HOQNO interaction with cytochrome *b* in succinate:menaquinone oxidoreductase from *Bacillus subtilis*

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Received 27 November 1994

Abstract 2-*n*-Heptyl 4-hydroxyquinoline-*N*-oxide (HOQNO) inhibits the succinate:quinone oxidoreductase activity of isolated and membrane-bound succinate:menaquinone oxidoreductase of *B. subtilis*. The inhibition pattern resembles closely that observed for α-thenoyltrifluoroacetone and carboxins in the mitochondrial succinate:ubiquinone oxidoreductase: ca. 90% of the activity is highly sensitive to HOQNO (K_i ca. 0.2 μM for the isolated enzyme) whereas the rest 10% proves to be resistant to the inhibitor. HOQNO binding is shown to perturb the absorption spectrum of the ferrous di-heme cytochrome *b* of the *B. subtilis* succinate:quinone oxidoreductase both in the α and Soret bands. In addition, the inhibitor is shown to bring about a negative shift of E_m of the low-potential heme *b*. It is suggested that HOQNO interacts with a menasemiquinone binding site near the low-potential heme and suppresses the $MQ^{\bullet-}$-to-MQH_2 step of the quinone reductase reaction but allows partly for the MQ-to-$MQ^{\bullet-}$ transition to occur; dismutation of $MQ^{\bullet}$ formed in the latter reaction to MQ and MQH_2 may account for the 10% of the enzyme activity insensitive to HOQNO.

Key words: 2-*n*-Heptyl 4-hydroxyquinoline-*N*-oxide; Succinate quinone reductase; Cytochrome *b*, *Bacillus subtilis*; Menasemiquinone; Complex II

1. Introduction

Succinate:quinone oxidoreductase from *B. subtilis* (SQR; EC 1.3.5.1) transfers reducing equivalents from succinate to menaquinone-7 (reviewed in [1,2]). The enzyme is an integral component both of the Krebs' citric acid cycle and of the respiratory chain. It consists of a two subunit water-soluble dehydrogenase part carrying a dicarboxylate binding site, one FAD and three iron sulfur centres, and of a membrane anchor subunit which is a transmembrane protein with 5 hydrophobic α-helical segments spanning the phospholipid bilayer.

The 23 kDa anchor subunit of *B. subtilis* SQR contains two tightly bound redox active protohemes with different optical, EPR and redox characteristics [3,4] and thus is a di-heme cytochrome *b*. In some other bacteria or in mitochondria from higher organisms, the corresponding subunit contains only one heme (see [2,3] for review and references).

Activity of the mammalian mitochondrial SQR has been long known to be blocked specifically by a α-thenoyltrifluoroacetone (TTFA) and carboxins [1,5,6]. These inhibitors are believed to act at the ubiquinone (UQ) reduction site, interrupting electron transfer between the high-potential iron–sulfur centre S-3 and UQ [1,7] and destabilizing the tightly bound ubisemiquinone radical [8–11]. *B. subtilis* SQR is not sensitive to these compounds but is inhibited by *n*-heptyl 4-hydroxyquinoline-*N*-oxide (HOQNO) [4,12]. HOQNO is a potent UQ and menaquinone (MQ) antagonist acting on many respiratory cytochrome *b* containing quinone-reactive redox enzymes in various organisms. In particular, HOQNO is known as a classical inhibitor of the mitochondrial cytochrome bc_1 complex [13], binding at the quinone reductase site of this enzyme (so-called, centre *i*) and bringing about a spectral perturbation of the high-potential heme *b* [14] and a positive shift of its E_m [15].

In this work we have studied effects of HOQNO on the purified and membrane-bound *B. subtilis* SQR. The HOQNO inhibition pattern is rather similar to the effect of TTFA and carboxins on the mitochondrial SQR. In addition HOQNO perturbs the optical absorption spectrum of cytochrome *b* in SQR and brings about a negative shift of the low-potential heme (heme b_L) of the cytochrome. Implications of these findings for the mechanism of HOQNO inhibitory action are discussed.

2. Materials and methods

Membranes were isolated from the *B. subtilis* SQR overproducing strain 3G18/pBSD1200 grown as described before [4]. SQR was purified from the membranes according to [4].

