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# The antioxidant functions of cytochrome *c*

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**Abstract** **Low ($C_{1/2} = 1.5 \times 10^{-7}$ M) concentrations of horse cytochrome *c* strongly inhibit $H_2O_2$ production by rat heart mitochondria under conditions of reverse electron transfer from succinate to $NAD^+$. The effect is abolished by binding of cytochrome *c* with liposomes and is not prevented by SOD. Yeast cytochrome *c* is much less effective than the horse protein whereas acetylated horse cytochrome *c* is without effect. $H_2O_2$ formation stimulated by antimycin A is resistant to added cytochrome *c*. In inside-out submitochondrial vesicles, $H_2O_2$ production is suppressed by all three cytochrome *c* samples tested, but at higher concentrations ($C_{1/2}$ is about $5 \times 10^{-7}$ M). In vesicles, SOD abolishes the cytochrome *c* inhibition. We conclude that extramitochondrial cytochrome *c* is competent in down-regulation of the Complex I $H_2O_2$ production linked to the reverse electron transfer. Such an effect is absent in the inside-out submitochondrial vesicles where another antioxidant cytochrome *c* function can be observed, i.e. the oxidation of $O_2^{-\bullet}$ to $O_2$. A possible role of cytochrome *c* in the antioxidant defence is discussed.**

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*Key words:* Cytochrome *c*; Mitochondrion; Reactive oxygen species; Superoxide oxidation; Antioxidant

## 1. Introduction

For many years, the biological function of cytochrome *c*, a small water-soluble mitochondrial hemoprotein, was assumed to be confined to electron transfer from cytochrome $c_1$ to cytochrome oxidase. However, in 1996 it was found that cytochrome *c*, when released from mitochondria to cytosol, activates a programmed cell death cascade (apoptosis) [1]. In 1997–1998, this observation was confirmed and extended by many laboratories (for review, see [2]).

In 1994 we suggested that one of the functions inherent in apoptosis is to purify a tissue from cells that produce large amounts of reactive oxygen species (ROS) [3] (see also [4]). Since mitochondria are one of the main sources of ROS in the cell, it is not surprising that they are involved in apoptosis. Mitochondria are presumably equipped not only by systems quenching already formed ROS, such as superoxide dismutase, catalase, and glutathione peroxidase, but also by several mechanisms preventing ROS formation. Among them the following lines of defence were postulated: (i) mild uncoupling which prevents protonic potential from being above a threshold level required for ROS formation by Complexes I and III [5,6]; (ii) permeability transition pore (PTP) allowing mitochondria to lower intracellular oxygen concentration by means of uncoupled high-rate respiration [3,4]; (iii) ROS-induced, PTP-mediated mitoptosis, i.e. purification of the mitochondrial population from ROS-overproducing mitochondria [4,7]; (iv) ROS-induced release of pro-apoptotic proteins (namely cytochrome *c*, the apoptosis-inducing factor and some caspases) from the mitochondrial intermembrane space to the cytosol [2,8].

Recently it was hypothesized that cytochrome *c* per se can operate in vivo as an antioxidant [2,9]. In fact, soluble cytochrome *c* is well known to oxidize superoxide back to $O_2$, an effect widely used by researchers as a method to measure $O_2^{-\bullet}$ [10,11]. It was proposed that also in the cell, solubilized cytochrome $c^{3+}$ can react with $O_2^{-\bullet}$, so that $O_2$ and cytochrome $c^{2+}$ are formed:

$$O_2^{-\bullet} + \text{cytochrome } c^{3+} \rightarrow O_2 + \text{cytochrome } c^{2+} \quad (1)$$

