Biochemistry (Moscow), Vol. 68, No. 12, 2003, pp. 1349-1359. Translated from Biokhimiya, Vol. 68, No. 12, 2003, pp. 1674-1686.

Mechanism of Superoxide Anion Generation in Intact Mitochondria in the Presence of Lucigenin and Cyanide

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Received February 22, 2003
Revision received July 7, 2003

Abstract—In the presence of cyanide and various respiratory substrates (succinate or pyruvate + malate) addition of high concentrations of lucigenin (400 μM; Luc^{2+}) to rat liver mitochondria can induce a short-term flash of high amplitude lucigenin-dependent chemiluminescence (LDCL). Under conditions of cytochrome oxidase inhibition by cyanide the lucigenin-induced cyanide-resistant respiration (with succinate as substrate) was not inhibited by uncouplers (FCCP) and oligomycin. Increase in transmembrane potential (Δφ) value by stimulating F_0F_1-ATPase functioning (induced by addition of MgATP to the incubation medium) caused potent stimulation of the rate of cyanide-resistant respiration. At high Δφ values (in the presence of MgATP) cyanide resistant respiration of mitochondria in the presence of succinate or malate with pyruvate was insensitive to tenoyltrifluoroacetone (TTFA) or rotenone, respectively. However, in both cases respiration was effectively inhibited by myxothiazol or antimycin A. Mechanisms responsible for induction of LDCL and cyanide resistant mitochondrial respiration differ. In contrast to cyanide-resistant respiration, generation of LDCL signal, that was suppressed only by combined addition of Complex III inhibitors, antimycin A and myxothiazol, is a strictly potential-dependent process. It is observed only under conditions of high Δφ value generated by F_0F_1-ATPase functioning. The data suggest lucigenin-induced intensive generation of superoxide anion in mitochondria. Based on results of inhibitor analysis of cyanide-resistant respiration and LDCL, a two-stage mechanism of autooxidizable lucigenin cation-radical ($Luc^{\bullet+}$) formation in the respiratory chain is proposed. The first stage involves two-electron Luc^{2+} reduction by Complexes I and II. The second stage includes one-electron oxidation of reduced lucigenin (Luc(2e)). Reactions of Luc(2e) oxidation involve coenzyme Q-binding sites of Complex III. This results in formation of autooxidizable $Luc^{\bullet+}$ and superoxide anion generation. A new scheme for lucigenin-dependent electron pathways is proposed. It includes formation of fully reduced form of lucigenin and two-electron-transferring shunts of the respiratory chain. Lucigenin-induced activation of superoxide anion formation in mitochondria is accompanied by increase in ion permeability of the inner mitochondrial membrane.

Key words: mitochondria, lucigenin, cyanide resistant respiration, superoxide anion, respiratory chain Complexes I, II, and III, ion permeability of the inner mitochondrial membrane

It is generally accepted that mitochondria, which possess powerful redox systems, represent one of the most potent generators of superoxide anion and hydrogen peroxide in cells. In many studies, e.g., [1-3], the mechanism of superoxide anion generation was studied using submitochondrial particles or partially damaged mitochondria and indirect methods of registration (by H_2O_2 generation). Intact mitochondria exhibit high activity of endogenous superoxide dismutases (SOD) and catalase. They represent the main factors limiting the use of direct (chemical superoxide anion scavengers) or indirect (enzymatic determination of H_2O_2) methods of detection of superoxide generation. These factors may cause a sharp decrease in the steady-state superoxide anion concentrations below detectable limits.

In this connection, use of mitochondrial permeable chemical probes that can directly detect superoxide anion within sites of its production inaccessible to SOD action is the most adequate approach. Relatively lipophilic lucigenin cation, Luc^{2+}, which can be accumulated in mitochondria in a potential-dependent manner, has recently been introduced for specific detection of superoxide anion production by appearance of chemiluminescence signal [4-7].

Abbreviations: LDCL) lucigenin-dependent chemiluminescence; Luc^{2+}) oxidized lucigenin; $Luc^{\bullet+}$) lucigenin cation radical; Luc(2e)) fully reduced lucigenin; Δφ) electric potential difference on the inner mitochondrial membrane; SOD) superoxide dismutase.

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According to generally accepted ideas, generation of lucigenin-dependent chemiluminescence (LDCL) is a multistage process. The key reaction (3) of LDCL in mitochondria involves interaction of lucigenin cation-radical ($Luc^{\bullet+}$) with superoxide anion. Both reactants are presumably formed at the same sites of the respiratory chain in reactions (1) and (2). Decomposition of the unstable reaction product, dioxetane ($LucO_2$), yields two molecules of N-methylacridone (MA), one of which is in an electronically excited state (reaction (4)):

$$Luc^{2+} + e^- \rightarrow Luc^{\cdot+}; \quad (1)$$

$$O_2 + e^- \rightarrow O_2^{\cdot-}; \quad (2)$$

$$Luc^{\bullet+} + O_2^{\cdot-} \rightarrow LucO_2; \quad (3)$$

$$LucO_2 \rightarrow 2\ MA + h\nu. \quad (4)$$

It has recently been recognized that in simple aqueous enzymatic systems generating superoxide and H_2O_2, lucigenin used at higher concentrations (5-20 μM) can catalyze a reaction of oxygen reduction [8-10]. This is accompanied by formation of additional quantities of superoxide anion (reaction (5)). The possibility of a strong shift of this reaction to superoxide anion formation is still discussed [11, 12] because there are no experimental data on determination of redox potential for $Luc^{2+}/Luc^{\cdot+}$ couple in polar aqueous media.

Appearance of cyanide-resistant respiration (under conditions of cytochrome oxidase inhibition by cyanide) is a consequence of activation of superoxide generation in mitochondria by lucigenin [5-7]. This is a result of the following reactions (5)-(7):

$$Luc^{\bullet+} + O_2 \rightarrow O_2^{\cdot-} + Luc^{2+}; \quad (5)$$

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ + SOD \rightarrow H_2O_2 + O_2; \quad (6)$$

$$H_2O_2 + CATALASE \rightarrow H_2O + 1/2\ O_2. \quad (7)$$

Although the phenomenon of cyanide-resistant oxidation of substrates in mitochondria in the presence of lucigenin has been known for several years, no indications exist in the literature on the mechanism responsible for formation of autooxidizable $Luc^{\bullet+}$. The possible interrelationship between cyanide-resistant respiration and chemiluminescence also requires detailed investigation.

In the present study, we have investigated the properties of lucigenin as a superoxide generator. This is important for elucidation of endogenous and inducible mechanisms of superoxide anion production in mitochondria and cells and, consequently, for regulation of intracellular processes involving superoxide anion-dependent reactions.

The main goal of this study consisted of investigation of the mechanism responsible for superoxide anion generation in intact mitochondria in the presence of high concentrations of Luc^{2+} and cyanide. We also found that under conditions of maximal reduction of respiratory chain components by cyanide, lucigenin-induced generation of superoxide anion increases the permeability of the inner mitochondrial membrane.

