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Unfolding of bacteriophage P22 tailspike protein: enhanced thermal stability of an N-terminal fusion mutant

X. Carbonell, A. Villaverde*

Institut de Biologia Fonamental and Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, E-08193 Barcelona, Spain

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Abstract The tailspike protein (TSP) of bacteriophage P22 is a homotrimeric multifunctional protein responsible for cell attachment and hydrolysis of the *Salmonella typhimurium* host cell receptor. Despite the folding of TSP involves the formation of thermolabile intermediates, the mature protein is extremely resistant to heat and detergent denaturation. We have analyzed the thermal resistance and unfolding pathway of two mutant, functional TSPs carrying end-terminal peptide fusions. Whereas the C-terminal fusion has minor effects on the TSP stability, the presence of a 23-mer foreign peptide at the N terminus (protein ATSP) results in a significant enhancement of the thermal resistance by retarding the first transition step of the unfolding process. At 65°C and in 2% SDS, the unfolding rate constant for the transition from the native to the unfolding intermediate is 9.3×10^{-4} s^{-1} for ATSP versus 1.7×10^{-3} s^{-1} for wild-type TSP. On the other hand, the electrophoretic mobility of ATSP intermediates is greatly affected, proving structural modifications induced by the fused peptide. These results suggest a critical participation of the N-terminal domain in the unfolding kinetic barriers generated during the TSP denaturation pathway.

Key words: P22; TSP; Folding; Protein denaturation; Unfolding intermediate; Kinetic barrier

1. Introduction

The tailspike protein (TSP) of *Salmonella typhimurium* phage P22 is a homotrimeric protein of 666 amino acid residues, non-covalently attached to the virion neck and essential for the infection of the host cells. Its carboxy terminus is responsible for the hydrolysis of the oligosaccharide receptor at the outer cell membrane [1], allowing the phage particles to positionate for DNA injection, while the amino terminal domain connects the TSP to the phage neck [2]. The native TSP is unusually resistant to proteolysis and to temperature- and detergent-mediated denaturation, whereas the folding intermediates are extremely thermolabile [3]. The crystallographic analysis of a truncated TSP (amino acids 109 to 666) provides a rationale for this high stability, and also reveals that the main bodies of the subunits fold into a compact interdigitated fish-shaped β-coil structure with close intersubunit contacts [4]. Furthermore, by using a recombinant telluromethionine TSP derivative, the N-terminal domain has been crystallographically solved, its structure being different from that of the main body, and showing a high flexibility in segments spanning residues from 1 to 4 and from 109 to 124 [2].

Many mutations hampering the folding of TSP (temperature-sensitive folding, *tsf*) have been identified [5,6], as well as suppressor mutations reverting the Tsf^+ phenotype [7,8]. By analyzing the folding intermediates derived from these mutants, the folding pathway of TSP has been subjected to an exhaustive analysis. The resulting data have been useful to model protein-protein interactions during protein assembling [9,10] and aggregation [11,12]. On the contrary, the denaturation pathway of TSP has been less extensively studied. However, it is known that unfolding occurs via intermediates, which are different from those involved in the folding process [13]. The unfolding of TSP initiates at its amino terminus [13], the carboxy moiety remaining still folded in the transient unfolding intermediates. The existence of a sequential unfolding pathway can be explained by the axial disposition of the amino and carboxy termini, these terminal domains being, in addition, structurally different. To investigate in more detail the involvement of the TSP termini in the transition states during TSP denaturation, we have explored here the thermal stability and unfolding pathway of two fusion TSP mutants. In these proteins, a foreign peptide has been joined at either the amino or the carboxy termini of TSP without disturbing its functionality [14,15]. Surprisingly, the presence of the additional peptide at the amino terminus significantly increases the thermal stability of TSP by enhancing the kinetic barriers during the first transitional unfolding step of the denaturation pathway.

2. Material and methods

2.1. Production and purification of recombinant TSP proteins

Recombinant ATSP and TSPA proteins contain a 23-mer foot-and-mouth disease virus (FMDV) peptide fused at either the amino or the carboxy termini of TSP respectively [14,15]. On the FMDV virion surface, this peptide appears as a disordered, highly mobile protrusion [16], whose structure has been solved either by chemical reduction of the virus [17] or by complexing a synthetic peptide with an anti-virus, neutralizing antibody [18]. In solution, this protein segment is largely unstructured [19]. The peptide added to TSP extends 32 residues in ATSP and 31 in TSPA. This small difference is caused by the introduction of different linker DNA segments during the cloning strategy. These fusion proteins are able to associate with phage heads to render infectious particles, which are indistinguishable from native P22 virions [15], and in both cases the FMDV peptide remains solvent-exposed. Recombinant TSP, ATSP and TSPA were produced in BL26 *E. coli* strain from the IPTG-inducible p_{trc} promoter in plasmids pTTSP, pTATSP and pTTSPA respectively. Details of these vectors are given elsewhere [14,15]. In cultures of 500 ml growing in LB at 37°C, gene expression was induced by the addition of 1 mM IPTG, when the OD_{550} reached 0.8 units. After 2 h of incubation under the same conditions, cells were harvested and pellets resuspended in 5 ml of buffer B25 [20]. After one freezing and thawing step, cell extracts were sonicated, and pH was titrated to 4.0 with glacial acetic acid. After a 30-min incubation at 65°C, extracts were centrifuged, and supernatants were titrated to pH 7.6 with 2 M Tris base. Proteins