Heme content of the SQR preparation was determined from the pyridine hemochromogen difference spectra [16]. Protein was measured with Folin reagent. HOQNO (Sigma) was dissolved in twice-distilled ethanol.

Optical absorption spectra were recorded in the Shimadzu UV-3000 or UV-2101PC spectrophotometers using semi-micro 1 cm optical pathway cells.

Quinone reductase activity of SQR was measured at 30°C in the presence of Q_2 (decylubiquinone) or Q_1 (both from Sigma) with 2,6-dichlorophenol indophenol (DCPIP) as the final electron acceptor. In case of experiments with membranes, 2 mM KCN was added; for more details, see [4].

Anaerobic redox titrations in the presence of a set of redox mediators were carried out in 100 mM MOPS-NaOH buffer, pH 7.4, as described previously [4]. Data were processed in PC/AT computers with the aid of a program package GIM (Graphic Interactive Management) developed by Dr. Alexander L. Drachev.

3. Results

3.1. Inhibitory action

Fig. 1 shows typical concentration dependence of the HOQNO inhibitory effect on the succinate:quinone reductase activity of isolated (A) and membrane-bound *B. subtilis* SQR

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Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; HOQNO, 2-*n*-heptyl 4-hydroxyquinoline-*N*-oxide; MQ, menaquinone; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMS, phenazine methosulfate; UQ, ubiquinone; SQR, succinate:quinone oxidoreductase; TTFA, α-thenoyltrifluoroacetone.

SSDI 0014-5793(94)01442-6

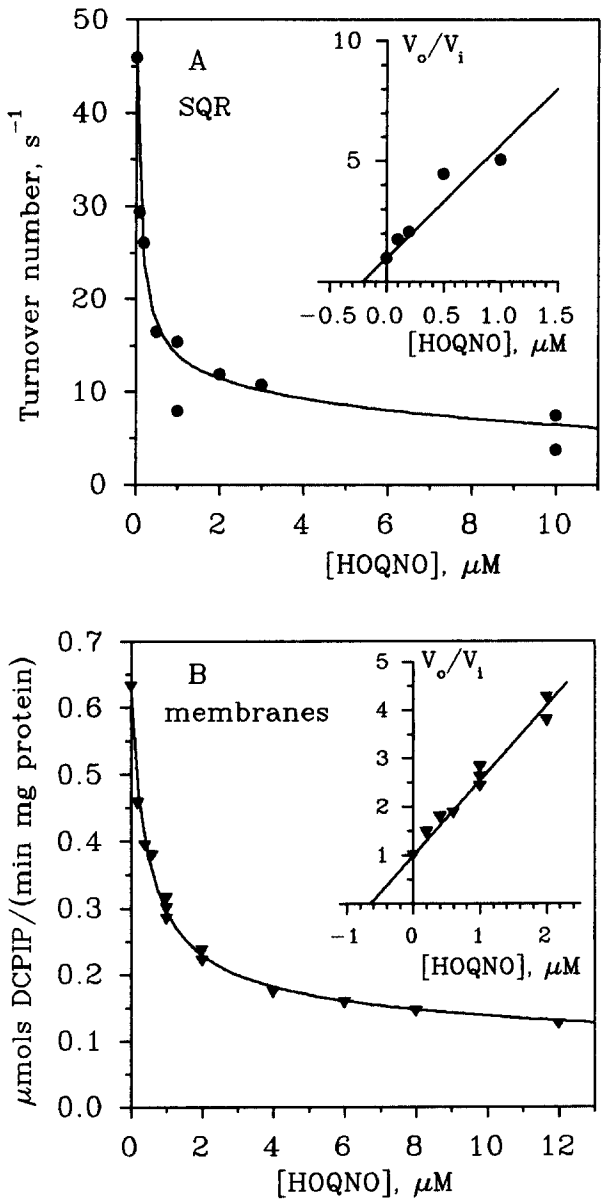

Fig. 1. HOQNO inhibition of the succinate:quinone oxidoreductase activity of isolated (A) and membrane-bound *B. subtilis* SQR (B). The measurements were carried out with 200 μM Q_1 (A) or 100 μM Q_2 (B) in 50 mM KH_2PO_4-KOH (pH 7.4) with 20 μg/ml of sodium 2,6-dichlorophenol-indophenolate as the final electron acceptor. The enzyme was activated before the measurements (5 min preincubation with 20 mM succinate at 30°C). (A) SQR concentration, 20 nM. (B) Membrane protein concentration, 12 μg/ml; the reaction mixture has been supplemented with 2 mM KCN. The insets in A and B give Dixon linearization of the inhibition curves after subtraction of the HOQNO-resistant part of the activity.

