Ubiquinone Binding Capacity of the *Rhodobacter capsulatus* Cytochrome bc₁ Complex: Effect of Diphenylamine, a Weak Binding Q₀ Site Inhibitor†

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ABSTRACT: Diphenylamine (DPA), a known inhibitor of polyene and isoprene biosynthesis, is shown to inhibit flash-activatable electron transfer in photosynthetic membranes of *Rhodobacter capsulatus*. DPA is specific to the Q₀ site of ubihydroquinone:cytochrome c oxidoreductase, where it inhibits not only reduction of the [2Fe-2S]⁺⁺ cluster in the FeS subunit and subsequent cytochrome c reduction but also heme b₁ reduction in the cytochrome b subunit. In both cases, the kinetic inhibition constant (Kᵢ) is 25 ± 10 μM. A novel aspect of the mode of action of DPA is that complete inhibition is established without disturbing the interaction between the reduced [2Fe-2S]⁺⁺ cluster and the Q₀ site ubiquinone complement, as observed from the electron paramagnetic resonance (EPR) spectral line shape of the reduced [2Fe-2S]⁺⁺ cluster, which remained characteristic of two ubiquinones being present. These observations imply that DPA is behaving as a noncompetitive inhibitor of the Q₀ site. Nevertheless, at higher concentrations (>10 mM), DPA can interfere with the Q₀ site ubiquinone occupancy, leading to a [2Fe-2S]⁺⁺ cluster EPR spectrum characteristic of the presence of only one ubiquinone in the Q₀ site. Evidently, DPA can displace the more weakly bound of the two ubiquinones in the site, but this is not requisite for its inhibiting action.

Ubihydroquinone:cytochrome c oxidoreductase (cyt₁ bc₁ complex in most organisms, cyt bc₁f in chloroplasts) comprises the central portion of electron transfer chains in all energy-transducing organelles. The Q₀ site is the locus of the primary energy conversion steps within the cyt bc₁ complex and initiates the conversion of the free energy between ubihydroquinone (QH₂) and ferricytochrome c into a transmembrane electrochemical gradient of protons. In prokaryotes, this site is located at the periplasmic face of the cytoplasmic membrane and in eukaryotes on the cytosolic side of the mitochondrial inner membrane (facing the intermembrane space). QH₂ oxidation is catalyzed by cooperation of two single-electron transfer chains which flank the Q₀ site and transport the electrons in different directions. The redox cofactors closest to the site are the [2Fe-2S]⁺⁺ cluster, the first cofactor in the high-potential chain which guides electrons to cyt c₁ and then to cytochromes c in the periplasm (or the mitochondrial intermembrane space); and cyt b₁, the first in the low-potential chain that through cyt b₁ and the Q₀ site drives electrons across the membrane to convert the redox potential differences into a transmembrane electrochemical gradient (1, 2).

For one complete turnover of the cyt bc₁ complex to occur, two QH₂ molecules must be oxidized at the Q₀ site (3, 4). The conventional model depicts the Q₀ site as binding one QH₂ at a time and performing two separate, serial oxidations. However, there is some controversy with regard to the Q₀ site reaction dynamics, as well as the actual number of Q₀ site ubiquinone occupants. The advent of crystal structures of cyt bc₁ complexes from various species with bound Q₀ site specific inhibitors has assisted in defining the general Q₀ site locality; however, in the native structures without inhibitors present, no electron density was identified that could be ascribed to ubiquinone (5–8). There are several plausible mechanisms for bifurcation of the electron transfer reaction in the Q₀ site (9). These include (a) a double-Q occupancy model (10, 11) invoking formation of a highly unstable ubisemiquinone transition state (11, 12) or a quinhydrone-like intermediate (11, 13); (b) a proton-gated charge transfer mechanism, where the activation barrier for the reaction is deprotonation of QH₂ (14, 15); (c) a protonated affinity change mechanism implicating a stable ubisemiquinone intermediate (7, 16); and (d) a model proposing formation of a single unstable ubisemiquinone diffusing within the Q₀ site to facilitate bifurcated electron transfer by a catalytic switching movement (7, 12, 13). The recent structural demonstration that the [2Fe-2S]⁺⁺ cluster moves over a distance of about 15 Å between the Q₀ site and cyt c₁ adds another dynamic feature to the mechanisms for achieving efficient bifurcation of electron transfer (5–8).
Inhibition of the Cytochrome $bc_1$ Complex $Q_O$ Site by DPA

