Mg^{2+} induces intermembrane electron transport by cytochrome *c* desorption in mitochondria with the ruptured outer membrane

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Received 14 February 2000; received in revised form 10 March 2000

Edited by Vladimir Skulachev

Abstract The mechanism of external NADH oxidation in rat liver mitochondria is not clear yet, but it evidently includes the intermembrane electron transport (IMET). We showed that Mg^{2+} significantly stimulated IMET insensitive to rotenone–antimycin A–myxothiazol in mitochondria with the hypotonically damaged outer membrane, even in the absence of exogenous cytochrome *c*. This effect was more specific than simply ionic strength increase. Direct evidence for cytochrome *c* desorption from mitochondrial membranes under 4 mM Mg^{2+} influence was obtained to explain the stimulatory effect of Mg^{2+} on external NADH oxidation that was coupled to the inner membrane potential generation. Obtained data suggest that cytochrome b_5 is normally oriented towards the cytosol in the outer membrane, and can be accessible for endogenous cytochrome *c* reduction only through the outer membrane rupture or permeabilization, to activate external NADH oxidation.

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

1. Introduction

The NADH–cytochrome *c* reductase electron transport system of the outer membrane of rat liver mitochondria is known to have a very high activity [1]. Free cytochrome *c* concentration in the mitochondrial intermembrane space has been estimated to be sufficiently high under physiological ionic strength ([2] and references therein). Additional desorption of cytochrome *c* from the inner membrane was assumed to explain an increased rate of external NADH oxidation in liver mitochondria of cold-adapted rats [3], although some degree of the outer membrane rupture due to possible swelling of mitochondria cannot be excluded as well [4]. In any case, the rate of the intermembrane electron transport (IMET) in intact mitochondria is known to be very low, which has to be explained.

We have previously shown that the outer membrane rupture was an obligatory condition for a relatively high rate of rotenone-insensitive oxidation of external NADH and that Mg^{2+} ions significantly stimulated this oxidation in the presence and even in the absence of exogenous cytochrome *c* [4,5]. Recently, these findings were confirmed in Skulachev's laboratory [6]. On the other hand, according to Marzulli et al. [7], 'intact (but not damaged) mitochondria are able to promote the oxidation of exogenous cytochrome *c*' under external NADH oxidation.

This work provides direct evidence that in mitochondria with the damaged outer membrane, Mg^{2+} stimulates IMET due to cytochrome *c* desorption from mitochondrial membranes. Obtained data suggest that external NADH oxidation insensitive to rotenone–antimycin A–myxothiazol (RAM) in rat liver mitochondria may be activated only through the outer membrane rupture or permeabilization, when outer membrane cytochrome b_5, oriented towards the cytosol, becomes accessible for endogenous cytochrome *c* reduction.

2. Materials and methods

Rat liver mitochondria were isolated by the standard procedure of differential centrifugation using the medium composed of 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM HEPES adjusted to pH 7.4 with Trizma base. Mitochondria were washed and finely suspended in the medium composed of 210 mM mannitol, 70 mM sucrose, 0.02 mM EGTA, 5 mM HEPES/Trizma, pH 7.4 (MSHG medium). The final concentration of mitochondria in suspension was 40–50 mg of mitochondrial protein/ml, determined by the biuret method.

Cytochrome *c* binding to mitochondrial membranes was investigated by measuring its redistribution between supernatant and mitochondrial pellet. Mitochondria were initially suspended in the final concentration of 0.6 mg of mitochondrial protein/ml in 3 ml of 5 mM HEPES/Trizma base buffer, 0.02 mM EGTA, pH 7.4 (HG medium) or MSHG medium, both supplemented with 1.5 or 2 μM exogenous cytochrome *c*, at 25°C for 3 min. After addition of 4 mM $MgCl_2$ or a corresponding volume of the medium, the samples were centrifuged at $10\,000\times g$ for 15 min at 2–4°C to obtain the supernatant and the pellet. Cytochrome *c* was determined by the method of differential spectrophotometry using the double beam spectrophotometer Specord UV VIS (Germany). The pellet was solubilized with 0.4% Triton X-100 in 100 mM potassium phosphate buffer, pH 7.4. Potassium phosphate concentration in the supernatant was adjusted to 100 mM by adding the adequate quantity of 3.0 M potassium phosphate buffer, pH 7.4. A differential reduction–oxidation spectrum of cytochrome *c* was obtained 5 min after addition of 50 mM ascorbate and a few crystals of dithionite in the first cuvette and 50 mM ferricyanide in the second.