(B). In both cases the effect is clearly biphasic. The major part of the activity is inhibited in a hyperbolic fashion giving linear Dixon plots with the apparent K_i values of 0.2 μM for the isolated enzyme and 0.7 μM for the membrane-bound SQR at pH 7.5. The remaining part of the activity is not inhibited even at 20–40 μM HOQNO and constitutes about 10% of the overall rate for the isolated and membrane-bound enzyme. Such a biphasic inhibition pattern was observed earlier for the effects of α-thenoyl trifluoroacetone and carboxins on the mitochondrial succinate:ubiquinone reductase activity [5,7]. The HOQNO inhibitory efficiency is lower at alkaline pH, the apparent K_i value for purified SQR being about 1 μM at pH 8.5 [3].

3.2. *Cytochrome* b *spectral shift*

As shown in Fig. 2, HOQNO added to the isolated SQR reduced with dithionite brings about a perturbation of the cytochrome *b* optical absorption spectrum. In the Soret band, the lineshape of the HOQNO-induced difference spectrum with λ_{min} = 425 nm, λ_{max} = 433 nm and $\Delta\varepsilon_{max-min}$ of ca. 5 $mM^{-1} \cdot cm^{-1}$ is very similar to a red shift of the cytochrome *b* spectrum induced by this inhibitor in the mitochondrial complex bc_1 [14]. This is accompanied by rather small but reproducible changes in the visible range dominated by increased extinction at 562 nm. The magnitude of this response ($\Delta\varepsilon$ ca. 0.4 $mM^{-1} \cdot cm^{-1}$) is also close to that observed for the mitochondrial cytochrome *b* α-band [14]. Notably, the HOQNO-induced peak at 562 nm is rather narrow and is red-shifted with respect to the α-maximum of the cytochrome *b*; such a lineshape would be consistent with a small increase in the Q_y component of the Q_{00} band, rather than with a red shift of the overall α-peak of the cytochrome.

The spectral effect of HOQNO suggests that the inhibitor binds to SQR at or near cytochrome *b*. Spectral characteristics of the two hemes at room temperature are rather similar [4] and at the moment we are not in a position to attribute the spectral perturbation induced by HOQNO specifically to heme b_H or b_L. However, evidence for the inhibitor binding at heme b_L is provided by the results presented below.

3.3. *HOQNO-induced shift of the heme* b_L *midpoint potential*

Fig. 3 shows reversible redox titrations of cytochrome *b* in isolated SQR in the absence and in the presence of HOQNO. The titration curve of the cytochrome in the uninhibited SQR shows the presence of two single-electron components (hemes b_H and b_L) with almost equal contributions to the absorbance at 558 nm and E_m values of +65 and −96 mV at pH 7.4 which is in close agreement with the data in [4]. It can be seen, that HOQNO causes a significant negative shift of the heme b_L midpoint potential without affecting the E_m of the high-potential heme.

4. Discussion

Our data on *B. subtilis* SQR show that HOQNO (i) inhibits the quinone reductase activity of both isolated and membrane-bound enzyme, (ii) perturbs the optical absorption spectrum of the di-heme cytochrome *b* in the enzyme and (iii) shifts the E_m of heme b_L to the negative by ca. 50 mV. The simplest explanation would be that all these effects are manifestations of the inhibitor binding at the same site, although more data is required to verify this postulate.

