Inhibition of Electron Transfer by 3-Alkyl-2-hydroxy-1,4-naphthoquinones in the Ubiquinol-Cytochrome c Oxidoreductases of Rhodopseudomonas sphaeroides and Mammalian Mitochondria

INTERACTION WITH A UBIQUINONE-BINDING SITE AND THE RIESKE IRON-SULFUR CLUSTER*

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3-Alkyl-2-hydroxy-1,4-naphthoquinones (alkyl-HNQ) inhibit Rieske iron-sulfur cluster (Rieske FeS) oxidation and cytochrome b reduction in ubiquinol-cytochrome c oxidoreductase. The effects are the same as those of 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole. Concentrations for 50% inhibition in chromatophores of Rhodopseudomonas sphaeroides (at 0.4 μM reaction center) are 2 μM for undecyl-, 3 μM for octyl-, and 40 μM for pentyl-substituted hydroxy-naphthoquinones. The ethyl-substituted and unsubstituted derivatives do not inhibit electron transfer below 2 mm. In chromatophores in which the ubiquinone is partially extracted by iso-octane (leaving 4 ubiquinones/reaction center), undecyl-HNQ is effective at 2.5 times lower concentration than in normal chromatophores (30 ubiquinones/reaction center). This observation suggests that the binding of the inhibitor is competitive with ubiquinone. Undecyl-HNQ eliminates the effect that the ubiquinone redox state has on the line shape of the EPR signal of Rieske FeS. This supports the idea that alkyl-HNQ shares a common binding site with ubiquinone which is closely associated with Rieske FeS. The ubiquinone inhibition has a midpoint oxidation-reduction potential at pH 7 of 90 mV with a -60 mV/pH unit dependency. This value matches that of the ubiquinone pool rather than that of ubiquinone Z, which is functionally recognized as a component "between" cytochrome b and Rieske FeS. When Rieske FeS is oxidized, a 20 times higher concentration of undecyl-HNQ is required for the electron transfer inhibition. This is consistent with the observation that the binding of the inhibitor shifts the midpoint oxidation-reduction potential of Rieske FeS about 60 mV higher, which in turn means that the inhibitor binds about 10 times stronger to the site when Rieske FeS is reduced than when it is oxidized. The observations suggest that 3-alkyl-2-hydroxy-1,4-naphthoquinones inhibit electron transfer by acting as ubiquinone antagonists at a site closely associated with Rieske FeS.

Q-c oxidoreductase is a multiredox center protein complex.

* This work was supported by Grant GM 27309 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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The abbreviations used are: Q-c oxidoreductase, ubiquinol-cytochrome c oxidoreductase; alkyl-HNQ, 3-alkyl-2-hydroxy-1,4-naphthoquinone; Rieske FeS, Rieske iron-sulfur cluster; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TMPD, N,N,N',N'-tetramethylphenylenediamine.

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yeast mitochondria at submicromolar concentrations without affecting NADH-ubiquinone oxidoreductase activity. Bowyer and co-workers (14–16) have shown in R. sphaeroides and Rhodopseudomonas capsulata that UHDBT inhibits cytochrome c1 reduction by Rieske FeS and cytochrome b reduction and that UHDBT changes the EPR spectral shape and midpoint potential of Rieske FeS (15). Similar findings have been found in mammalian mitochondrial preparations (8, 17–19). These results, together with the structural similarity of UHDBT to ubiquinone, suggest that UHDBT might bind to a ubiquinone-binding site which is closely associated with Rieske FeS.

3-Alkyl-2-hydroxynaphthoquinones have also been known as electron transfer inhibitors between cytochromes b and c1 in mitochondria (20–25). However, the site of action is not well characterized. In this paper, we report that the alkyl-HNQ inhibition site is the same as the UHDBT site in R. sphaeroides and mitochondrial preparations. We also describe properties of the ubiquinone bound at the site to which the alkyl-HNQ appears to competitively bind.

**MATERIALS AND METHODS**

*R. sphaeroides* Ga was grown photosynthetically and chromatophores were prepared as described previously (26). Partial extraction of ubiquinone from chromatophores was performed with isoctane in lyophilized chromatophores (27). Ubiquinol-cytochrome c oxidoreductase was isolated from beef heart mitochondria (28) and reaction centers were isolated from chromatophores of *R. sphaeroides* R 26 (29).

Flash-induced redox changes of cytochromes b and c were measured in a dual wavelength spectrophotometer at controlled redox potential (26). EPR measurements were made with a Varian E109 spectrometer equipped with a flowing helium cryostat. EPR samples were taken from a redox-poised chromatophore suspension in the dark and frozen rapidly using methylcyclohexane/isopentane (1:5, v/v) at 81 K. 3-Alkyl-2-hydroxy-1,4-naphthoquinones were purchased from Aldrich Chemical Co. and added as a solution in ethanol or dimethyl sulfoxide.