MATERIALS AND METHODS

The following chemicals were used in the study: Tris-HCl, Hepes, bovine serum albumin (BSA), and dimethylsulfoxide (DMSO) from Serva (Germany); EDTA, lucigenin, succinate, pyruvate, malate, antimycin A, rotenone, myxothiazol, tetramethylphosphonium (TPP^+), and tenoyltrifluoroacetone (TTFA) from Sigma (USA). ATP and *p*-trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP) were from Calbiochem (USA). Cyclosporin A was produced by Sandoz (Switzerland). The dye Dis-C_2-(5) was kindly provided by Prof. V. P. Zinchenko (Institute of Cell Biophysics, Russian Academy of Sciences). Other preparations and reagents of "chemically pure" or "specially pure" grade were produced by Russian suppliers. Lucigenin was dissolved in DMSO; antimycin A, rotenone, myxothiazol, and TTFA were dissolved in bidistilled ethanol.

Mitochondria were isolate from livers of Wistar rats by differential centrifugation using the standard method of Johnson and Lardy [13]. The isolation medium contained 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris-HCl, 1 mM EDTA, 0.5% BSA, pH 7.4. Isolated mitochondria were washed and resuspended in the isolation medium lacking EDTA and BSA. Mitochondrial protein was determined by the method of Lowry [14].

The registration medium contained 125 mM KCl, 2 mM KH_2PO_4, 10 mM Hepes, and 5 mM succinate with rotenone (2 μg/mg protein) or 5 mM pyruvate + 5 mM malate, pH 7.4. All measurements were carried out at 37°C. The rate of oxygen consumption by mitochondrial suspension was registered using a Clark type electrode. Mitochondrial transmembrane potential was evaluated using a TPP^+-selective electrode as described by Kamo et al. [15]. In some experiments the dye Dis-C_2-(5) was used for determination of $\Delta\varphi$ value. Its luminescence was excited at 651 nm and registered at 675 nm [16] using an MPF 44B spectrofluorimeter (Perkin-Elmer, USA). LDCL was registered using a Lucifer-02M chemiluminometer (Nika, Russia).

The respiration rate and $\Delta\varphi$ value (measured by TPP^+-selective electrode) were registered simultaneously in a specially constructed multi-electrode cell (total volume 2 ml) at concentrations of mitochondrial protein 1.5-2 mg/ml. In these experiments higher concentrations

of mitochondrial protein were used for reliable registration of changes of cyanide-resistant respiration rates. Changes in luminescence of Dis-C_2-(5) were evaluated using the same mitochondrial preparations (at protein concentration 0.6-1 mg/ml).

High concentrations of lucigenin (400 μM) were used in the study. These concentrations of Luc^{2+} (in the presence of cyanide) detect significant changes in the rate of mitochondrial cyanide-resistant respiration, and generation of the chemiluminescence signal in response to cyanide addition occurs without a lag-period (e.g., Fig. 8a, curve *1*).

At relatively low concentrations of Luc^{2+} (50 μM), the increase in LDCL after cyanide addition requires prolonged preincubation of the mitochondria in medium containing lucigenin and oxidizing substrate (Fig. 1, curve *3*). During short-term incubation of mitochondria with 50 μM Luc^{2+}, addition of cyanide to mitochondria inhibits LDCL (Fig. 1, curves *1* and *2*).

In the presence of cyanide, mitochondrial membrane potential was maintained by adding F_0F_1-ATPase substrate, 2 mM MgATP. Figure 2 shows that addition of MgATP after cyanide caused rapid restoration of Δφ value on the mitochondrial membrane. This effect was sensitive to oligomycin (an F_0F_1-ATPase inhibitor) and the protonophore FCCP. The figure shows results of typical experiments, which were repeated at least 3-5 times using various mitochondrial preparations.

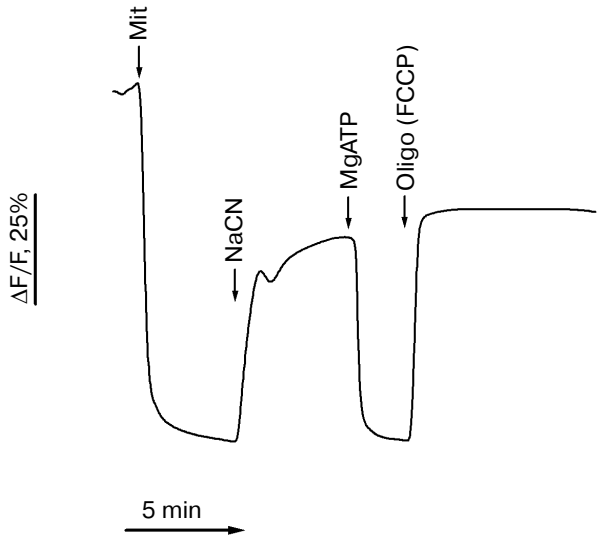

Fig. 2. Effects of cyanide, MgATP, and oligomycin or FCCP on mitochondrial membrane Δφ value. The incubation medium was the same as in Fig. 1; 400 μM Luc^{2+} and 0.1 μM Dis-C_2-(5) were added to the medium containing 5 mM succinate and rotenone (2 μg/mg protein) before addition of mitochondria. Arrows show the following additions: Mit) mitochondria, 0.6 mg protein/ml; NaCN) 1.2 mM NaCN; MgATP) 2 mM MgATP; Oligo) oligomycin, 5 μg/mg protein; FCCP) 0.1 μM FCCP.

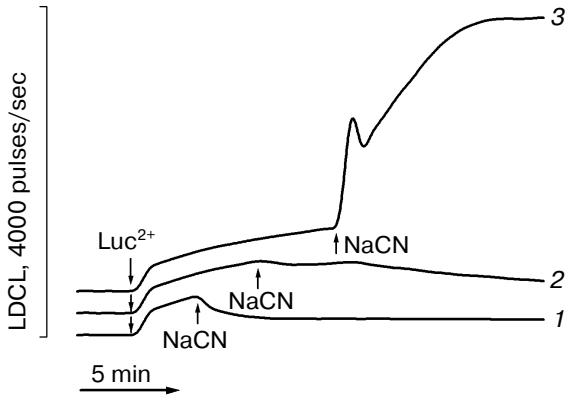

Fig. 1. Effect of preincubation of mitochondria with a low concentration of lucigenin on cyanide-induced LDCL. The composition of the incubation medium is given in "Materials and Methods". The respiratory substrate was 5 mM succinate (in the presence of rotenone, 2 μg/mg protein). The content of mitochondrial protein was 1 mg/ml. Arrows show the additions of 50 μM Luc^{2+} and 1.2 mM NaCN. The latter was added to mitochondria at different time intervals after addition of Luc^{2+} (curves *1-3*).

RESULTS

Cyanide-resistant mitochondrial respiration induced by lucigenin. As stated above, at relatively low (50 μM) Luc^{2+} concentrations lucigenin accumulation by energized (by oxidizing substrate) mitochondria is a rather slow process, which may limit generation of the chemiluminescence signal in response to cyanide addition (see Fig. 1). Taking into consideration this fact, we have used high concentrations of Luc^{2+} (400 μM) for registration of LDCL and cyanide-resistant respiration. For energization of mitochondria in the presence of respiratory substrates and cyanide, we added 2 mM MgATP (F_0F_1-ATPase substrate) to the incubation medium (Fig. 2).

