Accumulation of partly folded states in the equilibrium unfolding of the pneumococcal choline-binding module C-LytA

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Choline-binding modules are present in some virulence factors and many other proteins of Streptococcus pneumoniae (Pneumococcus). The most extensively studied choline-binding module is C-LytA, the C-terminal moiety of the pneumococcal cell-wall amidase LytA. The three-dimensional structure of C-LytA is built up from six loop-hairpin structures forming a left-handed β-solenoid with four choline-binding sites. The affinity of C-LytA for choline and other structural analogues allows its use as an efficient fusion tag for single-step purification of hybrid proteins. In the present study, we characterize the folding and stability of C-LytA by chemical and thermal equilibrium denaturation experiments. Unfolding experiments using guanidinium chloride at pH 7.0 and 20°C suggest the existence of two partly folded states (I1 and I2) in the following model: N (native) → I1 ↔ I2. The N → I1 transition is non-co-operate and irreversible, and is significant even in the absence of a denaturant. In contrast, the I1 ↔ I2 transition is co-operative and reversible, with an associated free-energy change (ΔG°) of 30.9 ± 0.8 kJ·mol⁻¹. The residual structure in the I2 state is unusually stable even in 7.4 M guanidinium chloride. Binding of choline stabilizes the structure of the native state, induces its dimerization and prevents the accumulation of the I1 species ([N]I1 = [I1], ΔG° = 50.1 ± 0.8 kJ·mol⁻¹). Fluorescence and CD measurements, gel-filtration chromatography and limited proteolysis suggest that I1 differs from N in the local unfolding of the N-terminal β-hairpins, and that I2 has a residual structure in the C-terminal region. Thermal denaturation of C-LytA suggests the accumulation of at least the I1 species. These results might pave the way for an effective improvement of its biotechnological applications by protein engineering.

Key words: affinity tag, choline-binding module, C-LytA, partly folded state, protein folding, β-solenoid.

INTRODUCTION

LytA amidase, the major murein hydrolase from Streptococcus pneumoniae, catalyses the cleavage of the N-acetylMuramoyl-L-alanine bond of the peptidoglycan backbone [1]. It is involved in the separation of the daughter cells at the end of cell division [2] and in cellular autolysis, where it mediates the release of toxins that damage the host tissues and allows the entry of pneumococcal cells, leading to pneumonia, meningitis or bacteraemia [3–5]. LytA amidase LytA. The three-dimensional structure of C-LytA is built from six loop-hairpin structures forming a left-handed β-solenoid with four choline-binding sites. The affinity of C-LytA for choline and other structural analogues allows its use as an efficient fusion tag for single-step purification of hybrid proteins. In the present study, we characterize the folding and stability of C-LytA by chemical and thermal equilibrium denaturation experiments. Unfolding experiments using guanidinium chloride at pH 7.0 and 20°C suggest the existence of two partly folded states (I1 and I2) in the following model: N (native) → I1 ↔ I2. The N → I1 transition is non-co-operative and irreversible, and is significant even in the absence of a denaturant. In contrast, the I1 ↔ I2 transition is co-operative and reversible, with an associated free-energy change (ΔG°) of 30.9 ± 0.8 kJ·mol⁻¹. The residual structure in the I2 state is unusually stable even in 7.4 M guanidinium chloride. Binding of choline stabilizes the structure of the native state, induces its dimerization and prevents the accumulation of the I1 species ([N]I1 = [I1], ΔG° = 50.1 ± 0.8 kJ·mol⁻¹). Fluorescence and CD measurements, gel-filtration chromatography and limited proteolysis suggest that I1 differs from N in the local unfolding of the N-terminal β-hairpins, and that I2 has a residual structure in the C-terminal region. Thermal denaturation of C-LytA suggests the accumulation of at least the I1 species. These results might pave the way for an effective improvement of its biotechnological applications by protein engineering.

Key words: affinity tag, choline-binding module, C-LytA, partly folded state, protein folding, β-solenoid.

Abbreviations used: ANS, 8-anilinothalene-1-sulphonic acid; ChBD, choline-binding domain; ChBR, choline-binding repeat; DSC, differential scanning calorimetry; GdmCl, guanidinium chloride; GdmSCN, guanidinium thiocyanate.

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Purified protein was subsequently dialysed at 4°C to improve the biotechnological potential of C-LytA. Finally, we discuss the possible application of these results to another intermediate that accumulates on heat denaturation [13].

