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Phage P22 tailspike protein: Removal of head-binding domain unmasks effects of folding mutations on native-state thermal stability

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Abstract

A shortened, recombinant protein comprising residues 109–666 of the tailspike endorhamnosidase of Salmonella phage P22 was purified from *Escherichia coli* and crystallized. Like the full-length tailspike, the protein lacking the amino-terminal head-binding domain is an SDS-resistant, thermostable trimer. Its fluorescence and circular dichroism spectra indicate native structure. Oligosaccharide binding and endoglycosidase activities of both proteins are identical. A number of tailspike folding mutants have been obtained previously in a genetic approach to protein folding. Two temperature-sensitive-folding (tsf) mutations and the four known global second-site suppressor (su) mutations were introduced into the shortened protein and found to reduce or increase folding yields at high temperature. The mutational effects on folding yields and subunit folding kinetics parallel those observed with the full-length protein. They mirror the in vivo phenotypes and are consistent with the substitutions altering the stability of thermolabile folding intermediates. Because full-length and shortened tailspikes aggregate upon thermal denaturation, and their denaturant-induced unfolding displays hysteresis, kinetics of thermal unfolding were measured to assess the stability of the native proteins. Unfolding of the shortened wild-type protein in the presence of 2% SDS at 71 °C occurs at a rate of 9.2×10^{-4} s^{-1}. It reflects the second kinetic phase of unfolding of the full-length protein. All six mutations were found to affect the thermal stability of the native protein. Both tsf mutations accelerate thermal unfolding about 10-fold. Two of the su mutations retard thermal unfolding up to 5-fold, while the remaining two mutations accelerate unfolding up to 5-fold. The mutational effects can be rationalized on the background of the recently determined crystal structure of the protein.

Keywords: bacteriophage tail protein; parallel beta-helix; protein folding intermediates; protein stability; temperature-sensitive mutants

Theoretical studies and recent experimental observations with small proteins indicate that the folding of an isolated protein domain may happen as a rapid nucleation-condensation process without detectable kinetic intermediates (Dill & Chan, 1997). In contrast, the much slower formation of the native three-dimensional structure of large, multidomain, oligomeric proteins generally can be described as a series of first-order and second-order reactions (Jaenicke, 1987) and is frequently compromised by competing off-pathway aggregation reactions (Jaenicke & Seckler, 1997). Little is known about the structure of the intermediates involved in productive folding, on the one hand, and aggregation, on the other. This is because of their transient nature and their inaccessibility to high-resolution methods like multidimensional NMR spectroscopy.

In a genetic approach to the problem, King and Yu (1986) have identified a large number of amino acid substitutions affecting the yield of folding and assembly of the homotrimeric bacteriophage P22 tailspike endorhamnosidase (Betts et al., 1997). Temperature-sensitive-folding (tsf) mutations reduce tailspike folding yields at high temperature and enhance aggregation, while mutations isolated as intragenic second-site suppressors (su) of the tsf phenotype have the opposite effect (Haase-Pettingell & King, 1988; Mitraki et al., 1991). Effects of both types of mutations on the refolding of purified tailspike protein after denaturant-induced unfolding in vitro mirror the respective phenotypes observed in vivo (Mitraki et al., 1993; Seckler, 1997). A thorough analysis of the refolding pathways of wild-type tailspikes and of a number of single- and double-mutant proteins at varied temperature has confirmed that the mutations act by altering the thermal stability of folding intermediates (Danner & Seckler, 1993; Beißinger et al., 1995).

Initial observations on the kinetics of thermal inactivation and on the irreversible thermal unfolding observed by differential scanning calorimetry suggested that the mutations did not affect the stability of the folded conformation of the tailspike protein (Gold-

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enberg & King, 1981; Sturtevant et al., 1989; Mitraki et al., 1991). Thus, the sites of tsf mutations were proposed to mark interactions important in early folding intermediates and necessary to direct productive folding but not present in the native, completely folded conformation (Yu & King, 1984, 1988). More detailed investigation later proved the thermal denaturation reaction to occur in two consecutive steps and showed tsf and su mutations to affect the rate of the second kinetic phase of unfolding (Chen & King, 1991a, 1991b; Danner & Seckler, 1993; Beißinger et al., 1995). The unfolding intermediate affected by the mutations was probed by chemical modification and proteolysis (Chen & King, 1991a; Danner et al., 1993). The results showed the amino-terminal part of the tailspike protein, some 110 residues of each chain responsible for binding the tailspike trimer to the bacteriophage head, to unfold in the first kinetic phase of thermal denaturation, while the major part of the protein appeared to remain stably folded.

Prompted by these observations, we have prepared amino-terminally shortened tailspikes corresponding to the major, C-terminal part (residues 109–666) with amino acid sequences of the wild-type and six folding mutants. The shortened, recombinant tailspikes, purified from *Escherichia coli*, are thermostable and SDS-resistant and their substrate binding and enzymatic activities are identical to those of the full-length proteins. The shortened wild-type protein was crystallized and its structure was determined at atomic resolution both in unliganded form and in complexes with oligosaccharide fragments from its cell surface receptor, Salmonella lipopolysaccharide (Steinbacher et al., 1994, 1996, 1997). Our analysis of folding and unfolding reactions of the shortened tsf and su mutant proteins prove that the folding mutations not only affect the stability of folding intermediates, but also affect the resistance of the native protein to thermal denaturation.

Results

In previous experiments, the head-binding domain of the tailspike protein had been removed by proteolytic digestion of a trimeric thermal unfolding intermediate (Chen & King, 1991a). The resulting N-terminally shortened protein, purified by gel filtration after tryptic digestion, was an SDS-resistant, enzymatically active trimer, which could be refolded and reassembled upon acid-urea denaturation (Danner et al., 1993). Because the yields of the proteolytic preparation were low and because tsf mutations were observed to reduce the population of the trimeric unfolding intermediate, the source of the preparation (Danner & Seckler, 1993), we attempted to produce the shortened protein by recombinant expression in *E. coli*.

Expression, purification, and biophysical characterization

The N-terminal residues of the proteolytically shortened protein are Tyr108-Asp109-..., resulting from tryptic cleavage of the unfolding intermediate after Lys107 (Chen & King, 1991a). Tyrosine at the N-terminus is supposed to render recombinant proteins labile to degradation in *E. coli* (Tobias et al., 1991). Thus, the primers we used to amplify a fragment of P22 gene 9 from phage DNA by the polymerase chain reaction were designed for expression of a protein beginning with Asp109 preceded by methionine (TSPΔN). Upon induction of protein synthesis at 26–37 °C, the shortened tailspike chains efficiently assembled into soluble, SDS-resistant trimers, as shown by SDS polyacrylamide gel electrophoresis (Danner et al., 1993; Miller, 1995). Purification of TSPΔN yielded about 30 mg of electrophoretically homogeneous protein from 1 L of bacterial culture.