Effect of initial state of mitochondria and changes in Δφ values on kinetics of cyanide-resistant respiration. Figure 3b shows that addition of Luc^{2+} to mitochondria in the presence of respiratory substrate (succinate) and cyanide induced cyanide-resistant respiration. The kinetics of the cyanide-resistant respiration depended on the order of additions of lucigenin and cyanide to the mitochondria. Under conditions of initial Luc^{2+} accumulation of mitochondria energized by succinate (Fig. 3a, curve *2*) cyanide addition caused oxygen release. The latter suggests possible hydroperoxide formation in the system. The intensity of this process depended on time of mitochon-

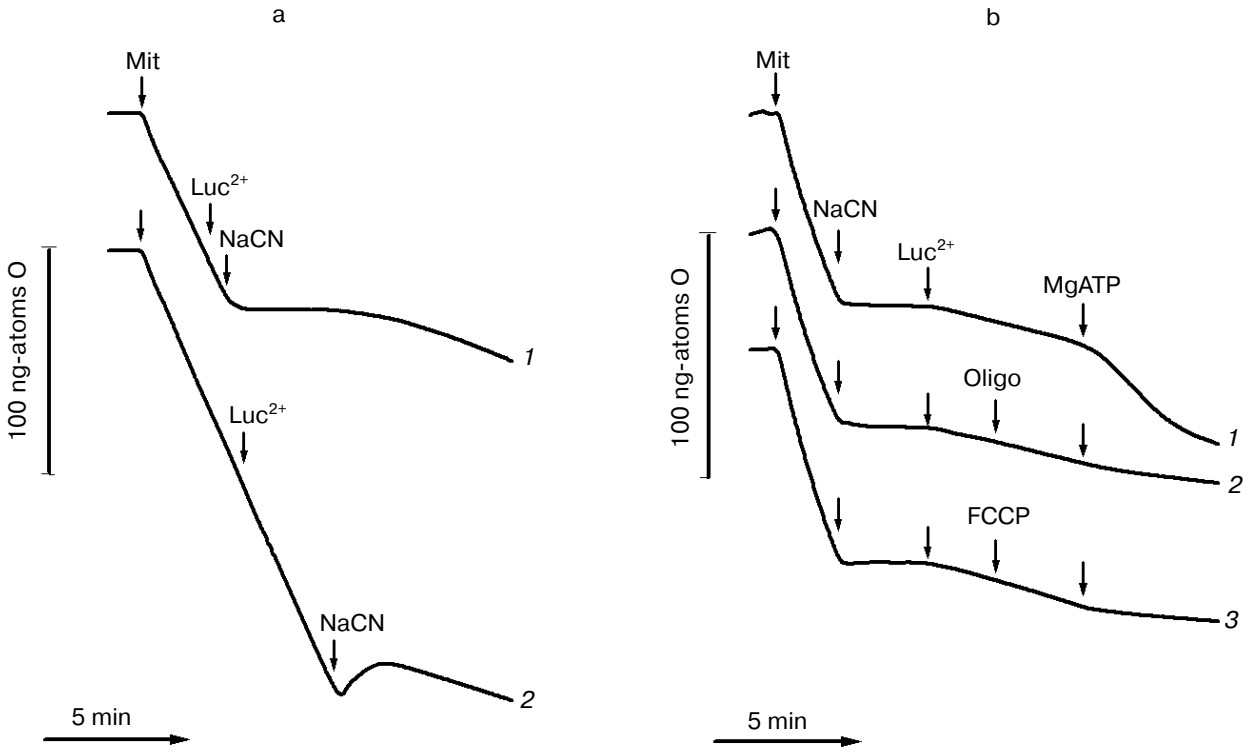

Fig. 3. Effect of order of addition of Luc^{2+}, cyanide (a, b), MgATP, oligomycin, and FCCP (b) on kinetics of mitochondrial oxygen consumption with succinate as respiratory substrate. The incubation medium was the same as in Fig. 1. a) Luc^{2+} was added to mitochondria energized by succinate before NaCN; b) Luc^{2+} was added to mitochondria after their deenergization by NaCN, but before addition of MgATP. Arrows indicate the following additions: Mit) mitochondria, 2 mg protein/ml; NaCN) 1.2 mM NaCN; Luc^{2+}) 400 μM Luc^{2+} (a, b); MgATP) 2 mM MgATP ((b), curves *1-3*); Oligo) oligomycin, 5 μg/mg protein ((b), curve *2*); FCCP) 0.1 μM FCCP ((b), curve *3*).

dria preincubation in the medium containing lucigenin (Fig. 3a, curves *2* and *1*).

When Luc^{2+} was added to deenergized mitochondria (after cyanide) oxygen release was not observed. Under these conditions, it was possible to register cyanide-resistant respiration (Fig. 3b, curve *1*).

Addition of MgATP to the deenergized mitochondria caused increase in Δφ value (see Fig. 2). This was accompanied by significant stimulation of mitochondrial respiration followed by spontaneous inhibition of oxygen consumption (Fig. 3b, curve *1*). The stimulatory effect of MgATP on the rate of mitochondrial respiration was inhibited by F_0F_1-ATPase inhibitor, oligomycin (Fig. 3b, curve *2*) or uncoupler FCCP (Fig. 3b, curve *3*). Their addition caused a decrease in Δφ value (see Fig. 2). At low Δφ values (in the medium without MgATP), neither oligomycin nor FCCP influenced the rate of cyanide-resistant respiration.

Effect of respiratory chain inhibitors on cyanide-resistant respiration. Figure 4 (curves *2-4*) shows results of experiments studying effects of specific inhibitors of the respiratory chain on the rate of cyanide-resistant respiration of mitochondria in the presence of succinate. These experiments were carried out under conditions of membrane potential maintenance by MgATP addition (before Luc^{2+}).

Under conditions of high Δφ value, the succinate dehydrogenase inhibitor TTFA insignificantly changed (or slightly stimulated) the rate of mitochondrial respiration in the presence of cyanide and lucigenin (Fig. 4, curves *1* and *2*). Complex III inhibitors, myxothiazol and antimycin A, strongly inhibited mitochondrial oxygen consumption (Fig. 4, curves *3* and *4*). Thus, in the presence of succinate and lucigenin electron transport in the respiratory chain is insensitive to TTFA. It should be noted that after addition of Complex III inhibitors to mitochondria energized by MgATP the effects of increase in oxygen content in the incubation medium appeared (Fig. 4, curves *3* and *4*). They were the same as in the case of long-term preincubation of mitochondria (before cyanide addition) with succinate but without MgATP and Complex III inhibitors (cf. with Fig. 3a, curve *2*).

