Membrane potential generation coupled to oxidation of external NADH in liver mitochondria

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Received 31 July 1998

Abstract Oxidation of added NADH by rat liver mitochondria has been studied. It is found that exogenous NADH, when oxidized by rat liver mitochondria in sucrose hypotonic medium supplemented with Mg^{2+} and EGTA, generates a membrane potential ($\Delta\Psi$) even in the absence of added cytochrome *c*. ADP and phosphate decrease $\Delta\Psi$, the effect being reversed by oligomycin. Rotenone and myxothiazol do not inhibit $\Delta\Psi$ generated by oxidation of exogenous NADH. Added cytochrome *c* increases the rate of the exogenous NADH oxidation and coupled $\Delta\Psi$ formation. In sucrose isotonic medium, or in hypotonic medium without Mg^{2+}, exogenous NADH fails to stimulate respiration and to form a membrane potential. In the presence of Mg^{2+}, exogenous NADH appears to be effective in $\Delta\Psi$ generation in isotonic sucrose medium if mitochondria were treated with digitonin. In isotonic KCl without Mg^{2+}, oxidation of exogenous NADH is coupled to the $\Delta\Psi$ formation and $MgCl_2$ addition before mitochondria prevents this effect. In hypotonic (but not in isotonic) sucrose medium, Mg^{2+} makes a portion of the cytochrome *c* pool reducible by exogenous NADH or ascorbate. It is assumed that (i) hypotonic treatment or digitonin causes disruption of the outer mitochondrial membrane, and, as a consequence, desorption of the membrane-bound cytochrome *c* in a Mg^{2+}-dependent fashion; (ii) incubation in isotonic KCl without Mg^{2+} results in swelling of mitochondrial matrix, disruption of the outer membrane and cytochrome *c* desorption whereas Mg^{2+} lowers the K^+ permeability of the inner membrane and, hence, prevents swelling; (iii) desorbed cytochrome *c* is reduced by added NADH via NADH-cytochrome b_5 reductase and cytochrome b_5 or by ascorbate and is oxidized by cytochrome oxidase. The role of desorbed cytochrome *c* in oxidation of superoxide and cytoplasmic NADH as well as possible relations of these events to apoptosis are discussed.

Key words: Exogenous NADH; Cytochrome *c*; Membrane potential; Liver mitochondrion; Apoptosis; Superoxide

1. Introduction

Oxidation of exogenous NADH by hypotonically-treated liver mitochondria in the presence of added cytochrome *c* has been described by Lehninger [1]. Oxidation was shown to be insensitive to rotenone, antimycin A, and was suppressed by cyanide. The external NADH-cytochrome *c* reductase system was found to be located in the outer mitochondrial membrane [2]. It was proposed that the external pathway of NADH oxidation may also function without added cytochrome *c*, by interaction of NADH-cytochrome *c* reductase (composed of NADH-cytochrome b_5 reductase and cytochrome b_5) in the outer membrane with cytochrome *c* oxidase in the inner membrane via the intermembrane cytochrome *c* [3,4]. This pathway was assumed to be activated by cytochrome *c* desorption to the intermembrane space of mitochondria in animals subjected to cold exposure [3]. In line with this assumption, in liver mitochondria of cold exposed rats, activation of the external pathway of NADH oxidation and a partial desorption of mitochondrial cytochrome *c* were found [5]. Slow oxidation of exogenous NADH without cytochrome *c* added was also found in liver mitochondria from non-exposed rats [5–7,10]. ATP synthesis coupled to NADH oxidation via the external pathway was shown only if cytochrome *c* was added [8,9].

In the present paper, it is shown that in hypotonic sucrose medium supplemented with magnesium ions, exogenous NADH oxidation is coupled to generation of membrane potential even in the absence of added cytochrome *c*. This potential is sufficiently high to support ADP phosphorylation since additions of ADP and phosphate decrease the transmembrane electric potential difference ($\Delta\Psi$) in an oligomycin-sensitive fashion. Digitonin and Mg^{2+} or isotonic KCl without Mg^{2+} effectively substitute for hypotonic sucrose and Mg^{2+}.

