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The site of production of superoxide radical in mitochondrial Complex I is not a bound ubisemiquinone but presumably iron–sulfur cluster N2

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Abstract The mitochondrial respiratory chain is a powerful source of reactive oxygen species, considered as the pathogenic agent of many diseases and of aging. We have investigated the role of Complex I in superoxide radical production in bovine heart submitochondrial particles and found, by combined use of specific inhibitors of Complex I and by Coenzyme Q (CoQ) extraction from the particles, that the one-electron donor in the Complex to oxygen is a redox center located prior to the binding sites of three different types of CoQ antagonists, to be identified with a Fe–S cluster, most probably N2 on the basis of several known properties of this cluster. Short chain CoQ analogs enhance superoxide formation, presumably by mediating electron transfer from N2 to oxygen. The clinically used CoQ analog, idebenone, is particularly effective in promoting superoxide formation. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Coenzyme Q; Complex I; Iron–sulfur cluster; Rotenone; Submitochondrial particle; Superoxide

1. Introduction

Reactive oxygen species (ROS) is a cumulative designation for the products of partial reduction of molecular oxygen and comprehend both free radicals and neutral molecular species. There are several reactions in cells that are able to give rise to superoxide anion radical and/or to hydrogen peroxide; the latter can react with a reduced metal ion (such as Fe^{2+} or Cu^{+}) and give rise to the most aggressive hydroxyl radical.

There is almost no area of human pathology where oxidative stress has not been implicated since ROS are considered as the main pathogenic agents of most diseases [1]. Moreover, it is currently believed that ROS are involved in the progressive deterioration of cell structures accompanying ageing [2].

Within a cell, mitochondria largely contribute to the production of ROS via the respiratory chain [3]. The relevance of mitochondrial production of ROS within a cell is indirectly revealed by the results of deficiency of mitochondrial antioxidant enzymes. The effect of knock-out of the superoxide dismutase (SOD)-2 gene for mitochondrial SOD in transgenic mice is dramatic, with inability to survive for more than 2 weeks after birth and development of a series of biochemical defects ascribed to oxidative stress [4]. This means that the enzyme function is vital in the normal cell for preventing the toxic effect of superoxide radical generated in the mitochondria. Similarly, the lack of mitochondrial glutathione peroxidase is deleterious in that the accumulation of hydrogen peroxide in the presence of reduced metal ions leads to the extremely toxic hydroxyl radical [5].

It has long been understood that the major sites of superoxide formation in the respiratory chain are within respiratory complexes I and III [6]. Early experiments proved the involvement of Complex I (NADH Coenzyme Q (CoQ) reductase) in ROS production [7]; addition of either NADH at low concentration or of NADPH, which feeds the electrons at decreased rate into the Complex, led to copious ROS production detected by lipid peroxidation; on the other hand, addition of NADH at high concentration, but in presence of rotenone, also induced peroxidation. In another study [8] water-soluble CoQ homologs used as electron acceptors from isolated Complex I-stimulated H_2O_2 production in the order $CoQ_1 > CoQ_0 > CoQ_2$, whereas CoQ_6 and CoQ_{10} were inactive; the rate of H_2O_2 production was partly inhibited by rotenone, indicating that water-soluble quinones may react with oxygen when reduced at sites both prior and subsequent to the rotenone block. There is evidence that the one-electron donor to oxygen in Complex I is a non-physiological quinone reduction site different from the physiological site [9,10]; the former, hydrophilic site reduces several quinones to the corresponding semiquinone forms, which are unstable and can reduce oxygen to superoxide. This mechanism is shared by several quinones, including such drugs as anthracyclines [11] and the clinically employed CoQ analog, idebenone [12]. In view of the experiments of Takeshige et al. [7], the hydrophilic, rotenone-insensitive site can apparently reduce oxygen to superoxide in absence of intermediate acceptors. A series of recent studies by Barja and coworkers confirmed that Complex I is a major source of superoxide production in several types of mitochondria [13] and localized the oxygen reducing site between the ferricyanide reducing site and the rotenone block [14].

The presence of antioxidant enzymes in the mitochondrial matrix [4,5,15] makes it difficult to localize the site(s) of ROS production in intact mitochondria. Although the first product of oxygen reduction by the respiratory chain is certainly

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Abbreviations: CoQ, Coenzyme Q; DB, decyl-ubiquinone (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone); EDTA, ethylenediaminetetraacetate; HPLC, high performance liquid chromatography; ROS, reactive oxygen species; SMP, submitochondrial particles; SOD, superoxide dismutase

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superoxide, this radical has a short life and is rapidly converted into hydrogen peroxide by mitochondrial SOD or by spontaneous disproportionation, or can attack other molecules, such as lipids, before being able to escape the mitochondrion. For this reason identification of the site(s) of superoxide production can best be achieved in submitochondrial particles (SMP) which are devoid of matrix (antioxidant) enzymes and do expose the respiratory enzymes to the external medium.