Figure 5 (curves *2-4*) shows the effects of respiratory chain inhibitors on cyanide-resistant respiration of mito-

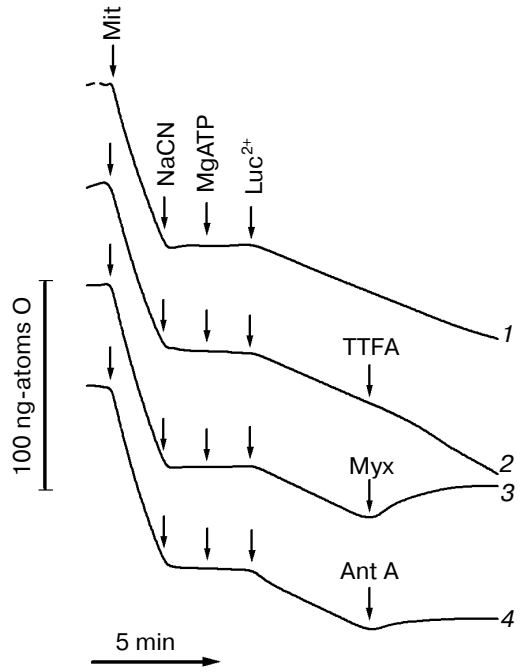

Fig. 4. Effect of TTFA, antimycin A, and myxothiazol on mitochondrial oxygen consumption in the presence of succinate, MgATP, cyanide, and lucigenin. The composition of the incubation medium was essentially the same as in Fig. 3b, but MgATP was added to mitochondria before Luc^{2+}. Arrows show the following additions: Mit) mitochondria, 2 mg protein/ml; NaCN) 1.2 mM NaCN; MgATP) 2 mM MgATP; Luc^{2+}) 400 μM Luc^{2+} (curves *1-4*); TTFA) 25 μM TTFA (curve *2*); Myx) 2 μM myxothiazol (curve *3*); Ant A) antimycin A, 0.5 μg/mg protein (curve *4*).

chondria using NADH-dependent respiratory substrates pyruvate + malate. (Other conditions were the same as in Fig. 4.) Rotenone, an NADH-dehydrogenase inhibitor, did not inhibit mitochondrial respiration; moreover, it slightly stimulated respiration, which was inhibited by subsequent addition of myxothiazol (Fig. 5, curves *1* and *2*). Separate addition of myxothiazol or antimycin A to the incubation medium (in the absence of rotenone) also caused potent inhibition of mitochondrial respiration (Fig. 5, curves *3* and *4*).

In another series of experiments without cyanide, lucigenin addition to mitochondria pretreated with the NADH-dehydrogenase inhibitor rotenone resulted in stimulation of mitochondrial respiration, which was effectively inhibited by myxothiazol (Fig. 6).

Lucigenin-dependent chemiluminescence and registration of Δφ value. *Dependence of LDCL on changes in Δφ.* Figure 7 (curve *1*) shows that in the presence of Luc^{2+} and cyanide, addition of MgATP to mitochondria (succinate present in the medium) induces generation of a high amplitude chemiluminescence signal. This suggests increased level of superoxide anion production in the system. In contrast to cyanide-resistant respiration, generation of LDCL by mitochondria required potential on the inner mitochondrial membrane. The uncoupler (FCCP) and oligomycin reduced Δφ value and completely abolished generation of MgATP-dependent chemiluminescence (Fig. 7, curve *2*).

In the absence of exogenous MgATP, addition of cyanide to mitochondria can also result in initial generation of the chemiluminescence signal (Fig. 8a, curve *1*), which is effectively abolished by pretreatment of mitochondria with oligomycin or FCCP (Fig. 8a, curves *2* and *3*, respectively). Using Dis-C_2-(5) in parallel experiments on registration of Δφ changes revealed that the presence of cyanide did not eliminate transmembrane potential completely (Fig. 8b). The remaining Δφ value may be attributed to hydrolysis of endogenous MgATP by F_0F_1-ATPase. This potential was further reduced after addition of uncouplers and oligomycin to mitochondria.

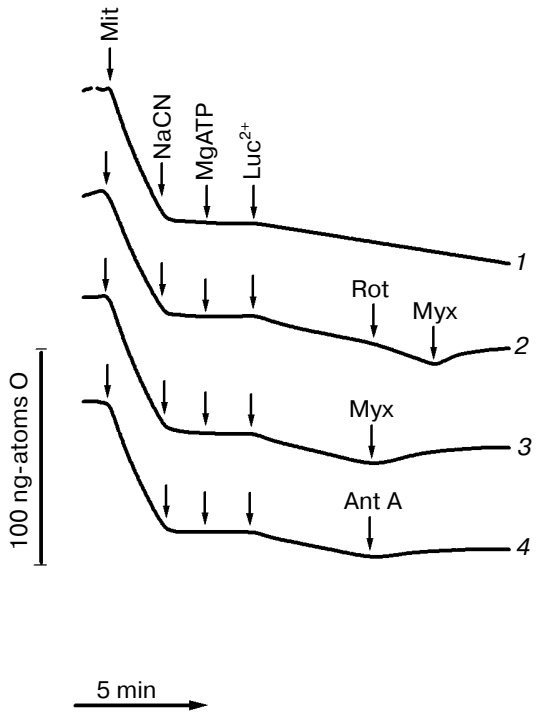

Fig. 5. Effect of rotenone, antimycin A, and myxothiazol on mitochondrial oxygen consumption in the presence of pyruvate + malate, MgATP, cyanide, and lucigenin. The composition of incubation medium and experimental conditions were the same as shown in Fig. 3b. The concentration of both substrates (pyruvate and malate) was 5 mM. Arrows show the following additions: Mit) mitochondria, 2 mg protein/ml; NaCN) 1.2 mM NaCN; MgATP) 2 mM MgATP; Luc^{2+}) 400 μM Luc^{2+} (curves *1-4*); Rot) rotenone, 2 μg/mg protein (curve *2*); Myx) 2 μM myxothiazol (curve *3*); Ant A) antimycin A, 0.5 μg/mg protein (curve *4*).

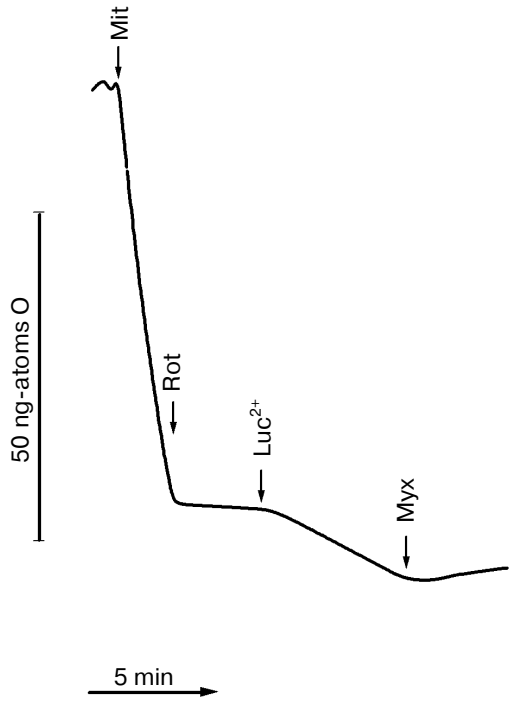

Fig. 6. Effect of rotenone and myxothiazol on mitochondrial oxygen consumption in the presence of pyruvate + malate and lucigenin. The composition of the incubation medium was the same as in Fig. 5. Arrows show the following additions: Mit) mitochondria, 2 mg protein/ml; Rot) rotenone, 2 μg/mg protein; Luc^{2+}) 400 μM Luc^{2+}; Myx) 2 μM myxothiazol.