GDmCl, GDmSCN (guanidinium thiocyanate) and potassium dichromate were purchased from VWR International Eurolab (Mollet del Valles, Spain). ANS (8-anilinonaphthalene-1-sulphonic acid) was obtained from Sigma (Madrid, Spain). Choline chloride, trypsin, chymotrypsin, Dextran Blue and DEAE-cellulose were from Sigma. Owing to the hygroscopic properties of choline, concentrated stock solutions were always prepared from a freshly opened bottle and stored in aliquots at −20°C.

Protein purification

C-LytA was purified by affinity chromatography from the overproducing Escherichia coli strain RB791 [pCE17] as described previously [7]. Purified protein was subsequently dialysed at 4°C against 20 mM sodium phosphate buffer (pH 7.0) plus 50 mM NaCl to remove the bound choline. Dialysis performed at 4°C resulted in reversible precipitation of the protein inside the dialysis bag, especially when the sample was concentrated. Protein concentration was determined spectrophotometrically as described previously [7] using a molar absorption coefficient of 280 nm of 62.540 M−1·cm−1.

CD spectroscopy

CD experiments were performed in a Jasco J-810 spectropolarimeter fitted with a thermostatically controlled cell holder and interfaced with a Neslab RTE-111 water bath. Isothermal wavelength spectra were acquired at a scan speed of 50 nm·min−1 with a response time of 2 s and averaged over at least six scans at 20°C. Protein concentration was 6.3 μM and the cuvette path length was 1 or 2 mm. For GDmCl titrations, aliquots from an 8.0 M denaturant stock solution were added stepwise and incubated for 5 min before recording the wavelength spectra. Ellipticities ([θ]) are expressed in units of degrees·cm2·dmol−1, using the residue concentration of the protein. With GDmCl present, spectra could not be recorded below 215 nm, due to the high absorbance of the sample.

CD-monitored thermal denaturation experiments were performed in a 1 mm path cell. The sample was layered with mineral oil to avoid evaporation, and the heating rate was 60°C·h−1. When a second scan was required, the heated sample was cooled down in the same cuvette and left for low-temperature equilibration for at least 1 h.

Fluorescence spectroscopy

Fluorescence measurements were performed on an Aminco SLM8000 spectrofluorimeter using a 5 mm × 5 mm path-length cuvette and a protein concentration of 6.3 μM. Tryptophan emission spectra were obtained using an excitation wavelength of 280 nm, with excitation and emission slits of 4 nm and a scan rate of 60 nm·min−1. We monitored the ratio of intensities at 325 and 365 nm to avoid errors caused by slight changes in protein concentration. For GDmCl or GDmSCN titrations, aliquots from an 8.0 M denaturant stock solution were added stepwise and incubated for 5 min before starting measurements. ANS fluorescence spectra were recorded on excitation of the probe at 380 nm and emission was registered between 400 and 600 nm, using excitation and emission slits of 4 nm and a scan rate of 60 nm·min−1. Final ANS concentration was 0.1 mM.

Data analysis

Equilibrium unfolding was fitted by the method of least squares to the corresponding two-state process according to eqn (1) [20]:

$$\Delta G = \Delta G^0 - m \text{ [denaturant]}$$

where ΔG and ΔG0 are the free energies of unfolding in the presence and absence of denaturant respectively and m represents the dependence of ΔG with respect to [denaturant].

Size-exclusion chromatography

Samples of 65 μl containing 200 μg of C-LytA were loaded on to a Sephadex G-75 column (26 cm × 0.9 cm) (Sigma) or a Toyopearl HW-55F column (50 cm × 2.5 cm) (Tosoh, Tokyo, Japan) equilibrated in 20 mM sodium phosphate buffer (pH 7.0) plus 200 mM NaCl for different GDmCl and/or choline concentrations, and were run with the same buffer at a flow rate of 0.5 ml·min−1 at 20°C. Fractions of 500 μl were collected and their absorbance was measured at 280 nm. Exclusion (6.3 ml for Sephadex, 28.6 ml for Toyopearl) and total (21.0 ml for Sephadex, 68.0 ml for Toyopearl) volumes were determined with Dextran Blue and potassium dichromate respectively.

