Effect of Inhibitors on the Ubiquinone Binding Capacity of the Primary Energy Conversion Site in the *Rhodobacter capsulatus* Cytochrome bc_1 Complex†

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ABSTRACT: A key issue concerning the primary conversion (Q_O) site function in the cytochrome bc_1 complex is the stoichiometry of ubiquinone/ubihydroquinone occupancy. Previous evidence suggests that the Q_O site is able to accommodate two ubiquinone molecules, the double occupancy model [Ding, H., Robertson, D. E., Daldal, F., and Dutton, P. L. (1992) *Biochemistry 31,* 3144−3158]. In the recently reported crystal structures of the cytochrome bc_1 complex, no electron density was identified in the Q_O site that could be ascribed to ubiquinone. To provide further insight into this issue, we have manipulated the cytochrome bc_1 complex Q_O site occupancy in photosynthetic membranes from *Rhodobacter capsulatus* by using inhibitor titrations and ubiquinone extraction to modulate the amount of ubiquinone bound in the site. The nature of the Q_O site occupants was probed via the sensitivity of the reduced [2Fe-2S] cluster electron paramagnetic resonance (EPR) spectra to modulation of Q_O site occupancy. Diphenylamine (DPA) and methoxyacrylate (MOA)-stilbene are known Q_O site inhibitors of the cytochrome bc_1 complex. Addition of stoichiometric concentrations of MOA-stilbene or excess DPA to cytochrome bc_1 complexes with natural levels of ubiquinone elicits the same change in the [2Fe-2S] cluster EPR spectra; the g_x resonance broadens and shifts from 1.800 to 1.783. This is exactly the same signal as that obtained when there is only one ubiquinone present in the Q_O site. Furthermore, addition of MOA-stilbene or DPA to the cytochrome bc_1 complex depleted of ubiquinone does not alter the [2Fe-2S] cluster EPR spectral line shapes, which remain indicative of one ubiquinone or zero ubiquinones in the Q_O site, with broad g_x resonances at 1.783 or 1.765, respectively. The results are quite consistent with the Q_O site double occupancy model, in which MOA-stilbene and DPA inhibit by displacing one, but not both, of the Q_O site ubiquinones.

Ubihydroquinone:cytochrome *c* oxidoreductase (cyt[1] bc_1 complex or cyt b_6f in chloroplasts) comprises the central portion of electron-transfer chains in all energy-transducing organelles. The key primary energy conversion reaction of this complex is the two-electron oxidation of ubihydroquinone (QH_2) to ubiquinone (Q), which occurs at the Q_O site. This QH_2 oxidation involves obligatory bifurcation of electron transfer along a high- and low-potential redox chain by the cooperation of two one-electron redox centers which flank the Q_O site: the [2Fe-2S] cluster and cyt b_L, respectively (Figure 1A) (*1*, *2*). This bifurcated electron transfer is the only known reaction of its kind in biology, and despite intensive investigation of Q_O site functionality, the detailed mechanistic features remain obscure (*3*−*12*). Recently, the crystal structures of cyt bc_1 complexes from higher eukaryotes have been determined, providing insights into how bifurcated electron transfer may be effectively achieved from the demonstration that the FeS subunit can occupy different positions in different crystal forms (approximately 15 Å movement of the [2Fe-2S] cluster), either close to the Q_O site (Q_O proximal) or close to cyt c_1 (Q_O distal) (Figure 1A). Since the rate of electron transfer is strongly distance-dependent, the two FeS subunit positions are mutually exclusive in terms of the electron-transfer reactions that they can support (*13*−*16*). Structures of cyt bc_1 complexes cocrystallized in the presence of Q_O site specific inhibitors have assisted in defining the general Q_O site locality (*11*, *15*−*18*). However, in the native cyt bc_1 structures without inhibitors present, no electron density that can be ascribed to ubiquinone has been identified, leaving the question of the actual Q_O site ubiquinone stoichiometry open, at least concerning the available structural information.

A crucial part of the progress toward investigating the Q_O site substrate binding capacity has been provided by the electron paramagnetic resonance (EPR) of the cyt bc_1 complex reduced [2Fe-2S] cluster (*8*, *9*). This has been shown to be highly sensitive to the stoichiometry and type of Q_O site occupant, whether it be Q, QH_2, or inhibitors (*8*, *9*, *19*−*22*). Previous studies of the cyt bc_1 complex in *Rhodobacter capsulatus* chromatophore membranes, in which the Q_O site ubiquinone occupancy was modulated by Q-extraction and utilizing the subsequent change in the [2Fe-

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[1] Abbreviations: cyt, cytochrome; cyt b_L, low-potential cytochrome *b*; cyt b_H, high-potential cytochrome *b*; DPA, diphenylamine; EPR, electron paramagnetic resonance; [2Fe-2S], FeS protein iron−sulfur cluster; MOA, methoxyacrylate; Q, ubiquinone; QH_2, ubihydroquinone; RC, photosynthetic reaction center.

