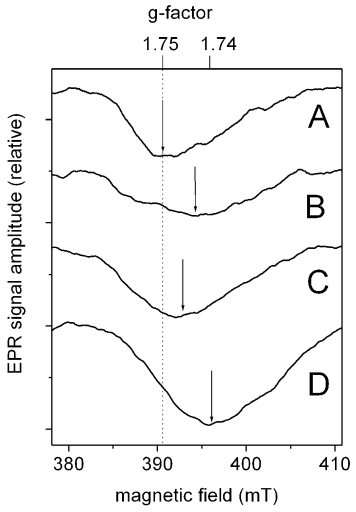

FIGURE 2: Effects of inhibitors in the Rieske 2Fe−2S cluster g_x transition. Traces were taken with samples containing no inhibitors (A), 50 μM DNP-INT (B), 20 μM DBMIB (C), and 20 μM stigmatellin (D). EPR spectrometer settings were the same as those listed in the legend of Figure 1.

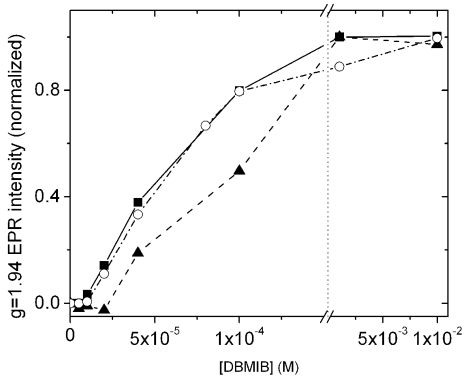

FIGURE 3: Titration of DBMIB effects on the $g = 1.94$ EPR signal of the 2Fe−2S cluster. Samples of the isolated cyt b_6f complex (■) or intact thylakoid membranes (○) were treated with a range of DBMIB concentrations. The isolated complex was suspended at 5 μM cyt *f*, while the thylakoids were suspended at 10 μM cyt *f*. The mixtures with data represented by the black squares received no further additions, while the mixture with the data represented by the black triangles (▲) was preincubated with 50 μM DNP-INT before titration. Thylakoid membranes (○) were pretreated with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to prevent photosystem II-mediated photoreduction of DBMIB. The magnitudes of the $g = 1.94$ signal were normalized to the highest observed values for each sample. EPR parameters were the same as those listed in the legend of Figure 1.

Figure 3 shows the DBMIB concentration dependence of the EPR transitions in the isolated cyt b_6f complex. At 5 μM [Figure 3 (■)] and 50 μM cyt b_6f complex (data not shown), the amplitude of the $g = 1.94$ signal increased only at superstoichiometric concentrations of DBMIB. At a >1:1 DBMIB:cyt b_6f complex ratio, the magnitude of the $g = 1.94$ signal increased essentially monotonically (Figure 3), with a concomitant decrease in the $g = 1.89$ signal (data not shown). When the cyt b_6f complex was preincubated with 50 μM DNP-INT, a significantly higher concentration of DBMIB was required for the onset of the $g = 1.94$ signal or the disappearance of the $g = 1.89$ signal [Figure 3 (▲)]. These are consistent with the earlier results by Malkin (*60*), who found that DNP-INT reversed the 2Fe−2S EPR changes induced by DBMIB, indicating that DBMIB and DNP-INT competed for the Q_o site.

We interpreted these results to indicate that two separate sites within the Q_o pocket can bind DBMIB, a high-affinity (or tight) site, and a lower-affinity (or weak) site. The binding of DBMIB at the high-affinity site induced shifts in the EPR spectra that were similar to shifts induced by stigmatellin and DNP-INT, suggesting a close interaction with the 2Fe−2S cluster. The binding of DBMIB at the lower-affinity site produced large characteristic shifts in the 2Fe−2S EPR spectra, indicating significant distortions of the 2Fe−2S cluster ligand geometry and electronic structure. Thus, we propose that the second, low-affinity DBMIB binds near the Q_o site proximal niche, but also interacts with DBMIB prebound at the high-affinity site near the distal niche.

These two DBMIB binding sites could act independently or sequentially. In either case, the strong and weak sites should have K_D values for DBMIB of <1 and ~50 μM, respectively. Binding to the tight site appeared to inhibit the complex, since K_I values for DBMIB in thylakoid membranes of considerably less than 1 μM have been reported (*80*−*83*). On the other hand, Q_o site inhibitors have been found to be relatively ineffective in the isolated cyt b_6f complex, presumably because detergent micelles interfere with partitioning (*84*). We thus repeated DBMIB titrations on intact thylakoid membranes with essentially identical results [Figure 3 (○)]. Thus, we conclude that binding of DBMIB at the strong binding site inhibits activity.

In preliminary experiments, similar phenomena were observed with the DBMIB analogues, 2,3-diiodo-5-*tert*-butylbenzoquinone (DIBB) and 2-bromo-5-*tert*-butylbenzoquinone (BBB), though the binding affinities differed (see also ref *60*). We also found that the quinol form of DBMIB and its analogues did not bind tightly or cause the large $g =$ 1.94 EPR shift as shown in refs *31* and *58*. Thus, it appears that at least one halogen (Cl, Br, or I) on the oxidized parent benzoquinone structure is required for the effect (see refs *60* and *61*).

The readily observable inhibitor double occupancy in the cyt b_6f complex represents an excellent model system with which to study the architecture and function of the Q_o site, and lends significant support to models incorporating such features (*43*−*46*). The above data and conclusions are consistent with the X-ray crystal structures of the cyt bc_1 complex, which show that the Q_o site was large enough to accommodate two quinone headgroups, although no resolvable quinones have been found at the site and there was some question whether two isoprenoid tails of native quinones can simultaneously occupy the entrance to the Q_o site without significantly distorting it (see refs *3*, *16*, *28*, and *29*). It is tempting to assign the strong and weak DBMIB binding sites on the cyt b_6f complex to the Q_{OS} and Q_{OW} sites proposed by Dutton and co-workers (*43*, *45*, *46*). Indeed, the proposed positions of these sites within the complex are consistent with our proposal, where a strong site lies near the 2Fe−2S cluster and a weak site lies near the cyt b_L heme (see also ref *45*). However, because it is an inhibitor rather than a substrate, the relevance of DBMIB double occupancy to the native catalytic cycle needs to be further explored.

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