**RESULTS**

**Effects of Alkyl-HNQ on Light-induced Electron Flow in Chromatophores**—Fig. 1 shows the oxidation-reduction kinetics of cytochromes b and c induced by flash illumination of chromatophores. The ambient redox potential of the chromatophore suspension was adjusted to optimal values (e.g. 40 mV at pH 8.0) for rapid electron transfer through the Q-c oxidoreductase; i.e. Qc reduced before activation (30–32). The absorbance difference, measured at 560–570 nm, represents changes of cytochrome b560 of which about half was oxidized before activation under the conditions of Fig. 1 (33, 34). The absorbance difference measured at 550–540 nm represents changes of cytochromes c1 and c2 since both were fully reduced before activation. Because cytochromes c1 and c2 react rapidly with each other and have similar spectra and midpoint potentials (35), we describe the 550–540-nm changes as redox changes of both cytochromes c1 + c2. In the traces in Fig. 1, the upward direction corresponds to the reduction of the cytochromes. In the absence of electron transfer inhibitors, the traces show (a) flash-induced rapid oxidation of cytochromes c1 + c2 followed by subsequent dark re-reduction, and (b) flash-induced reduction of cytochrome b560 followed by re-oxidation. All changes virtually relaxed within 5 ms after the flash; furthermore, another flash delivered 8 ms after the first elicits the same redox changes, implying very rapid return to the pre-activated condition. In contrast, when 10 μM undecyl-HNQ is present (Fig. 1, + Undecyl-HNQ) the amplitude of the redox changes of cytochrome b560 is considerably diminished while with cytochromes c1 + c2 there is an enhancement of the amplitude of photooxidation and the re-reduction becomes very slow. The presence of antimycin inhibits cytochrome b560 re-oxidation and cytochrome c1 + c2 re-reduction. Antimycin alone causes an increase in the amplitude of cytochrome c1 oxidation which reaches a maximum value after one flash. The extent of cytochrome c1 + c2 oxidation after the first flash is similar to that without antimycin but the re-reduction is very slow. The second flash elicits a further increase in the level of cytochromes c1 + c2 oxidized, again with slow re-reduction kinetics. Almost all cytochromes c1 + c2 are oxidized after the second flash because subsequent flashes induce little further oxidation as reported previously (26). When undecyl-HNQ is added together with antimycin (Fig. 1, + Antimycin Undecyl-HNQ), no reduction of cytochrome b560 occurs following the flash and the extent of cytochrome c1 + c2 oxidation on the first flash is almost doubled, approaching the same level as that which required two flashes with antimycin alone.

These observations with undecyl-HNQ are the same as those reported by Bowyer and co-workers (14–16) using UHDBT. The enhancement by UHDBT of cytochromes c1 + c2 oxidation was explained by the existence of Rieske FeS which, in the absence of the inhibitor, is in rapid redox equilibrium with the roughly isopotential cytochromes c1 + c2. Thus, during the oxidation of cytochromes c1 + c2 by the photooxidized bacteriochlorophyll dimer, re-reduction by Rieske FeS is occurring concurrently. Therefore, the actual full extent of oxidation of cytochromes c1 + c2 following a flash cannot formally be observed. Inhibition by UHDBT or undecyl-HNQ of the electron transfer from Rieske FeS to cytochromes c1 + c2 explains the observed enhancement of the
oxidation of cytochromes $c_1 + c_2$. This inhibition of Rieske FeS oxidation was shown directly by EPR measurements of chromatophores frozen after flash excitation with and without UHDBT (15).

The concentrations of undecyl-HNQ required for the inhibition of cytochrome $b_{550}$ reduction and cytochrome $c_1 + c_2$ re-reduction and the enhancement of cytochrome $c_1 + c_2$ oxidation are identical as was shown previously with UHDBT (16). Although this is not proof, it certainly suggests that binding of the inhibitor to one site in the Q-c oxido-reductase induces these multiple effects of alkyl-HNQ or UHDBT. No inhibition of electron transfer from the primary quinone (designated $Q_1$ or $Q_4$) to secondary quinone ($Q_2$ or $Q_3$) in the reaction center was observed by undecyl-HNQ below 200 $\mu$M in R. sphaeroides chromatophores (data not shown).

When the $E_a$ is lowered so that most of the cytochrome $b_{550}$ is reduced before activation, the observed flash-induced oxidation of cytochrome $b_{550}$ (34) is also inhibited by undecyl-HNQ. The electrogenic reaction associated with electron transfer through Q-c oxido-reductase (carotenoid band shift phase II) (36) is also abolished by undecyl-HNQ. The effect on the carotenoid band shift is not an unrelated uncoupling effect because the rate of decay of the membrane potential ($t_{1/2} = 2$ s) as indicated by the carotenoid band shift (phases I and II only) was not affected by fully inhibiting levels (e.g. 10 $\mu$M) of undecyl-HNQ. The inhibitor only induced about a 30% acceleration of the decay when added at a large excess concentration of 200 $\mu$M.