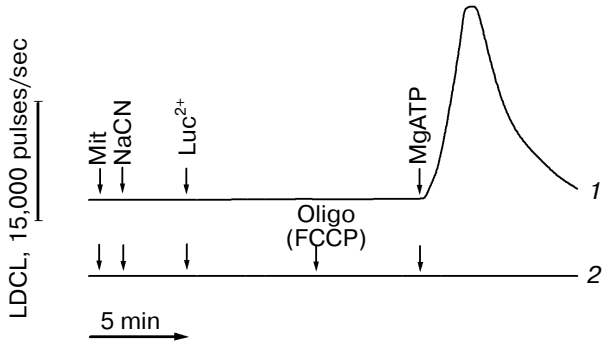

Fig. 7. Effect of oligomycin and FCCP on MgATP-dependent stimulation of mitochondrial LDCL in the presence of cyanide. The composition of the incubation medium was the same as in Fig. 3b. Arrows show the following additions: Mit) mitochondria, 1 mg protein/ml; NaCN) 1.2 mM NaCN; Luc^{2+}) 400 μM Luc^{2+}; MgATP) 2 mM MgATP (curve *1*); Oligo) oligomycin, 5 μg/mg protein; FCCP) 0.1 μM FCCP (curve *2*).

Experiments on registration of kinetics of LDCL and mitochondrial Δφ values in the presence of succinate and cyanide (Fig. 9, a and b) revealed that in the presence of Luc^{2+} addition of MgATP induced a biphasic process: initial increase followed by spontaneous decrease in Δφ value (Fig. 9b, curve *2*). Figure 9a (curve *2*) shows that during the same time interval corresponding phases of increase and spontaneous decrease in LDCL were also observed. In a control experiment (without addition of Luc^{2+} into the incubation medium), mitochondrial Δφ value remained unchanged during the same time interval (Fig. 9b, curve *1*).

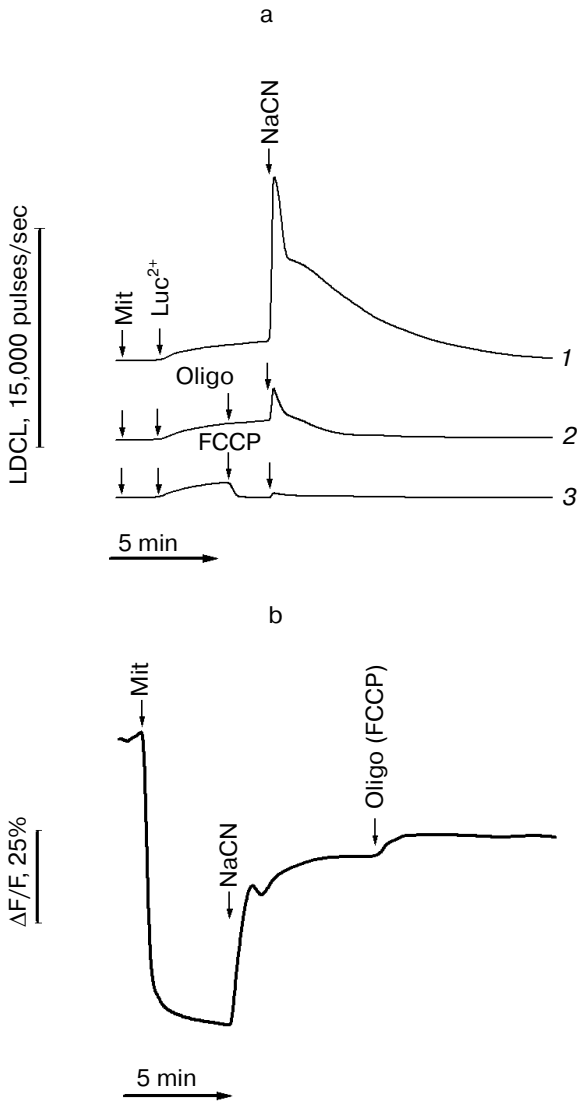

Fig. 8. Effect of oligomycin and FCCP on cyanide induced changes in LDCL (a) and Δφ (b) values in mitochondria incubated without MgATP. The composition of the incubation medium and experimental conditions were the same as in Fig. 1. a) Arrows show the following additions: Mit) mitochondria, 1 mg protein/ml; Luc^{2+}) 400 μM Luc^{2+}; NaCN) 1.2 mM NaCN (curves *1-3*); Oligo) oligomycin, 5 μg/mg protein (curve *2*); FCCP) 0.1 μM FCCP (curve *3*). b) Incubation medium contains 400 μM Luc^{2+} and 0.1 μM Dis-C_2-(5). Arrows show the following additions: Mit) mitochondria, 0.6 mg protein/ml; NaCN) 1.2 mM NaCN; Oligo) oligomycin, 5 μg/mg protein; FCCP) 0.1 μM FCCP.

It was reasonable to suggest that rapid spontaneous decrease of Δφ was related to time-dependent increase in membrane permeability. Figure 9 (a and b) (curve *3*) shows that addition of the nonspecific pore inhibitor cyclosporin A to the incubation medium significantly increased initial rates of generation of Δφ and LDCL after addition of MgATP. However, cyclosporin A addition did not prevent subsequent rapid decrease of Δφ and LDCL.

Effects of respiratory chain inhibitors on LDCL. Study of the effects of respiratory chain inhibitors on generation of LDCL revealed that during succinate oxidation in the presence of MgATP, TTFA insignificantly influenced chemiluminescence (Fig. 10, curves *2* and *1*, respectively). In contrast to cyanide-resistant respiration, total inhibition of LDCL was achieved only by combined addition of Complex III inhibitors, myxothiazol and antimycin A (Fig. 10, curve *3*). Separate addition of these inhibitors to the incubation medium insignificantly influenced generation of MgATP-dependent LDCL (data not shown).

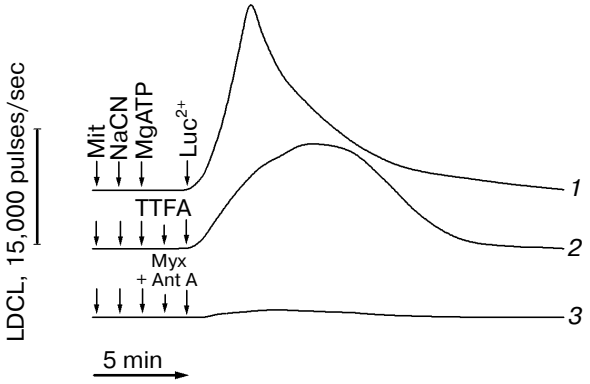

Fig. 10. Effects of TTFA, antimycin A, and myxothiazol on MgATP-dependent stimulation of LDCL of mitochondrial suspension. The composition of the incubation medium and experimental conditions were the same as shown in Fig. 4. Arrows show the following additions: Mit) mitochondria, 1 mg protein/ml; NaCN) 1.2 mM NaCN; MgATP) 2 mM MgATP; Luc^{2+}) 400 μM Luc^{2+} (curve *1*); TTFA) 25 μM TTFA (curve *2*); Myx + Ant A) myxothiazol (2 μM) with antimycin A (0.5 μg/mg protein) (curve *3*).

