Pressure dissociation studies provide insight into oligomerization competence of temperature-sensitive folding mutants of P22 tailspike

BRIAN G. LEFEBVRE, NOELLE K. COMOLLI, MATTHEW J. GAGE, AND ANNE SKAJA ROBINSON

Department of Chemical Engineering, University of Delaware, Newark, Delaware 19716, USA

(RECEIVED December 17, 2003; FINAL REVISION February 24, 2004; ACCEPTED February 24, 2004)

Abstract

Several temperature-sensitive folding (*tsf*) mutants of the tailspike protein from bacteriophage P22 have been found to fold with lower efficiency than the wild-type sequence, even at lowered temperatures. Previous refolding studies initiated from the unfolded monomer have indicated that the *tsf* mutations decrease the rate of structured monomer formation. We demonstrate that pressure treatment of the tailspike aggregates provides a useful tool to explore the effects of *tsf* mutants on the assembly pathway of the P22 tailspike trimer. The effects of pressure on two different *tsf* mutants, G244R and E196K, were explored. Pressure treatment of both G244R and E196K aggregates produced a folded trimer. E196K forms almost no native trimer in in vitro refolding experiments, yet it forms a trimer following pressure in a manner similar to the native tailspike protein. In contrast, trimer formation from pressure-treated G244R aggregates was not rapid, despite the presence of a G244R dimer after pressure treatment. The center-of-mass shifts of the fluorescence spectra under pressure are nearly identical for both *tsf* aggregates, indicating that pressure generates similar intermediates. Taken together, these results suggest that E196K has a primary defect in formation of the β -helix during monomer collapse, while G244R is primarily an assembly defect.

Keywords: P22 tailspike; temperature-sensitive mutations; protein folding kinetics; aggregation; hydrostatic pressure

Protein folding and aggregation are extremely important competing processes, yet the determinants that commit a polypeptide chain to the folding pathway remain largely unknown. Without sufficient information to select conditions that favor folding and disfavor aggregation, in vivo production of recombinant protein, recovery of native protein from aggregates, and dissecting the molecular origin of protein aggregation-related diseases are difficult tasks.

We are using the P22 tailspike protein from bacteriophage P22 as a model system for aggregation studies because the folding and aggregation pathways are well char-

acterized and the structure is known. The tailspike protein forms a homotrimer of 666 amino acid residues per monomer, and four to six of the tailspike trimers attach to the baseplate of the phage during its maturation. The structure of the protein has been solved in two segments (Fig. 1A; Sauer et al. 1982; Steinbacher et al. 1994, 1997). The in vivo and in vitro folding and aggregation pathways for the P22 tailspike protein (wtTSP) have been identified through native and SDS gel electrophoresis (Fig. 1B; Goldenberg and King 1982; Haase-Pettingell and King 1988; Seckler et al. 1989; Fuchs et al. 1991; Speed et al. 1995, 1997; Robinson and King 1997). In vitro aggregation of the tailspike can occur between protein assemblies of any size and is not limited to sequential addition of monomers (Speed et al. 1995, 1997). Covalent interactions do not play a role in tailspike aggregation, as SDS in the absence of reducing agents is able to dissociate aggregates into monomers

Reprint requests to: Anne Skaja Robinson, Colburn Laboratory, Department of Chemical Engineering, University of Delaware, Newark, DE 19716, USA; e-mail: robinson@che.udel.edu; fax: (302) 831-1048.

Article published online ahead of print. Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.03579304>.

Figure 1. (A) Structure of P22 tailspike. Ribbon diagram of the structure of P22 tailspike protein. The three subunits are colored red, blue, and yellow. The structure was solved in two parts: the main body in 1994 (Steinbacher et al. 1994), and the head binding domain in 1997 (Steinbacher et al. 1997). The positions of E196 and G244 on the dorsal loop are shown in ball-and-stick representation in the inset. (B) Tailspike folding pathway (Goldenberg and King 1982; Haase-Pettingell and King 1988; Seckler et al. 1989; Fuchs et al. 1991; Speed et al. 1995, 1997; Robinson and King 1997; Speed et al. 1997). *M* represents monomeric intermediates; *D*, dimeric intermediates. Asterisks (*) distinguish aggregation-prone intermediates from properly folded intermediates.