To allow a direct comparison with the shortened protein, the full-length gene 9 coding for the tailspike protein including its N-terminal head-binding domain was cloned into the same vector, expressed, and purified by the same methods used for TSPΔN.

Fluorescence emission spectra of TSPΔN were identical to those determined previously for the proteolytically shortened protein and, except for a small decrease in intensity around 300 nm, were very similar in shape and intensity to the spectra of TSP measured at identical molar concentration (Danner et al., 1993). This is to be expected if TSPΔN assumes the native conformation and if the six tyrosines removed with the N-terminal domain exhibit minimal energy transfer to the tryptophans in the full-length tailspike protein.

Sensitive quantitative assays for the receptor binding and enzymatic activities using defined, purified oligosaccharides have recently been developed (Baxa et al., 1996). We used these assays to compare the biological activities of TSPΔN and TSP and found identical dissociation equilibrium constants for octasaccharides from *S. enteritidis* and enzymatic turnover numbers determined with a coumarin-labeled dodecasaccharide substrate (0.8 ± 0.2 μM and 0.01 ± 0.002 s^{-1}, both measured at 10 °C).

Crystallization of the full-length tailspike protein had been attempted by a number of groups including ours (T. Alber and J. King, pers. comm.; McPherson et al., 1986). The only crystal form obtained consisted of thin plates with high mosaicity. In contrast, TSPΔN readily crystallized in a rapid vectorial screen for crystallization conditions (Jancarik & Kim, 1991). Orthorhombic crystals obtained around pH 4.6 in the presence of 0.2–0.4 M $CaCl_2$ with isopropanol as a precipitant diffracted to better than 2.5 Å, but had a very large lattice constant of 420 Å (Steinbacher, 1996). A more systematic variation of conditions led to a second, cubic crystal form that was used for structure determination. These results have been published (Steinbacher et al., 1994, 1997) and some of the structural features of the protein are illustrated in the kinemages enclosed in the electronic appendix.

Folding and unfolding reactions

The pathway of refolding and reassociation of TSP upon denaturation in urea solutions at acid pH has been characterized by spectroscopic and hydrodynamic techniques (Fuchs et al., 1991; Danner & Seckler, 1993). It comprises three major phases: (1) chain folding into highly structured subunits, as observed by fluorescence and CD spectroscopy; (2) the formation of a trimeric intermediate ("protrimer"), in which the chains are stably associated but which lacks the thermostability of the native protein; (3) a rate-limiting folding reaction at the trimer level, in which the protein becomes thermostable and SDS-resistant and which is associated with a very high activation enthalpy. Experiments with the proteolytically shortened protein have shown that the folding-association pathway of the tailspike lacking the N-terminal head-binding domain mirrors the pathway of the full-length protein, with slightly accelerated subunit and trimer folding reactions (Danner et al., 1993). These observations were confirmed with the recombinant proteins purified from *E. coli* (compare Table 1 and Materials and methods).

A new observation is a fluorescence change during trimer maturation. Because of the very high activation enthalpy of the trimer folding reaction, the protrimer can be trapped in the cold and its maturation to the SDS-resistant trimer can be observed after a temperature shift to 30 °C (Danner et al., 1993). Formation of

Table 1. *Effect of point mutations on subunit folding and thermal denaturation kinetics*[a]

	Subunit folding		Thermal unfolding	
Mutant	10 °C $10^3 \cdot k$ (s^{-1})	25 °C $10^2 \cdot k$ (s^{-1})	68 °C $10^4 \cdot k$ (s^{-1})	71 °C $10^4 \cdot k$ (s^{-1})
TSPΔNwt	3.1 ± 0.1	1.9 ± 0.1	1.3	9.2
TSPΔNts238S	2.2 ± 0.1	0.9 ± 0.1	12	n.d.[b]
TSPΔNts244R	2.5 ± 0.1	1.1 ± 0.1	13	n.d.[b]
TSPΔNsu331A	3.2 ± 0.1	2.6 ± 0.2	n.d.[b]	1.7
TSPΔNsu331G	3.3 ± 0.1	2.0 ± 0.1	n.d.[b]	3.3
TSPΔNsu334V	3.1 ± 0.1	2.2 ± 0.1	n.d.[b]	13
TSPΔNsu334I	2.8 ± 0.1	2.1 ± 0.1	6.7	40

[a]Subunit folding after dilution from acid urea and thermal denaturation in the presence of SDS were measured as described in Materials and methods. Subunit folding rates were determined from nonlinear fits to single exponentials and are averages (± standard deviation) of at least three independent measurements. Unfolding rates were determined as described in the legend to Figure 3.

[b]Not determined; unfolding of tsf mutant proteins at 71 °C was too fast and unfolding of su331 mutant proteins at 68 °C was too slow to be reliably quantified by the techniques used.

SDS-resistant trimers and the change in tryptophan fluorescence after such a temperature shift are depicted in Figures 1A and 1B, respectively. Trimer maturation, as measured by densitometry of Coomassie-stained gels, could be described by a single-exponential rise with a half-time of 5 ± 1 min, identical to that observed previously with the proteolytically shortened protein, and slightly, but consistently smaller than the maturation half-time of TSP (7 ± 1 min) under the same conditions (Danner et al., 1993). In the fluorescence experiment, the signal decreased by around 20% during the first 20 min. This signal change was not observed with a sample of native protein preincubated and diluted at the same temperatures. In contrast, a slow decrease in fluorescence that did not come to completion over the next hour was observed with both samples, and probably corresponded to photobleaching. When the fluorescence change was fit to a biexponential decay, the first phase ($k = 2.2 \times 10^{-3}$ s^{-1}, $t_{1/2} = 5.3$ min) coincided with the formation of SDS-resistant trimers.

Like TSP, TSPΔN could not be denatured by high concentrations of urea at neutral pH, and its unfolding and refolding transitions induced by varying the concentrations of GdmCl and observed by fluorescence spectroscopy did not coincide even after incubation for many days (Fuchs et al., 1991; Danner & Seckler, 1993). Thus, the thermodynamic stability of TSPΔN could not be determined from denaturant-induced unfolding equilibria and we had to resort to unfolding kinetics to obtain information about its stability.

