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Porin and cytochrome oxidase containing contact sites involved in the oxidation of cytosolic NADH

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Abstract

Cytochrome *c* (cyto-c) added to isolated mitochondria promotes the oxidation of extra-mitochondrial NADH and the reduction of molecular oxygen associated to the generation of an electrochemical membrane potential available for ATP synthesis. The electron transport pathway activated by exogenous cyto-c molecules is completely distinct from the one catalyzed by the respiratory chain. Dextran sulfate (500 kDa), known to interact with porin (the voltage-dependent anion channel), other than to inhibit the release of ATP synthesized inside the mitochondria, greatly decreases the activity of exogenous NADH/cyto-c system of intact mitochondria but has no effect on the reconstituted system made of mitoplasts and external membrane preparations. The results obtained are consistent with the existence of specific contact sites containing cytochrome oxidase and porin, as components of the inner and the outer membrane respectively, involved in the oxidation of cytosolic NADH. The proposal is put forward that the bi-*trans*-membrane electron transport chain activated by cytosolic cyto-c becomes, in physio-pathological conditions: (i) functional in removing the excess of cytosolic NADH; (ii) essential for cell survival in the presence of an impairment of the first three respiratory complexes; and (iii) an additional source of energy at the beginning of apoptosis.

Keywords: Mitochondria; Mitoplasts; Contact sites; Respiratory chain; Cytosolic NADH oxidation; Cytochrome *c*; Cytochrome oxidase; Porin (VDAC); Dextran sulfate; Ascorbic acid

A catalytic amount of cytochrome *c* (cyto-c),[1] added to rat liver mitochondria incubated in isotonic media, supports a cyanide-sensitive and a membrane potential ($\Delta\Psi_m$) generating oxygen uptake, given the presence of a source of external NADH [1–6]. This electron transport system, being insensitive to inhibitors of respiratory Complexes I–III, is independent from the activity of the respiratory chain even if both systems share cytochrome oxidase as common component for the reduction of molecular oxygen. Starting from 1991 [1] we continue to maintain that intact mitochondria oxidize both ferrocyto-c present in the intermembrane space, responsible for the oxidation of matrix substrates mediated by the respiratory chain and ferrocyto-c present outside the mitochondria for cytosolic substrates oxidation catalyzed by the above-mentioned NADH/cyto-c system.

Owing to the impermeability of the outer mitochondrial membrane to cyto-c, we have first hypothesized [1] and then obtained experimental data [7] that the electron

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[1] *Abbreviations used:* cyto-c, cytochrome *c*; cyto-b_5, cytochrome b_5; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide translocator; DS_{500}, dextran sulfate 500 kDa molecular weight; AP_5A, P^1,P^5-di(adenosine-5′)pentaphosphate; TMPD, *N*,*N*,*N*′,*N*′,-tetramethyl-*p*-phenylendiamine; FCCP, carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone; Adk, adenylate kinase; $\Delta\Psi_m$, mitochondrial membrane potential.

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transfer from cytosolic cyto-c to cytochrome oxidase, spanning the inner membrane of intact mitochondria, may take place at the level of contact sites, where the two mitochondrial membranes are juxtaposed [8]. Specific contact sites may constitute an appropriate solution where the bi-*trans*-membrane pathway, catalyzing the electron transfer from cytosolic cyto-c to molecular oxygen inside the mitochondria, can be localized. Some authors have sponsorized the simple hypothesis that ferrocyto-c, added to isolated mitochondria, is oxidized exclusively by those mitochondria which are completely broken or with a damaged external membrane [5,6,9]. The increased rate of exogenous NADH oxidation, observed inducing damage or an increased permeability of the external membrane, has been extensively discussed in [4]. Further support will be given that a direct correlation between the respiratory rate and the entity of induced permeability cannot be utilized to assume that the activity of NADH/cyto-c system is to be ascribed exclusively to the damaged mitochondria. However, the data presented to support the hypothesis of damaged mitochondria were either indirect [9] or obtained from experiments made in unphysiological highly hypo-osmotic incubation media [5,6], in both cases not corroborated by specific integrity tests. This hypothesis was emphasized by the finding that in hypotonic but not in an isotonic medium, the addition of magnesium ions promotes the oxidation of exogenous NADH even in the absence of added cyto-c [5,6,10]. We will discuss this aspect in more detail in a forthcoming paper concerning the role of magnesium ions on the binding of cyto-c.

