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The thermodynamics of the unfolding of an isolated protein subdomain

The 255–316 C-terminal fragment of thermolysin

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Abstract

Differential scanning calorimetry has been used to study the thermal unfolding of the 255–316 C-terminal fragment of thermolysin. The concentration effect on the calorimetric transitions of the fragment in 0.1 M NaCl and 20 mM phosphate buffer, pH 7.5, shows that it behaves as a highly stable dimer in solution, whithin the concentration range 0.19–4.55 mg/ml, undergoing a reversible two-state thermal unfolding process. The thermodynamic parameters of unfolding (ΔG = 60 ± 6 kJ/(mol of dimer) at 20°C) are similar to those normally observed for small, compact, globular proteins. This and previous studies [1989, Eur. J. Biochem. 180, 513–518] show that the 255–316 fragment folds into a stable, native-like globular structure.

Key words: Differential scanning colorimetry; Thermolysin; Protein stability; Protein domain; Domain folding

1. Introduction

That relatively large proteins have a discrete structure is nowadays well accepted, since proteins of more than 100 amino acid residues are usually composed of domains and subdomains. This can be seen by X-ray [1,2] or computational [3–6] analysis, as well as by studying the folding properties of protein fragments corresponding to domains in the intact protein ([7] and references cited therein). The 316-residue chain of thermolysin [EC 3.4.24.4] was shown by X-ray analysis to be composed of two similar structural domains (1–157 and 158–316) [8] and computational approaches described the hierarchic architecture of the protein ranging from secondary-structure, element-folding units to whole protein subdomains [9–11]. The location of domains and subdomains in thermolysin has been studied experimentally by examining the folding and stability properties of its chemical and proteolytic fragments [12–15]. Circular dichroism and immunochemical measurements showed that some C-terminal fragments adopt stable globular conformations in water, similar to the corresponding regions in the native protein [16]. The 255–316 thermolysin fragment was shown to fold independently into a stable, native-like conformation [17]. This fragment dimerizes when dissolved in aqueous buffer at concentrations higher than 0.1 mg/ml [18]. Circular dichroism studies show, however, that dimerization does not lead to alterations of fragment secondary structure, thus signifying that the association process is not critical for dictating the native-like folding of the fragment [18].

High-sensitivity differential scanning calorimetry (DSC) is a very appropriate technique for characterizing the energetics of the thermal unfolding of proteins, and may lead to the analysis of association processes, as long as the overall denaturation process occurs under equilibrium conditions [19,20]. DSC has been used here to study the thermal stability of the 255–316 thermolysin fragment at pH 7.5. The thermal unfolding of the 25_ 316 fragment has proved to be a highly reversible process. An analysis of the concentration effect on the DSC traces is consistent with a dimeric fragment undergoing two-state unfolding with no intermediate states according to the scheme $F_2 \rightleftarrows 2U$. The thermodynamic parameters of the unfolding process are characteristic of a compact globular structure for the dimer.

2. Materials and methods

Thermolysin was bought from Sigma as a crystallized and lyophilized powder. The 255–316 thermolysin fragment was obtained by limited proteolysis of the 205–316 fragment with subtilisin as described elsewhere [17]. The homogeneity of the fragment, further purified by re-

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verse phase HPLC, was at least 95%. The concentration of the 255–316 fragment was determined using the $E^{0.1\%}$ coefficient 0.52 at 280 nm [17]. Before the calorimetric experiments the sample solutions were dialyzed for 24 h at 4°C against two changes of a large volume of the dialysis buffer. For all DSC measurements sample concentrations were in the range of 0.19–4.55 mg/ml. Fragment solutions contained 0.1 M NaCl and 20 mM phosphate buffer, pH 7.5.

DSC experiments were performed in a computer-interfaced DASM-4 model [21] with 0.47-ml cells under a constant pressure of 2.5 atm. The reversibility of the transitions was checked by reheating the solution in the calorimeter cell after the first run. The scan rates used were in the range of 0.5–2.0 K/min. The excess heat capacity of the transition, $C_p^{ex}(T)$, was obtained as described in [22]. The thermograms were corrected for the effect of the time response of the instrument [23]. In calculating the molar quantities we used a molecular mass of 6,630 Da for the fragment, estimated from its known sequence. The uncertainty in the enthalpy values was between 5–10% and that of the melting temperatures, T_m, was ± 0.2°C.

