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THE SITE OF INHIBITION BY 5,5′-DITHIOBIS(2-NITROBENZOATE) IN UBIQUINOL:CYTOCHROME *c* OXIDOREDUCTASE

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In 5,5′-dithiobis(2-nitrobenzoate) (DTNB)-treated succinate:cytochrome *c* reductase, the electron transfer from duroquinol to cytochrome *c* is inhibited due to the fact that the Rieske Fe-S cluster and, consequently, cytochrome *c*, are no longer reducible by substrate. The finding that, after this treatment, cytochrome *b* is still reducible by substrate in the absence of antimycin, but not in its presence, is consistent with a Q-cycle mechanism for the electron transfer through QH_2:cytochrome *c* oxidoreductase. The inhibitory effect of DTNB and its effect on the EPR spectrum of the [2Fe-2S] cluster suggest that it prevents either the binding of ubiquinone in the vicinity of this cluster or the interaction between the Fe-S protein and a ubiquinone-binding protein.

Baum et al. [1] and Gellerfors et al. [2] showed that the thiol reagents, mersalyl and iodoacetamide, inhibit QH_2:cytochrome *c* oxidoreductase (EC 1.10.2.2). We shall report elsewhere that the enzyme is also inhibited by the thiol reagents *p*-hydroxymercuribenzoate, 2-nitro-5-thiocyanobenzoate and DTNB. The present report deals with the localization of the inhibition by DTNB.

Succinate:cytochrome *c* reductase was prepared by the method of Yu et al. [3]. Incubation of succinate:cytochrome *c* reductase with the thiol reagent was carried out as described by Baum et al. [1], with a final pH of the reaction mixture of 7.1. The reaction was stopped by removal of unreacted DTNB by centrifugal gel filtration [4]. All spectral measurements were carried out in an Aminco-Chance spectrophotometer (DW-2), supplied with a thermostatically controlled cuvette holder and a magnetic stirrer. EPR measurements were performed as described in Ref. 5. 7-(*n*-Heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone (HMHQQ), like 7-(*n*-hexadecyl)mercapto-6-hydroxy-5,8-quinolinequinone [6] an inhibitory Q analogue (Zhu, Q.S., Berden, J.A., Slater, E.C., Folkers, K. and Porter, T., unpublished observations), was a gift from Dr. K. Folkers to Dr. A.W. Linnane.

In order to localize the site of inhibition by DTNB in QH_2:cytochrome *c* oxidoreductase, we followed the reduction of cytochromes c_1 (Fig. 1A) and *b* (Fig. 1B) by DQH_2, in the absence and presence of antimycin. In the absence of antimycin, the reduction of cytochrome c_1 (Fig. 1A, a–d) is inhibited in the DTNB-treated enzyme, as is also the reduction of the Rieske Fe-S protein (determined by EPR, not shown), whereas cytochrome *b* is still rapidly reducible (Fig. 1B, a–d). In the presence of antimycin, the reduction of cytochrome *b* is also inhibited (Fig. 1B, e–h). The results in Table I indicate a close correlation between the degree of inhibition and the extent of reduction of cytochrome *b* in the presence of anti-

Abbreviations: BAL (British Anti-Lewisite), 2,3-dimercaptopropanol; DTNB, 5,5′-dithiobis(2-nitrobenzoate); Q, ubiquinone including all redox states; QH_2, ubiquinol; DQH_2, duroquinol; HMHQQ, 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone; Me_2SO, dimethyl sulfoxide.

