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Impact of adenosine nucleotide translocase (ANT) proline isomerization on Ca^{2+}-induced cysteine relative mobility/mitochondrial permeability transition pore

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Abstract Mitochondrial membrane carriers containing proline and cysteine, such as adenine nucleotide translocase (ANT), are potential targets of cyclophilin D (CyP-D) and potential Ca^{2+}-induced permeability transition pore (PTP) components or regulators; CyP-D, a mitochondrial peptidyl-prolyl *cis-trans* isomerase, is the probable target of the PTP inhibitor cyclosporine A (CsA). In the present study, the impact of proline isomerization (from *trans* to *cis*) on the mitochondrial membrane carriers containing proline and cysteine was addressed using ANT as model. For this purpose, two different approaches were used: (*i*) Molecular dynamic (MD) analysis of ANT-Cys^{56} relative mobility and (*ii*) light scattering techniques employing rat liver isolated mitochondria to assess both Ca^{2+}-induced ANT conformational change and mitochondrial swelling. ANT-Pro^{61} isomerization increased ANT-Cys^{56} relative mobility and, moreover, desensitized ANT to the prevention of this effect by ADP. In addition, Ca^{2+} induced ANT "c" conformation and opened PTP; while the first effect was fully inhibited, the second was only attenuated by CsA or ADP. Atractyloside (ATR), in turn, stabilized Ca^{2+}-induced ANT "c" conformation, rendering the ANT conformational change and PTP opening less sensitive to the inhibition by CsA or ADP. These results suggest that Ca^{2+} induces the ANT "c" conformation, apparently associated with PTP opening, but requires the CyP-D peptidyl-prolyl *cis-trans* isomerase activity for sustaining both effects.

Keywords Adenine nucleotide translocase · Mitochondrial carriers · Cyclophilin D · Calcium · Permeability transition pore · Molecular dynamic analysis

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Introduction

Mitochondria are important coordinators of apoptotic cell death signaling. The increase of mitochondrial Ca^{2+} levels, observed in many pathological situations, opens the mitochondrial permeability transition pore (PTP) followed by impairment of oxidative phosphorylation (Hajnoczky et al. 2006; Grimm and Brdiczka 2007). In a previous study we have demonstrated, by means of molecular dynamic (MD) analysis, that Ca^{2+} interacts with ANT-surrounding cardiolipins (CDL) (bound to ANT H4 helix) and weakens CDL/ANT associations. In the ANT "c" conformation, Ca^{2+} interaction destabilizes the initial ANT-Cys^{56} residue resulting in the increase of its relative mobility, with potential critical involvement in Ca^{2+}-induced PTP opening. The binding of ADP, in turn, stabilizes the ANT "m" conformation and prevents increase of ANT-Cys^{56} relative mobility (Pestana et al. 2009).

ANT catalyzes the reversible exchange of ADP for ATP across the inner mitochondrial membrane, accounting for approximately 10% of the protein content of this membrane (Fiore et al. 1998). Besides its physiological role in the transport of adenine nucleotides, ANT, which shares a high

homology with other mitochondrial membrane carriers, is a probable component or regulator of PTP (Kokoszka et al. 2004; Leung et al. 2008). Indeed, PTP opening may be (*i*) stimulated by atractyloside (ATR) and carboxyatractyloside (CAT), which stabilize the "c" conformation of ANT and (*ii*) inhibited by bongkrekic acid, which stabilizes the "m" conformation (Klingenberg 1989, 2008). In addition, the ability of nucleotides to inhibit PTP opening correlates with their role as ANT substrates (Gizatullina et al. 2005).