Thermal denaturation kinetics were measured by SDS gel electrophoresis, monitoring the decrease in intensity of the detergent-resistant trimer band. The thermal unfolding reactions of TSP and of TSPΔN, measured under rigorously identical conditions, are compared in Figures 1C and 1D. Thermal unfolding of TSP occurs in a biphasic reaction via an intermediate that runs slightly faster than the native trimer on the SDS gels and in which the amino-terminal domains are sensitive to proteolysis (Chen & King, 1991a; Danner et al., 1993). For the present experiments, conditions were optimized to rapidly reach the target temperature and to keep it in a narrow range throughout the course of the reaction. This was achieved by using small sample volumes in thin-wall reaction vessels that were completely immersed into a large volume thermostatted water bath at the start of the reaction and transferred into an ice-water mixture to stop unfolding. Under these improved conditions, the trimeric unfolding intermediate was formed rapidly, the two kinetic phases were well separated, and the observed first-order rate of denaturation of the intermediate ($k = 7 \pm 1 \times 10^{-4}$ s^{-1} at 71 °C) was highly reproducible. Denaturation of TSPΔN, occurring after a short apparent lag of ≈2 min, was well described by a first-order reaction with $k = 9 \pm 1 \times 10^{-4}$ s^{-1} at 71 °C, identical, within error, to the rate of unfolding of the intermediate in the full-length protein. The short lag at the beginning of the reaction was significantly prolonged when standard reaction vessels were substituted for the thin-wall PCR cups used in the optimized procedure (data not shown). When the temperature was reduced to 68 °C, the unfolding rate was reduced 8-fold to $k = 1.2 \times 10^{-4}$ s^{-1} with little effect on the duration of the lag phase (Fig. 3). These results suggest that the observed lag corresponded to the time required for temperature equilibration. Because a large part of the first kinetic phase of unfolding of TSP, i.e., of the unfolding of the amino-terminal domain, occurred in this heating period, we did not attempt a quantitative analysis of its kinetics.

Amino-terminally shortened folding mutant proteins

Six single amino acid substitutions were selected for transfer into TSPΔN. TsfG244R and tsfD238S are two temperature-sensitive-folding mutations of which D238S is the more defective one. They have been subject to a number of in vivo and in vitro folding studies with the full-length tailspike protein and represent the two most completely characterized tsf mutations (Yu & King, 1984, 1988; Haase-Pettingell & King, 1988; Villafane & King, 1988; Sturtevant et al., 1989; Stroup & Gierasch, 1990; Mitraki et al., 1991; Danner & Seckler, 1993; Mitraki et al., 1993; Lee & Yu, 1997). SuV331A and suA334V are the two mutations originally identified as global suppressors of tsf mutations (Fane et al., 1991); they increase tailspike folding yields in vivo and in vitro at high temperature (Mitraki et al., 1991, 1993; Danner & Seckler, 1993). SuV331G and suA334I, two other substitutions at the same sites

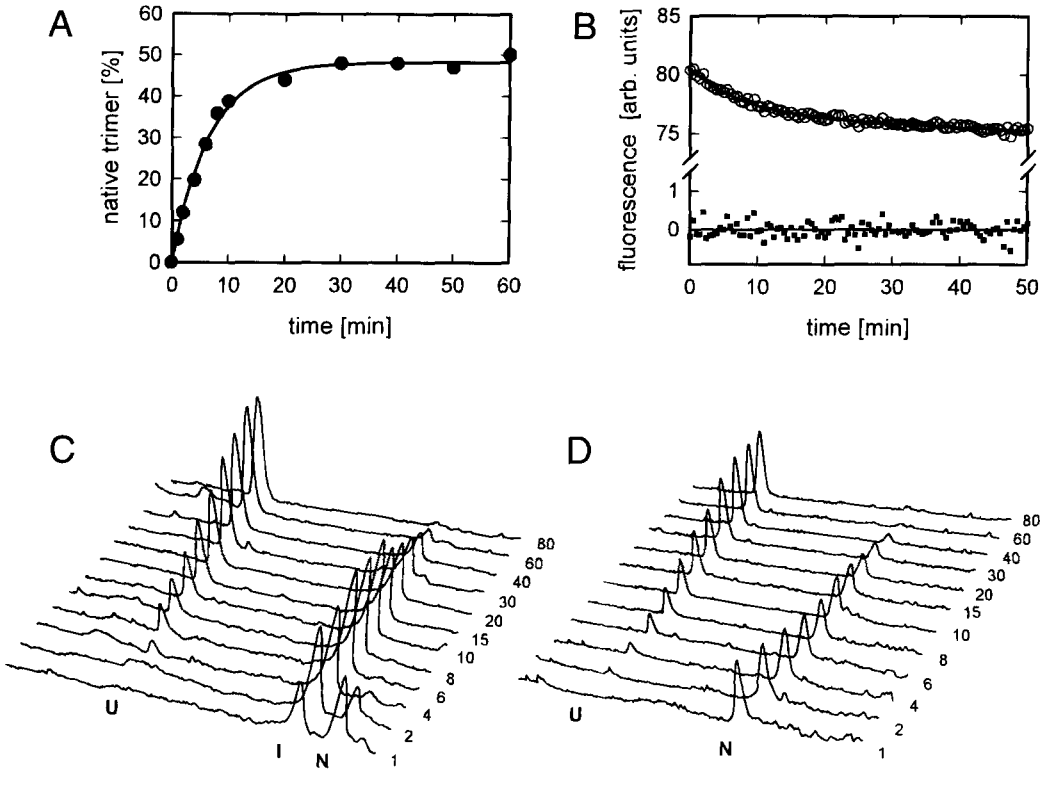

Fig. 1. Folding and unfolding of TSPΔN. **A** and **B:** Trimer maturation at 30 °C quantified by SDS-PAGE and silver staining (**A**) and tryptophan fluorescence at 342 nm with excitation at 295 nm (**B**). Refolding of TSPΔN was initiated at 10 °C and a protein concentration of 50 μg/mL and was allowed to continue for 30 min. At this time, polypeptide chains (>90%) are stably associated, but not yet SDS-resistant. Aliquots of the sample were further diluted 10-fold into buffer preincubated at 30 °C to initiate the maturation reaction. Maturation was followed by SDS gel electrophoresis or by recording the fluorescence at 342 nm in a Spex Fluoromax. Lines represent single exponential (**A**) or double exponential (**B**) fits to the data (■, residuals). Trimer band intensities are given relative to a native control. **C** and **D:** Thermal unfolding of TSP (**C**) and TSPΔN (**D**) at 71 °C in the presence of SDS. After 1–80 min the reaction was stopped by rapid cooling on ice and the samples were analyzed by SDS gel electrophoresis and densitometry. *N* and *U* correspond to native trimers and denatured polypeptides, respectively; *I* designates the intermediate observed in the unfolding of TSP.

generated by site-directed mutagenesis (Lee et al., 1991), also act as tsf suppressors in vivo and in vitro (Beißinger et al., 1995). All six mutant polypeptides folded and assembled into SDS-resistant trimers at permissive temperature in *E. coli* and were purified by the techniques used for TSPΔN with the wild-type sequence (TSPΔNwt).

