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IDENTIFICATION OF TWO DIFFERENT Q-BINDING SITES IN QH_2-CYTOCHROME *c* OXIDOREDUCTASE, USING THE Q ANALOGUE *n*-HEPTADECYLMERCAPTO-6-HYDROXY-5,8-QUINOLINEQUINONE

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The p*K* and mid-point redox potential of the Q-analogue 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone (HMHQQ) in aqueous medium are so low that under the experimental conditions used for studying the inhibition of electron transfer in submitochondrial particles only the oxidized, anionic form is present. The K_D of the analogue, determined by comparing its inhibitory effect with that of *n*-heptyl-4-hydroxyquinoline *N*-oxide, is (0.003 + 0.24 × mg protein/ml) μM. The inhibition of succinate oxidation is pH dependent, due to a pH-dependent change in the overcapacity of the QH_2-oxidizing system above the Q-reducing system. If the terminal part of the respiratory chain is reduced with ascorbate, the analogue inhibits the reduction of cytochrome *b* by substrate in the presence of antimycin with a similar K_D value. In the absence of ascorbate the K_D value is 100-times higher. The reduction of cytochrome *b* by substrate in particles treated with 2,3-dimercaptopropanol (BAL) + O_2 is also sensitive to HMHQQ, with a K_D value in between the two values given above. It is concluded that the QH_2 oxidase system contains two different sites for interaction with ubiquinone. The site responsible for the inhibition of steady-state electron transfer is near the Fe-S cluster, as is shown by the sensitivity to the redox state of this cluster and by the effect of HMHQQ on the EPR signal of the reduced cluster. The second site, which is similar to the antimycin-binding site, is occupied only at higher concentrations of inhibitor. The affinity of HMHQQ for this site is not affected by the redox state of the Fe-S cluster.

Introduction

In mitochondrial respiration the transfer of reducing equivalents from the dehydrogenases to the cytochrome system is mediated by ubiquinone [1]. In a previous paper [2] we have shown that both the mobile pool of ubiquinone [3] and direct collision [4,5] between Q-loaded Q-reducing and QH_2-oxidizing enzymes contribute to the electron transfer. The mobile pool is the more important factor except when the concentration of ubiquinone in the membrane is diminished. In the present paper, we report studies directed to the question of the site of interaction of ubiquinol with the QH_2-oxidizing system, the QH_2:cytochrome *c* oxidoreductase.

A considerable body of evidence has now been brought forward indicating that the reduction of

Abbreviations: HQNO, *n*-heptyl-4-hydroxyquinoline *N*-oxide; HMHQQ, 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole; BAL (British Anti-lewisite), 2,3-dimercaptopropanol; Mops, 4-morpholinepropanesulphonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

cytochrome *b* can occur via two pathways, one inactivated by treatment with BAL + O_2 [6,7], or by extraction of the Rieske Fe-S protein [8], the other inhibited by antimycin or heptyl- (or nonyl-) hydroxyquinoline *N*-oxide [2,9]. The recently reported inhibitor myxothiazol [10,11] mimics the effect of BAL (+O_2) treatment on the reduction of cytochrome *b* and cytochrome $c + c_1$ (unpublished observations). The two types of inhibitors have different effects on the redox level of cytochrome *b*: inhibitors acting at the site of antimycin cause an increased reduction of cytochrome *b* (in the presence of oxidant), those acting at or near the Fe-S cluster do not. With a combination of the two types of inhibitors all reduction of cytochrome *b* is inhibited.

In addition, two forms of bound semiquinone have been reported: one present under conditions of equilibrium at suitable redox potentials and sensitive to antimycin [12–14], the other present under non-equilibrium conditions in the presence of antimycin and oxygen, and sensitive to BAL (+O_2) treatment [15]. These results can most easily be explained with a Q-cycle mechanism [16] of the type proposed in Refs. 2 and 17. According to this model, one pathway for reduction of cytochrome *b* is reversible (QH_2 + cytochrome $b^{3+} \leftrightarrows Q^{\cdot-}$ + cytochrome $b^{2+} H^+ + H^+$) due to the high stability of the bound semiquinone at this site. During steady-state oxidation the flux is in the direction of the reduction of $Q^{\cdot-}$ by reduced cytochrome *b*. The second pathway for reduction of cytochrome *b* is not readily reversible in uncoupled systems, owing to the low stability of the semiquinone at this site. In steady-state oxidation the net flux is in the direction of reduction of cytochrome *b* by the semiquinone that is formed via the oxidation of ubiquinol by the Rieske Fe-S cluster.