(Speed et al. 1995; Robinson and King 1997). Tailspike aggregates have been successfully dissociated by high pressure, leading to the formation of trimer from pressure-generated intermediates (Foguel et al. 1999; Lefebvre and Robinson 2003).

The stability and refolding behavior of a number of tailspike mutants has been examined previously. Temperature-sensitive folding mutations (*tsf*) prevent the protein chains from assembling to the native trimeric state at higher temperatures by destabilizing intermediates in the productive folding pathway, preventing subunit recognition and association (Smith and King 1981; Goldenberg et al. 1983). Trimer formation can occur *in vivo* at reduced temperatures, with purified mutant tailspike trimers displaying thermostability similar to that of the wild type (Goldenberg and King 1981; Sturtevant et al. 1989). *tsf* mutations are generally associated with surface β -turns, suggesting that these mutants may interfere with the correct folding of the β -helix

domain (Villafane and King 1988; Yu and King 1988; Stroup and Gierasch 1990).

At low protein concentrations, *tsf* mutants appear to fold *in vitro* to wild-type levels at temperatures lower than 25°C, but enhanced aggregation was observed at higher concentrations as monitored by classical light scattering (Mitraki et al. 1993). Fluorescence spectroscopy has shown that the *tsf* mutations reduced the rate of structured monomer formation at high temperatures. In the full-length tailspike, the *tsf* mutations lead to small changes in stability that strongly affected marginally stable monomeric intermediates, with smaller effects observed later in the pathway (Danner and Seckler 1993).

The G244R *tsf* mutation lies on the outside of the dorsal fin loop (Fig. 1A). Structured monomer formation in G244R resembles wild-type folding (0.0043 ± 0.0002 [wt] versus 0.0033 ± 0.0003 [G244R] sec^{-1} at 20°C), as observed by intrinsic tryptophan fluorescence (Danner and Seckler

1993). Thermal unfolding experiments have shown that the rate of trimer dissociation is faster in the G244R than in the wild type, both in the full-length and N-terminal truncated tailspike (Danner and Seckler 1993). The E196K *tsf* mutation is positioned at the base of the dorsal fin in the β -helix domain (Fig. 1A), and unlike G244R, is not on the surface of the protein (Haase-Pettingell and King 1997). The effects of the E196K mutant on the in vitro folding pathway have not been characterized. Hydrostatic pressure has been used to dissociate oligomeric proteins without denaturing the secondary and tertiary structure of the subunits (Silva et al. 1996). Quaternary structure of oligomeric proteins usually dissociate to monomers between 1 and 3 kbar, while secondary and tertiary structures require 5 kbar of pressure to denature (Silva and Weber 1993). Recently, it has been shown that tailspike aggregates can be dissociated to structured folding intermediates by high hydrostatic pressure (Lefebvre and Robinson 2003). High pressure has been shown to slow aggregation of rhodanese, to disrupt small aggregation intermediates in the presence of urea (Gorovits and Horowitz 1998), and to induce the dissociation and aggregation of recombinant human IFN- γ (Webb et al. 2001). Pressure has also been used to dissociate or solubilize aggregates of lysozyme, recombinant human growth hormone, and β -lactamase, with some increase in yield of functional protein (St. John et al. 1999). However, pressures of 200 MPa do not induce the dissociation or denaturation of native subtilisin or lysozyme (Webb et al. 2000).

With the P22 tailspike, the wild-type trimer formation from pressure-treated aggregates takes place after depressurization, as on-pathway dimers produced during pressure treatment rapidly associate. Previous studies with *tsf* mutant tailspike proteins have investigated the mutation through two means: unfolding of the native trimer, or refolding of the denatured monomer. In this study, pressure is used to generate monomeric and dimeric folding intermediates from aggregates, allowing the refolding process to be monitored from a new starting condition, past the formation of structured monomers. When the behavior of these postmonomer *tsf* intermediates are compared with the behavior of similarly formed wild-type folding intermediates, a direct evaluation of the importance of the dimer intermediate in *tsf* tailspike assembly is obtained.

