Biochimica et Biophysica Acta 1787 (2009) 176–182

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Ca^{2+} binding to c-state of adenine nucleotide translocase (ANT)-surrounding cardiolipins enhances (ANT)-Cys^{56} relative mobility: A computational-based mitochondrial permeability transition study

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ARTICLE INFO

Article history:
Received 27 October 2008
Received in revised form 18 December 2008
Accepted 29 December 2008
Available online 7 January 2009

Keywords:
Mitochondrion
Calcium
ADP
Mitochondrial permeability transition
Permeability transition pore
Adenine nucleotide translocase
Cardiolipin
Computational chemistry
Molecular interaction field
Molecular dynamics

ABSTRACT

The oxidation of critical cysteines/related thiols of adenine nucleotide translocase (ANT) is believed to be an important event of the Ca^{2+}-induced mitochondrial permeability transition (MPT), a process mediated by a cyclosporine A/ADP-sensitive permeability transition pores (PTP) opening. We addressed the ANT-Cys^{56} relative mobility status resulting from the interaction of ANT/surrounding cardiolipins with Ca^{2+} and/or ADP by means of computational chemistry analysis (Molecular Interaction Fields and Molecular Dynamics studies), supported by classic mitochondrial swelling assays. The following events were predicted: (i) Ca^{2+} interacts preferentially with the ANT surrounding cardiolipins bound to the H4 helix of translocase, (ii) weakens the cardiolipins/ANT interactions and (iii) destabilizes the initial ANT-Cys^{56} residue increasing its relative mobility. The binding of ADP that stabilizes the conformation "m" of ANT and/or cardiolipin, respectively to H5 and H4 helices, could stabilize their contacts with the short helix h56 that includes Cys^{56}, accounting for reducing its relative mobility. The results suggest that Ca^{2+} binding to adenine nucleotide translocase (ANT)-surrounding cardiolipins in c-state of the translocase enhances (ANT)-Cys^{56} relative mobility and that this may constitute a potential critical step of Ca^{2+}-induced PTP opening.

1. Introduction

Mitochondrial permeability transition (MPT) is a Ca^{2+}-dependent, cyclosporine A (CsA)-sensitive permeabilization of inner mitochondrial membrane, which is mediated by the opening of permeability transition pores (PTP). MPT is usually associated with oxidative stress and has been implicated as being involved in cell necrosis and some types of cell apoptosis [1–10].

The PTP structure appears to include the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane, the voltage-dependent anion channel (VDAC) in the outer membrane and cyclophilin D in the matrix. VDAC, a highly conserved protein responsible for the access of metabolites to the mitochondrial intermembrane space, may regulate the cytosolic stimuli for PTP opening. Indeed, Ca^{2+}-induced MPT is inhibited by anti-VDAC antibodies as well as by the cytosolic pro/anti-apoptotic members of the Bcl-2 family of proteins [10]. The participation of cyclophilin D is inferred mainly by the well-established inhibition of MPT by the immunosuppressant CsA (for review see [8]).

Due to their predominance in the inner mitochondrial membrane and common structural features, the members of the mitochondrial carrier family have been proposed as potential PTP constituents. The ANT has been considered for a long time as a central PTP component [3]; however, recent knock-out studies have reduced its impact [11] and the mitochondrial phosphate carrier has gained evidence [12]. In fact, PTP composition is believed to be very complex and to change depending on the cell physiological state [7].

ANT contains three cysteine residues, including the ANT-Cys^{56}, proposed as accounting for the sensitization of PTP to Ca^{2+} by oxidative

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doi:10.1016/j.bbabio.2008.12.013

stress. This carrier displays two distinct conformational states according to the cytosolic ("c") or matrix ("m") location of the nucleotide binding site. MPT is inhibited by ADP and stimulated by atractyloside/carboxyatractyloside, responsible for stabilizing the conformational states "m" and "c" of ANT, respectively. The MPT inhibition by ADP is believed to be mediated by the decrease of the ANT affinity for Ca^{2+} [3].

Six molecules of cardiolipin are tightly bound to ANT, arranged at three in each subunit of translocase. This phospholipid, present mostly in the mitochondrial membranes of eukaryotic cells, is essential for the transport of adenine nucleotides by ANT [13], and may both (i) participate in the mitochondrial apoptosis signaling by releasing cytochrome *c*, to which it is bound [14], and (ii) modulate conformational changes of ANT, inducing MPT [15]. In regard to the involvement of cardiolipin/ANT in MPT, it has been reported that (i) Ca^{2+} weakens this interaction and opens channels within each or between two subunits of translocase [15]; (ii) Ca^{2+} sequesters cardiolipins aggregating membrane proteins, including ANT, and favoring intermolecular oxidation of thiols [16]; (iii) cardiolipins mediate interaction between two subunits of ANT [17]; (iv) peroxidized cardiolipins induce MPT with the involvement of ANT [14]; and (v) cardiolipins interfere with the alkylation of ANT-Cys^{56} in purified ANT, apparently by occluding the surface domain that encompasses this residue [18].

