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Extensive unfolding of the C-LytA choline-binding module by submicellar concentrations of sodium dodecyl sulphate

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Abstract **We have investigated the stability of the choline-binding module C-LytA against sodium dodecyl sulphate (SDS)-induced unfolding at pH 7.0 and 20 °C. A major intermediate with an unfolded N-terminal region accumulates at around 0.75 mM SDS, whereas 2.0 mM SDS was sufficient for a complete unfolding. This might be the first report of a protein being extensively unfolded by submicellar concentrations of SDS, occurring through formation of detergent clusters on the protein surface. All transitions were reversible upon SDS complexation with β-cyclodextrin, allowing the calculation of thermodynamic parameters. A model for the unfolding of C-LytA by SDS is presented and compared to a previous denaturation scheme by guanidine hydrochloride.**

Keywords: Choline-binding module; SDS denaturation; Protein stability; Repeat proteins; Affinity tag

1. Introduction

Choline-binding proteins (CBPs) constitute a family of prokaryotic polypeptides that can be found in a number of microorganisms such as the pathogen *Streptococcus pneumoniae* [1]. They account for a wide range of functions [2,3], but all of them share the property of recognizing the presence of choline in the cell wall by means of a choline-binding module (CBM) (Pfam ID code PF01473: http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF01473). The major representative of the CBM family is C-LytA, the C-terminal module of the pneumococcal LytA autolysin. This 135-aa polypeptide is a repeat protein, built up from six conserved β-hairpins that configure four choline-binding sites [4]. Binding of choline induces a significant stabilization, together with dimerization through the C-terminal hairpin [4–6]. The structure of the unligated form is not yet know. The affinity of C-LytA for choline and structural analogs allows its use as an affinity tag for single-step purification of recombinant proteins in amine-containing chromatographic resins upon specific elution with choline [7–9].

The stability of the C-LytA module has been studied by thermal [5] and chemical [6] denaturation experiments. Both approaches showed co-operativity in unfolding and unveiled the accumulation of partly folded intermediates, allowing the calculation of thermodynamic stability parameters. However, despite the absence of a definite hydrophobic core, the protein cannot be completely unfolded by guanidine hydrochloride (Gdn-HCl) or guanidine isothiocyanate at neutral pH and room temperature [6]. This has been ascribed to the unusual stability of the C-terminal hairpins. Therefore, in order to calculate the overall stability of C-LytA, the use of a stronger denaturant is necessary. In this sense, sodium dodecyl sulphate (SDS) is a very well known surfactant that is mostly used for the thorough denaturation of proteins and their analysis by polyacrylamide gel electrophoresis (SDS–PAGE) [10]. Protein–SDS interaction has been well established by binding isotherms studies. It has been described that an specific, non-cooperative binding takes place at low SDS concentrations, mainly guided through ionic interactions. An increase in surfactant concentration is subsequently responsible for the unfolding of the protein by means of hydrophobic forces [11,12]. There are several models accounting for the structure of unfolded protein–SDS complexes, although the "necklace" model seems to be supported by most experimental techniques [12,13]. According to this model, the unfolded polypeptide chain wraps around SDS micelles like beads in a string. On the other hand, it should be remarked that many proteins are highly resistant to SDS denaturation [14], and in some cases, partly folded states may be stabilized by the detergent (e.g. [15,16]). There are only few examples of thermodynamic analyses on the equilibrium unfolding of proteins by SDS [17–19], despite the fact that SDS denaturation has been demonstrated to be reversible by the addition of cyclodextrins [20] and that unfolding kinetics are similar to those obtained with other chemical denaturants [21].

In this work we carry out a thermodynamic equilibrium study on the unfolding of C-LytA by SDS. We show that submicellar concentrations of SDS are able to extensively unfold the protein, and that partly folded states also accumulate at intermediate concentrations of surfactant. Moreover, we propose a thermodynamic model for the SDS-induced equilibrium denaturation of this choline-binding module.

2. Materials and methods

2.1. Materials

β-Cyclodextrin and pyrene were purchased from Fluka. Sodium dodecyl sulphate, 1,6-diphenyl-1,3,5-hexatriene (DPH),

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Abbreviations: CBM, choline-binding module; Gdn-HCl, guanidine hydrochloride; CD, circular dichroism; c.m.c., critical micellar concentration; DPH, 1,6-diphenyl-1,3,5-hexatriene; LEM, linear extrapolation method

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N-acetyltryptophanamide, choline chloride and DEAE-cellulose were from Sigma–Aldrich. Due to the hygroscopic properties of choline, concentrated stock solutions were always prepared from a freshly opened bottle and stored in aliquots at −20 °C.