A crucial aid in defining the function of the cyt $bc_1$ complex has been the use of specific, tight binding inhibitors, classified according to whether they affect catalysis at the $Q_O$ or $Q_b$ site (17). Another essential part of the progress into the $Q_O$ site character has been provided by the electron paramagnetic resonance (EPR) spectral line shape of the reduced, paramagnetic [2Fe-2S] cluster. This has proven to be highly sensitive to the degree and nature of the $Q_O$ site occupants ($Q/QH_2$ or inhibitors) (10, 11). Dutton and co-workers have proposed a working model, based on $Q_O$ site occupancy and kinetic analysis of wild-type and mutant cyt $bc_1$ complexes, that is entirely consistent with the basic precedents of the Q-cycle hypothesis (10, 11). These studies indicated that the $Q_O$ site is able to accommodate two ubiquinone molecules within two distinct binding domains, one of which was determined to have a high affinity and the other a lower affinity for $Q/QH_2$ and were accordingly designated the $Q_{OS}$ (strong) and $Q_{OW}$ (weak) domains, respectively. On the basis of this, a plausible model for one complete turnover of the cyt $bc_1$ complex was presented which only required exchange of ubiquinone in the $Q_{OW}$ domain with the $Q_{pool}$.

Diphenylamine (DPA) is a well-known inhibitor of carotenoid (18, 19) and ubiquinone (20) synthesis whose action is clearly observed during the growth of photosynthetic prokaryotes. It has also been demonstrated that DPA can act as an inhibitor of the cyt $bc_1$ complex in vitro (21), as well as an effective inhibitor of photosystem II, but not photosystem I in chloroplasts (22). This report focuses on the nature of DPA inhibition of the cyt $bc_1$ complex in cytoplasmic membranes of the photosynthetic prokaryote Rhodobacter capsulatus. We show that DPA is most likely acting as a noncompetitive inhibitor of the $Q_b$ site and discuss how the inhibiting action lends support to the double-occupancy mechanism.

EXPERIMENTAL PROCEDURES

Chromatophore preparation and extraction of ubiquinone from lyophilized chromatophores using isooctane were performed as previously described (10).

EPR measurements were performed on a Bruker ESP300E spectrometer (23). 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 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. Sample concentrations were typically 10 $\mu$M reaction concentration. Further specific experimental details are provided in the figure legends.

Flash-activated turnover of the cyt $bc_1$ complex was performed on a Biomedical Johnson Foundation single-wavelength spectrophotometer (University of Pennsylvania) fitted with an anaerobic redox cuvette as previously described (23).

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI), except for the cyt $bc_1$ inhibitors stigmatellin and antimycin, which were purchased from Sigma Chemical Co. (St. Louis, MO). 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 spectrum (10).

RESULTS

Inhibition of $Q_O$ Site Function by DPA with the $Q_{pool}$ Fully Oxidized Prior to Activation. When the $Q_{pool}$ is fully oxidized prior to flash activation, the $Q_O$ site contains only oxidized ubiquinone (Q) at the time of activation and the substrate $QH_2$ is provided by the $Q_b$ site of the photosynthetic reaction center. Under these conditions, the $QH_2$ released from the $Q_b$ site must diffuse to the $Q_O$ site and displace the resident $Q$ prior to being oxidized itself. Figure 1 shows the effect of DPA upon the kinetics of the cyt $bc_1$ complex, where incremental additions of DPA exert a parallel effect upon the re-reduction of cytochromes $b$ and $c$.

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In both cases, DPA decreases the rate constants but not the overall amplitude of the reduction (cyt $b$) or re-reduction (cytochromes $c$) processes, respectively. These observations imply that DPA appears to be behaving as a typical competitive inhibitor of the $Q_b$ site. Figure 2 shows the inhibition profile of DPA fitted to a simple inhibition
half-reduced. In this case, upon flash activation, QH₂ either identical within experimental error (25 ± 10 μM).

Inhibition of Qₐ Site Function by DPA with QH₂ in the Q_pool Prior to Activation. Figure 3 shows the effect of DPA upon the kinetics of the cyt bc₁ complex when the Q_pool is half-reduced. In this case, upon flash activation, QH₂ either is initially present in the Qₐ site or is derived directly from the Q_pool. Hence, the overall kinetics are faster than those requiring diffusion of QH₂ generated at the Qₐ site, as described above. Figure 3A illustrates the behavior of the total cyt c re-reduction kinetics under multiple flash turnover conditions. From a comparison of the kinetic traces presented in Figures 1 and 3, it is apparent that the inhibition of cytochromes b and c follows the same pattern and is independent of the Q_pool redox state. This observation is confirmed in Figure 4, where the inhibition profile of DPA is fitted to the inhibition equation for both cyt b and c reduction data when the Q_pool is fully oxidized prior to flash activation. It is clear that DPA inhibits cyt b reduction and cyt c re-reduction in the same fashion, and the inhibition constants (Kᵢ) are identical within experimental error (25 ± 10 μM).