In this paper we make use of the Q-analogue 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone (HMHQQ) to discriminate between the two ubiquinone-binding sites in QH_2:cytochrome *c* oxidoreductase. It will be shown that the site with the highest affinity that is responsible for inhibition of electron transfer at low concentrations of inhibitor is located near the Fe-S cluster, whereas a second site of inhibition similar to the antimycin-binding site, is occupied only at higher concentrations of inhibitor. The affinity of the former site is very sensitive to the redox state of the Fe-S cluster, while that of the latter is not.

Materials and Methods

Submitochondrial particles were prepared according to Ref. 18, except that during the washing procedure the particles were incubated for 20 min at 30°C with 2 mM malonate to activate succinate dehydrogenase. The BAL (+O_2) treatment was carried out as described previously [2] and extraction of lyophilized particles with pentane and pentane plus acetone according to Ref. 19.

Antimycin was obtained from Nutritional Biochemical Corp. and HQNO from Sigma. Both were added as ethanolic solutions. The concentrations were determined spectrophotometrically at 420 nm [20] and at 448 nm [9], respectively. HMHQQ was synthesized [21] and added from a stock solution in ethanol/2 M Tris-HCl buffer, pH 8 (9:1, v/v). Particles were incubated with HMHQQ for 5 min before the reaction to be measured was started. The ethanol added to the reaction mixture never exceeded 1% of the total volume.

Oxidase activities were measured at 25°C using an Oxygraph equipped with a Clark oxygen electrode. Absorbance changes of cytochromes were measured with an Aminco DW-2 spectrophotometer equipped with a thermostatically controlled cuvette holder and spectra were recorded on a Cary 17 spectrophotometer at room temperature.

EPR measurements were performed as described in Refs. 7 and 14. Potentiometric titrations were carried out anaerobically in a special cuvette containing platinum and reference electrodes.

Results

Properties of HMHQQ

The Q-analogue, HMHQQ, used in this study is similar to compound 1 of Roberts et al. [22], the only difference being the number of methylene groups in the alkyl side chain (17 instead of 16). The compound is only slightly soluble in aqueous media, but is sufficiently soluble in ethanol/2 M Tris-HCl buffer, pH 8 (9:1, v/v). The absorption

spectra of the basic and acidic forms in ethanol are shown in Fig. 1. The pK measured in dilute aqueous solution (25 μM) is 5.5 (see Fig. 2), which is 1 pH unit lower than that of UHDBT, the analogue that has been studied intensively by Trumpower and colleagues [23]. Thus, at physiological pH the compound is in its anionic form. It can be reduced by dithionite or potassium borohydride, but not by succinate or NADH (in the presence of submitochondrial particles). The mid-point redox potential at pH 7.2, determined by redox titrations under anaerobic conditions in the absence of additional mediators (the analogue itself reacts with the electrode), is -132 mV (Fig. 3). Due to this low value of the E_m, the analogue HMHQQ is more suitable as an inhibitor than UHDBT of which the E_m value at neutral pH equals -40 mV [23], so that under anaerobic conditions the reduced form of UHDBT may provide reducing equivalents to the QH_2 oxidase system. HMHQQ is fluorescent, with the excitation maximum at 275 nm and emission maximum at 385 nm. Although the compound binds quite strongly to bovine serum albumin, its fluorescence, unlike that of antimycin or HQNO, does not change upon binding. The binding parameters to serum albumin could not be measured fluorimetrically, since the excitation maximum coincides with the absorption maximum of serum albumin.

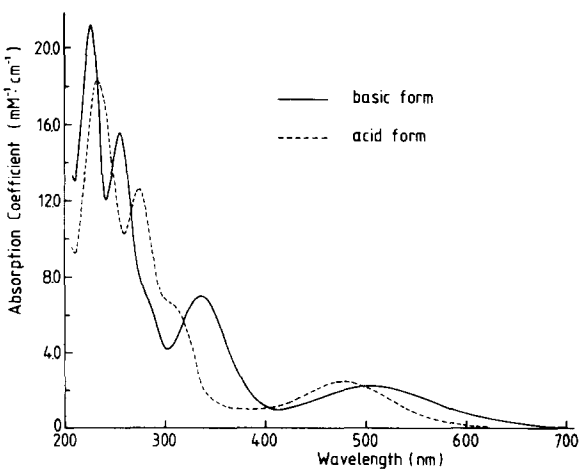

Fig. 1. Absorbance spectra of HMHQQ in ethanol. One drop of concentrated NH_4OH was added to the solution to obtain the basic form and one drop of concentrated HCl for the acid form. The concentration of HMHQQ was 50 μM.