In this study we provide further evidence pertaining to the site of superoxide production in Complex I in bovine heart SMP by exploiting a series of inhibitors acting at different sites in the route of electrons and by studying the behavior of CoQ-depleted and -reconstituted mitochondrial particles. In particular we have used *p*-hydroxy-mercuribenzoate, which inhibits at the level of iron–sulfur clusters of the Complex [16], and three classes of quinone antagonists according to the nomenclature of Degli Esposti [17], acting at three different hydrophobic sites in the Complex. The results of this investigation provide evidence that the source of one-electron reduction of oxygen in the Complex is not endogenous bound ubisemiquinone but is an iron–sulfur cluster, presumably N2 in view of its physico-chemical properties and its ability to interact with endogenous ubiquinone [18].

2. Materials and methods

Most chemicals were obtained from Sigma Co., St. Louis, MO, USA. Idebenone was a gift of Dr. M. Degli Esposti, University of Glasgow, UK. Rolliniastatin-1 and -2 were gifts of Dr E. Estornell of the University of Valencia, Spain. Mucidin (strobilurin A) was a gift from Dr. J. Subik of the University of Bratislava, Slovakia.

SMP were prepared from bovine heart mitochondria as described elsewhere [19]. CoQ-depleted SMP were prepared by pentane extraction of lyophilized particles according to Szarkowska [20], and reconstitution with CoQ_{10} was achieved by adding the quinone in pentane to the dried sample as described by Norling et al. [21] The CoQ content of different types of particles was determined by high performance liquid chromatography (HPLC) as described elsewhere [22].

NADH oxidase, NADH CoQ reductase (using decyl-ubiquinone (DB) as acceptor), and ubiquinol cytochrome *c* reductase activities were assayed as described previously [23]. All inhibitors used were at least 95% effective at the concentrations used, except *p*-hydroxy-mercuribenzoate and myxothiazol that were employed at concentrations giving ca. 90% and 82% inhibition respectively.

Superoxide production was assayed by exploiting the oxidation of epinephrine to adrenochrome by the superoxide radical [24] in a Sigma-Biochem ZWS2 double-wavelength spectrophotometer thermostated at 25°C, at 485−575 nm, using an extinction coefficient of 2.96 mM^{-1} cm^{-1}. A standard curve was obtained by using a xanthine–xanthine oxidase system for the generation of superoxide. The system contained 10 mM Tris–HCl, 50 mM KCl and 1 mM ethylenediaminetetraacetate (EDTA), pH 7.8, 0.5 μM catalase, 1 mM epinephrine and 0.5 mg SMP, in presence of different inhibitors as described in the text; the reaction was started by addition of 125 μM NADH (occasionally by 12.5 μM DBH_2 for estimation of superoxide production by Complex III); after 15 min recording, eventual addition of Complex I acceptors (DB, CoQ_1, CoQ_2) was performed and the trace was recorded for 5 additional min. At the end, SOD (Sigma, from bovine liver), 13 μg, was added and the trace was recorded for 10 min The rate was usually inhibited by SOD by 90% or more. The results are expressed as SOD-sensitive activity by subtracting the activity in presence of SOD, unless otherwise specified.

Table 1
Effect of Complex III inhibitors on superoxide production in bovine heart SMP

Inhibitor	Activity −SOD	Activity +SOD
None	0.27 ± 0.16	0.01 ± 0.02
Mucidin (1.8 μM)	0.23 ± 0.03	0.04 ± 0.06
Antimycin A (0.2 mg ml^{-1})	2.41 ± 0.31	0.21 ± 0.17

Superoxide production was assayed as described in Section 2. The activity was determined with 12.5 μM DBH_2 as a substrate. Activity is expressed as nmol min^{-1} (mg protein)$^{-1}$.