Results

Initial refolding events of E196K and G244R *tsf* mutants are different

In vitro refolding studies at 20°C were performed on both the E196K and G244R *tsf* mutants to characterize the effects of each mutation on the refolding pathway of the P22 tailspike. Purified trimer was denatured for 1 h, diluted into a

refolding buffer to 100 μ g/mL, and incubated for 4 h. In vitro refolding of E196K showed extremely low levels of trimer formation compared to both G244R and wtTSP (Fig. 2, lanes 2,4,6). This result indicates that the E196K mutation forces the majority of the protein to off-pathway aggregation at what is a permissive temperature for most *tsf* mutants (Haase-Pettingell and King 1997). However, when the refolding samples were pretreated on ice, the mutant was capable of forming trimer (Fig. 2, lane 5) at levels comparable to wild-type tailspike and G244R (Fig. 2, lanes 1,3,5). Ice incubation has been shown to allow the accumulation of on-pathway monomer and dimer folding intermediates (Betts and King 1998; Danek and Robinson 2003). Ice incubation appears to completely compensate for the E196K folding defect, while the enhancement of G244R trimer production is comparable to the enhancement seen for wild-type tailspike. These results indicate that the folding defect of E196K may lie in a different location in the folding pathway than the G244R defect.

To further characterize the location of the defects in the folding pathway of each of the mutants, structured monomer formation kinetics of both E196K and G244R were studied by monitoring the intrinsic fluorescence of a 10 μ g/mL refolding sample at both 20°C and 30°C (Table 1). As can be seen, the wild-type tailspike, G244R, and E196K had similar rates at 20°C. At 30°C, the wild-type tailspike and the G244R *tsf* mutant had similar rates of structured monomer formation, but it was impossible to measure a rate for the E196K *tsf* mutant because the sample immediately aggregates (data not shown). This indicates that the E196K defect affects formation of the folding competent monomer species at 30°C. In contrast, G244R, which was incapable of

Figure 2. Native PAGE shows lethal effect of E196K in vitro. Wild-type tailspike, G244R, and E196K protein was refolded at 100 μ g/mL at 20°C. For the samples in lanes 1, 3, and 5, refolding was initiated on ice and incubated for 20 min prior to incubation at 20°C. WtTSP and G244R form comparable amounts of protein at 20°C without ice incubation. In contrast, E196K forms almost no trimer without the initial ice incubation, indicating the presence of a more severe defect due to the mutation.

Table 1. Structured monomer formation rates for wild-type tailspike, E196K and G244R

	20°C (sec ⁻¹)	30°C (sec ⁻¹)
Wild type	0.0085 ± 0.0013	0.021 ± 0.005
G244R	0.0099 ± 0.0004	0.016 ± 0.004
E196K	0.0087 ± 0.0008	N.D.

Refolding was initiated by dilution of denatured tailspike protein to a final concentration of 10 µg/mL into prewarmed buffer at either 20° or 30°C. Values are an average of a minimum of two trials. N.D. indicates that aggregation was sufficient enough to prevent accurate determination of the monomer folding rate.

forming trimer in vitro at 30°C, could form a folding-competent monomer, indicating that the folding defect occurs beyond the formation of the structured monomer intermediate.

Aggregated tsf protein dissociates to structured monomer under pressure similar to wtTSP

Previous work on the wild-type tailspike has shown that pressure treatment can dissociate aggregates to early folding intermediates (Lefebvre and Robinson 2003). Hydrostatic pressure dissociates the wild-type aggregates into primarily monomer and dimer intermediates, and leaves some residual aggregates that cannot be dissociated by the application of pressure. Therefore, pressure treatment serves as another method of probing the locations in the folding pathway that are affected by the E196K and G244R mutations.

