AS-48: a circular protein with an extremely stable globular structure

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Abstract The unfolding thermodynamics of the circular enterocin protein AS-48, produced by *Enterococcus faecalis*, has been characterized by differential scanning calorimetry. The native structure of the 70-residue protein is extremely thermally stable. Thus, at pH 2.5 and low ionic strength thermal denaturation occurs under equilibrium at 102°C, while the unfolded state irreversibly aggregates at neutral and alkaline pH. Calorimetric data analysis shows that the specific enthalpy change upon unfolding is unusually small and the heat capacity change is quite normal for a protein of this size, whereas the Gibbs energy change at 25°C is relatively high. At least part of this high stability might be put down to entropic constraints induced by the circular organization of the polypeptide chain.

Key words: AS-48 protein; Protein unfolding; Protein stability; Calorimetry; Circular protein

1. Introduction

The influence of many structural factors on the overall stability and folding of globular protein is still not well understood. For example, although intuitively it would seem that cross-links (S–S bridges first of all) introduced into proteins should increase their Gibbs energy of unfolding, through entropic destabilization of the unfolded state, the experiments on designing artificial S–S bridges have not given so far clear results [1–3]. A similar case would be that of a circular protein, where again the entropic loss in the unfolded state should stabilize the folded conformation. The magnitude of such an effect is here unknown due to a lack of clear experimental evidence.

Recently some natural circular proteins have been reported in the literature [4–8], including enterocin AS-48 [9–11]. So far, however, no systematic study has been made to discover the contribution of this circular structure to the stability of these proteins.

AS-48 is a 70-residue cyclic peptide produced by *Enterococcus faecalis* subsp. *liquefaciens* S-48, which shows a broad antimicrobial spectrum against both Gram-positive and Gram-negative bacteria [12–14]. It is encoded by the 68 kb pheromone-responsive plasmid pMB2 [15], and the gene cluster involved in its production and immunity has been identified [16]. The amino acid composition of the purified protein reveals the absence of cysteine residues and shows a high proportion of hydrophobic and basic amino acids, making AS-48 extremely basic with an isoelectric point at about 10.5.

In a previous work using nuclear magnetic resonance, we have solved the three-dimensional structure in solution of AS-48 [17], which consists of a globular arrangement of five α-helices enclosing a compact hydrophobic core. Interestingly enough, the head-to-tail peptide link between Trp-70 and Met-1 lies in the middle of α-helix 5, which is shown to have a pronounced effect on the stability of the three-dimensional structure.

In this work we have explored different pH and ionic strength conditions to analyze the stability of AS-48 by differential scanning microcalorimetry (DSC). Under most conditions, however, the thermal unfolding of the protein is irreversible and followed by the aggregation of the denatured state. Furthermore, the linear peptide obtained by cleaving the peptide bond between Trp-70 and Met-1 with BrCN does not keep its folded conformation. Finally, equilibrium conditions for the unfolding of AS-48 were found at acid pH and low ionic strength, which have allowed us to characterize the thermodynamics of its thermal denaturation. Even under these extreme conditions the folded conformation of this cyclic polypeptide shows great thermal stability, one of the highest reported in the literature.

2. Materials and methods

Bacteriocin AS-48 (70 amino acids, $M_r = 7.2$ kDa) was purified as described elsewhere [9]. The concentrations were measured spectrophotometrically at 280 nm using the extinction coefficient 12 350 M^{-1} cm^{-1} according to Gill and von Hippel [18].

DSC was done as described elsewhere [19,20] using a VP-DSC microcalorimeter (MicroCal Inc., USA) with 0.52 ml cell volume, at heating rates of 1 or 1.5 K/min and protein concentrations of around 1 mg/ml (100–200 μM). Before calorimetric experiments the samples were thoroughly dialyzed against the appropriate buffer. The specific volume used to transform the calorimetric data into temperature-dependent molar heat capacities was 0.73 ml/g.

The DSC data were analyzed by applying the two-state unfolding model to the temperature dependencies of the heat capacity as described elsewhere [20].

