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Thermal stability of CopA, a polytopic membrane protein from the hyperthermophile *Archaeoglobus fulgidus*

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Abstract

Despite recent progress in understanding membrane protein folding, little is known about the mechanisms stabilizing these proteins. Here we characterize the kinetic thermal stability of CopA, a thermophilic P_{IB}-type Cu^+-ATPase from *Archaeoglobus fulgidus*. When heterologously expressed in *Escherichia coli*, purified and reconstituted in mixed micelles, CopA retained thermophilic characteristics with maximum activity at 75 °C. Incubation of CopA in the absence of substrates at temperatures in the 66–85 °C range led to an irreversible exponential decrease in enzyme activity suggesting a two-state process involving fully-active and inactive molecules. Although CopA inactivated much slower than mesophilic proteins, the activation energy was similar to that observed for mesophilic P-type ATPases. The inactivation process was found to be associated with the irreversible partial unfolding of the polypeptide chain, as assessed by Trp fluorescence, Phe UV spectroscopy, far UV circular dichroism, and 1-aniline-8-naphtalenesulfonate binding. However, the inactive thermally denatured protein still conserves large hydrophobic regions and considerable secondary structure.

Keywords: Cu-ATPase; Thermophilic; Denaturation; Unfolding

Folding and stability are determinant elements of protein biological functions. Membrane proteins are a large fraction of proteomes (20–30%) [1,2] and a large number of pathologies appear to be associated with mutations leading to altered folding/stability [3,4]. However, the study of membrane protein stability has not received as much attention as that of soluble proteins [5–8]. Progress in understanding membrane protein stability has been hindered by several factors. A relatively small number of membrane proteins can be obtained from natural sources or by heterologous expression in quantities and forms suitable for stability studies [9,10]. Many are unstable when removed from the lipid environment during solubilization by detergents, a step required for purification [11]. The irreversible unfolding that most membrane proteins undergo under various experimental conditions impedes quantitative thermodynamical analysis [6–8]. Finally, adding an extra layer of complexity, the transmembrane region of these proteins appears significantly more stable than water exposed domains [5–8].

The discovery of extremophilic organisms, hyperthermophiles in particular, and the subsequent characterization of their proteins have produced relevant information on soluble protein stability [12–15]. On the contrary, membrane protein stability studies have not taken advantage of naturally occurring hyperthermostable proteins that work at temperatures over 70 °C. This lack of information is curious considering that molecules from thermophiles and hyperthermophiles have proved instrumental in elucidating the structure of several major classes of membrane proteins (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Toward understanding the stability of thermophilic membrane proteins, we initiated the characterization of CopA, a Cu^+ transporting ATPase from

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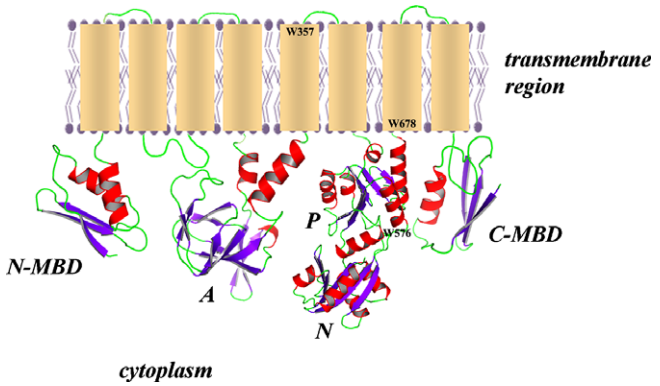

Fig. 1. Schematic illustration of *A. fulgidus* CopA topology and cytoplasmic domains. The structure of A (PDB # 2HC8) and ATP binding (PDB # 2B8E) domains are presented. The structures N- and C-terminus metal binding domains were obtained by homology modeling using Helicobacter pylori CopZ (1YG0) as a template. The locations of the three Trp residues present in CopA (W357, W576 and W678) are indicated.

Archaeoglobus fulgidus. We hypothesize that this protein might serve as a model to study stabilizing mechanisms, folding/unfolding determinants, and the contributions of various protein domains to these properties.

A. fulgidus CopA is a member of the P_{IB} subfamily of P-type ATPases [16–18]. These transport heavy metals (Cu^+, Zn^{2+}, Cd^{2+}, Co^{2+}, etc.) across biological membranes. They confer metal tolerance to bacteria [19,20], and are essential for the absorption, distribution, and bioaccumulation of metal micronutrients by higher organisms [20,21]. Mutations in the two human P_{IB}-type ATPases (Cu^+-ATPases) are responsible for Menkes' and Wilson's diseases [21,22]. CopA can be expressed in *Escherichia coli* in mg quantities and purified in a micellar form [23]. The enzyme retains Cu^+ dependent ATPase activity and it can, alternatively, use Ag^+ as transported ion. Analysis of the enzyme reconstituted in mixed dodecyl-β-D-maltoside (DDM)[1] /asolectin micelles shows that heterologously expressed CopA has maximum activity at 75 °C and an activation energy of 103 kJ/mol [23]. There is significant structural-functional information on this protein. It essentially follows the classical Post's E1/E2 cycle that describes the ATP-coupled metal transport by P-type ATPases [23,24]. CopA has eight transmembrane (TM) helices [16–18] (Fig. 1). Three of these (TM6, TM7, and TM8) contribute invariant residues to form the transmembrane metal binding site [25]. The structures of the major cytoplasmic regions of the enzyme have been established. CopA contains an ATP binding loop (N and P-domains) linking TM6 and TM7 and an actuator domain (A-domain) between TM4 and TM5 [26,27]. The enzyme also has two cytosolic metal Cu^+ binding domains (N- and C-MBDs) that are homologous to those of known structures present in eukaryote and prokaryote Cu^+-ATPases [24,28,29] (González–Guerrero and Argüello unpublished results).