The peptidyl-prolyl *cis-trans* isomerase activity of CyP-D is believed to be involved in Ca^{2+}-induced PTP opening by promoting conformational change in mitochondrial membrane proteins (Halestrap and Davidson 1990); the classical PTP opening inhibitor, CsA, prevents this action (Crompton et al. 1988; Broekemeier et al. 1989). ANT (Halestrap and Brenner 2003; Li et al. 2004) and/or the mitochondrial phosphate carrier (PiC) (Leung et al. 2008) are probable targets of CyP-D, whose binding to the inner mitochondrial membrane occurs in parallel with PTP opening. ANT-Pro^{61} residue, in turn, is the probable direct CyP-D target in ANT. It is located in the loop 1 facing the mitochondrial matrix and is broadly conserved in the ANT isoforms of mammals, whose mitochondria undergo CsA-sensitive PTP opening, but is not present in the isoforms of yeast, in which every observed PTP opening is CsA-insensitive (Jung et al. 1997; Manon et al. 1998).

In the present study, the impact of proline isomerization (from *trans* to *cis*) on the mitochondrial membrane carriers containing proline and cysteine was addressed using ANT as a model. For this purpose, two different approaches were used: (*i*) MD analysis of ANT-Cys^{56} relative mobility, in the absence or presence of Ca^{2+} and ADP, and (*ii*) light scattering techniques employing rat liver isolated mitochondria to assess both Ca^{2+}-induced ANT conformational change and mitochondrial swelling, in the absence or presence of ATR, CsA and ADP.

Materials and methods

Molecular modeling procedures—molecular dynamic (MD) analysis

Trajectories resultant from MD analysis were performed in order to predict ANT-Cys^{56} relative mobility behavior applying the structure of ANT in complex with CDL, retrieved from the Protein Data Bank (PDB entry: 1OKC), after removal of all the ligands (Pebay-Peroula et al. 2003). MD analysis was carried out using the Discover module of the Insight II software, with the Consistent Valence Force Field (CVFF). ANT-Pro^{61} isomerization (*trans* to *cis*) was modeled in order to mimic the proposed CyP-D isomerase activity on ANT, followed by its combination with our previous reported Ca^{2+} placement bound to CDL (Pestana et al. 2009). Before MD analysis, hydrogen atoms were added and oriented in ANT, and the energy of the complex was minimized using 1500 steps of a combined Steepest-Descent/Conjugate Gradient algorithm protocol. The systems were energy-minimized until the maximum derivative was lower than 0.001 kcal/mol/Å. Implicit solvent condition with a dielectric constant of 80 (water) was employed. ADP molecule was further positioned in the ANT active site, and long trajectories (1000–1500 ps) MD analysis were performed with the models, at a constant temperature of 298 K and equilibration phase of 80 ps. A cut-off radius of 12 Å for both non-bonded electrostatic and van der Waals interactions was employed. Atomic charges for the receptor atoms were obtained using the CVFF force field. The coordinates of the system were saved every 15 ps for further analysis. From the molecular trajectories generated by the MD (four simulations), we analyzed the root mean square deviation (RMSD) of ANT-Cys^{56} atoms, as well as the total energy for (*i*) ANT-Pro^{61} *trans*, (*ii*) ANT-Pro^{61} *cis* and (*iii*) ANT-Pro^{61} *cis* plus Ca^{2+} in the absence or presence of ADP complexes as a time function.

Isolation of rat liver mitochondria

Mitochondria were isolated by standard differential centrifugation (Pedersen et al. 1978). Male Wistar rats (200 g) were sacrificed by decapitation; livers (10–15 *g*) were immediately removed, sliced in a medium (50 ml) consisting of 250 mM sucrose, 1 mM EGTA and 10 mM HEPESKOH, pH 7.2, and homogenized three times for 15 s at 1 min intervals with a Potter-Elvehjem homogenizer. Homogenates were centrifuged (580 *g*, 5 min) and the resulting supernatant was further centrifuged (10,300 *g*, 10 min). Pellets were then washed in a medium (30 ml) consisting of 250 mM sucrose, 0.3 mM EGTA and 10 mM HEPES-KOH, pH 7.2, and centrifuged (3,400 *g*, 15 min). The final mitochondrial pellet was suspended in a medium (1 ml) consisting of 250 mM sucrose and 10 mM HEPES-KOH, pH 7.2, and used within 3 h. Mitochondrial protein content was determined by the biuret reaction. The isolated mitochondria, energized with succinate (in the presence of rotenone), presented respiratory control ratio (RCR) and ADP/O in the 4.5–5.5 and 1.3–1.7 ranges, respectively.

