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Reversible Unfolding of a Thermophilic Membrane Protein in Phospholipid/Detergent Mixed Micelles

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Folding mechanisms and stability of membrane proteins are poorly understood because of the known difficulties in finding experimental conditions under which reversible denaturation could be possible. In this work, we describe the equilibrium unfolding of *Archaeoglobus fulgidus* CopA, an 804-residue α-helical membrane protein that is involved in transporting Cu^+ throughout biological membranes. The incubation of CopA reconstituted in phospholipid/detergent mixed micelles with high concentrations of guanidinium hydrochloride induced a reversible decrease in fluorescence quantum yield, far-UV ellipticity, and loss of ATPase and phosphatase activities. Refolding of CopA from this unfolded state led to recovery of full biological activity and all the structural features of the native enzyme. CopA unfolding showed typical characteristics of a two-state process, with $\Delta G_w^\circ = 12.9$ kJ mol^{-1}, $m = 4.1$ kJ mol^{-1} M^{-1}, $C_m = 3$ M, and $\Delta Cp_w^\circ = 0.93$ kJ mol^{-1} K^{-1}. These results point out to a fine-tuning mechanism for improving protein stability. Circular dichroism spectroscopic analysis of the unfolded state shows that most of the secondary and tertiary structures were disrupted. The fraction of Trp fluorescence accessible to soluble quenchers shifted from 0.52 in the native state to 0.96 in the unfolded state, with a significant spectral redshift. Also, hydrophobic patches in CopA, mainly located in the transmembrane region, were disrupted as indicated by 1-anilino-naphtalene-8-sulfonate fluorescence. Nevertheless, the unfolded state had a small but detectable amount of residual structure, which might play a key role in both CopA folding and adaptation for working at high temperatures.

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Introduction

Membrane proteins constitute a large fraction of proteomes, and a number of pathologies appear to be related to alterations in their folding and stability.[1] However, there is limited knowledge on associated molecular events and forces involved.[2] The little attention that these aspects have apparently received is likely rooted on the experimental difficulties linked to their characterization, such as low-yield purification, scarcity of adequate heterologous expression systems, poor stability in solubilization detergents, and others.[3] In addition, their intrinsic physicochemical properties required for adaptation to the complexity of the heterogeneous membrane–water environment also appear to hamper efforts to characterize their reversible unfolding.

Reversible solvent denaturation has been extensively used to characterize the thermodynamic stability of water-soluble proteins.[4] Among various chemical agents, urea and guanidine hydrochloride (GndHCl) have proven particularly successful. It has been proposed that both denaturants preferentially bind to the unfolded state, solvating charged residues or engaging in hydrogen bonds with the protein backbone.[5] Consequently, the folding equilibrium would be shifted toward the

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Abbreviations used: GndHCl, guanidine hydrochloride; DDM, dodecylmaltoside; pNPPase, *p*-nitrophenyl phosphatase; ANS, 1-anilino-naphtalene-8-sulfonate.

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unfolded form. The denatured state is characterized by an ensemble of multiple conformations with a very low content of secondary and tertiary structures. Indeed, the presence of long-range contacts in this ensemble has been reported even in the presence of high denaturant concentrations.[6]

Helical membrane proteins appear to be resistant to chemical denaturation[7] or, alternatively, treatment with denaturant frequently leads to irreversible unfolding.[8] Nevertheless, reversible unfolding and thermodynamic characterization have been achieved for a few of these proteins: bacteriorhodopsin,[9,10] diacylglycerol kinase,[11] the KcsA potassium channel,[12] and the disulfide-bond-reducing protein DsbB.[13] Equilibrium and, in some cases, kinetic characterization of the unfolding of these proteins have provided an initial view of this process. For instance, observed unfolding free energies ($\Delta G_w°$) and their derivatives with respect to the denaturant concentration (m) were similar to those measured in soluble proteins of comparable size,[14] with transition states closer to the unfolded state than to the folded form.[2,10] Contrary to the energetic similarities, these helical membrane proteins, compared to soluble ones, seem to retain significant secondary structure in the unfolded states.[15]