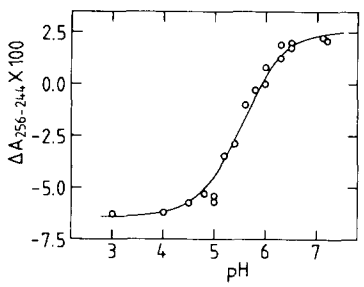

Fig. 2. Determination of the pK of HMHQQ. The buffer contained 20 mM succinate (pK_1 and pK_2 4.21 and 5.63, respectively). The concentration of HMHQQ was 25 μM. The absorbance differences at the wavelength pair 356–344 nm were plotted against pH. The solid line is a theoretical acid-base titration curve, with a pK of 5.5.

Inhibition of electron transfer

The oxidation of succinate or NADH in the presence of submitochondrial particles is inhibited at relatively low concentrations of HMHQQ. Even after preincubation the inhibition sets in only after a few turnovers and does not become completely constant with time. In an Oxygraph experiment the initial lag phase cannot be seen clearly but in the experiment of Fig. 4 it can be seen that the inhibition requires time, when the inhibitor is incubated with oxidized enzyme, but decreases with

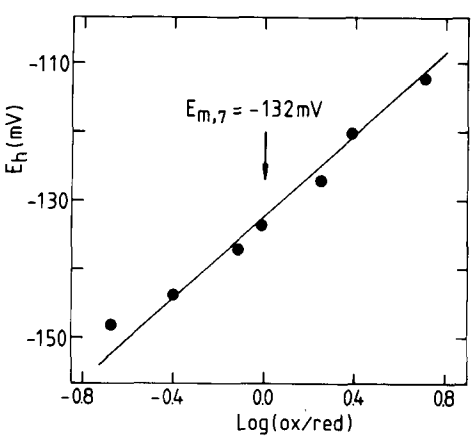

Fig. 3. Determination of the mid-point redox potential of HMHQQ. HMHQQ (100 μM) was dissolved in 50 mM Mops-Tris buffer (pH 7), 1% Triton X-100. The cuvette was equipped with a platinum and a reference electrode. HMHQQ itself reacted with the electrode. Increasing oxidation or reduction was obtained by addition of anaerobic solutions of $K_3Fe(CN)_6$ or $Na_2S_2O_4$, respectively. Absorption spectra were measured at each point of the titration.

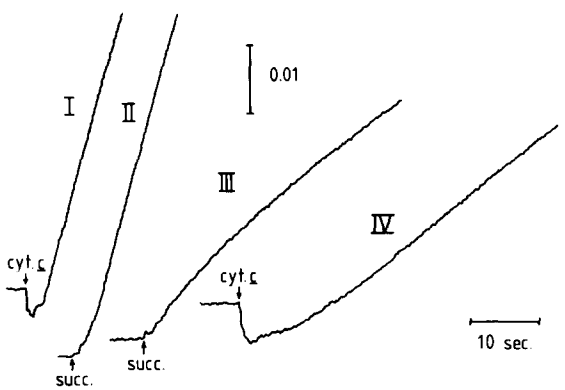

Fig. 4. Inhibition of succinate-cytochrome *c* reductase activity by HMHQQ. Submitochondrial particles (50 μg/ml) were incubated in a medium containing 0.25 M sucrose, 50 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA, 10 mM $MgCl_2$ and 2 mM KCN. (II and III) 10 μM cytochrome *c* was present during the preincubation (5 min) and the reaction was started with 10 mM succinate (succ.). (I and IV) 10 mM succinate was present during the preincubation and the reaction was started with 10 μM cytochrome *c*. (I and II) Preincubation was performed in the absence of HMHQQ and (III and IV) in the presence of 0.5 μM HMHQQ. The cytochrome *c* reduction was measured at the wavelength pair 550−539 nm.