The above scenario prompted us to address, by means of computational chemistry analysis (Molecular Interaction Fields and Molecular Dynamics studies) the ANT-Cys^{56} relative mobility status resulting from the interaction of ANT/surrounding cardiolipins with Ca^{2+} and/or ADP, supported by classic mitochondrial swelling assays. Our findings suggest that Ca^{2+} binding to the adenine nucleotide translocase (ANT)-surrounding cardiolipins in c-state of the translocase enhances (ANT)-Cys^{56} relative mobility and may constitute a critical step of Ca^{2+}-induced PTP opening.

2. Materials and methods

2.1. Isolation of rat liver mitochondria

Mitochondria were isolated by standard differential centrifugation. Male Wistar rat weighing approximately 200 g were sacrificed by decapitation; liver (10–15 g) was immediately removed, sliced in medium (50 ml) consisting of 250 mM sucrose, 1 mM ethylene glycol bis (-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 10 mM N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)-KOH (HEPES-KOH) 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 medium (30 ml) consisting of 250 mM sucrose, 0.3 mM EGTA and 10 mM HEPES-KOH, pH 7.2, and centrifuged (3400 *g*, 15 min). The final mitochondrial pellet was suspended in medium (1 ml) consisting of 250 mM sucrose and 10 mM HEPES-KOH, pH 7.2, and used within 3 h. Mitochondrial protein contents were determined by the biuret reaction.

Table 1
Effects of classic MPT inhibitors on Ca^{2+}-elicited swelling (Δ apparent absorbance at 540 nm) on succinate-energized isolated rat liver mitochondria

Agent	25 μM Ca^{2+}	100 μM Ca^{2+}
Ca^{2+}	0.08 ± 0.011	0.30 ± 0.032
Ca^{2+} + 1 μM CsA	0.01 ± 0.004	0.08 ± 0.010
Ca^{2+} + 25 μM NEM	0	0
Ca^{2+} + 50 μM ADP	0.03 ± 0.008	0.20 ± 0.022
Ca^{2+} + 200 μM ADP	0	0.11 ± 0.017

The conditions are described in Materials and methods. Values represent extent of swelling (mean ± SD from at least three different preparations) estimated after 10 min of time scan.

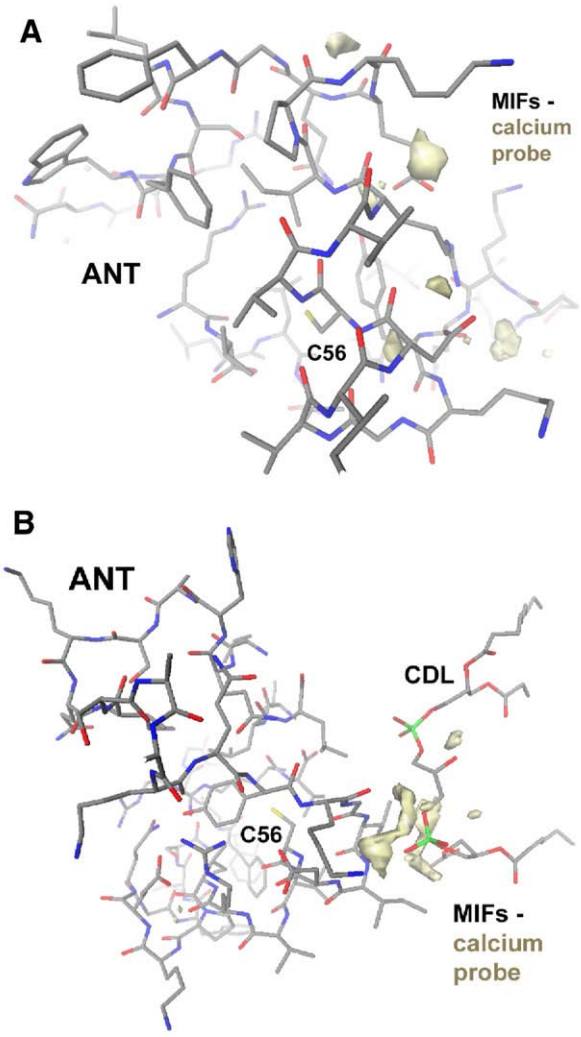

Fig. 1. Isocontour plots of MIFs computed with a Ca^{2+} probe for adenine nucleotide translocase (ANT) at −2.0 kcal/mol in (A) absence or (B) presence of cardiolipin. Selected residues, including Cys^{56} (in yellow) are shown.