2.2. Proteins

C-LytA was purified from crude extracts of the overproducing *Escherichia coli* strain RB791 [pCE17], following the details previously described [22] and optimized using the materials and protocols contained in the C-LYTAG™ expression and purification kit (Biomedal). Purified samples were subsequently dialyzed at 20 °C against 20 mM sodium phosphate buffer, pH 7.0, to remove the choline used for elution. Protein concentration was determined spectrophotometrically [23]. The C-LytA(Δ32) truncated form was obtained by limited proteolysis as described before [6].

2.3. Circular dichroism

Circular dichroism (CD) experiments were carried out in a Jasco J-810 spectropolarimeter equipped with a Peltier PTC-423S system. Isothermal wavelength spectra were acquired at a scan speed of 50 nm/min with a response time of 2 s and averaged over at least six scans at 20 °C. Protein concentration was 12 μM unless otherwise stated, and the cuvette path-lengths were 0.1 cm (far-UV) or 1 cm (near-UV). Ellipticities ([θ]) are expressed in units of deg cm^2 (dmol of residues)$^{-1}$.

2.4. Fluorescence

Emission scans were performed at 20 °C on an Aminco SLM8000 spectrofluorimeter using a 5 × 5 mm path length cuvette and a protein concentration of 12 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}. The critical micellar concentration (c.m.c.) of SDS in 20 mM sodium phosphate, pH 7.0, at 20 °C was determined according to the procedure of Chattopadhyay and London [24], using DPH as a fluorescence probe. The cuvette path length was 10 × 10 mm, and excitation and emission slits were set to 1 nm. Formation of SDS clusters or micelles was followed by measuring the ratio of I_3 to I_1 fluorescence bands of pyrene at 385 and 373 nm, respectively, using the method described by Turro et al. [12]. Excitation wavelength was 335 nm, with excitation and emission slits of 0.4 and 1 nm respectively.

2.5. Thermodynamic analysis

For SDS titrations, aliquots from a 4.0 mM stock solution of detergent in 20 mM phosphate buffer, pH 7.0 (plus the corresponding additions), were added stepwise and incubated for 5 min prior to record the spectra (this waiting time was sufficient for the system to reach equilibrium). Experiments were repeated at least three times. Unfolding of monomeric C-LytA (*i.e.*, in the absence of choline) was assumed to occur through a three-step process:

$$F \leftrightarrows I \leftrightarrows U \tag{1}$$

where F, I and U represent the folded, intermediate and unfolded species. Data were fitted by least squares to two consecutive two-state processes according to the linear extrapolation method of Greene and Pace [25], using the SigmaPlot utilities (SPSS Science):

$$\Delta G_{XY} = \Delta G^0_{XY} - m_{XY}[SDS] \tag{2}$$

where ΔG_{XY} and ΔG^0_{XY} are the free energies of unfolding of state X relative to state Y in the presence and absence of denaturant, respectively, and m_{XY} represents the dependence of ΔG_{XY} with respect to [SDS]. From Eq. (2), it follows:

$$\Delta G^0_{XY} = m_{XY}[SDS]_{(1/2)XY} \tag{3}$$

being $[SDS]_{(1/2)XY}$ the denaturant concentration at which the equilibrium constant K_{XY} equals 1. In this case, it corresponds to the midpoint of the transition.

As an alternative to the linear extrapolation method, we attempted data fitting using the denaturant binding model by Aune and Tanford [26], assuming discrete, equivalent and noninteracting binding sites for the denaturant:

$$\Delta G_{XY} = \Delta G^0_{XY} - \Delta nRT\ln(1 + ka) \tag{4}$$

where Δn is the difference in the number of binding sites between Y and X, k is the SDS binding constant and a is the activity of the denaturant.

Unfolding of dimeric, choline-ligated C-LytA was assumed to occur via a dimeric intermediate that further denatures following an unfolding-dissociation coupled equilibrium:

$$(F)_2 \leftrightarrows (I)_2 \leftrightarrows 2U \tag{5}$$

In this case, the unfolding of the intermediate depends on protein concentration according to

$$f_u = [(K^2_{UI} + 8K_{UI}P_t)^{1/2} - K_{UI}]/4P_t \tag{6}$$

where f_u is the fraction of unfolded protein, K_{UI} is the equilibrium constant and P_t is the total protein concentration. f_u can be calculated from the relative change in ellipticity at any SDS concentration with respect to the total change in ellipticity upon denaturation. On the other hand, ΔG_{UI} is related to K_{UI} and, therefore, the m_{UI} and $[SDS]_{(1/2)UI}$ parameters (Eqs. (2) and (3)) can be directly calculated:

$$K_{UI} = \exp(-\Delta G_{UI}/RT) = \exp\{-m_{UI}([SDS]_{(1/2)UI} - [SDS])/RT\} \tag{7}$$

It should be noted that, in this case, due to the change in molarity upon denaturation, $[SDS]_{(1/2)UI}$ does not correspond to the midpoint of the transition.