In this work, we describe the characterization of CopA thermal inactivation and denaturation. This thermophilic protein appears more stable than its mesophilic counterparts independently of the presence of archaeal lipids, other stabilizing molecules, or archaeal cell derived conditions. These results represent the first quantitative analysis of a thermophilic membrane protein stability and indicate that the irreversible enzyme inactivation is likely associated with the exposure of hydrophobic regions of the protein to the solvent.

Materials and methods

cDNA constructs and protein expression

A. fulgidus CopA cDNA, cloned into pBADTOPO/His vector (Invitrogen, Carlsbad, CA), was used in this study [24]. *E. coli* Top10 cells (Invitrogen, Carlsbad, CA) carrying the chloramphenicol resistant plasmid CP encoding for rare tRNAs (tRNA ^{arg}AGA/AGG and tRNA ^{ile}AUA) [30] were transformed with this construct and expression was induced with 0.002% L-arabinose.

Enzyme preparation

The enzyme was purified as described [23]. Briefly, membranes were isolated from cells expressing CopA and solubilized with 0.75% dodecyl-β-D-maltoside (DDM). CopA was isolated by affinity chromatography using a Ni^{2+}-nitrilotriacetic acid column. After purification, the protein (3–4 mg/ml) was stored in 25 mM Tris–HCl (pH 8.0 at 20 °C), 100 mM sucrose, 50 mM NaCl, 0,01% DDM and 1 mM dithiothreitol (DTT) at −80 °C until use. Protein concentration was determined in accordance to Bradford [31]. For all the experiments, the enzyme was diluted to 0.4 mg/ml (≈5 μM) in a medium containing 25 mM Tris–HCl (pH 6.1 at 75 °C), 50 mM NaCl and 250 μM asolectin/250 μM DDM. During thermal treatments, the enzyme was maintained in close-sealed tubes to avoid evaporation and in the absence of light to prevent photooxidation. After incubation, protein aliquots were placed in an ice-water bath until determinations were performed.

ATPase activity assays

ATPase activity was measured at 75 °C as the initial velocity of release of P_i from ATP in a medium containing: 50 mM Tris–HCl (pH 6.1 at 75 °C), 3 mM $MgCl_2$, 3 mM ATP, 20 mM Cys, 0.01% asolectin, 0.01% DDM, 400 mM NaCl, 100 μM $AgNO_3$, 2.5 mM DTT and 10 μg/ml purified enzyme [23]. Ag^+-ATPase activity was measured as the difference between the activity in the aforementioned medium and that measured in the same medium without Ag^+. Released inorganic phosphate (P_i) was determined in accordance to Lanzetta et al. [32]. The measured activity remained practically constant up to at least 60 min (Bredeston et al, unpublished results).

Polyacrylamide gel electrophoresis

Prior to electrophoresis, samples were mixed at room temperature for 5–10 min in sample buffer. SDS–PAGE was carried out as described by Schägger [33] using 10% T and 1% C. PAGE using non-denaturing blue gels was performed as described by Wittig et al. [34]. Gels were stained with colloidal Coomassie brilliant blue [35].

Fluorescence measurements

Steady state fluorescence measurements were performed at 25 °C in a 3 × 3 mm quartz cuvette using a SLM-Aminco Bowman Series 2 spec-

[1] *Abbreviations used:* DDM, dodecyl-β-D-maltoside; ANS, 1-aniline-8-napthalenesulphonate; TM, transmembrane.

trofluorometer as described previously [36]. Both excitation and emission bandwidths were set at 4 nm. CopA emission spectrum was registered between 305 and 400 nm after excitation at 295 nm. 1-Aniline-8-naphtalenesulfonate (ANS) fluorescence was registered between 420 and 550 nm following excitation at 380 nm. The spectra were corrected for background emission. Total intensity I_t was calculated as:

$$I_t = \sum_i I_{\lambda_i} \tag{1}$$

and the wave number $\bar{\nu}$ corresponding to the center of spectral mass was determined according to Silva et al. [37] as:

$$\bar{\nu}_{CM} = \frac{\int I_{CopA(\bar{\nu})}\bar{\nu}d\bar{\nu}}{\int I_{CopA(\bar{\nu})}d\bar{\nu}} \cong \frac{\sum_i I_{CopA(\bar{\nu}_i)}\bar{\nu}_i\Delta\bar{\nu}_i}{\sum_i I_{CopA(\bar{\nu}_i)}\Delta\bar{\nu}_i} \tag{2}$$

1-Aniline-8-naphtalenesulfonate binding

The binding of ANS to CopA was evaluated at 25 °C as described by Daniel and Weber [38] with some modifications. Briefly, after the incubation the enzyme was titrated with increasing volumes of a 700 μM ANS stock solution. After each ANS injection and 2 min equilibration, the fluorescence spectra of ANS were registered and corrected for dilution effects. ANS total intensity (I_{ANS}) was calculated using Eq. (1). The dependence of ANS total intensity with ANS concentration can be described by:

$$I_t = \frac{[ANS]I_{max}}{K_{0.5}[ANS]} \tag{3}$$

where I_{max} is the maximal ANS fluorescence intensity and $K_{0.5}$ is the affinity constant between ANS and the hydrophobic regions of the protein.