Evidence for Specific Inhibition of the Qₐ Site by DPA. It is clear from the total cyt c re-reduction traces presented in Figure 3A that DPA inhibition over the concentration range investigated is manifested at the level of the Qₐ site and not at the Qₐ or Qₙ site of the reaction center. Protocols facilitating a closer examination of the inhibition at the Qₐ site are well-established (24, 25). If DPA had affected the function of the Qₐ or Qₙ site, then upon addition of increasing amounts of DPA under multiple saturating flash turnover of the reaction center (as is occurring in Figure 3A), the amplitude of the total cyt c oxidation signal with successive light flashes would dramatically decrease, which is clearly not the case. Analysis of the rate constants for charge recombination to the bacteriochlorophyll special pair (P⁺) provides direct information with regard to the reaction center Qₙ site occupancy by ubiquinone. The charge recombination is more rapid when the Qₙ site is inhibited, since the process occurs directly from ubiquinone in the Qₙ site (14.4 vs 0.9 s⁻¹) (10, 26). Addition of 1 mM DPA has no effect on the charge recombination kinetics, providing further evidence that DPA does not inhibit Qₙ site function (data not shown).

EPR Spectral Changes of the [2Fe-2S] Cluster Induced by Inhibiting Amounts of DPA. Figure 5 shows the concentration dependence of the DPA effect upon the EPR spectral line shape of the [2Fe-2S] cluster. In the absence of DPA,
the [2Fe-2S] cluster EPR line shape is characteristic of the native QO site fully occupied by ubiquinone with a gₓ resonance at 1.800. Significantly, there is no detectable change in the [2Fe-2S] EPR spectrum at DPA concentrations which completely inhibit the QO site kinetics (20-fold higher than the Ki value). However, the high concentrations of DPA (>10 mM) is very similar to that obtained for Q-extracted chromatophore membranes, when there is only one Q present in the QOS domain of the QO site (gₓ, resonance at 1.783). In both cases, the line shape and position of the gₓ resonance are the same and are at a value of 1.783. There are two possible explanations for this. (a) At high concentrations, the DPA-induced EPR spectral change could be due to DPA itself interacting directly with the [2Fe-2S] cluster and coincidentally yielding the same line shape. (b) Alternatively, DPA binding in the QO site and displacing Q from the QOW domain results in an EPR spectral line shape reporting the occupancy of only the QOS domain by Q. One line of evidence that DPA does not interact directly with the [2Fe-2S] cluster, even at high concentrations, is that in extensively Q-extracted chromatophores (such that the QO site is devoid of Q), no change occurs in the EPR spectral line shape of the [2Fe-2S] cluster (gₓ, resonance at 1.765), even at 100 mM DPA. Furthermore, as shown in Figure 6, addition of up to 100 mM DPA has no effect on the [2Fe-2S] cluster EPR spectral line shape in partially Q-extracted chromatophores, such that only the QOS domain of the QO site is occupied (gₓ, resonance at 1.783), thus demonstrating that DPA does not mimic the EPR effect of Q in the QOW domain. In light of this result, it seems clear that explanation (b) is correct. Although DPA appears to displace Q from the QOW domain at high concentrations, it does not do so at sub-millimolar concentrations, since if this were to occur, the [2Fe-2S] cluster EPR spectral line shape would be indicative of one Q in the QOS domain of the QO site (gₓ, resonance at 1.783), which is clearly not the case (Figure 6). Under all conditions, addition of the tight binding QO site specific inhibitor, stigmatellin to chromatophores produced the characteristic stigmatellin-induced [2Fe-2S] EPR spectral line shape with a prominent gₓ resonance at 1.785 (10, 28), implying that stigmatellin overrides the DPA effect, even in the presence of 100 mM DPA (Figure 6). The effect of high concentrations of DPA (>10 mM) upon the [2Fe-2S] cluster EPR spectral line shape is not
The effect of saturating amounts of DPA (100 mM) on the cyt bc₁ complex redox midpoint potential is illustrated in Figure 7. The midpoint potential of the one-electron [2Fe-2S]²⁺/²⁺ couple is slightly elevated by about 30 mV in the presence of DPA (28). This implies that DPA binds with an about 5-fold greater affinity to the Q₀ site of the cyt bc₁ complex when the [2Fe-2S] cluster is in the reduced state.