NADH was measured fluorometrically [5]. Oxygen consumption was determined using a Clark-type oxygen electrode. Inner membrane electrical potential was estimated by measuring 10 μM safranin O fluorescence [8]. The following media were used in the experiments: isotonic MSHG medium, strongly hypotonic HG medium and moderately hypotonic medium composed of MSHG and HG media mixed in the v/v ratio of 15:85. The media were kept at 37°C and mixed with a magnetic stirrer during measurements.

All used reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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Abbreviations: IMET, the intermembrane electron transport; MSHG, the medium composed of 210 mM mannitol, 70 mM sucrose, 0.02 mM EGTA, 5 mM HEPES/Trizma, pH 7.4; HG, the medium composed of 5 mM HEPES/Trizma, 0.02 mM EGTA, pH 7.4; RAM, rotenone–antimycin A–myxothiazol

PII: S0014-5793(00)01419-8

3. Results

Oxygen consumption by rat liver mitochondria under RAM-insensitive oxidation of external NADH in MSHG medium was extremely low (Fig. 1A, curve a). Addition of 4 mM Mg^{2+} almost did not stimulate the oxidation unless exogenous cytochrome *c* was also added, in accordance with Sottocasa et al. [1] and Marzulli et al. [7]. In HG medium, addition of 4 mM Mg^{2+} significantly stimulated IMET even in the absence of exogenous cytochrome *c* (Fig. 1A, curve b). Addition of 4 μM exogenous cytochrome *c* instead of 4 mM Mg^{2+} stimulated IMET nearly to the same level (data not included). Strong, but not additive activation of IMET was observed when 4 mM Mg^{2+} and 4 μM exogenous cytochrome *c* were present together. Strong activation of the Mg^{2+}–cytochrome *c*-dependent, RAM-insensitive oxidation of external NADH by mitochondria in hypotonic HG medium was also observed using the fluorimetric method (Fig. 1B), in good agreement with our previous data for the rotenone-insensitive NADH oxidation [4,5].

The stimulatory effect of Mg^{2+} on IMET (Fig. 1) seems to be caused by cytochrome *c* desorption from mitochondrial membranes, taking into account our data about IMET saturation under titration by mitochondria additions in the presence of exogenous cytochrome *c* in strongly hypotonic medium [9]. This saturation effect may be interpreted as cytochrome *c* adsorption to mitochondrial membranes. Indeed, mitochondria suspended in HG medium bound most of the added cytochrome *c* (1.5 μM initial concentration, Fig. 2A). Most of the portion of bound cytochrome *c* was liberated after addition of 4 mM Mg^{2+} in the medium. In experiments with 2 μM cytochrome *c* and 0.6 mg/ml of mitochondrial protein, 77 ± 2% ($n = 8$) of the total cytochrome *c* bound with mitochondrial pellet in HG medium without Mg^{2+}. On the other hand, only 21 ± 1% ($n = 8$) of the total cytochrome *c* was bound with the pellet in the presence of 4 mM Mg^{2+}. The binding capacity of mitochondria was lower in isotonic MSHG medium (Fig. 2B) which could be related to the fraction of mitochondria with the ruptured outer membrane.

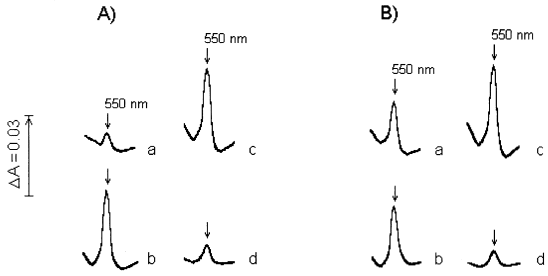

Fig. 2. Differential reduction–oxidation spectra of cytochrome *c* in the supernatant (a, c) and the pellet (b, d) of rat liver mitochondria initially suspended (0.6 mg mitochondrial protein/ml) in HG (A) or MSHG (B) media in the presence of 1.5×10^{-6} M cytochrome *c*, followed by addition of $MgCl_2$ to the final concentration 4×10^{-3} M $MgCl_2$ (c, d) or corresponding volume of the medium (a, b).

