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

Beatriz Maestro, Jesús M. Sanz*

Instituto de Biologia Molecular y Celular, Universidad Miguel Hernández, Av. Universidad, s/n 03202 Elche, Alicante, Spain

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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),

*Corresponding author. Fax: +34 966 658 758.
E-mail address: jmsanz@umh.es (J.M. Sanz).

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,