2.2. Mitochondrial swelling assay

Mitochondrial swelling was estimated by the decrease of apparent absorbance at 540 nm of a mitochondrial suspension (0.5 mg protein/ml) exposed to MPT inhibitors followed by Ca^{2+}. Inhibitors were incubated with mitochondria energized with 5 mM succinate (+1 μM rotenone) in a standard medium consisting of 125 mM sucrose, 65 mM KCl and 10 mM HEPES-KOH, pH 7.4, at 30 °C. Ca^{2+} was added 1 min after the reaction started and the extent of swelling in the absence or presence of inhibitor was estimated after 10 min of time scan in relation to the tracings in the absence (control) of Ca^{2+}.

2.3. Molecular modeling procedures — Molecular Interaction Fields (MIFs) studies

The process of ligand–receptor interaction has been successfully represented with the help of Molecular Interaction Fields (MIFs) [19–23]. MIFs identify regions where certain chemical groups favorably interact, suggesting positions where a ligand could place

similar chemical groups. By using ALMOND 3.3 [19] implemented in the Sybyl 8.0 package [20] and GRID v.22 software [21], a Ca^{2+} probe predicted potential Ca^{2+}-binding sites in ANT structure in the absence and presence of cardiolipin. For ALMOND analysis, the grid the grid spacing was set to 0.5 Å and the smoothing window to 0.8. The number of filtered nodes was set to 100, with 35% relative weights. In GRID, a phosphate probe was used in order to predict nucleotides potential binding sites in ANT. A box was placed in the ANT cavity in order to encompass the overall active site, and its grid spacing was set to 0.33 Å. Default parameters were used. We have used the structure of ANT in complex with carboxyatractyloside and cardiolipin, retrieved from the Protein Data Bank (PDB entry: 1OKC) [24], after removal of all the ligands.

2.4. Molecular modeling procedures — flexible docking simulation

Previous to docking simulations, one ADP molecule was built and energy minimized using the Discover module of the **Insight II** package [25], with an all-atom consistent valence force field (CVFF) and subsequent semi-empirical quantum-chemical AM1 method. Flexible docking simulations were performed using the **GOLD 4.0** software [26] in order to propose a binding mode for ADP inside the ANT active site, which employs a genetic algorithm to model the flexibility of the ligand. The software classifies the orientations of the molecules in the ANT active site by a decreasing affinity order (fitness) with the translocator, using an empirical energy function (the GoldScore), originally parameterized against the experimental binding affinities for a test set of 82 protein-ligand complexes [26]. We used populations of 100 conformers, 100,000 operations, 95 mutations and 95 crossovers as genetic algorithm parameters. All the simulations were performed inside a sphere of 12 Å radius centered at the Lys^{32} zeta-nitrogen of ANT. Among 5 orientations of highest score, we selected a high score orientation containing the pyrophosphate moiety of ADP in phase with the phosphate binding sites predicted by **GRID**.

2.5. Molecular modeling procedures — Molecular Dynamics (MD) simulations

Molecular Dynamics (MD) simulations were performed based on three systems: a) ANT/cardiolipin/Ca^{2+}, b) ANT/cardiolipin/Ca^{2+} plus ADP and c) ANT in the absence of cardiolipin and Ca^{2+}. All the molecular mechanics and dynamics simulations were carried out using the Discover module of **Insight II**, with the CVFF force field. Ca^{2+} was placed in the two different sites (along with ANT and bound to cardiolipin) based on MIF analyses. Before this simulation, hydrogen atoms were added and oriented in ANT, and the energy of the complex was minimized using 2000 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^{-1}/$Å^{-1}$. Implicit solvent condition with a dielectric constant of 80 (water) was employed. ADP molecule was