2. Materials and methods

Mitochondria were isolated from liver of white rats of 180–200 g weight. The isolation medium contained 250 mM sucrose, 2 mM EGTA, 5 mM MOPS-KOH (pH 7.4). The homogenate was centrifuged at $700\times g$ for 10 min. Mitochondria were sedimented at $9000\times g$ for 10 min, resuspended in 1 ml of isolation medium supplemented with bovine serum albumin (BSA) (2 mg/ml), diluted with 30 ml isolation medium without BSA, and centrifuged at $12\,000\times g$ for 10 min. The mitochondrial pellet was resuspended in the isolation medium supplemented with BSA. The final mitochondrial suspension contained about 100 mg mitochondrial protein/ml and about 0.3 mg BSA/ml. In the experiments where the salt incubation medium was used, EGTA concentration in isolation medium was reduced to 1 mM, and EGTA was excluded from the washing medium and mitochondrial suspension medium.

Oxygen consumption was recorded by a Clark-type oxygen electrode and LP-7E polarograph. The sucrose incubation medium contained 250 mM sucrose, 0.5 mM EGTA, and 5 mM MOPS-KOH, pH 7.4; $t° = 25°C$. In hypotonic incubation media, sucrose concentration varied from 5 to 60 mM. The salt incubation medium contained 125 mM KCl, 5 mM MOPS, pH 7.4, with or without 0.5 mM EGTA.

Membrane potential was estimated by tetraphenylphosphonium (TPP^+) distribution, using a TPP^+-sensitive electrode. In this case, the above incubation media were supplemented with 1.6 μM TPP^+ chloride.

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Abbreviations: $\Delta\Psi$, transmembrane electric potential difference; BSA, bovine serum albumin; DNP, 2,4-*p*-dinitrophenol; ROS, reactive oxygen species; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylene diamine; TPP^+, tetraphenylphosphonium

PII: S0014-5793(98)01072-2

Cytochrome difference spectra were recorded with a split-beam Aminco DW-2000 spectrophotometer. Experimental conditions were similar to those used for oxygen consumption recording.

Mitochondrial protein was measured with the biuret method.

MOPS, cytochrome *c* (C 3131, prepared without using trichloroacetic acid), oligomycin, rotenone, antimycin A, succinate, myxothiazol, gramicidin D, glutamate, malate, digitonin and delipidized BSA were from Sigma; EGTA was from Serva; 2,4-*p*-dinitrophenol (DNP) and TPP^+ chloride were from Merck, NADH and ADP were from Fluka. Sucrose was twice precipitated from a concentrated solution in bidistilled water with distilled ethanol. Digitonin was twice recrystallized from distilled absolute ethanol.

3. Results

Results of polarographic measurement of oxygen consumption of rat liver mitochondria are shown in Fig. 1. It is seen that addition of Mg^{2+} causes stronger stimulation of oxidation of the added NADH in the hypotonic medium than in the isotonic medium. Additions of cytochrome *c* and DNP result in further marked stimulation of the respiration only in the hypotonic medium.

In the same experiments, addition of NADH entails no membrane potential ($\Delta\Psi$) generation in isotonic medium supplemented with Mg^{2+}, rotenone and myxothiazol (Fig. 2A). On the other hand, in a hypotonic Mg^{2+}-containing medium, added NADH causes generation of $\Delta\Psi$ which is only slightly lower than that generated by oxidation of glutamate and malate. This $\Delta\Psi$ is decreased by ADP and phosphate and is increased by subsequent addition of oligomycin. Further addition of antimycin A entails some $\Delta\Psi$ decrease which is apparently not due to inhibition of oxidation of NAD^+-linked substrates since rotenone and myxothiazol are already present. Rather, it is explained by a small increase in the ion conductance of the mitochondrial membrane, which is known to accompany the effect of antimycin A. Final addition of gramicidin D abolishes the NADH oxidation-supported $\Delta\Psi$ (Fig. 2B).