UV spectrophotometry

UV spectra were collected at 25 °C using a Jasco 7850 spectrophotometer with a slit width of 1 nm, a scan rate of 20 nm/min, a time constant of 1 s and a 1 cm path length cuvette. Data were taken every 0.2 nm. The fourth derivative spectrum was calculated as described by Padros et al. [39] from the average of 5 accumulated absorption spectra by applying two successive cycles of second-order derivation:

$$\frac{\Delta^2 A}{\Delta\lambda^2} = \frac{(A_{i+20} - 2A_i + A_{i-20})}{2\Delta\lambda^2} \tag{4}$$

with a derivative interval ($\Delta\lambda$) of 8 nm.

Circular dichroism

CD spectra of CopA were registered at 25 °C in the wavelength region of 200–250 nm on a Jasco J-810 spectropolarimeter. Data were collected in a 1 mm path length cuvette using a scan speed of 20 nm/min with a time constant of 1 s. An average of three independent measurements was used to calculate the molar residue ellipticity $[\theta]$ as:

$$[\theta] = \frac{(\theta \cdot 100 \cdot M_r)}{(c \cdot l \cdot N_A)} \tag{5}$$

where $[\theta]$ is the mean residue molar ellipticity in deg cm^2 dmol^{-1}, θ is the experimental ellipticity in millidegree, M_r is the molecular mass of the protein, c is protein concentration in mg/ml, l is the cuvette path length in centimeters, and N_A is the number of amino acid of the protein.

Data analysis

At least three independent preparations of purified CopA were evaluated for each experiment. The equations were fitted to the experimental data using a non-linear regression procedure based on the Gauss–Newton algorithm [40]. The dependent variable was assumed to be homoscedastic (constant variance), and the independent variable was considered to have negligible error. The "equation of best-fit" was deemed that which gave the minimal Akaike index [41] and the least biased fit. Parameters were expressed as means ± standard error.

Results

It has been previously shown that heterologously expressed *A. fulgidus* CopA displays maximal activity at 75 °C when reconstituted in mixed DDM/asolectin micelles [23]. Although these data indicated that the enzyme retains its thermophilic characteristics when heterologously expressed, an open question was how long this active conformation persists; i.e., whether the enzyme has significant thermostable characteristics. To explore this matter, we decided to monitor the enzyme Ag^+ dependent ATPase activity as a function of the incubation time at 75 °C in the absence of substrates. After various time periods, samples were taken, momentarily placed in an ice-water bath until all aliquots were collected and the ATPase activity at 75 °C was determined. Fig. 2 shows that the enzyme activity decreased as a function of the incubation time, and after 70 min a complete inactivation was observed. Because the ATPase activity measured at 75 °C was not restored after the cooling step at 4 °C, it can be assumed that CopA thermal inactivation is an irreversible process. The best fit to the experimental data was obtained with a single exponential function:

$$\frac{v}{v_0} = e^{-k_{inact}t} \tag{6}$$

where v and v_o are the remaining and initial activities, t is the incubation time, and k_{inact} is the kinetic coefficient for the thermal inactivation. The observed exponential decrease in CopA activity suggests a two-state process involving only fully active and inactive molecules.

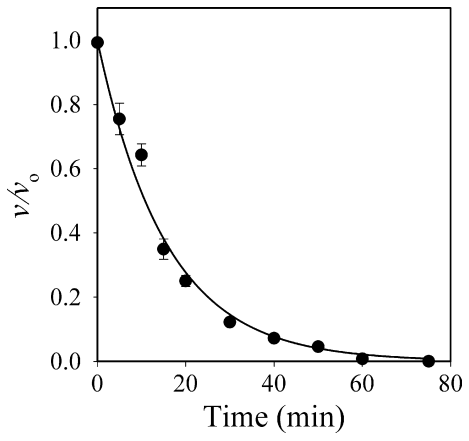

Fig. 2. Time course of CopA thermal inactivation. The purified enzyme was incubated at 75 °C. After incubation, the relative Ag^+-ATPase activity was determined and plotted as a function of the incubation time. The initial ATPase activity was 20.5 μmol mg^{-1} h^{-1}. Continuous line is the graphical representation of Eq. (6) with the best fit parameter value $k_{inact} = 0.056 \pm 0.002$ min^{-1}.