ANT conformational change in isolated mitochondria

Conformational change of ANT (from "m" to "c") was assessed as previously described (Das et al. 2003; Leung et al. 2008). Mitochondria (5 mg protein/ml, final concentration) were incubated (30 °C) in a medium consisting of 125 mM KCl, 20 mM MOPS, 10 mM TRIS, 0.5 mM EGTA, 5 mM L-glutamate, 2 mM L-malate and 7.5 μM oligomycin,

pH 7.2. Light scattering was monitored at 520 nm after the addition of Ca^{2+} and/or ATR, in the absence or presence of ADP and CsA, in a Cary 50-BIO Varian spectrophotometer.

Swelling of isolated mitochondria

Mitochondria (0.5 mg protein/2 ml, final concentration) were incubated (30 °C) in a medium consisting of 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, 5 mM succinate, 1 μM rotenone, pH 7.4. Light scattering was monitored at 540 nm after the addition of Ca^{2+} and/or ATR, in the absence or presence of ADP and CsA, in a MultiSpec-1501 Shimadzu spectrophotometer.

Results

The impact of ANT-Pro^{61} isomerization on the relative mobility of ANT-Cys^{56} was assessed by MD analysis, both in the absence and presence of Ca^{2+} and ADP. RMSD intensities of ANT-Cys^{56} indicated an increase of its relative mobility after *trans* to *cis* ANT-Pro^{61} isomerization (Fig. 1). In addition, this isomerization intensified the relative mobility increase of ANT-Cys^{56} promoted by Ca^{2+} (Fig. 2).

We had previously predicted the potential phosphate binding sites of ANT using the GRID software, followed by a flexible docking procedure carried out for ADP, inside the ANT active site. This was performed to select the highest scored orientation (GoldScore of 67.7) of ADP, which showed the pyrophosphate moiety in phase with the phosphate-binding site predicted by GRID Molecular Interaction Fields (MIF) (Pestana et al. 2009). In the present study, we have not observed restoration of the ANT-Cys^{56} relative mobility after ANT-Pro^{61} isomerization in the presence of ADP (Fig. 3). The results obtained from MIF studies suggest Arg^{234} and Arg^{79} as the most energetically favorable and important ANT residues which bind the pyrophosphate moiety of ADP. Accordingly, R96H mutation in the *Saccharomyces cerevisiae* carrier drastically diminishes ADP binding affinity, whose corresponding residue in the bovine structure is Arg^{79} (Jung et al. 1997). MIF were energy contoured for the phosphate probe at −19.0 kcal/mol and the main contribution was due to Arg^{79}. These MD results suggest that, similarly to Ca^{2+}, ANT-Pro^{61} isomerization increases ANT-Cys^{56} relative mobility and, moreover, desensitizes ANT to the prevention of this effect by ADP.

Next, we assessed (*i*) the conformational changes of ANT induced by 0.5 mM Ca^{2+}, which in the presence of 0.5 mM EGTA yields ~1 μM free Ca^{2+} concentration (Leung et al. 2008), in glutamate/malate-energized rat liver isolated mitochondria and (*ii*) the mitochondrial swelling