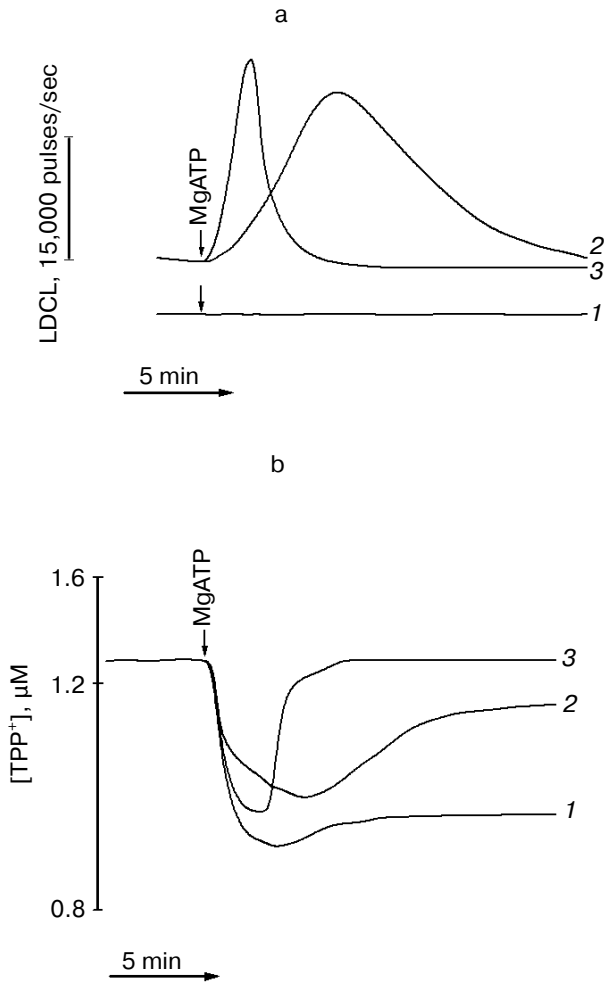

Fig. 9. Effects of lucigenin and cyclosporin A on MgATP-dependent changes of LDCL (a) and Δφ (b) values in mitochondria incubated in the presence of succinate and cyanide. The composition of the incubation medium and experimental conditions were the same as in Fig. 3b. Mitochondria (1 mg protein/ml), 1.2 mM NaCN, and 400 μM Luc^{2+} (a, b) were added just before measurements. The arrow shows addition of 2 mM MgATP. Curves: *1*) without Luc^{2+}; *2*) in the presence of 400 μM Luc^{2+}; *3*) in the presence of 400 μM Luc^{2+} and 1 μM cyclosporin A. b) All conditions were the same as in (a), but in the presence of 2 μM TPP^+.

DISCUSSION

Data of Figs. 3, 4, and 5 obtained in the presence of various respiratory substrates and a cytochrome oxidase inhibitor, cyanide, demonstrate that a high concentration of lucigenin (400 μM) induced cyanide-resistant respiration. Induction of cyanide-resistant respiration and results of direct chemiluminescence assays of superoxide anion formation (Figs. 7, 8, 9, and 10) suggest abnormally high level of superoxide production in mitochondria and its dependence on the physiological state of these organelles.

It was reasonable to suggest that increased superoxide anion production in mitochondria in the presence of high Luc^{2+} concentrations might be attributed to oxygen reduction catalyzed by lucigenin (reaction (5)). According to reaction (6), this would result in hydrogen peroxide formation. Indeed, studying the dependence of kinetics of cyanide-resistant respiration on changes in initial state of mitochondria (energized or deenergized), which was determined by an order of addition of Luc^{2+}

and cyanide to mitochondria (Fig. 3, a and b), we found in some cases increase in oxygen content in the incubation medium instead of expected oxygen consumption. According to reactions (5) and (6), short-living superoxide anion formed during cyanide-resistant respiration may be involved in hydrogen peroxide formation. Under our experimental conditions, it was possible to detect oxygen release only when cyanide was added after prolonged incubation of mitochondria with Luc^{2+} and a respiratory substrate (Fig. 3a, curves *2* and *1*). Decomposition of accumulated hydrogen peroxide (reaction (7)) should increase oxygen concentration in the closed polarographic cell. This effect we actually observed in our experiments (Fig. 3a, curve *2*).

To reduce effects of accumulation and decomposition of hydrogen peroxide on the initial rate of cyanide-resistant respiration, in most experiments we added lucigenin to mitochondria after cyanide (or after cyanide and MgATP). Under these conditions, there was no preliminary accumulation of Luc^{2+} by mitochondria, and the reaction of oxygen release was minimal.

Studying the dependence of the rate of cyanide-resistant respiration on $\Delta\varphi$ changes, we found that the increase in $\Delta\varphi$ value by adding MgATP caused significant stimulation of cyanide-resistant respiration (Fig. 3b, curve *1*) which was suppressed during $\Delta\varphi$ decrease by oligomycin (Fig. 3b, curve *2*) or FCCP (Fig. 3b, curve *3*). However, at low $\Delta\varphi$ values (in the medium without MgATP) oligomycin and FCCP did not influence the rate of cyanide resistant respiration. Possible reasons underlying this phenomenon will be considered below during consideration of the problem of localization of redox reactions of lucigenin in the mitochondrial membrane.

Good evidence exists that the effect of potential-dependent redistribution (accumulation/release) of Luc^{2+} in mitochondria, which was observed during energization (by respiratory substrates or MgATP) or deenergization (by oligomycin, uncouplers, and inhibitors of electron transport chain such as cyanide) of these organelles [6], can strongly influence kinetics of cyanide-resistant respiration. This was demonstrated in our experiments under conditions of generation of high $\Delta\varphi$ values on mitochondrial membranes.

To elucidate the mechanism of superoxide anion production in the mitochondrial respiratory chain, we employed inhibitory analysis of cyanide-resistant respiration and LDCL. Membrane potential was maintained by adding MgATP to the incubation medium. The results of inhibitory analysis of cyanide-resistant respiration of mitochondria oxidizing succinate or pyruvate + malate (Figs. 4 and 5) suggest that lucigenin forms electron transporting shuttles. These shuttles bypassing TTFA and rotenone blockades transfer electrons from dehydrogenases to Complex III. It is clear that Luc^{2+} can be reduced at sites positioned between substrate-binding regions of dehydrogenases (Complexes II and I) and sites of TTFA and rotenone action, whereas lucigenin oxidation might occur in coenzyme Q-binding sites of Complex III. It is possible that the reaction of Luc^{2+} reduction follows the two-electron mechanism:

$$Luc^{2+} + 2e^- \rightarrow Luc(2e). \quad (8)$$

If reduction were to yield the autooxidizable lucigenin cation radical, Complex III inhibitors would not inhibit respiration. It is important to emphasize that formation of the two electron reduction product, Luc(2e), was registered by a spectral method during electrochemical Luc^{2+} reduction in hydrophobic solvents [17].