Fig. 2 shows the concentration dependency of several alkyl-HNQ homologues on the cytochrome $c_1 + c_2$ re-reduction rate. The undecyl-HNQ concentration required for the 50% inhibition is about 2 $\mu$M, which is similar to the reported value for UHDBT (16). Octyl-HNQ is a little less effective and pentyl-HNQ is effective at about 20 times higher concentrations for the inhibition of cytochrome $c_1 + c_2$ re-reduction. Similarly, higher concentrations of pentyl-HNQ were needed for the inhibition of cytochrome $b_{550}$ reduction. Ethyl-HNQ and HNQ without an alkyl chain do not inhibit flash-induced electron transfer below 2 or 4 mM, respectively. 3-Undecyl-2-methyl-1,4-naphthoquinone has no effect on the electron transfer below 200 $\mu$M (data not shown). Thus, it appears that the hydroxy substituent is required for inhibition and that increasing the length of the alkyl side chain has a marked effect on the 50% inhibition value, probably due to the increased hydrophobicity it conferns on the HNQ. The fact that the length of the alkyl side chain has a similar effect on the concentration dependencies of inhibition on both cytochrome $c_1 + c_2$ re-reduction and cytochrome $b_{550}$ reduction further supports the proposal that the inhibitor affects both reactions by binding to a single site.

**Dependence of Electron Transfer Inhibition by Undecyl-HNQ on the Redox State of the Rieske FeS Cluster**—Fig. 3 shows the electron transfer inhibition by undecyl-HNQ under various conditions. When Rieske FeS is oxidized before activation (Fig. 3, 380 mV, $\bullet$), the flash-induced reduction of cytochrome $b_{550}$ (antimycin present) is much less sensitive to undecyl-HNQ than when Rieske FeS is reduced before activation; in fact, about 20 times more undecyl-HNQ is required to inhibit the cytochrome $b_{550}$ reduction than when Rieske FeS is reduced (Fig. 3, 180 mV, $\bullet$). This suggests that binding of the inhibitor to the inhibitory site when Rieske FeS is oxidized is about 20 times weaker than when Rieske FeS is reduced.

**Dependence of Electron Transfer Inhibition on the Total Ubiquinone Content of the Chromatophores**—The effect of partial quinone extraction on the concentration of undecyl-HNQ required for inhibition is also shown in Fig. 3. In chromatophores which have 4 ubiquinones/reaction center, the inhibitor is effective at 2.5 times lower concentration ($\bigcirc$) compared to when studied under the same conditions in normal chromatophores ($\bigtriangleup$) which have 30 ubiquinones/reaction center (Fig. 3). This difference can be explained by the idea that the binding of undecyl-HNQ is competitive with the ubiquinone pool; however, the fact that the extraction may have simply removed hydrophobic material (quinone pool included) into which the undecyl-HNQ would partition obviously cannot be ruled out.

**The Dependence of Electron Transfer Inhibition on the Redox State of the Ubiquinone Pool and $Q_{2}$**—The inhibitory effect appears to be independent of the redox state of the ubiquinone pool as also shown in Fig. 3. The sensitivity of cytochrome $c_1 + c_2$ reduction to undecyl-HNQ is the same when 90% of the pool ubiquinone is reduced before activation ($E_a$, 0 mV, pH 8.0, $\bigtriangleup$) as when most of the pool ubiquinone is oxidized but $Q_2$ is reduced ($E_a$, 60 mV, pH 8.0, $\bigtriangleup$). Similarly, there is no measurable difference in the inhibitor sensitivity when, in addition to the ubiquinone pool, $Q_2$ is also oxidized ($E_a$, 180 mV, pH 8.0, $\bigtriangleup$). This shows that, if the binding of
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Undecyl-HNQ is competitive with ubiquinone, reduced and oxidized forms of ubiquinone are quite similarly competitive with undecyl-HNQ.

Effects of Undecyl-HNQ on the Midpoint Potential and EPR Spectrum of Rieske FeS—Undecyl-HNQ shifts the midpoint oxidation-reduction potential (E_m) of the Rieske FeS cluster as was shown with UHDBT (15). The amplitude of the g 1.90 EPR signal, which represents the Rieske FeS in the reduced state, is shown in Fig. 4, as a function of ambient redox potential (pH 8.0). The E_m (pH 8.0) of Rieske FeS is 295 mV in the chromatophores with 200 μM undecyl-HNQ. This is 45 mV higher than that determined under the same conditions but without the inhibitor. The addition of 300 μM more inhibitor shifted the E_m (pH 8.0) only 10 mV further, indicating that a 200 μM concentration of inhibitor had almost saturated the shift. The direction and the value of the shift are similar to that reported with UHDBT (ΔE_m + 70 mV) (15). One possible explanation for the E_m shift is that undecyl-HNQ binds stronger to the reduced Rieske FeS than to the oxidized form. Simple thermodynamics predicts that, if a ligand binds to the reduced form of an n = 1 redox component 10 times stronger than to the oxidized form, the E_m value becomes 60 mV more positive at a saturating concentration of ligand (37). This prediction is consistent with the above finding that when Rieske FeS is oxidized before activation the 50% inhibition concentration is approximately 20-fold higher than that found when the Rieske FeS is reduced.