Reduced cytochrome *c* can then be reoxidized by cytochrome oxidase or (in yeast and some bacteria) by cytochrome *c* peroxidase localized in the intermembrane space [2]. During apoptosis, when the outer mitochondrial membrane is no longer a barrier for cytochrome *c*, this hemoprotein might perform its superoxide-oxidizing function also in cytosol. It is obvious that this qualitative consideration should be supplemented with a quantitative analysis to answer the question whether the cytochrome *c* concentrations in the mitochondrial intermembrane space and cytosol are sufficient to carry out a sufficiently fast rate of $O_2^{-\bullet}$ oxidation. According to Forman and Azzi [9], the total amount of cytochrome *c* in the intermembrane space should be as high as 0.5–5 mM. However, it is not clear what portion of the cytochrome *c* pool is dissolved in the intermembrane water and what is bound to the membranes. $O_2^{-\bullet}$ is a hydrophilic anion. It seemed probable that only solubilized but not membrane-bound cytochrome *c* should be competent in the $O_2^{-\bullet}$ oxidation (just as in the case of oxidation of ascorbate, another hydrophilic anion). In this paper, we report that liposome-bound cytochrome *c*, in contrast to its water-soluble form, cannot be reduced by $O_2^{-\bullet}$ generated by xanthine oxidase. In submitochondrial vesicles, small amounts of added cytochrome *c* inhibit $H_2O_2$ formation under conditions of reverse electron transfer or in the presence of antimycin A. In mitochondria, even smaller concentrations of cytochrome *c* proved to be inhibitory under the reverse electron transfer conditions. However, the antimycin A-treated mitochondria were shown to produce $H_2O_2$ in a cytochrome *c*-resistant fashion. Further analysis of these relationships revealed that cytochrome *c* added to mitochondria

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*Abbreviations:* DNP, 2,4-*p*-dinitrophenol; FCCP, *p*-trifluoromethylcarbonylcyanide phenylhydrazone; PTP, permeability transition pore; ROS, reactive oxygen species; SOD, superoxide dismutase; $TPP^+$, tetraphenyl phosphonium

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prevents $O_2^{-\bullet}$ formation by Complex I rather than scavenges already formed $O_2^{-\bullet}$.

## 2. Materials and methods

Rat heart mitochondria were isolated as described previously [5] but the procedure of pre-treatment of mitochondria by $H_2O_2$ and aminotriazole was omitted. Beef heart submitochondrial vesicles were prepared as described by Kotlyar and Vinogradov [12]. Liposomes were formed by sonication of the asolectin suspension (40 mg lipid/ml) for six 30 s periods with 1 min intervals. The medium contained 10 mM MOPS, 0.25 M sucrose and 0.5 mM EGTA, pH 7.3.

$H_2O_2$ generation was measured by the scopoletin/horseradish peroxidase method [13]. Scopoletin fluorescence was calibrated with glucose and glucose oxidase [14] and by adding known amounts of $H_2O_2$.

The mitochondrial membrane potential was monitored by measuring the $TPP^+$ concentration in the incubation medium using a $TPP^+$-sensitive electrode.

To follow reverse electron transfer in mitochondria, fluorescence of endogenous reduced nicotinamide nucleotides was excited at 366 nm and measured at 460 nm.

Cytochrome *c* reduction was measured with an Aminco DW-2000 spectrophotometer (dual wavelength mode), using 550–540 nm light absorption difference.

EDTA, EGTA, DNP, rotenone and scopoletin were from Serva; oligomycin, MOPS, the horse and yeast cytochrome *c*, $K_2HPO_4$, Tris, succinate, bovine serum albumin, antimycin A, xanthine oxidase, superoxide dismutase and partially acetylated horse cytochrome *c* were from Sigma, 30% $H_2O_2$ was from Merck; $TPP^+$ was from Aldrich.

## 3. Results

In the first series of experiments, we addressed the question whether the membrane-bound cytochrome *c* is competent in $O_2^{-\bullet}$ oxidation. To this end, we compared rates of reduction of oxidized cytochrome *c* by $O_2^{-\bullet}$ and by ascorbate in the absence and in the presence of liposomes that are known to bind cytochrome *c*. It is seen in Fig. 1A, B that liposomes completely inhibit the cytochrome *c* reduction by ascorbate, although it remains reducible by dithionite. A similar effect is observed when a superoxide-generating system, xanthine and xanthine oxidase, substitutes for ascorbate as the cytochrome *c* reductant (Fig. 1C, D). The inhibiting effect of liposomes is attenuated when 125 mM KCl was added to decrease the cytochrome *c* binding to the liposomal membrane (cf. Fig. 1C and D).

Fig. 2 shows effects of cytochrome *c* and liposomes on the $H_2O_2$ production by rat heart mitochondria oxidizing succinate. Cytochrome *c* ($2\times10^{-7}$ M) is found to decelerate this production and such an action is abolished by subsequent addition of liposomes (Fig. 2A, B). Liposomes added before cytochrome *c* slightly decrease the $H_2O_2$ generation and completely prevent the cytochrome *c* inhibition (Fig. 2C).