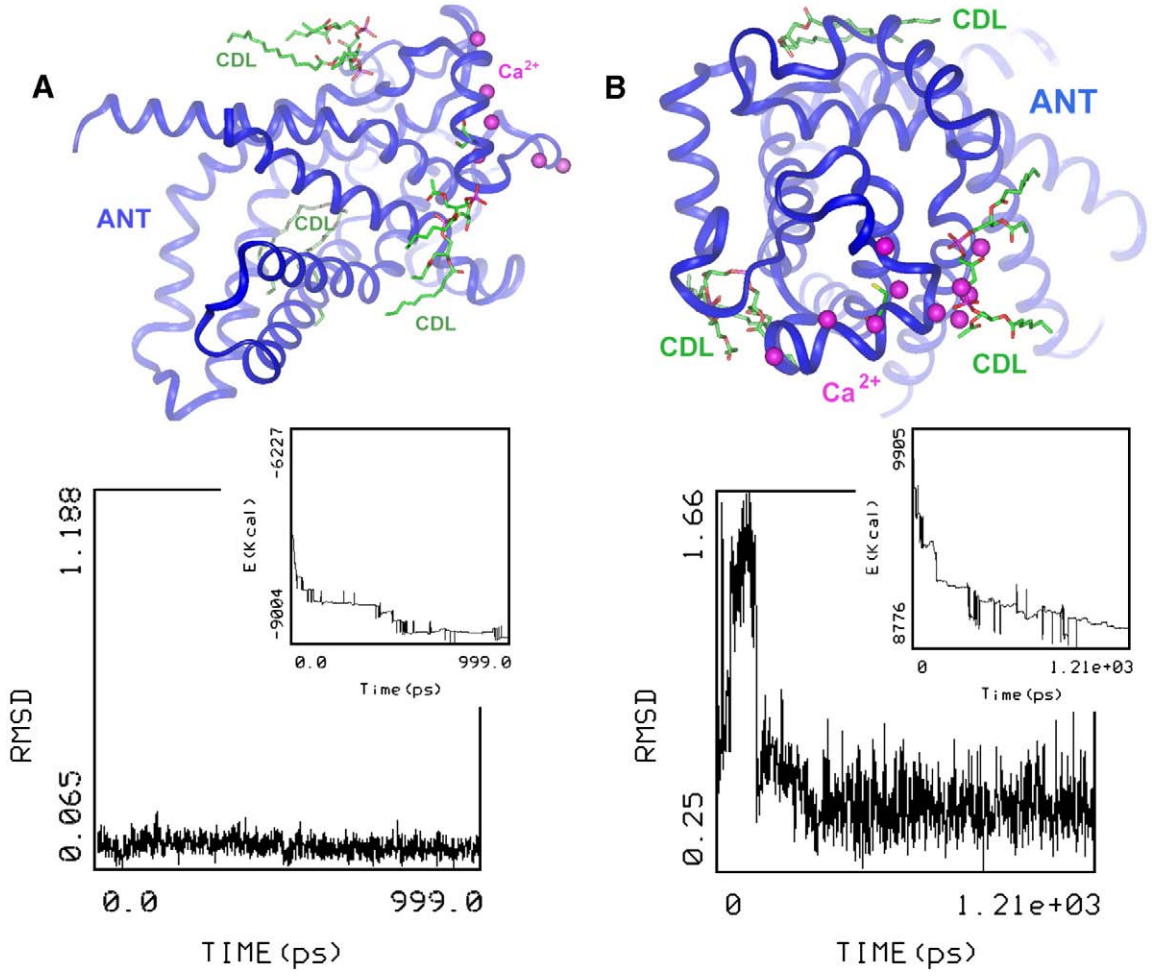

Fig. 2. Plots of RMSD (in angstroms) of the Cys^{56} atoms (in yellow) and energy *vs.* time (simulation time) for the ANT (ribbons diagram, in blue) with (A) Ca^{2+} probe (6 ions, in magenta) along with adenine nucleotide translocase or (B) Ca^{2+} (10 ions, in magenta) bound to cardiolipin (in green).

positioned in the ANT active site, using long trajectories (1000–1500 ps) MD simulation for the models, with a temperature constraint 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 during the simulation for further analysis. From the molecular trajectories generated by the MD simulations, we analyzed the root mean square deviation (RMSD) of the atoms of Cys^{56}, as well as the total energy of the ANT/ADP/cardiolipin/Ca^{2+} and ANT/ADP complexes as a time function.

3. Results

3.1. Effects of MPT inhibitors on Ca^{2+}-elicited swelling in succinate-energized isolated rat liver mitochondria

Table 1 shows the effects of classic MPT inhibitors (cyclosporine A–CsA, N-ethylmaleimide-NEM and ADP) on Ca^{2+}-elicited swelling in succinate-energized isolated rat liver mitochondria as an *in vitro* biological support for the next computational chemistry findings. The data show an overview regarding these inhibitors. The extent of swelling induced by 100 μM Ca^{2+}, a condition near to the maximum effect (not shown), was more than three times higher as compared to 25 μM Ca^{2+}; the inhibition by CsA was lower for the former condition. At both Ca^{2+} concentrations swelling was completely inhibited by NEM and, as for CsA, the degree of inhibition by ADP was inversely related to the Ca^{2+} concentration.