Normal functioning of Q-cycle of Complex III results in specific separation of electron pair and, hence, oxidation of reduced ubiquinone (QH_2) in *o*-center of Q-cycle is accompanied by formation of semiquinone form of ubiquinone.

It is possible that during cyanide-resistant respiration in the presence of Luc(2e) the formation of readily oxidizable lucigenin cation radical is a consequence of a similar reaction (reaction (9)):

$$Luc(2e) \rightarrow Luc^{\bullet+} + e^-_1. \quad (9)$$

Our interpretation of lucigenin cation radical formation in the mitochondrial respiratory chain as a two-stage process qualitatively modifies the known scheme of Luc^{2+} redox reactions in biological systems (see the introductory section, reaction (1)). It basically coincides with the scheme of QH_2 oxidation in *o*-center of Complex III Q-site with only the exception that instead of cytochrome oxidase, excess of oxidized lucigenin (Luc^{2+}) acts as an oxidant (reaction (10)):

$$Luc^{2+} + e^-_1 \rightarrow Luc^{\bullet+}. \quad (10)$$

Inhibition of cyanide-resistant respiration (in the presence of succinate and TTFA) by myxothiazol and antimycin A suggests that generation of autooxidizable lucigenin cation radical involves both *o*- and *i*-centers of the Q-cycle of Complex III. Since cyanide-resistant respiration is sensitive to antimycin A, it is possible that division of the electron couple of Luc(2e) in *o*-center occurs via the following mechanism (Fig. 11). One electron (e_1) from Luc(2e) enters cytochrome *b* and then move to the *i*-center, where one electron reduction of the Luc^{2+} molecule (reaction (10)) occurs. Resultant lucigenin cation radical ($Luc^{\bullet+}$) is further oxidized by oxygen (reaction (5)). Another autooxidizable lucigenin cation radical formed in the *o*-center can also react with oxygen (reaction (5)).

The scheme of Fig. 11 shows that potential-dependent activation of Luc^{2+} accumulation in the membrane at *i*-center positively influences reactions of $Luc^{\bullet+}$ (reaction (9)) and superoxide anion (reaction (5)) at the *o*-center.

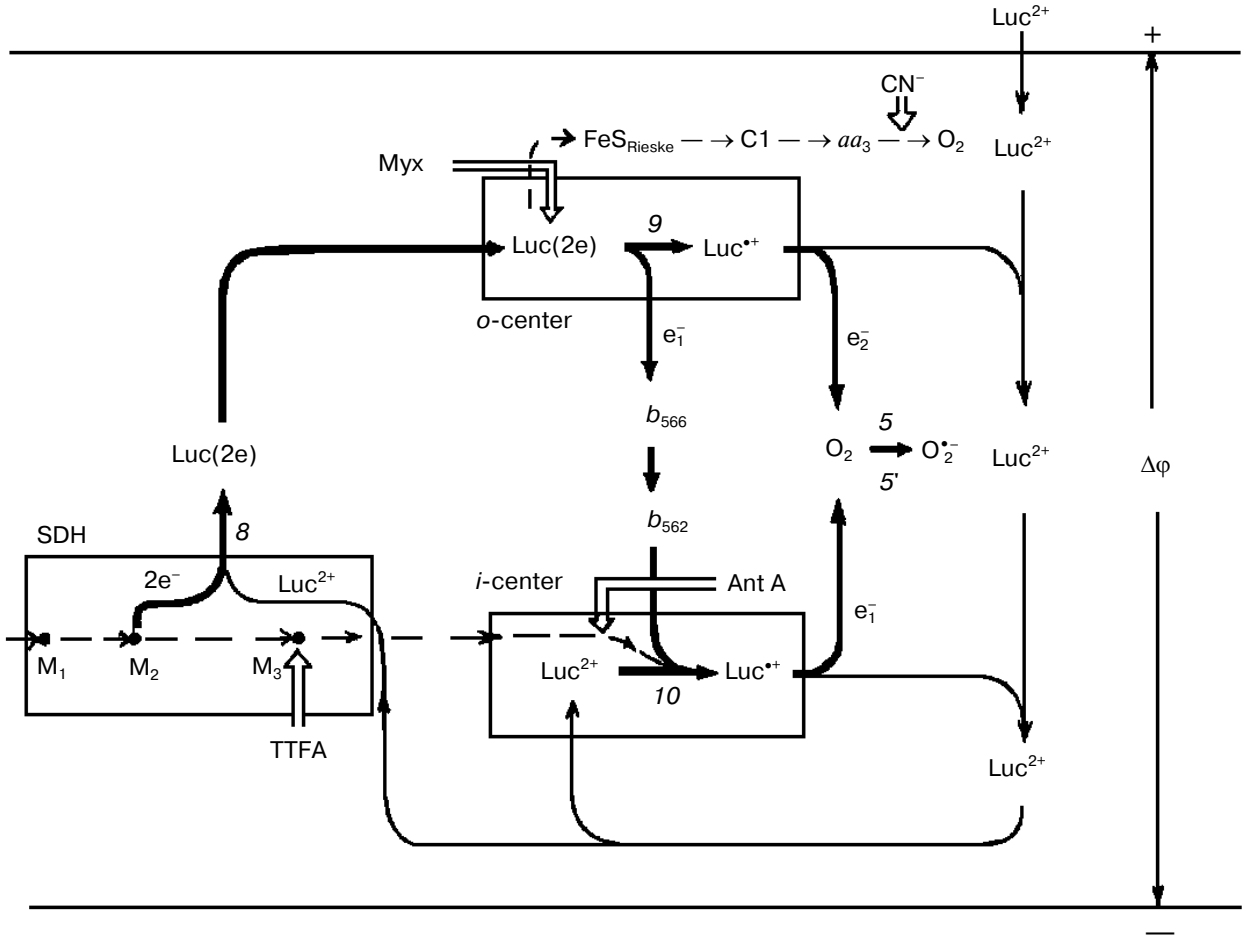

Fig. 11. Lucigenin shuttle and the scheme of Luc(2e) oxidation in the Q-cycle of Complex III of the mitochondrial respiratory chain. SDH is succinate dehydrogenase; M_1, M_2, and M_3 represent sites of substrate binding, two-electron reduction of Luc^{2+} (reaction (8)), and TTFA action, respectively. TTFA) tenoyltrifluoroacetone; Myx) myxothiazol; Ant A) antimycin A; CN^-) cyanide; e^-_1 and e^-_2 are electrons transported to oxygen during Luc(2e) oxidation which occurs at the *o*-center (reactions (9) and (5)) of the Q-cycle and involves cytochrome *b* (reactions (10) and (5′)). Broken arrows show the main pathway of electron transfer of the respiratory chain to cytochrome oxidase and oxygen (in the absence of cyanide and lucigenin). Solid bold arrows show reactions of electron transfer which involve lucigenin under conditions of reduction of respiratory chain components by cyanide (with indication of sites for action of the main inhibitors). Solid thin arrows show Δφ-dependent reactions of transmembrane and intramembrane distribution of oxidized form of lucigenin.