time when the inhibitor is incubated with reduced enzyme. Direct measurement of the binding constant was not possible, since the inhibitor is completely taken up by the particles and nothing is left in the water phase after spinning down the particles. This distribution can be changed by the addition of bovine serum albumin to the medium and, under these conditions the distribution can be measured, in principle, by measuring the inhibition of the succinate oxidation (by submitochondrial particles) by the supernatant in the presence of a standard amount of serum albumin, compared with a reference curve for the inhibition by known amounts of analogue in the presence of the same amounts of bovine serum albumin. In practice, however, the data were not very accurate and also the binding to serum albumin itself (needed to calculate the real binding constant) could not be measured sufficiently accurately. Therefore, we measured the binding to the inhibitory site by comparing the inhibition by the analogue with that caused by HQNO, for which the binding parameters and the concentration of binding sites (equal to that of the QH_2:cytochrome *c* oxidoreductase) can be measured [9]. Thus, for each point on the HQNO-inhibition curve the saturation of the binding site with HQNO can be calculated. If we assume that the inhibition by HMHQQ is also caused by binding to a single specific site in the enzyme, we can determine the saturation of this site with HMHQQ for each point of the inhibition curve, by comparison with the HQNO-inhibition curve. The precise location of the inhibitory site within the enzyme is not relevant, since we have shown previously [2] that the level of inhibition of the oxidation of substrate by an inhibitor of the QH_2:cytochrome *c* oxidoreductase is independent of its site of interaction.

Fig. 5 shows that at the protein concentration used (0.28 mg protein/ml) the inhibition curve for HMHQQ is nearly the same as that for HQNO, indicating that the apparent dissociation constant for the two inhibitors is about equal at this protein concentration. As shown in Fig. 6 the value for the K_D, calculated from titrations such as that shown in Fig. 5 and the separately determined K_D of HQNO, is strongly dependent on the protein concentration, which reflects the high affinity of the inhibitor for the membrane phase, resulting in a high level of aspecific binding (see Ref. 17). The aspecific binding of HMHQQ is much higher than that of HQNO. The value of the measured K_D

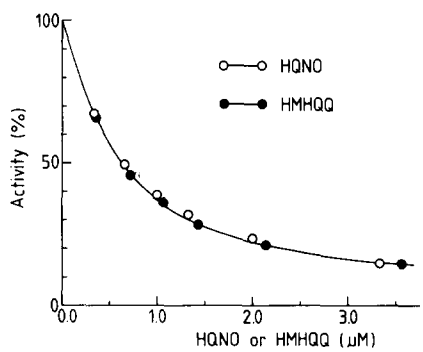

Fig. 5. Inhibition of succinate oxidation by HMHQQ and HQNO. Submitochondrial particles (0.28 mg/ml) were suspended in an Oxygraph vessel in a medium containing 0.25 M sucrose, 50 mM Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 4 μM cytochrome *c* and variable amounts of HQNO or HMHQQ. After 5 min at 25°C 10 mM succinate was added and the oxygen uptake measured. The succinate oxidation in the absence of inhibitor was 0.52 μmol/min per mg protein.

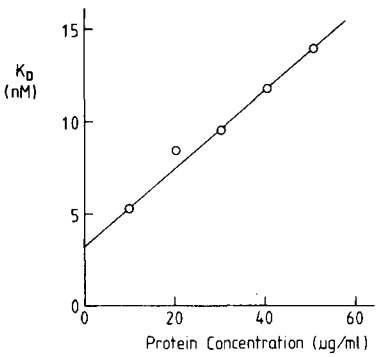

Fig. 6. Dependence of K_D of HMHQQ on protein concentration. From experiments similar to that shown in Fig. 5, the K_D of HMHQQ was calculated by assuming that the saturation of the HMHQQ-binding site with HMHQQ equals the saturation of the HQNO-binding site with HQNO, at the same degree of inhibition of the oxidation of succinate.

equals $(0.003 + 0.24 \times$ mg protein/ml) μM, where 0.003 μM is the K_D relative to the aqueous medium, but in fact this value is much less important than the concentration of particles. This relation of K_D to the protein concentration is valid for concentrations of protein up to more than 1 mg/ml. Isolated succinate-cytochrome *c* oxidoreductase gave similar results.