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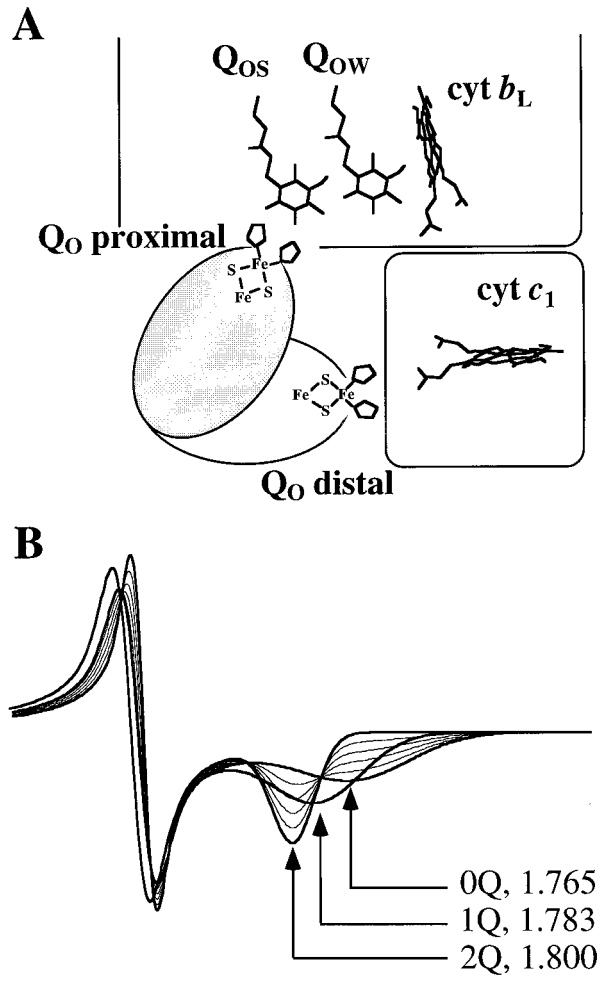

FIGURE 1: Correlation of the Q_O site occupancy with the reduced [2Fe-2S] cluster EPR spectra. (A) Schematic representation based on the crystal structure data for the region of the cyt bc_1 complex surrounding the Q_O site, illustrating the different positions of the FeS subunit. Also shown in the schematic is the proposed double ubiquinone occupancy of the Q_O site, based on biochemical evidence. (B) The characteristic [2Fe-2S] cluster EPR spectra, which have previously been shown to be dependent upon the number of ubiquinone occupants in the Q_O site. When two ubiquinones are in the Q_O site, the EPR spectrum has a narrow g_x resonance centered at 1.800; with only one ubiquinone in the Q_O site, the EPR spectrum g_x resonance broadens and shifts upfield to 1.783, which has been shown to be indicative of occupancy of only the Q_{OS} domain by Q, and when the Q_O site is completely devoid of ubiquinone, the [2Fe-2S] cluster EPR g_x resonance is shifted further upfield to 1.765. Panel B also illustrates the fact that the EPR spectrum with the g_x resonance centered at 1.783 is not the average of the 1.800 and 1.765 signals. The lighter spectral traces between the 1.800 and 1.765 limits are generated by combing these signals in the following proportions: 1:0 (1.800:1.765), 4:1, 3:2, 2:3, 1:4, and 0:1, respectively. The spectra were generated from simulating the experimental data using the program EPRSim XOP for Igor Pro (J. Boswell, Oregon Graduate Institute of Science & Technology, Portland, OR).

2S] cluster EPR signal as a spectral probe, suggested the presence of two ubiquinones located in separate domains of the Q_O site, one strongly bound (Q_{OS}) and the other weakly bound (Q_{OW}) (*9*). Furthermore, kinetic analysis coupled to the sensitivity of the [2Fe-2S] cluster EPR spectra of cyt bc_1 complexes with Q_O site mutations has facilitated putative functional assignments for the Q_{OS} and Q_{OW} domains in Q_O site catalysis (*9*). Figure 1B shows the generic [2Fe-2S] cluster EPR spectra which have been ascribed to the two ubiquinone binding domains in the Q_O site. In native chromatophores with the Q_{pool} oxidized, the g_x resonance has a narrow line shape and is centered at 1.800; partial Q-extraction results in broadening and an upfield shift of the g_x resonance to 1.783, and finally, upon complete Q-extraction, further broadening and an upfield shift of the g_x resonance to 1.765 occurs (*8*). The data depicted in Figure 1B also show the linear combinations of the two [2Fe-2S] cluster EPR spectra, with g_x resonances at 1.800 (native Q_{pool} levels) and 1.765 (fully Q-extracted) to illustrate the fact that the spectrum with the broad g_x resonance at 1.783 is not the average of the 1.800 and 1.765 signals, as has been suggested (*23*). Thus, these three [2Fe-2S] cluster EPR spectra must reflect a genuine three-state transition, which would be expected for a binding site that accommodates two ubiquinone ligands with different binding affinities.

To provide greater insight into the nature of ubiquinone occupancy in the Q_O site, we have introduced a third experimental component into the analysis of Q_O site function, use of Q_O site specific inhibitors (*24*). This yields a powerful combination for modulation of Q_O site occupancy by both Q-extraction and addition of Q_O site inhibitors, which can be monitored by the sensitivity of the [2Fe-2S] cluster EPR spectral signature to the stoichiometry and nature of the Q_O site occupants. Taking the lead from previous studies of the inhibitor diphenylamine (DPA) (*22*), and with investigation of methoxyacrylate (MOA) stilbene inhibition (*25*−*28*), we show that these two inhibitors specifically affect only the Q_{OW} domain ubiquinone occupancy in the Q_O site, under all Q-modulated experimental conditions. This provides additional support for the Q_O site double occupancy model and implies that these two inhibitors achieve full inhibition without completely displacing ubiquinone from the Q_O site.

EXPERIMENTAL PROCEDURES

Chemicals and Buffers. All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI), except for the cyt bc_1 complex inhibitors stigmatellin and antimycin, which were purchased from Sigma Chemical Co. (St. Louis, MO) and MOA-stilbene which was kindly provided by P. R. Rich (University College, London). All mediators and inhibitors were added as solutions in dimethyl sulfoxide; at the concentrations used, this solvent has no effect upon the cyt bc_1 complex kinetic activity or the [2Fe-2S] cluster EPR spectral properties (*8*, *21*). The buffer for all experiments was 50 mM MOPS and 100 mM KCl (pH 7.0).