It is known that the irreversible inactivation of soluble proteins can be due to covalent modifications [42] or non-covalent changes such as aggregation or kinetically trapped conformations [43,44]. To evaluate the integrity of CopA after thermal inactivation, SDS–PAGE analysis was performed (Fig. 3A). No changes were detected in the electrophoretic pattern and mobility of CopA following thermal inactivation, suggesting that inactivation was not associated with either major fragmentation or significant formation of SDS stable protein aggregates. Analogous information was obtained from non-denaturing gels (Fig. 3B). Two well differentiated bands were observed when CopA was analyzed in Blue Native gels. This indicates that CopA is composed of at least two different oligomeric states in its native form: a monomeric one and a dimer (or trimer). The indetermination in the assignment of the number of CopA subunits associated in the oligomer comes from the error in the estimation of molecular masses from native gels using soluble proteins as standards. Interestingly, neither significant aggregation nor significant changes in CopA oligomeric distribution were associated with thermal inactivation.

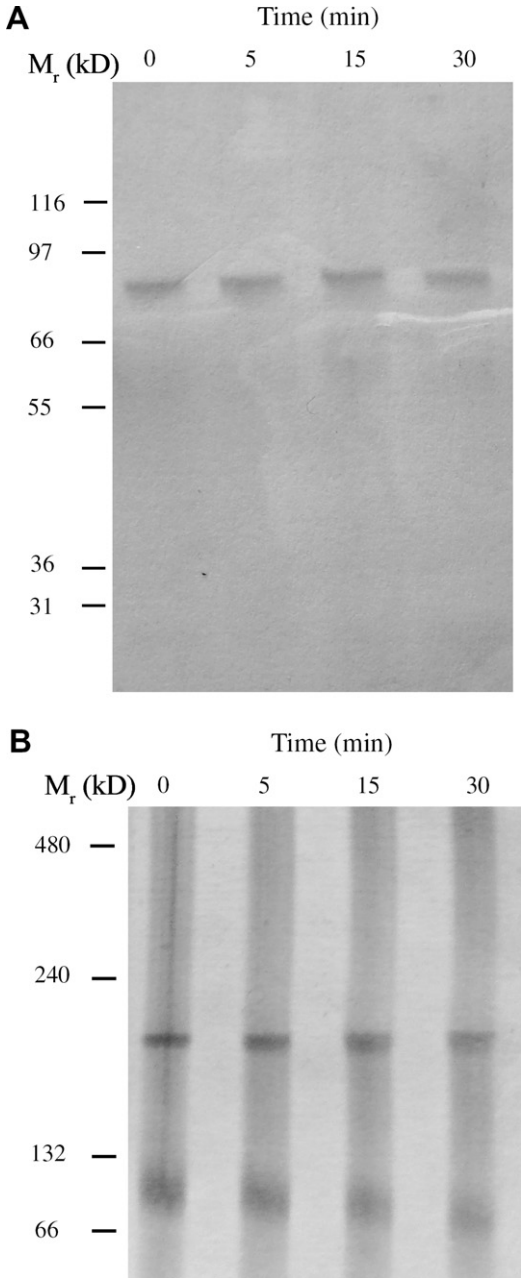

Fig. 3. PAGE analysis of native and thermally inactivated CopA. The enzyme was incubated at 75 °C. After the indicated incubation times, aliquots were taken and run on SDS–PAGE (A) or blue native PAGE (B). Aliquots were 2 μg CopA per lane for SDS–PAGE and 12 μg CopA per lane for blue native PAGE.

Some CopA structural properties were analyzed to further understand the inactivation mechanism. To explore possible changes in hydrophobic regions during CopA thermal inactivation, we first evaluated the binding of ANS to the protein. The fluorescence quantum yield of this naphthalene dye in water is very low and strongly increases when the probe locates in hydrophobic protein pockets [45]. Mirroring ANS binding to CopA, fluorescence intensity followed a hyperbolic function of ANS concentration as the protein was titrated (Fig. 4A). The best-fit values for the binding parameters were plotted as a function of the incubation time to evaluate the kinetics of the structural change monitored by the probe. These clearly show that ANS fluorescence intensity (I_{max}) decreased with increasing the incubation time (Fig. 4B). This analysis indicates that CopA thermal inactivation is paralleled by a large decrease of hydrophobic cavities within the protein. The correlation of both, the structural change and the inactivation, is further supported by the similar values of k_{inact} and k_{ANS} that describe the exponential decrease in enzyme activity and ANS binding. Additionally, it can be observed that the affinity between CopA and ANS increases significantly when increasing the incubation time, reaching a constant value after 50 min of incubation (Fig. 4C). This suggests that the hydrophobic pockets remaining after the inactivation are, on average, more hydrophobic than those accessible to the probe in the native state [46].

The observed alterations in CopA hydrophobic regions may also be associated with changes in the environment of aromatic residues. The Trp fluorescence spectrum of native CopA excited at 295 nm was centered on 340 nm, which is characteristic of Trp in moderated hydrophobic environments as those of the water-membrane interface. During thermal inactivation, spectral changes in CopA fluorescence spectra were detected (Fig. 5). The center of spectral mass was calculated using Eq. (2), and a slight red shift (about 2 nm) was observed upon enzyme inactivation. This suggests that Trp residues, partially buried in the native state, were becoming slightly exposed to the solvent as a function of the incubation time [47]. More evident, the Trp fluorescence intensity decayed exponentially until a constant value as function of the incubation time (Fig. 5B). This kinetic behavior was similar to that of the inactivation process (Fig. 2).