Yields of refolding of TSPΔN mutants are depicted in Figure 2. They were measured as the formation of SDS-resistant trimers and are plotted relative to the refolding yield observed for TSPΔNwt at the respective temperatures. The refolding yield of TSPΔNwt decreased from 80 ± 5% at 10 °C to below 1% at 42 °C. Both tsf mutations reduced refolding yields at high temperatures but had little effect at low temperature. The substitution D238S, which causes the more defective phenotype in vivo, more severely affected refolding of TSPΔN. These results parallel those obtained previously with TSP (Mitraki et al., 1993). Among the su mutations, V331G increased refolding yields at high temperature most strongly, V331A and A334V were somewhat less effective, and A334I actually decreased refolding yields at temperatures above 35 °C. Again, these observations mirror those made with the full-length protein, where suA334I only increases refolding yields when present as a second substitution in a tsf background (Beißinger et al., 1995).

Previous refolding experiments with TSP have shown that tsf mutations decrease the apparent rate of subunit folding at moderately high, but not at low temperature. This effect is partly alleviated by a su mutation in tsf/su double mutants and is probably caused by both types of mutations altering the thermal stability of folding intermediates (Danner & Seckler, 1993; Beißinger et al., 1995). Subunit folding kinetics of TSPΔN proteins were measured by fluorescence spectroscopy at 10 and 25 °C and were analyzed as first-order reactions (Danner et al., 1993; Danner & Seckler, 1993). The data are depicted in Table 1. Both tsf mutations decreased the rate of subunit folding at 25 °C close to 2-fold, and the amplitude of the fluorescence change was significantly reduced. As observed previously with TSP, the tsf mutations had much smaller effects at low temperature, and the su mutations, present as single substitutions in the wild-type background, did not significantly affects subunit folding kinetics at 10 and 25 °C (Danner & Seckler, 1993).

Thermal unfolding of tsf and su mutant proteins

Kinetics of thermal denaturation of TSPΔN with wild-type and mutant sequences are compared in Figures 3A and 3B and Table 1. The substitutions from both temperature-sensitive mutants, G244R

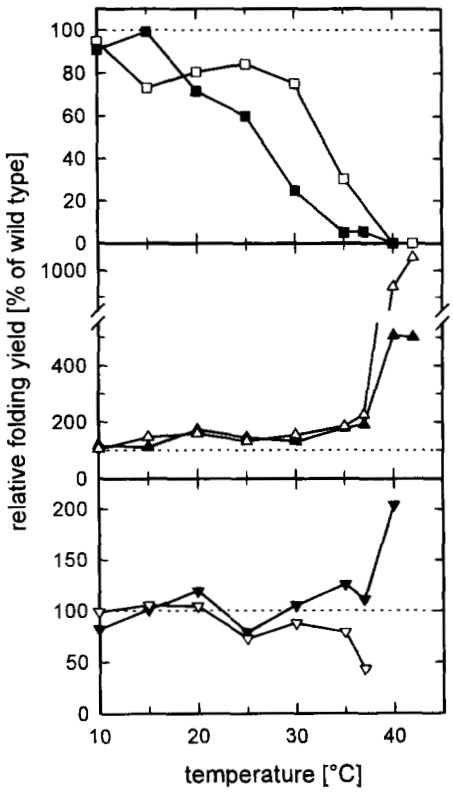

Fig. 2. Temperature-sensitivity of TSPΔN refolding. Yields of renaturation of TSPΔNts244R (□), TSPΔNts238S (■), TSPΔNsu331A (▲) and TSPΔNsu331G (△), TSPΔNsu334V (▼), and TSPΔNsu334I (▽) are plotted relative to the yields observed for TSPΔN with the wild-type sequence (TSPΔNwt) at the same temperature. Reconstitution yields of TSPΔNwt trimers decrease from ~80% at 10 °C to ~1% at 42 °C.

and D238S, accelerated thermal denaturation about 10-fold. Among the su mutations, both substitutions at position 331 (V331G and V331A) retarded thermal unfolding of TSPΔN 2.5–5-fold. SuA334V weakly accelerated unfolding (1.6-fold), while the acceleration by the suA334I mutation approached those observed for the tsf mutations (5-fold relative to wild-type TSPΔN).

The effects of the folding mutations on the thermal stability of TSPΔN also became apparent when the loss of the native secondary structure with increasing temperature was followed by CD spectroscopy. During a temperature scan in neutral buffer, TSPΔN aggregates upon unfolding above 83 °C, leading to high light-scattering and preventing spectroscopic analysis (data not shown). When temperature scans were done in the presence of SDS, the protein remained soluble. The CD amplitude increased upon denaturation, when the native β-sheet secondary structure was lost and replaced by the helical secondary structure proteins assumed in association with SDS micelles (Mattice et al., 1976; Ibel et al., 1990). Thermal unfolding transitions so determined for TSPΔN wild-type and two mutants are depicted in Figure 3C. For TSPΔN with wild-type sequence, irreversible unfolding occurred around a transition midpoint of 76 °C at a temperature scan rate of 1 K/min. Under the same conditions, transition midpoints were 66 °C for the protein with the tsfG244R substitution and 77 °C for TSPΔN proteins carrying the suV331G or suV331A mutations.