We hypothesized that characterizing the stability of membrane proteins from hyperthermophilic organisms might provide further insights on this matter.[16] To test this, we have studied *Archaeoglobus fulgidus* CopA, a well-characterized Cu^+-transporting ATPase.[17,18] CopA belongs to the subfamily of P_{IB}-type ATPases involved in transporting heavy metals (Cu^+, Zn^{2+}, Cd^{2+}, etc.) throughout biological membranes.[18] It can be heterologously expressed in *Escherichia coli*, purified by affinity chromatography, and reconstituted in dodecylmaltoside (DDM)/asolectin micelles in functional form with maximum activity at 75 °C.[17] This single-chain 804-amino-acid-long protein has eight transmembrane helices.[18] Three transmembrane segments (TM6, TM7, and TM8) contribute invariant residues to form two transmembrane metal binding sites involved in metal translocation.[19] The major cytoplasmic regions of the enzyme include the ATP binding loop (N-domain and P-domain) linking TM6 and TM7, and the actuator domain (A-domain) connecting TM4 with TM5.[20,21] The N-terminal and C-terminal regions of CopA face the cytoplasm and contain regulatory Cu^+ binding domains.[18,22] The atomic-resolution structures of cytoplasmic domains,[20,21] together with whole-protein low-resolution structures, have contributed to the current model of CopA and similar P_{1B}-type ATPases.[23,24]

Here, we report the study of CopA unfolding upon treatment with GndHCl and its subsequent refolding to yield an active enzyme that is functionally and structurally undistinguishable from the native protein. The characteristics of these phenomena are distinct from those observed in other membrane proteins and provide additional insights toward understanding membrane protein folding and stability.

Results

Reversible denaturation of CopA

To characterize CopA unfolding, we incubated the enzyme (isolated in DDM/asolectin micelles) with increasing concentrations of GndHCl at 25 °C. Structural changes upon addition of denaturant were monitored by registering Trp fluorescence and ellipticity. CopA contains three Trp residues—one (Trp576) located in the ATP binding domain and two (Trp357 and Trp678) located in its fifth and seventh transmembrane segments—as well as a significant content of helical structures in its cytoplasmic domains and particularly in its transmembrane region.[20–24] A steep decrease in spectroscopic signals was observed within the first seconds (Fig. 1). No further changes were detected following this initial decay, even after an overnight incubation. Thus, a 2-h incubation time was chosen for equilibrium denaturation experiments. Figure 2a shows the Trp fluorescence spectra of native and fully denatured CopA. The fluorescence quantum yields for the native and denatured CopA were 0.18 and 0.10, respectively. In addition to the decrease in fluorescence intensity, the center of spectral mass shifted from 342 nm to 350 nm, indicating a major exposure of Trp residues to the solvent. Figure 2b shows the circular dichroism (CD) spectra of native and denatured CopA. The native spectrum has an ellipticity minimum at 222 nm, which is lost upon incubation with a high denaturant concentration. To test the reversibility of the observed transition, we decreased GndHCl concentration by dilution in the same micellar system. After 2 h of incubation, both the fluorescence spectra and the CD spectra of CopA recovered all the features of the native state (Fig. 2, blue lines). No further changes were detected upon longer incubation. These results suggest that GndHCl-induced unfolding of CopA

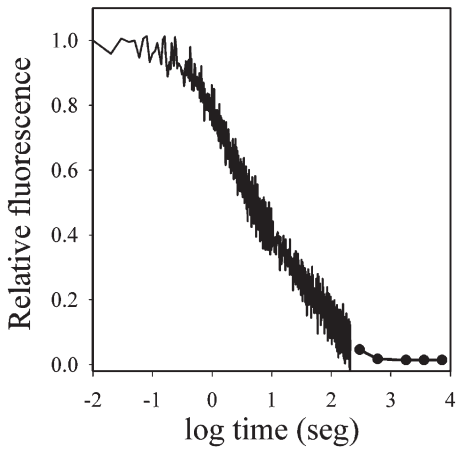

Fig. 1. Unfolding kinetics of CopA. Time course of CopA Trp fluorescence after mixing the protein with 7.5 M GndHCl at 25 °C.