pH dependence of the inhibition by HMHQQ

Trumpower and Haggerty [23] have concluded from the pH dependence of the inhibition of succinate-cytochrome *c* oxidoreductase by UHDBT that the protonated form of the inhibitor has a much higher affinity for the enzyme than the non-protonated form. Since it is known [14] that only the anionic form of ubisemiquinone is stabilized by binding, we might expect a higher affinity of the non-protonated form for the Q-binding site, especially if Rich [24] is right in his conclusion that QH^- and not QH_2 is the substrate for the QH_2 oxidase system. In fact, the increase of the inhibition by UHDBT by decreasing pH reported by Trumpower and Haggerty [23] does not fit exactly the pH dependence of the protonated form of the inhibitor. Since the degree of inhibition by inhibitors of this type depends upon the relative capacities of the Q-reducing and QH_2-oxidizing systems, indicated by V_1 and V_2, respectively (see Refs. 3 and 2), the effect of pH on V_1 and V_2, measured as described previously [2], was determined using both HQNO and HMHQQ as inhibitor. It was found that V_2 decreases about 10-fold on decrease in the pH by 1 unit, while V_1 decreases less than 2-fold with duroquinol as substrate, and about 3.5-fold with succinate as substrate. The resulting decrease in overcapacity (V_2/V_1) with decreasing pH results in an increased inhibition by a certain concentration of inhibitor, which is a sufficient explanation for Trumpower and Haggerty's [23] findings, without invoking a pH-dependent change in the affinity. The 10-fold increase in the QH_2 oxidase activity with an increase in the pH by 1 unit also favors Rich's [24] proposal that the QH^- is the substrate for the enzyme.

Inhibition of reduction of cytochrome b in antimycin-treated particles

To determine the site of inhibition we measured the effect of HMHQQ on the reduction of cytochrome *b* in submitochondrial particles upon addition of succinate in the presence of antimycin. At low concentrations, sufficient to inhibit substrate oxidation, HMHQQ alone does not inhibit the reduction of cytochrome *b*, nor does antimycin alone. In the presence of antimycin, reduction of cytochrome *b* is inhibited by HMHQQ, but half-maximal inhibition occurs only at 40–50 μM HMHQQ, a concentration 100-times higher than that needed for inhibition of substrate oxidation. However, when succinate is added after a previous addition of ascorbate, which reduces the high-potential components of the respiratory chain (Fe-S cluster, cytochromes c_1, *c*, aa_3), the reduction of cytochrome *b* in the presence of antimycin and cyanide (which is relatively slow under these conditions [17]) is inhibited at low concentrations of HMHQQ. In the presence of HMHQQ the reduction becomes biphasic and the fraction of the cytochrome *b* that is still reduced at the same rate as in the absence of HMHQQ (the 'fast phase') may be taken as the percentage of binding sites not containing bound HMHQQ. In Fig. 7 the extent of the disappearance of the fast phase is plotted against the concentration of HMHQQ. The large effect of reducing the terminal part of the respiratory chain is very clear. From the plot of Fig. 8, a K_D of 0.30 μM for the inhibitory

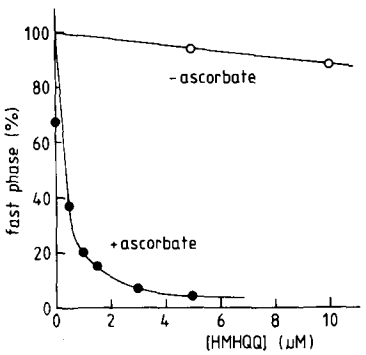

Fig. 7. The effect of ascorbate on the inhibition by HMHQQ of the reduction of cytochrome *b* by succinate in the presence of 2.8 μM antimycin. The wavelength pair used was 563–575 nm. The percentage of fast phase was obtained from semi-logarithmic plots as described in Ref. 2. The buffer used was the same as in Fig. 5 but without cytochrome *c*. The concentration of ascorbate, when used, was 5 mM, together with 0.1 mM TMPD and 2 mM KCN. The protein concentration was 0.5 mg/ml. For the definition of fast phase in the presence of ascorbate, see text.

complex can be derived. Exactly the same value for the K_D was found when, instead of measuring the reduction of cytochrome *b* in the presence of antimycin upon addition of substrate, the further reduction, in the presence of substrate, upon addition of antimycin was determined. These K_D values are of the same order as the value determined from the inhibition of the steady-state electron transfer at the same protein concentration (0.12 μM).