Notably, the HOQNO inhibition pattern observed for *B. subtilis* SQR is similar to that described for TTFA and carboxins in mitochondrial succinate:ubiquinone reductase. Like the latter two inhibitors, HOQNO blocks electron transfer to Q without affecting greatly the dehydrogenase activity of the enzyme as measured in the PMS/DCPIP assay [4], and displays the same peculiar biphasic concentration dependence (this

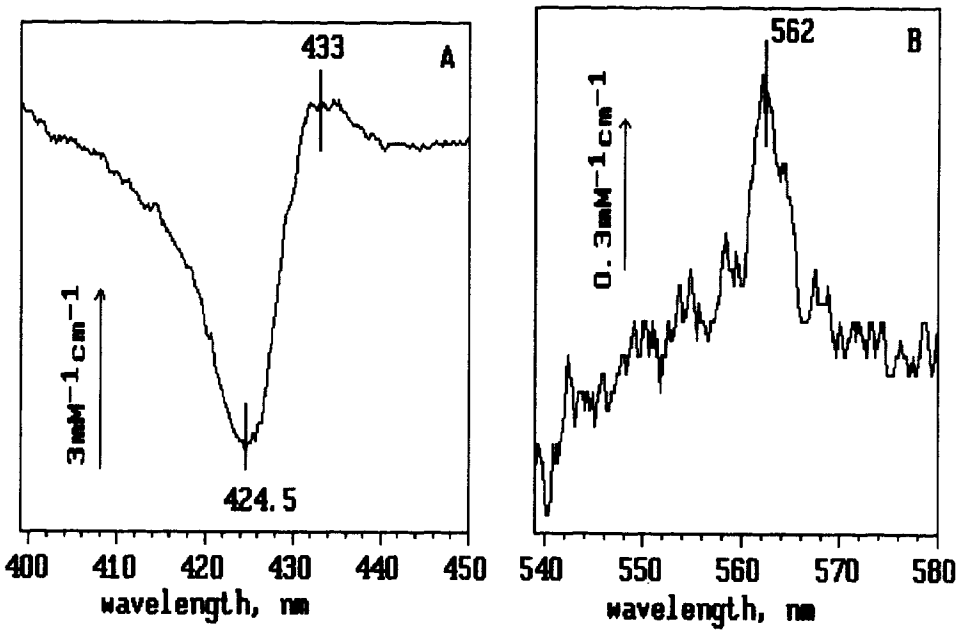

Fig. 2. Effect of HOQNO on the optical absorption spectrum of cytochrome *b* in isolated *B. subtilis* SQR. The sample and reference contained 2.8 μM SQR in 100 mM MOPS-NaOH (pH 7.4) reduced with 1–2 mg of solid sodium dithionite. Baseline recorded, 40 μM HOQNO was added to the sample and an equal volume of ethanol to the reference, and the difference spectrum was scanned. The Soret band part of the difference spectrum (A) has been corrected for the contribution of HOQNO to the absorbance.

work). It is likely that the inhibitors affect Q reduction by a common or at least similar mechanism. Nevertheless, *B. subtilis* SQR is not inhibited by TTFA or carboxins, whereas the mitochondrial enzyme is resistant to HOQNO. It would be of great interest to test a nature of these differences in the enzyme sensitivity to the inhibitors by molecular genetics methods.

Since HOQNO is a structural analog of MQ, the inhibitor binding with cytochrome *b* implies involvement of the latter in MQ binding.

Kostyrko and co-workers [17–19] revealed a preference of the Q-reducing site in mitochondrial SQR for the anionic forms of inhibitors and concluded the ubisemiquinone binding site of the enzyme to be the target [19] in line with the inhibitory effect of TTFA and carboxins on the stable ubisemiquinone(s) EPR signal(s) in the mitochondrial SQR [8–10,20–22]. It is then of particular interest that in *B. subtilis* SQR, HOQNO shifts the E_m of heme b_L to the *negative*, i.e. binds more tightly to the *oxidized* form of b_L. Since the heme ferric state bears a positive charge, whereas the reduced one is neutral, this may indicate preferential binding of the HOQNO anion which is obviously a close analog of the anionic form of menasemiquinone.

We propose that in *B. subtilis* SQR, cytochrome *b* participates in the binding and stabilization of the menasemiquinone anion, and that the inhibitory effect of HOQNO is due to interaction with cytochrome *b* at the $MQ^{\bullet-}$ binding site at or near heme b_L, analogous to one of the two $UQ^{\bullet-}$ binding sites in mitochondrial SQR [23,24] or the two putative MQ binding sites in *E. coli* fumarate-reductase [25]. These two sites seem to be arranged across the membrane [3,24,25]. Magnetic interaction of ubisemiquinone(s) with the single heme *b* in the *E. coli* SQR (corresponding probably to heme b_H of *B. subtilis* SQR [3]) has been discussed by Salerno [26] and may correspond to the proximal $UQ^{\bullet-}$ site (with respect to the dehydrogenase part of SQR). The heme b_L-associated HOQNO-sensitive site would