Chromatophore Preparation. Growth of *R. capsulatus*, chromatophore preparation, and Q-extraction were performed as previously described (*8*). The concentration of the cyt bc_1 complex in the chromatophore suspensions was deduced by quantitative titration of the antimycin-induced spectral shift in the α-band of the reduced cyt b_H absorption spectrum (*29*, *30*). The experiment was performed on a Johnson Foundation dual-wavelength spectrophotometer, constructed at the University of Pennsylvania.

EPR Spectroscopy. EPR measurements were performed on a Bruker ESP300E spectrometer as previously described (*20*). Temperature control was maintained by an Oxford ESR model 900 continuous flow cryostat interfaced with an Oxford model ITC4 temperature controller. The frequency was measured by a Hewlett-Packard model 5350B frequency

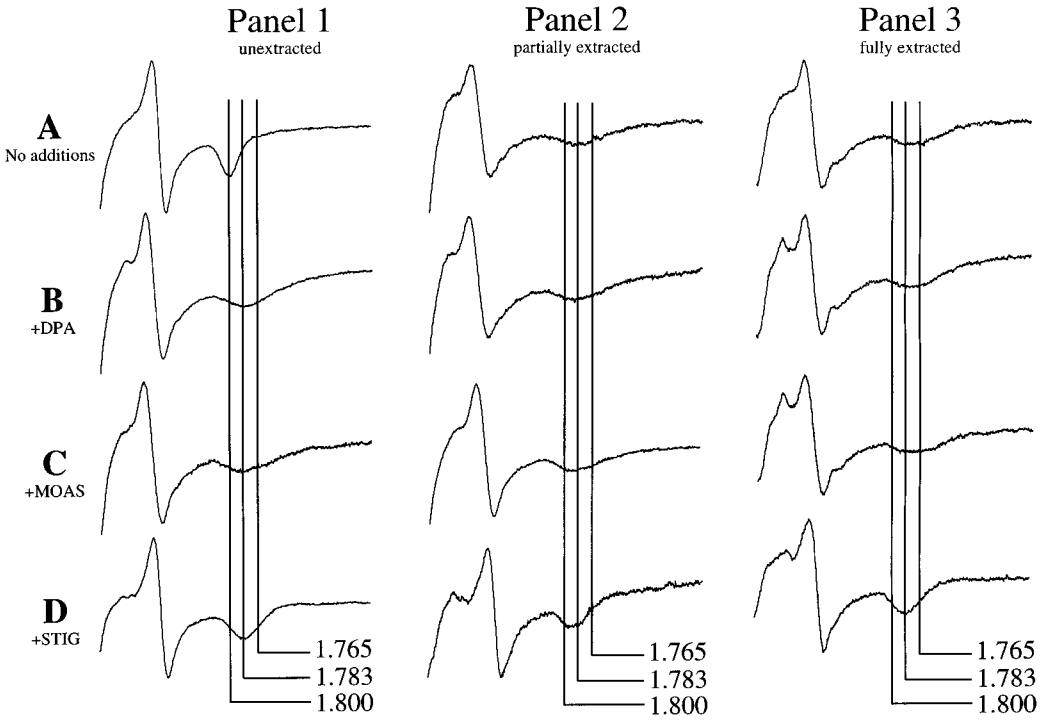

FIGURE 2: Effect of Q_O site inhibitors and Q-extraction on the reduced [2Fe-2S] cluster EPR spectra in *R. capsulatus* chromatophores. Experimental conditions are described in Experimental Procedures. All samples were suspended to a cyt bc_1concentration of 20 μM, and the redox poise was adjusted to 200 mV. At this potential, the [2Fe-2S] cluster is >95% reduced and the Q_{pool} is fully oxidized. Each spectrum is the sum of five successive acquisitions, except for the data depicted in panel 3 which are the sum of 15 acquisitions: panel 1, unextracted chromatophores with a native level of ubiquinone (≈30 mM in the membrane); panel 2, partially Q-extracted chromatophores with the g_x resonance centered at 1.783; and panel 3, fully Q-extracted chromatophores with the g_x resonance centered at 1.765. (A) No additions. (B) Addition of 100 mM DPA. (C) Addition of 25 μM MOA-stilbene. (D) Addition of 25 μM stigmatellin.

counter. Typical operating parameters were as follows: sample temperature, 20 K; microwave frequency, 9.474 GHz; microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 19.8 G; and time constant, 164 ms. Further details are provided in the appropriate figure legends.

Redox Titrations. These were performed essentially as previously described (*31*). For measurement of the $[2Fe\text{-}2S]^{2+/+}$ cluster midpoint potential, high-potential mediators covering a 300 mV range were used to maintain electrochemical equilibrium between the electrodes and redox centers: 2,3,5,6-tetramethylphenylenediamine, 1,2-naphthoquinone, 1,2-naphthoquinone 4-sulfonate, 1,4-benzoquinone, ferrocyanide, and 2,3,5,6-tetrachlorohydroquinone each at a concentration of 100 μM. Measurement of the cyt b_L and b_H midpoint potentials required the use of a wide range of mediators spanning 600 mV in potential (*32*): 2,3,5,6-tetramethylphenylenediamine, 2-hydroxy-1,4-naphthoquinone, and 1,2 naphthoquinone each at a concentration of 25 μM, *N*-methyldibenzopyrazine methosulfate, *N*-ethyldibenzopyrazine ethosulfate, pyocyanin, and phenazine each at a concentration of 20 μM, and 50 μM duroquinone. Additional experimental details are provided in the appropriate figure legends.

Kinetic Measurements. Flash-activated turnover of the cyt bc_1 complex was performed on a Johnson Foundation single-wavelength spectrophotometer (University of Pennsylvania) fitted with an anaerobic redox cuvette, as previously described (*20*).