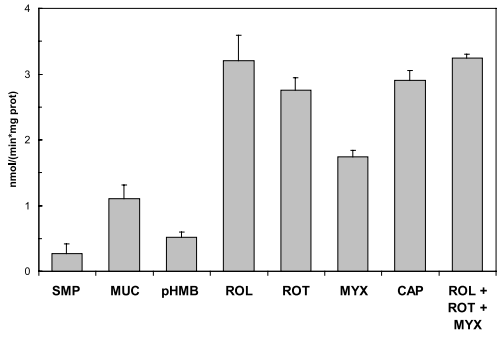

Fig. 1. Superoxide radical production by Complex I in bovine SMP in the presence of 125 μM NADH and no Complex I acceptor; mucidin (MUC) (1.8 μM) was added to inhibit Complex III. The concentrations of the other inhibitors on top of mucidin were: *p*-hydroxy-mercuribenzoate (pHMB) (Fe–S cluster inhibitor), 59 μM; rolliniastatin-2 (ROL) (Center A inhibitor), 0.2 nmol mg^{-1} protein; rotenone (ROT) (Center B inhibitor), 0.2 nmol mg^{-1} protein; capsaicin (CAP) (Center C inhibitor), 4 μmol mg^{-1} protein; myxothiazol (MYX) (Center C inhibitor), 170 nmol mg^{-1} protein. Inhibitor classes are according to the nomenclature of Degli Esposti [17] (cf. Fig. 3). The lower effect of myxothiazol with respect to capsaicin may be related to its lower extent of Complex I inhibition at the concentration used (82% vs. 99%).

3. Results

In order to functionally isolate superoxide production by Complex I only, its formation by Complex III was prevented using mucidin, an inhibitor of center o. We avoided to use antimycin A, since this center i inhibitor is known to enhance superoxide formation [25], and myxothiazol, another center o inhibitor that however also inhibits Complex I [26]. The effect of antimycin A and mucidin on superoxide production by Complex III is shown in Table 1: as expected, antimycin A strongly stimulated superoxide production, however mucidin had no effect. For this reason, all subsequent experiments were performed in presence of 1.8 μM mucidin.

At difference with the findings of Barja [14] we observed very low superoxide production after NADH addition to non-inhibited SMP. Addition of NADH to mucidin-inhibited SMP enhanced superoxide formation 4-fold; the mucidin-stimulated activity was inhibited by *p*-hydroxy-mercuribenzoate and was further enhanced to similar extents (about 3-fold) by Complex I inhibitors belonging to all three classes of quinone antagonists and by combinations thereof (Fig. 1). Addition of short chain analogs and homologs of CoQ (CoQ_1, CoQ_2, DB) enhanced superoxide formation of non-inhibited Complex I (in presence of mucidin) and this enhancement was further stimulated by the hydrophobic Complex I inhibitors (Fig. 2): the three acceptors were effective in the order $CoQ_1 > DB > CoQ_2$. The Complex I activity in SMP, measured as NADH-DB reductase under the same conditions, was 299 ± 47 nmol (i.e. 598 electron equivalents) min^{-1} (mg protein)$^{-1}$; thus, the electrons escaping to oxygen in presence of DB were 0.4% in absence of Complex I inhibitors and 1.4% in presence of hydrophobic inhibitors.

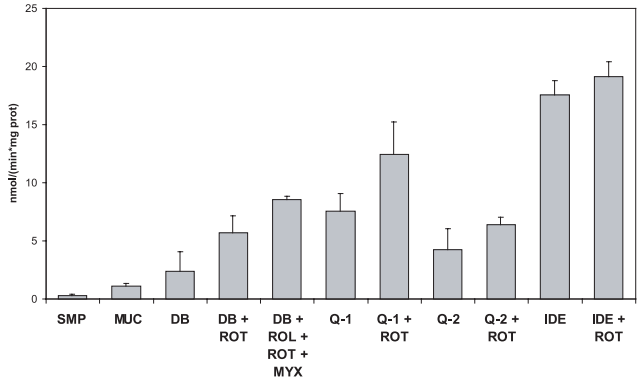

Fig. 2. Superoxide radical production by Complex I in the presence of 125 μM NADH, 1.8 μM mucidin and 60 μM acceptor, either DB; CoQ_1, or CoQ_2. Inhibitor concentrations were the same as in the legend of Fig. 1. Idebenone (IDE) was 2 μmol (mg protein)$^{-1}$.

The behavior of idebenone was particularly striking, as it stimulated superoxide production in mucidin-inhibited SMP almost 20-fold; idebenone is a type A inhibitor but is also an electron acceptor from the Complex [17].