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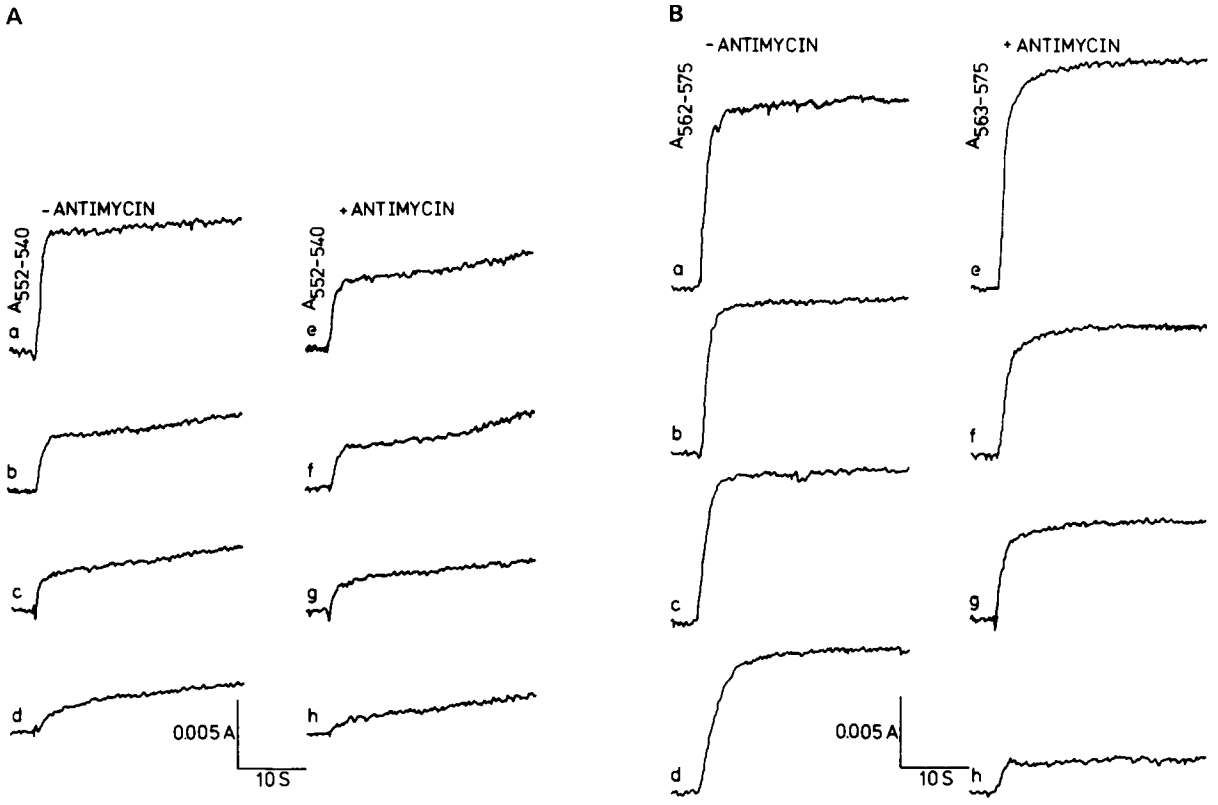

Fig. 1. Reduction of cytochrome c_1 (A) and cytochrome b (B) by 40 μM DQH_2, in the absence (a–d) and presence (e–h) of antimycin, in control (a and e) and DTNB-inhibited preparations of succinate: cytochrome c reductase. The remaining activities were: b and f, 57%, obtained with 5 mM DTNB, incubated for 10 min; c and g, 45%, obtained with 5 mM DTNB, incubated for 20 min; d and h, 16%, obtained with 20 mM DTNB, incubated for 15 min. Prior to the addition of DQH_2, the enzyme was oxidized with 9 μM ferricyanide. The biphasic reduction of cytochrome c_1 in the presence of antimycin (cf. traces e and a in A), in which the fast reduction phase consists always of 50% of the total cytochrome c_1, has been previously observed by De Vries et al. [12,14]. Temperature, 5°C; slit width, 2 nm. The decrease in the rate of reduction of cytochrome b (trace d) is only seen at high concentrations of DTNB and may be due to the reaction with other -SH groups.

mycin and of cytochrome c_1 in its absence.

A similar behaviour of cytochromes b and c_1 has been reported for BAL($+O_2$)-treated submitochondrial particles [7] and for Fe-S protein-depleted succinate: cytochrome c reductase [8]. As has been pointed out [7,9], this reduction be-

TABLE I

CORRELATION BETWEEN THE INHIBITION BY DTNB AND THE EXTENT OF CYTOCHROME b AND c_1 REDUCTION BY DQH_2 (40 μM) IN SUCCINATE: CYTOCHROME c REDUCTASE

DTNB (mM)	Incubation time (min)	% DQH_2: cytochrome c reductase activity	% cytochrome b reduced (+antimycin)	% cytochrome c_1 reduced (−antimycin)
0	10 and 60	100	100	100
0.2	60	86	86	85
4	60	38	39	42
20	10	19	18	19