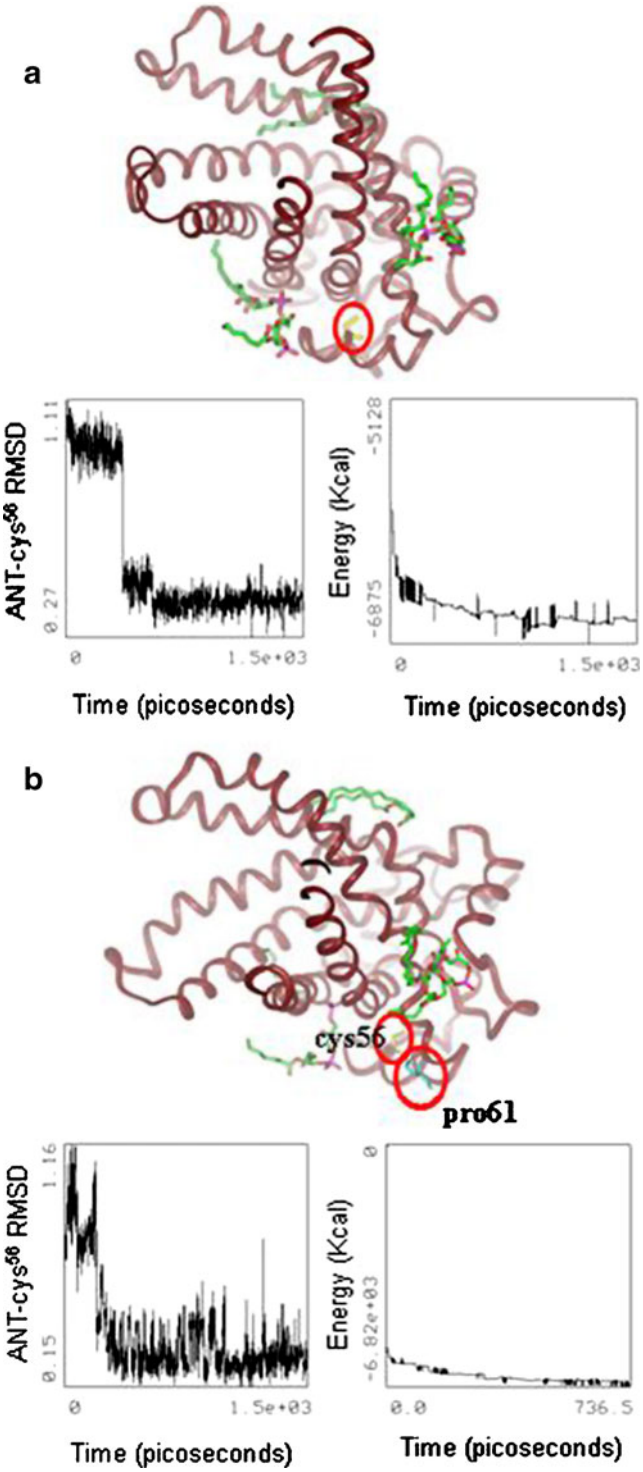

Fig. 1 ANT-Pro^{61} isomerization (*trans* to *cis*) results in the increase of ANT-Cys^{56} root mean square deviation (RMSD), assessed by MD analysis. Left chart: plots of RMSD (in angstroms) *vs.* time (in picoseconds) of the ANT-Cys^{56} residue (highlighted in yellow in the ribbon diagram), and right chart: energy (Kcal) *vs.* time (in picoseconds), of ANT-Pro^{61} isomers (**a**) *trans* and (**b**) *cis* (highlighted in blue)