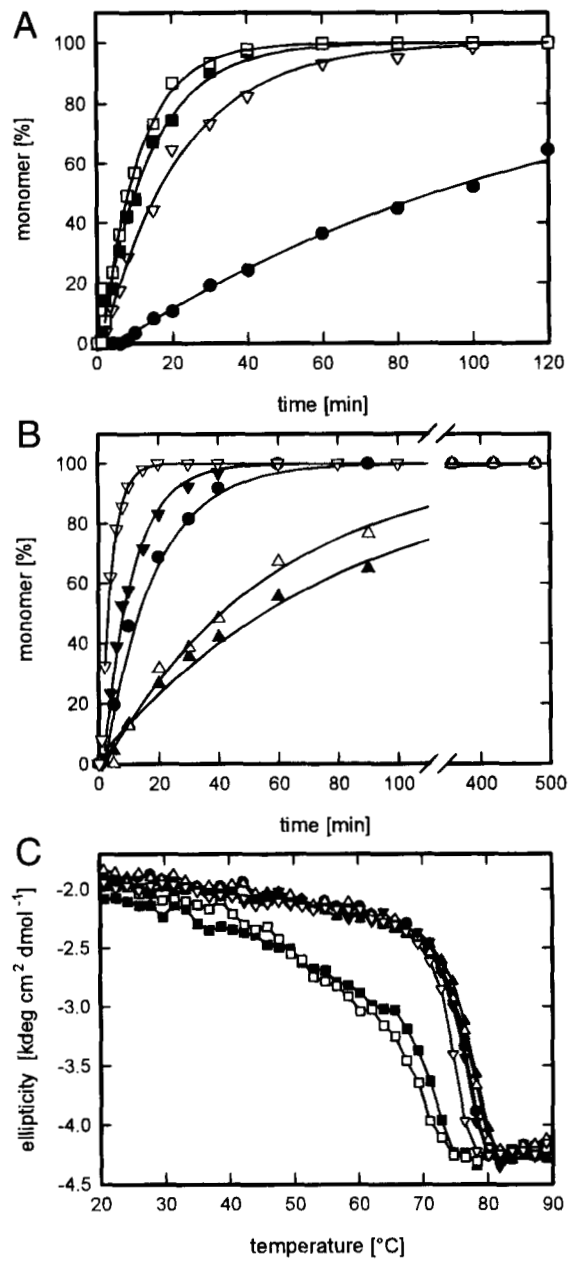

Fig. 3. Mutation effects on thermal unfolding of TSPΔN. **A** and **B**: Thermal unfolding kinetics of TSPΔNwt (●), TSPΔNts244R (□), TSPΔNts238S (■), TSPΔNsu331A (▲) and TSPΔNsu331G (△), TSPΔNsu334V (▼), and TSPΔNsu334I (▽) measured at 68 °C (**A**) or 71 °C (**B**) as illustrated in Figures 1C and 1D. The fractional peak area corresponding to the SDS-sensitive denatured polypeptide (U) is plotted. Solid lines represent single exponentials fitted to the data for $t \geq 2$ min, i.e., omitting the lag period. Rate constants obtained from such fits are depicted in Table 1. They were identical within experimental error to those resulting from fits to a uni-unimolecular sequential model ($N \rightarrow I \rightarrow U$). **C**: Irreversible thermal melting transitions of TSPΔN wild-type and mutants observed by far-UV CD at 230 nm in the presence of SDS (symbols as in **A** and **B**).

Discussion

In an attempt to facilitate crystallization and X-ray analysis of the phage P22 tailspike endorhamnosidase and to simplify the analysis

of the effects of amino acid substitutions on its folding and unfolding, we have generated a recombinant fragment of the protein lacking the amino-terminal head-binding domain. The shortened protein, encompassing residues 109–666 (TSPΔN) folds and trimerizes efficiently in *E. coli*. Its thermostability, resistance to denaturation by SDS, oligosaccharide binding and hydrolysis, and its spectral properties prove that the amino-terminally shortened protein assumes the native three-dimensional structure. This structure, which has been determined by X-ray analysis of TSPΔN crystals (Steinbacher et al., 1994, 1996, 1997), is illustrated in Figure 4 and Kinemage 1. The major part of a tailspike subunit (residues 143–540) consists of a right-handed parallel β-helix of 13 complete turns into which several loops and a small, irregularly structured domain (residues 197–259) are inserted. The β-helices of the three subunits are associated side-by-side through hydrophilic subunit interfaces, whereas the C-terminal segments of the three polypeptide chains (residues 541–666) are intertwined; in this part of the trimer, the secondary and tertiary structure and the hydrophobic core are formed between the subunit polypeptides.

The folding pathway of TSPΔN reflects that of TSP with successive subunit folding, association and trimer maturation reactions (Fuchs et al., 1991; Danner & Seckler, 1993). Trimer maturation likely involves major structural rearrangements of the C-terminal parts of the chains. This is suggested by the crystal structure, and is supported by the large activation energy of the maturation reaction (halftimes decrease from ~10 h at 10 °C to ~1 h at 20 °C, and ~0.1 h at 30 °C, which results in $E_a \approx 165$ kJ/mol assuming Arrhenius behavior), and by the finding that trimer maturation is prevented by chemical modification of cysteine residues located in the C-terminal segment (Sather & King, 1994; Robinson & King, 1997; S. Miller, unpubl. obs.). The fluorescence quench associated with trimer maturation (Fig. 1B) may be due to Trp640, which is located at the subunit interface in the extreme C-terminal part of the protein. A significant increase in the rate of trimer maturation is observed upon removal of the N-terminal head-binding domain, indicating that the conformational rearrangement required for folding of the C-termini is propagated through the whole protein.