3. Results and discussion

3.1. Equilibrium denaturation of C-LytA by SDS

Far-UV CD is a suitable technique for monitoring the degree of structure of C-LytA [6,27]. The spectrum of the protein at 20 °C in 20 mM sodium phosphate, pH 7.0, is dominated by positive aromatic contributions centered around 223 nm (Fig. 1A), as described before [23]. As shown in the figure, addition of 2.0 mM SDS induces the loss of the positive peak, rendering an spectrum comparable to that of the thermally, fully unfolded protein. This suggests that such a concentration of surfactant might induce the unfolding of the polypeptide. On the other hand, the intrinsic fluorescence emission spectrum of C-LytA displays a peak around 342 nm (Fig. 1B). In this case, the presence of 2.0 mM SDS induces a decrease in the intensity together with a small blue-shift to 339 nm, that reflects both an enhanced quenching by the solvent and a decrease in the polarity of the environment surrounding the tryptophan residues. An inspecific effect of the SDS-containing solvent on the intrinsic tryptophanyl fluorescence can be ruled out since a spectrum of *N*-acetyltryptophanamide recorded in the presence or in the absence of 2.0 mM SDS showed no significant differences (data not shown). To check whether the mentioned blue-shift in fluorescence might arise from the burial of tryptophanyl side chains in a hydrophobic core, we recorded the near-UV CD spectrum of the protein in the same conditions. It can be seen in Fig. 1C that 2.0 mM SDS yields a featureless spectrum throughout the wavelength range, again similar to that of the unfolded protein, indicating the absence of a rigid environment around any aromatic residues despite the abundance of these and their regular distribution throughout the sequence. The spectrum did not change upon addition of 20 mM SDS (data not shown). Therefore, we can assume that the protein is extensively unfolded in 2.0 mM SDS, and that the blue-shifted fluorescence spectrum may be originated from the influence of nearby hydrophobic SDS molecules adsorbed onto the polypeptide chain. Moreover, the c.m.c. of SDS in the above conditions was determined experimentally to be 3.25 mM. To our knowledge, this is the first reported case of a compact,

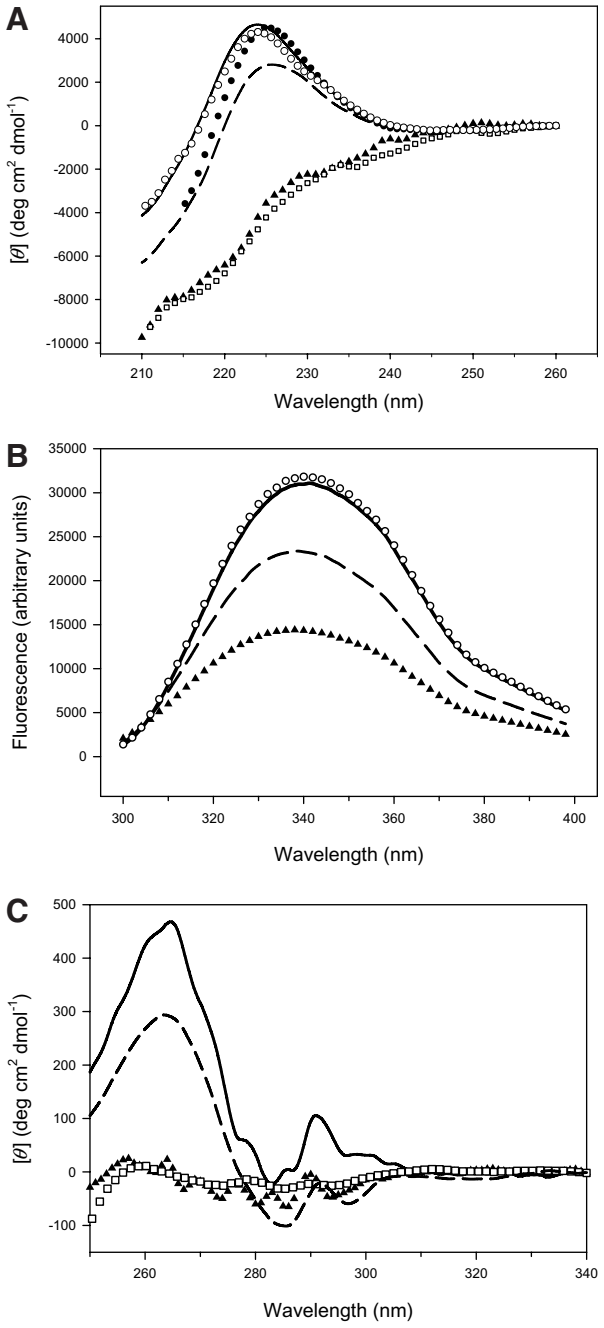

Fig. 1. Spectroscopical features of C-LytA. (A) Far-UV CD spectra of C-LytA in phosphate buffer (——) and upon addition of 0.75 mM SDS (– – –), 2.0 mM SDS (▲) and 2.0 mM SDS plus 4.0 mM β-cyclodextrin (○). The spectra of thermally unfolded C-LytA at 95 °C (□) and the I_1 intermediate accumulated in 2.0 M Gdn-HCl (●) [6] are also displayed. (B) Intrinsic fluorescence spectra of C-LytA in phosphate buffer (——) and upon addition of 0.75 mM SDS (– – –), 2.0 mM SDS (▲) and 2.0 mM SDS plus 4.0 mM β-cyclodextrin (○). (C) Near-UV CD spectra in phosphate buffer at 20 °C (——), upon addition of 0.75 mM SDS (– – –), upon addition of 2.0 mM SDS (▲), and in phosphate buffer at 95 °C (□).