The effect of CoQ extraction and reconstitution from lyophilized mitochondria is shown in Table 2: apart from the low extent of rotenone stimulation of superoxide production in all particles that had undergone lyophilization, the superoxide production in mucidin-inhibited particles was not changed by extraction of the endogenous CoQ_{10}. The stimulation by DB was about the same as in non-extracted SMP. The extent of extraction of endogenous CoQ was checked by HPLC: the extracted particles were found to contain a residual amount of 83 pmol CoQ (mg protein)$^{-1}$, in accordance with previous results [22], vs. an amount of 3.03 nmol mg^{-1} in the parent lyophilized particles and an excess of 15.7 nmol mg^{-1} in the reconstituted ones.

4. Discussion

Bovine heart SMP respiring with NADH under non-inhibited conditions produce low amounts of superoxide radical at 25°C (0.27 ± 0.16 nmol min^{-1} (mg protein)$^{-1}$), nevertheless comparable with the results of Herrero and Barja [14] in cow SMP (2.5 ± 0.3 nmol min^{-1} mg^{-1}, however at 37°C). In order to functionally isolate Complex I from the downstream segments of the respiratory chain in membranes containing the entire respiratory chain, one has to add a respiratory inhibitor acting at the level of Complex III. This manipulation has the further advantage of maintaining the respiratory chain components upstream of Complex III more reduced, thus favoring interaction with oxygen of the auto-oxidizable components (cf. [27]). It is known that addition of antimycin A to mitochondria respiring on either NAD-linked substrates or succinate enhances ROS production at the level of Complex III [25,28]: this inhibitor is therefore not suitable for the purpose. According to the Q-cycle mechanism, center o inhibitors should inhibit superoxide production by inhibiting semiquinone formation [29]. However myxothiazol, in absence of antimycin, may induce ROS production [30]; moreover myxothiazol is also an inhibitor of Complex I [26], therefore we have used it in connection with our survey of Complex I inhibitors. We have found that mucidin (or strobilurin A), a center o inhibitor [31], does not stimulate superoxide generation by Complex III in SMP supplemented with ubiquinol (Table 1). We have therefore used mucidin in most experiments in order to functionally isolate Complex I.

Table 2
Superoxide production in SMP after pentane extraction (SMP −Q) and reconstitution with CoQ_{10} (SMP +Q) (cf. Section 2)

Particle	Addition	−DB	+DB
SMP −Q	–	0.57 ± 0.05	1.91 ± 0.51
SMP −Q	rotenone (2 nmol mg^{-1})	0.73 ± 0.01	2.64 ± 0.93
SMP +Q	–	0.60 ± 0.05	1.41 ± 0.03
SMP +Q	rotenone (2 nmol mg^{-1})	0.57 ± 0.05	2.87

The endogenous CoQ_{10} content was 83 pmol mg^{-1} in the extracted particles and 15.7 nmol mg^{-1} in the reconstituted ones. Activities were assayed in the presence of NADH and mucidin at the usual concentrations and are expressed in nmol min^{-1} (mg protein)$^{-1}$. [DB] when added was 60 μM.

Addition of NADH to mucidin-inhibited SMP results in a 4-fold enhancement of superoxide production with respect to non-inhibited particles: this is expected, since these conditions are bound to keep Complex I in a more reduced state, thus favoring interaction of one or more redox centers with oxygen [27]. Subsequent addition of rotenone and other inhibitors, belonging to all three classes of antagonists of the ubiquinone-binding sites [17], further enhances superoxide formation by about 3-fold. Thus the enhancing effect of rotenone (and the other hydrophobic inhibitors) on superoxide production over that of non-inhibited SMP is more than 10-fold, compared with a less than 2-fold effect found by Herrero and Barja [14]; this striking difference might be explained by a higher superoxide production by Complex III in absence of Complex III inhibitors in the study by Herrero and Barja, perhaps due to the different assay temperature (cf. above).

Addition of *p*-hydroxy-mercuribenzoate inhibits superoxide generation, in agreement with the finding of Herrero and Barja [14], who also found inhibition by ethoxyformic anhydride. While the former compound acts before the ferricyanide site, the latter acts after the same site [32], so that flavin can be discarded as the free radical generator [14]. The enhancement of superoxide generation by the quinone antagonists belonging to all three classes of Degli Esposti's classification [17] and by a combination thereof demonstrates that the free radical generator is located before the quinone-binding sites. It is known that rotenone quenches the semiquinone signal of CoQ bound to Complex I [33], and it is extremely likely that a combination of inhibitors of the three classes prevents formation of ubisemiquinone, thus excluding its being the direct electron donor to oxygen. The oxygen radical generator is therefore presumably an iron–sulfur cluster, in agreement with the conclusion of Herrero and Barja [14]. This assumption was directly demonstrated in this study by the observation that CoQ depletion has no effect on superoxide production in mucidin-inhibited SMP. To this purpose it has to be noted that even a large excess of CoQ_{10}, as in the CoQ-reconstituted particles, has no effect on superoxide production by Complex I, although the short chain analog DB added to the reconstituted particles enhances its generation to similar extents as in the extracted particles (cf. Table 2).

