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# Differential effects of mitochondrial Complex I inhibitors on production of reactive oxygen species

Romana Fato [a], Christian Bergamini [a], Marco Bortolus [c], Anna Lisa Maniero [c], Serena Leoni [a], Tomoko Ohnishi [b], Giorgio Lenaz [a,*]

[a] Dipartimento di Biochimica "G. Moruzzi", University of Bologna, Via Irnerio 48, 40126 Bologna, Italy
[b] Johnson Research Foundation, Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[c] Dipartimento di Scienze Chimiche, University of Padova, via Marzolo 1, 35131 Padova, Italy

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ABSTRACT

We have investigated the production of reactive oxygen species (ROS) by Complex I in isolated open bovine heart submitochondrial membrane fragments during forward electron transfer in presence of NADH, by means of the probe 2′,7′-Dichlorodihydrofluorescein diacetate. ROS production by Complex I is strictly related to its inhibited state. Our results indicate that different Complex I inhibitors can be grouped into two classes: Class A inhibitors (Rotenone, Piericidin A and Rolliniastatin 1 and 2) increase ROS production; Class B inhibitors (Stigmatellin, Mucidin, Capsaicin and Coenzyme $Q_2$) prevent ROS production also in the presence of Class A inhibitors. Addition of the hydrophilic Coenzyme $Q_1$ as an electron acceptor potentiates the effect of Rotenone-like inhibitors in increasing ROS production, but has no effect in the presence of Stigmatellin-like inhibitors; the effect is not shared by more hydrophobic quinones such as decyl-ubiquinone. This behaviour relates the prooxidant $CoQ_1$ activity to a hydrophilic electron escape site. Moreover the two classes of Complex I inhibitors have an opposite effect on the increase of NADH–DCIP reduction induced by short chain quinones: only Class B inhibitors allow this increase, indicating the presence of a Rotenone-sensitive but Stigmatellin-insensitive semiquinone species in the active site of the enzyme. The presence of this semiquinone was also suggested by preliminary EPR data. The results suggest that electron transfer from the iron–sulphur clusters (N2) to Coenzyme Q occurs in two steps gated by two different conformations, the former being sensitive to Rotenone and the latter to Stigmatellin.



## 1. Introduction

Complex I is a very large enzyme catalyzing at the entry point of the mitochondrial electron transport chain [1–3]. The total number of subunits in the bovine heart enzyme is 45 [4] for a molecular mass of about 1000 KDa. Seven subunits are products of the mitochondrial genome [5,6] that correspond to hydrophobic subunits named ND1–ND6 and ND4 L. The molecular mechanism of catalysis of this enzyme is not completely understood. The main reason is the lack of detailed structural information of the membrane part of Complex I, although X-ray structure of the extramembrane part was determined recently by Sazanov and Hinchliffe [7] utilizing *Thermus thermophilus* HB-8 enzyme. The minimal active form of Complex I is that found in bacteria, composed of 14 subunits, all of which are homologous to their mitochondrial counterparts. Based on this comparison, all other subunits are called "accessory subunits" and their functional role in the mitochondrial enzyme is not yet clear. The Complex I enzyme oxidizes NADH transferring electrons to a lipid soluble electron carrier, namely Ubiquinone or Coenzyme Q (CoQ). Based on the thermodynamic profiles of redox active groups, the FMN is considered to be the direct electron acceptor of NADH and subsequently electrons are transferred to the iron–sulphur clusters. Bovine heart Complex I contains 8 distinct iron–sulphur clusters (cluster N1a, N3, N1b, N4, N5, N6a, N6b, N2). Clusters N3–N6 are considered to share the same midpoint redox potential ($E_m$) values (−250 mV), and are called the isopotential group. Two clusters have different characteristics: N1a, that is the [2Fe–2S] type cluster, and has the lowest midpoint potential ($E_m$ = −370 mV) and cluster N2, that is the [4Fe–4S] type cluster which has the highest $E_m$ value (between −150 mV and −50 mV), and is located close to the interface between the peripheral and the