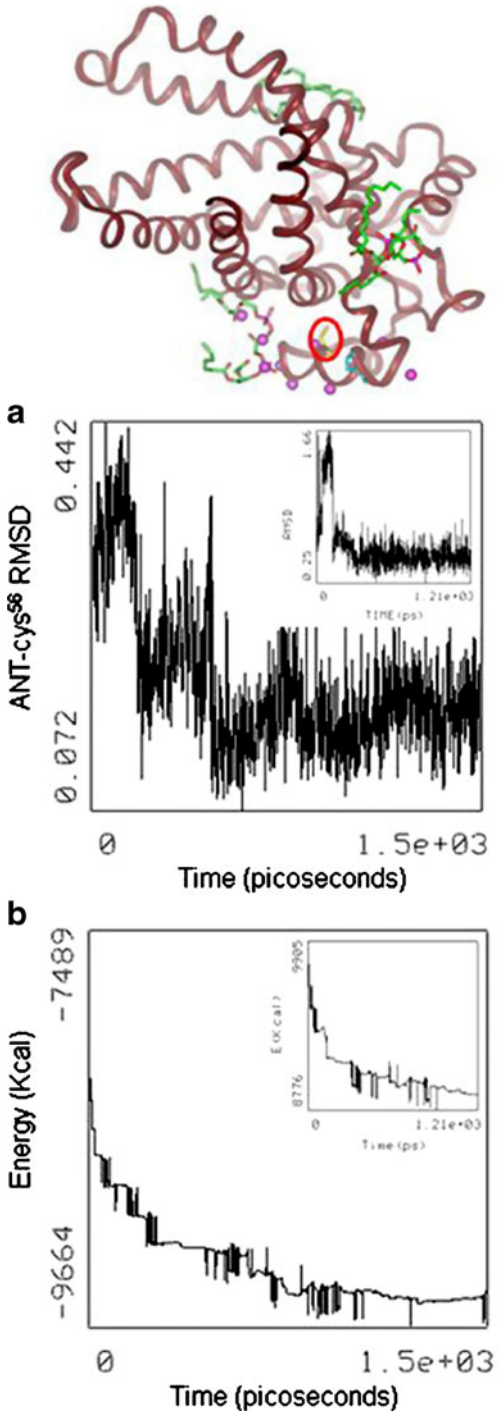

Fig. 2 ANT-Pro^{61} isomerization and ANT-Cys^{56} root mean square deviation (RMSD) promoted by Ca^{2+}, assessed by MD analysis. **a** Plots of RMSD (in angstroms) *vs.* time (in picoseconds) of the ANT-Cys^{56} residue (in yellow, in the ribbon diagram), and **b** energy *vs.* time (in picoseconds), of ANT-Pro^{61} *cis* isomer + 10 Ca^{2+} ions (spheres in magenta) bound to ANT-CDL (colored by atom) compared to ANT-Pro^{61} *trans* isomer + 10 Ca^{2+} ions (inserts)

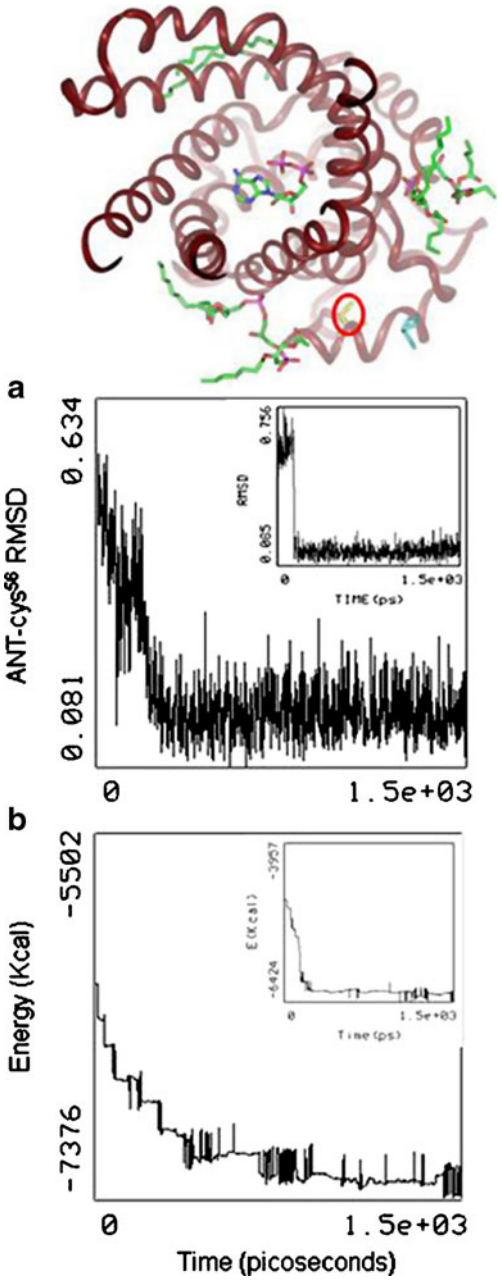

Fig. 3 ANT-Pro^{61} isomerization and ADP effect on ANT-Cys^{56} root mean square deviation (RMSD), assessed by MD analysis. ADP placement inside ANT was performed according to flexible docking results obtained for ADP (colored by atom, in the center of the ribbon diagram) followed by (**a**) plots of RMSD (in angstroms) *vs.* time (in picoseconds) of the ANT-Cys^{56} residue (in yellow), and (**b**) energy *vs.* time (in picoseconds), of ANT-Pro^{61} *cis* isomer compared to ANT-Pro^{61} *trans* isomer (inserts)