3. Results and discussion

We have studied the thermal unfolding of the 255–316 fragment by DSC in 0.1 M NaCl and 20 mM phosphate buffer, pH 7.5. The process has been found to be highly reversible (> 90%) and independent of scan rate within the range 0.5–2.0 K/min, thus taking place under equilibrium conditions in the calorimeter cell. Fig. 1 shows the concentration effect of the fragment, within the range 0.19–4.55 mg/ml, on the position of the thermograms on the temperature scale. Table 1 includes the characteristic parameters of the transitions. The specific calorimetric enthalpy at the corresponding T_m values (Table 1), as well as the specific heat capacity increase of unfolding, 0.36 ± 0.08 J/K·g (0.087 ± 0.018 cal/K·g), compare well

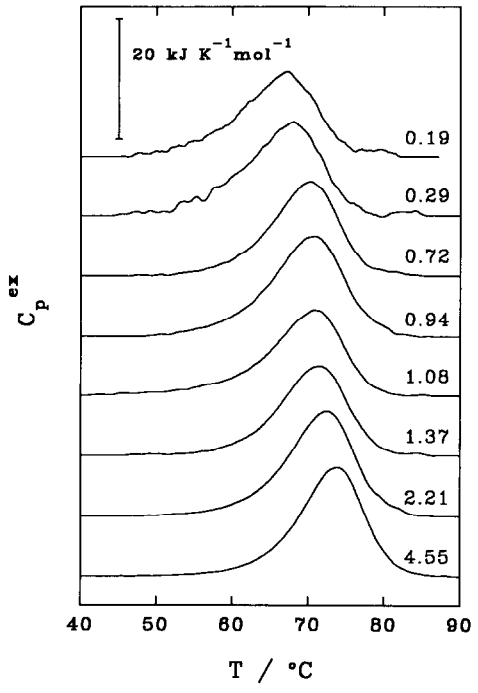

Fig. 1. Effect of sample concentration (mg/ml) on the $C_p^{ex}(T)$ curves of the 255–316 thermolysin fragment at pH 7.5. Scan rate 2.0 K/min, except for curve at 1.37 mg/ml, which is at 0.5 K/min.

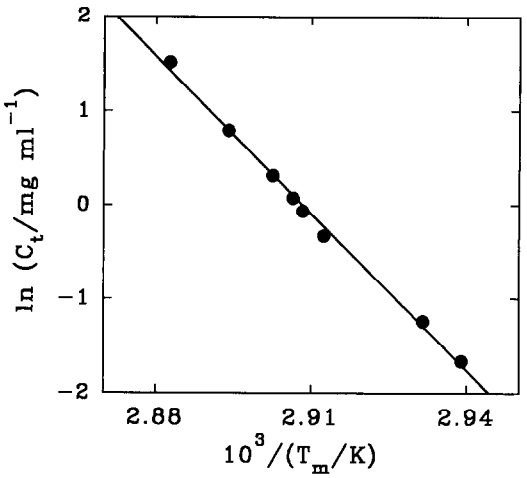

Fig. 2. Plot of ln C_t vs. $1/T_m$ for the 255–316 thermolysin fragment, pH 7.5. Values are taken from Table 1.

with values reported for compact globular proteins [19]. This shows the capacity of the 62-amino acid chain to fold into a compact globular structure.

The ratio of the calorimetric enthalpy, ΔH (obtained from the area under the calorimetric peak), to the van't Hoff enthalpy, ΔH^{vH} (obtained from the well known equation $\Delta H^{vH} = 4RT_m^2 \cdot C_p^m/\Delta H$, where C_p^m is the C_p^{ex} value at T_m), is equal to 0.55 ± 0.04 (Table 1). This value clearly suggests that the fragment undergoes dissociation upon thermal unfolding, which agrees with [18] concerning the dimeric character of the fragment within our experimental concentration range, as well as with the effect of concentration on the T_m values (Table 1).

In a general coupling between unfolding and dissociation processes, $F_n \rightleftarrows nU$, where n associated molecules of the folded fragment, F, give rise to n molecules of the unfolded fragment, U, equilibrium thermodynamics predicts the following relationship between the total fragment concentration, C_t, and the unfolding temperature, T_m [22,24],

$$\ln C_t = \text{constant} - n\Delta H/(n-1)RT_m$$

where ΔH stands for the unfolding enthalpy per monomer. The plot of ln C_t vs. $1/T_m$ (Fig. 2) for a dimer ($n = 2$) gives a straight line with a slope equal to $-2\Delta H/R$. The value of ΔH obtained from the slope is 231 ± 6 kJ/mol, close to the calorimetric enthalpy, 192 ± 13 kJ/mol (Table 1). The use of the above equation for $n > 2$ leads to much higher ΔH values.