In line with data previously obtained in this group [5], rotenone causes 4–5-fold inhibition of the $H_2O_2$ production by the succinate-oxidizing mitochondria. The residual $H_2O_2$ formation is shown to be cytochrome *c*-resistant (Fig. 2D) and cannot be stimulated by SOD (Fig. 2E).

In Fig. 3A one can see titration of the mitochondrial $H_2O_2$ production by cytochrome *c*. As is shown in the figure, measurable inhibition of the $H_2O_2$ production by the oligomycin-treated mitochondria oxidizing succinate is already seen at $1\times10^{-7}$ M cytochrome *c*. A 4-fold inhibition occurs at $4\times10^{-7}$ M cytochrome *c*. Addition of SOD increases the rate of the $H_2O_2$ formation. However, the cytochrome *c* inhibition still takes place, the $C_{1/2}$ for cytochrome *c* being as low as without SOD, i.e. $1.5\times10^{-7}$ M.

Submicromolar cytochrome *c* appears to be ineffective when $H_2O_2$ generation is stimulated by antimycin A (Fig. 3B). Nevertheless, SOD stimulates the $H_2O_2$ production in the antimycin A-inhibited mitochondria (Fig. 3B).

The above cytochrome *c* effect can hardly be explained by its superoxide-oxidizing activity since it is SOD-resistant. On

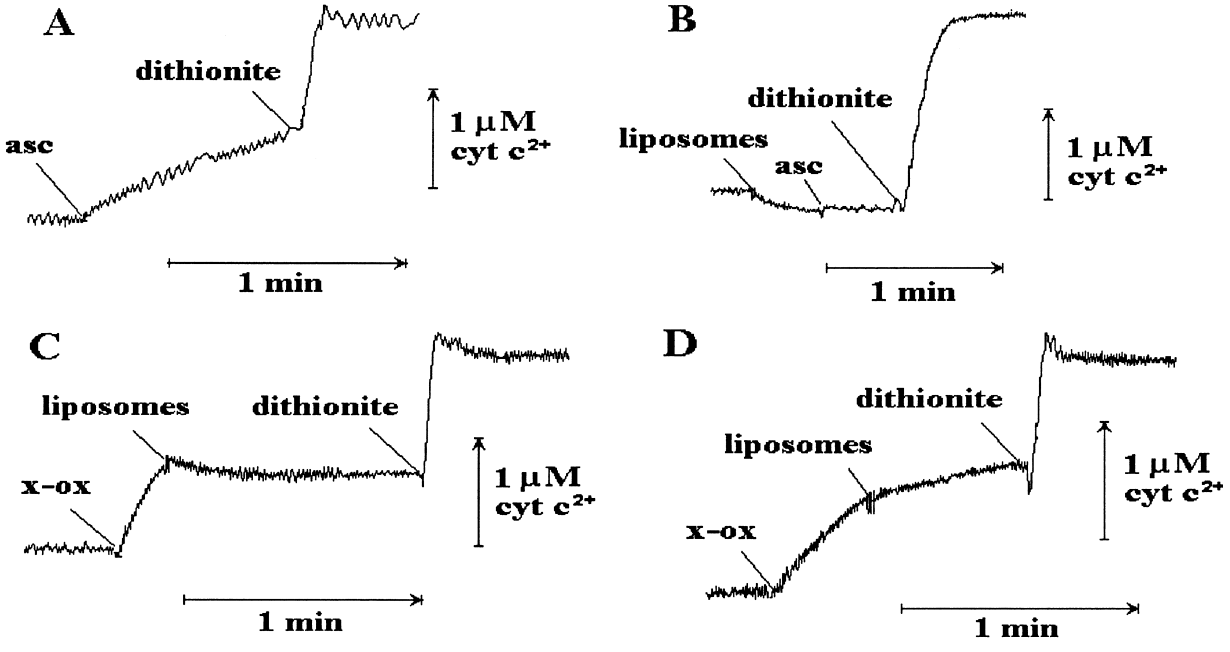


Fig. 1. Effect of liposomes on reduction of cytochrome *c* by ascorbate (A, B) or by superoxide anion (xanthine plus xanthine oxidase as a source of superoxide) (C, D). Incubation medium: 2 μM cytochrome *c*, 10 mM MOPS, 0.25 M sucrose, pH 7.3 (A, B); 10 mM MOPS, 0.25 M sucrose, 0.5 mM EDTA, 0.04 mM xanthine, pH 7.3 (C); 10 mM MOPS, 125 mM KCl, 0.5 mM EDTA, 0.04 mM xanthine, pH 7.3 (D). Additions: 5 mM ascorbate, liposomes (1 mg lipid), xanthine oxidase (0.005 mg).