3.2. The preferential Ca^{2+} binding to ANT-surrounding cardiolipins weakens their interactions and unmasks/increases relative mobility of ANT-Cys^{56}

To determine the influence of cardiolipin and Ca^{2+} on ANT-Cys^{56} residue relative mobility we performed MD simulations of ANT in the absence or in the presence of these ligands. First, **Sybyl/ALMOND** descriptor was used to predict the potential Ca^{2+} binding sites in ANT in the absence (Fig. 1A) and in the presence (Fig. 1B) of cardiolipin. Based on these results of MIF studies, we placed Ca^{2+} ions close to Ala^{45}, Glu^{46}, Glu^{48}, Gly^{51}, Asp^{54}, Arg^{58}, Pro^{60}, Lys^{61} and Glu^{62} residues (depicted from ANT without cardiolipin) and closely bound to the cardiolipin molecule (depicted from ANT with cardiolipin). After an initial energy minimization of the system in water followed by a 1000 ps MD simulation, we analyzed the RMSD of the atoms of Cys^{56} as well as the total energy of both systems (Fig. 2A and B). For the first situation (Ca^{2+} dispersedly bound to ANT), we also carried out a MD simulation, but in the absence of cardiolipin (Fig. 3). The RMSD level of Cys^{56} residues increased after approximately 500 ps in comparison to the initial coordinates used as inputs of the simulations only for the situation in which Ca^{2+} is bound close to cardiolipin, indicating an increase in the mobility of this residue along 1200 ps. The higher affinity of the Ca^{2+} probe for cardiolipin than for ANT residues suggests that the cation binds preferentially to the phospholipids and only the direct binding of Ca^{2+} to the cardiolipin phosphate headgroups would be capable to weaken the cardiolipin/ANT interactions destabilizing the initial ANT-Cys^{56} and increasing its relative mobility. Theoretically, the absence of cardiolipin in the structure also destabilized the ANT-Cys^{56} mainly due to the interactions between the backbone of the translocator and cardiolipin (Fig. 3).

3.3. The presence of ADP in the ANT structure protects against the increase of ANT-Cys^{56} relative mobility due to Ca^{2+} binding to ANT-surrounding cardiolipins

Similar simulations were conducted for ANT/cardiolipin/Ca^{2+} in the presence of ADP in order to determine restoration in the relative

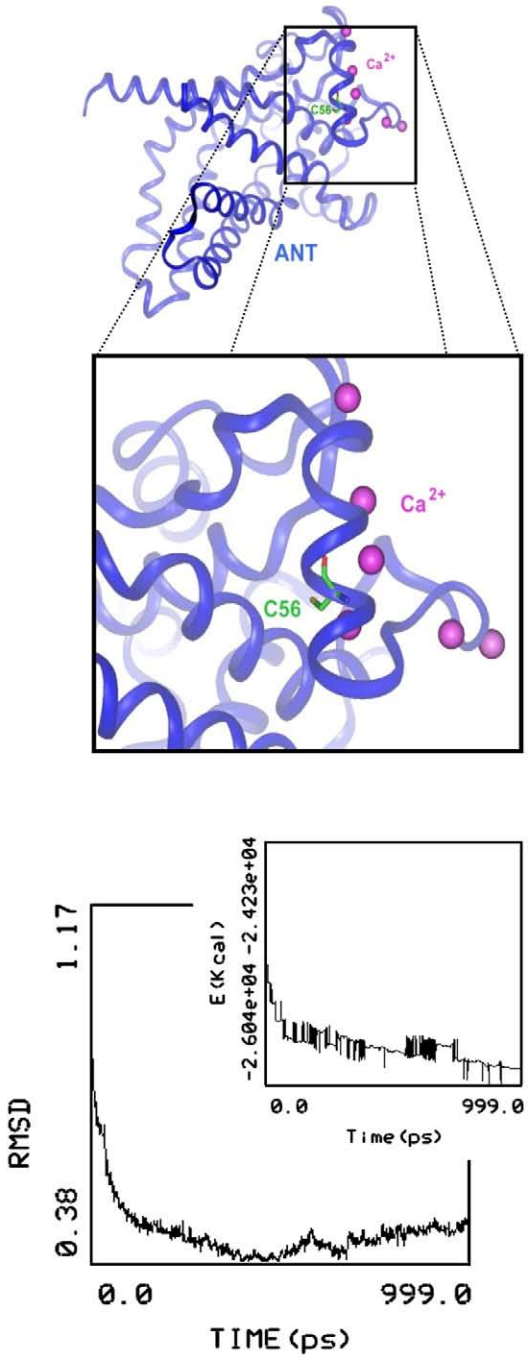

Fig. 3. Plots of RMSD (in angstroms) the Cys^{56} atoms (in green) and energy *vs.* time (simulation time) for the ANT (ribbons diagram, in blue) with Ca^{2+} probe (6 ions, in magenta) along with adenine nucleotide translocase, in the absence of cardiolipin. Detail of the Ca^{2+} ions surrounding Cys^{56} is shown.

mobility of the ANT-Cys^{56} residue. As a preliminary step, we used **GRID** to predict the potential phosphate binding sites in ANT. Subsequently, we performed a flexible docking for ADP with the ANT active site, and selected a high scored orientation (GoldScore of 67.7) containing the pyrophosphate moiety of ADP in phase with the phosphate binding site predicted by **GRID** MIFs (Fig. 4).