induced by 0.5 mM Ca^{2+} (without EGTA) in succinate-energized mitochondria; both assays were performed in the absence or presence of ATR, CsA and ADP. Ca^{2+} decreased the apparent absorbance at 520 nm of the mitochondrial suspension (Fig. 4b compared with a) in a way that such light-scattering is not attributed to mitochondrial swelling,

but rather to the ANT change from "m" to "c" conformation (Das et al. 2003); indeed, although detection of ANT conformational change relies on decrease in the light scattering (A_{520}), the increase in matrix volume measured isotopically is either negligible (Das et al. 2003) or absent (Klingenberg 1980). Even with a short delay, CsA or ADP fully reversed this effect (Figs. 4c and d, respectively). ATR (10 μM) did not change ANT conformation (Fig. 4e) unless 0.5 mM Ca^{2+} concentration was present; moreover, it stabilized ANT "c" conformation induced by Ca^{2+} (Fig. 4f), resulting in a lower inhibition by CsA or ADP (Fig. 4g and h, respectively). The effects of ATR, CsA and ADP on Ca^{2+}-induced swelling in succinate-energized mitochondria were close similar to those on ANT conformational change (Fig. 5).

Discussion

An extensive literature on PTP points to a relationship between Ca^{2+} and ANT, either *via* interaction of the cation with ANT surrounding cardiolipins (Hoffmann et al. 1994; Beyer and Nuscher 1996; Nury et al. 2005) or *via* changes in ANT conformation and reactivity status of cysteine residues (Klingenberg 1989; Costantini et al. 2000; Mcstay et al. 2002). ANT has for the long time been considered the probable target of CyP-D on the mitochondrial membrane, with implications in PTP opening whereas ANT-Pro^{61} isomerization has been pointed as the probable mechanism involved (Woodfield et al. 1998; Griffiths and Halestrap 1991). ANT-Cys^{56} oxidation by Ca^{2+}-induced ROS generated in mitochondria, followed by thiol cross-linking involving

