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Mechanism of phage P22 tailspike protein folding mutations

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Abstract

Temperature-sensitive folding (*tsf*) and global-*tsf*-suppressor (*su*) point mutations affect the folding yields of the trimeric, thermostable phage P22 tailspike endorhamnosidase at elevated temperature, both in vivo and in vitro, but they have little effect on function and stability of the native folded protein. To delineate the mechanism by which these mutations modify the partitioning between productive folding and off-pathway aggregation, the kinetics of refolding after dilution from acid-urea solutions and the thermal stability of folding intermediates were analyzed. The study included five *tsf* mutations of varying severity, the two known *su* mutations, and four *tsf/su* double mutants.

At low temperature (10 °C), subunit-folding rates, measured as an increase in fluorescence, were similar for wild-type and mutants. At 25 °C, however, *tsf* mutations reduced the rate of subunit folding. The *su* mutations increased this rate, when present in the *tsf*-mutant background, but had no effect in the wild-type background. Conversely, *tsf* mutations accelerated, and *su* mutations retarded the irreversible off-pathway reaction, as revealed by temperature down-shifts after varied times during refolding at high temperature (40 °C). The kinetic results are consistent with *tsf* mutations destabilizing and *su* mutations stabilizing an essential subunit folding intermediate.

In accordance with this interpretation, *tsf* mutations decreased, and *su* mutations increased the temperature resistance of folding intermediates, as disclosed by temperature up-shifts during refolding at 25 °C. The stabilizing and destabilizing effects were most pronounced early during refolding. However, they were not limited to subunit-folding intermediates and were also observable during thermal unfolding of the native protein.

Keywords: bacteriophage P22; folding intermediates; inclusion body; protein folding; tailspike protein; temperature-sensitive mutations

Experimental evidence accumulated over more than 50 years has demonstrated conclusively that protein folding is a spontaneous and reversible process directed by the amino acid sequence of the folding polypeptide (Anson, 1945; Anfinsen, 1973). However, the rules by which the linear polypeptide sequence determines the folding pathway and the final conformation have remained elusive (Jaenicke, 1988; Seckler & Jaenicke, 1992). Three-dimensional structure detected in folding intermediates of a number of proteins, by virtue of experimental techniques developed recently, generally appears stabilized by a subset of the interactions present in the native folded protein (Roder et al., 1988; Udgaonkar & Baldwin, 1988; Blond-Elguindi & Goldberg, 1990; Bycroft et al., 1990; Weissman & Kim, 1991; Radford et al., 1992). Because of technical limitations, however, most such experiments have focused on small monomeric proteins and, with the exception of the trapping of disulfide-bonded intermediates, the experimental techniques are biased toward detecting native interactions. For large, multidomain proteins, the question of kinetic vs. thermodynamic control of the folding process is still open (Sugihara & Baldwin, 1988). In principle, the amino acid sequence of such proteins may include information that is important to direct the polypeptide chain along the productive folding pathway but unimportant in the final, folded structure.

An alternative experimental strategy, not connected with the limitations described above, may be a genetic approach to the folding problem, i.e., the analysis of amino acid substitutions affecting folding (King & Yu, 1986; King, 1989; Lecomte & Matthews, 1993). In the case of the tailspike endorhamnosidase from *Salmonella typhimurium* phage P22, King and coworkers have character-

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ized a large number of mutations that affect the folding efficiency of the protein but appear to have no significant effect in the final native structure (King, 1989; King et al., 1990; Mitraki & King, 1992). The tailspike protein is a highly thermostable homotrimer of 666-residue polypeptides with known amino acid sequence (Goldenberg & King, 1982; Sauer et al., 1982; Seckler et al., 1989). Despite the high stability of the native protein, a large number of sites in the tailspike gene are the targets of point mutations leading to a temperature-sensitive phenotype (Yu & King, 1984; Villafane & King, 1988). These mutations have been designated *tsf* (temperature-sensitive folding) because they reduce the folding yield at high temperature (39 °C), but once matured at the permissive temperature (≤30 °C), the mutant proteins are enzymatically active, thermostable, and resistant to the ionic detergent sodium dodecyl sulfate (SDS) as is the wild-type protein (Goldenberg & King, 1981; Smith & King, 1981; Sturtevant et al., 1989; Thomas et al., 1990). A second class of mutations, isolated as intragenic suppressors of the *tsf* phenotype (*su* mutants) has the opposite effect, i.e., the amino acid substitutions increase the yield of folding at high temperature and suppress the formation of inactive aggregates, both in the wild-type and in the *tsf* mutant genetic backgrounds (Fane & King, 1991; Fane et al., 1991; Mitraki et al., 1991).