*Abbreviations:* DCFDA, 2′,7′-Dichlorodihydrofluorescein diacetate; NADH, β-Nicotinamide adenine dinucleotide; ROS, Reactive oxygen species; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; FMN, Flavin mononucleotide; BHM, Bovine heart mitochondria; BSA, Bovine serum albumine; DOC, Deoxycholate; SMP, Submitochondrial particles from bovine heart; DPI, Diphenylene iodonium; DB, Decyl-ubiquinone; DCIP, 2,6-dichloroindo-phenol; $CoQ_1$, 2,3-Dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone; FCCP, Carbonylcyanide-p-trifluoromethoxyphenylhydrazone; AAPH, α,α′-Azodiisobutyramidine dihydrochloride

* Corresponding author. Tel.: +39 051 209 1229; fax: +39 051 209 1217.
*E-mail address:* giorgio.lenaz@unibo.it (G. Lenaz).

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membrane arms [7]. Thus cluster N2 is considered to be the direct electron donor to ubiquinone. In the tightly coupled bovine heart SMP, initially three distinct EPR semiquinone (SQ) signals were proposed as Complex I components [8], but subsequently revised to two species of SQ signals [9]; one is uncoupler sensitive the other is insensitive. In the presence of reduced cluster N2, the former SQ species shows extremely fast spin relaxation (thus designated as $SQ_{Nf}$) while the latter shows much slower spin relaxation (designated as $SQ_{Ns}$). Direct spin–spin interaction between cluster N2 and $SQ_{Nf}$ was demonstrated and their mutual distance was estimated to be 12 Å [10,11]. In uncoupled SMP only the slowly relaxing SQ species is observed [8,9].

Complex I is inhibited by more than 60 different families of compounds [12] starting from Rotenone, the prototype of this series, to a number of synthetic insecticides/acaricides. These inhibitors were grouped into three classes based on their effects on the kinetic behaviour of the enzyme: Class I/A (the prototype of which is Piericidin A), Class II/B (the prototype of which is Rotenone) and Class C (the prototype of which is Capsaicin). Nevertheless, from kinetic studies it has not been possible to assign different binding sites for these three classes of inhibitors. Thus it is commonly accepted that they share the same large hydrophobic pocket in the enzyme [13].

Complex I is also involved in the formation of the trans-membrane proton gradient with a stoichiometry of $4H^+/2e^-$. The limited knowledge about the 3D-structure and the function of the whole Complex I makes it difficult to predict the proton pumping mechanism of Complex I across the inner mitochondrial membrane [9,14].

Besides its well known redox role in the electron transport chain, Complex I is also considered to be one of the main sites of reactive oxygen species (ROS) production; electrons leaked at Complex I can reduce oxygen and give rise to superoxide anion [15]. The mechanism of superoxide production by Complex I is not yet clear probably because of the lack of knowledge on the exact sequence of the electron carriers and how electron transfer is coupled to proton translocation. The sites of ROS production in the mitochondrial electron transport chain have been localized in Complex I and Complex III [16]. Whereas the site of electron escape in Complex III has been identified in the so called center "o", the direct oxygen reductant site in Complex I has not been established yet.

Recently, using different Complex I inhibitors to functionally dissect the enzyme, it was suggested that iron–sulphur cluster N2 could be the site of the electron leak [17], but N2–$SQ_{Nf}$ region [18], ubisemiquinone ($SQ_{Nf}$) [19], FMN [15,20,21] and iron–sulphur cluster N1a [22] have also been proposed as electron donors to oxygen. In addition, it was found that defective Complex I produces more reactive oxygen species (ROS) [16], suggesting that structural modifications of the enzyme may play a crucial role in the ROS production process.

The superoxide production by Complex I is much higher during the reverse electron transport from succinate to $NAD^+$ [19,23], than during the forward electron transport. The reasons of this discrepancy are still not understood.

An understanding of the detailed mechanism of reaction of Complex I is required not only for advancement in basic knowledge but also in biomedical research. In fact, a number of devastating neurodegenerative disorders are associated with Complex I deficiency, resulting in a decline of energy production by the respiratory chain and in increased production of reactive oxygen species (ROS) (for reviews see [24–28]).

