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CYTOCHROME C IS A HYDROGEN PEROXIDE SCAVENGER IN MITOCHONDRIA

Zhi-Bo Wang, Min Li, Yungang Zhao and Jian-Xing Xu*

National Laboratory of Biomacromolecule, Center for Molecular Biology, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

Abstract: The ability of succinate–cytochrome c reductase (SCR) reduced cytochrome c to scavenge H_2O_2 was investigated. H_2O_2, whether added or produced by SCR, was efficiently removed when cytochrome c was reduced by SCR. On the other hand, ferrocytochrome c underwent re-oxidization when H_2O_2 was added. Thus, these results indicate that cytochrome c reduced by succinate–cytochrome c reductase has the ability to regulate H_2O_2 in mitochondria.

Key words: cytochrome c; hydrogen peroxide; mitochondria; succinate-cytochrome c reductase.

INTRODUCTION

There have been several reviews about cytochrome c [1-3]. Cytochrome c is an electron carrier, situated between Complex III (ubiquinone-cytochrome c reductase) and Complex IV (cytochrome oxidase) of electron transport chain of eukaryotic mitochondria. It is a peripheral membrane protein of approximately 12.5 kDa, lying on the outer surface of inner mitochondrial membrane, in the intermembrane space. It is reduced by electrons entering the chain either from NADH via Complex I (NADH-ubiquinone reductase) or through the Kreb's cycle via succinate and Complex II (succinate-ubiquinone reductase), but it is extremely rapidly oxidized by Complex IV, with the steady state of reduction towards the oxidized state. The redox function of cytochrome c is mediated by a single heme group (protophorphyrin IX), switching between the Fe^{2+} (ferro) and Fe^{3+} (ferri) states. In 1996, it was found that cytochrome c, when released from mitochondria to cytosol, activated an apoptosis [4]. Later on it was hypothesized that cytochrome c could be an antioxidant in vivo [5, 6].

Mitochondria from various aerobic organisms have been recognized as effective sources of H_2O_2 [7]. During mitochondrial respiration, as much as 1-2% of oxygen undergoes incomplete reduction to

superoxide anion ($O_2^{\cdot-}$) [8-10]. Produced at Complex I and III, sites of the electron transport chain, superoxide is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase or spontaneously [11]. $O_2^{\cdot-}$ and H_2O_2 are the primary sources of reactive oxygen species (ROS),and they play prominent roles in diverse pathophysiological progresses, such as neurodegeneration, aging, and heart and lung toxicity. Interaction of H_2O_2 with metal ions, such as Fe^{2+}, can result in the formation of hydroxyl radical ($OH^{\cdot}$). These species are highly reactive and can oxidatively damage protein, lipids and DNA [12, 13]. To maintain the delicate balance between production and removal of oxygen radicals, mitochondria maintain a vast array of antioxidant enzymes and low molecular weight scavengers including Mn-superoxide dismutase, gluathione peroxidase and vitamin E [7, 14, 15].

The purpose of this study was to gain further insight into the potential role of cytochrome c in the regulation of ROS level in mitochondria. Highly purified succinate-cytochrome c reductase was used, which could transfer electrons from succinate to cytochrome c.

2. MATERIALS AND METHODS

2.1. Materials

3-aminophthalhydrazide (luminol) was purchased from Acros, dimethyl-1-pyrroline-N-oxide (DMPO) from Sigma, succinic acid (disodium salt) from Aldrich, horseradish peroxidase (HRP) from Roche Diagnostics. Cytochrome c was prepared according the method of Margoliash and Walasek [16]. All other chemicals were commercial samples of high purity and used as supplied.

2.2. Preparation of succinate-cytochrome c reductase

Succinate-cytochrome c reductase (SCR) was prepared according to the procedure of Yu *et al.* [17]. The pig heart muscle preparation was used as starting materials instead of bovine heart muscle preparation. SCR contained 7.7 nmol of cytochrome b/mg of protein and 2.7 nmol of cytochrome c_1/mg of protein. The SCR activity was 2.1 μmol cytochrome c reduced/min/mg protein.

2.3. Measurement of H_2O_2

For the detection of hydrogen peroxide, the chemiluminescence (CL) method with luminol and horseradish peroxidase (HRP) was employed [18]. A modification is made with a dialysis tube (6 kDa MWCO) to separate the reactive system and the detective system. The inside of the tube contains 0.1 M phosphate buffer, pH 7.4, 0.3 mM EDTA and cytochrome c, SCR and H_2O_2 (if needed). The outside of the dialysis tube contains 0.1 M phosphate buffer, pH 7.4, 0.3 mM EDTA, 5 uM luminol and 10 ug/ml HRP. Reaction is started by adding succinate, and HRP was then added to detect H_2O_2 after 5 minutes.