Limited proteolysis

Trypsin and chymotrypsin digestion of C-LytA (0.3 mg·ml−1) was performed at 25°C in 20 mM phosphate buffer (pH 8.0) using a 1:50 (w/w) protease/protein ratio. Aliquots were withdrawn at different times and the reaction was stopped by the addition of SDS/PAGE loading buffer [21]. Samples were stored frozen at
– 20°C until electrophoresis. Purification of the chymotrypsin-resistant C-LytA fragment [C-LytA(Δ32)] was performed after a preparative 16 h digestion for the same protease/protein ratio. The sample was applied on to a DEAE-cellulose column and purified as described for the full-length protein. The chymotrypsin-resistant fragment was dialysed against 20 mM phosphate buffer (pH 7.0), and subsequently characterized by Edman sequencing in a 477A analyser (Applied Biosystems) and matrix-assisted laser-desorption ionization–time-of-flight MS. The laser desorption/ionization experiments were performed on a BIFLEX III time-of-flight instrument (Bruker-Franzen Analytik, Bremen, Germany) operated in the positive mode. Peptides were analysed in the reflectron mode after external calibration using myoglobin as the standard using a saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/water (1:2, v/v) with 0.1% trifluoroacetic acid as the matrix, and the samples were analysed in the linear mode with SA [sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid)] as the matrix. Typically, 50–100 laser shots were summed into a single mass spectrum. Equal volumes (0.5 µl) of the sample solution and the matrix were spotted on the target and air-dried.

RESULTS

Equilibrium GdmCl titrations monitored by CD and fluorescence

The influence of GdmCl on the structure of C-LytA was first analysed by CD. It should be pointed out now that, although some specific dimerization of C-LytA may take place at neutral pH even in the absence of choline, this event is negligible at the concentrations used in our experiments (6.3 µM), where the predominant species is the monomer (> 90%) [13]. As shown in Figure 2(A), the far-UV CD spectrum of C-LytA at pH 7.0 and 20°C displays a maximum at 223 nm and a shoulder centred at 235 nm. This unusual spectrum does not reflect the real content of secondary structure, but accounts instead for a high contribution of aromatic side chains in the far-UV region [7,12]. Surprisingly, we found that the CD spectrum of C-LytA under these conditions was not completely reproducible, with moderate differences in the ellipticity depending on the age of the sample and/or the number of freeze–thaw cycles. This point will be discussed below. On the other hand, addition of saturating amounts of choline (150 mM) induces a positive ellipticity centred at 230 nm (Figure 2A). The spectrum is very different from that of a heat-unfolded C-LytA, which shows the characteristic features of a random coil (Figure 2A). Any possible CD artifact due to the high ionic strength provided by GdmCl can be ruled out, since the addition of 1.5 M NaCl to heat-unfolded C-LytA barely changes its CD spectrum (results not shown). Therefore, the residual structure remaining at high GdmCl concentrations was taken as indicative of a second, partly folded, state that was termed I₂.

Figure 2B depicts the intrinsic fluorescence properties of C-LytA at 20°C and pH 7.0. The spectrum displays a maximum at 344 nm. In the presence of 150 mM choline, the intensity of the spectrum increases and the maximum is shifted to 333 nm, reflecting the burial of the aromatic side chains involved in the binding of the ligand [10]. The change in fluorescence signal of C-LytA after GdmCl titration is also shown in Figure 3(A). The curve displays a sigmooidal shape, with a GdmCl midpoint of 3.52 M (close to that obtained on monitoring [θ]_226), with virtually no variation in the fluorescence spectrum from 0 to 2.0 M GdmCl (populated by the I₁ species) (Figure 2B). On the other hand, in 7.4 M GdmCl, the fluorescence spectrum shows a decreased intensity and a red shift to 354 nm, reflecting the exposure of tryptophan residues to the solvent on unfolding (Figure 2B).
150 mM choline. Symbol scheme as in (C). Solid lines are the fittings of fluorescence results to eqn (1).

phosphate buffer (pH 7.0) by monitoring the ellipticity signal at 219 nm (H17033), i.e. conformations with near-native secondary structure. ANS is normally used to check the presence of ‘molten globule’ states [22], i.e. conformations with near-native secondary structure but with a disrupted tertiary structure, allowing the binding of the probe to the solvent-accessible hydrophobic cores of the protein and therefore modifying its spectral features. However, we did not observe any significant binding of ANS to C-LytA at any GdmCl concentration (results not shown), suggesting that I₁ and I₂ may not be molten globule-type intermediates.

From the above experiments, it follows that two partly folded states, I₁ and I₂, accumulate in the presence of approx. 2.0 and 7.4 M GdmCl respectively at 20 °C and pH 7.0. Other species between I₁ and the fully unfolded form cannot be detected at this temperature with this denaturant. An increase in temperature from 20 to 95 °C in the presence of 7.4 M GdmCl results in the progressive non-co-operative loss of the positive CD signal (results not shown), suggesting that the residual structure of I₁ is of decreased compactness. In an attempt to further unfold I₁ at 20 °C, a stronger denaturant such as GdmSCN was used [23]. A GdmSCN titration monitored by fluorescence spectroscopy showed a single sigmoidal transition with a midpoint of 1.1 M, followed by a linear, slightly sloping baseline up to 5.6 M denaturant (Figure 3B). This could mean that the I₁ structure persists even under these conditions, that the unfolding transition is not co-operative, or that it is barely detectable by fluorescence spectroscopy. Unfortunately, these alternatives could not be tested by CD, since a suitable CD-monitored transition could not be obtained due to the high absorbance of GdmSCN.