Based upon the latter observations we have studied the effect of different Complex I inhibitors on the ROS production to elucidate the mechanism by which Complex I transfers electrons to molecular oxygen, with the additional aim to exploit superoxide generation to shed light on the mechanism of electron transfer to the natural acceptor, Coenzyme $Q_{10}$.

## 2. Materials and methods

### 2.1. Materials

2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) was purchased from Molecular probes, Invitrogen, Milano Italy. Mucidin (Strobilurin A) was a kind gift from Dr. F. Nerud of the Academy of Sciences in Prague, Czech Republic. Rolliniastatin-1 and -2 were gifts from Dr E. Estornell of the University of Valencia, Spain. Piericidin A and Stigmatellin were purchased from Fluka, Sigma-Aldrich, Milano, Italy. All other chemicals were purchased from Sigma-Aldrich, Milano, Italy.

### 2.2. Preparations

Submitochondrial particles (SMP) were prepared from bovine heart mitochondria (BHM) by sonic irradiation of the frozen and thawed BHM [29]; the particles were essentially broken membrane fragments [30]. Protein was evaluated by the Biuret method of Gornall et al. [31] with addition of 10% sodium deoxycholate and using bovine serum albumin (BSA) as the standard.

#### 2.2.1. Measure of hydrogen peroxide production

The method used to measure $H_2O_2$ production in submitochondrial particles (SMP) is based on the fluorogenic probe 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA or $H_2$DCFDA) which emits an intense green fluorescence only after deacylation and subsequent oxidation [32,33]. The advantage to use this probe is that it does not inhibit the activity of Complex I [34]. Alternatively $H_2O_2$ production was measured using Amplex Red. ROS production by SMP was measured in a fluorescence plate reader using a 96-well microtiter plate. In each well were present 0.5 mg/ml SMP (pretreated with 1.8 μM Mucidin) and 5 μM DCFDA or 10 μM Amplex Red to a final volume of 0.2 ml with KCl, 10 mM TRIS, 1 mM EDTA buffer, pH 7.5, 25 °C. The reaction was started by the addition of 150 μM NADH, in presence and in absence of different respiratory inhibitors and/or quinone acceptors.

#### 2.2.2. Enzyme assays

NADH–CoQ reductase was assayed essentially as described by Yagi [35] and modified by Degli Esposti et al. [36] in the presence of 2 mM KCN and 2 μM Antimycin A to block Complexes IV and III, respectively. Determination of the kinetic constants was accomplished at saturating concentration of NADH (150 μM) and 150 μM of $CoQ_1$ following the decrease in absorbance at 340 minus 380 nm, in a Jasco V550 spectrophotometer equipped with dual wavelength device, using an extinction coefficient of 3.5 $mM^{-1}$ $cm^{-1}$.

NADH–$O_2$ reductase activity was assayed essentially in the same conditions avoiding only KCN and Antimycin A in the assay mixture. To compare the inhibition effect of different Complex I inhibitors with ROS production, we performed the NADH–$CoQ_1$ reductase assays with high protein concentration (0.25 mg/ml of SMP).

NADH–DCIP reductase activity was assayed as above, following the reduction of DCIP absorbance at 748 nm using an extinction coefficient of 0.8 $mM^{-1}$ $cm^{-1}$ with 40 μg/ml of SMP. Activity was recorded in the presence and absence of Complex I inhibitors and $CoQ_1$ (25 μM) or DB (25 μM).

#### 2.2.3. EPR sample preparation

EPR samples were prepared as follows. Submitochondrial particles were suspended in the reaction buffer (Sucrose 0.25 M, TRIS 10 mM, EDTA 1 mM) to be 30 mg/mL. The suspension in a glass test tube was kept on ice. Antimycin A 5 μM, Carboxin 100 μM and Mucidin 1.8 μM were added and the mixtures were incubated on ice for at least 5 min.

SMP samples were treated with different Complex I inhibitors to completely block the enzyme: 10 μM Rotenone and 80 μM Stigmatellin. The reaction was initiated by adding 150 μM NADH.