Further evidence of an interaction between undecyl-HNQ and Rieske FeS is the effect of the inhibitor on the EPR spectrum of the reduced Rieske FeS. As shown in Fig. 5, at E_m 0 and 95 mV (pH 8.0), undecyl-HNQ shifts the g, and g, bands of about -300 mV at pH 8.0 (data not shown). This value is in good agreement with the E_m of ethyl-HNQ in aqueous solution (~270 mV, determined polarographically by R. C. Prince). Therefore, the reduced quinol form of undecyl-HNQ may not bind as strongly as the oxidized quinone form to the Rieske site; alternatively, if it binds similarly, the interaction between reduced undecyl-HNQ and Rieske FeS cluster may be very different from that between oxidized undecyl-HNQ and the cluster. It is difficult to examine the inhibitory effect of the reduced undecyl-HNQ on electron transfer activities in chromatophores because the E_m of alkyl-HNQ is much lower than that of Q_b of the reaction center. Thus, at potentials when alkyl-HNQ is reduced, Q_b will also be reduced which in turn inhibits useful photochemistry in the reaction center. However, UHDBT has an E_m value of ~40 mV in aqueous solution (38) which is more suitable to test the inhibitory effect of the reduced form of the alkylhydroxyquinone inhibitor. Bowyer et al. (15) reported that the disappearance of the UHDBT effect on the EPR spectrum of Rieske FeS had an E_m of ~50 mV, an effect analogues to that described above for undecyl-HNQ. We find that, in chromatophores of R. sphaeroides (0.4 μM reaction center) at pH 6.0, 12 μM UHDBT (E_m, 20 mV) has a 90% inhibitory effect on cytochrome c1, c c1 reduction and cytochrome b reduction and oxidation at E_m 150 mV but little effect at 40 mV (data not shown). This observation, by analogy, suggests that alkyl-HNQ would also become less effective as an inhibitor when it is reduced.

The Midpoint Potential of the Ubiquinone Which Affects the EPR Spectral Shape of Rieske FeS—It is known that the EPR spectral shape of Rieske FeS in subtomochondrial particles and isolated Q-c oxidoreductases is dependent on the redox state of a component in the complex (39, 40). Recently, J. R. Bowyer and T. Ohnishi1 showed by ubiquinone-10 extraction and reconstitution that this component appeared to be ubiquinone in beef heart submitochondrial particles. A similar spectral change of the g, band around g 1.81 is shown in Fig. 5 in R. sphaeroides chromatophores between 95 and ~2 mV. At 95 mV, there is a peak at g 1.81, but at ~2 and ~450 mV, this peak becomes very broad around g 1.79. A slight broadening of the g, signal is also observed. The spectral change in chromatophores induced by the potential change is diminished reversibly by ubiquinone extraction and reconstituted with ubiquinone-10 (data not shown). The spectral change was also eliminated by the addition of undecyl-HNQ (Fig. 5). The observations suggest that the binding site for the ubiquinone and undecyl-HNQ is the same or closely related.

Fig. 4 shows redox titrations of this ubiquinone at various pH values. The titration data at pH 8.0 and 9.0 best fit n = 2 curves. We note, however, that the data at pH 6.0 and 7.0 deviate somewhat from n = 2 curves, but further work would be required to establish this with more certainty. The mid-point potentials have a ~60 mV/pH unit dependency and the E_m is 90 ± 10 mV. The E_m value is very similar to pool ubiquinone in the chromatophore membrane (27) and is about 60 mV lower than that of Q_b which has been shown to be a functionally important ubiquinone bound in the Q-c oxidoreductase of R. sphaeroides (30–32).

In chromatophores from which 85% of the ubiquinone is extracted (leaving 4 ubiquinones/reaction center), the reduced Rieske FeS still displayed the same spectral change dependent

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1 R. C. Prince, personal communication.

1 J. R. Bowyer and T. Ohnishi, manuscript in preparation.
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on the ubiquinone redox state (data not shown). On the other hand, in chromatophores from which 93% of the ubiquinone is extracted (leaving 1.9 ubiquinones/reaction center; QA is fully active and Q$_b$ is 40% active), no change in the reduced Rieske FeS spectrum was observed when the $E_0$ was changed over the same range. These observations, although preliminary, suggest that the ubiquinone in question binds to the Rieske FeS site in the absence of the most of the pool ubiquinone, although the binding affinity is weaker than those of the Q$_b$ and QA sites in the reaction center complex.

Effects of Undecyl-HNQ in Isolated Ubiquinol-Cytochrome c Oxidoreductase (bc$_1$ Complex) from Beef Heart Mitochondria—Recently, a light-induced cyclic electron transfer system similar to that in membranes of _R. sphaeroides_ has been constructed in detergent solution with isolated Q-c oxidoreductase (beef heart), cytochrome c (horse heart), and reaction center (_R. sphaeroides_) (7, 8). UHDBT was shown to have a similar effect on the flash-induced cytochrome b and c kinetics as that in chromatophores (8). Fig. 7 shows the effect of undecyl-HNQ on flash-induced cytochrome b and c. The change in this hybrid system is similar to that shown for chromatophores in Fig. 1. A positive shift in midpoint potential of Rieske FeS and an EPR spectral change similar to that shown in Figs. 4 and 5 were also observed in mitochondrial preparations using another long chain alkyl-HNQ, 10-(4-cyclohexyloxy)decyl-HNQ (data not shown).
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**Fig. 7.** Effect of undecyl-HNQ on the flash-induced oxidation-reduction kinetics of cytochromes b and c in a mixture of mitochondrial Q-c oxidoreductase, cytochrome c, and reaction centers from *R. sphaeroides*. 4 μM (in c) Q-c oxidoreductase, 8 μM cytochrome c, 1 μM reaction centers were mixed in 10 mM Tris-HCl, pH 8.0, containing 19 μM DAD, 10 μM pyocyanine at E0 40 mV. 50 μM undecyl-HNQ was added where indicated.