The results obtained with the MIFs generated by **GRID** indicate Arg^{234} and Arg^{79} as the most important residues to bind to pyrophosphate moiety of ADP. In agreement with our results, it has been shown that R96H mutation (named *op1*) *Saccharomyces cerevisiae* carrier drastically diminishes ADP binding affinity, whose corresponding residue in the

bovine structure is Arg^{79} [24]. MIFs were energy contoured for the phosphate probe at −19.0 kcal/mol and the main contribution was due to Arg^{79}.

Docking analysis revealed that, as previously described, ADP theoretically interacts with Asn^{276} and Arg^{279} of the H4 helix as well as Lys^{32} of the H5 helix of ANT. Yet, in the solvated translocase structure, cardiolipin has both polar and hydrophobic contacts to the H4 helix of ANT, via main and side chains, as previously described [24]. Both helices have contacts with the short α-helical stretch h56, which contains the Cys^{56} residue. Here we added Ca^{2+} bound to cardiolipin in the presence of ADP and monitored the mobility of Cys^{56} residue. After an initial energy minimization of the system in water, followed by a 1500 ps MD simulation, we analyzed the RMSD of the atoms of Cys^{56} and the total energy of ANT/cardiolipin//Ca^{2+}/ADP complex as time functions (Fig. 5A). Comparing these results to those previously obtained in the same system, but in the absence of ADP (Fig. 2B), we found that the conformational stability of Cys^{56} is guaranteed in the presence of ADP, suggesting that the mobility of Cys^{56} residues is much lower in the presence of ADP. Moreover, this complex showed faster stabilization at low values of energy and more stable values of RMSD for Cys^{56} atoms. Notably, the absence of cardiolipin, even in the presence of ADP, can confer instability to Cys^{56}, as shown by the RMSD of the coordinates of Cys^{56}, at least twice as large as that obtained in the ANT/cardiolipin/ADP/Ca^{2+} system (Fig. 5B). This suggests that ADP or cardiolipin binding respectively to H5 and H4 helices stabilize their contacts with the short helix h56 in the bundle, avoiding broader vibrational movements of Cys^{56} that would subsequently expose it to oxidation.

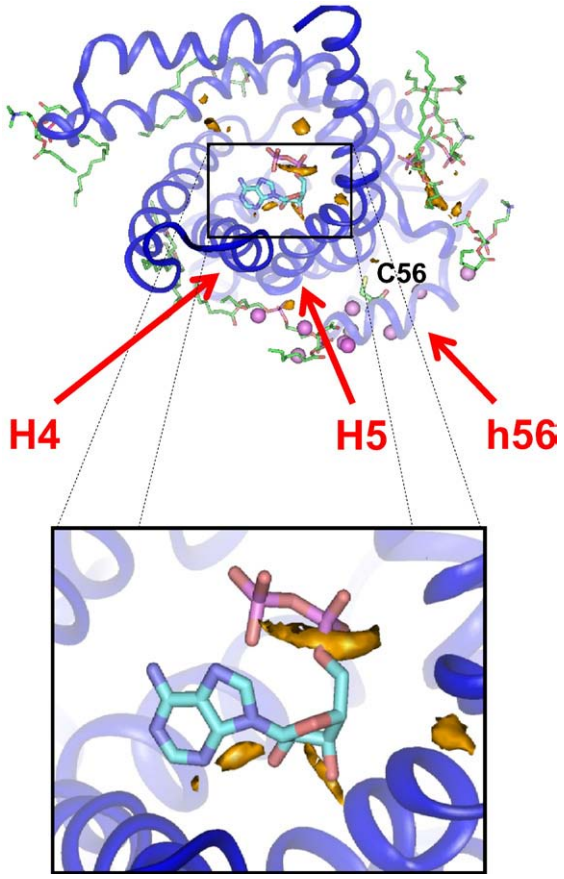

Fig. 4. Isocontour plot of a **GRID** MIF map (in orange) computed with the phosphate probe for adenine nucleotide translocase — ANT (ribbons diagram, in blue). The energy is contoured at −19.0 kcal/mol. Top-ranked solution obtained with flexible docking for ADP (carbon atoms in cyan) is shown. Red arrows indicate the helices H4, H5 as well as h56, a short α-helical stretch that contains Cys^{56} (in color by atom). Ca^{2+} is represented in magenta spheres. Cardiolipin molecules are shown with carbon atoms in green.