Aggregates of both E196K and G244R were generated by refolding freshly denatured protein at 37°C for 60 min. Aggregates were pressure treated for 90 min at 240 MPa, and subsequently allowed to refold at 20°C. The effect of pressure treatment on the *tsf* aggregates is shown in Figure 3. Each of the samples contained no trimer prior to pressure treatment (lanes 1,2,3). Immediately following pressure treatment, the samples contain a mixture of monomer and dimer (lanes 4,5,6), which forms trimer during incubation at 20°C (lanes 7,8,9). There is also a significant amount of large recalcitrant aggregates that are not visible, as they may be too large to enter the separating gel, but were observed by size-exclusion chromatography. As can be seen, pressure dissociated the *tsf* aggregates in a manner similar to wild-type aggregates, promoting formation of a trimer.

Pressure produces E196K folding intermediates located past the lethal defect

Refolding of dissociated E196K aggregates following pressure treatment was followed over time to determine if the intermediates formed during pressure treatment were folding-competent. Aggregates were made from freshly denatured E196K by refolding at 100 µg/mL at 37°C for 60 min.

The aggregates were then pressure treated for 90 min at 240 MPa, and subsequently allowed to refold at 20°C. Samples were quenched by placing on ice and by addition of 3X-SDS sample buffer, and were analyzed by gel electrophoresis. A representative gel is shown in Figure 4. After 90 min, postpressure oligomerization (lane 5), E196K produces appreciable amounts of the trimer.

Trimer concentrations were determined from comparisons to standards on the silver-stained SDS-PAGE as described in Materials and Methods, and could therefore be compared to wild-type postpressure refolding. Figure 5 is a plot of the trimer concentration as a function of refolding time after pressure treatment. E196K and the wild type display an increased amount of trimer over time; however, E196K does not achieve the wild-type level of trimer formation at the same postpressure conditions. The final yield of the E196K trimer postpressure is about 50% of the wild-type postpressure yield of the trimer after 3.5 h.

Pressure-generated G244R folding intermediates are not folding-competent

When the initial (immediately after pressure treatment) and final (after incubation of the pressure-treated sample at 20°C) species concentrations for G244R are compared to the wild type, it is evident that the refolding process of

Figure 3. Pressure recovers trimer from *tsf* aggregates in a similar manner to wtTSP. Native PAGE analysis of the effects of pressure on wtTSP (T), E196K (E), and G244R (G) aggregates. Aggregates were generated by diluting freshly denatured protein into refolding buffer prewarmed to 37°C and incubating at 37°C for 60 min. Lanes 1, 2, and 3 show the wtTSP, G244R, and E196K samples, respectively, prior to pressure treatment. Samples were pressurized to 240 MPa for 90 min and then depressurized and incubated at 20°C for 4 h. Lanes 4, 5, and 6 show the wtTSP, G244R, and E196K samples immediately following pressure treatment, while lanes 7, 8, and 9 show wtTSP, G244R, and E196K pressure-treated samples following incubation at 20°C. Aggregate samples (prepressure) show no evidence of trimer formation. Following pressure treatment, the samples are a mixture of monomer and dimer and undissociated aggregates. Incubation at 20°C allows the monomer and dimer species to form trimer for the wtTSP and the two *tsf* mutants.

Figure 4. E196K trimer forms over time following pressure treatment. Samples of aggregated E196K were pressure treated at 240 MPa for 90 min. Following pressure treatment, samples were collected over time and quenched on ice. Shown is a nondenaturing PAGE gel demonstrating the increasing concentration of E196K trimer over time.

G244R must be different (Table 2). Immediately after pressure treatment, the species concentrations are very similar. G244R aggregate concentration is somewhat higher after pressure treatment, and monomer concentration lower, than the wild type, but dimer and trimer concentrations are very similar. For the wild type, the folding intermediates assemble productively to the trimer, while for G244R, these intermediates predominantly aggregate (yield of trimer from pressure-generated intermediates after 3.5 h: G244R = 7.2%, wt = 32.0%).

To characterize the refolding process, the kinetics of the G244R tailspike folding from pressure-generated intermediates was examined. After release of pressure, the tailspike mixtures were placed in a water bath at 20°C. Samples were taken at various times to examine the association of tailspike species, with time = 0 occurring when the pressure equaled 7 MPa on the return to atmospheric pressure. Trimer concentration was determined through native PAGE and aggregate, dimer, and monomer concentrations were determined by size-exclusion chromatography, as described in Materials and Methods.