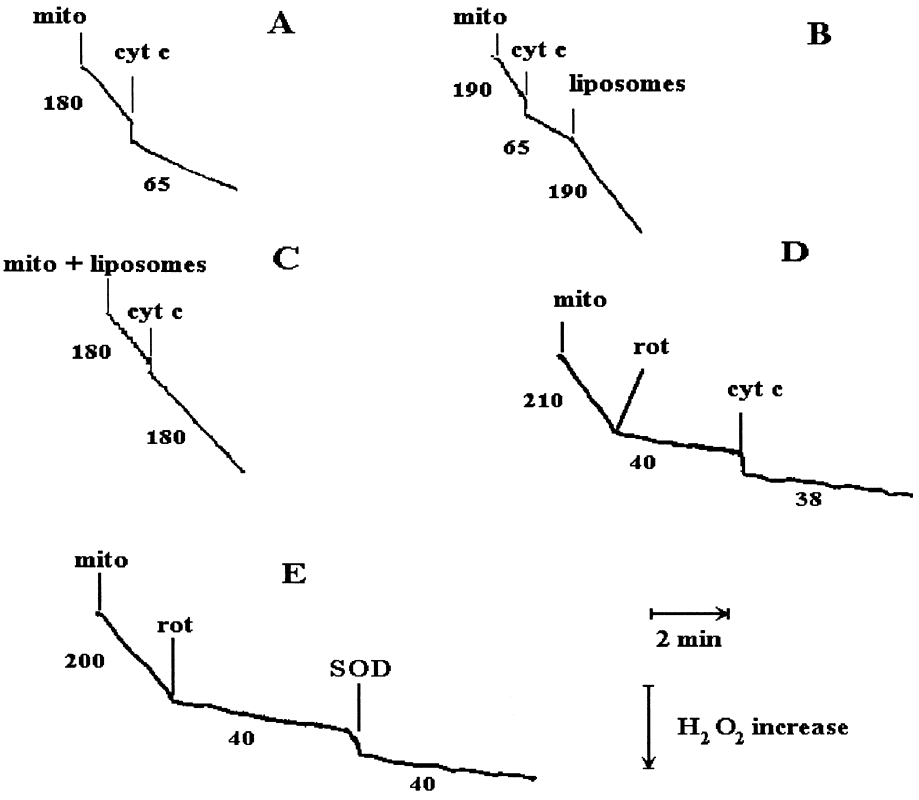


Fig. 2. Effect of cytochrome *c* on $H_2O_2$ generation by rat heart mitochondria oxidizing succinate. Incubation medium: 10 mM MOPS, 0.25 M sucrose, 0.5 mM EDTA, 6 mM succinate, pH 7.3. Additions: mitochondria (1 mg protein/ml), cytochrome *c* (A–C, 200 nM; D, 400 nM), liposomes (1 mg lipid), 0.002 mM rotenone, $1\times10^{-6}$ M SOD. Figures near curves are rates of $H_2O_2$ production, pmol/(mg protein×min).

the other hand, it is known that uncouplers arrest the $H_2O_2$ production by mitochondria oxidizing succinate in State 4 [15]. To such an action, very low uncoupler concentrations slightly decreasing membrane potential but already inhibiting the reverse electron transfer in the respiratory chain proved to be necessary [5,6]. Thus one might suggest that cytochrome *c* added to mitochondria causes some uncoupling. The data shown in Fig. 3C and D exclude such a possibility. It is seen that submicromolar cytochrome *c* fails to inhibit the membrane potential formation and the reverse electron transfer.

In Fig. 3E, effects of the horse and yeast cytochromes *c* on $H_2O_2$ formation by succinate-oxidizing mitochondria are demonstrated. It is obvious that the yeast cytochrome *c* is much less efficient than the horse enzyme. On the other hand, experiments with xanthine oxidase revealed that both cytochromes have identical $O_2^{-\bullet}$-oxidizing activity (not shown). Acetylation of the horse cytochrome *c* completely abolished its effect on $H_2O_2$ generation (Fig. 3E), but does not arrest the $O_2^{-\bullet}$-oxidizing activity (not shown).