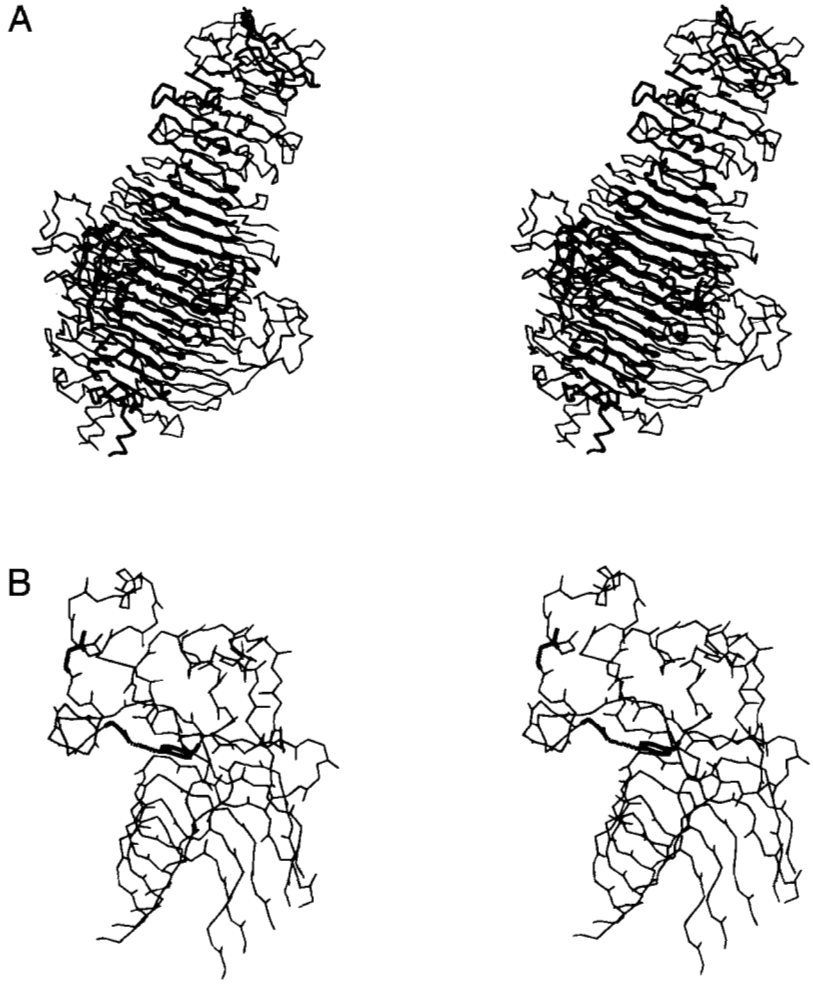

Fig. 4. Structural background of temperature-sensitive-folding mutations (Kinemages 1–3). **A:** Alpha-carbon trace of the TSPΔN trimer (Steinbacher et al., 1994; PDB entry 1TSP). One subunit is traced with bold lines. **B:** Section through the central part of the parallel β-helix showing the polypeptide backbone of the "dorsal fin" domain and the turn regions connecting β-sheets B and C of the 3rd to 8th β-helix winding as viewed from the C-terminus. Bold lines highlight the sites of tsf mutations discussed in this study, i.e., the backbone of Gly244 (upper left) and the side chains of Asp238 and Tyr232 (center) which are hydrogen-bonded (bold dashed line).

Six single amino acid substitutions were introduced into TSPΔN, the resultant mutant TSPΔN trimers were purified, and their folding and unfolding was analyzed. In all six cases, the observations on their in vitro refolding mirror the results obtained previously with the same mutations in the full-length tailspike protein in vitro and in vivo (Danner & Seckler, 1993; Mitraki et al., 1993; Beißinger et al., 1995). The results are consistent with the tsf mutations destabilizing and the su mutations stabilizing subunit folding intermediates (Danner & Seckler, 1993; Beißinger et al., 1995).

Thermal denaturation of TSPΔN and TSP are irreversible reactions and for both proteins the unfolding and refolding transitions induced by varying the concentration of GdmCl do not coincide. Thus, the thermodynamic stabilities of wild-type and mutant TSP and TSPΔN proteins cannot be determined from unfolding equilibria, and one has to resort to unfolding kinetics to obtain information about the stability of the native state. Protein unfolding kinetics reflect the difference in stability between the native state and the rate-limiting transition state of unfolding, and may be affected by changes in either state. However, because many native interactions are broken in the transition state, unfolding kinetics are very sensitive to changes in the native structure and there is a good correlation between the unfolding rates and the free energies of folding for a series of mutants of a given protein (Klemm et al., 1991).

Because native TSP and TSPΔN trimers are resistant to denaturation by the ionic detergent SDS at ambient temperature, their thermal unfolding could be assayed by gel electrophoresis. Thermal unfolding of TSPΔN in the presence of SDS around 70 °C can be described as a single exponential decay. Its rate is identical to the unfolding rate of the trimeric intermediate observed during thermal denaturation of TSP, confirming that the major part of the tailspike protein remains stably folded in this intermediate (Chen & King, 1991a; Danner et al., 1993).

Both tsf mutations introduced into TSPΔN accelerate its thermal unfolding, consistent with these mutations destabilizing the native state. Thermal unfolding experiments with full-length tailspikes have been performed for a number of tsf mutant proteins in comparison with the wild-type (Chen & King, 1991b; Danner & Seckler, 1993). Although a kinetic analysis is more reliable with TSPΔN, the previous results with the full-length proteins provide a qualitative estimate of the effects of a much larger number of tsf mutations. In all cases, the tsf mutations accelerate denaturation of the trimeric unfolding intermediate, and there is a good correlation between the observed acceleration of thermal unfolding and the severity of the temperature-sensitive-folding phenotype. This strongly suggests that the tsf mutations affect the same interactions in thermolabile subunit folding intermediates and in the native, folded protein. As the known sites of tsf mutations are spread throughout the β-helix domain of the tailspike, we propose that this major domain is largely folded in the subunit folding intermediates destabilized by tsf mutations.

Can the destabilizing effect of tsf mutations be rationalized on the basis of the high-resolution crystal structure of TSPΔN? Figure 4 and Kinemage 2 illustrate the environment of Gly244 and Asp238, the residues which are the sites of the tsf mutations subject to the folding and unfolding studies presented in this paper. Also highlighted is Tyr232, whose substitution by Gln leads to an extremely temperature-sensitive mutant with a restrictive temperature around 25 °C (Fane & King, 1991; C. Haase-Pettingell & J. King, pers. comm.). All three residues are part of the large "dorsal fin" insertion packed against the central part of the β-helix (compare Kinemage 1). Gly244 is a surface-exposed residue and part of a type-II β-turn with backbone dihedral angles of $\phi = 96°$ and $\psi = -7°$. Its replacement by Arg is expected to cause local rearrangements and/or lead to steric strain and destabilize the β-turn, in agreement with peptide studies (Stroup & Gierasch, 1990). Asp238 and Tyr232, the sites of the more defective mutants, are both completely buried at the interface between the "dorsal fin" insertion and the parallel β-helix and are connected by a hydrogen bond. Replacement of either of the two by smaller polar residues, as in tsfD238S and tsfY232Q, may be expected to lead to unsatisfied hydrogen bonds or create a water-filled cavity. In addition, Tyr232 makes contacts with a number of surrounding residues that will be disturbed upon its removal. The mechanism of action of tsf substitutions in the P22 tailspike protein suggested by our observations differs from that of mutations that cause a similar phenotype in the heterodimeric bacterial luciferase. The luciferase mutations affect the kinetics of heterodimer assembly and do not alter the stability of the native state (Clark et al., 1997; T.O. Baldwin, pers. comm.).