Reconstitution experiments with the purified P22 tailspike protein, starting from unfolded polypeptides in guanidinium chloride (GdmCl) or acid-urea solutions, have shown that the in vitro folding reaction mirrors closely the in vivo folding pathway of the protein (Seckler et al., 1989; Fuchs et al., 1991). Similar to the biogenesis of tailspikes in the infected *Salmonella* cell, tailspike folding yields in vitro decrease strongly with increasing temperature (Brunschier et al., 1993; Danner et al., 1993), and both the *tsf* and the second-site suppressor mutations affect refolding yields in a manner corresponding to their respective in vivo phenotypes (Mitraki et al., 1993). Thus, an analysis of mutant tailspike folding kinetics and of the stability of folding intermediates should allow delineation of the mechanism by which the amino acid substitutions affect the partitioning between the "correct" folding pathway and "wrong" aggregation.

This study attempts such an analysis with a number of *tsf* and second-site suppressor mutations, using fluorescence spectroscopy to trace subunit-folding kinetics and temperature shifts during refolding, in order to compare the stabilities of wild-type and mutant folding intermediates. The observations are consistent with the *tsf* mutations destabilizing and the suppressor mutations stabilizing essential folding intermediates. The small effects of the substitutions on intermediate stability are most evident early during refolding, become less apparent in the more stable later intermediates, but remain detectable during thermal unfolding of late species, including the native protein.

Results

The in vitro folding pathway of the P22 tailspike protein has been characterized previously in detail only at low temperature (10 °C), where tailspike folding yields are high and where subunit folding, association, and trimer maturation reactions are well separated kinetically (Fuchs et al., 1991). Because the folding yields of tailspike *tsf* and *su* mutants differ from wild-type yields only at elevated temperature, both in vivo and in vitro (Mitraki et al., 1993) wild-type and mutant folding pathways had to be compared at higher temperature in order to locate the steps affected by the mutations.

Tailspike refolding pathway at varied temperatures

The consecutive pathway of folding, association, and folding reactions by which native tailspikes formed after dilution of unfolded wild-type polypeptides from acid-urea solutions at 25 °C is illustrated in Figure 1. Similar to tailspike folding at low temperature, upon initiation of refolding at 25 °C, the intrinsic fluorescence intensity around 340 nm increased and reached its final value in a reaction that was essentially protein-concentration independent in the range of 1.5–20 μg/mL (Fig. 1A). The biphasic kinetics comprised a rapid burst of fluorescence intensity, followed by a further signal increase in the minutes time range that fit fairly well to a single exponential with a time constant of ≈130 s, corresponding to a half-time of 1.5 min (compare also Fig. 2, Table 2, and Danner et al. [1993]).

The spectroscopically observed changes preceded subunit association, which was detected by the formation of hybrid trimers (Fig. 1B). When tailspike renaturation intermediates from wild-type and from a mutant with altered net charge (G244R) are mixed at different times after initiation of refolding, hybrid trimers can form only from subunits not yet associated into homotrimers at the time of mixing. Thus, subunit association kinetics can be estimated from the relative band intensities of hybrids and homotrimers after completion of reconstitution and electrophoretic analysis (Goldenberg & King, 1982; Fuchs et al., 1991; Danner et al., 1993). The half-time of association ($t_{1/2} \approx 12$ min) was essentially independent of protein concentration between 5 and 30 μg/mL, suggesting that a spectroscopically undetected folding reaction rather than subunit collision is rate-limiting for association. Although no difference in association kinetics had been observed previously at 10 °C, the distribution of band intensities in Figure 1B indicates somewhat slower trimerization of the G244R mutant compared to the wild-type polypeptides. The wild-type homotrimer appeared to form faster, whereas the hybrid composed of one wild-type and two mutant chains was dominant in samples mixed later (15–30 min) during refolding. This is most likely a consequence of the slower subunit folding in the G244R mutant (see below, Fig. 2, Table 2).