This should stimulate the rate of oxygen consumption during cyanide-resistant substrate oxidation. This was actually observed in our experiments using MgATP-energized mitochondria.

For simplicity only one of Luc(2e) shuttles appearing in the respiratory chain during succinate oxidation (in the presence of TTFA) by Complex II is shown in the scheme. In the absence of TTFA, formation of Luc(2e) may also occur in the *i*-center due to $Luc^{\bullet+}$ reduction by succinate dehydrogenase; this involves the main pathway of electron transfer.

Earlier it was shown that during pyruvate + malate oxidation (in the presence of rotenone) Q_0 and menadione could also form shuttles in the respiratory chain. Their reduced forms were effectively oxidized by oxygen in the Q-cycle of Complex III, and this was accompanied by superoxide anion formation [18, 19]. Additional experiments revealed that in medium containing pyruvate, malate, and rotenone (without cyanide) lucigenin as well as menadione induced formation of electron-transferring shuttle to Complex III, bypassing the rotenone block (Fig. 6). Under these conditions, addition of myxothiazol effectively suppressed stimulation of respiration by lucigenin.

Experiments on the effects of altered Δφ values and specific inhibitors of the respiratory chain on LDCL revealed that the mechanism of LDCL differs from the mechanism responsible for induction of cyanide-resistant

respiration. First, LDCL required the presence of Δφ (Figs. 7 and 8); second, in contrast to cyanide-resistant respiration chemiluminescence was inhibited only during combined addition of inhibitors of *o*- and *i*-centers of the Q-cycle of Complex III (Fig. 10, curve *3*). The latter suggests that reactions responsible for LDCL under conditions of cytochrome oxidase inhibition by cyanide and also during normal substrate oxidation [6, 20] involve both centers of Complex III.

It should be noted that initial stages of Luc^{2+} reduction during cyanide-resistant respiration and during LDCL reduction are obviously the same, because TTFA did not inhibit cyanide-resistant succinate oxidation (Fig. 4, curve *2*) and caused only much weaker inhibition of LDCL than myxothiazol and antimycin A (Fig. 10, curve *2*).

However, details of the mechanism responsible for LDCL induction during cyanide-resistant respiration, especially reasons for strict potential dependence of LDCL generation, remain unclear.

Results of quantitative calculations of the ratio between intensity of superoxide anion utilization during cyanide-resistant respiration and LDCL generation revealed that the proportion of superoxide anion interacting with $Luc^{\bullet+}$ (reactions (3) and (4)) is very low; it is approximately 5-6 orders of magnitude less than the amount of superoxide anion involved in the process of cyanide-resistant respiration (reactions (5)-(7)). These calculations were made using assumptions that quantum output of reaction (4) was 0.01% [21], strong effect of fluorescent quenching was absent, and the number of photons interacting with the registering device did not exceed 10% of the total number of all emitted photons.

We do not have simple explanation for the low effectiveness for interaction of $Luc^{\bullet+}$ with superoxide anion under conditions of intensive cyanide-resistant respiration. One possible reason consists in different localization of key reactions resulting in formation of superoxide anion (reactions (5)-(7)) and LDCL generation (reactions (3) and (4)) in the mitochondrial membrane. By analogy with known mechanism of autooxidation of menadione radical by oxygen [18, 19], it is plausible to assume that $Luc^{\bullet+}$ formation and its oxidation by oxygen involve polar zones of the membrane, whereas the reaction between $Luc^{\bullet+}$ and superoxide anion may occur in a hydrophobic zone of the membrane. Strict potential-dependence of this reaction is consistent with our suggestion. In fact, high Δφ values should result in increase in concentrations of lucigenin di-cation and $Luc^{\bullet+}$ in mitochondrial matrix; this might also be accompanied by sharp increase in equilibrated concentrations of $Luc^{\bullet+}$ in the hydrophobic phase of the membrane. The effect has been studied in detail using membrane permeable cations, for example, Dis-C_2-(5). At Δφ value of 180-200 mV, the increase in mitochondrial matrix concentration of this cation can reach three orders of magnitude.

In this study, we have presented evidence for an increase in ion permeability of the inner mitochondrial membrane under conditions of lucigenin-induced superoxide production: in the presence of lucigenin and cyanide there was the effect of spontaneous dissipation of Δφ generated by F_0F_1-ATPase (Fig. 9b, curve *2*). It is clear that the effect of inhibition of cyanide-resistant respiration, which occurs after stimulation by MgATP (Fig. 3b, curve *1*), may be attributed to time-dependent increase in mitochondrial membrane permeability. The time-reduction of the respiration rate correlates well with the decrease in Δφ and LDCL shown at Fig. 9 (a and b, curve *2*).

It is known that activation of superoxide formation by menadione is accompanied by opening of mitochondrial inner membrane non-selective pore, which is inhibited by cyclosporin A [22, 23]. In our experiments addition of cyclosporin A to mitochondria (before MgATP) significantly increased the rate of Δφ generation and LDCL, suggesting a coupling effect in the initial period of time (Fig. 9, a and b, curve *3*). However, subsequently, cyclosporin A caused more pronounced decrease in Δφ value. The latter suggests the existence of a feedback mechanism in the system of superoxide anion production in mitochondria: conditions of closed pore (in the presence of cyclosporin A) favor faster increase in critical Δφ values and corresponding Luc^{2+} accumulation in matrix, and this is accompanied by cyclosporin A-insensitive uncoupling of mitochondria—fall of Δφ value and reduction in chemiluminescence intensity.

Thus, based on results of the present study we have come to the following conclusions.

1. In intact mitochondria under conditions of lucigenin-induced cyanide-resistant oxidation of succinate or pyruvate + malate, lucigenin can form shuttles transferring electrons from Complex I and II to Complex III. These shuttles bypass TTFA and rotenone binding sites. Lucigenin reduction within Complexes II or I involves the two-electron mechanism followed by formation of the non-autooxidizable form Luc(2e).

2. Use of specific inhibitors of electron transfer revealed involvement of coenzyme Q-binding sites of Complex III in the reactions of one electron oxidation of Luc(2e), which was accompanied by formation of autooxidizable lucigenin cation radical and massive generation of superoxide anion.

3. Mechanisms of induction of cyanide-resistant respiration and LDCL differ. In contrast to cyanide-resistant respiration, generation of LDCL is a strictly potential-dependent process, which is suppressed only by combined addition of myxothiazol and antimycin A.

4. In the presence of lucigenin and cyanide, the high rate of superoxide anion production in mitochondria causes two effects: a) opening of nonspecific pore; b) induction of cyclosporin A-insensitive ion permeability of the inner mitochondrial membrane (the latter occurs during increase in lucigenin concentration in matrix).

This work was supported by the Russian National Program "Integration" (No. A0108), OURC "Life Sciences", and a NWO grant for a Joint Russian–Holland project (No. 047-006-005).

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