Among the four mutations acting as suppressors of the tsf phenotype in the tailspike protein, the substitutions at position 331 (suV331A and suV331G) clearly retarded, whereas those at position 334 (suA334V and suA334I) accelerated thermal unfolding of TSPΔN. Qualitatively, the results reflect those obtained previously with the same mutations in the full-length protein (Beißinger et al., 1995). However, the effects of the mutations were more clearly visible during unfolding of TSPΔN, and their quantitative analysis by densitometry of two rather than three differently staining bands on electrophoresis gels was more reliable. As discussed previously (Steinbacher et al., 1994; Beißinger et al., 1995) and illustrated in Kinemage 3, the two mutations that retard thermal unfolding (V331A and V331G) may stabilize the native structure by removing backbone strain, as Val331 has unfavorable backbone dihedrals ($\phi = -121°$, $\psi = -144°$). Although alanine and, even more so, glycine introduced at position 331 both stabilize the native tailspike protein against thermal unfolding and increase its folding efficiency, the energetically more unfavorable valine residue is present at this position in wild-type P22 phage isolates (J. King, pers. comm.). Residue 331 is located in the immediate vicinity of the receptor binding site of the tailspike protein (Steinbacher et al., 1996), and both substitutions reduce its oligosaccharide binding affinity (U. Baxa & R. Seckler, unpubl. obs.).

In conclusion, the removal of the amino-terminal head-binding domain from the tailspike protein not only was prerequisite to the determination of the crystal structure, but also facilitated the analysis of the effects of folding mutations on its thermal stability. The results of our denaturation experiments prove that tsf and su mutations alter the rate of unfolding of the native structure in this major part of the tailspike protein. The observed effects of the mutations on refolding yields and kinetics are compatible with the mutations affecting the thermal stability of the parallel β-helix in largely structured subunit folding intermediates. Future experiments will be aimed at the recombinant expression of the isolated β-helix domain, in order to establish a model for such an intermediate.

Materials and methods

Materials

Ultrapure urea and guanidinium chloride (GdmCl) were obtained from ICN Biomedicals. Concentrations of urea and GdmCl solu-

tions were determined by refractive index measurements (Pace, 1986). Polyoxyethylenesorbitan monolaurate (Tween 20) and Coomassie Brilliant Blue (Serva Blue R) were obtained from Serva (Heidelberg, Germany). Oligonucleotides were supplied by MWG-Biotech (Munich, Germany) and enzymes were from Boehringer Mannheim (Mannheim, Germany), Pharmacia (Uppsala, Sweden), or Promega Inc. (Madison, Wisconsin). Other chemicals were of analytical grade and quartz-bidistilled water was used throughout.

Spectroscopy and quantitative electrophoresis

UV absorption spectra were recorded on a Cary 1 spectrophotometer (Varian, Palo Alto, California). Protein extinction coefficients were determined according to Pace et al. (1995). Molar absorbances of unfolded proteins in 6 M GdmCl were calculated from the amino acid composition to 76,620 $cm^2\ mmol^{-1}$ for TSP and 68,250 $cm^2\ mmol^{-1}$ for TSPΔN. Those of the native proteins were determined from the spectra of quintuplicate dilutions into buffer or denaturant, respectively. In both cases, absorbance spectra taken in buffer and denaturant, respectively, exhibited an isosbestic point near 278 nm. Thus, the experimentally determined extinction coefficients (76,300 $cm^2\ mmol^{-1}$ for TSP and 68,100 $cm^2\ mmol^{-1}$ for TSPΔN) and specific absorbances (1.06 and 1.13 $cm^2\ mg^{-1}$, respectively) in neutral buffer were close to the calculated values for the denatured proteins.

Fluorescence was measured in a Spex Fluoromax, and far-UV circular dichroism (CD) was recorded in Jasco 715 or Aviv 62A-DS spectropolarimeters. Rectangular fused silica cells were used and temperature was controlled by circulating water (Spex) or peltier elements (Jasco, Aviv).

The procedures for SDS-polyacrylamide (10% acrylamide) gel electrophoresis, silver staining, and densitometry have been described (Fuchs et al., 1991; Brunschier et al., 1993). As dithiothreitol as well as β-mercaptoethanol have been shown to produce artificial bands during SDS-gelelectrophoresis and silver staining with mobilities corresponding to 58 and 68 kDa (Kumar et al., 1993)—two bands with mobilities near monomeric TSPΔN—we used Coomassie rather than silver staining to avoid problems during densitometry. For Coomassie staining, the method of Fairbanks et al. (1971) was used with slight modifications. Prior to the steps of the original procedure, gels were soaked in 50% (v/v) methanol, 10% (v/v) acetate, 0.1% (w/v) Coomassie blue R for 10 min for fixation and removal of SDS; staining and destaining was accelerated by heating the solutions in a microwave.

Molecular cloning procedures

Purification of phage DNA

Bacteriophage P22 DNA was purified from phage lysates prepared as described (King & Yu, 1986). Phage particles were collected by centrifugation at 38,000 × *g*, extracted with phenol (2×) and chloroform (1×) to remove phage proteins, and DNA was precipitated with ethanol, dried and resuspended in 10 mM Tris/HCl pH 8.0, 1 mM EDTA. The concentration was determined spectrophotometrically at 260 nm and 10 ng of wild-type or mutant DNA were used as templates in PCR reactions.

Polymerase chain reaction and cloning of PCR fragments

Nucleic acids coding for the N-terminally shortened wild-type tailspike protein TSPΔNwt (pTSF1), the shortened tsf-mutant proteins TSPΔNts238S (pTSF5) and TSPΔNts244R (pTSF6), and the full-length tailspike protein (pTSP1) were amplified from purified phage DNA using the polymerase chain reaction. The oligonucleotide primers used for the shortened genes were TSF-forward:

5′-TCAGGTTGACACTAGTTAACGAGGGCAAAAAATGGATCC
AGATCAATATTCAATAGAAGC-3′

and TSF-reverse:

5′-CCCATTTTTATAAGCTTACTAAAGTGTTGCC
AAGGTTAATCTA-3′.

For the complete gene, the forward primer (TN1) was

5′-TCAGGTTGACACTAGTTAACGAGGGCAAAAATGAC
AGACATCACTGCAAACGTAGTTGTT-3′.

The PCR fragments were cloned via XbaI and HindIII restriction sites into pASK31 (Skerra, 1989) a derivative of pASK40 (Skerra et al., 1991) containing the lacUV5- instead of the lac-promotor. As the tailspike gene contains a XbaI site, we used a newly introduced SpeI site (isoschizomer of XbaI) for cloning. Nucleic acid sequences were verified by chain termination sequencing. All sequences contained a single substitution in codon 513 GGT→AGT leading to a Gly → Ser exchange relative to the published sequence (Sauer et al., 1982). This substitution was also found in the parent phage DNA and has since been observed in other phage stocks (C. Haase-Pettingell, pers. comm.). In the crystal structure, residue 513 is located at the end of a disordered loop, distant from the active site (Kinemage 1), in agreement with the observation that its substitution has no phenotype.