To check the reversibility of the unfolding transitions, a sample of C-LytA was incubated in 2.0 or 7.4 M GdmCl for 30 min and subsequently dialysed against phosphate buffer. In all the cases, the resulting CD spectrum turned out to be fully coincident with the spectrum recorded at 2.0 M GdmCl, i.e. that corresponding to I₁ (results not shown). Moreover, accumulation of I₁ may also occur under non-denaturing conditions, with aged or repeatedly freeze-thawed samples explaining the lack of reproducibility of the native CD spectrum described above. In accordance with this, the fact that the [θ]₂₂₆ titration within the 0–2.0 M GdmCl range is linear and shows no pre-transitional baseline strongly suggests the existence of a pre-established N → I₁ transition which is very sensitive to low denaturant concentrations.

The results shown so far suggest that the N → I₁ transition is non-co-operative and essentially irreversible in the absence of choline, involving the unfolding of a part of C-LytA that either involves solvent-exposed tryptophans or lacks these residues. This would explain why the absorption of I₁ equilibrium without interference from the N → I₁ transition, fluorescence results were fitted into a two-state unfolding equation using the linear extrapolation model (eqn 1). Results of the fitting are shown in Figure 3A and Table 1, with a calculated free energy of unfolding [ΔG°(I₁)]₂₂₆ of (30.9 ± 0.8) kJ · mol⁻¹. Results obtained with GdmSCN (Figure 3B) yielded similar ΔG° values [(28.4 ± 2.1) kJ · mol⁻¹], although the fits are characterized by larger errors, especially in the m value (Table 1).

![Figure 3](image-url)

The measurement of the fluorescence of the hydrophobic probe ANS is normally used to check the presence of ‘molten globule’ states [22], i.e. conformations with near-native secondary structure but with a disrupted tertiary structure, allowing the binding of the probe to the solvent-accessible hydrophobic cores of the protein and therefore modifying its spectral features. However, we did not observe any significant binding of ANS to C-LytA at any GdmCl concentration (results not shown), suggesting that I₁ and I₂ may not be molten globule-type intermediates.

Table 1 Thermodynamic quantities of the equilibrium denaturation of C-LytA

<table>
<thead>
<tr>
<th>Protein</th>
<th>Denaturant</th>
<th>m (kJ · mol⁻¹ · M⁻¹)</th>
<th>[Denaturant]I₁/M (M)</th>
<th>Transition</th>
<th>ΔG° (kJ · mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>GdmCl</td>
<td>8.8 ± 0.4</td>
<td>3.5 ± 0.1</td>
<td>I₁ ↔ I₂</td>
<td>30.9 ± 0.8</td>
</tr>
<tr>
<td>Native</td>
<td>GdmSCN</td>
<td>25.9 ± 2.1</td>
<td>1.1 ± 0.1</td>
<td>I₁ ↔ I₂</td>
<td>28.4 ± 2.1</td>
</tr>
<tr>
<td>Native + choline (150 mM)</td>
<td>GdmCl</td>
<td>10.4 ± 0.8</td>
<td>4.8 ± 0.1</td>
<td>N₁ • ch ↔ (I₁) • ch</td>
<td>50.1 ± 0.8</td>
</tr>
<tr>
<td>Native + choline (150 mM)</td>
<td>GdmSCN</td>
<td>21.7 ± 1.3</td>
<td>2.0 ± 0.1</td>
<td>N₁ • ch ↔ (I₁) • ch</td>
<td>43.5 ± 1.3</td>
</tr>
</tbody>
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Effect of choline on unfolding

Figure 3(C) shows the GdmCl-induced unfolding curves of C-LytA in the presence of 150 mM choline, a concentration sufficient to saturate all the choline-binding sites [13]. In this case, the initial drift of the \([\theta]_\text{219}\) signal was drastically reduced and, as a consequence, the CD denaturation curves were nearly independent of the wavelength monitored and virtually coincident with the fluorescence titration. A significant increase in the \([\theta]_\text{226}\) transition midpoint is evident (4.70 M). The GdmCl denaturation turned out to be completely reversible on dialysis of the denaturant, while keeping the choline concentration at 150 mM (results not shown). The final conformational state accumulated at 7.4 M GdmCl displays a CD spectrum that is superimposable with that of I2 (Figure 2A). These results would indicate the relative destabilization of I1, so that unfolding proceeds mostly from the dimeric native state to the I2 species following an apparent two-state transition. The fact that the fluorescence spectrum recorded at 7.4 M GdmCl plus 150 mM choline displays a modest (10%) but very reproducible increase in intensity (not ascribed to experimental error) when compared with that of the unligated form (Figure 2B) suggests that the residual structure in the I2 state is able to retain some ligand molecules bound to it.

