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Discrimination between two steps in the mitochondrial permeability transition process

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Abstract

It is well known that a lag phase generally elapses between the addition of inducers of the mitochondrial permeability transition and the opening of the pore. To advance our present understanding as regards the significance of this phenomenon, we used experimental approaches which are sensitive to different aspects of the permeability transition process. The pore conformation was sensed by the fluorescence anisotropy changes of hematoporphyrin-labelled mitochondria. Membrane permeabilization was ascertained by following the matrix swelling consequent to external solute equilibration. We show that the anisotropy changes of mitochondria-bound hematoporphyrin precede both membrane depolarization (proton permeation) and matrix swelling (solute permeation), thus sensing a step of the permeability transition process that involves the pore in its closed state. We suggest that the opening of the pore is preceded by a structural remodelling of mitochondrial domains containing hematoporphyrin-near, pore-regulating histidines. Such a perturbation is strongly inhibited at acidic matrix pH and completely blocked by cyclosporin A. In sucrose-based media the opening of the pore can be strongly delayed, as compared to salt-based media, a fact which probably reflects perturbation of mitochondrial membranes by sugar. We conclude that the mitochondrial permeability transition could be described as an at least two-step process which is mainly regulated by conformational changes of the pore components.

Keywords: Mitochondria; Permeability transition; MPT pore; Lag time; Hematoporphyrin

Abbreviations: ANT, adenine nucleotide translocase; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; CsA, cyclosporin A; CypD, cyclophilin D; DEPC, diethyl pyrocarbonate; DIA, diamide; $\Delta\psi_m$, membrane potential; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraaceticacid; FCCP, carbonylcyanide-*p*-(trifluoromethoxy)phenylhydrazone; His, histidine; HP, hematoporphyrin IX; Mops, 4-morpholinepropanesulfonic acid; MPT, mitochondrial permeability transition; NH_2OH, hydroxylamine; PhAsO, phenylarsine oxide; PBR, (peripheral-type) benzodiazepine receptor; PyrG, pyronin G; r, fluorescence anisotropy; VDAC, voltage-dependent anion channel

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1. Introduction

In isolated mitochondria, excessive Ca^{2+} accumulation induces matrix swelling and Ca^{2+} release. According to a widely accepted hypothesis, both phenomena are mediated by the opening of the mitochondrial permeability transition (MPT) pore; this voltage-dependent, cyclosporin A-sensitive, high-conductance, inner membrane proteinaceous channel allows diffusion of solutes with molecular mass up to ~1500 Da (Zoratti & Szabò, 1995). Different events occur during the MPT, including mitochondrial uncoupling, collapse of membrane potential ($\Delta\psi_m$), matrix swelling, Ca^{2+} efflux and loss of matrix components (Bernardi, Scorrano, Colonna, Petronilli, & Di Lisa, 1999; Gunter & Pfeiffer, 1990; Halestrap, McStay, & Clarke, 2002; Zoratti & Szabò, 1995), which were recently assumed to play an important pathogenic role in both necrotic and apoptotic cell death (Bernardi, 1999; Bernardi et al., 1999; Halestrap et al., 2002; Lemasters et al., 1998).

Although it is generally accepted that the MPT pore is a supramolecular structure involving (or regulated by) more than one protein, its molecular nature is still little understood. A current model suggests that MPT pores form at contact sites between the inner and outer membranes by association of the matrix cyclophilin D (CypD), the inner membrane adenine nucleotide translocase (ANT), the outer membrane voltage-dependent anion channel (VDAC), and possibly other proteins (Crompton, Virji, & Ward, 1998; Halestrap et al., 2002; Woodfield, Ruck, Brdiczka, & Halestrap, 1998). The mechanism through which normal components of mitochondria participate in the pore assembly to form a non-selective Ca^{2+}-activated pore is also being debated. According to some authors, the pore complex probably pre-exists as a permanent entity under normal physiological conditions, and Ca^{2+} activates an allosteric action allowing the pore to flicker into an open state (Crompton, 1999). Other authors suggest that the MPT is rather a consequence of the assembly of individual membrane proteins following conformational modifications (Kowaltowski, Castilho, & Vercesi, 2001).

Little attention has been focused on the lag time that precedes the development of permeabilization after Ca^{2+} accumulation. The lag time could reflect the time needed for assembly of the MPT pore from components, or for development of a larger pore from a smaller one. Alternatively, it could be correlated with some processes of membrane reorganization, e.g., contact site formation (see, for a review, Zoratti & Szabò, 1995).

The investigations described in this paper are aimed at getting further information on the causes which delay the opening of the pore, by using experimental approaches which follow different, pore-related phenomena. Thus, the pore structure was monitored by the fluorescence anisotropy changes of hematoporphyrin (HP)-labelled mitochondria which were shown to accompany the MPT (Ricchelli, Gobbo, Moreno, & Salet, 1999). These changes reflect modifications in the HP microenvironment which are linked to conformational variations of the pore, probably as a consequence of a specific localization of the dye in protein sites of the inner mitochondrial membrane participating in pore formation or regulation (Ricchelli et al., 1999, 2003). The matrix swelling as an index of membrane permeabilization was followed by the decrease in light scattering intensity at 540 nm. We show that the opening of the pore is a multistep process regulated by conformational changes of the protein constituents, which are activated by the MPT-inducer and depend on the medium components.

2. Materials and methods

2.1. Preparation of mitochondria

Liver mitochondria from Wistar rats were prepared by standard differential centrifugation (Salet, Moreno, & Vinzens, 1982). The final pellet was suspended in 0.25 M sucrose to give a protein concentration of 80–100 mg/ml, as measured by the biuret method. The functionality of mitochondria was established by measuring the value of the respiratory control ratio (RCR), that is, the ratio between the rate of oxygen consumption in state 3 (in the presence of 0.3 mM ADP) and in state 4 (in the absence of ADP) in a thermostated ($T = 25\,^{\circ}C$), water-jacketed vessel, using a Clark electrode connected to a recorder. The incubation buffer contained 10 mM Tris–Mops, 100 mM sucrose, 2 mM $MgCl_2$, 50 mM KCl, 10 mM KH_2P0_4, 1 mM EDTA, 2 μM rotenone, pH 7.4. succi-