Any procedure to analyze DSC data is based on a reasonable approximation of the partial heat capacities of all macroscopic states populated during unfolding (in our case of the two-state transition, $C_{p,N}$ and $C_{p,U}$). It is generally accepted that the heat capacity of the native state can be well represented by a linear temperature function, while the heat capacity of the unfolded state has a more complex temperature dependence [21]. It is known from our own experience,

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as well as from a number of other DSC studies, that the heat capacity functions are usually remarkably curved below 40°C [20–23]. Privalov and Makhatadze [24] showed that this curvature is caused by a strong temperature dependence of the partial heat capacity of the hydrophilic side chains and peptide bonds, and developed an empirical algorithm (we will call it PM) for estimating $C_{p,U}(T)$ from the amino acid composition. Häckel and coworkers have recently proposed a new set of parameters for a similar algorithm (H method, [25]). Applying each of these two methods allows us to present $C_{p,U}$ as a polynomial:

$$C_{p,U}(t_r) = a_U + b_U t_r + c_U t_r^2 + d_U t_r^3 + \ldots \quad (1)$$

where $t_r = T - T_r$ (we have selected here T_r as 293.15 K). The PM method generally gives smooth functions, which might be adequately represented by a second-order polynomial, whereas a third-order regression was necessary to approximate $C_{p,U}$ for AS-48 by the H method. The coefficients of the corresponding polynomials are given in Table 1.

Although the heat capacity of the native state is well expressed by a linear temperature function [21,22], in some cases it has proved to be a quadratic function [20,26]. In general,

$$C_{p,N}(t_r) = a_N + b_N t_r + c_N t_r^2 + \ldots \quad (2)$$

Thus, the temperature dependence of ΔC_p is expressed as

$$\Delta C_p = C_{p,U} - C_{p,N} = \Delta a + \Delta b \cdot t_r + \Delta c \cdot t_r^2 + \Delta d \cdot t_r^3 + \ldots \quad (3)$$

To decrease the number of adjustable parameters during curve fitting, the shapes of the $C_{p,U}$ approximations were conserved and only the temperature-independent term, a_U, was allowed to change. When the shape of $C_{p,U}$ was represented by a quadratic function and $C_{p,N}$ was a linear one, the parameter Δc became c_U. In the case of the H approximation the parameter Δc was adjustable but Δd was fixed equal to d_U, which means that in this case $C_{p,N}$ was a second-order polynomial and $C_{p,U}$ a third-order function. Therefore in addition to ΔH_m and T_m with the PM method three more parameters were changed to adjust the baselines, a_U, Δa and Δb, while with the H method four more parameters were fitted: a_U, Δa, Δb and Δc.

The areas exposed to the solvent in the native and unfolded states were calculated using NACCESS software kindly provided by Dr. S. Hubbard. The program is based on Lee and Richards' method [27]; the probe size was 1.4 Å, the slice size was 0.05 Å and the van der Waals radii were those suggested by Chothia [28]. The native structure coordinates were taken from the PDB file 1e68.ent. The unfolded state of the protein was taken to be an extended β-structure, simulated with the Biopolymer module of INSIGHTII software on an INDIGO O_2 Workstation. We used formulas deduced elsewhere [29–31] to estimate the ΔC_p value from changes in solvent-accessible areas (ΔASA).

3. Results and discussion

It is known that AS-48 has a strong tendency to form reversibly soluble oligomers at neutral pH [32]. As expected, the heat-induced unfolding of AS-48 around pH 7.0 resulted in irreversible aggregation. On the other hand, it was found by ultracentrifugation that the protein is monomeric at acid pH [17] and indeed DSC experiments at pH 2.5 and low ionic strength gave rise to a highly reversible unfolding transition, although at very high temperatures (Fig. 1). In an attempt to destabilize the structure, while keeping the reversibility of unfolding, we tried raising the pH above 10. As can be seen in Fig. 1, the stability of the structure is then somewhat lower but once again the transition is irreversible and accompanied by exothermal aggregation. Further attempts to decrease the stability and obtain a linear polypeptide by hydrolyzing the Tyr-70–Met-1 peptide bond with BrCN failed since the resulting open chain was unfolded at any pH (results not shown), probably because the chain was cut not in a loop but in the middle of α-helix 5.