RESULTS

EPR Spectral Line Shape Changes Induced by Q-Extraction and Q_O Site Inhibitors. The effect of Q-extraction and Q_O site specific inhibitors is presented in Figure 2 and summarized in Table 1. Panel 1 of Figure 2 depicts the effect of Q_O site inhibitors on the reduced [2Fe-2S] cluster EPR spectrum in chromatophores with native complements of ubiquinone. Panel 1 of Figure 2A shows the familiar [2Fe-2S] cluster EPR spectrum characteristic of the native Q_O site fully occupied by ubiquinone with a narrow g_x resonance centered at 1.800 (*8*, *9*). Panel 1 of Figure 2B confirms that addition of excess DPA (>10 mM) alters the [2Fe-2S] cluster EPR spectrum, broadening the g_x resonance, and shifting it upfield to 1.783 (*22*). Panel 1 of Figure 2C demonstrates that the presence of stoichiometric amounts of MOA-stilbene (1:1 with respect to the cyt bc_1 concentration in the chromatophores) induces the same EPR spectral change as excess DPA (broad g_x resonance at 1.783). Addition of excess MOA-stilbene up to a concentration of 1 mM does not cause any further spectral changes (data not shown). The pronounced change in the [2Fe-2S] cluster EPR spectrum upon addition of MOA-stilbene to native chromatophore membranes is illustrated in Figure 3A, which shows a difference spectrum of spectrum A minus spectrum C of panel 1 of Figure 2.

Panel 2 of Figure 2 shows the effects of the same inhibitors on the EPR spectra of partially Q-extracted chromatophores, such that the g_x resonance is already broad and centered at 1.783, prior to the addition of inhibitors. The addition of excess DPA or MOA-stilbene to these chromatophores does not result in any further changes to the EPR spectrum (Figure 3B), where the g_x resonance remains centered at 1.783 (panel 2 of parts B and C of Figure 2). This has previously been interpreted as being due to the fact that under these conditions

Table 1: Summary of the Effect of Ubiquinone Extraction and Addition of Q_O Site Inhibitors to *R. capsulatus* Chromatophores Containing the Cyt bc_1 Complex (except where indicated)

	[2Fe-2S] cluster EPR g_x resonances			prosthetic group redox potentials (mV)		
	native Q-levels[a]	partially Q-extracted[b]	fully Q-extracted[c]	[2Fe-2S] cluster	cyt b_L and b_H	inhibitor binding constants (μM)
native cyt bc_1 complex	2Q, 1.800	1Q, 1.783	0Q, 1.765	320 ± 10	-120 ± 10 60 ± 10	−
native complex with 1 molar equiv of stigmatellin[d]	1.785	1.785	1.785	>500[e]	ND	$\ll 1$[e]
native complex with excess DPA[f]	1.783	1.783	1.765	350 ± 10	-120 ± 10[g] 60 ± 10	25 ± 10[h]
native complex with 1 molar equiv of MOA-stilbene	1.783	1.783	1.765	290 ± 10[i]	-120 ± 10 60 ± 10	$\ll 1$[j]

[a] Two ubiquinone molecules in the Q_O site. [b] One ubiquinone molecule in the Q_O site. [c] Zero ubiqunone molecules in the Q_O site. [d] One molar equivalent per cyt bc_1 complex. [e] Isolated bovine cyt bc_1 complex [von Jagow and Ohnishi (*37*)]. [f] Greater than 10 mM. [g] With 1 mM DPA added. [h] Sharp et al. (*22*). [i] With 0.1 mM MOA-stilbene added. [j] With 10 μM MOA-stilbene added.

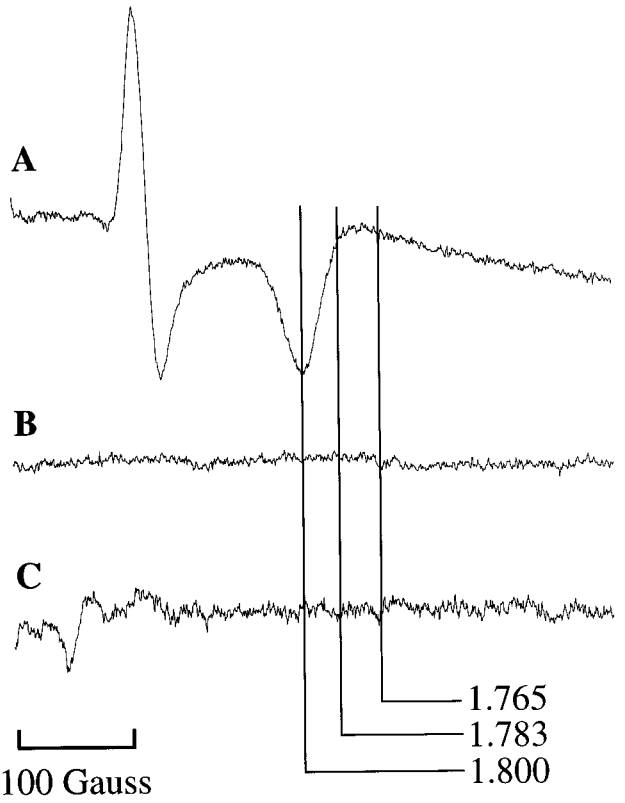

FIGURE 3: [2Fe-2S] cluster difference EPR spectra, illustrating the effect of addition of MOA-stilbene to chromatophores which have (A) a full complement of ubiquinone (determined from spectrum A minus spectrum C of panel 1 of Figure 2), (B) been partially extracted of ubiquinone, such that there is only one ubiquinone in the Q_O site (determined from spectrum A minus spectrum C of panel 2 of Figure 2), and (C) been fully Q-extracted, such that there are no ubiquinones in the Q_O site (determined from spectrum A minus spectrum C of panel 3 of Figure 2).