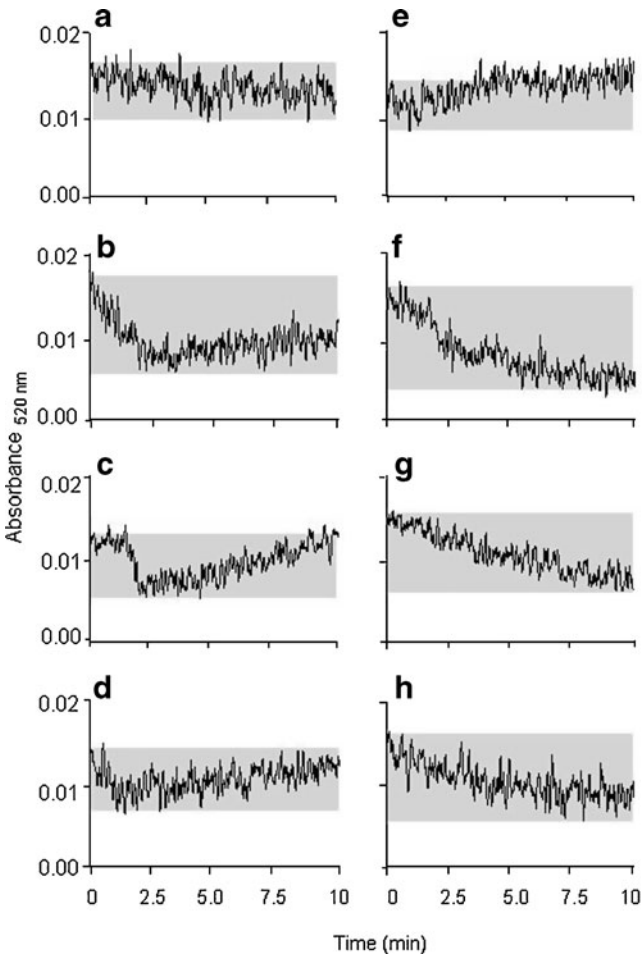

Fig. 4 Ca^{2+}-induced conformational change (from "m" to "c") of ANT in rat liver isolated mitochondria, assessed as described in Materials and methods. Mitochondria (5 mg protein/ml, final concentration) were incubated (30°C) in a medium consisting of 125 mM KCl, 20 mM MOPS, 10 mM TRIS, 0.5 mM EGTA, 5 mM L-glutamate, 2 mM L-malate and oligomycin, pH 7.2 and light-scattering was monitored at 520 nm. **a** None (control); **b** 500 μM Ca^{2+}; **c** 500 μM Ca^{2+} + 5 μM CsA; **d** 500 μM Ca^{2+} + 500 μM ADP; **e** 10 μM ATR; **f** 10 μM ATR+500 μM Ca^{2+}; **g** 10 μM ATR+500 μM Ca^{2+} + 5 μM CsA; **h**) 10 μM ATR+500 μM Ca^{2+} + 500 μM ADP

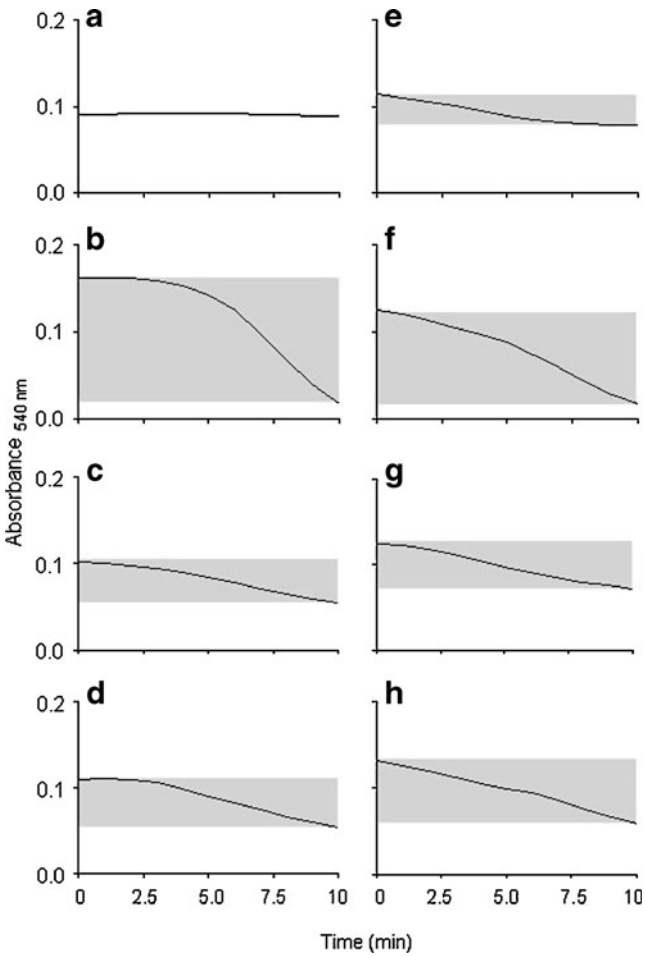

Fig. 5 Ca^{2+}-induced swelling of rat liver isolated mitochondria, assessed as described in Materials and methods. Mitochondria (0.5 mg of protein/2 ml) were incubated (30°C) in a standard medium consisting of 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, 5 mM succinate, 1 μM rotenone, pH 7.4 and light-scattering was monitored at 540 nm. **a** None (control); **b** 100 μM Ca^{2+}; **c** 100 μM Ca^{2+} + 1 μM CsA; **d** 100 μM Ca^{2+} + 100 μM ADP; **e** 10 μM ATR; **f** 10 μM ATR + 10 μM Ca^{2+}; **g** 10 μM ATR + 10 μM Ca^{2+} + 1 μM CsA; **h** 10 μM ATR + 10 μM Ca^{2+} + 100 μM ADP

other cysteine residues from ANT itself or from other mitochondrial membrane carriers, have also been considered to be implicated (Hashimoto et al. 1999; Mcstay et al. 2002). However, a CyP-D-facilitated/Ca^{2+}-induced conformational change of PiC has recently gained evidence as a potential PTP opening mechanism, either *per se* or in association with ANT "c" conformation as a regulatory unit (Leung et al. 2008).