Size-exclusion chromatography

This technique provides information on the overall shape of polypeptides, and has been usually employed to track the equilibrium unfolding of proteins [24]. Figure 4(A) shows the elution profile of C-LytA in a Sephadex G-75 column at pH 7.0 and 20°C equilibrated in different GdmCl concentrations. Experiments could not be performed at denaturant concentrations higher than 5.6 M due to severe flow restrictions in the column. In the absence of a denaturant, C-LytA is eluted as a single broad peak. It should be mentioned here that gel-filtration chromatography is not useful to obtain information about the molecular mass of C-LytA, as described before [25], probably due to the non-globular elongated shape of the protein [10]. On increasing the concentration of GdmCl, the elution peak shifts to lower volumes, in accordance with a concomitant higher proportion of extended molecules. Interestingly, we observed, in some cases, a minor peak centred at a lower volume in the range between 1.0 and 3.0 M GdmCl.
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comparable with the full-length protein despite the loss of one

cellulose suggests that its overall affinity to choline and DEAE is

MS analysis of the fragment that indicated a molecular mass of

12 209.8 Da. The proteolytic-resistant species was named C-LytA-

after a 16 h incubation (Figure 5, top panel), which was further

purified by affinity chromatography on DEAE-cellulose (see the

Experimental section above). Five cycles of Edman degradation

yielded the sequence MLADR, coincident with residues 33–37

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scopy. The far-UV CD spectrum of the C-LytA(Δ32) is remark-

ably similar to that of I1, (Figure 5, middle panel). Moreover, a

GdmCl titration of C-LytA(Δ32) followed by [θ]219 lacks the char-

acteristic low-co-operativity decrease in the signal up to 2.0 M

assigned to the N → I1 transition, but preserves the co-operative

change ascribed to the I1 ⇔ I2 equilibrium as monitored by [θ]226

(Figure 5, bottom panel). These results strongly suggest that

C-LytA(Δ32) may represent the I1 species, and therefore points to

the unfolding of the N-terminal part of C-LytA as leading to

the accumulation of this state.

Limited proteolysis

Our results show that the accumulation of I1 in the absence of

choline can be achieved either spontaneously or under mild de-
naturing conditions (2.0 M GdmCl). Therefore the stability of

the region of the protein that is unfolded in I1 must be low when

compared with the rest of the protein and probably more accessible
to proteases. In an attempt to further characterize this inter-

mediate, a limited proteolysis experiment was performed on

C-LytA using trypsin and chymotrypsin at 20°C. Whereas the

protein is fairly resistant to trypsin under these conditions (results

not shown), a chymotrypsin digest yielded a stable core fragment

after a 16 h incubation (Figure 5, top panel), which was further

purified by affinity chromatography on DEAE-cellulose (see the

Experimental section above). Five cycles of Edman degradation

yielded the sequence MLADR, coincident with residues 33–37

of the full-length protein. This demonstrates that there is a site

especially accessible to chymotrypsin in the middle of the loop

corresponding to the ChBR2 (Figure 1), giving rise to a fragment

of theoretical mass 12 197.3 Da. This is in accordance with an

MS analysis of the fragment that indicated a molecular mass of

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(Δ32). The fact that C-LytA(Δ32) could be purified on DEAE-

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Effect of low choline concentration on the unfolding equilibria

The three-dimensional structure of choline-saturated C-LytA

reveals that the C-terminal repeat (ChBR6) is atypical with respect
to the others, as it lacks tryptophans, its position deviates from

the solenoid axis and constitutes the choline-induced dimerization

surface [10,11]. In the monomeric form (i.e. with choline absent),

this hairpin may have few tertiary contacts with the rest of the

molecule and so would be only marginally stable. It could there-

fore be another candidate for that part of the protein that unfolds

in the N → I1 transition. This hypothesis is consistent with the

unfolding being insensitive to Trp fluorescence or [θ]226 measure-

ments as this part is tryptophan-deficient, but contrasts with the

results from limited proteolysis discussed above. To decide

between these possibilities, we performed fluorescence- and CD-

monitored GdmCl unfolding titrations at low concentrations of

choline (1.5 and 3.0 mM), where the occupation of the high-

affinity-binding sites takes place exclusively [13]. This induces
dimerization (up to 75 and 90 % respectively [13]) that should