In Fig. 4, results obtained on the inside-out bovine heart submitochondrial vesicles are summarized. It is seen that cytochrome *c* lowers the $H_2O_2$ formation by the vesicles both in the absence and in the presence of antimycin A (Fig. 4A), and this effect is inherent in the horse as well as yeast cytochromes *c* (Fig. 4B–E) and acetylated cytochrome *c* (not shown). In contrast to mitochondria, SOD completely abolishes the cytochrome *c* inhibition of $H_2O_2$ generation by the vesicles (Fig. 4B–E).

## 4. Discussion

The above data clearly indicate that very small (submicromolar) concentrations of water-soluble cytochrome *c* added to isolated mitochondria can strongly decrease the mitochondrial $H_2O_2$ production in the resting state (succinate oxidation in the absence of ADP). On the face of it, such an effect is consistent with the suggestion [2,9] that the superoxide-oxidizing activity of cytochrome *c* can represent a line of the antioxidant defence of aerobic cell. In apparent agreement with this suggestion, it was shown that only water-soluble, but not membrane-bound cytochrome *c* was competent in the oxidation of $O_2^{-\bullet}$ in the water phase. In fact, adsorption of cytochrome *c* on liposomes strongly inhibited its activity towards xanthine oxidase-generated superoxide (see Fig. 1). In succinate-oxidizing mitochondria, inhibition of the $H_2O_2$ formation by added cytochrome *c* was abolished by liposomes (Fig. 2A–C).

On the other hand, several observations made in the further experiments, proved to be inconsistent with the assumption that the $O_2^{-\bullet}$-oxidizing activity of cytochrome *c* is responsible for the above effects of this protein on mitochondria.

1. Cytochrome *c* failed to inhibit generation of $H_2O_2$ by the antimycin A-treated mitochondria.
2. SOD, which stimulated $H_2O_2$ production both in the absence and in the presence of antimycin A, could not abolish the inhibitory effect of cytochrome *c*.
3. Yeast cytochrome *c* which showed the same $O_2^{-\bullet}$-oxidizing

