The Irreversibility of Inner Mitochondrial Membrane Permeabilization by Ca\(^{2+}\) plus Prooxidants Is Determined by the Extent of Membrane Protein Thiol Cross-linking

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INTRODUCTION

Inner mitochondrial membrane permeabilization caused by Ca\(^{2+}\) is generally referred to as mitochondrial membrane permeability transition and is assumed to be associated with the opening of a Ca\(^{2+}\)-induced pore, the mitochondrial membrane permeability transition pore (MTP) (for reviews, see Gunter and Pfeiffer, 1990; Bernardi et al., 1994; Gunter et al., 1994; Zoratti and Szabó, 1995). This Ca\(^{2+}\) effect is potentiated by various agents or conditions, such as inorganic phosphate, thiol, or pyridine nucleotide oxidants, low membrane potential, and oxidative stress (for reviews, see Gunter and Pfeiffer, 1990; Vercesi, 1993; Zoratti and Szabó, 1995).

It is known that mitochondrial membrane potential (ΔΨ) drop due to MTP opening is reversed by pyridine nucleotide reductants (Vercesi, 1984), ethylene glycol-bis(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA) (Al-Nasser and Crompton, 1986; 1990; Bernardi et al., 1994; Gunter et al., 1994; Zoratti and Szabó, 1995). This Ca\(^{2+}\) effect is potentiated by various agents or conditions, such as inorganic phosphate, thiol, or pyridine nucleotide oxidants, low membrane potential, and oxidative stress (for reviews, see Gunter and Pfeiffer, 1990; Vercesi, 1993; Zoratti and Szabó, 1995).

We have previously shown that mitochondrial membrane potential (ΔΨ) drop promoted by prooxidants and Ca\(^{2+}\) can be reversed but not sustained by ethylene glycol-bis(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA) unless dithiothreitol (DTT), a disulfide reductant, is also added [Valle, V. G. R., Fagian, M. M., Parentoni, L. S., Meinicke, A. R., and Vercesi, A. E. (1993). Arch. Biochem. Biophys. 307, 1-7]. In this study we show that catalase or ADP are also able to potentiate this EGTA effect. When EGTA is added long after (12 min) the completion of swelling or ΔΨ elimination, no membrane resealing occurs unless the EGTA addition was preceded by the inclusion of DTT, ADP, or catalase soon after ΔΨ was collapsed. Total ΔΨ recovery by EGTA is obtained only in the presence of ADP. The sensitivity of the ADP effect to carboxyatractyloside strongly supports the involvement of the ADP/ATP carrier in this mechanism. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of solubilized membrane proteins shows that protein aggregation due to thiol cross-linkage formed during ΔΨ drop continues even after ΔΨ is already eliminated. Titration with 5,5'-dithio-bis(2-nitrobenzoic acid) supports the data indicating that the formation of protein aggregates is paralleled by a decrease in the content of membrane protein thiols. Since the presence of ADP and EGTA prevents the progress of protein aggregation, we conclude that this process is responsible for both increased permeability to larger molecules and the irreversibility of ΔΨ drop. The protective effect of catalase suggests that the continuous production of protein thiol cross-linking is mediated by mitochondrial generated reactive oxygen species.

KEY WORDS: Calcium; cyclosporin A; mitochondria; mitochondrial permeability transition pore; protein oxidation; reactive oxygen species.
Crompton et al., 1987; Valle et al., 1993; Bernardes et al., 1994; Vercesi et al., 1993), by the reductant dithiothreitol (DTT) (Valle et al., 1993), or by the immune suppressor cyclosporin A in the presence of Mg$^{2+}$ or ADP (Novgorodov et al., 1994). Due to these characteristics, the MTP has been proposed to be a regulated channel, compatible with the maintenance of mitochondrial integrity and possibly related to specific functions not yet elucidated (for reviews, see Bernardi et al., 1994 and Gunter et al., 1994). However, results from our laboratory (Valle et al., 1993; Castilho et al., 1995a, b; Kowaltowski et al., 1995, 1996a) indicate that MTP opening is the result of membrane damage due to the oxidative attack of reactive oxygen species (ROS) to membrane protein thiols. This causes thiol cross-linkage and high-molecular-weight protein aggregate production (Fagian et al., 1990; Valle et al., 1993; Castilho et al., 1995a).

It has been shown that MTP opening takes place during various pathological states such as ischemia/reperfusion or prolonged hypoxia (Nazareth et al., 1991; Pastorino et al., 1993) and may be an important event in the mechanism of cell death. The aim of this work was to establish conditions that permit mitochondrial recovery from the state of membrane permeabilization and detect alterations that may lead to irreversible mitochondrial injury.

**MATERIALS AND METHODS**

**Isolation of Rat Liver Mitochondria**

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar strain rats fasted overnight.

**Standard Incubation Procedure**

The experiments were carried out at 30°C in a standard reaction medium containing 130 mM KCl, 10 mM Hepes (2-hydroxyethyl-1-piperazine ethane-sulfonic acid) buffer, pH 7.2, 2 mM succinate, 10 μM Ca$^{2+}$, and 4 μM rotenone. Other additions are indicated in the figure legends. The results shown are representative of a series of at least three experiments.

**Determination of Mitochondrial Swelling**

Mitochondrial swelling was estimated from the decrease in the absorbance at 520 nm measured in an SLM Aminco DW2000 spectrophotometer.

**Measurements of Mitochondrial Transmembrane Electrical Potential (ΔΨ)**

Mitochondria were incubated in standard reaction medium containing 3 μM tetrphenylphosphonium (TPP$^+$). The concentration of TPP$^+$ in the extramitochondrial medium was continuously monitored with a TPP$^+$-selective electrode prepared in our laboratory according to Kamo et al. (1979). The membrane potential was then calculated assuming that the TPP$^+$ distribution between mitochondria and medium follows the Nernst equation (Muratsugu et al., 1977). Corrections due to the binding of TPP$^+$ to the mitochondrial membranes were made according to Jensen et al. (1986). No corrections were made to compensate ΔΨ values for the continuous alteration in mitochondrial volume that occurs during the experiments.

**Sodium Dodecyl Sulfate-Polyacrylamide Slab Gel Electrophoresis (SDS-PAGE)**

Aliquots of mitochondria were taken and the matrix proteins were released by three subsequent freeze–thawing procedures. Electrophoresis of the solubilized membrane proteins was performed according to Laemmli (1970) and as described by Fagian et al. (1990).

**Determination of Protein Thiol Groups Content**

The mitochondrial suspension incubated in standard reaction medium was submitted to three subsequent freeze–thawing procedures to release matrix proteins and centrifuged 2 min at 10,000 rpm. The pellet was treated twice with 200 μl of 6.5% trichloroacetic acid and centrifuged at 10,000 rpm during 2 min in order to precipitate the protein. The final pellet was resuspended in 1 ml of medium containing 100 μM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 0.5 mM EGTA, and 0.5 M Tris, pH 8.3. Absorption was measured at 412 nm, using cysteine for calibration.