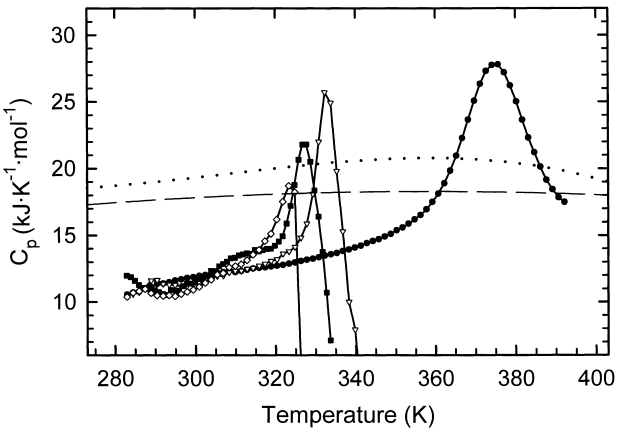

Fig. 1. Temperature dependencies of the partial molar heat capacity, C_p, of AS-48. Filled circles correspond to pH 2.5, 10 mM phosphate buffer; diamonds, pH 11.5, 10 mM phosphate; filled squares, pH 11.0, 50 mM phosphate; upside-down triangles, pH 11.0, 20 mM glycine. Dashed and dotted lines show the $C_{p,U}$ functions calculated by the PM and H methods respectively before curve fitting. Solid lines through the data-points serve only to guide the eye.

Therefore, our equilibrium DSC experiments were restricted to acid pH, where the position of the unfolding transition did not appear to depend upon pH below 4.0. The DSC curve of AS-48 at pH 2.5 exhibits a single, symmetric, endothermic peak, with a T_m of about 374.9 K and, since the peak is rather broad, the transition is not completed even at 393 K. The results of the analysis for one of the DSC curves recorded under these conditions are shown in Fig. 2. The C_p function fits the two-state model very well with the parameters listed in Table 1. $\Delta C_{p,m}$ is close to zero, which is not surprising bearing in mind that it decreases with temperature and T_m is very high.

As far as the baseline approximations are concerned, since

Table 1
The results of DSC data analysis for AS-48 at pH 2.5, 10 mM phosphate buffer

	Coefficients of $C_{p,U}(t_r)$ polynomial				Coefficients of $\Delta C_p(t_r)$ polynomial			ΔH_m (kJ/mol)	T_m (K)	$\Delta G(298)$ (kJ/mol)
	a_U (kJ/K/mol)	b_U (kJ/K^2/mol)	$c_U \cdot 10^4$ (kJ/K^3/mol)	$d_U \cdot 10^6$ (kJ/K^4/mol)	Δa (kJ/K/mol)	$\Delta b \cdot 10^3$ (kJ/K^2/mol)	$\Delta c \cdot 10^4$ (kJ/K^3/mol)			
PM	(17.7)									
	15.9 ± 0.5	*0.018*	*1.38*	–	**4.45**	**−44.4**	*1.38*	**231.6 ± 10**	**374.9 ± 0.5**	**34.6 ± 5**
H	(19.1)									
	15.2 ± 0.5	*0.032*	*2.09*	*−4.64*	**3.74**	**−5.66**	**−4.79**	**228.6 ± 10**	**374.9 ± 0.5**	**31.6 ± 5**

The parameters in bold letters correspond to the polynomial coefficients representing the best approximations to $C_{p,U}$ and ΔC_p found by fitting the experimental heat capacity curve (Fig. 2) to the two-state model. Other numbers correspond to the $C_{p,U}$ approximations by the PM and H methods, while those in italics were fixed during curve fitting.