2.4. Measurement of hydroxyl radical By ESR

Spin trapping with DMPO was used to detect hydroxyl radical. Succinate (0.2 mM), cytochrome c (1 μM), SCR (4 μg/ml) or $FeSO_4$ (10 μM) were incubated at room temperature for 1 min with DMPO (100

mM) in the Kreb's buffer prior to the addition of H_2O_2 (30 μM). All ESR spectra were recorded on a Varian E-109 ESR spectrometer. The conditions are: microwave power 20 mW. X-band, 100 kHz modulation with amplitude 1G, central magnetic field 3250 G, scan width 200 G, time constant 0.128 second, receiver gain 1×10^4, room temperature.

2.5. Measurement of cytochrome c reduction

The redox state of cytochrome c was determined by measurement of the 550 nm absorbance. The medium contained 0.1 M phosphate buffer, pH 7.4, 0.3 mM EDTA, 4 μg/ml SCR, 10 μM cytochrome c, 0.2 mM succinate. Different concentrations of H_2O_2 were added to the reaction system.

3. RESULTS AND DISCUSSION

In this study it is shown that the SCR-reduced cytochrome c efficiently scavenges H_2O_2 regardless of if added or produced by SCR itself.

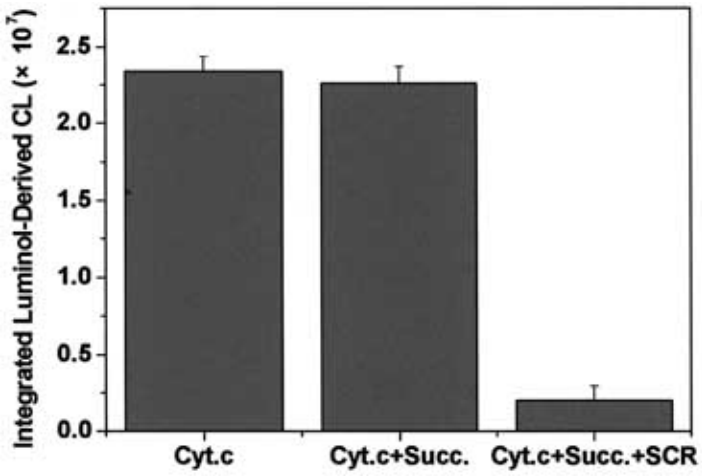

Figure 1. Scavenging of added H_2O_2 by cytochrome c. The reaction medium contains 0.1M phosphate buffer, pH 7.4, 0.3 mM EDTA, 5 μM luminol, 10 μg/ml HRP (out of the dialyze tube); 0.3 mM H_2O_2 and 0.01 mM cytochrome c, 0.2 mM succinate and 4 μg/ml SCR (inside the dialysis tube). CL: chemiluminescence. Data were means ± SEM, N=3.

As shown in Figure 1, the exterior H_2O_2 can be scavenged only when SCR transfers electrons from succinate to cytochrome c. That means the electrons transferred from succinate to cytochrome c can be further delivered to the exterior H_2O_2. Yu proved that in purified SCR an electron leak point is located in Q-cycle to generate $O_2^{\cdot-}$ [19]. In Figure 2 we show that the H_2O_2 produced by succinate-cytochrome c reductase could also be removed by succinate-reduced cytochrome c. If adding succinate to the purified succinate-

cytochrome c reductase in the absence of cytochrome c, a significant amount of H_2O_2 was produced. The addition of cytochrome c causes a dramatic decrease of H_2O_2 production (Figure 2). Our previous results demonstrate that there was dose-dependent removal of H_2O_2 by addition of cytochrome c back to the assay medium containing cytochrome c-depleted submitochondrial particle [20]. These observations indicated that the cytochrome c may act as a scavenger of hydrogen peroxide.

