Effect of electron transfer inhibitors on superoxide generation in the cytochrome bc_1 site of the mitochondrial respiratory chain

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Antimycin, 2-nonyl-4-hydroxyquinoline *N*-oxide and funiculosin induce $O_2^{\cdot-}$ generation by submitochondrial particles oxidizing succinate, whereas KCN, mucidin, myxothiazol or 2,3-dimercaptopropanol inhibit $O_2^{\cdot-}$ generation. Thenoyltrifluoroacetone does not induce superoxide production by itself but slightly stimulates the reaction initiated by antimycin. The results indicate that auto-oxidation of unstable ubisemiquinone formed in centre *o* of the Q-cycle generates most of the $O_2^{\cdot-}$ radicals in the cytochrome bc_1-site of the mitochondrial respiratory chain.

Electron-transfer inhibitor *Superoxide generation* *Respiratory chain, of mitochondria*
Cytochrome bc_1 *site* *Q cycle, center* o *Ubisemiquinone auto-oxidation*

1. INTRODUCTION

Superoxide and H_2O_2 production in the bc_1 segment of the mitochondrial respiratory chain is one of the several well established but not yet fully understood specific phenomena intimately associated with electron transfer at coupling site 2 [1–9]. In the presence of uncouplers, H_2O_2 and/or $O_2^{\cdot-}$ generation with succinate as electron donor is virtually absent and the reaction can be switched on by antimycin [1–9]. Whether the initiation of 'active oxygen' production is a specific effect of antimycin or merely a consequence of electron-transfer inhibition between cytochromes *b* and c_1 is not clear.

In addition to antimycin, several other compounds block electron flow through the bc_1 site; they include BAL [10,11], NoHOQnO [12,13], mucidin [14], funiculosin [15], myxothiazol [16]. Preliminary experiments with NoHOQnO [8], BAL [17] and mucidin [18], as well as the reported effect of cyanide [8,9], suggested that oxygen radical generation at site 2 might be related to the mechanism of QH_2 oxidation in center *o* of the Q cycle [19,20].

We have previously described a very simple and sensitive method for studying $O_2^{\cdot-}$ generation based on the use of Tiron (1,2-dihydroxybenzene-3,5-isulfonate) as an EPR-visible superoxide scavenger [8]; earlier, Tiron had been used to detect $O_2^{\cdot-}$ formation in chloroplasts [21]. Here, we have taken advantage of the Tiron method to compare the effects of several specific inhibitors of mitochondrial electron transfer 'between *b* and c_1 cytochromes' on the succinate-dependent superoxide generation in beef heart SMP. Three of the inhibitors studied are found to stimulate $O_2^{\cdot-}$ production (antimycin,

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Abbreviations: NoHOQnO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; BAL, British Anti-Lewisite (2,3-dimercaptopropanol); SMP, sub-mitochondrial particles; SOD, superoxide dismutase; TTFA, α-thenoyltrifluoroacetone

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NoHOQnO and funiculosin) whereas 3 others have proved inhibitory (BAL, mucidin and myxothiazol). The data obtained corroborate the hypothesis [8,20] that in the absence of a physiological electron acceptor (ferricytochrome *b*-566), ubisemiquinone formed in center *o* of the Q cycle can react with oxygen, yielding superoxide.

2. METHODS

Antimycin and NoHOQnO were from Serva. Mucidin was kindly supplied by Dr V. Musilek (Inst. Microbiology, Acad. Sci. ČSSR, Prague), myxothiazol was a generous gift of Dr W. Trowitzsch (Gesellschaft für Biotechnologische Forschung, Braunschweig) and funiculosin was obtained through the courtesy of Dr P. Bollinger (Sandoz, Basel) and Professor P. Walter (University of Basel). SOD (superoxide dismutase grade I from bovine erythrocytes) and BAL (2,3-dimercaptopropanol) were purchased from Sigma. Tiron was either from Reachim ('pure for analysis' grade) or from Serva (reagent grade).