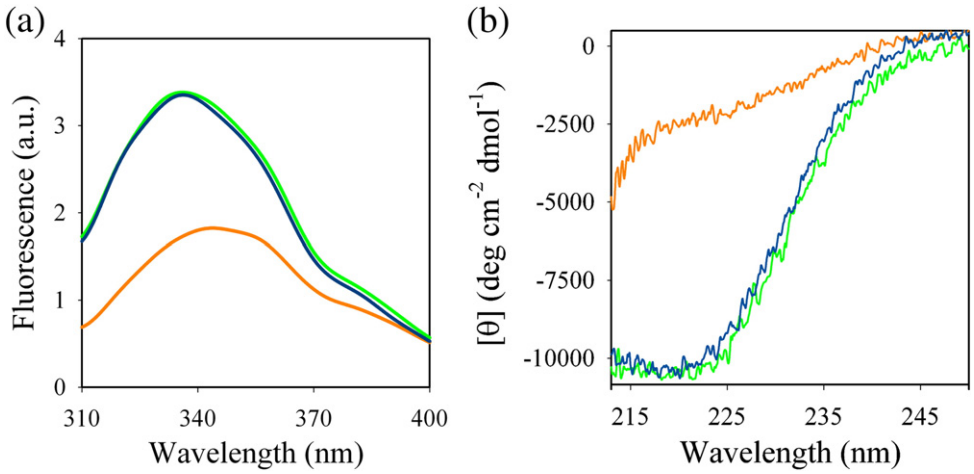

Fig. 2. Spectroscopic characterization of CopA unfolding. (a) Trp fluorescence of 2.5 μM CopA native (green line), denatured in 7.5 M GndHCl (orange line) and refolded by 1:12 dilution of denaturant (blue line). (b) Far-UV ellipticity of samples treated as in (a).

involves significant structural changes and, remarkably, is fully reversible.

In addition, the effect of GndHCl on the functionality of CopA was also assessed, in this case by measuring ATPase and *p*-nitrophenyl phosphatase (pNPPase) activities in the presence of different concentrations of denaturant. Figure 3 shows that both ATPase and pNPPase activities decreased to undetectable levels as GndHCl concentration increased. GndHCl half-maximal inhibitory concentrations were 0.42 M (ATPase) and 0.86 M (pNPPase). These were significantly lower than those required to induce structural changes (see the text below). It is remarkable that, as in the case of structural parameters (Fig. 2), both enzymatic activities, even after exposure to 6 M GndHCl, were fully restored following refolding of the protein by dilution of the denaturant.

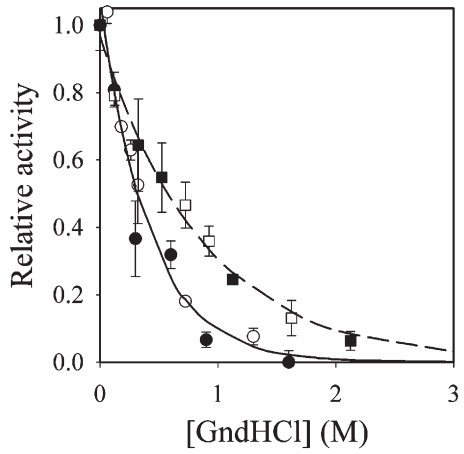

Fig. 3. ATPase and pNPPase activities after treatment with GndHCl. Two CopA aliquots (,) were incubated for 2 h at 25 °C with the indicated GndHCl concentrations, and then half-diluted in the reaction medium, keeping the GndHCl concentration constant. The other two (,) were identically treated but diluted in a medium without GndHCl, yielding a half dilution of denaturant before activity determination. After 2 h of preincubation at 25 °C, ATPase (,) and pNPPase (,) activities were measured. Lines in the figure are a guide to the eye. The activities are represented as a function of the final denaturant concentration and expressed relative to the values observed in the absence of denaturant: ATPase activity, 13.55±0.03 μmol mg^{-1} h^{-1}; pNPPase activity, 76.1±0.1 μmol mg^{-1} h^{-1}.

Thermodynamic analysis of equilibrium unfolding

The reversible unfolding of CopA made possible the thermodynamic analysis of the folding process. Unfolded fractions at various denaturant concentrations were calculated from fluorescence and CD data (Fig. 4a). The calculated thermodynamic parameters for the reversible cooperative transition at 25 °C were as follows: the standard Gibbs free-energy change extrapolated to water, $\Delta G_w^\circ = 12.9 \pm 2.8$ kJ mol^{-1}; its derivative with respect to the denaturant concentration, $m = 4.1 \pm 1.2$ kJ mol^{-1} M^{-1}; and the denaturant concentration at which the standard Gibbs free-energy change is zero, $C_m = 3.0 \pm 0.4$ M. A small decrease in ΔG_w° was observed as temperature increased (Fig. 4b). Fitting Eq. (5) to these data yielded a standard heat capacity change at constant pressure, $\Delta Cp_w^\circ = 0.93 \pm 0.07$ kJ mol^{-1} K^{-1}. The values obtained for the change in the thermodynamic functions corresponding to CopA unfolding appear lower than those expected for mesophilic soluble proteins of similar molecular mass;[14] however, the C_m value is right shifted compared to the same set.