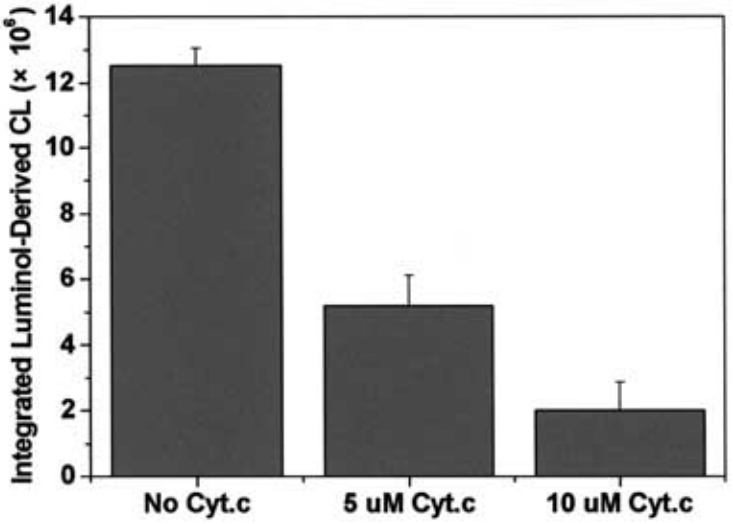

Figure 2. Scavenging of SCR-produced H_2O_2 by cytochrome c. Reaction medium contains 0.1M phosphate buffer, pH 7.4, 0.3 mM EDTA, 5 μM luminol, 10 μg/ml HRP (outside the dialysis tube); 4 μg/ml SCR, 0.2 mM succinate and 5/10 μM cytochrome c (inside the dialysis tube). Data were means ± SEM, N=3.

Cytochrome c contains Fe, and H_2O_2 reacts preferentially with transition-metal ions to form hydroxyl radical ($OH^{\cdot}$) by the Fenton reaction. Does reduced cytochrome c scavenge H_2O_2 by forming $OH^{\cdot}$? In order to exclude this possibility, DMPO was used to trap $OH^{\cdot}$ and no $OH^{\cdot}$ signal was observed in above reactive system (Figure 3).

We also detected the effect of H_2O_2 on succinate-reduced cytochrome c by observing the 550 nm absorbance change. In a system containing cytochrome c and trace amount of SCR, the addition of succinate causes the reduction of cytochrome c shown as the increase of absorbance at 550 nm. In this experiment lower concentration of succinate was used (20 uM) to reduce the reductive rate of cytochrome c in order to observe the effect of H_2O_2 on the oxidation of ferrocytochrome c. In the middle of cytochrome c reduction, the addition of H_2O_2 causes a decrease of absorbance at 550 nm, which means the reduced cytochrome c been oxidized by H_2O_2 (Figure 4). This observation implies that the electrons donated by the substrate could be transferred to H_2O_2 by cytochrome c. Figure 5 shows the oxidized cytochrome c can be re-reduced by the addition of succinate which suggests that cytochrome c indeed was oxidized not destroyed by H_2O_2.

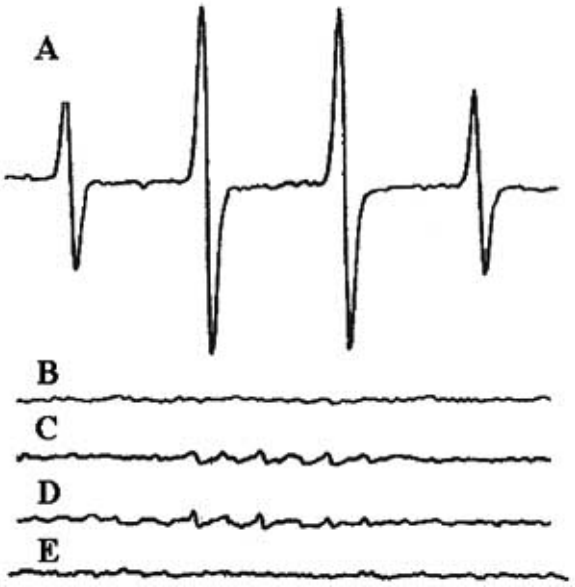

Figure 3. Measurement of hydroxyl radical trapped with DMPO. A: 10 μM $FeSO_4$, no SCR; B: No $FeSO_4$ and SCR; C: 1 μM cytochrome c; D: 1 μM cytochrome c and 0.2 mM succinate; E: 1 μM cytochrome c, 0.2 mM succinate and 4 μg/ml SCR. Succinate (0.2 mM), cytochrome c (1 μM), SCR (4 μg/ml) were incubated at room temperature for 1 min with DMPO (100 mM) in the Kreb's buffer prior to the addition of H_2O_2 (30 μM).