The equation for $C_p^{ex}(T)$, which gives the shape of the thermal transitions, as reported in [15] for the unfolding of multimeric proteins, leads to excellent fittings of our experimental data for $n = 2$, whereas similar fittings for $n > 2$ were increasingly poor (Fig. 3).

Therefore, it can be concluded that the 255–316 fragment behaves as a dimer in solution, which undergoes a two-state reversible thermal unfolding under our experi-

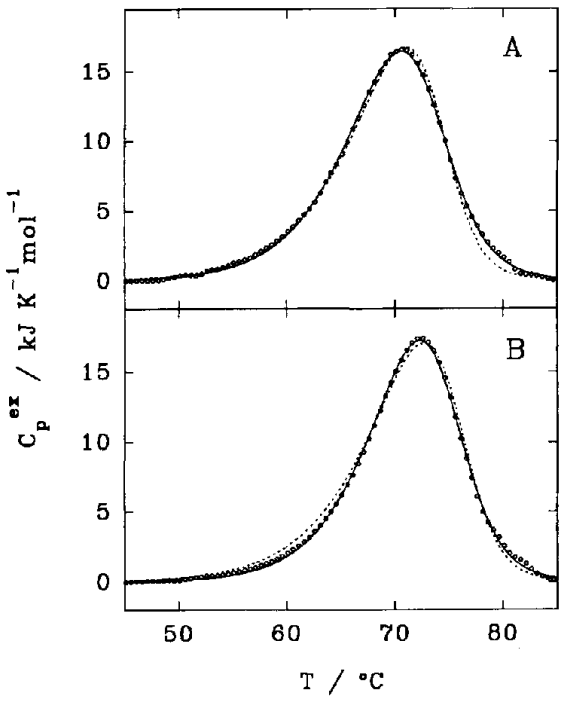

Fig. 3. Non-linear least-square fittings of the C_p^{ex} curves to the $F_n \rightleftarrows nU$ model for the 255–316 thermolysin fragment, pH 7.5. (A) 0.94 mg/ml; (B) 2.21 mg/ml. (○) Experimental data; solid line, best fitting for $n = 2$; dotted line, best fitting for $n = 3$.

mental conditions. This dimerization agrees with previous studies [18], where an association constant at 20°C equal to $(2 \pm 1) \cdot 10^4$ M^{-1} was obtained, from which the Gibbs energy of dimerization results in -24 ± 2 kJ/mol of dimer. This is quite a large value compared to the Gibbs energy of folding of the dimer we can calculate here at 20°C from our DSC data, -60 ± 6 kJ/mol of dimer (i.e. with a dimer stability similar to that of larger globular proteins [19]). Hence, the energetics of the interactions at the contact surface between the two monomers must be particularly significant, bearing in mind the small size of the fragment.

Table 1
Thermodynamic parameters for the DSC thermal unfolding of 255–316 thermolysin fragment at pH 7.5, different sample concentrations and scan rate 2 K/min

C_t (mg/ml)	ΔH (kJ/mol)	Δh (J/g)	ΔH^{vH} (kJ/mol)	T_m (°C)	ΔC_p (kJ/K·mol)
0.19	177	26.6	307	67.1	–
0.29	200	30.2	306	68.0	–
0.72	176	26.5	352	70.2	–
0.94	204	30.8	320	70.7	1.8
1.08	178	26.8	318	70.9	3.2
1.37[a]	170	25.6	349	71.4	2.0
2.21	199	30.0	346	72.4	2.4
4.55	199	30.0	363	73.7	2.4
Average[b]:	192 ± 13	29 ± 2	347 ± 22		2.4 ± 0.5

[a]Scan rate 0.5 K/min.
[b]Average values weighed by sample concentration.

Recently the solution structure of the dimeric fragment has been elucidated by proton NMR techniques (manuscript in preparation), where it is shown that the fragment acquires a secondary and tertiary structure very similar to that of the corresponding chain in the intact protein. Therefore, the results of all these studies decisively show that domains and subdomains in relatively large globular proteins can be viewed as independent co-operative folding units. Since thermolysin is made up of two structural domains of similar size, these studies clearly show that folding units can be substantially smaller than entire structural domains.

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