Since the rate of oxidation of added NADH is rather low even in the presence of Mg^{2+} (see Fig. 1), a small concentration of uncoupler (2×10^{-6} M DNP) strongly decreases the $\Delta\Psi$ level. Subsequent addition of cytochrome *c* increases $\Delta\Psi$. Half-maximal effect was obtained with 2×10^{-7} M cytochrome *c*. Now much higher concentrations of DNP are required to lower $\Delta\Psi$ (Fig. 2D). This observation can be accounted for by the fact that added cytochrome *c* significantly stimulates exogenous NADH oxidation by mitochondria in hypotonic media (see above, Fig. 1). In the same manner one may explain much smaller $\Delta\Psi$ decrease by antimycin A when both NADH and cytochrome *c* are added (Fig. 2C).

In Fig. 2B–C and D, 45 and 30 mM sucrose concentrations were used, respectively. Decrease in sucrose concentrations below 20 mM resulted in a lowering of the membrane potential generation coupled to the NADH oxidation with either endogenous or exogenous cytochrome *c* (not shown). It should be noted that gradual decrease in sucrose concentration from 250 to 20 mM (Mg^{2+} is present) resulted in parallel elevations of $\Delta\Psi$ generated by oxidation of external NADH via either endogenous or added cytochrome *c* (not shown).

On the face of it, oxidation of added NADH via endogenous cytochrome *c* might occur in contact areas of the outer and inner membranes as suggested by Jasaitis et al. [11,12]. Really, it is known that under hypotonic conditions these areas increase and volume occupied by the intermembrane space decreases. However, this explanation is hardly sufficient since it fails to account for the fact that under the same conditions added cytochrome *c* is rapidly oxidized by mitochondria. Apparently, swelling of mitochondria results in damage of the outer membrane so cytochrome oxidase becomes available for added cytochrome *c*. Moreover, the damage in question entails dilution by the incubation mixture of endogenous cytochrome *c*, desorbed from its binding sites on the inner mitochondrial membrane. Calculations showed that after such a dilution concentration of the desorbed cytochrome *c* in the medium is still sufficient to explain observed $\Delta\Psi$ formation coupled to oxidation by cytochrome oxidase of this

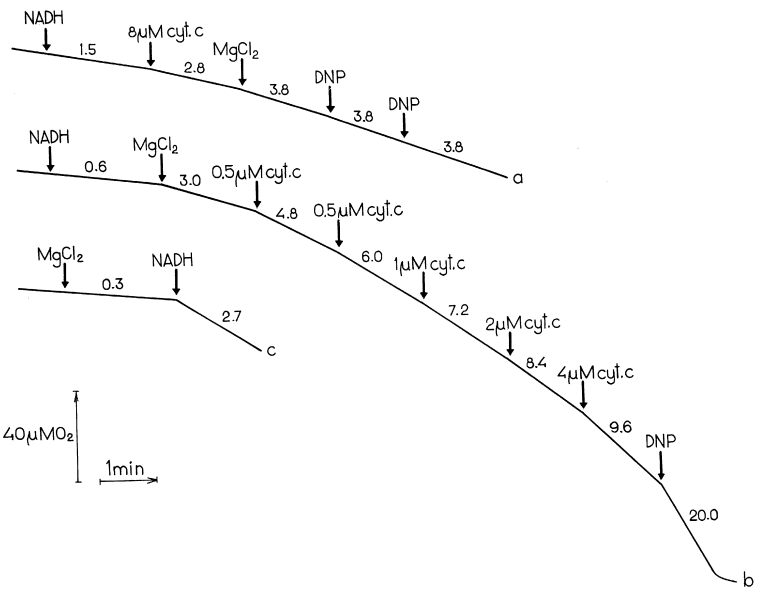

Fig. 1. Oxidation of external NADH by rat liver mitochondria in incubation media of different tonicity. Incubation medium contained 250, 60 or 45 mM sucrose (curves a, b and c, respectively), 2×10^{-6} M rotenone, 0.5 mM EGTA, 5 mM MOPS-KOH, pH 7.4, and 2.2 (curves a and b) or 4 (curve c) mg mitochondrial protein/ml. Additions: 0.5 mM NADH, 3 mM $MgCl_2$, 0.02 mM DNP, and various concentrations of cytochrome *c*. Figures near curves, rates of O_2 consumption, nmol O_2/mg protein/min.