*Corresponding author. Fax: +34 (3) 5812011.
E-mail: a.villaverde@cc.uab.es

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were then precipitated with 40% ammonium sulfate. Pellets were resuspended in 50 mM acetate buffer, pH 4.0. Protein samples were finally dialyzed against 10 mM phosphate buffer, pH 7.6.

2.2. Thermal denaturation of recombinant TSP proteins

Denaturation experiments were performed as described [13]. Briefly, pure proteins were diluted to a final concentration of 1 μM (about 0.2 g/l) in 50 mM Tris-HCl, pH 8.0, plus 2% SDS and further incubated at 65°C for 200 min. Aliquots were withdrawn at different times, mixed with 1 volume of SDS-PAGE loading buffer (62.5 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.03% bromophenol blue, pH 6.8) and kept in ice until electrophoresis. Samples of equal volume were ran in a 7.5% SDS-PAGE at 100 V for about 2 h. Finally, gels were submitted to Coomassie blue-staining and bands were quantified by densitometric analysis. Densitometric values from different experiments were adjusted by non-linear regression analysis, using SigmaPlot for Windows, v. 3.0 (Jandel Scientific) software. Then, parameters describing the unfolding process were obtained and their significance was tested by one-way ANOVA and post-hoc comparisons of the means (Newman-Keuls test).

3. Results and discussion

3.1. Unfolding kinetics of mutant TSPs

Fig. 1 shows the disappearing of the native form (N) during the denaturation of either TSP or mutant TSPs, and the evolution of the folding intermediates (I) and the unfolded, monomeric chains (M). Data from four independent experiments are shown. The quantitative analysis of protein bands allows the kinetic analysis of the three-step sequential unfolding reaction (Fig. 2) as previously described [13]:

$$N \xrightarrow{k_1} I \xrightarrow{k_2} M$$

by finding the parameters of the equations that describe the time-dependent evolution of these forms during the unfolding process [13]. The two rate constants for the transitions between N and I, and I and M (k_1 and k_2 respectively) are shown in Table 1. Note that both the carboxy and amino terminal fusions exhibit a transition rate to the unfolding intermediate (k_1) lower than that of the native TSP, being this reduction especially dramatic for ATSP. Therefore, the presence of the added peptide at the amino terminus could create a kinetic barrier for the oligomer dissociation, probably caused by the imposition of sterical impediments to the unfolding process that initiates at the amino terminus of TSP [13]. Therefore, the basis for the enhancement of the thermal resistance in ATSP is mechanistically distinct of that of the *tsf* suppressor mutations, in which the destabilizing effects of specific amino acid substitutions are structurally compensated. During the unfolding of these mutants, there are not changes in the transition to the unfolding intermediates comparing with the wild-type TSP [21].

3.2. Structure of ATSP unfolding intermediates

Interestingly, the electrophoretic mobility of unfolding intermediates is greatly affected in ATSP, showing an apparent shift from 126 ± 2 to 167 ± 3 kDa (Fig. 1). Moreover, the transition rate from the trimeric, partially unfolded intermediate to monomeric chains is higher in TSP (Table 1). In wild-type TSP and also in TSPA, the unfolding intermediates migrate faster than the folded trimer ([13], Fig. 1). The solvent-exposure of larger protein segments in partially denatured TSPs might permit the binding of a higher number of SDS molecules, thus increasing the net negative charge of the proteins and therefore its mobility with respect to native trimers. Therefore, the aberrant migration of partially unfolded ATSP trimers could be explained by important modifications induced by the peptide, affecting the whole structure of the intermediate. Since a reduction of the net negative charge with respect to the native protein is not possible, we would favor the hypothesis that the extent of unfolded polypeptide chain is much larger in ATSP, imposing sterical impediments to the electrophoretic migration. This would be in agreement with the enhanced unstability of these ATSP intermediates during the denaturation process (Table 1), which could be much more kinetically related to the unfolded monomer than those appearing in the wild-type TSP and other TSP mutants. The slight shift observed in TSPA intermediates (Fig. 1) could be accounted by an increase of the predicted p*I* in the fusion mutants (5.8 and 5.9 for TSPA and ATSP