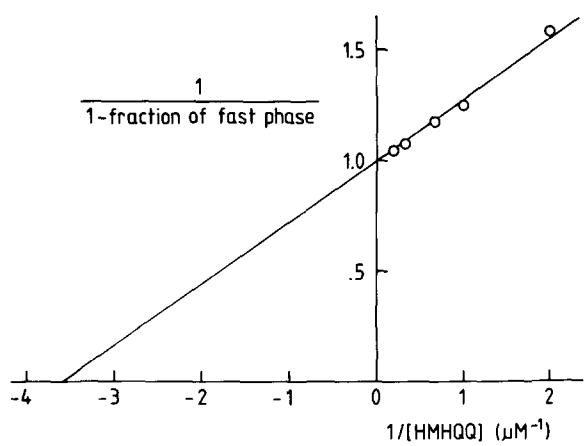

Fig. 8. Determination of the K_D of HMHQQ for the inhibition of the reduction of cytochrome *b* in the presence of antimycin. The data are taken from Fig. 7, in the presence of ascorbate. the K_D equals 0.28 μM.

Inhibition of cytochrome b reduction in BAL (+ O_2)-treated particles

To test whether HMHQQ also binds to the antimycin-binding site, the reduction of cytochrome *b* by succinate in BAL (+O_2)-treated particles was measured. It has been shown before [2] that in these particles the reduction of cytochrome *b* by substrate is fully sensitive to antimycin. When inhibition of cytochrome *b* reduction in BAL (+O_2)-treated particles was measured, the degree of saturation of the inhibitory site with HMHQQ was taken from the change in $t_{\frac{1}{2}}$ for the reduction of cytochrome *b*, since the on and off rates for the binding of the inhibitor are at least of the same order of magnitude as the rate of electron transfer. The inhibition by HMHQQ of reduction of cytochrome *b* by succinate is shown in the reciprocal plot of Fig. 9, from which a K_D of 3 μM can be determined, clearly different from the values found for inhibition of cytochrome *b* reduction in the presence of antimycin (0.3 and 50 μM in the presence and absence of ascorbate, respectively).

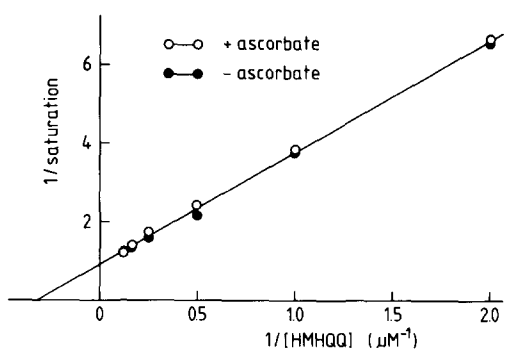

Fig. 9. Determination of the K_D of HMHQQ for the inhibition of the reduction of cytochrome *b* by succinate in BAL (+O_2)-treated submitochondrial particles. The reaction conditions were the same as in Fig. 7 except that BAL (+O_2)-treated particles were used and no antimycin was present. In the presence of HMHQQ, the reduction of cytochrome *b* by succinate was still nearly monophasic, but with a longer half-time. The extent of saturation by HMHQQ was obtained according to the proposed formula $k' = k_0$ (1 − saturation), where k' and k_0 are the first-order rate constants in the presence and the absence of HMHQQ, respectively. The K_D thus obtained is 3.0 μM, both in the presence and absence of ascorbate.

Inhibition by HMHQQ of cytochrome *b* reduction in BAL-treated particles is not affected by reduction of the terminal part of the respiratory chain by ascorbate.

Identical results are obtained when NADH is used as substrate instead of succinate, showing that the site of inhibition is at the level of the reduction of cytochrome *b* by ubiquinol, and not at the level of the succinate: Q oxidoreductase.

Effect of HMHQQ on the Rieske Fe-S cluster

It has been shown previously that the line shape of the EPR signal of one of the two Fe-S clusters in the dimeric QH_2:cytochrome *c* oxidoreductase is sensitive to the presence and redox state of Q [25,26]. It is 'sharp' when oxidized ubiquinone is present and broad when it is absent. This is shown also in Fig. 10. After extraction of ubiquinone (or at low potentials), the sharp signal is replaced by a broad signal. The sharp signal is recovered again by incorporation of ubiquinone [26] or by addition of the analogue HMHQQ. However, the signal with HMHQQ is not only sharp but also shifted to lower *g* values, demonstrating a close interaction between bound HMHQQ and the Rieske Fe-S cluster.