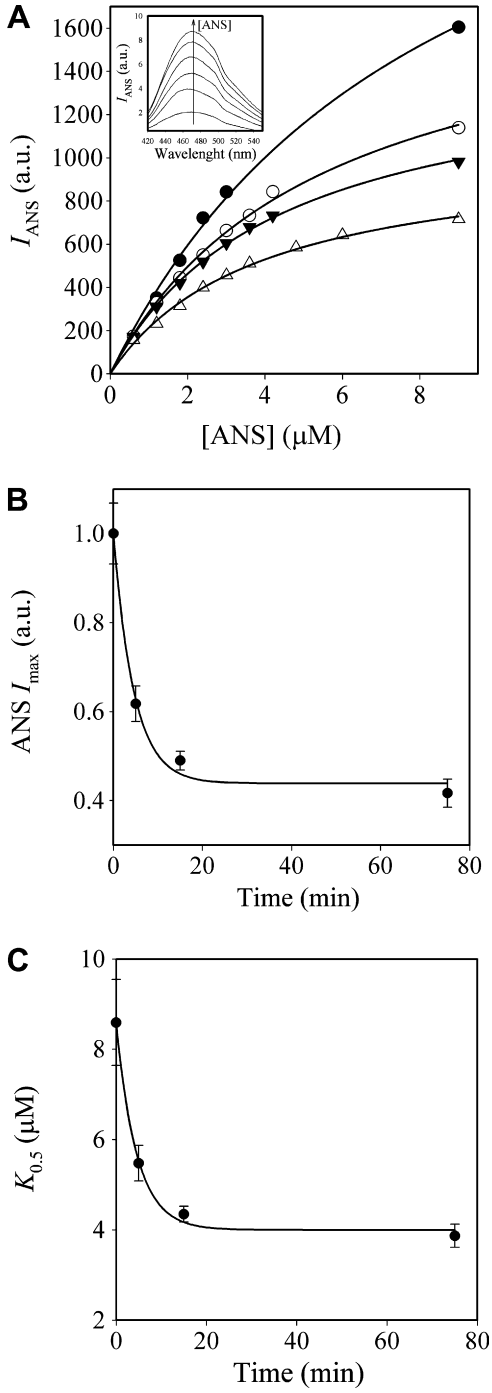

Fig. 4. ANS binding to native and thermally inactivated CopA. (A) CopA was incubated 0 (●), 5 (○), 15 (▼), and 70 (Δ) min at 75 °C and subsequently titrated with ANS. After each ANS addition, the fluorescence spectrum was recorded (figure inset shows the raw spectra of native CopA titrated with ANS). Total fluorescence intensity was calculated using Eq. (1) and plotted as a function of the incubation time. The Eq. (3) was fitted to each set of the experimental data, obtaining a set of best-fit parameter values for I_{max} and $K_{0.5}$. ANS I_{max} (B) and $K_{0.5}$ (C) were plotted as a function of the incubation time. An exponential dependence of I_{max} and $K_{0.5}$ on the incubation time was included in Eq. (3) for the global fitting to the experimental data. The best fit value for the kinetic coefficient for the change on both parameters was $k_{ANS} = 0.11 \pm 0.01\ min^{-1}$.

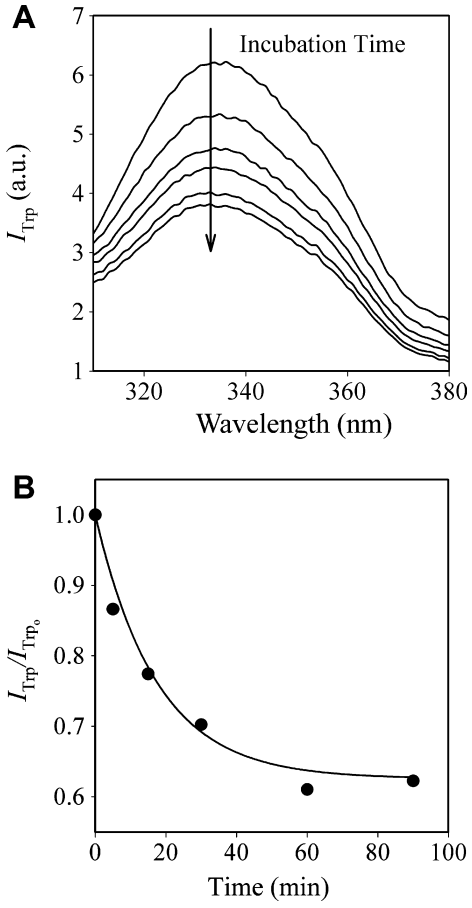

Fig. 5. Trp fluorescence of native and thermally inactivated CopA. (A) Purified enzyme was incubated at 75 °C, aliquots were taken, and the Trp emission fluorescence spectra were recorded. (B) Total fluorescence Intensity (I_t) was calculated from each spectrum using Eq. (1) and plotted as a function of the incubation time. The continuous line represents an exponential function decreasing towards a constant value with the best-fit parameter value $k_{Trp} = 0.058 \pm 0.011\ min^{-1}$.