Figure 6 shows the trimer and dimer concentration as a function of time at 20°C after pressure treatment for the G244R and wild type. The initial trimer and dimer concentrations for the G244R are very similar to those observed for the wild type. For the wild type, the dimer concentration rapidly drops and trimer concentration increases. This behavior has led to the conclusion that the wild-type dimers present after pressure treatment are on-pathway, enabling rapid formation of the trimer from these folding intermediates (Lefebvre and Robinson 2003). For G244R, there is

only a slight increase in trimer concentration, and the dimer concentration slowly drops, suggesting that the nature of the G244R postpressure dimer is different from the wild type.

Fluorescence center of mass is similar among wtTSP, G244R, and E196K under pressure

Pressure treatment of E196K and G244R aggregates has significantly different effects on trimer recovery. One possible explanation for this difference is that pressure treatment results in formation of different intermediate conformations. E196K may populate on-pathway intermediates, while pressure treatment of G244R aggregates may result in off-pathway intermediates. One way to investigate this possibility is by measuring the fluorescence center of mass of aggregate samples before, during, and after pressure treatment.

Aggregate samples of wtTSP, E196K, and G244R were prepared by diluting the denatured tailspike protein into refolding buffer at 37°C. Fluorescence spectra were measured before pressurization, while the sample was pressurized at 240 MPa and then after returning the sample to atmospheric pressure. The center of mass was calculated as described in the Materials and Methods.

Figure 7 shows the center of mass for each sample during the course of the experiment. For comparison, the center of mass of the wild-type tailspike trimer under identical conditions has been added to the figure (identical measurements were performed on both *tsf* trimers as well, which looked identical to the plot for the wtTSP trimer; data not shown). As can be seen, the center of mass of both *tsf* aggregates

Figure 5. E196K forms trimer postpressure similar to wtTSP. Samples of E196K (open circles) and wild-type (filled squares) were aggregated at 37°C and pressure treated for 90 min at 240 MPa. Trimer formation during refolding at 20°C was monitored by taking samples and visualizing on silver-stained SDS PAGE. The error bars show the standard deviation from the average of three independent experiments.

Table 2. Initial species concentration and ultimate refolding yield for pressure treated G244R and wild-type tailspike aggregates

Species	Initial species concentration ($\mu\text{g/mL}$)		Final species concentration ($\mu\text{g/mL}$)	
	Wild type	G244R	Wild type	G244R
Aggregate	39.4 ± 5.5	52.4 ± 15.0	61.3 ± 10.1	76.9 ± 9.5
Trimer	2.5 ± 0.3	0	19.4 ± 0.9	3.4 ± 0.8
Dimer	11.2 ± 2.6	14.1 ± 4.9	-3.8 ± 5.9	6.3 ± 3.5
Monomer	47.0 ± 5.8	33.4 ± 10.2	23.1 ± 4.4	13.2 ± 3.4

Tailspike aggregates were created by refolding at 37°C (wild type) or 30°C (G244R) for 1 h, at which time all tailspike chains are in the aggregate state. These aggregates were subjected to 240 MPa for 90 min, and placed in a 20°C bath following extraction from the pressure cell. Initial species concentration corresponds to a sample obtained immediately after depressurization, and final species concentration corresponds to a sample incubated at 20°C for 210 min. Species concentrations determined as described in Materials and Methods.

exhibits a larger shift than the wtTSP aggregates under pressure. However, all three samples show similar effects when returned to atmospheric pressure, indicating that wtTSP, G244R, and E196K form folding intermediates with similar tertiary structure following pressure treatment, and that the differences in the postpressure assembly are due to the different effects of the mutants on the folding pathway.