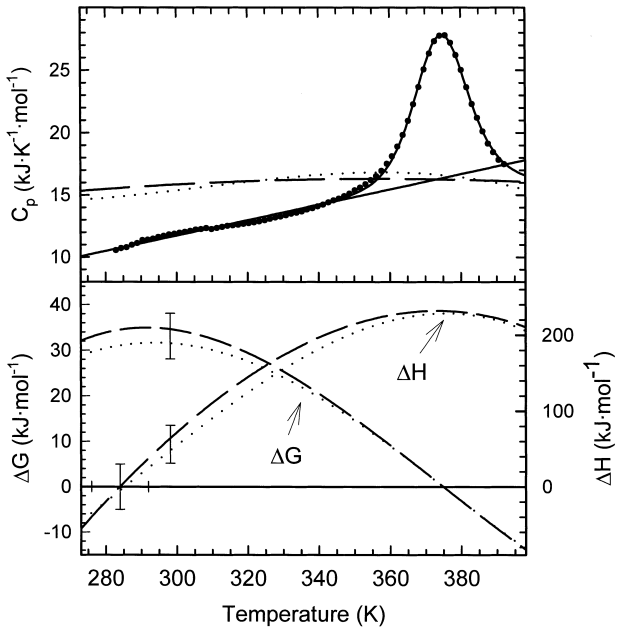

Fig. 2. Temperature dependencies of thermodynamic parameters of unfolding for AS-48 at pH 2.5, 10 mM phosphate buffer. Upper panel: partial heat capacity measured by DSC (solid dots) and its best fitting to the two-state model (solid line) assuming a temperature-dependent ΔC_p. The solid straight line below the thermal transition shows the linear function of the partial heat capacity of the native state, $C_{p,N}$, while dashed and dotted lines show the $C_{p,U}$ functions, corresponding to the PM and H methods, after adjusting their positions by curve fitting. Lower panel: the heat effects (right axis) and Gibbs energy changes of unfolding (left axis) found by curve fitting. Dashed lines correspond to the functions approximated by the PM method, while dotted lines to the H approximation. In both cases $\Delta C_p(T)$ are quadratic functions of temperature, as specified in the text. The bars show the extrapolation errors.

the protein is native over a wide temperature range, no problem arose with the $C_{p,N}$ extrapolations; in fact the $C_{p,N}$ functions which fitted best were always very close to the linear ones. The position, a_U, for $C_{p,U}$ is well adjusted during the curve fitting, but its temperature dependence cannot be unambiguously deduced from our data because below the transition peak the heat capacity curve does not give any information about $C_{p,U}$, as the population of the unfolded state is close to zero. The $C_{p,U}$ value estimated by the PM method is about 10% higher than the value observed at 390 K (Fig. 1), which is similar to what we have found for other small proteins [20,22,23,26], whereas the H method leads to deviations of about 20%. This overestimation could well be explained by the fact that the real unfolded chain is not identical to the short peptide models used for establishing the empirical heat capacities of amino acids and peptide groups. Nevertheless, our estimations of the $C_{p,U}$ function were supported by direct and independent experimental data. In our attempts to obtain an open form of the protein (see above), the open polypeptide chain of AS-48 at the single Met residue was found to be unfolded. Its partial molar heat capacity at 25°C was about 14 kJ/K/mol, only slightly lower than 15 kJ/K/mol, the average between the two estimations for $C_{p,U}$ of the circular form (Fig. 2).

As seen from Fig. 2 and Table 1, the shapes of two $C_{p,U}$ estimations are somewhat different and lead to two ΔC_p functions, which practically coincide at around T_m but differ by as much as 20% at room temperature. Nevertheless, the temperature dependencies of the unfolding enthalpy cross zero at almost the same temperature, which explains the rather small difference between the Gibbs energy extrapolations at 25°C. Since this difference is smaller than the uncertainty of extrapolation due to error propagation, it might be concluded that the two methods for obtaining the shape of $C_{p,U}$ result in an average $\Delta G(298) = 32.8 \pm 5$ kJ/mol.

According to Privalov [33], the average specific heat effect of unfolding, $\Delta h = \Delta H/M_r$, of globular proteins at 110°C is about 50 J/g, which is only a little higher than the values measured for proteins with a size similar to that of AS-48 [20,22,23,26,34–37]. Since AS-48 has a highly thermostable structure and unfolds above 100°C, even at extreme pH conditions, there is no need to extrapolate the measured heat effect to 110°C. It turns out, however, that its specific heat of unfolding is 32.2 J/g at 102°C, much lower than the 'consensus' value proposed by Privalov. Another exception would be barnase, which shows, however, a much higher Δh value [38]. We cannot currently suggest any plausible structural interpretation for this discrepancy.