Structural characterization of CopA unfolded state

Evaluating the structure of the unfolded state is critical to understanding the folding reaction.[25] Soluble quenchers of Trp fluorescence are widely used to evaluate protein unfolded states.[26] In the native enzyme, two populations of fluorophores were detected (Fig. 5): one, representing a 0.48±0.02 fraction, was not accesible to the quencher, while a

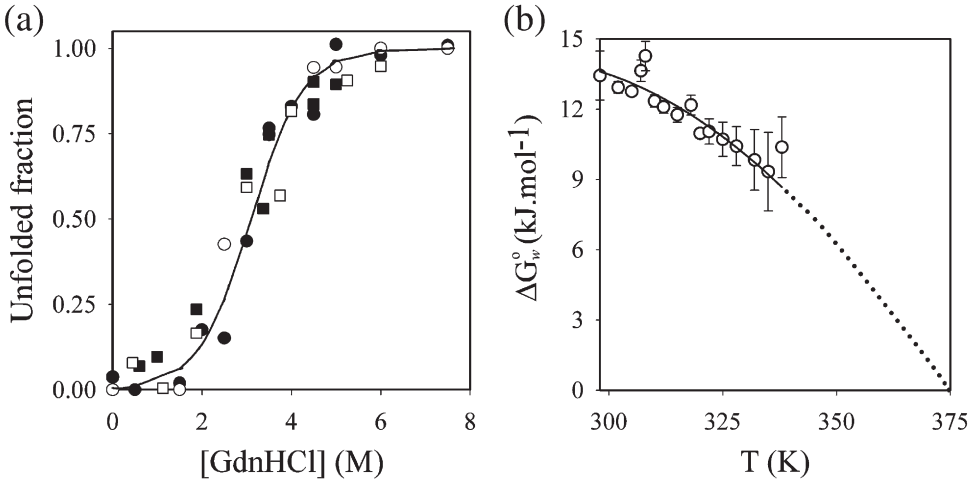

Fig. 4. Thermodynamics of CopA unfolding. (a) CopA (2.5 μM) was incubated with increasing concentrations of denaturant for 2 h at 25 °C, and Trp fluorescence and ellipticity were registered. Then, samples were diluted four times and, after 2 h of equilibration at 25 °C, Trp fluorescence and ellipticity were registered. Unfolded fractions were calculated from Trp fluorescence (,) and far-UV CD (,) data for unfolding (,) and refolding (,), assuming a two-state model. The continuous line is the graphical representation of the two-state model, with the best-fit parameter values indicated in the text. (b) Changes in ΔG_w° in the temperature range 25–65 °C calculated from Trp fluorescence data. The line is the graphical representation of Eq. (5) fitted to the experimental data.

fraction of accesible fluorescence (f_a) of 0.52±0.02 was quenched by NaI with a Stern–Volmer constant of 1.096±0.04 M^{-1}. GndHCl-denatured CopA fluorescence was quenched, to a greater extent, by NaI. The linear relation between the reciprocal of fluorescence intensity and the quencher concentration suggests a single Trp population (f_a=0.96±0.02) whose fluorescence is quenched with a Stern–Volmer constant of 2.47±0.02 M^{-1}. These results, together with the redshift of the Trp fluorescence spectra (Fig. 2a), suggest that Trp residues are largely exposed to the solvent in the structure of the denatured CopA.

The size of hydrophobic patches in the native and denatured states were explored using 1-anilinonaphtalene-8-sulfonate (ANS), an environment-sensitive fluorescent probe.[27] The enzyme was incubated with increasing concentrations of GndHCl and, after addition of ANS, the fluorescence spectra of the probe were recorded (Fig. 6). As expected from a process where tertiary structure is lost reducing the hydrophobic areas in the protein, ANS fluorescence decreased with increasing GndHCl concentration until a value close to zero. The concentration of the denaturant where half of the ANS fluorescence