Mg^{2+}–cytochrome *c*-stimulated IMET was inhibited by cyanide (Fig. 1), indicating that electrons finely flow through cytochrome *c* oxidase of mitochondria in this process. Hence, inner membrane potential may be generated under IMET, as it was shown in [6]. In our experiments, the change in safranin O fluorescence intensity, related to membrane potential generated under Mg^{2+}–cytochrome *c*-dependent, RAM-insensitive oxidation of external NADH by mitochondria in the moderately hypotonic medium, was nearly the same as that induced by succinate oxidation in the absence of respiration inhibitors (Fig. 3A). It means that high potential was generated under external NADH oxidation in the moderately hypotonic medium, where the fraction of mitochondria with the ruptured outer membrane was expected to be higher than 80–90%, according to the tests of IMET [5] and adenylate kinase release [10]. In MSHG medium, the change in safranin O fluorescence intensity induced by Mg^{2+}–cytochrome *c*-dependent IMET in mitochondria was significantly lower than that in the moderately hypotonic medium (Fig. 3B). The amplitude of this change was comparable with that caused by addition of only 1 μM cytochrome *c* (Fig. 3A) or only 4 mM Mg^{2+}

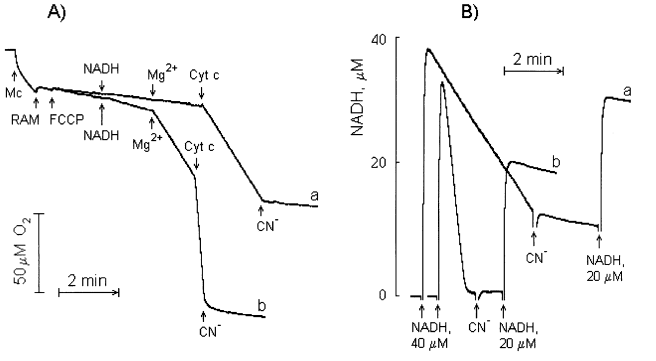

Fig. 1. Oxidation of external NADH by rat liver mitochondria in MSHG (a) and HG (b) media. (A) Oxygen consumption; (B) NADH fluorescence. Additions: (A) Mc, mitochondria, 2 mg mitochondrial protein/ml; RAM, 2.5×10^{-6} M rotenone, 10^{-6} M antimycin A, 10^{-6} M myxothiazol; FCCP, 10^{-6} M carbonyl-cyanide-*p*-trifluoromethoxy phenylhydrazone; NADH, 7.5×10^{-4} M NADH; Mg^{2+}, 4×10^{-3} M $MgCl_2$; Cyt c, 4×10^{-6} M cytochrome *c*; CN^-, 10^{-3} M KCN; (B) CN^-, 10^{-3} M KCN; initially mitochondria (0.3 mg mitochondrial protein/ml), 4×10^{-3} M $MgCl_2$, 10^{-6} M cytochrome *c*, RAM and 10^{-6} M FCCP were added in incubation medium.

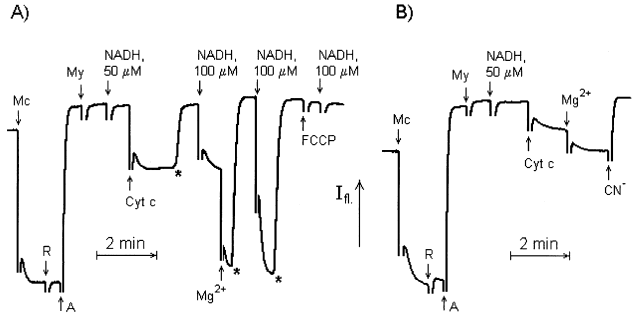

Fig. 3. Safranin O fluorescence intensity of rat liver mitochondria suspensions in moderately hypotonic medium composed of MSHG and HG media mixed in the v/v ratio of 15:85 (A) and in MSHG medium (B) supplemented with 2.5 mM succinate-Tris and 10 μM safranin O. Additions: Mc, mitochondria (0.6 mg mitochondrial protein/ml); R, 2.5×10^{-6} M rotenone; A, 10^{-6} M antimycin A; My, 10^{-6} M myxothiazol; Cyt c, 10^{-6} M cytochrome *c*; Mg^{2+}, 4×10^{-3} M $MgCl_2$; FCCP, 10^{-6} M carbonyl-cyanide-*p*-trifluoromethoxy phenylhydrazone; CN^-, 10^{-3} M KCN. *[NADH] = 0. The cell is open and the medium is stirred to be accessible for additional oxygen entrance.

(data not included) to mitochondria suspended in the moderately hypotonic medium. Generation of membrane potential in MSHG medium seems to be related to the fraction of mitochondria with the ruptured outer membrane.