Additional information on the environment of aromatic amino acids can be obtained from the fourth derivative absorption spectra of these residues [48]. Phe presents a maximum peak around 258 nm, a spectral region far from the absorption peaks of Tyr and Trp [39]. It was observed a significant decrease in the amplitude and a slight blue shift from 258.8 nm to 258.4 nm in the Phe peak of the fourth derivative spectrum of inactive CopA compared with native CopA spectrum (Fig. 6). In agreement with the exposure of hydrophobic regions as the enzyme is inactivated, these data indicate that the average environment surrounding Phe residues in the inactive state is less hydrophobic and more heterogeneous than those in the native state [39]. Changes in the spectra can be also observed in the range of absorption of Tyr and Trp, this is also indicative of modifications in the environment of these residues but the overlapping among the absorption bands of both

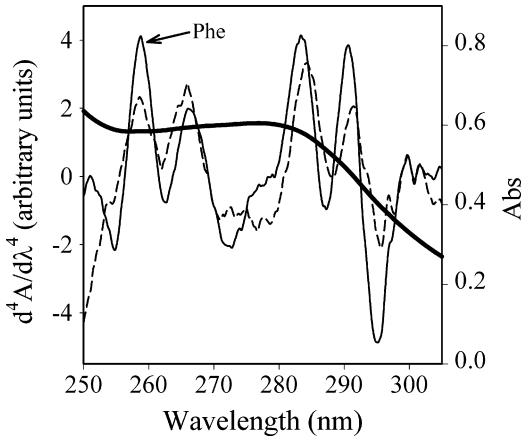

Fig. 6. Absorption and fourth derivative UV spectra of native and thermally inactivated CopA. Purified enzyme was incubated 0 and 120 min at 75 °C. After that, aliquots were taken and the absorption spectra from 250 to 300 nm were recorded. The thick solid line shows the raw spectrum corresponding to the native enzyme. The derivative spectra of native (continuous line) and inactivated CopA (dashed line) were calculated as described in Materials and methods.

residues [48] makes difficult the assignment of the peaks in this region of the fourth derivative spectra.

Toward determining whether structural alterations during CopA inactivation included changes in protein secondary structure, far UV CD spectroscopy was employed. Fig. 7A shows the CD spectra of CopA upon incubation at 75 °C during various times. A significant decrease in the ellipticity as a function of the incubation time was observed. The mean residue molar ellipticity evaluated at 222 nm exponentially decreased until a constant value (65% of the initial ellipticity value) indicating an important loss of α helix structure of CopA during inactivation (Fig. 7B).

Finally, the influence of temperature on CopA stability was explored by determining the kinetics of inactivation in a range of temperatures. Fig. 8A shows the exponential time course of the enzyme inactivation at 66–85 °C. The influence of temperature on the rates of inactivation was analyzed in terms of the Arrhenius equation (Fig. 8B). The linearity of Arrhenius plot suggests that a single common mechanism is responsible for CopA inactivation over the range of temperatures assayed.

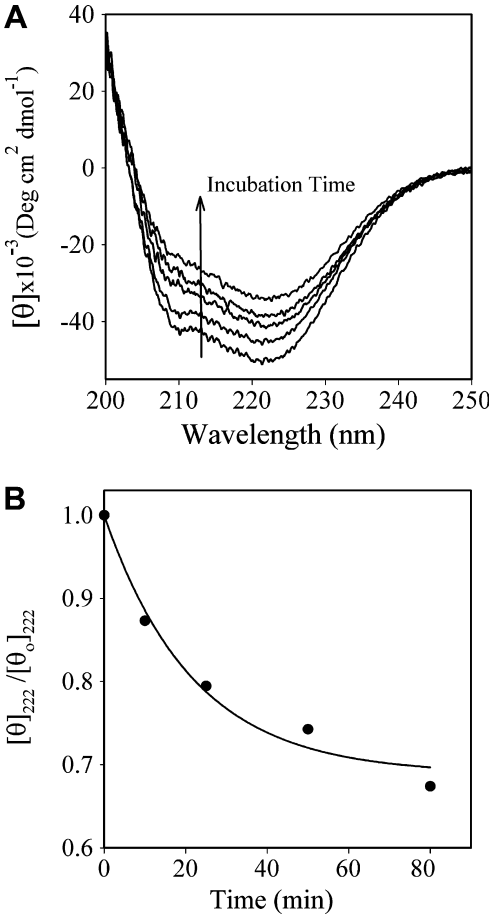

Fig. 7. Far UV CD spectra of native and thermally inactivated CopA. (A) The enzyme was incubated at 75 °C and after 0, 10, 20, 25, 50 and 80 min of incubation aliquots were taken and the CD spectra were collected. Each spectrum is the average of three consecutive spectra of the same sample. (B) The mean residue molar ellipticity at 222 nm was calculated using Eq. (5) and plotted as a function of the incubation time. The continuous line is the graphical representation of an exponential function decreasing towards a constant value, with the best-fit value of the kinetic coefficient $k_{[\theta]} = 0.046 \pm 0.011\ \text{min}^{-1}$.