folded protein that is fully denatured below the c.m.c. It should be mentioned that Gudiksen et al. [28] by means of an exhaustive study using capillary electrophoresis, have previously suggested the possibility of the denaturation of several proteins below the c.m.c., although in their study there is no biophysical characterization of the degree of unfolding.

Fig. 1A and B also show that SDS denaturation is promptly reversible by the addition of a 2:1 molar ratio of β-cyclodextrin, as described for other proteins [21]. This makes the SDS unfolding of C-LytA amenable to be analyzed by equilibrium thermodynamics. It should be pointed out that detergent dilution was also an effective method for renaturation, although in a much higher time scale (data not shown). Fig. 2 displays the equilibrium denaturation profiles monitored by far-UV CD and fluorescence. In both cases a clear biphasic transition is detected, with a plateau around 0.75 mM SDS, suggesting the accumulation of an intermediate (I_{SDS}). The CD and fluorescence spectra of I_{SDS} are shown in Fig. 1. The spectral characteristics are somehow intermediate between those of the native and the fully denatured protein, indicating a significant loss of secondary and tertiary structure. We also performed a near-UV CD-monitored titration, with similar results to those showed in Fig. 2 when plotting the ellipticity at 265 nm (data not shown). However, trying to follow the tyrosine and tryptophanyl signals in the 280–295 range failed to yield good curves, due to the low difference in intensity between the spectra of the folded and unfolded states in this region (Fig. 1C). The unfolding transitions were independent of protein concentration in the range 2–12 μM (data not shown), indicating that the [SDS]/[C-LytA] ratio is not a significant parameter. Moreover, as described above, the far-UV CD spectrum of C-LytA contains contributions from aromatic side-chains. Nevertheless, we checked that the CD-monitored denaturation transitions were independent of the wavelength at least in the 210–235 nm range (data not shown). Since the aromatic contributions strongly depend on the wavelength [27], this result suggests that secondary and tertiary structures unfold jointly.

Free energies of unfolding were determined by the linear extrapolation method (LEM) described by Greene and Pace [25]. The results of the fittings are shown in Table 1. As an alternative, we tried to use the Eq. (4) developed by Aune and Tanford [26], that assumes the presence of a discrete number of binding sites in the protein for the denaturant. However, in this case, fittings were always poor and very dependent on initial estimates when applying least squares methods (data not shown). According to our calculations (Table 1) both CD and fluorescence-monitored transitions display similar energetics. While the denaturation midpoints are precisely calculated, the major errors affect to the *m* values, which affects the calculation of the partial free energies (ΔG^0_{IF} and ΔG^0_{UI})

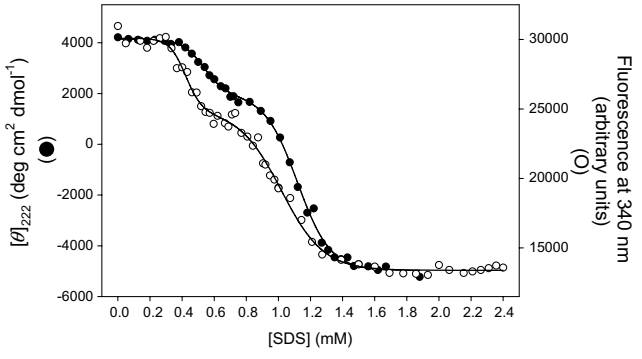

Fig. 2. Equilibrium denaturation of C-LytA by SDS. (●), ellipticity at 222 nm; (○), fluorescence intensity at 340 nm. Solid lines are least square fits to a three-state model according to Eq. (2).