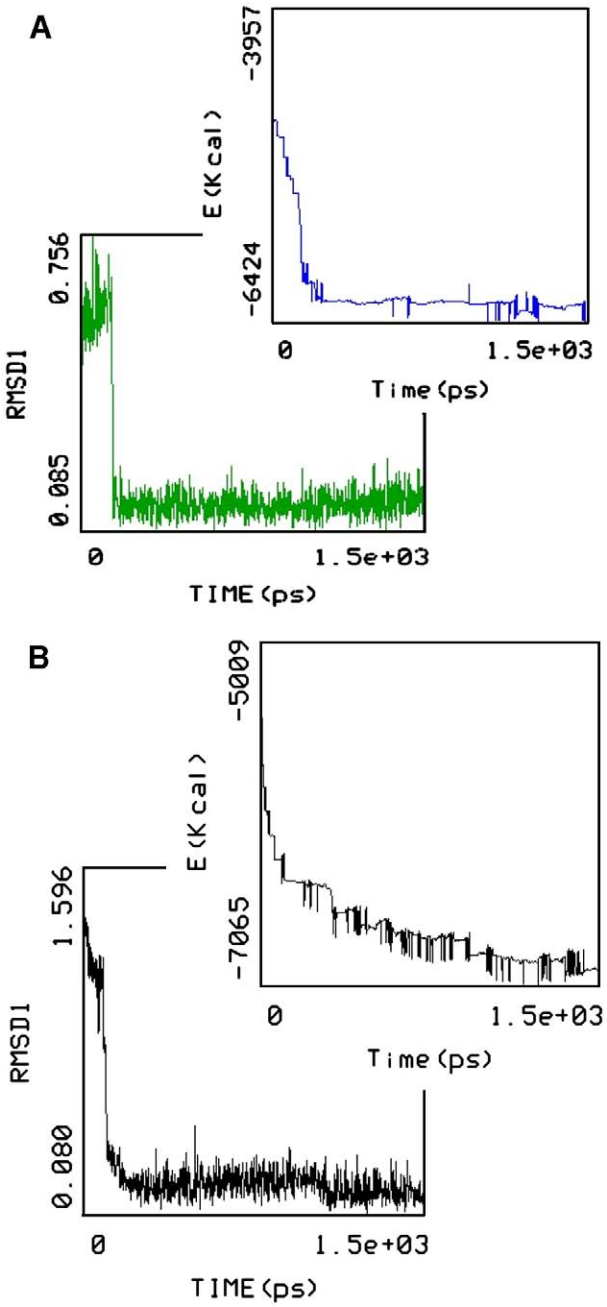

Fig. 5. Plots of RMSD (in angstroms) of the Cys^{56} atoms and energy *vs.* time (simulation time) for (A) ANT/cardiolipin/Ca^{2+} system in the presence of ADP or (B) ANT/ADP/Ca^{2+} system in the absence of cardiolipin.

4. Discussion

The ANT has been considered for a long time as a central PTP component, mostly due to sensitivity of mitochondrial permeability transition (MPT) to physiological (ADP), or non-physiological ligands, which determines the ANT conformational states “c” and “m”. It is a consensus that oxidation of mitochondrial membrane protein thiols is involved in MPT. Indeed, the reactivity of the hydrophobic thiol reagent NEM with the ANT-Cys^{56} (see for example [3,18]), as well as its well documented mitochondrial swelling inhibiting ability, suggest that the oxidation of this specific translocase residue is critical for PTP opening.

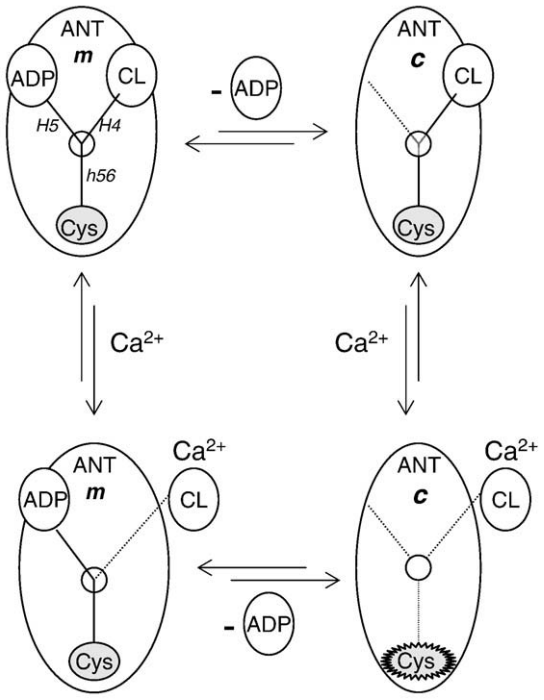

Fig. 6. Representative scheme based on computational chemistry analysis – Molecular Interaction Field and Molecular Dynamic studies – for ANT-Cys^{56} relative mobility status resulting from the interaction of ANT/ANT surrounding cardiolipins with Ca^{2+} and/or ADP. See Discussion section for description.