**DISCUSSION**

Mechanism of Q₀ Site Inhibition by DPA. Figure 8 shows a schematic representation of DPA interacting with the Q₀ site and depicts a possible mechanism for the inhibition process, based on the two-Q/QH₂ site model of Ding et al. (10, 11). In part A, the arrow indicates the rapid equilibrium for exchange of Q/QH₂ in the Q₀w domain of the Q₀ site with the Qpool (10, 11), with the rate of exchange governing the turnover kinetics of the cyt bc₁ complex. Part B illustrates the noncompetitive Q₀ site inhibition exerted by sub-millimolar concentrations of DPA, where the interaction of Q/QH₂ in the Q₀ site with the [2Fe-2S] cluster is not disrupted, but QH₂ oxidation is impeded by DPA binding at a tertiary position in or near the Q₀ site. As yet, we are unsure of the actual physical mechanism for Q₀ site inhibition by DPA; however, it cannot solely be due to DPA impeding exchange of Q/QH₂ in the Q₀w domain with the Qpool, since the inhibition kinetics are independent of the oxidation state of ubiquinone in the Q₀ site. Part C shows displacement of Q/QH₂ in the Q₀w domain by high concentrations of DPA, where the [2Fe-2S] cluster EPR spectral line shape reports the occupancy of the Q₀s domain by Q/QH₂ and an empty Q₀w domain. It should be noted from the data summarized in Figure 7 that the model of DPA inhibition is also compatible with a high degree of plasticity with respect to the number of Q/QH₂ occupants of the Q₀w domain within the Q₀ site. In light of the obtained results, the most important point to arise from this report is that the effect of DPA upon Q₀ site catalysis is quite readily explained in terms of the double-occupancy model as originally proposed by Ding et al. (10).

Comparison of Q₀ Site Inhibition by DPA with Other Inhibitors. Q₀ site specific cyt bc₁ complex inhibitors have been conveniently grouped according to their inhibiting action (17). Group I includes the β-methoxyacrylates, for
example, myxothiazol (29) and methoxy-acrylate-stilbene (30–33), which typically bind with sub-nanomolar dissociation constants and inhibit electron transfer from QH$_2$ to the [2Fe-2S] cluster of the FeS subunit and onto cyt $b_6$ but, in the case of myxothiazol at least, do not inhibit reduction of cytochromes $c$ by the [2Fe-2S] cluster (not yet determined for MOA-stilbene). Group II includes the hydroxyquinone analogues, for example, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) (34) and 3-alkyl-2-hydroxy-1,4-naphthoquinones (alkyl HQN) (35), which bind with a lower affinity than the group I inhibitors and specifically affect the properties of the [2Fe-2S] cluster but not cyt $b_6$. They act by inhibiting electron transfer from the [2Fe-2S] cluster to cyt $c_1$, as well as onto cyt $b_6$. The chromone inhibitors typified by stigmatellin exhibit properties of both group I and II inhibitors, binding tightly with sub-nanomolar $K_d$ values and inhibiting electron transfer from QH$_2$ to the [2Fe-2S] cluster and cyt $b_6$, and [2Fe-2S] to cyt $c_1$. (36). From the properties of DPA presented in this paper, it is clear that this inhibitor cannot be exclusively classified into any one of these classic groups since it appears to have properties in common with several of the above-mentioned inhibitors from different groups, as well as possessing unique inhibiting characteristics of its own. Like DPA, MOA-stilbene also has been shown to bind noncompetitively to the Q$_0$ site of the mitochondrial cyt $b_1$ complex (30). Moreover, it is interesting to note that when the complex is fully reduced, two MOA-stilbene molecules are able to bind to the Q$_0$ site. However, the Q/QH$_2$ occupancy of the Q$_0$ site was not determined under these conditions, and the identity of the cofactor whose reduction controls this binding change is currently unknown (33). It is not yet established whether binding of one or two MOA-stilbene molecules at the Q$_0$ site affects the [2Fe-2S] cluster EPR spectral properties; this is presently under investigation (R. E. Sharp and P. L. Dutton, experiments in progress).

**Comparison of cyt $b_1$ Complex Inhibition by DPA with the Effect of Q$_0$ Site Mutations.** Site-directed mutations of Y147 $\rightarrow$ S/A in the cyt $b$ subunit of the *R. capsulatus* cyt $b_1$ complex severely disrupt Q$_0$ site catalysis, but from the appearance of the [2Fe-2S] cluster EPR spectral line shape, they do not induce loss of ubiquinone from the Q$_0$ site (37). The effect of these mutations appears to be very similar to that of DPA described in this paper. Electron transfer from QH$_2$ in the Q$_0$ site to the [2Fe-2S] cluster and cyt $b_6$ is impeded by these mutations. In the cyt $b_1$ complex crystal structures, Y147 is located close to heme $b_6$ and at one end of the presumed Q$_0$ site (5–8). The effect of the mutations was interpreted as a strict requirement for tight side chain packing and a high degree of hydrophobicity in establishing efficient electron transfer from QH$_2$ to the [2Fe-2S] cluster and cyt $b_6$ (37). In light of these findings, we suggest that DPA may inhibit by binding in the vicinity of Y147 and perhaps disrupt the protein packing, hindering efficient QH$_2$ oxidation in the Q$_0$ site.

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