4. Discussion

The external pathway of NADH oxidation has been postulated by Sottocasa et al. [1]. Endogenous cytochrome *c* shuttling in the intermembrane space of mitochondria has been considered as a possible mechanism of electron transport in this pathway [3,6,11–14], based on the assumption that cytochrome b_5, located in the outer membrane, may be oriented towards the mitochondrial intermembrane space [14]. If this is the case, than it is not clear why the rate of external NADH oxidation by intact mitochondria is extremely low, while the concentration of cytochrome *c* in the intermembrane space has been estimated to be about 0.7 mM, and the major portion of this protein is believed to be in the three-dimensionally diffusible state at physiological ionic strength ([2] and references therein). To explain this paradox, a 'bi-trans-membrane' electron transfer mechanism, with participation of extramitochondrial cytochrome *c*, was recently proposed ([7,15] and references therein). According to the authors, 'intact (but not damaged) mitochondria are able to promote the oxidation of exogenous cytochrome *c*' and 'catalytic amount of exogenous but not endogenous cytochrome *c*' (5 μM!) 'is required to promote the oxidation of external NADH' [7]. Our data contradict these conclusions, because oxidation of external NADH by rat liver mitochondria was observed even with endogenous cytochrome *c* in the presence of 4 mM Mg^{2+}, when the outer membrane was damaged (Fig. 1). Addition of 50 mM KCl instead of 4 mM $MgCl_2$ was needed to cause almost the same degree of the IMET stimulation (data not included), indicating that the effect of Mg^{2+} is more specific than simply the ionic strength increase. Low IMET observed in isotonic medium may be related to the fraction of mitochondria with the damaged outer membrane that is generally about 10% [16,17].

The test of mitochondria integrity proposed by Lofrumento et al. [15] indicated that only 1.0–1.5% of mitochondria used in the work of Marzulli et al. [7] were damaged, according to the authors. Unfortunately, the test did not allow to determine exactly the fraction of mitochondria with the damaged outer membrane, because it was based on measuring the ratio of the rate of external NADH oxidation by mitochondria in isotonic medium over that of the water-treated mitochondria (without rotenone and uncoupler addition). That means, denominator in the formula used by the authors included the rate of NADH oxidation through complex I of the respiratory chain in addition to the rate of the external pathway of NADH oxidation. Both types of oxidation have to increase due to the inner and the outer membrane damage in water-treated mitochondria. Such an approach does not seem to be adequate for estimation of the fraction of mitochondria with damaged outer membrane. In addition, according to their own measurements of succinate–cytochrome *c* reductase activity in control and water-treated mitochondria [15], the degree of outer membrane damage in control mitochondria may be estimated as 9%, which contradicts their 'integrity test' result 1.0–1.5%.

Hence, we may conclude that Mg^{2+}–cytochrome *c*-stimulated IMET depends on the outer membrane rupture and may serve as a simple test for evaluation of the outer membrane damage of rat liver mitochondria, using the protocol on Fig. 1, with 4–5 mM Mg^{2+} and only 4–5 μM exogenous cytochrome *c* instead of 50–100 μM reduced form of exogenous cytochrome *c* [16].

The stimulatory effect of Mg^{2+} on IMET may be explained by cytochrome *c* desorption from mitochondrial membranes in the presence of this ion, as it was also assumed by Bodrova et al. [6] and directly demonstrated in this work (Fig. 2). When the outer membrane is damaged and 4 mM Mg^{2+} is present, even endogenous desorbed cytochrome *c* may shuttle between the outer side of the ruptured outer membrane and the outer side of the inner membrane, thereby transporting electrons to the cytochrome *c* oxidase from cytochrome b_5. Cytochrome b_5, outside-oriented in the outer membrane [18], is in turn reduced by NADH through the outside-oriented NADH cytochrome b_5 reductase of the outer membrane [19]. Instead of the rupture, any permeabilization of the outer membrane, by proapoptotic protein Bax (see [20] for review) for example, seems to be able to stimulate IMET. It is important that IMET can generate inner membrane potential even in mitochondria with the ruptured outer membrane (Fig. 3), and so may be coupled to ATP synthesis.

Stimulatory effect of 4 mM Mg^{2+} on the cytochrome *c* release from mitochondria in the presence of Bax has been observed recently [21]. These experiments have been done in the low ionic strength medium. So, the obvious explanation of this finding is that Mg^{2+} ions caused cytochrome *c* desorption from mitochondrial membranes at low ionic strength, with the following release of cytochrome *c* through the Bax-permeabilized outer membrane. The degree of cytochrome *c* desorption under physiological ionic strength seems to be relatively high even without Mg^{2+}. Therefore, any mechanism that would make the outer membrane of mitochondria to be permeable for cytochrome *c* may activate IMET, switching on some not yet clarified redox-signaling mechanisms of apoptosis [22], in addition to the well-known activation of the caspases by cytochrome *c* released into the cytosol.

Acknowledgements: This study was supported by Grant # 1118-05-261-97 of Colciencias.

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