Discussion

The hypothesis driving these initial studies is that uncovering the stabilizing mechanisms of extremophilic thermostable polytopic membrane proteins might contribute to understanding the folding/stability of similar mesophilic proteins. The large yield of pure active *A. fulgidus* CopA produced by heterologous expression, the significant available structural-functional information, and the feasibility of simple functional assays and suitable structural analysis highlight the convenience of CopA as a model protein. The stability of proteins is characterized by the resistance of native folded conformations against their disruption by environmental factors conducive to denatured states. Protein stability can be evaluated by measuring the thermodynamic parameters that characterize the reversible unfolding process (thermodynamic stability) or the time persistence of the native folded conformation (kinetic stability). In this work CopA stability was first characterized studying its inactivation at 75 °C. Initially the enzyme was incubated at 75 °C in the absence of ligands, then cooled and supplemented with its ligands for the ATPase activity measurement at optimal temperature. As described in Materials and methods no further inactivation occurs during the activity measurement. Our results show that activity of CopA decreases as a function of the incubation time. Considering that any reversible step occurring in the system during the initial incubation at 75 °C was reverted during the cooling step at 4 °C, our results indicate that thermal inactivation of CopA was, at least in part, irreversible.

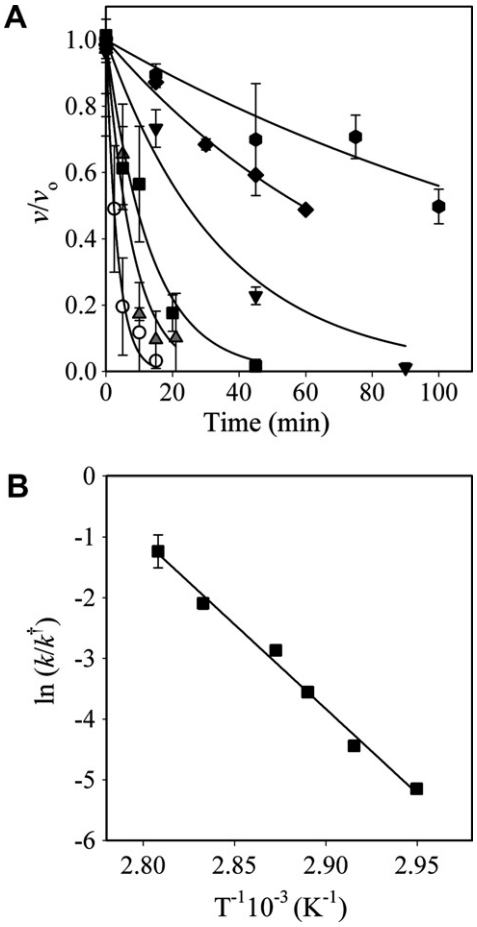

Fig. 8. Time course of thermal inactivation of purified CopA at different temperatures. (A) The enzyme was incubated at 66 °C (●), 70 °C (◆), 73 °C (▼), 75 °C (■), 80 °C (▲) and 85 °C (○). At selected incubation periods aliquots were taken, Ag^+-ATPase activity was determined and the relative activity was plotted as a function of the incubation time. The continuous line is the graphical representation of the Eq. (6) with the following best-fit values for the inactivation coefficients: $0.006 \pm 0.001\ min^{-1}$ (66 °C), $0.012 \pm 0.002\ min^{-1}$ (70 °C), $0.029 \pm 0.001\ min^{-1}$ (73 °C), $0.056 \pm 0.002\ min^{-1}$ (75 °C), $0.089 \pm 0.003\ min^{-1}$ (80 °C) and $0.330 \pm 0.007\ min^{-1}$ (85 °C). Initial ATPase activity was in the range of 20 to 25 μmol $mg^{-1}\ h^{-1}$ (B) Arrenhius plot showing the temperature dependence of the kinetic coefficients of thermal inactivation of CopA. The solid line is the graphical representation of the Arrhenius equation $\ln\frac{k_i}{k^\dagger} = \ln A - \frac{E_a}{R \cdot T}$, where $k^\dagger = 1\ min^{-1}$, $R = 8.314\ J\ K^{-1}\ mol^{-1}$, E_a = Activation Energy, A = pre-exponential factor, the best-fit parameter values were $E_a = 231 \pm 12$ kJ/mol and $\ln A = 77 \pm 14$.

The time course of this process is well described by an exponential function. This is characteristic of first order reactions and indicates that thermal inactivation of CopA can be kinetically described as a two state process involving only fully active and inactive molecules. As expected, the half-life of CopA at 75 °C (10.80 ± 0.35 min) is much longer than that observed for other P-type ATPases (e.g. $t_{0,5} = 10$ s at 51 °C for purified plasma membrane H^+-ATPase from yeast [49]), and in general for other membrane and soluble mesophilic proteins.

It is known that the irreversible inactivation of soluble proteins is due, in many cases, to either covalent modifications (side-chain oxidation, hydrolysis of peptide bonds, etc.) or non-covalent changes leading to aggregation [42,50]. PAGE analysis indicates that CopA inactivation is apparently not associated with these phenomena, and then other structural perturbations should be involved (e.g. kinetically trapped conformational changes [51]). It is known that many P-type ATPases form oligomeric structures [52,53] and it has been reported that some of these arrangements contribute to the enzyme stability [54]. In this work, we show that purified CopA exists as two oligomeric states: a more abundant monomeric form and a higher order structure (likely a dimer), and no major changes in the distribution of oligomeric forms of CopA were observed during the inactivation at 75 °C.