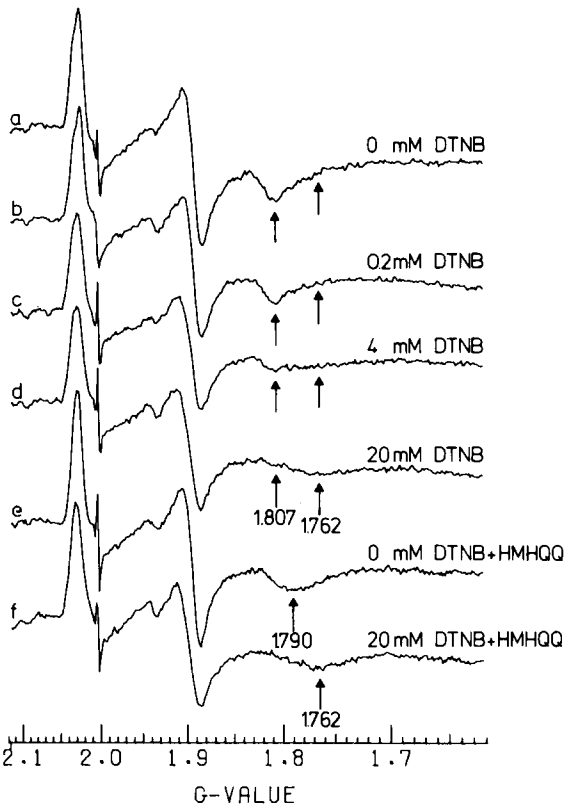

Fig. 2. Effect of DTNB on the EPR signal of the Rieske [2Fe-2S] cluster of succinate:cytochrome *c* reductase. Same samples as described in Table I. The protein concentration was the same in all six traces. The [2Fe-2S] cluster was reduced with 10 mM ascorbate. Traces a–d show the effect of increasing inhibition by DTNB. Traces e and f were obtained after addition of the Q analogue (100 μM in Me_2SO). EPR conditions: frequency, 9.26 GHz; modulation amplitude, 0.63 mT; power 5 mW; temperature, 36 K; The gain is the same for all traces.

haviour of cytochrome *b* in the inhibited enzyme cannot be explained by a linear electron-transfer sequence, but is easily explained by the version of the Q cycle [10] favoured by Zhu et al. [11]. In BAL ($+O_2$)-treated and Fe-S protein-depleted preparations, the oxidation of ubiquinol is impaired by destruction or removal of its oxidant, the Fe-S cluster.

Unique for the DTNB-inhibited enzyme, however, is the presence of the [2Fe-2S] cluster, as observed in EPR spectra of the ascorbate-reduced enzyme, although the g_x resonance at g 1.807 of the untreated preparation is broadened and shifted to g 1.762 in the DTNB-inhibited sample (cf. Fig. 2, traces a and d). As De Vries et al. [5] showed, the EPR spectrum of the Rieske Fe-S cluster in ascorbate-reduced submitochondrial particles and purified QH_2 : cytochrome *c* oxidoreductase is composed of two different EPR signals, originating from Fe-S cluster 1 and Fe-S cluster 2. Both the shape and the g values of the two clusters are different. This is most clearly seen in the g_x region where a sharp signal with g_x 1.807 (cluster 1) and a broad signal with g_x 1.762 (cluster 2) can be distinguished (cf. Fig. 2, trace a). More recently, it was shown [12] that cluster 1 has a sharp line shape with g_x 1.807 only in the presence of oxidized Q. In the absence of oxidized Q, either after extraction [12] or after reduction of Q [6], the line shape of cluster 1 is identical to that of cluster 2 (g_x 1.762). The line shape of cluster 2 is independent of the presence of Q or the latter's redox state.

Fig. 2, traces a–d, shows that as the degree of inhibition increases, the g_x resonance of cluster 1 (1.807) gradually broadens and shifts to 1.762. Since the total area under the g_z peak is equal in all four spectra we can conclude that cluster 1 is not destroyed by DTNB treatment, but that its line shape changes to become eventually identical to that of cluster 2. In control experiments, in which the DTNB-inhibited enzyme was oxidized with ferricyanide, prior to the addition of ascorbate, which selectively reduces the Fe-S cluster and cytochrome c_1 (but not Q), the same broad line shape of cluster 1 was observed. This indicates that the apparent absence of (oxidized) Q is not due to direct reduction of Q by thionitrobenzoate, formed during the reaction of DTNB with thiol groups.

Addition of the oxidized Q analogue (HMHQQ) to the control preparation induces a change in the line width and peak position of all three resonances of both cluster 1 and 2, in such a way that the two Fe-S clusters become indistinguishable (cf. traces e and a in Fig. 2). This is best seen in the g_x resonance line which, after addition of HMHQQ, is at g 1.790 for both clusters. In contrast, the EPR spectrum of the Fe-S clusters in the DTNB-treated preparation was not affected by the addition of the Q analogue (Fig. 2, traces d and f), indicating that not only cluster 1, but both clusters, are

affected by DTNB treatment.

The inhibition by DTNB and its effect on the EPR spectrum of the [2Fe-2S] clusters indicate that modification of a cysteine residue in the Q-binding site results in the prevention, directly or indirectly, of the binding of ubiquinol (and ubiquinone and ubisemiquinone) and, consequently, of electron transfer from QH_2 to the [2Fe-2S] clusters.

Whether the Fe-S protein itself contains the Q-binding site has not been unambiguously determined [13]. If there exists a separate Q-binding protein, it is possible that a modification of either this protein or the Fe-S protein causes a loss of interaction between the two proteins, resulting in an impaired electron transfer from QH_2 to the Fe-S cluster, and in a loss of response of the EPR signal of the Fe-S cluster 1 to the binding of oxidized Q and of both clusters to the binding of the Q analogue.

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