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**DISCUSSION**

The Role of the Quinone Ring Substituents of Alkyl-HNQ and UHDBT in the Inhibition of Electron Transfer

Alkyl-HNQ is a potent inhibitor of electron transfer in the Q-c oxidoreductase of the photosynthetic bacteria and mitochondria. The inhibitory pattern of alkyl-HNQ is essentially identical with that of UHDBT, and is different from that of antimycin. This similarity between alkyl-HNQ and UHDBT is understandable from the similarity of their chemical structures. Some points on these similarities and differences yield the following conclusions.

The Nature of the Adjacent Ring—The results presented in this paper indicate that nitrogen and sulfur atoms in the thiazole ring of UHDBT do not have any inhibitory role for the inhibitory action.

The Hydroxyl Group—The hydroxyl group ortho to the quinone oxygen seems to be essential for inhibition. When the hydroxyl group was substituted by a methyl group, no inhibitory effects were observed even at 100 times higher concentrations.

The Alkyl Group—The role of the alkyl group may be to raise the hydrophobicity of the molecule and to promote access and binding in the inhibition site. Whether an isoprenoid chain will prove to have a structural contribution to inhibition beyond the simple hydrophobic effect remains to be determined.

The 1,4-Dione Groups—There are indications that the quinone form of UHDBT makes a strong contribution to the inhibitory potency. Lowering the redox potential to a point where UHDBT would be expected to be predominantly in its quinol form releases the system from inhibition. The coincident loss of effect on the EPR signal of Rieske FeS suggests the existence of the EPR spectral shape of Rieske FeS is evident.

Although undecyl-HNQ has a similar effect to UHDBT, UHDBT may be a slightly more potent inhibitor than undecyl-HNQ. The concentration of UHDBT for 50% inhibition in *R. capsulata* chromatophores was reported to be 3.5 μM at a reaction center concentration of 1.5 μM (16). This concentration is 2 times lower than that of undecyl-HNQ shown in Fig. 2 when the numbers are compared for the same reaction center concentration. Roberts et al. (13) have reported that UHDBT is effective at 2.5 times lower concentrations than undecylthio-HNQ in inhibiting mitochondrial NADH oxidase activity.

**pH Dependencies of Inhibition**

Trumpower and Haggerty (17) reported that the inhibitory effect of UHDBT on mitochondrial succinate-cytochrome c oxidoreductase activity is dependent on pH, becoming less effective as the pH is raised, yielding an apparent pK in the region of 7.2-7.5. A similar pH dependence was reported with (2-methyloctyl)-HNQ and cyclohexylpropyl-HNQ in mitochondrial succinate oxidase activity (20). Because the pK of UHDBT is 6.5 (38), but that of alkyl-HNQ is around 5.8, it would be reasonable to consider that there may be a common ionizable group related to the effect, a group perhaps belonging to the Q-c oxidoreductase rather than to the inhibitors. However, such a pH effect on the effectiveness of inhibition was not observed in flash-activated experiments of chromatophores with undecyl-HNQ from pH 5.0 to 8.0. Similarly, Bowyer et al. (15) reported almost no pH dependence of the effect of UHDBT in chromatophores from pH 7.0 to 10.5. Furthermore, J. R. Bowyer and B. L. Trumpower (4) have observed that the effect of UHDBT on oxidant-induced reduction of cytochrome b (18) is less dependent on pH than the steady state electron flow in a mitochondrial preparation. Thus, it is conceivable that the reported pH dependencies of the inhibition by UHDBT or alkyl-HNQ of succinate oxidase activity may be related to the steady state condition of the measurements. One possible source for such apparent pH dependent effects on inhibition could be the pH-dependent redox state of Rieske FeS in the steady state electron flow conditions. The results in Fig. 3 indicate that, if most of the Rieske FeS remains oxidized at the higher pH during the steady state experiments, the requirement for higher concentrations of UHDBT or alkyl-HNQ is expected.

Because of the pK value of 5 on the oxidized form of alkyl-HNQ in aqueous solution, most of the alkyl-HNQ is in the ionized form at neutral pH in solution. This does not necessarily mean that the bound form of the inhibitor is also anionic. The results with various alkyl substituents (Fig. 2) suggest that the hydrophobicity of the molecule is important for the binding of inhibitors. If the forces governing the inhibitor binding in the site of the Q-c oxidoreductase are predominantly hydrophobic and uncharged, the binding form of alkyl-HNQ is more likely to be the neutral form. However, if the anionic charge of the head group of the inhibitor is neutralized or delocalized by protein groups at the binding site, the specific binding of the anionic form to a part of the otherwise hydrophobic site is also possible.