only the Q_{OS} domain of the Q_O site is occupied by Q (*8*, *22*). It is important to recognize that the [2Fe-2S] cluster EPR spectra generated upon addition of excess DPA or MOA-stilbene to native chromatophores depicted in panel 1 of parts B and C of Figure 2 are the same within experimental error as that for partially Q-extracted chromatophores (panel 2 of Figure 2). It should be noted that the [2Fe-2S] cluster EPR g_y resonances are also the same in the DPA-inhibited, MOA-stilbene-inhibited, and partially Q-extracted chromatophores; however, for convenience, we chose to solely focus on changes in the g_x resonance. Panel 3 of Figure 2 shows the effect of inhibitors on fully Q-extracted chromatophores, such that the g_x resonance is broadened and centered at 1.765, implying that the Q_O site is devoid of Q before addition of inhibitors. Again, addition of excess DPA or stoichiometric amounts of MOA-stilbene does not alter the [2Fe-2S] EPR spectral signature (panel 3 of parts B and C of Figure 2) and (Figure 3C).

The results depicted in panels 2 and 3 of Figure 2 demonstrate that the [2Fe-2S] cluster EPR spectra induced by excess DPA or 1:1 MOA-stilbene are not due to the inhibitors themselves interacting directly with the [2Fe-2S] cluster, since these inhibitors have no effect on the EPR spectrum in the absence of Q in either the Q_{OW} or Q_{OS} domain of the Q_O site. Given the data described above, it is clear that binding of stoichiometric amounts of MOA-stilbene to the Q_O site has the same effect as that of excess DPA. That is, in native chromatophores, binding of DPA or MOA-stilbene to the Q_O site causes displacement of Q from the Q_{OW} domain, resulting in [2Fe-2S] cluster EPR spectra only reporting the occupancy of the Q_{OS} domain by Q. Moreover, only 1 molar equiv of MOA-stilbene (per cyt bc_1 complex) is required to displace Q from the Q_{OW} domain under these experimental conditions because it is a much tighter binding inhibitor than DPA. The K_i values for MOA-stilbene and DPA are <1 μM (*25*, *26*) and 25 μM (*22*), respectively. In support of this Q-displacement hypothesis from the Q_{OW} domain of the Q_O site, previous experiments in which the capacity of binding of MOA-stilbene to bovine mitochondrial cyt bc_1 complex was examined have shown that the Q_O site is able to accommodate both ubiquinone and MOA-stilbene (*25*−*28*). Since the Q-extraction process is a harsh procedure, the physical integrity of the Q_O site under all the Q-extracted conditions was confirmed by addition of the tight binding Q_O site inhibitor stigmatellin, as binding of this inhibitor is sensitive to defects in the Q_O site (*33*−*36*). Regardless of the chromatophore ubiquinone content, stigmatellin always induces the same [2Fe-2S] cluster EPR spectrum with a diagnostic narrow g_x resonance centered at 1.785 (panels 1−3 of Figure 2D) (*37*), implying that the Q_O site remained intact under all experimental conditions.

We have previously reported that addition of ethanol to *R. capsulatus* chromatophore membranes results in a unique [2Fe-2S] cluster EPR spectrum with a broad g_x resonance centered at 1.773 (*20*, *21*). In contrast to the effect of

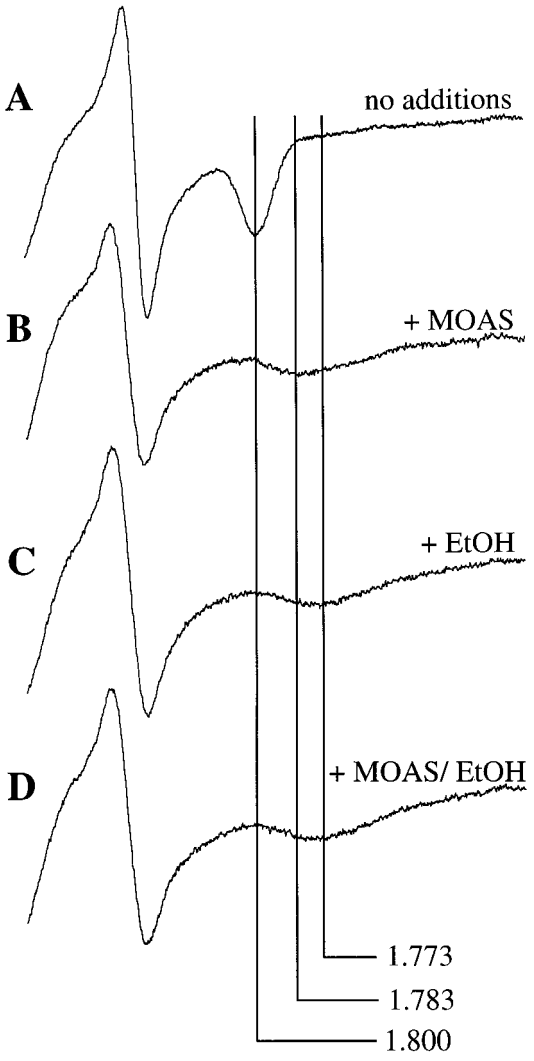

FIGURE 4: Dual effect of MOA-stilbene and ethanol on the reduced [2Fe-2S] cluster EPR spectrum in *R. capsulatus* chromatophores. Experimental conditions are as described in the legend of Figure 2. (A) Unextracted chromatophores with native levels of ubiquinone. (B) Addition of 25 μM MOA-stilbene. (C) Addition of 5% (v/v) ethanol (0.85 M). (D) Addition of 25 μM MOA-stilbene and 5% (v/v) ethanol. The order of the addition is not critical.