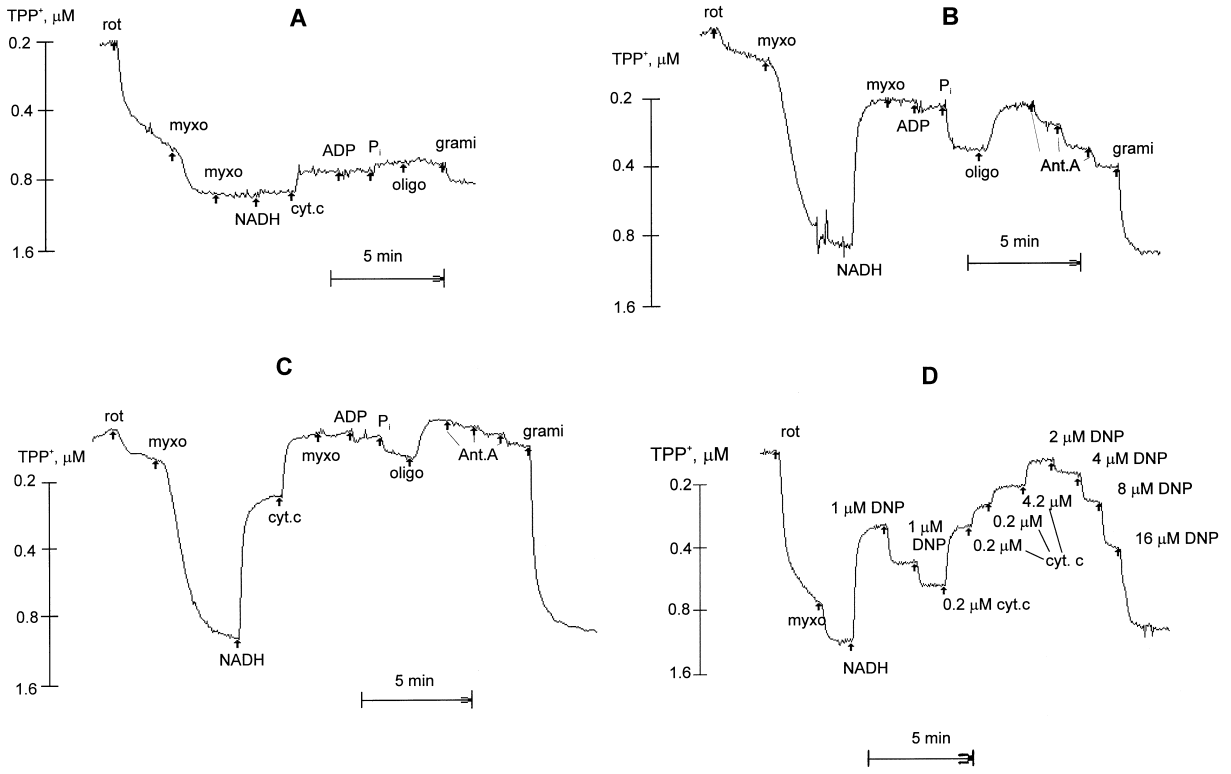

Fig. 2. Membrane potential generation coupled to oxidation of external NADH by rat liver mitochondria in hypotonic sucrose media. Incubation media contained 250 (A), 45 (B,C) or 30 (D) mM sucrose, 1.6×10^{-6} M TPP^+, 4 mM glutamate, 1 mM malate, 0.5 mM EGTA, 5 mM MOPS-KOH, pH 7.4, mitochondria (1.9 mg protein/ml), and 4 mM $MgCl_2$ which was added after mitochondria. Additions: rot: 1×10^{-6} (A–C) or 2×10^{-6} (D) M rotenone, respectively; ant.A: 100 nM antimycin A; 1 mM NADH; cyt c: 5×10^{-6} M cytochrome *c* (A,C); grami: gramicidin D (0.8 μg/ml); myxo: 0.8×10^{-6} M myxothiazol; 0.25 mM ADP; P_i: 1 mM potassium phosphate; oligo: oligomycin (2 μg/ml).

cytochrome *c* (which is reduced by exogenous NADH via cytochrome b_5 reductase and cytochrome b_5 localized in the outer mitochondrial membrane).