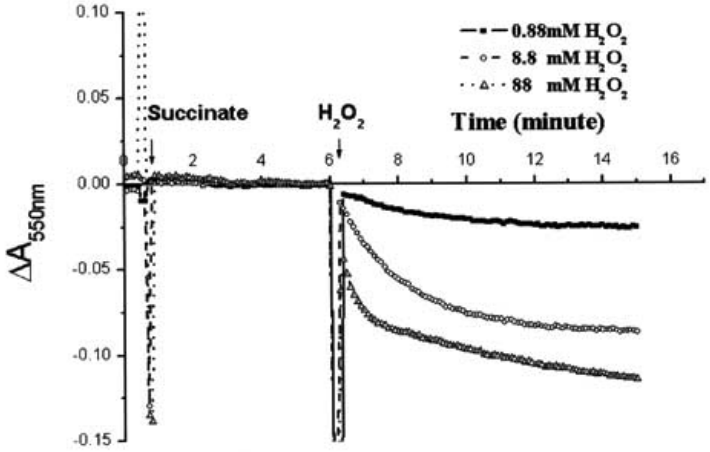

Figure 4. The difference spectra of cytochrome c absorbance at 550nm in the presence of H_2O_2 minus zero H_2O_2. Reaction medium contains 0.1 M phosphate buffer, pH 7.4, 0.3 mM EDTA, 4ug/ml SCR and 10 μM cytochrome c. Succinate 20 μM was added to start the reaction. Different amounts of H_2O_2 were added to show the oxidation of the reduced cytochrome c.

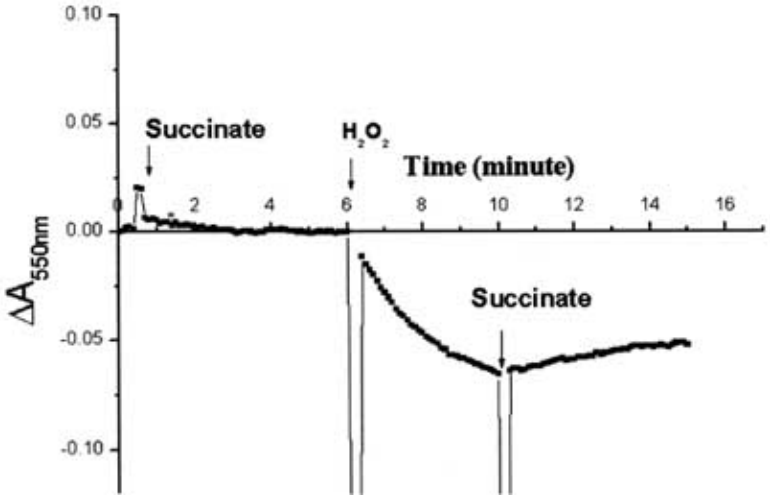

Figure 5. The difference spectra of cytochrome c absorbance at 550nm in the presence of H_2O_2 minus zero H_2O_2. Reaction medium contains 0.1 M phosphate buffer, pH 7.4, 0.3 mM EDTA, 4 ug/ml SCR and 10 μM cytochrome c. 20 μM Succinate and 8.8 mM H_2O_2 was added to observe the reduction and oxidation of cytochrome c.

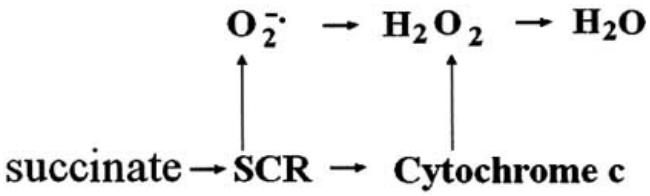

Figure 6. A proposal of electron leak bypass mediated by cytochrome c.

Mitochondria are the major producer of ROS in the cell [8-10]. These ROS are potentially dangerous not only to mitochondria itself but also to the other cellular organelles. So it is important that mitochondrion has an efficient system to scavenge ROS. Mitochondria contain Mn-superoxide dismutatase and glutathione peroxidase in matrix [7] and some antioxidant including V_E, which can eliminate most of ROS. These biomolecules were situated in the mitochondrial matrix, and could not eliminate ROS in the mitochondrial intermembrane space. The mitochondrial respiratory chain can be damaged by itself, if the generated $O_2^{-\cdot}$ and H_2O_2 can not be removed in time. Cytochrome c is abundant, and the concentration may reach mM level in the intermembrane space of mitochondria [5]. Here we use purified succinate-cytochrome c reductase and this *in vitro* biochemical system may not exactly react as *in vivo*. So our experimental results suggests the possibility that a scavenge H_2O_2 pathway as shown in Figure 6 may work in the respiratory chain. It is possible that ferrocytochrome c should be included to the mitochondrial antioxidant system, especially as a self-defense factor of the respiratory chain.

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