Table 1
Thermodynamic stabilities of C-LytA and C-LytA(Δ32) in 20 mM sodium phosphate buffer, pH 7.0, and 20 °C

Protein	Addition	Technique	m_{IF} (kJ mol^{-1} mM^{-1})	$[SDS]_{(1/2)IF}$ (mM)	ΔG^0_{IF} (kJ mol^{-1})	m_{UI} (kJ mol^{-1} mM^{-1})	$[SDS]_{(1/2)UI}$ (mM)	ΔG^0_{UI} (kJ mol^{-1})	ΔG^0_{UF} (kJ mol^{-1})[a]
C-LytA	None	CD	33.9 ± 10.9	0.5 ± 0.1	17.5 ± 7.9	23.0 ± 1.3	1.1 ± 0.1	25.1 ± 1.3	42.6 ± 7.9
	None	Fluorescence	48.9 ± 9.2	0.4 ± 0.1	20.5 ± 5.8	18.8 ± 1.7	1.0 ± 0.1	19.2 ± 1.7	39.7 ± 6.3
	100 mM NaCl	CD	71.3 ± 17.9	0.3 ± 0.1	21.4 ± 9.8	42.1 ± 2.6	0.6 ± 0.1	25.3 ± 2.1	46.7 ± 10.0
	3.0 mM choline[b]	CD	N.D.[c]	N.D.	N.D.	41.2 ± 5.0[d]	2.0 ± 0.1	41.2 ± 7.1[e]	N.D.
	3.0 mM choline[f]	CD	N.D.	N.D.	N.D.	40.1 ± 1.9[d]	2.3 ± 0.1	46.1 ± 3.0[e]	N.D.
C-LytA(Δ32)	None	CD	N.D.	N.D.	N.D.	21.3 ± 0.8	1.0 ± 0.1	21.3 ± 0.8	N.D.

[a] $\Delta G^0_{UF} = \Delta G^0_{IF} + \Delta G^0_{UI}$.
[b] Fittings using Eq. (5); [C-LytA] = 1.2 μM.
[c] N.D., not determined.
[d] Values are per mole of dimer.
[e] Values are per mole of monomer.
[f] Fittings using Eq. (5); [C-LytA] = 12.0 μM.

(Eq. (3)). An average value of 41.2 ± 9.7 kJ mol^{-1} was obtained for the overall free energy of unfolding (ΔG^0_{UF}), and, as expected, it was higher than the previous results on the partial unfolding of C-LytA by Gdn-HCl (30.9 kJ mol^{-1}) [6]. It could be argued that the difference in energies may also be due to differential charge screening by each denaturant. However, we have checked by thermal unfolding experiments that the stability of C-LytA is independent of ionic strength at least up to 1.5 M NaCl (data not shown), so that screening effects seem to have little effect on stability.

Previous results have revealed that the N-terminal part of C-LytA is especially susceptible to thermal and chemical unfolding [5,6]. To check this point in our study, we analyzed the denaturation of C-LytA(Δ32), a truncated form of C-LytA lacking the first β-hairpin and turns [6]. Fig. 3 shows that in this case the first co-operative transition is drastically diminished in intensity, whereas the second one fully remains (Table 1). This suggests that the I_{SDS} species involves the unfolding of the amino-terminal structure of C-LytA. In this sense, it has been shown that a similar intermediate (I_1), lacking structure in the N-terminal region, may accumulate in the presence of 2.0 M Gdn-HCl [6]. However, both intermediates must be of different nature, since the unfolding transition leading to the accumulation of I_{SDS} is co-operative (Fig. 2), unlike that of I_1 [6], and therefore should be more energetic. Moreover, the far-UV CD spectrum of I_{SDS} has a lower intensity than that of I_1 (Fig. 1A), suggesting a higher degree of unfolding.

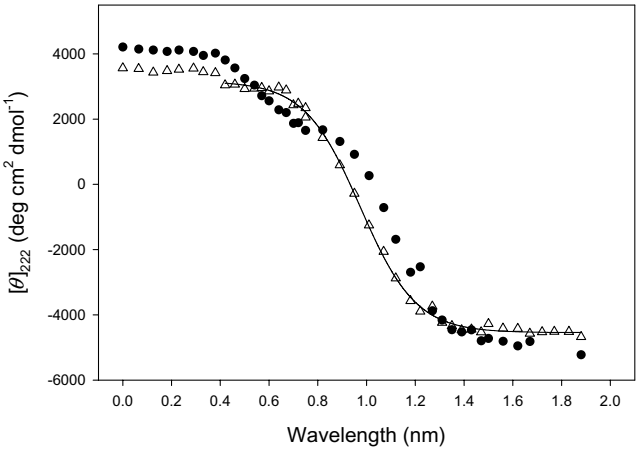

Fig. 3. Equilibrium denaturation of C-LytA(Δ32) by SDS, monitored by far-UV CD (Δ). Solid line represents the fit of the second transition to a two-state model using Eq. (2). Denaturation of full-length C-LytA (●) is shown for comparison purposes.