The Binding of Undecyl-HNQ and the EPR Interaction with Rieske FeS

The effects of undecyl-HNQ on the midpoint potential and the EPR spectral shape of Rieske FeS suggest the existence of an interaction, direct or indirect, between the bound inhibitor and the Rieske FeS cluster (Figs. 4 and 5). The shift of the g1 band (g 1.90) to a lower g value, 1.89, and g, band (g 2.03) to a higher g value, 2.04, represents an increase in the

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*4 J. R. Bowyer and B. L. Trumpower, unpublished work.*
rhombic distortion of the Rieske FeS cluster by the binding of the inhibitor (41, 42). The change of rhombic distortion could be caused by a configuration change of the FeS cluster or by a change in electrostatic effects of nearby charges on the cluster (42). It would be valuable to determine if the primary cause of the rhombic distortion and the $E_m$ shift are the same. Recently, Malkin (43) reported, using spinach chloroplast membranes, that DBMIB shifts the $E_m$ of Rieske FeS as well as the EPR g value by binding at a site in the chloroplast which probably corresponds to the undecyl-HNQ binding site in the Q-c oxidoreductase of mitochondria and R. sphaeroides. In contrast with the above, however, DBMIB shifts the $E_m$ of the Rieske FeS down from 310 to 180 mV, as well as moving the g band from g 1.89 to g 1.94 (43), which may correspond to a decrease in rhombicity. Thus, the directions of the shifts of the g band and the shift of the $E_m$ value induced by DBMIB are opposite and are of greater magnitude compared to the shifts induced by undecyl-HNQ reported in this paper. This correspondence in the opposite directions of the g value shift (or rhombicity) and the $E_m$ shift (the more positive the $E_m$, the higher the rhombicity) may be more than incidental. While this possible relationship does not hold between different species of two-iron, two-sulfur cluster (42), it might be true when modifying one particular cluster by different ligands.

The Binding of Undecyl-HNQ and the Redox Interaction with Rieske FeS

Undecyl-HNQ binds to oxidized Rieske FeS about 20 times weaker than to the reduced form (Fig. 3). This is roughly consistent with the $E_m$ shift of Rieske FeS by the inhibitor (Fig. 4), as described above (see Ref. 37 for a description of the ligand effect on $E_m$). It should be remembered that the $E_m$ change is induced in the presence of ubiquinone. The $E_m$ of Rieske FeS in mitochondrial membranes is unaffected by ubiquinone extraction, but the $E_m$ value in extracted membranes in the presence of undecyl-HNQ remains to be established.

Applying the same considerations to DBMIB, we would predict from the $E_m$ shift data (43) that DBMIB may bind to oxidized Rieske FeS in the chloroplast membrane about 100 times stronger than to the reduced Rieske FeS. If this is true, DBMIB may be a more potent inhibitor under the condition when Rieske FeS is oxidized in contrast to the case of alkyl-HNQ reported in this paper.

A final historical note is that the results relating to the different binding affinity of undecyl-HNQ, depending on the Rieske FeS redox state, confirm Estabrook’s prediction (22) that 2-methyl-6octyl-HNQ exerts its greatest influence on the electron transport chain when some component between the dehydrogenase and cytochrome c, is reduced.

The Nature of the Binding Site and the Ubiquinone Occupant

The foregoing discussions suggest that the alkyl-HNQ binding site is probably a ubiquinone-binding site. This is supported by the fact that the effect of the ubiquinone redox state on the Rieske FeS spectrum is eliminated by the addition of undecyl-HNQ (Fig. 5) and by the fact that the potency of alkyl-HNQ is increased in chromatophores 85% depleted of the ubiquinone complement (Fig. 3). Competitive binding was demonstrated by Tappel (23) in mitochondria by showing that the inhibition by alkyl-HNQ could be diminished by the addition of ubiquinone-10, -7, or -2.

The experiments with 85% ubiquinone-extracted chromatophores provide an indication of the affinity of the site for ubiquinone. In these preparations, a predominant occupancy of the site was still apparent as evidenced by the effect of redox potential on the EPR signal of Rieske FeS. An 85% ubiquinone-depleted preparation contains about 4 ubiquinones/reaction center. Each reaction center can be seen to have 2 functional ubiquinones so that the other 2/reaction center are available to the Q-c oxidoreductase. Since there is a 1.5- to 2-fold excess of reaction centers over Q-c oxidoreductase in the chromatophore (10, 31, 35), this leaves in the region of 3 or 4 ubiquinones available to each Q-c oxidoreductase instead of the usual 35-50 ubiquinones calculated on the same basis. Thus, despite the approximately 10-fold depletion of the total quinone complement, the presence of a quinone complement is felt by the Rieske FeS and we may presume that the quinone has a significant affinity for a binding site effectively close to the Rieske FeS.

The $E_m$ of this quinone appears to be 90 ± 10 mV and displays an $E_m$/pH dependence of -60 mV from pH 6 to 9 in chromatophores of R. sphaeroides. There is at present no evidence for any major effects that would stabilize the semiquinone form in the site. The redox titrations (Fig. 6) appear to be $n$ = 2 even at pH values as high as 9.0, when stability of a semiquinone species might be expected to be enhanced (see Ref. 45). Thus, it is evident that it is the transition from ubiquinone to ubiquinol that has the effect on Rieske FeS without involvement of the semiquinone. This is not inconsistent with the results of Siedow et al. (49) from experiments with yeast mitochondrial preparations.