Sonic SMP were prepared from beef heart mitochondria according to [22] in the absence of Mn^{2+} which interferes with the $O_2^{\cdot-}$ and H_2O_2 assays. Treatment of SMP with BAL was done following [10] as described [11]. Measurements of the $O_2^{\cdot-}$ generation in SMP by the Tiron method were done as in [8] in a Varian E-4 ESR spectrometer.

3. RESULTS

As reported in [8], the steady-state concentration of Tiron semiquinone is fairly sensitive to the rate of $O_2^{\cdot-}$ generation by the respiratory chain of SMP. Fig.1 shows typical recordings of the succinate-dependent $O_2^{\cdot-}$ generation in SMP as monitored by the Tiron method [8,21]. One can see that addition of antimycin (A), NoHOQnO (B), or funiculosin (C) to the aerobic succinate-supplemented particles gives rise to an intensive Tiron semiquinone ESR signal which is strongly inhibited (and even more strongly prevented, cf. table 1) by cyanide.

Incubation of SMP with Tiron alone, Tiron and succinate or Tiron and any of the inhibitors used did not induce any measurable Tiron semiquinone ESR signal.

In the presence of SOD added before the initiation of $O_2^{\cdot-}$ generation, at doses sufficiently high to compete for $O_2^{\cdot-}$ with excess Tiron (as calculated from the published rate constants of $O_2^{\cdot-}$ removal by the two scavengers [21,23], the Tiron ESR signal observed soon after the addition of succinate and antimycin was strongly depressed (expt. 1, table 1). However, the signal recovered in 10–15 min. We also found that SOD did not inhibit but rather slightly increased the preformed Tiron signal when added after antimycin + succinate (not shown). The latter effects agree well with the data in [24] and are due to Tiron semiquinone stabilization in a complex with SOD [24].

Some of the effects of a number of respiratory chain inhibitors on the succinate-dependent $O_2^{\cdot-}$ generation by submitochondrial particles are listed in table 1. The results are briefly discussed below.

3.1. *Antimycin, NoHOQnO and funiculosin*

These 3 inhibitors actively stimulated $O_2^{\cdot-}$ generation at concentrations consistent with their $[I]_{50}$ values, and at saturating doses gave rise to the Tiron signal of approximately the same size. In each case the reaction proved highly sensitive to cyanide.

We have noticed in [8] that NoHOQnO addition after antimycin partially inhibited H_2O_2 generation in SMP as monitored by the scopoletin method [1]. Such an effect was not observed in the experiments with Tiron, and we have found subsequently that NoHOQnO can interfere with the scopoletin/peroxidase H_2O_2 assay as checked with the glucose oxidase-catalyzed H_2O_2 generation.

In the experiments with funiculosin, preincubation with the inhibitor for 3–5 min was required for a full development of the inhibition of electron transfer and of the $O_2^{\cdot-}$ generation in agreement with [15].

3.2. *BAL, mucidin and myxothiazol*

These inhibitors did not induce $O_2^{\cdot-}$ generation themselves and strongly suppressed the effect induced by antimycin (expt.5–7), or NoHOQnO and funiculosin (not shown). The same results have been obtained with respect to H_2O_2 generation (not shown, [17,18]). Inhibition of the antimycin-stimulated $O_2^{\cdot-}$ production by mucidin and myxothiazol occurred in the same concentration range as the inhibition of the succinate oxidase activity.

Also with several BAL-treated SMP preparations, inhibition of succinate oxidase activity correlated with the diminution of the height of the antimycin-induced Tiron signal (not shown).

3.3. *α-Thenoyltrifluoroacetone*

Trumpower and Simmons [25] reported that the specific inhibitor of succinate:CoQ-reductase TTFA stimulated $O_2^{\cdot -}$ generation by antimycin-inhibited, purified succinate–cytochrome *c*-reductase. On the other hand, TTFA has been reported to inhibit antimycin-induced H_2O_2 production in mitochondria [9]. It was therefore interesting to test the effect of TTFA in the Tiron assay.