We have previously demonstrated by MD analysis an association between Ca^{2+}-induced increase of ANT-Cys^{56} relative mobility and Ca^{2+}-induced PTP opening (Pestana et al. 2009). Here, MD analysis was performed in order to mimic the postulated CyP-D action on ANT/PTP opening (Crompton et al. 1988; Broekemeier et al. 1989; Halestrap and Davidson 1990; Griffiths and Halestrap 1991), showing that the *trans* to *cis* ANT-Pro^{61} isomerization, *per se,* increases ANT-Cys^{56} relative mobility. However, in contrast to Ca^{2+}, this effect was insensitive to the inhibition by ADP. ANT conformational change and mitochondrial swelling analyses were performed in the same context, demonstrating that Ca^{2+} (*i*) induces ANT "c" conformation and (*ii*) opens PTP; while the first effect was fully inhibited, the second was only attenuated by CsA or ADP. ATR, in turn, stabilized Ca^{2+}-induced ANT "c" conformation, rendering the ANT conformational change and PTP opening less sensitive to the inhibition by CsA or ADP.

It should be emphasized that this ANT-based study provides a suitable model for any other mitochondrial membrane carrier which contains critical proline and cysteine residues and might be potentially involved in PTP opening mechanism. ANT was selected due to its importance in the context of the PTP literature, abundance in the inner mitochondrial membrane, and because it is the unique potential PTP component/regulator with solved crystallographic structure in the PDB. ANT-Cys^{56} was selected due to its proximity to cardiolipin, which enables dimerization of adjacent ANTs, itself or associated with Cys^{159}/Cys^{256} intramolecular cross-linking (Mcstay et al. 2002). In this regard, an expressive literature reports that ANT-Cys^{56} is involved in PTP opening (Beyer and Nuscher 1996; Hashimoto et al. 1999; Costantini et al. 2000).

Remarkably, the ADP-insensitive increase of ANT-Cys^{56} relative mobility due to ANT-Pro^{61} isomerization suggests that the transport of adenine nucleotides in mitochondria may occur simultaneously to PTP opening, or even that other mitochondrial proline-containing carriers are also involved. In fact, an ANT-independent role of ADP has been proposed based on the observation that PTP inhibition by ADP persists even in the presence of CAT, which prevents ANT binding to immobilized phenylarsine oxide; therefore, the inhibition by ADP may occur, at least in part, independently of its binding to ANT (Leung et al. 2008).

Our results suggest that Ca^{2+} induces ANT "c" conformation, apparently associated with PTP opening, but requires the CyP-D peptidyl-prolyl *cis-trans* isomerase activity for sustaining both effects. Additionally, they suggest that, *in vivo*, this isomerase activity may enable the increase of ANT-Cys^{56} relative mobility and PTP opening, even in the presence of the PTP inhibitor, ADP. In this case, it is possible that PTP opens even when part of ANT works as a carrier. Accordingly, a full PTP inhibition by ADP, *in vitro*, was achieved only in the presence of CsA (data not shown). Therefore, these findings may also open perspectives for understanding why in some conditions PTP opening neither requires Ca^{2+} nor is CsA-sensitive (Baines et al. 2005; Basso et al. 2005; Jung et al. 1997; Manon et al. 1998). It is possible that under specific conditions, such as the ATR action on ANT, a high CyP-D isomerase activity is sufficient to induce and stabilize the ANT "c" conformation or that the Ca^{2+}-induced "c" conformation is sufficiently stable to open PTP.

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