Discussion

Results from previous research have suggested that the *tsf* phenotype is the result of a destabilization of folding inter-

mediates on the productive folding pathway (Smith and King 1981; Goldenberg et al. 1983). Recovery of tryptophan fluorescence during refolding has shown that *tsf* mutations retard the rate of structured monomer formation from the denatured state. On a molecular level, *tsf* mutations have been thought to lead to small changes in stability that strongly affect early folding intermediates (Danner and Seckler 1993). Studies with the isolated β -helix domain have suggested that *tsf* mutations predominantly affect the monomeric folding intermediate (Danner and Seckler 1993). The kinetics of *tsf* association under aggregating conditions are less well understood, but it has been noted that differences in aggregation kinetics, as monitored by classical light scattering, occur at temperatures as low as 14°C (Mitraki et al. 1993).

All of these results were obtained by examining refolding from the unfolded monomer state. These studies have carefully determined the effect of *tsf* mutations on the early stages of tailspike folding. In this research, we sought to compare the effects of two *tsf* mutations on the later stages of trimer assembly by observing refolding from a different initial condition: the pressure-generated state. Application of high pressure dissociates wild-type tailspike aggregates and promotes the formation of an on-pathway monomer and dimer. This allows refolding to be studied from a new starting condition, and bypasses the most common *tsf*-induced folding defect, the “unfolded monomer-to-structured monomer” transition. Therefore, mutants with defects after the on-pathway monomer will not show improved folding from the pressure-dissociated state, and those with defects only before the on-pathway monomer formation will show improved productive assembly.

Importance of the monomer folding intermediate in the E196K temperature-sensitive folding tailspike

E196K is a near-lethal *tsf* mutation located in the β -helix domain (Lee and Yu 1997), which has been shown to be

Figure 6. G244R and wtTSP species show different effects following pressure treatment. Tailspike aggregates were created by refolding at 37°C (wild type) or 30°C (G244R) for 1 h, at which time all tailspike chains are in the aggregate state. These aggregates were subjected to 240 MPa for 90 min, and placed in a 20°C bath following removal from the pressure cell. Diamonds represent trimer concentration and squares represent dimer concentrations. Error bars are the standard deviation from the average of at least three independent experiments. Lines are first-order fits to the data. Solid lines and filled symbols represent wild-type tailspike, and dashed lines and open symbols represent the G244R tailspike. Wild-type tailspike data are from Lefebvre and Robinson (2003).

Figure 7. G244R and E196K fluorescence spectra exhibit similar effects during pressure treatment. (A) Fluorescence spectra of wtTSP, G244R, and E196K aggregates were measured before, during, and following pressure treatment, and the center of mass of the spectra was calculated. As can be seen, all three aggregate samples exhibited similar effects under pressure. The center of mass shift was slightly larger for the two *tsf* aggregates relative to wtTSP. Upon release of pressure, all three samples exhibited similar behavior, indicating that the dissociated proteins from all three samples are in similar conformations. (B) Fluorescence spectra of wtTSP aggregates before, during, and after pressure treatment. Arrows indicate the maximum in the spectrum. Note the redshift in the peak intensity of the spectrum under pressure.

critical in the formation and stability of the monomer folding intermediate. Proper formation of on-pathway monomer is critical to the production of trimer in the *in vitro* folding pathway of the P22 tailspike (Fuchs et al. 1991). This work has shown that the “unfolded monomer-to-structured monomer” step is inhibited in E196K at high temperatures, as

monitored by intrinsic fluorescence. Therefore, a lack of on-pathway monomer results in the inability to form a trimer at atmospheric conditions. The decreased folding efficiency of the E196K trimer is likely to be due to the charge change in a location near a turn of the β -helix in one of the parallel sheets.

The formation of E196K trimer at a reasonable rate under high protein concentrations after pressure treatment of aggregates can be explained by the nature of the E196K mutation. E196K inhibits the formation of the on-pathway monomer, while pressure generates the on-pathway monomer from aggregates, bypassing this limitation and allowing for productive folding. These results are further validated by the comparison of the postpressure fluorescence spectra of both the E196K and wild-type P22 tailspike. Pressure treatment of the wild-type P22 tailspike aggregates leads to formation of the on-pathway monomer and dimer intermediates (Lefebvre and Robinson 2003). Pressure-treated E196K aggregates show center-of-mass shifts similar to those of wtTSP, and native PAGE indicates that pressure also generates on-pathway monomer and dimer intermediates from E196K aggregates. Taken together, these results support the hypothesis that E196K affects folding by inhibiting the formation of folding-competent monomer species.