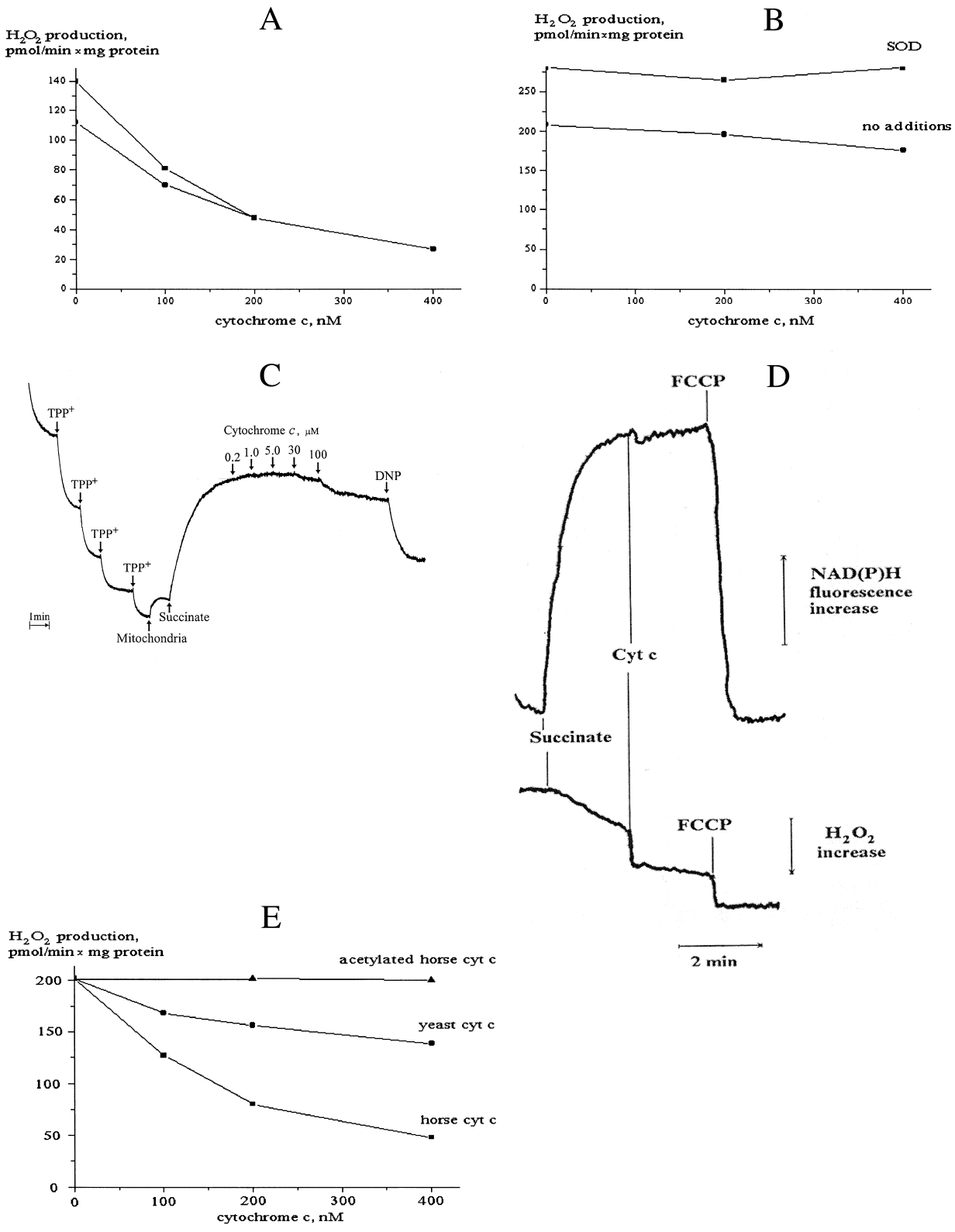


Fig. 3. Effects of cytochrome *c* and SOD on some parameters of rat heart mitochondria oxidizing succinate. Incubation medium: 10 mM MOPS, 0.25 M sucrose, 0.5 mM EDTA, pH 7.3, mitochondria (A, C, E, 1 mg protein/ml; B, 0.5 mg protein/ml) and 6 mM succinate (A, B, E). Additions: 6 mM succinate, 50 μM DNP, 100 nM FCCP, $1\times10^{-6}$ M antimycin A, $1\times10^{-6}$ M SOD (A, B).