stabilize the ChBR6 against GdmCl denaturation. Since the
efficacy of C-LytA in the absence of additives is variable depend-
ing on the age of the sample (see above), we performed a control
experiment on the same day using the same batch of protein but

without choline. As depicted in Figure 6(A), the curves obtained

by plotting [θ]226 are almost superimposable in all three cases

(0, 1.5 and 3.0 mM choline), together with a fluorescence titration

performed in 3.0 mM choline. In contrast, substantial differences

are seen at 219 nm (Figure 6B). In this case, the linear loss of
efficacy before the main co-operative change presents an en-
hanced amplitude on the addition of 1.5 and 3.0 mM choline,
are both dimers in the presence of choline. To verify the oligomerization state of the I₂, we performed a size-exclusion chromatography experiment in 6 M GdmCl in the absence and presence of 150 mM choline. Owing to the expanded nature expected for the I₁ state, the more suitable Toyopearl HW-55F resin was used instead of Sephadex G-75. As shown in Figure 4(D), binding of choline to I₁ causes a significant increase in the hydrodynamic radius that is compatible with the dimerization of the protein under these conditions. Hence, assuming GdmCl does not interfere with dimerization, spectroscopic results obtained in 150 mM choline (Figure 3C) were fitted to a two-state transition between dimers [N₂ and (I₁)₂] (eqn 1). The associated free-energy change was calculated to be 50.1 ± 0.8 kJ·mol⁻¹ (monomer)⁻¹ (Table 1), by assuming that the regions of C-LytA undergoing the unfolding transition behave as independent co-operative units within the dimer. Similar results were obtained with GdmSCN-induced denaturation (43.5 ± 1.3 kJ·mol⁻¹) (Figure 3B, Table 1).

Correspondence with thermal unfolding intermediates

DSC experiments have previously shown [13] that the thermal denaturation of C-LytA in the absence of choline involves the accumulation of two intermediates, which in the present study we refer to as I₁ and I₉. The N → I₁ transition is irreversible and displays a small broad peak centred at approx. 40°C, whereas the I₁ ↔ I₉ ↔ U transitions give rise to a single asymmetric peak centred at 61°C, I₉ being metastable at these temperatures. We examined the relationship between these heat-induced intermediates and those described in the present work.

Figure 7(A) shows the denaturation profile of C-LytA after increasing the temperature by monitoring the CD signal at 223 nm. This wavelength is the optimal wavelength for simultaneously following the unfolding of both secondary and tertiary structures. A first scan of a freshly purified sample displays a biphasic transition with temperature midpoints (Tₘ) at 43.6 ± 0.7 and 63.5 ± 0.4°C. These values are very close to those reported from calorimetric measurements (40 and 61°C respectively) [13], taking into account (i) that the second CD-monitored curve may comprise two overlapping transitions that can only be discerned by DSC; (ii) the necessarily higher precision of the DSC measurements and (iii) the fact that effects from any aggregation are more evident in DSC owing to the higher concentrations of protein used and tend to shift the temperature midpoints to lower values.

The CD spectrum recorded at 85°C is characteristic of a fully unfolded protein (Figure 2A). Cooling down the sample did not restore the native CD spectrum (Figure 7B), as described before [12]. However, there are many similarities between the recovered spectrum and that of I₁ accumulated at 2.0 M GdmCl (Figure 7B). The difference in intensity may be ascribed to the irreversible aggregation of a part of the sample [13]. A second temperature scan performed on the same sample yielded virtually only the second transition [Tₘ = (62.5 ± 0.3)°C] (Figure 7A). These results suggest that the calorimetric I₁ intermediate might correspond to the I₁ form, and that this is the predominant form of C-LytA on refolding from the heat-unfolded conformation, as also occurs with the removal of GdmCl (see above). To confirm this hypothesis, a GdmCl titration was performed at 20°C on a protein sample previously heated up to 55°C. As shown in Figure 7(C), the denaturation curve monitoring [θ]₁₂₃ certainly lacks the characteristic loss of a signal up to 2.0 M assigned to the N → I₁ transition. Nevertheless, the co-operative change ascribed to the I₁↔I₉ equilibrium is preserved as detected by [θ]₁₂₃; although, in this case, a moderately sloping pre-transition baseline can also be seen (Figure 7C). It could be argued that the 40°C DSC peak displays a very low intensity when compared with the 61°C