Importance of the dimer folding intermediate in the formation of the G244R trimer

Previous research with the wild-type tailspike has shown that pressure is able to dissociate aggregates and produce on-pathway assembly intermediates. After pressure is released, these pressure-generated intermediates rapidly associate to form trimer (Lefebvre and Robinson 2003). In this study, it was demonstrated that pressure is also able to dissociate G244R tailspike aggregates. However, the pressure-generated G244R intermediates did not display the same refolding behavior as pressure-generated wild-type intermediates; the G244R intermediates did not rapidly associate to form a trimer.

The reason for this difference cannot be explained with the current theories on the nature of *tsf* folding defects. Because the pressure-generated intermediates populate both the monomer and dimer state, they are past the “unfolded monomer-to-structured monomer” transition. In addition, the formation of structured monomer, as followed by intrinsic fluorescence, shows wild-type kinetics. We propose that the G244R *tsf* mutation gives rise to an altered dimer assembly intermediate. Because the G244 site is exposed on the dorsal fin of the native trimer, this mutation must change either the monomer β -helix domain conformation to inhibit oligomerization, or the dimer structure must position G244 in a position to interfere with assembly.

The presence of an altered dimer in G244R tailspike would account for the differences we observe in postpressure refolding. For the wild type, a dimer is present after

pressure treatment, and this species is on-pathway and able to rapidly associate to form a trimer. For G244R, a dimer is also present after pressure treatment, but this species is not able to rapidly form a trimer. Three possibilities could account for this difference: An on-pathway G244R dimer has a reduced tendency to advance along the folding pathway, relative to the wild type; the on-pathway G244R dimer is not present after pressure treatment; or the on-pathway G244R dimer is present after pressure treatment, but it rapidly shifts to an off-pathway form. Each of these explanations suggests that the dimer intermediate is affected by the *tsf* mutation, with the latter two implying that the on-pathway G244R dimer is thermodynamically destabilized.

This work has provided valuable insight into the nature of two P22 tailspike *tsf* mutants. By utilizing hydrostatic pressure, it has been possible to isolate individual steps in the folding pathway, demonstrating that the G244R mutation affects later steps in the folding pathway than the E196K mutation. As hydrostatic pressure is capable of dissociating protein–protein interactions without inducing significant unfolding of the protein, this technique may prove to be a powerful new tool to decipher the effects of mutations on folding intermediates in other protein systems.

Materials and methods

E196K site-directed mutagenesis

Mutagenesis was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Gene-specific primers containing either the specific mutations or degenerate bases to generate a series of mutations were designed. Mutagenesis was performed according to instructions using a pET11a vector containing the full-length P22 tailspike gene as a template. The presence of the mutation was verified by complete sequencing of the gene.

Tailspike protein production

G244R tailspike expression in *Salmonella typhimurium* by phage infection was performed as described by Yu and King (1988). G244R tailspike was purified as described by King and Yu (1986) to a concentration of 10.7 mg/mL. The wild-type and E196K tailspike proteins were prepared as described by Lefebvre and Robinson (2003).

In vitro refolding and aggregation reactions

Native tailspike trimer was denatured for approximately 60 min in 7 M urea, 50 mM Tris-HCl, and 1 mM EDTA (pH 3.0) at a final concentration of 1 mg/mL. Refolding or aggregation reactions were initiated by rapidly diluting the denatured protein to a final concentration of 100 µg/mL into Tris Refolding Buffer (50 mM Tris-HCl, 1 mM EDTA [pH 7.6]) prewarmed to the desired reaction temperature (20°C for refolding and 37°C for aggregation). At various times, 500-µL aliquots were removed and placed into an ice-water bath. Gel electrophoresis samples were created by mixing 30 µL of the chilled reaction mixture with 15 µL of 3× sample

buffer (15 mM Tris-HCl [pH 6.8], 120 mM glycine, 50% glycerol, bromophenol blue) preincubated to 0°C in an ice-water bath. The remaining sample was reserved for HPLC analysis.