The above reasoning was confirmed by the experiment shown on Fig. 3A,B. Here digitonin was used, instead of hypotonic treatment, to disrupt the outer mitochondrial membrane. It is seen (Fig. 3A) that, in line with data on Fig. 2A, isotonic mitochondria fail to generate measurable ΔΨ when NADH and cytochrome *c* are added even in the presence of Mg^{2+}. However, both NADH and cytochrome *c* appear to be effective in ΔΨ generation when isotonic mitochondria are treated with digitonin, a detergent attacking first of all the outer mitochondrial membrane [13]. This is shown in Fig. 3B. One can see that increase in concentration of digitonin added after NADH results in appearance of ΔΨ. Subsequent addition of cytochrome *c* causes further elevation of ΔΨ. The ΔΨ appearance was not accompanied by any significant changes in the light scattering of mitochondria (not shown in the figure).

It should be stressed that neither hypotonicity nor digitonin were effective if Mg^{2+} was not added after mitochondria. The first observation on activation of the exogenous NADH oxidation by Mg^{2+} was published by Lemeshko (for references see [14]). The mechanism of this effect consists, most probably, in facilitation by Mg^{2+} of desorption of the membrane-bound cytochrome *c*. If this is the case, high concentration of KCl should substitute for Mg^{2+}. This suggestion was confirmed by experiments when mitochondria were incubated in 125 mM KCl without Mg^{2+}. As is shown in Fig. 3C, addition of NADH under such conditions results in a ΔΨ formation which is increased by subsequent Mg^{2+} additions only slightly. If Mg^{2+} is added to isotonic KCl before mitochondria, it was found to prevent the ΔΨ generation coupled to oxidation of external NADH (Fig. 3D). These relationships can be explained by the fact that the K^+ permeability of the liver mitochondrial inner membrane is much higher in the absence of Mg^{2+} than in its presence (for references see [15]). Apparently, high KCl caused both swelling of mitochondria and desorption of cytochrome *c*.

In further experiments, it was found that ascorbate added instead of NADH supports some ΔΨ generation provided that the outer membrane is broken and cytochrome *c* is desorbed. The level of ΔΨ with ascorbate was always lower than with NADH (not shown in figures).

Since ascorbate is known to reduce water-soluble but not membrane-bound cytochrome *c*, it is possible to monitor the cytochrome *c* desorption measuring degree of reduction of the cytochrome *c* pool by ascorbate. As is seen in Fig. 4A, ascorbate fails to reduce a measurable amount by cytochrome *c* in mitochondria incubated in isotonic sucrose. Subsequent addition of $MgCl_2$ is practically without effect. External NADH causes reduction of cytochrome b_5 whereas *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylene diamine (TMPD) and cyanide strongly reduce mitochondrial cytochromes including cytochrome *c*. In hypotonic sucrose medium (Fig. 4B), a small portion of cytochrome *c* is reduced by ascorbate even without Mg^{2+}.