A number of authors have suggested empirical algorithms to relate solvent-accessible surfaces of the proteins with the ΔC_p [29–31], which in the majority of thermodynamic studies is assumed to be temperature-independent. The changes in the solvent-exposed areas upon the unfolding of AS-48 are $\Delta ASA_{pol} = 1541$ Å^2, $\Delta ASA_{np} = 4026$ Å^2 and $\Delta ASA_{tot} = 5567$ Å^2 for the polar, non-polar and overall surfaces correspondingly. The estimation of ΔC_p based on the empirical equation of Gomez and Freire [29] is equal to 5.9 kJ/K/mol, which considerably exceeds our estimations (4.2 and 3.7 kJ/K/mol for the PM and H approximations respectively), whereas the value of 3.5 kJ/K/mol calculated by the third approach [31] is a little below ours. Nevertheless, both estimations based on the formulas suggested by Myers et al. [30] practically coincide with our data. Since the latter formulas are based on the statistical analysis of data for more than 40 globular proteins, this coincidence not only validates our heat capacity approximations but also shows that AS-48 is a typical globular protein with respect to its $\Delta C_p(298)$ value.

Finally, there are clearly two factors, which should reduce the Gibbs energy of unfolding, i.e. the above-mentioned low unfolding heat effect and a certain asymmetry of charge distribution on the protein surface [17]. In fact, one of the sides of the structure displays a cluster of positive charges, while the opposite side is mostly hydrophobic. This clustering is probably important for the protein interaction with the membrane surface and its thermodynamic disadvantage is reduced either by such an interaction or by high ionic strength. The two destabilizing factors cannot, however, overcome a significant entropic gain caused by closing up the polypeptide chain. Our attempt to measure the magnitude of this effect by cutting the chain failed. A more rational opening of the chain (by cutting it within the loops, for example) has to be designed. This work, together with studies into the effect of urea and guanidinium hydrochloride on stability, and the design of specific mutants is currently in progress in our laboratories.