inhibitors, the addition of ethanol has no effect on the kinetic properties of the cyt bc_1 complex, but it does render the [2Fe-2S] cluster EPR spectrum insensitive to the Q_O site ubiquinone content. The addition of ethanol to fully Q-extracted chromatophores or to chromatophores containing a full complement of ubiquinone results in the same EPR signal (broad g_x resonance centered at 1.773) (*21*). For further confirmation that the [2Fe-2S] cluster EPR spectrum induced by binding of MOA-stilbene to the Q_O site is not due to a direct interaction with the inhibitor itself, but rather to displacement of ubiquinone from the Q_{OW} domain as implied above, we have examined the effect of a combination of ethanol and MOA-stilbene on the EPR spectral properties of the [2Fe-2S] cluster. Figure 4 establishes that the [2Fe-2S] cluster EPR spectra of chromatophores with native Q complement before (Figure 4A) and after individual additions of MOA-stilbene (Figure 4B) and 5% (v/v) ethanol (Figure 4C) show the expected broadening and upfield shifts in the g_x resonance from 1.800 to 1.783 and 1.773, respectively. Figure 4D shows that MOA-stilbene added in the presence of 5% ethanol yields a [2Fe-2S] cluster EPR spectrum with

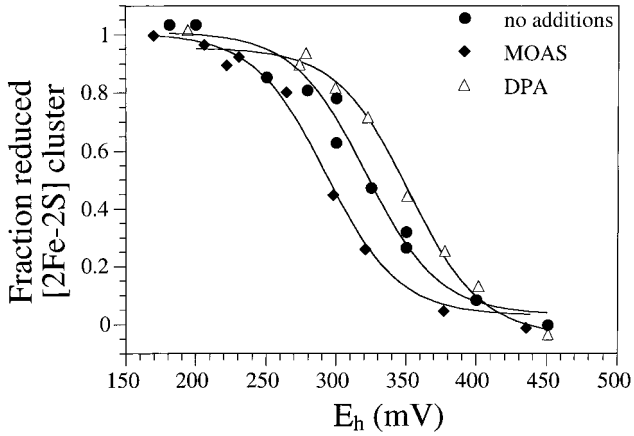

FIGURE 5: Effect of inhibitors on the redox midpoint potential of the cyt bc_1 complex [2Fe-2S] cluster. The fraction reduced was determined from the peak to trough amplitude difference of the g_y resonance, and the data were fit to the Nernst equation for a one-electron couple, with the indicated midpoint potentials (standard error $\pm$ 10 mV). Chromatophores were suspended to a cyt bc_1 complex concentration of 10 μM, and the EPR conditions are as reported in Experimental Procedures: (●) no addition, (◆) addition of 100 μM MOA-stilbene, and (△) addition of 100 mM DPA.

a broad g_x resonance at 1.773, not 1.783. Indeed, spectra C and D of Figure 4 are identical, showing that the effect of ethanol on the [2Fe-2S] cluster EPR spectrum is independent of the presence of MOA-stilbene. This implies that the [2Fe-2S] cluster EPR spectrum induced by MOA-stilbene is due to displacement of ubiquinone from the Q_{OW} domain.

To confirm that ethanol did not disrupt inhibition of Q_O site function by MOA-stilbene, the combined effect of inhibiting concentrations of MOA-stilbene in the presence of 5% (v/v) ethanol on cyt bc_1 complex activity was investigated. As previously demonstrated, addition of ethanol alone had no effect upon cyt bc_1 complex activity (*21*), but in the presence of 5 μM MOA-stilbene and 5% (v/v) ethanol, full inhibition occurred, demonstrating that ethanol does not disrupt MOA-stilbene binding to the Q_O site.

Effect of Inhibitors on the [2Fe-2S] Cluster Redox Midpoint Potential. The effect of excess DPA (100 mM) and MOA-stilbene (0.1 mM) on the cyt bc_1 complex [2Fe-2S] cluster redox midpoint potential is illustrated in Figure 5 and summarized in Table 1. The midpoint potential of the one-electron $[2Fe\text{-}2S]^{2+/+}$ couple is slightly elevated in the presence of DPA (E_{m7} = 350 mV) (*22*) and lowered by MOA-stilbene (E_{m7} = 290 mV) compared to that for the uninhibited cyt bc_1 complex (E_{m7} = 320 mV). This implies that excess DPA binds with about 5-fold greater affinity to the Q_O site of the cyt bc_1 complex when the [2Fe-2S] cluster is in the reduced state. In contrast to excess DPA, MOA-stilbene binds with a 5-fold greater affinity to the Q_O site when the [2Fe-2S] cluster is in the oxidized state.

Effect of Inhibitors on the Reduced Cytochromes b Absorbance Spectra. Figure 6 shows the absorbance difference spectra of the two *b*-type hemes (b_L and b_H) of the cyt bc_1 complex upon binding inhibitors. The figure includes the characteristic red shift in the absorbance spectrum of ferrous cyt b_H, induced by binding of antimycin to the Q_i site (*29*, *30*). Likewise, addition of DPA and MOA-stilbene also results in an apparent red shift in the ferrous heme α-band of cyt b_L, presumably due to DPA and MOA-stilbene binding in the Q_O site. This effect is independent of that caused by

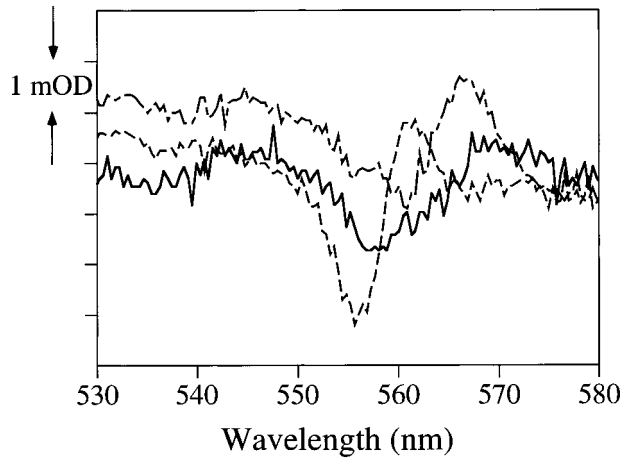

FIGURE 6: Effect of cyt bc_1 complex inhibitors on the reduced cyt *b* heme spectra. Chromatophores were suspended to a cyt bc_1 complex concentration of 0.5 μM, and excess sodium dithionite was added to fully reduce the complex. The traces displayed are absorbance difference spectra for inhibitor minus baseline and were recorded as described in Experimental Procedures: (− − −) addition of 10 μM antimycin, (−·−) addition of 1 mM DPA, and (−) addition of 10 μM MOA-stilbene.