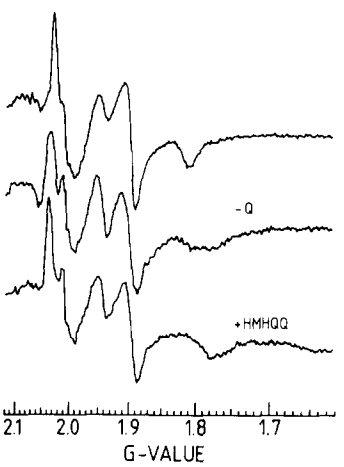

Fig. 10. The effect of HMHQQ on the EPR signal of Rieske Fe-S cluster in submitochondrial particles. Submitochondrial particles (50 mg/ml) were incubated in a buffer (pH 7.4, see Fig. 7) containing 10 mM ascorbate, 3 μM TMPD and 4 mM KCN. Upper trace, normal submitochondrial particles. Middle trace, ubiquinone-depleted submitochondrial particles. Lower trace, ubiquinone-depleted submitochondrial particles in the presence of 100 μM HMHQQ, added from a stock solution in Me_2SO. At the concentration used, Me_2SO does not affect the EPR spectrum of the Fe-S cluster [26].

Discussion

The experiments reported clearly show the presence of two different sites of inhibition for the ubiquinone analogue HMHQQ, corresponding to the two different pathways for reduction of cytochrome *b* by ubiquinol. The measured K_i for the effect of the analogue on cytochrome *b* reduction is dependent upon whether the antimycin- or BAL-sensitive pathway for cytochrome *b* reduction is tested, and only in the former case is it sensitive to reduction of the terminal part of the respiratory chain. The difference in the effect of ascorbate reduction shows that the different K_i values are not due to an effect of BAL treatment on the affinity of HMHQQ for its binding site. In fact, we have shown in an earlier paper that BAL ($+O_2$) treatment does not change the affinity of HQNO for its binding site [2], and we may expect that this is also the case for HMHQQ. The experiments described in the present paper confirm our previous conclusion that ubiquinol can reduce cytochrome *b* via two distinct pathways: one pathway is sensitive to antimycin, HQNO and HMHQQ (K_D 3 μM), the other is sensitive to BAL ($+O_2$) treatment, UHDBT, HMHQQ and to depletion of the Fe-S protein. The effect of reduction with ascorbate on the affinity of HMHQQ for its site in the first pathway can be interpreted as an increase by HMHQQ of the apparent E_m of the Rieske Fe-S cluster, similar to what is found with UHDBT [27].

The effect of HMHQQ on the EPR signal of the Rieske Fe-S cluster is in agreement with the assumption that it is the reduction of the Fe-S cluster that induces the high affinity of HMHQQ. Indeed, it is possible that HMHQQ increases the E_m of the Fe-S cluster to such an extent that electron transfer from the cluster to cytochrome c_1 is inhibited. As suggested in Ref. 28, the Q-binding site near the Fe-S cluster could well be the inhibitory site of 5,5'-dithiobis(2-nitrobenzoic acid) which also affects the EPR signal of the Fe-S cluster. The inhibition of reduction of cytochrome *b* is, according to this view, due to the stabilization of the reduced form of the Fe-S cluster, thereby

inhibiting the formation of semiquinone (by reduction of an oxidized Fe-S cluster by uibiquinol). Direct competition of HMHQQ with ubiquinol for its binding site, however, could also explain the inhibition. Due to the fact that the high affinity of HMHQQ for its inhibitory site in the region of the Fe-S cluster is only present after reduction of the cluster, we could not measure whether the analogue inhibits the reduction of the cluster by ubiquinol. If the change in redox state of the Fe-S cluster is also important for the binding of oxidized ubiquinone, it might well be that the oxidized Q dissociates from its binding site only when the Fe-S cluster has been oxidized again by cytochrome c_1.

The inhibition by HMHQQ of the reduction of cytochrome *b* by the second pathway can be most easily explained by a direct competition between the inhibitor and ubiquinol for the Q-binding site.

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