Site-directed mutagenesis

Plasmids coding for N-terminally shortened suppressor mutant tailspike proteins TSPΔNsu331A (pTSF9), TSPΔNsu331G (pTSF10), TSPΔNsu334V (pTSF11), and TSPΔNsu334I (pTSF12) were produced by site-directed mutagenesis according to Kunkel et al. (1987) with slight variations (Yuckenberg et al., 1991). Single-stranded DNA of the plasmid pTSF1 (coding for TSPΔNwt) was prepared upon infection with M13K07 helper phage, and the following oligonucleotide primers were used:

position 331(Ala or Gly, degenerate primer):

5′-TAAAAACTGGGCGCTCGAG(G/C)CTGACCC
ATAGCTGGT-3′ (Stm11);

position 334 (Val or Ile, degenerate primer):

5′-ATTACGTAAAAACTGAA(C/T)GGAGCTCACT
GACCCATAGCT-3′ (Stm12).

Protein expression and purification

Cells of *E. coli* JM83 (Yanisch-Perron et al., 1985) carrying the respective expression plasmid were grown in two l LB-medium containing ampicillin (100 μg/mL) at 30 °C (26 °C for the ts-mutants). At an optical density at 550 nm of 1.0, expression of the recombinant proteins was induced by adding IPTG to a final concentration of 1 mM and the cells were further grown for 16 h. The cells were harvested by centrifugation, resuspended in buffer A

(40 mM Tris/HCl, 2 mM EDTA, pH 8.0) and disrupted by high-pressure lysis.

The tailspike protein was precipitated from the soluble fraction of the cell lysate by adding solid ammonium sulfate to 35% saturation. The precipitate was again resuspended in buffer A, dialyzed against the same buffer (2 × 5 L) and applied to a DE52 anion exchange column (Whatman) equilibrated with buffer A. Fractions of a linear gradient (0–200 mM NaCl in buffer A) were pooled, brought to 0.2 M ammonium sulfate by addition of a concentrated solution, and applied to a phenyl sepharose FF column (Pharmacia, Uppsala, Sweden). The tailspike variants were eluted with a linear gradient of 0.2–0 M ammonium sulfate in buffer A and were concentrated by precipitation at 40% saturation with ammonium sulfate. Trace impurities were removed by gel filtration on a Superdex 200PG column (Pharmacia) in buffer B (10 mM potassium morpholinopropanesulfonate, pH 7.0). All recombinant tailspike proteins were judged to be at least 98% pure, as no additional bands were detectable by Coomassie Blue or silver staining after SDS gel electrophoresis at high sample loads.

Crystallization

Crystals were grown in hanging drops (McPherson, 1976) in Linbro cell culture plates (24 wells sealed with circular cover slips). Protein solutions were dialyzed against buffer B, concentrated to 5–10 mg/mL in Centricon 30 microconcentrators (Amicon, Beverly, Massachusetts) and centrifuged to remove aggregates prior to use. Drops were made of equal volumes (2 μL) of TSPΔN and precipitant solution (0.1 M $CaCl_2$, 0.1 M sodiumacetate, pH 4.6, 20% (v/v) isopropanol). The drops were suspended over 0.7 mL of precipitant solution at 4 °C and crystals appeared within a few days.

Denaturation and renaturation

Yields and kinetics of renaturation

Unfolding of tailspike proteins in acid urea and reconstitution in neutral buffer were done essentially as described (Seckler et al., 1989; Danner et al., 1993). Briefly, proteins were dissociated and unfolded in 5 M urea, pH 2.9–3.0 (50–65 mM H_3PO_4) for ≥30 min at room temperature and refolding was initiated by a 1:50 or 1:100 dilution of denatured protein into thermostatted, rapidly stirred reconstitution buffer (50 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0). Unless indicated otherwise, the total protein concentration during refolding was 25 μg/mL. To determine trimer reconstitution yields, reaction vessels were pretreated with Tween 20 (Danner & Seckler, 1993), and SDS gel electrophoresis with Coomassie staining was used. To measure reconstitution efficiencies at varied temperature, samples were incubated 70 h at 10, 15, and 20 °C, and 16 h at temperatures ≥25 °C.

Subunit folding kinetics were observed by following the fluorescence emission at 342 nm ($\lambda_{exc.}$ = 280 nm) after injecting 25 μL unfolded protein (1 mg/mL) into 2.475 mL thermostatted, stirred reconstitution buffer. Association of TSPΔN, i.e., the formation of protrimers, was tested by hybrid formation of polypeptide chains not yet associated (Fuchs et al., 1991; Danner & Seckler, 1993). Briefly, reconstitution of TSPΔNwt and TSPΔNts244R, a mutant protein with altered net charge, was started simultaneously in separate vessels, aliquots of both refolding samples were mixed at varied times, and reconstitution of the mixtures was allowed to proceed to completion (70 h). The time of 50% completion of TSPΔN protrimer formation so determined was 20 ± 5 min at 10 °C and 420 nM total subunit concentration, compared to ≈30 min observed previously for TSP under similar conditions.

Thermal unfolding

To elucidate the effect of point mutations on the stability of the native tailspike protein, thermal unfolding in the presence of SDS was analyzed by quantitative gel electrophoresis (Danner & Seckler, 1993) and by far-UV CD. For analysis of unfolding kinetics, thermal denaturation of the tailspike variants was performed in thin-walled PCR tubes (Roth), which were fully submerged in a thermostatted water bath (Haake) at 68 or 71 °C, as indicated. Reaction volume for each time point was 40 μL with a final protein concentration of 25 μg/mL in 50 mM Tris/HCl, adjusted to pH 7.0 at room temperature (Chen & King, 1991a; Danner et al., 1993), 150 mM β-mercaptoethanol, 20 mg/mL SDS. At different time points, vessels were transferred to an ice-water bath to stop unfolding. Native and denatured proteins were quantified by densitometry of trimer and monomer bands on Coomassie-stained SDS gels.

The midpoints of the irreversible thermal transition of wild-type and mutant TSPΔN were determined by far-UV CD. Changes in ellipticity at 230 nm (optical path length 5 mm) were recorded with a heating rate of 1 K/min. The protein concentration was 0.2 mg/mL in 50 mM sodium phosphate, 20 mg/mL SDS, pH 6.8.

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