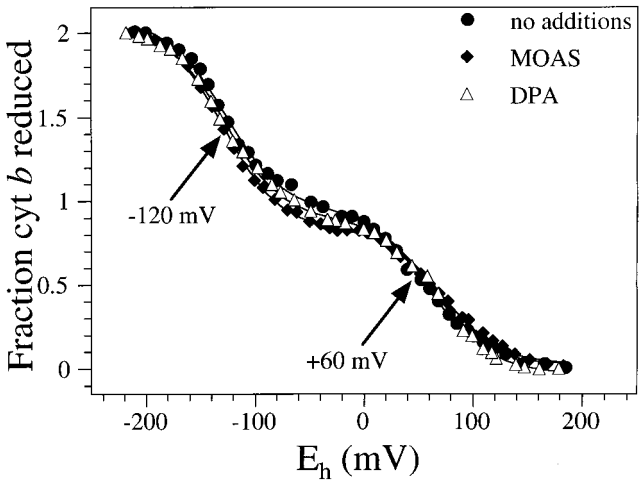

FIGURE 7: Effect of inhibitors on the redox midpoint potential of the cyt *b* hemes. The fraction reduced was determined from the gain in intensity of the ferrous heme α-band (560−540 nm), and the data were fit to the Nernst equation for two one-electron couples, with the indicated midpoint potentials (standard error ± 10 mV). Chromatophores were suspended to a cyt bc_1 complex concentration of 0.5 μM, and the experiments were performed as described in Experimental Procedures: (●) no addition, (◆) addition of 10 μM MOA-stilbene, and (△) addition of 1 mM DPA.

antimycin; however, the intensities of these spectral changes are less pronounced than that observed for binding of antimycin to the Q_i site. Although spectral shifts due to these Q_O site inhibitors are clear, deconvolution of the band shift into separate effects on cyt b_L and b_H is difficult given the signal-to-noise ratio of these rather turbid suspensions.

Effect of Inhibitors on Cytochrome b_L and b_H Redox Midpoint Potentials. The effect of 1 mM DPA and 10 μM MOA-stilbene (at which concentrations the cyt bc_1 complex is fully inhibited) on the cyt b_L and b_H redox midpoint potentials is shown in Figure 7 and summarized in Table 1. It is clear that neither inhibitor alters the midpoint potentials of either *b*-type heme from the wild-type values of −120 ± 10 mV for cyt b_L and 60 ± 10 mV for cyt b_H (*32*).

DISCUSSION

Figure 1A presents a schematic illustration of the region of the cyt bc_1 complex surrounding the Q_O site, based on the available crystal structure data (*11*, *14*, *15*, *17*, *19*) and biochemical evidence (*8*, *9*, *16*, *22*). As described in the introductory section, the structures reveal that the FeS subunit position is different in various structural forms. This is a key discovery, since it highlights a possible means for facilitating the obligatory bifurcated electron-transfer reaction that occurs in the Q_O site during the catalytic turnover of the enzyme (*6*, *7*). Another feature of the cyt bc_1 complex structures was the lack of any electron density in the Q_O site that could be ascribed to ubiquinone, presumably because of dissociation from the complex during the extensive purification procedure, a feature which has been previously observed (*38*). However, cocrystals obtained with Q_O site specific inhibitors have enabled the physical location of this site to be defined (*11*, *14*, *15*, *17*, *18*). Furthermore, the Q_O site inhibitors MOA-stilbene and 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) bind in different subsites within the Q_O pocket, with nonoverlapping electron density (*18*). Since MOA-stilbene and UHDBT are different types of Q_O site inhibitors (*24*−*27*), it seems reasonable to propose that perhaps these binding subsites (or domains) reflect the two possible ubiquinone binding sites. As for accommodation of ubiquinone isoprene tails, which would extrude from the Q_O site, given the present resolution of the cyt bc_1 complex crystal structures (about 3.0 Å), it seems premature to discuss the lack of any electron density due to the ubiquinone tail group(s).

Central to the description of the Q_O site mechanism is the number of ubiquinone molecules in this site and assignment of their function. From the dependence of the reduced [2Fe-2S] cluster EPR spectral line shape on the level of ubiquinone content in native, Q-extracted chromatophore membranes and cyt bc_1 complexes with Q_O site mutations that disrupt ubiquinone binding, it has previously been demonstrated that the EPR data could be deconvoluted and interpreted in terms of two ubiquinone species which bound to the Q_O site with strong and weak affinity, termed Q_{OS} ($K_d \approx 0.1$ mM) and Q_{OW} ($K_d \approx 1.0$ mM), respectively (*9*).