3.2. Mechanism of SDS-induced denaturation

Unfolding by SDS is thought to occur by formation of SDS clusters or "hemi-micelles" on the protein surface, onto which the polypeptide chain wraps [12,13]. To check whether this model could be applied to C-LytA denaturation below the SDS c.m.c., we used the pyrene assay described by Turro et al. [12]. Briefly, the insertion of pyrene in hydrophobic environments such as detergent micelles is detected by the increase in the ratio of fluorescence bands I_3/I_1 (Fig. 4A). As shown in Fig. 4B, the maximum of the I_3/I_1 ratio in phosphate buffer was achieved at approximately 3 mM SDS, in agreement with the c.m.c. value of 3.25 mM obtained with the DPH probe as mentioned above. On the other hand, when the titration was performed in the presence of C-LytA, the ratio I_3/I_1 increased even at the lowest SDS concentrations tested, and saturation was achieved at approximately 1.3 mM (Fig. 4B). This result is in agreement with the hypothesis of protein-induced SDS clusters as responsible for C-LytA denaturation, even though the bulk SDS concentration is below the c.m.c.

Investigation of the importance of ionic interactions in the SDS binding by C-LytA was first attempted by performing denaturation titrations at acidic pH. However, below pH 6 the solubility of the protein substantially decreased, so that spectroscopical measurements were not feasible. Alternatively, we tried to use cationic analogs of SDS at pH 7.0 such as dodecylamine and dodecyltrimethylammonium, but it was not useful either, due to the very low solubility of the former and the peculiar structure of the latter compound which, as a tertiary alkylamine, acted at low concentrations as a choline analog rather than as a detergent. Finally, we carried out unfolding experiments in the presence of 100 mM NaCl. We checked that the transitions occurred in a SDS range below the c.m.c. in the presence of the salt (1.25 mM) (data not shown). Table 1 shows that this moderate ionic strength induces a significant increase in the m_{IF} and m_{UI} parameters, concomitant with a

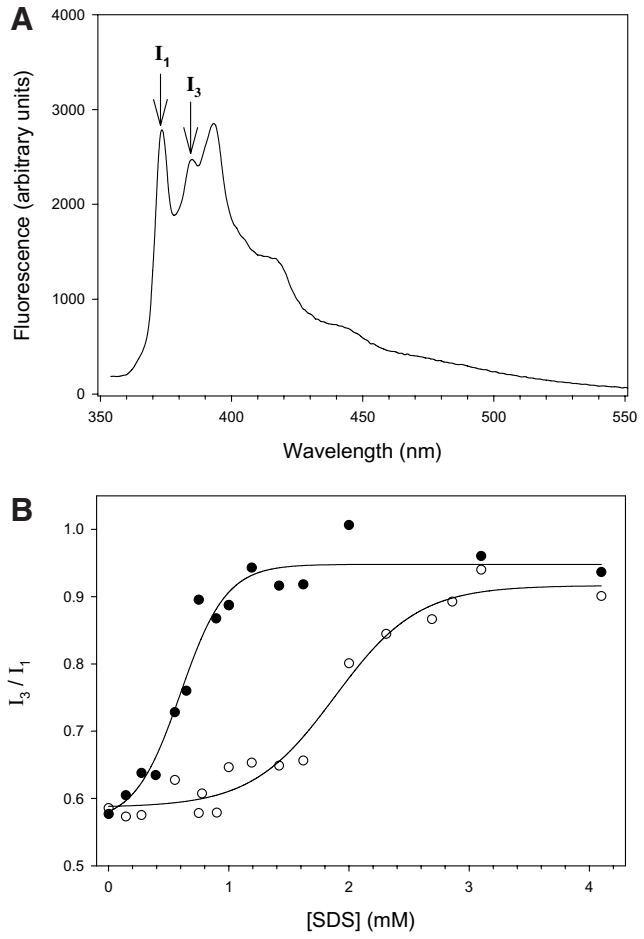

Fig. 4. Determination of SDS clusters using the pyrene probe. (A) Fluorescence spectrum of a pyrene-saturated solution in 20 mM sodium phosphate, pH 7.0, plus 12 μM C-LytA and 2.0 mM SDS, showing the I_1 and I_3 bands. (B) I_3/I_1 ratio of pyrene as a function of SDS concentration in the absence (○) and presence (●) of 12 μM C-LytA. Solid curves are fits for clarity of presentation.

decrease in the $[SDS]_{1/2}$ values. Nevertheless, the stabilization energies of both the native and the intermediate states remain unaltered within experimental error, in accordance with the stability of C-LytA not being affected by ionic strength (see above). This result seems to confirm the formation of SDS clusters or hemi-micelles on the surface that are promoted at lower concentrations of detergent due to the salt effect. Moreover, this finding supports the use of the LEM procedure for the calculation of unfolding energies using SDS, with the caution that the *m* and $[SDS]_{1/2}$ parameters may be extremely dependent on the experimental conditions (Table 1). The true importance of the protein-detergent electrostatic component should better be evaluated in the future using C-LytA mutants with a modified surface charge but with the same stability than the wild-type protein.