Addition of the commonly used acceptors for Complex I activity determination, such as CoQ_1, CoQ_2, and DB, in presence of NADH and mucidin, also enhances superoxide production, both in SMP and in CoQ-depleted SMP. This observation means that all of these acceptors, besides acting at the

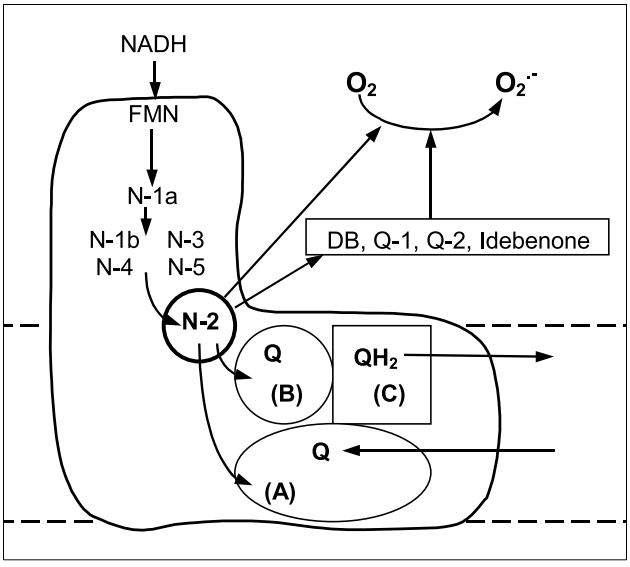

Fig. 3. A model of electron transfer in Complex I showing the site of superoxide production proposed on the basis of the results of the present study. The scheme follows the model of Degli Esposti [14] and depicts Fe–S cluster N2 as the source of electrons to bound ubiquinone (Center B) and to the ubiquinone molecule deriving from the pool (Center A). The two derived semiquinones dismutate so that Center B contains oxidized ubiquinone, while the reduced ubiquinone (ubiquinol) moves to Center C where from it is released to the pool. The effect of different inhibitors and acceptors (see text) is compatible with Fe–S cluster N2 as the source of one electron to oxygen or to exogenous quinones (in place of the endogenous bound CoQ_{10}), which, in turn, reduce oxygen to superoxide. Idebenone behaves both as an acceptor and as a type A inhibitor.

'physiological' site for CoQ_{10} of the pool, also interact at a site situated upstream with respect to the binding site of the ubiquinone antagonists, becoming reduced to semiquinones, which, in turn, reduce molecular oxygen to superoxide. This redox cycle is facilitated by the partially hydrophilic nature of these quinone acceptors.

The observation that short-chain quinone analogs enhance superoxide formation can be a hint on the identification of the redox center responsible for oxygen radical formation. It is likely, in fact, that this center is naturally a direct electron donor to protein-bound ubiquinone in the membrane, and that the quinone analogs compete with this interaction from within the water phase. Several reasons would point out that such a center is the tetranuclear iron–sulfur cluster N2 (Fig. 3): it has the highest mid-point potential among the Fe–S clusters of Complex I [34] and the redox potential is pH-dependent [18,35] and it appears to be located at the interface between the membrane subcomplex and the peripheral arm of the Complex [36]; in view of the magnetic interactions between the spins of N2 and bound ubisemiquinone, N2 is believed to be the direct donor to bound CoQ in the Complex [18,37] and it appears to be the immediate redox group prior to the rotenone block [18].

The effects of idebenone deserve a special comment. This quinone analog shares the property of being a Complex I inhibitor (of type A, according to Degli Esposti [17]) and an electron acceptor. It was already shown [12] that idebenone is strongly prone to stimulate oxygen radical generation by Complex I; this study quantitatively defines idebenone to be by far the most effective of the compounds tested in eliciting superoxide formation. It is remarkable that idebenone has a strong use in clinical experimentation [38] as a substitute of CoQ_{10} in therapy of mitochondrial cytopathies and of neurodegenerative diseases [39]. The observation that idebenone promotes oxidative stress on one hand may indicate that its behavior is different in vivo, on the other may suggest caution in its use as a drug.

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