Toward identifying the structural perturbations responsible for CopA thermal inactivation three approaches reporting the status of hydrophobic regions and associated aromatic residues were used: ANS binding, Trp fluorescence, and Phe fourth derivative absorbance analysis. ANS is a fluorescent probe frequently used to monitor conformational changes in proteins. The maximal value of ANS fluorescence is usually taken as a measure of the amount of hydrophobic pockets in the protein [55]. The titration experiment with ANS indicated that the extent of hydrophobic cavities of CopA diminished as the inactivation took place. The kinetic coefficient of the change in maximal ANS fluorescence intensity (k_{ANS}) is in the range of that of the inactivation process, supporting the idea that CopA inactivation is related to the exposition of hydrophobic regions to the solvent. On the other hand, the affinity for the probe of the remaining hydrophobic regions increased with the incubation time, indicating a larger hydrophobicity of these hydrophobic regions in the denatured state compared to those in the native protein.

CopA contains three tryptophan residues, two of them are located at the end of transmembrane helixes, perhaps slightly exposed to the solvent, whereas the third is in the helix connecting the N- and P-domains, with a moderated accessibility to the solvent (Fig. 1) The Trp fluorescence spectrum of native CopA excited at 295 nm is centered on 340 nm, in accordance with these structural details. As CopA is inactivated, a small red shift in the center of spectral mass was observed denoting a structural transition where the Trp becomes exposed, additionally the decrease in Trp fluorescence intensity suggests that Trp residues in native CopA are not in close proximity to quenching groups [56].

Near-ultraviolet absorption is also used to explore small changes in the environment of aromatic residues in proteins. In particular, Phe is easily observable in the fourth derivative UV spectra and shifts in the associated peak have been correlated to changes in the surrounding environment [39]. CopA has 26 Phe residues. The majority of these are located in transmembrane segments and only 7–8 are in cytoplasmic domains. The peak corresponding to Phe in the fourth derivative spectrum of native CopA was located at 258.8 nm, in agreement with the location of Phe in non-aqueous environments [39]. Upon inactiva-

tion at 75 °C, this maximum shifted towards the blue region of the spectra and the amplitude of the peak decreased significantly, indicating that Phe side chains become more exposed to the solvent and confirming the pattern observed in Trp fluorescence.

These findings are all indicative of the protein denaturation with the exposure of hydrophobic regions to the aqueous solvent. However, it is also evident that a large portion of structure is still conserved in the denatured state. This is reflected by the constant value (approximately 50% of that of the native state) that ANS binding and Trp fluorescence reached when extrapolated to infinite incubation time.

The circular dichroism spectrum in the far UV region of native CopA reported in this work is in good agreement with the structural information available from high resolution data [26,27] and bioinformatics [57–59], in which 39% of its residues are alpha helix structure (19% soluble and 20% transmembrane regions), 16% in β sheet and 45% in turns and unordered structure (Fig. 1). During the inactivation a large portion of the secondary structure of CopA is lost, although the inactivated state of CopA still retains considerable secondary structure. The CD spectrum of thermally inactivated CopA, compared to those of denatured proteins [60] and of all α transmembrane proteins [61], suggests that the major change occurs in the soluble regions of CopA, together with a slight unfolding of the transmembrane regions. The mean residue molar ellipticity at 222 nm, often considered indicative of alpha helix structure [62], significantly decreased as a function of the incubation time following an exponential function. The kinetic coefficient for this process is not significantly different from that obtained from the activity and fluorescence measurements. Thus, we can conclude that the changes observed in the enzyme activity, secondary and tertiary structure are all part of the same global change in CopA produced during the incubation at 75 °C.

As it was expected, CopA is more stable than its mesophilic counterparts at 75 °C. To further characterize the inactivation process the influence of temperature on CopA stability was studied. The relationship between the inactivation constants and temperature for elemental reactions is described by the Arrhenius equation [63]. The linear Arrhenius plot obtained for CopA thermal inactivation is compatible with a common inactivation mechanism in the studied range of temperatures (66–85 °C). The activation energy is a useful parameter in the characterization of kinetic enzyme stability [64]. Previous works postulated that if a protein becomes more stable in its folded state the E_a would be higher [65]. Since the rates of inactivation are measured at different temperature ranges in mesophilic and thermophilic proteins, E_a can be used to compare kinetic stability between these proteins [66]. Surprisingly the E_a value obtained for CopA inactivation is in the same order as that of mesophilic membrane proteins. Compare 231 ± 12 kJ/mol for CopA with 172 ± 14 kJ/mol for the photosynthetic reaction center from Rhodobacter sphaeroides [67], 222 ± 12 kJ/mol for the plasma membrane calcium pump [68], 300 kJ/mol for the sarcoplasmic reticulum Ca^{2+}-ATPase [69] and 444 ± 38 kJ/mol for the Na^+/K^+ ATPase (González–Lebrero et al, unpublished results). Thus, we hypothesize that the mechanism involved in stabilizing the structure of thermophilic membrane proteins has the same characteristics as those of mesophilic membrane proteins, but adjusted to confer larger resistance to high temperatures.

In summary, these studies show that CopA conserves a high temperature dependent activity and stability when it is heterologously expressed in a mesophilic organism. The irreversible inactivation characterized in this work was found to be associated with the partial unfolding of the polypeptide chain exposing hydrophobic regions of the enzyme and not significant changes in the oligomeric state of the enzyme. Thus, CopA appears as an attractive model to explore the forces and factors involved in membrane protein stability.

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