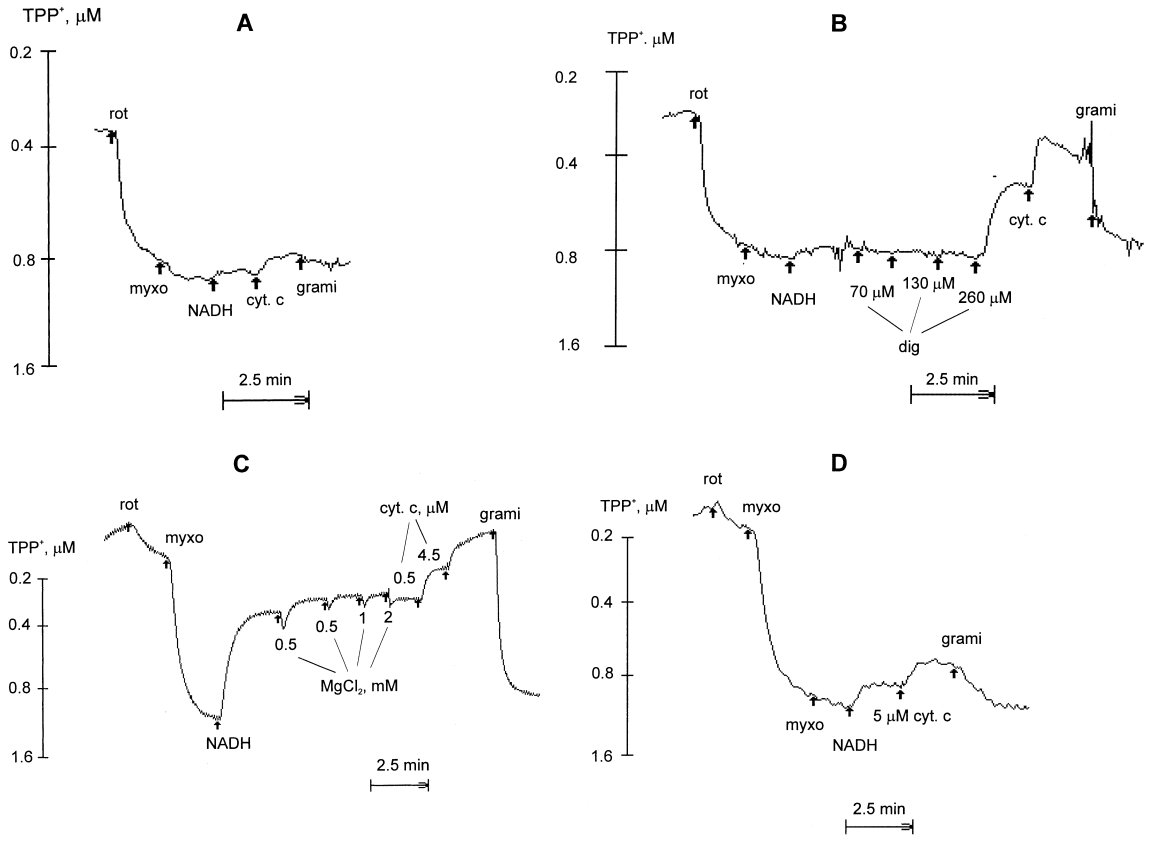

Fig. 3. Membrane potential generation coupled to oxidation of external NADH in isotonic sucrose with digitonin and in isotonic KCl. The incubation mixture contained 250 mM sucrose (A,B) or 125 mM KCl (C,D) and components listed in Fig. 2. In C the incubation mixture initially did not contain Mg^{2+}. In D 4 mM $MgCl_2$ was added before mitochondria. Additions: dig: digitonin. For other additions, see Fig. 2D.

Addition of Mg^{2+} strongly increases the degree of cytochrome *c* reduction which reaches approximately 50%. Subsequent NADH addition reduces cytochrome b_5 being almost without effect on cytochrome *c* reduction.

In the same experiments, it was found that NADH added before ascorbate reduced a portion of cytochrome *c* in hypotonic sucrose solution only if Mg^{2+} is present (not shown in the figure).

4. Discussion

The obtained data showed that two factors are required to activate energy-coupled oxidation by exogenous NADH by rat liver mitochondria, i.e. (i) disruption of the outer mitochondrial membrane and (ii) Mg^{2+} (or high K^+). It is noteworthy that the same requirements were revealed in the experiments where ascorbate was substituted for NADH as the