**Figure 6 GdmCl denaturation of C-LytA at low choline concentration**

The far-UV CD signal was followed at 226 nm (A) and 219 nm (B), in the absence of choline ( ), in 1.5 mM choline ( ) and in 3.0 mM choline ( ). A fluorescence-monitored titration at 3.0 mM choline is also overlaid in (A).

although it still takes place within approximately the same GdmCl range as in the absence of ligand. This suggests that binding of high-affinity choline does not change the observed unfolding scheme of unliganded C-LytA, that is, it does not preclude the accumulation of I₁ even though the ChBR6 motif involved in dimerization is stabilized. The augmented amplitude ascribed to the N → I₁ change is in accord with the increase in calorimetric enthalpy of the low-temperature endotherm observed by DSC (differential scanning calorimetry) at low choline concentration [13] (see below), which was explained in terms of more molecules undergoing such a transition. On the other hand, a size-exclusion chromatography experiment performed in the presence of 3.0 mM choline and 2.0 M GdmCl showed a single broad peak centred at 8.1 ml (Figure 4C), a lower elution volume compared with the unliganded I₁ (10.7 ml, Figure 4C), confirming that choline and 2.0 M GdmCl showed a single broad peak centred at approx. 40°C, whereas the I₁ ↔ I₉ ↔ U transitions give rise to a single asymmetric peak centred at 61°C, I₉ being metastable at these temperatures. We examined the relationship between these heat-induced intermediates and those described in the present work.

Characterization of the association state of I₂

Since no significant changes are observed in the main co-operative transition ascribed to the unfolding of I₁ when compared with the situation in the absence of choline (Figure 6A), this suggests that the GdmCl-induced transitions do not affect the dimerization interface in the I₁ ↔ I₂ equilibrium and that I₁ and I₂

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endotherm [13], while the two CD-monitored transitions are of comparable amplitudes (Figure 7A). However, we observed that the profiles obtained with aged samples contained a significantly decreased first transition, concomitant with our hypothesis that the native state irreversibly converts into I1 in a slow timescale (see above). On the other hand, due to the metastability of I1, a comparison with I2 is not possible. Finally, it should be mentioned that on the addition of 150 mM choline, the CD-monitored thermal unfolding of C-LytA displays a single co-operative curve at higher temperatures (results not shown), in accordance with the DSC experiments [13].

Figure 7 Correspondence of thermodynamic and thermal intermediates of C-LytA

(A) Thermal denaturation of C-LytA followed by ellipticity at 223 nm. First and second scans are represented by circles and squares respectively. Solid lines are sigmoidal fittings with no physical significance. (B) Far-UV CD spectra of C-LytA at 20°C after a first heating scan (dashed line) and a non-heated sample in 2.0 M GdmCl (solid line). (C) CD-monitored GdmCl titration of non-heated C-LytA (filled symbols) and protein heated and cooled down (open symbols) at 219 nm (circles) and 226 nm (squares).

DISCUSSION

ChBDs are found in many microbial enzymes that act on the rigid cell wall structure. They seem to have evolved to achieve a high-affinity, yet dynamic, mechanism of attachment to their substrate. With this, evolution might have selected the genetic concatenation of independent ChBRs for functioning rather than optimizing a single sequence with point mutations. Given this particular architecture, it would not be surprising if denaturation of ChBDs is a multistep process, arising from the independent unfolding of the ChBRs.

In the present study, we have investigated the structure and stability of the choline-binding module of the LytA amidase (C-LytA) by equilibrium denaturation experiments using GdmCl, GdmSCN and heat, in both the absence and presence of choline. To explain the complex unfolding equilibria, we propose a general scheme that is depicted in Figure 8. This scheme involves at least three structured species, the ‘native’ (fully folded) form (N) and two partly folded states (I1 and I2) that are able to dimerize with choline. The transition from N to I1 is irreversible in the absence of a ligand (Figure 8, left path), and I1 is the only species that accumulates on refolding of the chemical- or heat-denatured protein (Figure 7) or on storage of purified samples for more than 20 days at −20°C or overnight at room temperature (20°C). The fact that the N → I1 transition monitored by [θ]219 is not co-operative and lacks a defined pre-transitional baseline also suggests that C-LytA in 0 M GdmCl may already be in a partially unfolded form. Limited proteolysis experiments (Figure 5) together with denaturation experiments performed at low choline concentration (Figure 6) suggest ChBR1 and, probably, the ChBR2 motifs are the substructures in N whose unfolding results in I1. Since the tryptophan environments of N and I1 are very similar (Figure 2), these residues in the N-terminal region of C-LytA must be solvent-exposed in the absence of choline. In any case, I1 must preserve most of the C-LytA structure, since it unfolds in a co-operative manner with GdmCl (Figure 3A), GdmSCN (Figure 3B) or temperature increases (Figure 7A), with a free energy of unfolding of approx. 31 kJ·mol−1 at 20°C (Table 1). On the other hand, the residual structure at 7.4 M GdmCl (Figure 2A) is capable of interacting with choline (Figure 2B) and allows dimerization in the presence of the ligand (Figure 4D). Such structures may involve the C-terminal part of the protein, as discussed below.