In the absence of antimycin, TTFA did not induce $O_2^{\cdot -}$ generation, but a slight enhancement of the Tiron free radical signal could be observed upon TTFA addition after antimycin and succinate (expt.8). When TTFA was added to the antimycin-inhibited SMP before succinate, the development of the Tiron semiquinone spectrum was sometimes delayed (not shown), but the final size of the signal (after ~2–3 min) was usually ~20% higher than in the absence of the inhibitor. However, TTFA did not stimulate $O_2^{\cdot -}$ generation in the antimycin + KCN-inhibited SMP; i.e., under the conditions which are probably most close to those in [25], where cytochrome *c* oxidase-deficient succinate–cytochrome *c*-reductase was used.

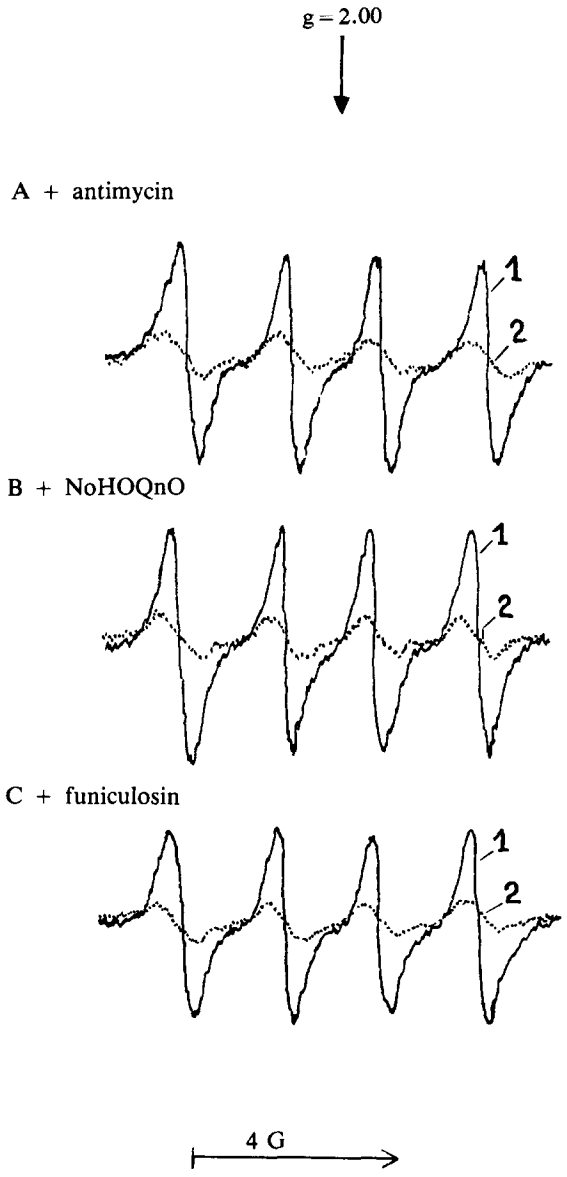

Fig.1. ESR spectra of Tiron semiquinone formed in the presence of beef heart SMP supplemented with succinate and antimycin (A), NoHOQnO (B) or funiculosin (C). Beef heart SMP (1 mg protein/ml) in the aerobic medium containing 0.2 M sucrose, 50 mM KCl, 20 mM Hepes–KOH (pH 7.5), 3 μM rotenone, 1 μM carbonyl cyanide *m*-chlorophenylhydrazone and 2 mM Tiron were preincubated for 3 min with (A) 1 μM antimycin, (B) 3 μM NoHOQnO or (C) 1 μg funiculosin/ml. Under these conditions no ESR signal could be observed (not shown). $O_2^{\cdot -}$ generation was then initiated by 5 mM succinate (spectra 1 in A–C) and subsequently inhibited by 1 mM KCN (spectra 2 in A–C). Experiments were performed as follows. The stock reaction mixture (total vol. 0.3 ml) was prepared in a short test tube. After appropriate additions, aliquots of the sample were transferred into a flat quartz cell for ESR spectroscopy (operative volume ~20 μl) and placed into the spectrometer; this took ~0.5 min. Spectra were subsequently scanned, one-by-one, to follow the dynamics of the Tiron signal; under most conditions, the steady-state was reached before the first spectrum was recorded. It was maintained for at least 10–15 min. The data in fig.1 and table 1 represent the measurements made 2 min after the addition of the final reagent, if not indicated otherwise. Conditions of ESR spectroscopy: modulation frequency, 100 kHz; modulation amplitude, 0.25 G; microwave power, 20 mW; scanning rate, 10 G/min; time constant, 0.1 s; T = 25°C.