3.3. Unfolding of choline-ligated C-LytA

The four choline-binding sites of C-LytA display both high- and low-affinity characteristics, being the high-affinity ones responsible for the dimerization of C-LytA through its C-terminal hairpin [5,27]. Fig. 5A displays the CD-monitored unfolding of C-LytA in the presence of 3.0 mM choline, a concentration that specifically saturates the high-affinity binding sites [5,27]. Two major transitions were detected, although shifted to higher SDS concentrations with respect to the free protein (Fig. 2), and in accordance with a significant stabilization of C-LytA by choline [5,6]. A plateau is visible at around 1.0 mM SDS, suggesting the accumulation of an intermediate (I_{SDScho}). On the other hand, when the experiment was repeated using a 10-fold less concentrated sample, only the transitions occurring at the higher SDS concentrations were affected (Fig. 5A). This suggests that the I_{SDScho} species is dimeric, like the choline-bound native state, and that its denaturation by SDS involves a coupled dissociation-unfolding process that should be dependent on protein concentration. This is also in agreement with the observation that the spectrum of the I_{SDScho} state shows distinct characteristics with respect to the unligated I_{SDS} (Fig. 5B and C), probably as a consequence of its oligomerization state and/or bound choline molecules, and reinforces the hypothesis that the C-terminal moiety of C-LytA, involved in dimerization, constitutes the region of maximum stability of the protein [5,6]. Whereas the Folded ⇒ I_{SDScho} transition is complex and reveals a series of kinks that suggests the accumulation of other minor intermediates, the denaturation of dimeric I_{SDScho} could be fitted to a coupled unfolding-dissociation model (Eqs. (6) and (7)). Results were similar when using two different concentrations of the protein (Table 1), adding a new argument in favour of the use of LEM for SDS denaturation. An average value of 43.7 ± 7.7 kJ(mol of monomer)$^{-1}$ for ΔG_{UI} accounts for the increased stability of the C-LytA intermediate, acquired upon dimerization together with the filling of the high-affinity choline-binding sites.

Fig. 5D shows the SDS denaturation of C-LytA in the presence of 25 mM choline, a concentration sufficient to saturate all choline-binding sites of C-LytA [5] and to induce a further increase in the thermal and chemical stability of the protein [5,6]. Nevertheless, contrary to the denaturation by Gdn-HCl [6], the unfolding pattern displays similar characteristics than in the presence of 3.0 mM ligand, i.e., accumulation of multiple intermediates followed by a cooperative transition, but all processes taking place at higher SDS concentrations. A denaturation carried out at 50 mM followed the same trend (data not shown). We did not attempt to fit the data to LEM equations as we determined that the c.m.c. of SDS at this concentration of choline was 1.0 mM, so that the state of aggregation of SDS in the bulk solution indeed varied throughout the titration range.

3.4. A model for C-LytA denaturation

We propose the scheme in Fig. 6 to account for the unfolding of the C-LytA protein. Partly folded intermediates accumulate at low concentrations of surfactant either in the presence or in the absence of choline. Such intermediates may contain an unfolded N-terminal moiety, similarly to those accumulated in low concentrations of Gnd-HCl [6]. SDS denaturation allowed the calculation of thermodynamic parameters, avoiding the uncomplete and irreversible steps that are found when using Gnd-HCl as denaturant [6]. The calculated overall stability of the unligated protein is 41.2 kJ mol^{-1}, a value that is in the usual range for compact, fully folded proteins [29]. However, despite its thermodynamic stability, extensive unfolding takes place below the SDS c.m.c (3.25 mM), in what