We were, in fact, surprised to find that the $E_m$ value of the quinone affecting the Rieske FeS is different from that of Qc; the $E_m$ value and pH relationships are the same as the Q pool ($E_m$ = 90 mV ($n$ = 2); -60 mV/pH unit from 5-9 (Ref. 27)). Qc has been recognized as the rate-limiting reductant for photooxidized cytochrome c$_2$ in the photosynthetic bacteria (30-32). The $E_m$ of Qc is 155 ± 10 mV at pH 7.0 and has a -60 mV/pH unit dependency from pH 5 to 11 in R. sphaeroides and the semiquinone stability constant is low (10, 27). Initially (see Ref. 31), Qc was assumed to be the direct reductant for ferriyctochrome c$_2$, but further studies have revealed two other components between Qc and cytochrome c$_2$, that is, Rieske FeS (14, 15) and cytochrome c$_1$ (35, 44). Qc is currently accepted to be the direct electron donor to the Rieske FeS. However, if the quinone affecting the line shape of the Rieske FeS EPR signal is a functional component which interacts with Rieske FeS on a redox basis, then we should re-evaluate our current views on the nature of the ubiquinone interaction with Rieske FeS. Two alternatives worth considering are as follows.

There Is a Single Binding Site for Ubiquinone Close to the Rieske FeS—The ubiquinone in this site is responsible for the Rieske FeS line shape alteration. It also interacts with the Rieske FeS cluster on a redox basis. These proposals can be reconciled with the experimental observations if the following conditions are met. The relative stability of quinol and quinone forms in the site is similar to that encountered in the Q pool yielding the similar $E_m$ value of 90 mV and the ubiquinone in the site is capable of exchange with the Q pool. In order to obtain the experimentally observed 155 mV $E_m$ value for the reduction of Rieske FeS, the pool size must be large and the rate of exchange with the pool must be much faster than rates of electron transfer. To obtain the observed positive shift of 65 ± 10 mV ($n$ = 2), an excess of about 75-fold quinones/site would be required, together with the capability of obtaining maximal rates of electron transfer when a very small fraction of the pool is in the quinol form (see Ref. 51). As already mentioned, there are in the region of 35-50 quinones/reaction center available to the Q-c oxidoreductase.
nones/oxidoreductase which does approach the experimental limits for the $E_m$ measurement. However, arguing against this is the finding that the measured $E_m$ value for the reduction of Rieske FeS is not altered when the quinones of the chromatophores are partially extracted to yield less than 10 quinones/oxidoreductase; more work is needed in this area.

There Are Multiple Sites for Ubiquinone Binding Close to the Rieske FeS—There is one site for $Q_s$ which, compared to the $Q_p$ pool, exhibits an approximately 100-fold higher affinity for the quinone with respect to the quinol form and leads to the observed $E_m$ value of 155 mV. This site houses the ubiquinone ($Q_2$) which interacts with Rieske FeS on a redox basis, but has no effect on the EPR line shape. Another site, which binds quinol and quinone forms with similar affinities compared to the $Q_p$ pool, affects the EPR line shape of the Rieske FeS. It should be stressed in this alternative that exchange between the sites and with the pool is not precluded, but exchange would be significantly slower than electron transfer rates. This alternative is unsatisfactory because there is no experimental basis to assign any function to the effect of the redox state of the quinone on the EPR line shape of Rieske FeS.

The recognition and definition of quinones in protein-binding sites are obviously important issues, especially with respect to their exchange dynamics about which very little is known. Electrochemical identity of quinones in protein-binding sites is simplified in certain cases by large enhancements of the stability of the semiquinone species compared with the $Q_p$ pool, which leads to easy detection by electron paramagnetic resonance spectroscopy. The primary ubiquinone of the bacterial reaction center is a clear case of a ubiquinone which forms a very stable semiquinone species; in this case, the quinone and semiquinone have very slow exchange kinetics with the site. Work with mitochondrial preparations using EPR has revealed several semiquinone species of quinones associated with the $Q_c$ oxidoreductase. $E_m$ values of 100 (45) and 84 mV (46) have been reported for the couple, $Q_c/QH_2$. The semiquinone signal becomes undetectable following the addition of antimycin, possibly because of displacement of the quinone by the inhibitor. Another semiquinone signal associated with the oxidoreductase was recently observed in the presence of antimycin (47). The functionally defined component $Q_s$ has also been detected in the mitochondrial $Q_c$ oxidoreductase, using the hybrid construction with reaction centers of $R$. sphaeroides (8). Because the methods of recognition of these three quinone species are all different, there is a possibility that some of them are identical. However, recent work (19) suggests that $Q_c$ and the quinone affecting the Rieske FeS EPR signal are different components based on redox titrations of submitochondrial particles with and without antimycin and UHDBT. Thus, while the existence of several quinone-binding sites in $Q_c$ oxidoreductases is very probable, we have much to learn about them.