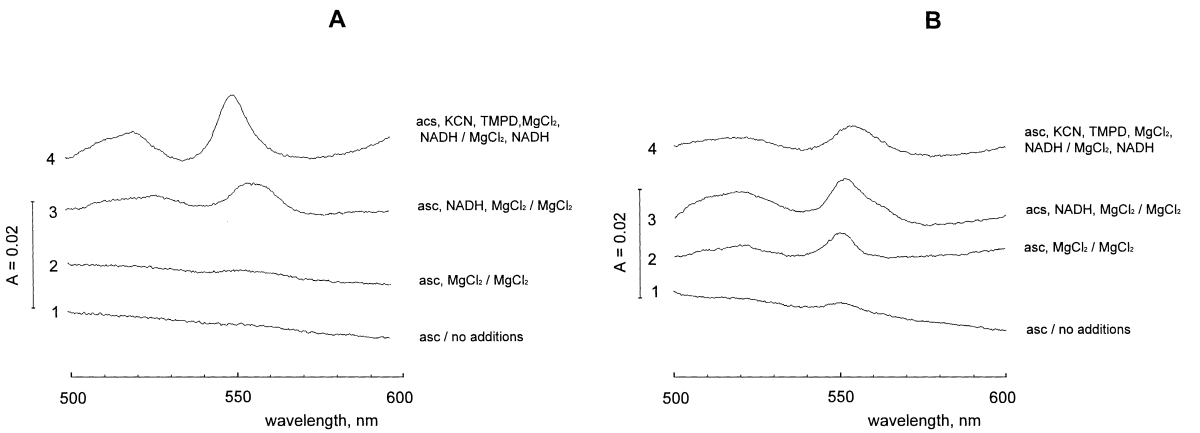

Fig. 4. Effect of magnesium and hypotonia on the difference spectra of cytochromes in rat liver mitochondria. The incubation mixture contained 250 mM (A) or 5 mM (B) sucrose, 0.5 mM EGTA, 10 mM MOPS-KOH, pH 7.4, mitochondria (2 mg protein/ml), 2×10^{-6} M rotenone and 2×10^{-6} M antimycin A. Additions: asc: 5 mM ascorbate; 4 mM $MgCl_2$; 0.5 mM NADH; 1×10^{-5} M TMPD; 2 mM KCN.

electron donor. This fact shows that the above results cannot be explained by mobilization of external NADH-cytochrome *c* reductase activities when the outer mitochondrial membrane is broken.

Formally, our data contradict those published quite recently by La Piana et al. [16]. The authors have reported that rat liver mitochondria incubated in isotonic sucrose medium with 5 mM Mg^{2+} could oxidize external NADH via added cytochrome *c* in a $\Delta\Psi$-generating fashion. No measurable $\Delta\Psi$ was formed without added cytochrome *c*. The incubation media in our and La Piana's experiments differed in pH and [KCl]. In experiments with sucrose media, we used pH 7.4 and about 2 mM K^+ whereas Piana et al. used pH 7.8 and 20 mM K^+. In the latter case, mitochondria were, most probably, swollen due to higher ion conductance of the inner membrane at high pH. As a result, the outer membrane was broken and cytochrome *c* was desorbed. On the other hand, $\Delta\Psi$ generation coupled to oxidation of the desorbed cytochrome *c* proved negligible because of high ion conductance.

Our observations may be related to the data published recently by Hackenbrock and coworkers [17]. The authors showed that cytochrome *c*, loosely (electrostatically) bound to mitochondrial or liposomal membrane, spontaneously converts to a form which is tightly bound to membrane. In this form, hydrophobic interactions are involved in cytochrome binding. The tight binding dramatically changes the cytochrome *c* conformation, namely, the α-helical content decreases down to 75% whereas the β-sheets content increases up to 135%. Release of the tightly-bound cytochrome *c* proved to be a very slow process.

Taking into account these data, we may assume the following scheme of interaction between cytochrome *c*, mitochondrial cytochrome oxidase and Mg^{2+}:

$$\mathrm{COX{\bullet}Mg^{2+}} + \mathrm{cyt.}c \underset{\mathrm{Mg^{2+}}}{\overset{(1)}{\leftrightarrows}} \mathrm{COX{\bullet}(cyt.}c)_{\mathrm{L}} \overset{(2)}{\longleftrightarrow} \mathrm{COX{\bullet}(cyt.}c)_{\mathrm{T}}$$