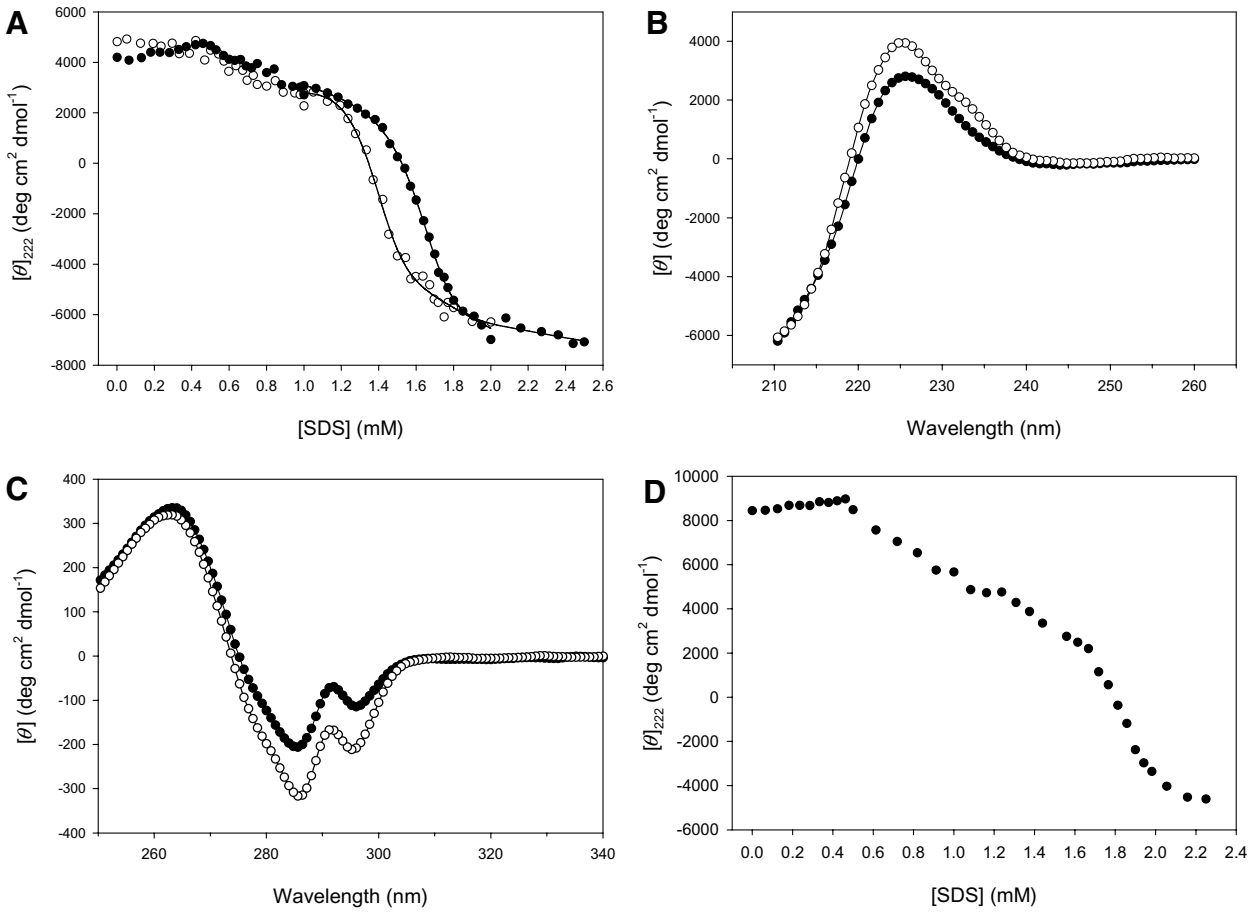

Fig. 5. Unfolding of dimeric C-LytA by SDS in the presence of choline. (A) Equilibrium titration monitored by far-UV CD in the presence of 3.0 mM choline. Protein concentration was 1.2 μM (○) or 12 μM (●). Solid lines indicate fits of the second transitions to Eqs. (6) and (7). (B) Far-UV CD wavelength spectrum of the I_{SDScho} intermediate stabilized in 1.0 mM SDS plus 3.0 mM choline (○). The spectrum of the I_{SDS} intermediate in 0.75 mM SDS (●) is shown for comparison. (C), Near-UV CD spectra of the I_{SDS} and I_{SDScho} intermediates. Same scheme as in (B); (D) Equilibrium titration monitored by far-UV CD in the presence of 25 mM choline. Protein concentration was 12 μM.

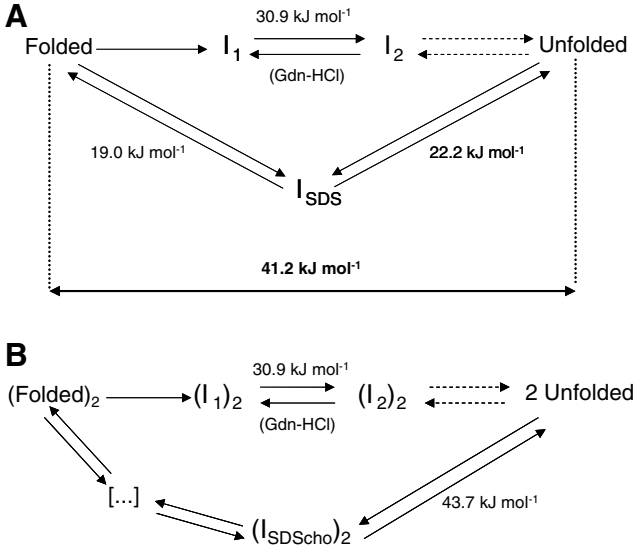

Fig. 6. A proposed denaturation scheme of C-LytA by SDS and Gdn-HCl. (A) in the absence of choline; (B) in the presence of 3.0 mM choline. Dashed arrows indicate steps that cannot be accomplished at 20 °C and pH 7.0. Data about Gdn-HCl denaturation are taken from [6].

it seems to be the first reported case of a full denaturation at such low concentrations of detergent. The mechanism of unfolding follows similar patterns than those described for other proteins, i.e., formation of SDS clusters on the protein surface [12,13]. This might represent a widespread mechanism of SDS-induced unfolding. We believe that C-LytA, and possibly other related choline-binding modules, may constitute good systems to study in detail the mechanism of protein denaturation by SDS.

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