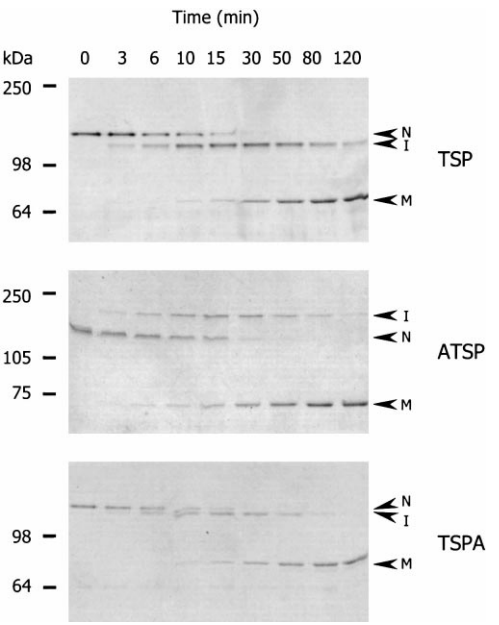

Fig. 1. Coomassie-stained polyacrylamide gels of TSP, ATSP and TSPA protein samples during denaturation in 2% SDS at 65°C. The figures at the left indicate the molecular masses (in kDa) of the markers. Native (N), unfolding intermediate (I) and totally unfolded monomer (M) bands are indicated.

Table 1
Non-linear regression values obtained for the unfolding transition rate constants (s^{-1})

Constant[a]	Protein TSP	ATSP	TSPA
k_1[b]	$1.7\times10^{-3}\pm1.2\times10^{-4}$	$9.3\times10^{-4}\pm1.1\times10^{-4}$	$1.2\times10^{-3}\pm1.2\times10^{-4}$
k_2[b]	$3.0\times10^{-4}\pm2.9\times10^{-5}$	$5.5\times10^{-4}\pm4.9\times10^{-5}$	$4.6\times10^{-4}\pm4.9\times10^{-5}$

[a]The presented values are the mean ± standard error of the mean of the parameters derived from the analysis of four independent experiments.
[b]A pairwise comparison of k_1 and k_2 values indicated significant differences for both constants between TSP and ATSP unfolding processes ($P<0.01$ for TSP-ATSP pair; $P<0.05$ for TSP-TSPA; $P>0.10$ for ATSP-TSPA).

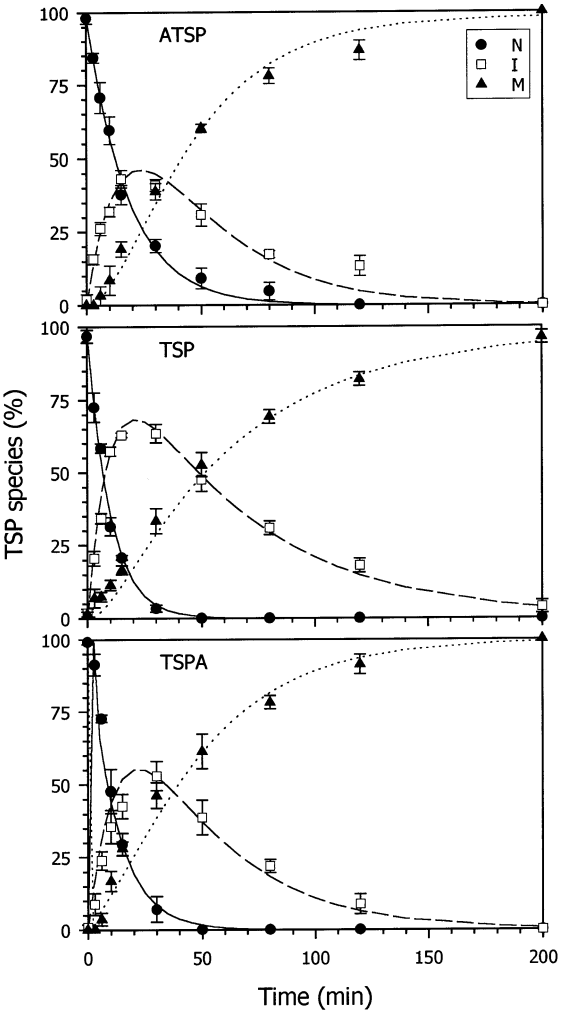

Fig. 2. Thermal denaturation kinetics for proteins TSP, ATSP and TSPA. Native (N), unfolding intermediate (I) and totally unfolded monomer (M) species are plotted as mean values and standard error of the mean (bars) from two independent experiments. Lines represent the theoretical values predicted from a non-linear regression adjust.

respectively versus 5.3 for TSP), due to the presence of the foreign peptide that alters the amino acid composition.

Protein ATSP, in which a foreign peptide is fused at the amino terminus of TSP, is the firstly described example of a new type of TSP mutants, showing an increase of its natural thermal stability. In addition, the unfolding of this protein progresses through the formation of unfolding intermediate species with aberrant electrophoretic mobilities. These intermediates are much more unstable than those found in the TSP denaturation, as reflected by a higher k_2 value in ATSP (Table 1). These observations, apart from providing an interesting tool to further explore the folding and unfolding kinetics of an unusually compact trimeric protein, confirms the critical role of the TSP amino terminal domain in the initiation of protein unfolding process during denaturation.

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