Specific contact sites, shown to be involved in the transport of adenine nucleotides, are complex structures containing at least porin, namely the voltage-dependent anion channel (VDAC) spanning the outer membrane, the adenine nucleotide translocator (ANT) of the inner membrane and additional proteins like cyclophilin-D lining out the inner side of the inner membrane (for review see [11]). Contact sites have been proposed to be also involved in the activity of NADH/cyto-c system, stimulated by atractylate + ADP and inhibited by glycerol [7], two conditions reported to promote, respectively, the increase and the decrease of the frequency of contact sites [8,12]. Porin is a relatively abundant proteinaceous structure of external membrane, accounting for 5% of total mitochondrial proteins [13]. This makes likely that porin could be also one component of the above-mentioned contact sites, involved in the oxidation of cytosolic cyto-c and containing cytochrome oxidase as inner membrane component. To address this issue, we have utilized dextran sulfate (DS) that interacts with mitochondrial VDAC activity increasing the voltage dependence of the channels so that they become completely closed at transmembrane voltage at which would normally have been open [14]. Here, we show that this synthetic polyanion, at the concentration as low as 200 nM, strongly inhibits either the translocation of adenine nucleotides or the oxidation of extra-mitochondrial cyto-c by intact mitochondria, while having no effect on the oxidation of cyto-c catalyzed by mitoplasts, where the VDAC is supposed to be not present. The possibility, therefore, exists that porin, among other activities, may also intervene in the interaction of extra-mitochondrial cyto-c with cytochrome oxidase at the level of respiratory contact sites, thus making the oxidation of cytosolic NADH, by intact mitochondria, a finely controlled process competent in the energy conservation, building up an $\Delta\Psi_m$ [2–4].

Materials and methods

Materials

Horse-heart cytochrome *c*, NADH, NAD^+, EDTA, and trypsin from bovine pancreas (E.C. 3.4.21.4) were from Roche Biochemicals (Milan, Italy). Succinic acid, ascorbic acid, ATP, ADP, $NADP^+$, hexokinase (E.C. 2.7.1.1), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), alcohol dehydrogenase (E.C.1.1.1.1), P^1,P^5-di (adenosine-5′)pentaphosphate (AP_5A), *N*,*N*,*N*′,*N*′,-tetramethyl-*p*-phenylendiamine (TMPD), rotenone, myxothiazol, oligomycin, and carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone (FCCP) were from Sigma (St. Louis, MO). The dextran sulfate and dextrans used in this study were obtained from Sigma. Antimycin A and digitonin (high purity) were obtained from Calbiochem (La Jolla, CA). All other reagents were of the highest purity available in commerce.

Isolation and incubation of mitochondria

Rat liver mitochondria, mitoplasts, and the external mitochondrial membrane fraction were isolated in mannitol–sucrose medium as previously described [2]. Protein content was determined by the biuret method. Incubations were carried out at 25 °C in a standard sucrose medium at pH 7.4 consisting of 220 mM sucrose, 20 mM KCl, 1 mM EDTA, and 20 mM Hepes–KOH. Intactness of mitochondrial membranes was routinely determined by two different and already described integrity tests: (a) the oxidation of exogenously added NADH in the absence of rotenone [1,2]; (b) the insensitivity of intermembrane adenylate kinase (Adk) to proteolytic attack by added trypsin [3,4,15]. The oxidation of exogenous NADH by NADH-dehydrogenase of Complex I (accessible to its substrate only from matrix side of the inner membrane) requires that both outer and inner membranes should be permeable to NADH. Therefore, the comparison has been made between the rate of NADH oxidation by mitochondria preincubated for 1 min in the standard medium and the oxidation rate