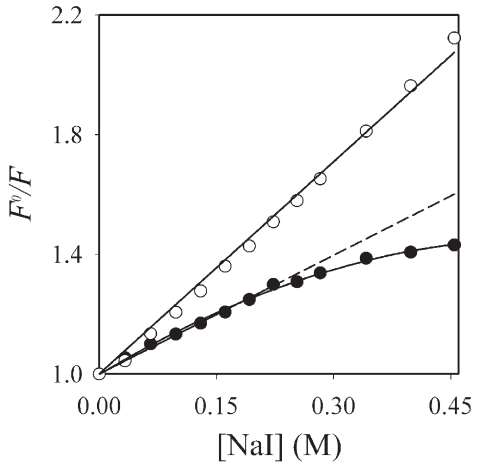

Fig. 5. NaI quenching of Trp fluorescence of CopA. The enzyme was incubated in the absence ( ) and in the presence ( ) of 7.5 M GndHCl for 2 h at 25 °C before the addition of the indicated amount of quencher. Continuous lines are the graphical representation of Eq. (4), with the best-fit parameter values indicated in the text. The broken line corresponds to the same representation with f_a=1.

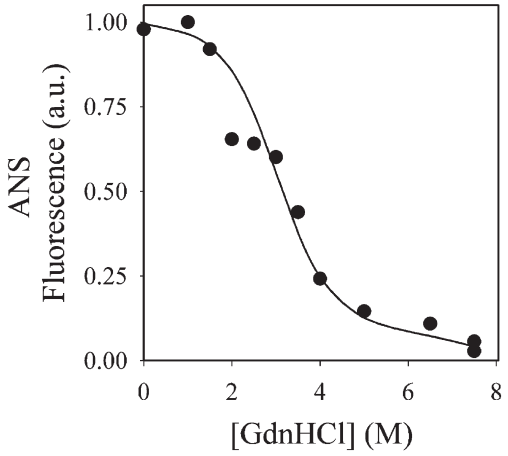

Fig. 6. Effect of GndHCl on the fluorescence of ANS–CopA complexes. CopA was incubated with increasing concentrations of GndHCl for 2 h at 25 °C before the addition of ANS (10:1 ANS:CopA mole ratio). The continuous line is the graphical representation of the unfolding equation for a two-state model, with thermodynamic parameter values estimated from fluorescence and CD data.

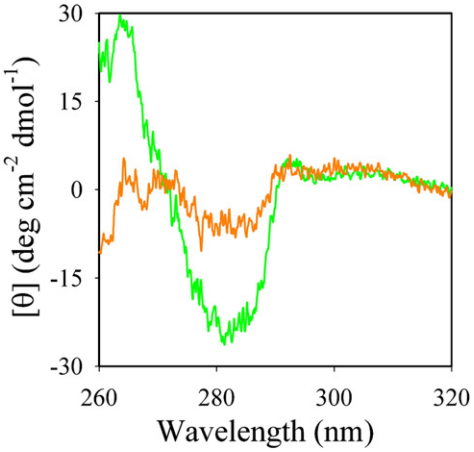

Fig. 7. Effect of GndHCl on the near-UV CD spectra of CopA. Two CopA samples of 6 μM native enzyme (green line) and 7.5 M GndHCl denatured enzyme (orange line) were incubated at 25 °C for 2 h, and then ellipticity in the range 260–320 nm was recorded.

persisted (C_m) was similar to those observed monitoring fluorescence and ellipticity (Fig. 4a). Further information on the tertiary structure of CopA after denaturation with GndHCl was obtained from the near-UV CD spectra of the protein (Fig. 7). Native enzyme ellipticity showed two major peaks at aproximately 260 nm and 280 nm. The first one corresponds to a structured environment around Phe residues, while the second reports the contibution of Tyr and Trp residues. Upon addition of the denaturant, these peaks significantly decreased, indicating a major disruption of tertiary structure.

The observed loss of the secondary and tertiary structures of CopA likely modifies its hydrodynamic properties. Evaluating this, native and denatured CopA were separated through a Superdex 200 column, and the absorbance and light scattering of protein fractions were monitored. The hydrodynamic radius at the elution peaks showed distributions of around 6 nm for native CopA and 11 nm for the denatured enzyme (Fig. 8), indicating a less compact conformation.