Speculation on the Mode of Inhibition

We can only speculate about the mechanism of inhibition of electron transfer by the binding of undecyl-HNQ at the Rieske site. There are several puzzling features of the effects of the inhibitor. The emphasis of the results is on the competition between alkyl-HNQ and quinones which in simple terms are viewed to operate on the low potential side of the Rieske FeS. Why then is electron transfer between the Rieske FeS and cytochromes $c_1$ + $c_2$ inhibited by UHDBT and the alkyl-HNQ? The positive shift of $E_m$ of Rieske FeS by alkyl-HNQ could result in some failure of Rieske FeS oxidation after a single flash because of the higher $E_m$ of Rieske FeS than cytochromes $c_1$ + $c_2$. However, even after several flashes spaced at 24 ms, electrons on Rieske FeS are not available to re-reduce bacteriochlorophyll dimer in reaction centers in spite of the almost complete oxidation of bacteriochlorophyll dimer whose $E_m$ is 450 mV. Therefore, the $E_m$ shift is not enough to explain the inhibition of Rieske FeS oxidation by alkyl-HNQ.

We have considered the possibility that there is a quinone operating between the Rieske FeS and cytochrome $c$, but thus far there is no evidence for this from quinone extraction experiments or from counting the reducing equivalents between Rieske FeS and bacteriochlorophyll dimer. However, more detailed work is needed before this possibility can be ruled out.

Another possibility is that the alkyl-HNQ acts as an oxidant for Rieske FeS following flash activation, but again there is no support for this; Rieske remains reduced during the inhibited state (15). A suggestion offered by Velthuys (48) is that if the protein machinery functions to prevent Rieske FeS oxidation when a semiquinone is present at the Rieske site (in order to prevent two-electron oxidation of $QH_2$ by the same oxidizing component) and if alkyl-HNQ mimics the semiquinone, Rieske FeS oxidation will be inhibited by the alkyl-HNQ.

The inhibition of cytochrome $b_{550}$ reduction by alkyl-HNQ when $QH_2$ is present before activation can be easily explained by a secondary effect of the inhibition of Rieske FeS oxidation, if $b_{550}$ reduction is caused by an “oxidant-induced” reduction mechanism (4, 5) as is probably the case. This probably also applies to the cytochrome $b_6$ reduction after the second flash in the chloroplast preparation (see Ref. 50).

There appears to be another route of cytochrome $b$ reduction in photosynthetic bacteria and mitochondria. This is supported in particular by experiments using mitochondrial $Q_c$ oxidoreductase activated by reaction centers without soluble cytochrome $c$. In these experiments, Rieske FeS is reduced before and during the cytochrome $b$ reduction following flash activation, and no oxidant-induced reduction is possible (5). UHDBT and alkyl-HNQ also interfered with this cytochrome $b$ reduction. Here again, however, an oxidized form of an $n = 2$ redox component, possibly a quinone bound to the $Q_c$ oxidoreductase, is shown to be necessary for this route of cytochrome $b$ reduction (5). How this route relates to the oxidant-induced reduction route with respect to the quinone(s) involved and the sites of inhibition is uncertain. This is obviously an elaborate and complicated system and much work remains to be done. However, the experimental system as a whole appears to offer useful access to the mode of inhibition and the mechanism of function of the $Q_c$ oxidoreductase.

Cautionary Notes on the Use of Alkylhydroxyquinones as Inhibitors

The Quinol as an Inhibitor—Reduced UHDBT is markedly less effective as an inhibitor than its oxidized form. This could not be checked directly with the alkyl-HNQs used in this present study. However, the loss of effect on the EPR spectrum of Rieske FeS following the reduction of UHDBT is also found with alkyl-HNQ indicating similar behavior. The $E_m$ of alkyl-HNQ has a value of around $-180$ mV at pH 7 and a $-90$ mV/pH unit dependency from pH 5 to 9 in aqueous...
solution. The $E_m$ (pH 7) of UHDBT is $-40$ mV and displays an almost $-60$ mV/pH unit dependency from pH 3 to 11 (38). Judicious choice of inhibitor and conditions may avoid problems; for example, alkyl-HNQ may be an advantage when a low potential reductant is used in inhibition experiments. The Effect of the Redox State of the Rieske FeS—There is a 20-fold decrease of potency in undecyl-HNQ when the Rieske FeS cluster is oxidized (Fig. 3). Since UHDBT induces a similar $E_m$ shift, it seems likely that this also applies to UHDBT. Uncoupling Effects—Howland (24, 25) has reported that alkyl-HNQ has an uncoupling effect in mitochondria. We also observed a slight uncoupling effect with alkyl-HNQ: 200 $\mu$M undecyl-HNQ is required to stimulate the rate of decay of the membrane potential when Rieske FeS is reduced, the effect on the membrane potential is used in inhibition experiments. An almost undecyl-HNQ is required to stimulate the rate of decay of the ubiquinone extraction. Observed a slight uncoupling effect with alkyl-HNQ: 200 $\mu$M undecyl-HNQ is needed for inhibition; thus, while the uncoupling effect is still relatively small with chromatophores, it may be significant in some experiments with certain organelles and therefore it cannot be dismissed entirely (see also Ref. 25).

Acknowledgments—We are grateful to Dr. Roger Prince for the polarographic information and to Dr. Daniel O'Keefe for his help in the ubiquinone extraction.

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