Like other potential critical cysteine residues on mitochondrial matrix facing loops of ANT, Cys^{56} has been proposed to undergo glutathione-mediated cross-linking followed by a decrease of ADP/increase of cyclophilin D binding to the molecule, and sensitization of PTP opening to Ca^{2+} by oxidative stress [3]. PTP opening sensitization, in turn, appears to depend on (i) conformation "c" of ANT, achieved in the absence of ADP [1,3], (ii) ANT-surrounding cardiolipins [14,16–18] and (iii) ANT-Cys^{56} relative mobility status [18].

Cardiolipin is involved in many mitochondrial processes, including modulation of MPT and cytochrome *c* release [27]. The influence of cardiolipin in the alkylation of ANT-Cys^{56} suggests that an eventual dissociation or weakening in the cardiolipin/ANT interaction changes the lability of critical mitochondrial membrane thiol residues [18]. Besides that, the requirement of Ca^{2+} for PTP opening by peroxidized cardiolipins suggests a synergistic effect between the cation and the phopholipid concerning MPT induction [14].

Computational chemistry has been here employed as a pioneer approach to address this issue, aiming to the mechanism of Ca^{2+}-induced PTP opening. For such approach, the recently described structure of ANT from bovine heart beef mitochondria, which shares a high degree of sequence similarity and properties with human organelles, was used [24]. The following events were predicted: (i) Ca^{2+} interacts preferentially with the ANT surrounding cardiolipins bound to the H4 helix of translocase, (ii) weakens the cardiolipins/ANT interactions and (iii) destabilizes the initial ANT-Cys^{56} residue increasing its relative mobility. The binding of ADP that stabilizes the conformation "m" of ANT and/or cardiolipin, respectively to H5 and H4 helices, could stabilize their contacts with the short helix h56 that includes Cys^{56}, accounting for reducing its relative mobility (Fig. 6).

It is believed that an enhancement in the ANT-Cys^{56} relative mobility predisposes it to oxidation by mitochondria-generated ROS, followed by thiol cross-linking involving other cysteine residues either from ANT itself or other mitochondrial membrane carriers including the Cys^{56} of an adjacent ANT molecule/subunit [15–17,18]. The prediction that Ca^{2+} itself is enough to provide such enhanced relative mobility suggests that it is rather an early step of MPT, instead of a proposed late effect in response to the sensitization of PTP opening to thiol oxidation [3]. Recent concepts consider that inner mitochondrial membrane PTP components may include (i) ANT (perhaps associated with VDAC/cyclophilin D) [3], (ii) ANT associated with other inner membrane transporters such as the phosphate carrier [12], or even (iii) clusters of non-specific integral membrane proteins, including ANT itself [9]. Therefore, the enhancement of ANT-Cys^{56} relative mobility here predicted fits into any of the three above-mentioned concepts and may be responsible, at least in part, for the well-documented intra/intermolecular thiol cross-linking associated with PTP opening.

In conclusion, the present study suggests that: (i) in the presence of ADP the ANT remains in the conformation "m", which is insensitive to Ca^{2+} binding to the ANT-surrounding cardiolipins for ANT-Cys^{56} relative mobility; (ii) in the absence of ADP, ANT remains in the conformation "c" and Ca^{2+} binding to ANT-surrounding cardiolipins enhances the ANT-Cys^{56} relative mobility. In an attempt to rationalize these counterparts, we propose that ANT-Cys^{56} relative mobility status depends on the relative concentration of ADP and Ca^{2+}, which in turn determine, respectively, the ANT conformation and the interaction of cardiolipins bound to the conformation "c" (Fig. 6). A study comprising classic concepts of PTP considers that the complexation of cardiolipins by Ca^{2+} destabilizes the dimeric active form of ANT, causing it to form a pore directly or via facilitation of any other interaction [15]. The present study supports this hypothesis and is in line with previous related concepts [1,3], while suggests that ANT switches between conformations "m" and "c" as a result of ADP binding. A novelty is the idea that when ANT is in the conformational state "c", ANT-Cys^{56} is more predisposed to oxidation via the binding of Ca^{2+} to ANT-surrounding cardiolipins, with potential critical involvement in the Ca^{2+}-elicited PTP opening.

Acknowledgements

This work was supported by grants from FAPESP, CAPEs and CNPq, Brazil. The authors thank Neife Aparecida Guinaim dos Santos for the careful revision of the manuscript.

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