Discussion

In this report, we describe that *A. fulgidus* CopA, a thermophilic protein with large cytoplasmic domains and eight α-helical transmembrane segments, can be largely unfolded by treatment with GndHCl, and that it refolds to a fully active form upon dilution of the denaturant. The remarkable reversibility of this system allowed us to characterize the thermodynamics of the process. These features appear distinct from those that have been obtained for the few helical membrane proteins previously characterized, providing a novel precedent for understanding the stability of large membrane proteins and the mechanisms involved in thermostability.

GndHCl-induced unfolding of CopA

The study of unfolding and refolding processes in α-helical membrane proteins is challenging. To date, there are only four members of this group for which reversible unfolding has been thermodynamically characterized: bacteriorhodopsin,[9,10] diacylglycerol kinase,[11] the potassium channel KcsA,[12] and the disulfide-bond-reducing protein DsbB.[13,28] None of them was unfolded following classical denaturing strategies usually employed with water-soluble proteins.[4] The anionic detergent SDS has been used to denature bacteriorhodopsin, DsbB, and diacylglycerol kinase, while reversible unfolding of the KcsA channel was achieved using trifluoroethanol. However, membrane proteins forming inclusion bodies when heterologously expressed can occasionally be recovered by solubilization with chaotropic agents and renatured into bicellar systems in functional form.[29] Hence, attempts to characterize the urea-driven unfolding of helical membrane proteins have been reported. For example, it was described that opsin loses about 50% of tertiary structure and fluorescence upon urea treatment.[8] This behavior was attributed to the presence of relatively large extracellular domains that could be denatured by the chaotrope, leading to subsequent unfolding of the transmembrane domain. However, urea-treated opsin was unable to refold from this denatured state.

Chaotropic agents appear to stabilize unfolded states of water-soluble proteins by engaging in hydrogen bonds with charged groups and backbone carbonyl groups.[5] In this way, their inefficiency as denaturants of helical membrane proteins has been linked to the structural organization of transmembrane segments as α-helix bundles. This retains a moderately hydrophobic core by exposing highly hydrophobic surfaces to membrane lipids, thus preventing the access of polar chaotropes.[30] In the

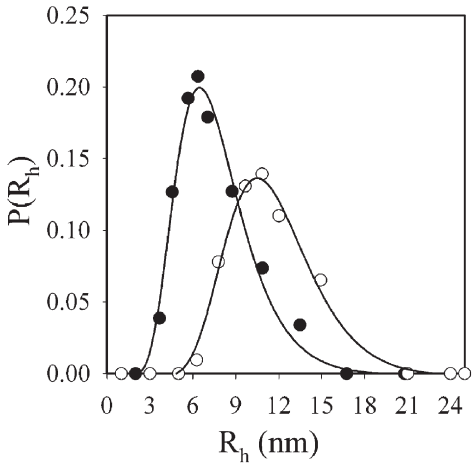

Fig. 8. Dynamic light-scattering characterization of CopA. Hydrodynamic radius distributions (normalized to unit area) for native ( ) and 7.5 M GndHCl denatured ( ) CopA.

case of CopA, it might be considered that it actively transports Cu^+ across biological membranes. The metal translocation mechanism involves the delivery of Cu^+ by the corresponding Cu^+ chaperone into binding sites at the cytoplasmic side of the membrane, followed by ATP-dependent ion occlusion inside the transmembrane domain and subsequent release at the extracellular side.[18,19,31] The docking of the Cu^+ chaperone with the ATPase requires a wide access path into transmembrane metal sites. Then, it can be hypothesized that Gnd^+ is able to access the transmembrane metal binding sites, and this initial step subsequently promotes the unfolding of the transmembrane region. Supporting this hypothesis, inhibition of the metal-ion-dependent ATPase activity is observed at GndHCl concentrations that do not produce significant changes in CopA structure.

The large unfolding of CopA following the interaction with GndHCl was evidenced by significant changes in spectroscopic properties: a large decrease in Trp fluorescence indicating their exposure to aqueous media, a shift in the center of the spectral mass of the fluorescence spectra from 342 nm to 350 nm with a quantum yield of 0.1 characteristic of unfolded proteins,[32] and a significant loss of secondary structure with a CD spectrum similar to that reported for soluble denatured proteins.[33] The fact that fluorescence and CD spectra recovered the native features upon dilution of denaturant shows that CopA unfolding is a reversible phenomenon.