Table 1

Succinate-dependent $O_2^{\cdot-}$ generation in beef heart submitochondrial particles as measured by the Tiron method

Expt. no., basic conditions	Other additions	Tiron semiquinone ESR signal height, arbitrary units
Expt.1 Control SMP	Antimycin	0
	Succinate	0
	Antimycin, succinate+ KCN	100
	KCN, antimycin + succinate	18
		<2
Expt.2 Control SMP	Antimycin, succinate	100
	SOD (150 μg/ml), antimycin and succinate	
	after 1 min	25
	after 10 min	85
Expt.3 Control SMP, succinate[b]	HOQNO	
	0	0
	0.1 μM	9
	0.5 μM	26
	1.0 μM	64
	4.0 μM	114
	42.0 μM	101
	KCN + 4 μM HOQNO	<2
Expt.4 Control SMP, succinate[b]	Funiculosin	
	0	0
	0.1 μM	20
	0.5 μM	62
	1.0 μM	110
	KCN + 1 μM funiculosin	<2
Expt.5 BAL-treated SMP[a]	Succinate	0
	Antimycin + succinate	25
Expt.6 Control SMP, succinate[b]	Mucidin (1 μM)	0
	Antimycin + mucidin	
	0	100
	80 nM	94
	160 nM	57
	320 nM	46
	630 nM	20
	780 nM	<2
Expt.7 Control SMP, succinate[b]	Myxothiazol (1 μM)	0
	Antimycin + myxothiazol	
	0	100
	20 nM	82
	40 nM	64
	60 nM	40
	80 nM	23
	150 nM	<2
Expt.8 Control SMP	Antimycin, succinate + 200 μM TTFA	100 130

[a] The particles subjected to the same procedure as the BAL-treated ones but omitting BAL from the reaction mixture were taken as the control; the succinate oxidase activity of the BAL-treated preparation was 30% of the control

[b] Succinate was the final addition which switched on $O_2^{\cdot-}$ generation

Experimental conditions were as in fig.1 unless indicated otherwise. In each experiment, the steady-state height of the Tiron free radical ESR signal observed in the presence of antimycin and succinate was taken as 100; in expt.3–5 this was measured in separate controls

4. DISCUSSION

There is much evidence that H_2O_2 and $O_2^{\cdot-}$ generation at site 2 of the respiratory chain results from auto-oxidation of ubiquinol or ubisemiquinone [3,5,6], which may be explained by reactions of the latter in center *o* of the Q cycle [19,20]. The present results are fully compatible with this idea, and demonstrate the usefulness of $O_2^{\cdot-}$- and/or H_2O_2-generating activity measurements for locating the sites of action of electron-transfer inhibitors of the cytochrome bc_1 complex (fig.2).