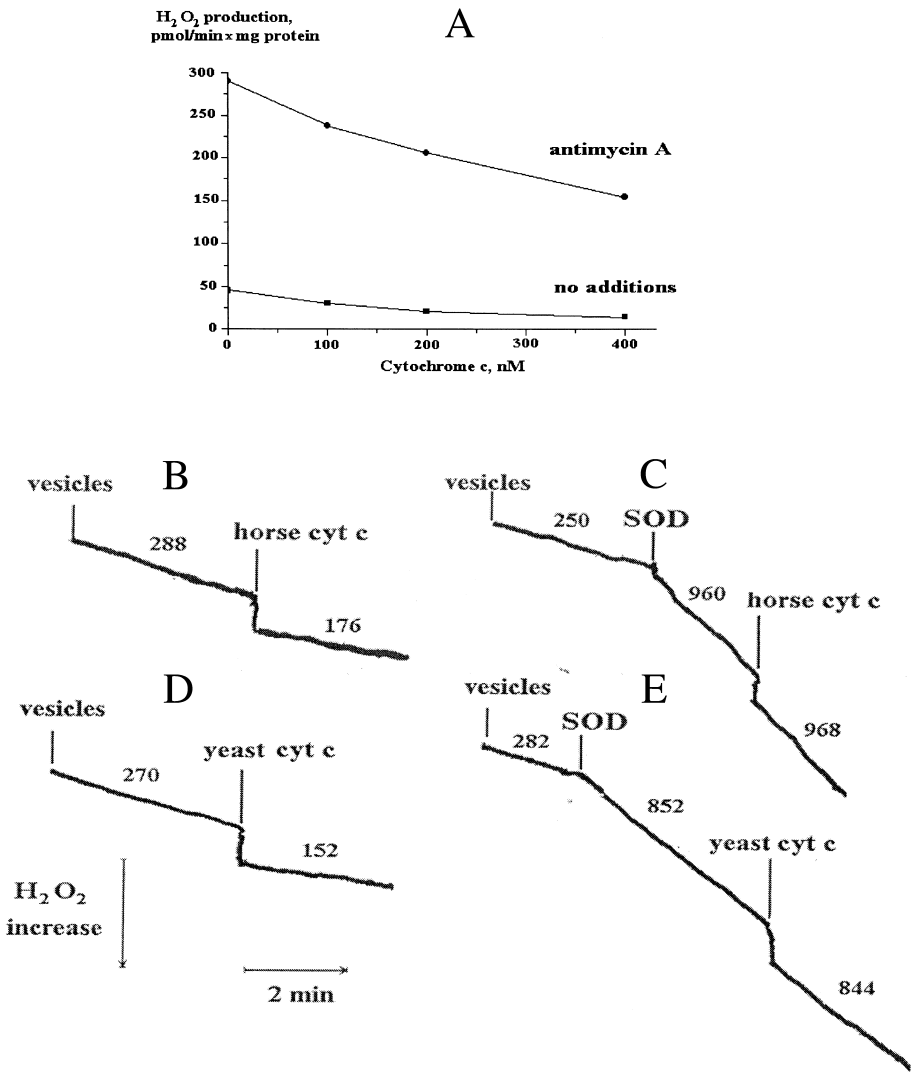


Fig. 4. Effect of cytochrome *c* on the $H_2O_2$ formation by the inside-out beef heart submitochondrial vesicles. Incubation mixture: 10 mM MOPS, 0.25 M sucrose, 0.5 mM EGTA, pH 7.3, submitochondrial vesicles (A, 0.25 mg protein, or B–E, 0.6 mg protein). Additions: $1\times10^{-6}$ M antimycin A, 400 nM cytochrome *c*. In A, upper curve, and B–E, $1\times10^{-7}$ M FCCP and antimycin A are present. Figures near curves are rates of $H_2O_2$ production, pmol/(mg protein × min).

activity as the horse enzyme was much less efficient in inhibiting the mitochondrial $H_2O_2$ formation (Fig. 3E).
4. Acetylated horse cytochrome *c* which was still competent in the $O_2^{-\bullet}$ oxidation was completely ineffective as inhibitor of $H_2O_2$ generation by mitochondria (Fig. 3E).

Quite other relationships were revealed with the inside-out submitochondrial vesicles.

1. Production of $H_2O_2$ proved to be inhibited by added cytochrome *c* not only in the absence of antimycin A, but also in its presence.
2. SOD abolished the cytochrome *c* effect.
3. Yeast cytochrome *c* and acetylated horse cytochrome *c* substituted for intact horse cytochrome *c* (Fig. 4).

Such relationships indicate that we deal with two different antioxidant actions of cytochrome *c* in mitochondria and vesicles. In the latter case, the behavior of cytochrome *c* exactly corresponds to that expected if cytochrome *c* operates as superoxide-oxidizing enzyme. As for the antioxidant effect of cytochrome *c* on mitochondria, it requires a different explanation. It is not an uncoupling since submicromolar cytochrome *c* did not affect membrane potential and reverse electron transfer. The effect in question does not require interaction of cytochrome *c* with the inner mitochondrial membrane as indicated by some probes on intactness of the outer mitochondrial membrane. In particular, we showed that under the conditions used, polylysine failed to inhibit mitochondrial respiration. Moreover, added cytochrome *c* and ascorbate could not be oxidized by mitochondria.

As a tentative explanation of the above effect, one may assume that there is a cytochrome *c* receptor on the outer surface of the outer mitochondrial membrane, and binding of extramitochondrial cytochrome *c* to this receptor generates a signal resulting in inhibition of the $H_2O_2$ formation by mitochondria. Since it is reverse electron transfer through Complex I that is responsible for 80% of ROS formation by the succinate-oxidizing mitochondria, Complex I must be the final target of the postulated regulatory system. As for the

receptor, this might be, for example, porin (or porin·Bax complex which seems to be responsible for the cytochrome *c* release at apoptosis [16]). There are some indications that in the contact sites of two mitochondrial membranes, porin forms a complex with the ATP/ADP antiporter localized in the inner membrane of mitochondria [17]. From the antiporter, the signal should be somehow transmitted to Complex I via the matrix space or along the inner mitochondrial membrane. One more possibility consists in that the signal in question is sent by a putative outer membrane cytochrome *c* receptor via intermembrane space to that part of Complex I which faces this space.

In any case, the fact that very low cytochrome *c* concentrations strongly and specifically inhibit the ROS formation under conditions of reverse electron transfer points to a functional significance of this process. For instance, one may speculate that Complex I operates as a generator of $H_2O_2$ which is then used as the intercellular mediator of apoptosis [18,19]. It is noteworthy that the yeast cytochrome *c* is of low efficiency both in inhibiting $H_2O_2$ formation (this paper) and activating apoptosis [20]. Acetylation of the horse cytochrome *c* abolishes its action on mitochondria (Fig. 3E).