Pressurization procedure

Pressure was generated using a manual piston (Model 37-5.75-60, High Pressure Equipment) with ethanol as the pressure-transmitting fluid. Samples were placed in polyethylene tubes and loaded in a 77 mL O-ring closure reactor rated to 414 MPa (Model R1-6-60, High Pressure Equipment). Samples were pressurized and depressurized at a rate of approximately 140 MPa per min, and all pressurizations were conducted at room temperature.

Gel electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed using a discontinuous buffer system (Davis 1964; Ornstein 1964). The resolving gel contained 0.37 M Tris buffer (pH 8.9) with 3.8 mM TEMED, 3.0 mM ammonium persulfate, and 7.0% acrylamide. The stacking gel contained 0.07 M Tris buffer (pH 6.7) with 7.5 mM TEMED, 2.5 mM ammonium persulfate, and 3.6% acrylamide. The gels were run at a constant voltage (150 V) for 16–24 h at 4°C. Bands were visualized by silver staining (Sather and King 1994). Quantitation of the tailspike trimer in silver-stained gels was determined by densitometry of dried gels, using at least five trimer concentrations electrophoresed on the same gel as the samples to determine a calibration curve. Only samples in the linear range of calibration were used in subsequent analysis.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was performed on a BioRad BioLogic system using a prepacked TSK 3000 SWxl column (Supelco). To prevent removal of large, but soluble, aggregates, no precolumn was used. The system was maintained at 4°C in a cold room and equilibrated with 25 mM Tris, 100 mM sodium acetate, and 0.5 M urea (pH 7.0) buffer at a flow rate of 0.5 mL/min. Urea was included to prevent partially folded proteins from sticking to the column. Sample elution was monitored by absorbance at 280 nm. Peak identity was confirmed using native PAGE, and peaks were divided by vertical lines dropped from the minimum of the troughs between neighboring peaks. Peak area was computed in a Microsoft Excel spreadsheet using the trapezoidal rule. Other approaches, such as fitting the peaks to Gaussian curves, did not improve the quality of reproducibility of the results.

Fluorescence experiments

Fluorescence experiments were performed using an ISS PC1 fluorimeter. Intrinsic fluorescence was measured by diluting denatured wtTSP, E196K, or G244R protein into Tris Refolding Buffer incubated at the desired temperature. Sample fluorescence was measured by exciting the sample at 280 nm, and the emission fluorescence intensity was measured at 340 nm. High-pressure fluorescence experiments were carried out using an ISS High-Pressure Fluorescence cell equipped with sapphire windows. Samples were pressurized at a rate of 70 MPa/min, and the initial pressurization spectra was measured immediately upon reaching maximal pressure. The samples were excited at 280 nm, and the emission was

recorded from 300 to 400 nm. The center of mass was calculated using the formula:

$$\text{Center of Mass} = \Sigma(I\nu)/\Sigma I$$

where I is equal to intensity and ν is equal to the wavenumber.

Model analysis

At the simplest level, trimer formation from folding intermediates can be fit to a first-order exponential. This analysis is adequate for detecting gross differences in trimer formation rates. The change in trimer concentration with time was fit with a first-order three-parameter exponential fit.

$$T = T_f - (T_f - T_0) * \exp [-k_T t] \quad (1)$$

In equation 1, the subscript f indicates final species concentration, and the subscript 0 indicates initial species concentration. T represents trimer, and k_T is the first-order rate constant for the trimer formation. The fit parameters were determined using the curve fitting routines in Kaleidagraph 3.0.

Acknowledgments

We thank Drs. Debora Foguel, Jerson Silva (Federal University of Rio de Janeiro, Brazil), and Cliff Robinson (University of Delaware) for helpful advice and discussions on hydrostatic pressure, and Dr. Jonathan King (MIT) for discussion on tailspike. Funding for this work was provided by NSF BES 99-84312.

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