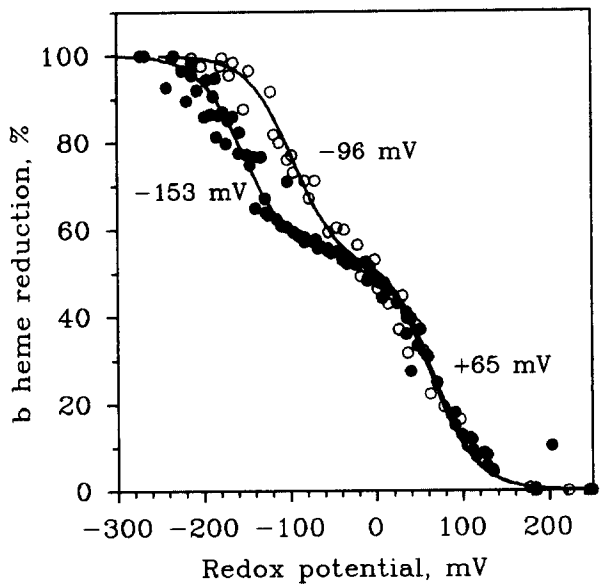

Fig. 3. Effect of HOQNO on the redox characteristics of the di-heme cytochrome *b* in isolated *B. subtilis* SQR. SQR (2.8 μM) was redox titrated anaerobically in the absence (○) and presence (●) of 40 μM HOQNO. Redox mediators: quinhydrone, diaminodurene, PMS, phenazine ethosulfate, 1,2-naphthoquinone, duroquinone, 1,4-naphthoquinone, and anthraquinone-1,5-disulfonic acid; duroquinone was used at 10 μM, all other mediators at 50 μM. Experimental points have been fitted by Nernst curves for 2 non-interacting single-electron components with unknown midpoint potentials (E_m) and contributions to the overall absorbance changes at the wavelength couple used (α). Computer analysis finds the best fit for the following parameters. *Without HOQNO:* Heme b_H: E_m = +64 mV, α = 0.52; heme b_L: E_m = − 96 mV, α = 0.48. *In the presence of HOQNO:* Heme b_H: E_m = +65 mV, α = 0.54; heme b_L: E_m = −153 mV, α = 0.46.

then possibly correspond to the distal semiquinone-binding domain since this heme is localized closer to the periplasmic side of the membrane [2,3]. The role of cytochrome *b* in semiquinone stabilization is further corroborated by the fact that the TTFA-sensitive stable ubisemiquinone EPR signal observed in mitochondrial membranes [8–10] is usually absent from purified SQR preparations depleted in cytochrome *b* [27]. It is tempting to suggest that the so-called Q-binding proteins [28], shown to stabilize ubisemiquinone when added to purified mitochondrial SQR [27], may be in fact parts of the cytochrome *b* apoprotein which splits into two polypeptides in the mammalian enzyme.

In the light of the above discussion, the inhibition mechanism of HOQNO on *B. subtilis* SQR could be analogous to that proposed for TTFA and carboxins in case of the mitochondrial enzyme [29]. Reacting with cytochrome *b*, HOQNO prevents $MQ^{\bullet-}$ binding but does not compete as strongly with MQ. Accordingly, the second reduction step (semiquinone to quinol) of the 2-electron quinone redox transition will be fully suppressed. The first $MQ \rightarrow MQ^{\bullet-}$ half-reaction can still remain viable, although probably at a decreased rate since the product (menasemiquinone) is destabilized and, being unable to bind tightly to the enzyme, will either disproportionate to MQ and MQH_2 or autoxidize giving rise to superoxide radicals (cf. [29,30]). This MQ to $MQ^{\bullet-}$ transition could then account for the ca. 10% of the *B. subtilis* SQR activity resistant to HOQNO.

Acknowledgements: This work has been supported in part by the grants from the Royal Swedish Academy of Sciences and the Swedish Natural Science Research Council to L.H. and by Grant 12726a from the Russian Fund for Fundamental Research to A.A.K. We gratefully acknowledge discussion of the results with Dr. V.A. Kostyrko.

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