In the presence of uncouplers but without site 2 inhibitors, ubisemiquinone formed in center *o* on QH_2 oxidation by FeS_{Rieske} [26], or by a special FeS_{Rieske}-associated CoQ molecule [20], is rapidly oxidized to Q by *b* cytochromes. Hence the steady state concentration of $SQ_o^{\cdot}$ and the rate of its auto-

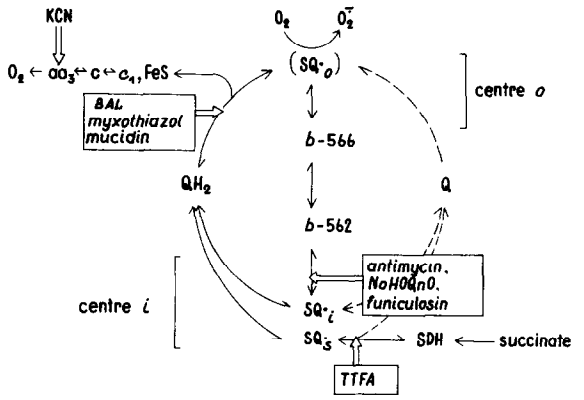

Fig.2. Possible mechanism of $O_2^{\cdot -}$ generation in the succinate:cytochrome *c*-reductase span of the mitochondrial respiratory chain. A 'Q cycle' [19] electron flow diagram is shown. Transfer of reducing equivalents is indicated by solid lines and migration of oxidized CoQ by dashed lines. SDH, succinate dehydrogenase. $SQ_s^{\cdot}$, the rapidly relaxing TTFA- and carboxin-sensitive stable ubisemiquinone [31–34] interacting with SDH; $SQ_i^{\cdot}$, the slowly relaxing antimycin-sensitive stable ubisemiquinone [32,35] associated with CoQ-reducing center *i* of complex bc_1; $SQ_o^{\cdot}$, a putative unstable ubisemiquinone presumed to be formed as an intermediate in QH_2-oxidizing center *o* of complex bc_1 [32,34,36]. Of these 3 ubisemiquinone species, the strongly reducing $SQ_o^{\cdot}$ is suggested to have the major contribution to superoxide generation stimulated by antimycin, NoHOQnO or funiculosin at low redox potential of the succinate/fumarate couple.

oxidation should be very low. When electron transfer via center *i* is inhibited by antimycin, NoHOQnO or funiculosin (or when the membrane is energized [3,9]), cytochrome *b*-566 becomes highly reduced in the aerobic steady state and can no longer compete with O_2 for $SQ_o^{\cdot}$. It follows that auto-oxidation of the latter will be greatly enhanced, as observed. In good agreement with this model, KCN suppresses antimycin- or NoHOQnO-stimulated $O_2^{\cdot -}$ generation by blocking oxidation of QH_2 to $SQ_o^{\cdot}$ via the cytochrome chain ([8,9] and here).

In contrast to antimycin, NoHOQnO and funiculosin, BAL, mucidin and myxothiazol inhibit center *o* [11,18,27]. This could occur at either of the two steps of QH_2 oxidation to Q (fig.2). Inhibition of QH_2 oxidation to $SQ_o^{\cdot}$ should inhibit $O_2^{\cdot -}$ production, whereas inhibition of $SQ_o^{\cdot}$ oxidation by cytochrome *b* should have an opposite antimycin-like effect. Accordingly, the abolishment of succinate-dependent $O_2^{\cdot -}$ production by BAL, mucidin and myxothiazol indicates that these agents interfere with oxidation of QH_2 to $SQ_o^{\cdot}$. In agreement with this, BAL has been reported to destroy the FeS_{Rieske} [28], which is believed to be involved in the above reaction [26].

TTFA affects $O_2^{\cdot -}$ generation by SMP only by inhibiting reduction of Q by succinate dehydrogenase. Indeed, our preliminary studies on the redox potential dependence of $O_2^{\cdot -}$ production [29] show that the subtle stimulation of the antimycin-induced reaction by TTFA in the presence of excess succinate may be an unspecific consequence of a decreased steady state $(QH_2)/(Q)$ ratio. In fact, malonate has been reported to have an effect similar to that of TTFA [30].

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