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References

[1] Scheraga, H.A., Denton, M.E., Konishi, Y. and Lin, S.H. (1984) Biochemistry 23, 5504.
[2] Goldenberg, D.P. and Creighton, T.E. (1984) J. Mol. Biol. 179, 527.
[3] Camarero, J.A., Fushman, D., Sato, S., Giriat, I., Cowburn, D., Raleigh, D.P. and Muir, T.W. (2001) J. Mol. Biol. 308, 1045–1062.
[4] Kawai, Y., Saito, T., Toba, T., Samant, S.K. and Itoh, T. (1994) Biosci. Biotechnol. Biochem. 58, 1218–1221.
[5] Kawai, Y., Saito, T., Suzuki, M. and Itoh, T. (1998) Biosci. Biotechnol. Biochem. 62, 887–892.
[6] Eisenbrandt, R., Kalkum, M., Lai, E.M., Lurz, R., Kado, C.I. and Lanka, E. (1999) J. Biol. Chem. 274, 22548–22555.
[7] Delgado, M.A., Solbiati, J.O., Chiuchiolo, M.J., Farías, R.N. and Salomón, R.A. (1999) J. Bacteriol. 181, 1968–1970.
[8] Blond, A., Péduzzi, J., Goulard, C., Chiuchiolo, M.J., Barthélémy, M., Prigent, Y., Salomón, R.A., Farías, R.N., Moreno, F. and Rebuffat, S. (1999) Wur. J. Biochem. 259, 747–755.
[9] Gálvez, A., Giménez-Gallego, G., Maqueda, M. and Valdivia, E. (1989) Antimicrob. Agents Chemother. 33, 437–441.
[10] Samyn, B., Martínez-Bueno, M., Devreese, B., Maqueda, M., Gálvez, A., Valdivia, E., Coyette, J. and van Beeumen, J. (1994) FEBS Lett. 352, 87–90.
[11] Martínez-Bueno, M., Maqueda, M., Gálvez, A., Samyn, B., van Beeumen, J., Coyette, J. and Valdivia, E. (1994) J. Bacteriol. 176, 6334–6339.
[12] Gálvez, A., Maqueda, M., Martínez-Bueno, M. and Valdivia, E. (1989) Res. Microbiol. 140, 57–68.
[13] Gálvez, A., Valdivia, E., Martínez-Bueno, M. and Maqueda, M. (1989) Can. J. Microbiol. 35, 318–321.
[14] Abriouel, H., Valdivia, E., Gálvez, A. and Maqueda, M. (1998) Appl. Environ. Microbiol. 64, 4623–4626.
[15] Martínez-Bueno, M., Gálvez, A., Valdivia, E. and Maqueda, M. (1990) J. Bacteriol. 172, 2817–2818.
[16] Martínez-Bueno, M., Valdivia, E., Gálvez, A., Coyette, J. and Maqueda, M. (1998) Mol. Microbiol. 27, 347–358.
[17] González, C., Langdon, G.M., Bruix, M., Valvidia, E., Maqueda, M. and Rico, M. (2000) Proc. Natl. Acad. Sci. USA 97, 11221–11226.
[18] Gill, S.C. and von Hippel, P.H. (1989) Anal. Biochem. 182, 319–326.
[19] Filimonov, V.V., Prieto, J., Martínez, J.C., Bruix, M., Mateo, P.L. and Serrano, L. (1993) Biochemistry 32, 12906–12921.
[20] Padmanabhan, S., Laurents, D.V., Fernández, A.M., Elias-Arnanz, M., Ruiz-Sanz, J., Mateo, P.L., Rico, M. and Filimónov, V.V. (1999) Biochemistry 38, 15536–15547.
[21] Privalov, P.L., Tiktopulo, E.I., Venyaminov, S.Yu., Griko, Yu.V., Makhatadze, G.I. and Khechinashvili, N.N. (1989) J. Mol. Biol. 205, 737–750.
[22] Viguera, A.R., Martínez, J.C., Filimonov, V.V., Mateo, P.L. and Serrano, L. (1994) Biochemistry 33, 2142–2150.
[23] Ruiz-Sanz, J., Simoncsits, A., Törö, I., Pongor, S., Mateo, P.L. and Filimonov, V.V. (1999) Eur. J. Biochem. 263, 246–253.
[24] Privalov, P.L. and Makhatadze, G.I. (1990) J. Mol. Biol. 213, 385–391.
[25] Häckel, M., Hinz, H.-J. and Hedwig, G. (1999) J. Mol. Biol. 291, 197–213.
[26] Martínez, J.C., Viguera, A.R., Berisio, R., Wilmanns, M., Mateo, P.L., Filimonov, V.V. and Serrano, L. (1999) Biochemistry 38, 549–559.
[27] Lee, B.K. and Richards, F.M. (1971) J. Mol. Biol. 55, 379–400.
[28] Chothia, C. (1976) J. Mol. Biol. 105, 1–14.
[29] Murphy, K.P. and Freire, E. (1992) Adv. Protein Chem. 43, 313–361.
[30] Myers, J.K., Pace, C.N. and Scholtz, J.M. (1995) Protein Sci. 4, 2138–2148.
[31] Khechinashvili, N.N., Janin, J. and Rodier, F. (1995) Protein Sci. 4, 1315–1324.
[32] Abriouel, H., Valdivia, E., Gálvez, A. and Maqueda, M. (2001) Curr. Microbiol. 42, 89–95.
[33] Privalov, P.L. (1982) Adv. Protein Chem. 35, 1–104.
[34] Filimonov, V.V., Azuaga, A.I., Viguera, A.R., Serrano, L. and Mateo, P.L. (1999) Biophys. Chem. 77, 195–208.
[35] Martínez, J.C., Filimonov, V.V., Mateo, P.L., Schreiber, G. and Fersht, A.R. (1995) Biochemistry 34, 5224–5233.
[36] O'Neil, K.T., Hoess, R.H., Raleigh, D.P. and DeGrado, W.F. (1995) Proteins Struct. Funct. Genet. 21, 11–21.
[37] Wintrode, P.L., Makhatadze, G.I. and Privalov, P.L. (1994) Proteins Struct. Funct. Eng. 18, 246–253.
[38] Martínez, J.C., El Harrous, M., Filimonov, V.V., Mateo, P.L. and Fersht, A.R. (1994) Biochemistry 33, 3919–3926.