Low concentrations of choline saturate the high-affinity sites in C-LytA and induce its dimerization through the binding of one molecule of ligand per monomer [13]. According to our results and on the basis of the three-dimensional structure of C-LytA, it seems reasonable to postulate (Figure 8, middle path) that at least one of these high-affinity sites is located in the vicinity of the C-terminal dimerization hairpin, i.e. between ChBRs 4 and 5 (Figure 5) together with denaturation experiments performed in a partially unfolded form. Limited proteolysis experiments (Figure 5) together with denaturation experiments performed at low choline concentration (Figure 6) suggest ChBR1 and, probably, the ChBR2 motifs are the substructures in N whose unfolding results in I1. Since the tryptophan environments of N and I1 are very similar (Figure 2), these residues in the N-terminal region of C-LytA must be solvent-exposed in the absence of choline. In any case, I1 must preserve most of the C-LytA structure, since it unfolds in a co-operative manner with GdmCl (Figure 3A), GdmSCN (Figure 3B) or temperature increases (Figure 7A), with a free energy of unfolding of approx. 31 kJ·mol−1 at 20°C (Table 1). On the other hand, the residual structure at 7.4 M GdmCl (Figure 2A) is capable of interacting with choline (Figure 2B) and allows dimerization in the presence of the ligand (Figure 4D). Such structures may involve the C-terminal part of the protein, as discussed below.

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the scheme would involve only the native and I₂ states (Figure 8, right path).

The I₁ and I₂ species seem to retain the aggregation state induced by choline (Figure 8). Size-exclusion chromatography experiments in the presence of choline show I₁ and I₂ eluting as higher-molecular mass species than in the absence of ligand (Figures 4C and 4D). Moreover, the GdmCl-induced I₁ \( \Rightarrow \) I₂ transition monitored by fluorescence or \([\theta]_{226}\) displays the same energetics as those obtained for the unligated form (Figure 6A). This may not be expected if a coupled unfolding + monomerization process were involved. Moreover, despite the similarities in the CD spectra (Figure 2A), the fluorescence characteristics of I₁ depend on the presence of choline (Figure 2B), confirming that the conformational state of this species is choline-dependent.

It should be pointed out that although the I₁ and I₂ states accumulate in equilibrium on unfolding, their precise role in the kinetic folding of the C-LytA protein from the unfolded state (i.e. whether I₁ proceeds sequentially to I₂, and this to N in the time course) remains to be established until CD- and fluorescence-monitored stopped-flow measurements are performed. On the other hand, the existence of a multistep process in the unfolding of C-LytA makes it necessary to revise the choice of the term ‘domain’ (e.g. choline-binding domain) to refer to this module, since the former expression should rather be reserved to represent a single co-operative structure.

As stated in the Introduction section, the C-LytA module is routinely used as an affinity tag for the single-step purification of fusion proteins ([15,17–19], and C-LYTAG® Purification System User’s Manual from Biomedal, http://www.biomedal.es). We believe that some of the results presented in this paper would help in the design of new variants with an expanded landscape of applications. For example, it is well known that partly folded intermediates are very prone to aggregation [26]. Thus, deletion of the first 32 amino acids of C-LytA, which are probably unfolded in the I₁ state, may improve both the resistance to proteases and the solubility of the module and, therefore, of the corresponding fusion protein, without affecting its affinity for DEAE-based resins (Figure 5). Size reduction would also contribute to diminish possible steric interactions with the fused proteins. Furthermore, our efforts are currently aimed to identify fully the residual structure in the I₁ state, which seems capable of recognizing choline (Figure 2B). This structure shows an unusual stability against GdmCl denaturation, with a few similar examples in the literature [27], and might serve as a model for the design of robust β-hairpins that could be used as a drastically minimized version of C-LytA or as scaffolds for new functions.

In summary, we hypothesize that C-LytA denatures at 20°C by highly independent unfolding of ChBRs. In the absence of choline, the protein is partially unfolded in water, probably around its N-terminal part. Finally, the addition of saturating choline concentrations stabilizes the native state and reduces the number of intermediate species in the unfolding scheme. These results will presumably be valuable in the design of C-LytA variants with an enhanced biotechnological potential.

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