In this paper, we have described the use of inhibitors which specifically displace ubiquinone from the Q_O site to further extend the analysis of the dependency of the [2Fe-2S] cluster EPR spectral line shape on the Q_O site occupancy. The data presented in Figures 2 and 3 and summarized in Table 1 show that addition of excess DPA or stoichiometric amounts of MOA-stilbene (relative to the concentration of the cyt bc_1 complex) to native chromatophores with natural ubiquinone levels results in the generation of [2Fe-2S] cluster EPR spectra, with a g_x resonance centered at 1.783 (*8*, *22*). These EPR spectra are identical to that for partially Q-extracted chromatophores where only the Q_{OS} domain of the Q_O site is occupied by ubiquinone (*8*). Addition of either excess DPA or MOA-stilbene to partially or fully Q-extracted chromatophores has no effect on the [2Fe-2S] cluster EPR spectrum, resulting in an unaltered g_x resonance at 1.783 or 1.765, respectively (Figures 2 and 3). These observations have important consequences for interpreting the original Q-extraction data, since they imply that the origin of the g_x resonance at 1.783 is not due to the inhibitors themselves

interacting with the [2Fe-2S] cluster, fortuitously generating the same EPR spectral line shape as the Q-extracted chromatophores, but rather as already suggested for the case of excess DPA, by specifically displacing ubiquinone from the Q_{OW} domain, resulting in a Q_O site in which only the Q_{OS} domain is occupied by ubiquinone. As has been previously discussed (*8*), in the fully occupied Q_O site, ubiquinone bound in the Q_{OW} domain may mediate its effect upon the [2Fe-2S] cluster EPR spectrum by interacting either directly with one of the cluster ligands or indirectly via an interaction with ubiquinone in the Q_{OS} domain. The lack of any effect upon the [2Fe-2S] cluster EPR spectrum when excess DPA or MOA-stilbene is bound in the Q_O site (presumably in the Q_{OW} domain) and ubiquinone is bound in the Q_{OS} domain may be due to the fact that these inhibitors cannot mimic the interaction which occurs when ubiquinone is present in both the Q_O domains. This is not altogether surprising, since DPA and MOA-stilbene have quite different structures with respect to the ubiquinone headgroup. To our knowledge, this is the first reported example of stoichiometric addition of an inhibitor to the Q_O site, in this case MOA-stilbene which specifically displaces ubiquinone from the Q_{OW} domain and not the Q_{OS} domain as well.

In a recent report in which the inhibition action of DPA was extensively characterized, comparisons were drawn between the mode of action of DPA in *R. capsulatus* chromatophore membranes (*22*) and that of MOA-stilbene inhibition in the purified bovine mitochondrial cyt bc_1 complex (*25*, *26*). In the latter case, Brandt and co-workers have shown that ubiquinone was still present in the MOA-stilbene-inhibited Q_O site and on the basis of this proposed that MOA-stilbene disrupted bovine cyt bc_1 complex activity in a noncompetitive manner (*25*−*27*). The quite different EPR data reported here completely agree with this conclusion concerning the nature of MOA-stilbene-mediated Q_O site inhibition. Cocrystals of MOA-stilbene bound in the Q_O site show that it is located toward the cyt b_L heme, but is not involved in any direct interaction, hydrogen bonding or otherwise, with either of the prosthetic groups that flank the Q_O site (*18*). In agreement with this, binding of MOA-stilbene to the Q_O site has very little effect on the thermodynamic properties of the flanking [2Fe-2S] cluster and cyt b_L (Table 1), with a 30 mV lowering in the [2Fe-2S] cluster midpoint potential (Figure 5), and has no effect on the cyt b_L heme midpoint potential (Figure 7). DPA also has similarly small effects on the [2Fe-2S] cluster midpoint potential, in this case raising it by 30 mV, and has no effect on the cyt b_L heme potential (Table 1). The effect of MOA-stilbene upon the [2Fe-2S] cluster midpoint potential correlates with a 5-fold greater binding affinity for this inhibitor when the [2Fe-2S] cluster is in the oxidized state. The [2Fe-2S] cluster redox-dependent difference in the binding affinity of MOA-stilbene for the Q_O site has also been previously demonstrated by a method entirely different from the one employed here (*26*, *27*). In the latter case, Q_O site MOA-stilbene binding affinity titrations were performed with the [2Fe-2S] in either the oxidized or reduced state, and the results showed a 2−3-fold difference in binding affinity between the two states, with the oxidized state having the lower binding affinity. These results along with other inhibitor studies of the Q_O site were interpreted as being due to the presence of inhibitors in the Q_O site locking the cyt bc_1 complex in different conformational substates (*26*, *27*). On the basis of this, a comprehensive model for Q_O site catalysis by the cyt bc_1 complex was proposed, involving a "catalytic switch" mechanism. While this is a useful model, especially in light of the crystal structure data which indicate that the FeS subunit is mobile, it may be a little tenuous to ascribe these rather small redox-dependent shifts in inhibitor binding affinity to different conformational substates of the cyt bc_1 complex.

CONCLUSIONS

Using the powerful combination of Q-extraction, Q_O site specific inhibitor binding, and the sensitivity of the reduced [2Fe-2S] cluster EPR spectra to the nature and extent of the Q_O site occupant(s), we have provided further evidence that when present in a native-like environment (chromatophore membranes), the primary energy conversion site of the cyt bc_1 complex is able to accommodate two ubiquinone molecules. Unless the extensive EPR data we report can be proven to arise from other sources, then the double ubiquinone Q_O site occupancy appears to be the best model for interpretation of our data (*8*, *9*, *22*). The crystal structures of the cyt bc_1 complex with stigmatellin and MOA-stilbene bound in the Q_O site are identified as binding these inhibitors proximal to the FeS subunit (stigmatellin and UHDBT) and proximal to cyt b_L (MOA-stilbene), respectively (*18*). On the basis of the data presented here, we propose that the stigmatellin and MOA-stilbene binding domains may very well correspond to the biochemically observed Q_{OS} and Q_{OW} ubiquinone binding domains, with the ubiquinone in the Q_{OS} domain behaving as a catalytic cofactor and that in the Q_{OW} domain as a substrate ubiquinone (*9*).

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