where COX is for mitochondrial cytochrome oxidase, $(\mathrm{cyt.}c)_{\mathrm{L}}$ and $(\mathrm{cyt.}c)_{\mathrm{T}}$ are for loosely and tightly bound cytochromes *c*, respectively. Apparently, in isotonic sucrose, equilibrium is shifted to the right so cytochrome *c* is mainly in the tightly bound form. Thus neither cytochrome b_5 nor ascorbate are oxidized. Addition of Mg^{2+} (or high K^+) shifts the equilibrium to the left and some cytochrome *c* is released to the intermembrane space. However, the volume occupied by this space is so small that desorption of little amounts of cytochrome *c* resulted in a strong increase in the soluble cytochrome *c* concentration. As a consequence, the equilibrium concentration of $\mathrm{COX{\cdot}Mg^{2+}}$ remains very low whereas that of $\mathrm{COX{\cdot}(cyt.}c)_{\mathrm{T}}$ is still very high.

Disruption of the outer mitochondrial membrane results in strong dilution of the soluble cytochrome *c* and, as a consequence, in further left shift of the above equilibrium. As a result, oxidation of cytochrome b_5 or ascorbate is activated since it requires (i) the soluble cytochrome *c* and (ii) cytochrome oxidase forms other than $\mathrm{COX{\cdot}(cyt.}c)_{\mathrm{T}}$.

It should be emphasized that desorption of the bound cytochrome *c* hardly occurs under in vivo conditions in normal cells (isotonic KCl, millimolar $MgCl_2$ and intact outer mitochondrial membrane). However, according to our data, disruption of the outer membrane or an increase of its permeability for cytochrome *c* must inevitably result in desorption of cytochrome *c*. The cytochrome *c* desorption from the inner mitochondrial membrane may be important in several aspects.

(i) According to our data [5,18], such a process occurs in liver mitochondria of cold-exposed rats being apparently involved in thermoregulatory response of warm-blooded animals [5]. This effect was found to be under thyroid hormone control [18].

(ii) Release of a moderate portion of the cytochrome *c* pool to cytosol, which does not inhibit the respiration rate yet, can be involved in the antioxidant system since the water-soluble cytochrome *c* catalyzes oxidation of superoxide to O_2 as was discussed elsewhere [19].

(iii) Recent observations point to massive cytochrome *c* release from mitochondria at early steps of apoptosis [19–22]. Calculations showed that concentrations of cytosolic cytochrome *c* released from mitochondria at apoptosis should be even higher than the concentration of endogenous cytochrome *c* in the incubation medium in our experiments on mitochondria with a broken outer membrane. This means that the released cytochrome can be involved in oxidation of cytosolic NADH by mitochondrial cytochrome oxidase during development of apoptosis. Decrease in cytosolic $NADH/NAD^+$ ratio must result in a positive shift of the cytosolic redox potential, an effect clearly related to control of a set of metabolic processes involved in apoptosis [23,24]. This may be one more function of cytochrome *c* in apoptosis, besides activation of procaspase 9 [25,26] and scavenging of superoxide [19]. In this way we may explain why cytochrome *c* has been chosen by biological evolution as a mediator of programmed cell death.

The chain of cytochrome *c*-related events, caused by [ROS] increase and taking part in the antioxidant system of the tissue, seems to be described as follows. (i) A moderate portion of the cytochrome *c* pool releases to cytosol from some mitochondria that are swollen because of the ROS-induced formation of pores in the inner membrane or for some other reasons. (ii) Superoxide is oxidized by the released cytochrome *c* which is, in turn, oxidized by mitochondrial cytochrome oxidase. (iii) If, nevertheless, [ROS] continues to increase, cytochrome *c* is released from a large portion of mitochondria, an event causing inhibition of cellular respiration at the cytochrome *c* level. (iv) This inhibition increases ROS production, as was recently shown by Cay and Jones [27], stimulates pore opening in the majority of mitochondria and causes further desorption of cytochrome *c*. (v) Cytosolic cytochrome *c* concentration reaches a critical level sufficient to (a) activate caspase 9 and (b) cause a positive shift of the cytosolic redox potential. (vi) Events (a) and (b) induce apoptosis which eliminates the ROS-producing cells.

Acknowledgements: This study was supported by Grants N 98-04-98327, N 96-15-98070 and N 96-04-00022 of the Russian Foundation for Basic Research.

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