While the significant spectral changes stress the large structural rearrangements occurring during unfolding and refolding of CopA, the full recovery of enzymatic activity highlights the total reversibility of the process. Catalytic activity is quite sensitive to enzyme unfolding (or misfolding). Examples of inactive proteins with native-like structures are well described.[34] Thus, biochemical activity clearly reports regaining of the fine structural features associated with the native form of an enzyme. CopA inactivates at GndHCl concentrations lower than those needed to produce spectroscopic changes. This suggests the existence of inactive CopA molecules with native spectroscopic signatures (i.e., inactivation is caused by small structural changes that are invisible to spectroscopic probes). However, CopA recovered its activity even after inactivation with 6 M GndHCl, indicating that fine secondary and tertiary native interactions are present. This is a strong evidence for the proper refolding of CopA.

Thermodynamics of CopA unfolding

CopA reversible unfolding is well described by a two-state model allowing the estimation of descriptive thermodynamic parameters. Surprisingly, the observed values of $\Delta G_w°$ and m were relatively lower than those expected of a previous analysis of mesophilic proteins of similar molecular masses that unfolds according to a two-state model.[35] However, the C_m value appears higher when compared to those from the same protein set. An approximately linear dependence of $\Delta G_w°$ on the number of protein residues is well established for the unfolding of small soluble monomeric proteins (smaller than 200 residues).[14] Booth and Curnow have indicated that bacteriorhodopsin, diacylglycerol kinase, and the potassium channel KcsA seem to fit within the linear relation observed for soluble proteins.[14] CopA $\Delta G_w°$ does not align with those of previously characterized membrane proteins. However, there is a small number of characterized large proteins (none of them is a membrane protein), and only in a few cases do they unfold following a two-state process. This set of large soluble proteins that reversibly unfold following a two-state model also shows unusually low values of $\Delta G_w°$ (e.g., human serum albumin for which $\Delta G_w°$ is a quarter of the value expected according its molecular mass).[36]

The pseudo-atomic models of CopA structure show five distinct structural domains or regions, including transmembrane α-helices.[18,23,24] Then, the description of unfolding in a two-state model has its limitations.[11] For instance, the transition region is likely to include an ensemble of unfolding intermediate states with closely related conformations indistinguishable by far-UV CD and Trp fluorescence. Moreover, as it was pointed out above, the region corresponding to low GndHCl concentrations contains inactive enzymes with an apparent native form, as suggested by spectroscopic characterization. The presence of these nondetectable unfolding intermediates could lead to a significant underestimation of both m and $\Delta G_w°$ values.[37]

The dependence of $\Delta G_w°$ on temperature, given by Eq. (5), results in so-called protein stability curves.[38] This analysis is particularly relevant given the thermophilic character of CopA.[16,17] The protein stability curve intercepts the temperature axis ($\Delta G_w° = 0$) at the thermal denaturation midpoint (T_m), and its convexity is given by the heat capacity change upon unfolding ($\Delta Cp_w°$). Protein stability curves can be altered to give larger T_m values in three main ways: (i) increasing enthalpic interactions ($\Delta H_w°$), which render a raised curve corresponding to a protein that is more stable at all temperatures; (ii) lowering $\Delta Cp_w°$, which produces a flatter curve characteristic of proteins with high T_m values without increased stability; and (iii) reducing the change in entropy for the folding transition ($\Delta S_w°$), which shifts the stability curve to higher temperatures.[39] In our case, there is only a small change in the apparent $\Delta G_w°$ for CopA unfolding between 25 °C and 65 °C, leading to a quite low $\Delta Cp_w°$ (0.93 kJ mol^{-1} K^{-1}). This stabilizing mechanism was also found for the thermophilic soluble protein RNase H.[40] Extrapolation of the CopA stability curve to higher temperatures provides an estimated T_m value of 376 K, which is consistent with the hyperthermophilic nature of this protein.

The structure of CopA denatured state

Considering the significance of the denatured state of proteins in understanding the folding process,[25] there have been increased efforts to improve its characterization. These have logically focused on small globular proteins. On the other hand, given the limited set of studied helical membrane proteins that reversibly unfolds, there is limited knowledge of their denatured state. The unfolded state obtained after the denaturation of the bacteriorhodopsin DsbB and diacylglycerol kinase with SDS conserves important amounts of secondary structure, although tertiary contacts seemed mostly disrupted. Conversely, KcsA loses almost all of its secondary structure upon denaturation with trifluoroethanol.

CopA appears to lose most of its secondary and tertiary structures content upon denaturation. This is evidenced by different spectroscopic techniques. For