On the other hand, the yeast cytochrome *c* and acetylated horse cytochrome *c* proved to be, according to our data, effective in oxidizing the xanthine oxidase-produced $O_2^{-\bullet}$ (not shown in figures) and inhibiting the $H_2O_2$ formation by submitochondrial vesicles (Fig. 4).

The $O_2^{-\bullet}$-oxidizing activity of cytochrome *c* requires higher cytochrome *c* concentrations to be revealed in the inside-out submitochondrial vesicles where there is neither superoxide dismutase nor the above described effect of cytochrome *c* on Complex I. Here $C_{1/2}$ was found to be around $5\times10^{-7}$ M cytochrome *c* in samples with antimycin A (cf. $C_{1/2}$ for effect on mitochondria equal to $1.5\times10^{-7}$ M; in fact, the difference in the $C_{1/2}$ values was even larger when we calculated the cytochrome *c* amount per mg protein or lipid).

For activation of apoptosis, larger concentrations of cytochrome *c* proved to be necessary ($C_{1/2}$ is about $5\times10^{-6}$ M cytochrome *c* injected into the cell [21]). Thus the following chain of events seems probable when the cytochrome *c* concentration in cytosol increases due to its release from mitochondria.

(1) Inhibition of the ROS-generating mechanism linked to the reverse electron transfer via Complex I. This effect is due to the regulatory action of extramitochondrial cytochrome *c* described in this paper. Like mild uncoupling (see above, Section 1), the Complex I-regulating activity of cytochrome *c* should be regarded as a mechanism preventing the $O_2^{-\bullet}$ formation.

(2) Conversion of $O_2^{-\bullet}$ back to $O_2$ (superoxide-oxidizing activity of cytochrome *c*). Such a process is activated when effect (1) appears to be insufficient to prevent a [ROS] increase. In fact, this is the most elegant way to get rid of $O_2^{-\bullet}$ which is formed from $O_2$. Here (i) $O_2$ is regenerated, (ii) electron removed from $O_2^{-\bullet}$ can be donated by cytochrome $c^{2+}$ to cytochrome oxidase to contribute to the convertible energy production, and (iii) the $O_2^{-\bullet}$ scavenger (cytochrome *c*) is also regenerated after its reoxidation by cytochrome oxidase.

(3) Induction of apoptosis by means of interaction of cytochrome *c* with Apaf-1. This process allows a tissue to be purified from those cells that still produce large amounts of ROS in spite of (1), (2) and other antioxidant measures.

It should be stressed that the cytochrome *c* concentrations required to effectively oxidize the respiratory chain-generated $O_2^{-\bullet}$ are still submicromolar. This means that desorption of, say, 1% of the mitochondrial cytochrome *c* pool from the inner membrane to the intermembrane space should be sufficient to utilize all the superoxide produced by the respiratory chain during reverse electron transfer or in the presence of antimycin A. Nevertheless, in both these cases mitochondria produce some $H_2O_2$ in a SOD-stimulated fashion (Fig. 3A, B), this fact pointing to the $O_2^{-\bullet}$ release from mitochondria to the extramitochondrial medium.

On the other hand, the State 4 $H_2O_2$ production by Complex III (succinate oxidation in the presence of rotenone) is not stimulated by SOD (Fig. 2D) and not inhibited by added cytochrome *c* (Fig. 2E). Apparently, in this case the rate of $O_2^{-\bullet}$ production is so slow that the amount of cytochrome *c* dissolved in the intermembrane water is sufficient to oxidize all the superoxide released from the inner membrane. Under these conditions, $H_2O_2$ diffusing from mitochondrial matrix seems to be the only source of ROS produced by the mitochondrial respiratory chain.

The above-listed antioxidant effects inherent at micro- and submicromolar cytochrome *c* concentrations give way to some pro-oxidant effects if the level of cytochrome *c* becomes $\geq10^{-5}$ M. According to Nantes et al. [22], $1\times10^{-5}$ M cytochrome *c* possesses peroxidase activity on diphenylacetaldehyde and 3-methylaceto-acetone, which is greatly stimulated by liposomes. Ozawa and coworkers [23] reported that cytochrome $c^{2+}$ activates production of $OH^{\bullet}$ from $H_2O_2$, being 20 times more effective than $Fe^{2+}$ ion. It might be possible that such kind of effects contribute to development of apoptosis when strong pro-apoptotic signals initiate release of all the cytochrome *c* molecules from all the mitochondria in the cell.

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