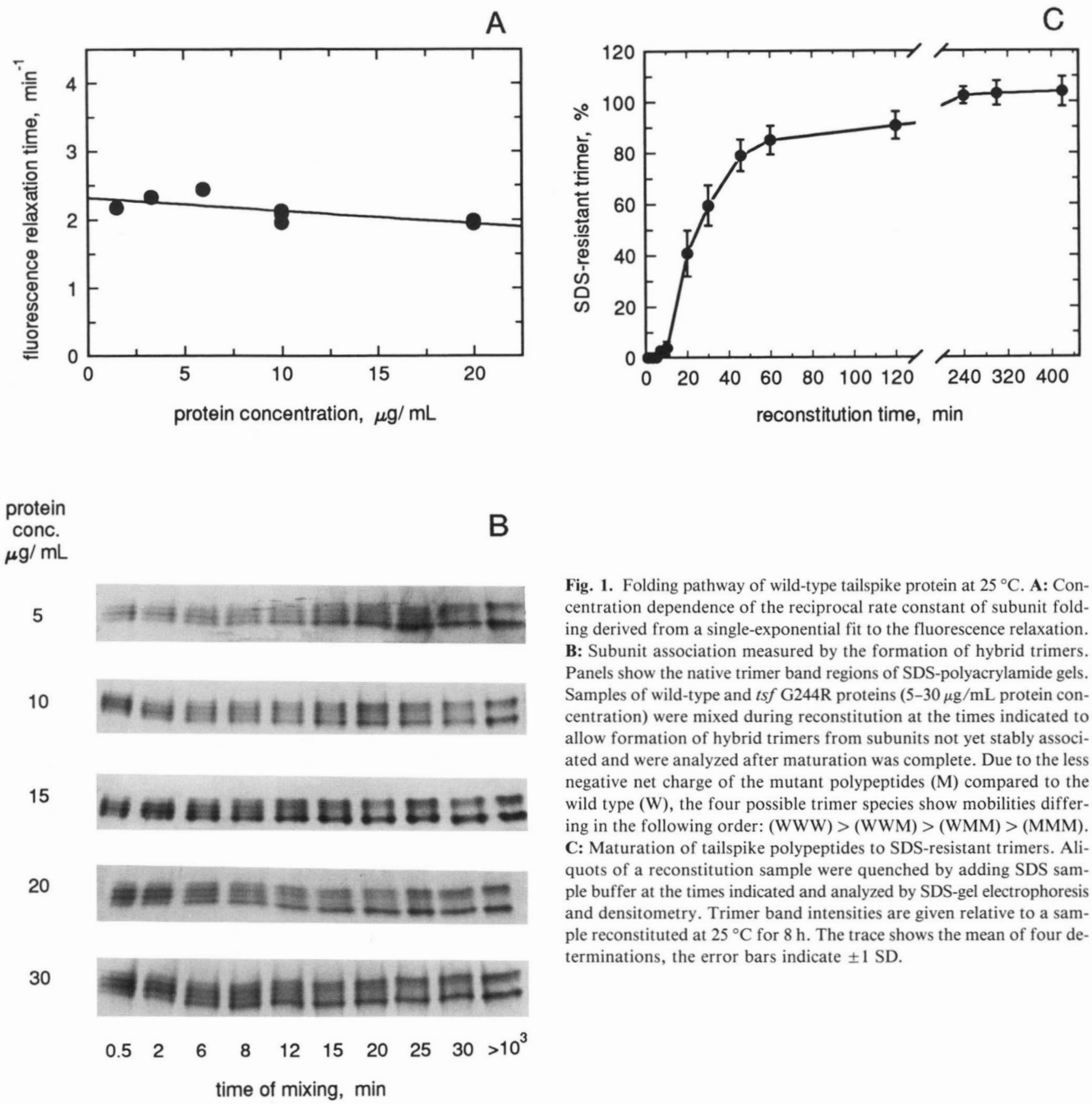

Fig. 1. Folding pathway of wild-type tailspike protein at 25 °C. **A:** Concentration dependence of the reciprocal rate constant of subunit folding derived from a single-exponential fit to the fluorescence relaxation. **B:** Subunit association measured by the formation of hybrid trimers. Panels show the native trimer band regions of SDS-polyacrylamide gels. Samples of wild-type and *tsf* G244R proteins (5–30 μg/mL protein concentration) were mixed during reconstitution at the times indicated to allow formation of hybrid trimers from subunits not yet stably associated and were analyzed after maturation was complete. Due to the less negative net charge of the mutant polypeptides (M) compared to the wild type (W), the four possible trimer species show mobilities differing in the following order: (WWW) > (WWM) > (WMM) > (MMM). **C:** Maturation of tailspike polypeptides to SDS-resistant trimers. Aliquots of a reconstitution sample were quenched by adding SDS sample buffer at the times indicated and analyzed by SDS-gel electrophoresis and densitometry. Trimer band intensities are given relative to a sample reconstituted at 25 °C for 8 h. The trace shows the mean of four determinations, the error bars indicate ±1 SD.

Similar to that which occurs at low temperatures, subunit association preceded the formation of native, SDS-resistant trimers by a significant period of time ($t_{1/2}$ = 25 ± 5 min), as detected by gel electrophoresis and densitometry (Fig. 1C). The overall kinetics of tailspike renaturation were not significantly different between the wild-type and the *tsf* G244R or the *tsf* D238S mutants. The temperature dependence of the trimer maturation reaction, with a half-time decreasing from $t_{1/2} \approx 10$ H at 10 °C to $t_{1/2} \approx 1$ h at 20 °C (data not shown) and $t_{1/2} \approx$ 7 min at 30 °C (Danner et al., 1993), corresponds to an Arrhenius activation energy of close to 165 kJ/mol.

Tsf and su mutations affect subunit folding

As stated previously, no significant differences were observed in the overall folding kinetics of tailspikes from the wild type and a *tsf* mutant (*tsf* G244R) at 10 °C (Fuchs et al., 1991), where both wild-type and mutant tailspikes refold with high yield (Mitraki et al., 1993). Differences became apparent when wild-type refolding kinetics were compared with those of sets of *tsf*, *su*, and *tsf/su* mutants at 25 °C, where the refolding yields begin to diverge. The fluorescence increase, signaling subunit folding of one such set of mutants (*tsf* D238S and *tsf* D238S/*su*

Table 1. *Mutations carried by P22 tailspike proteins used in the present study*

Amino acid substitution	Original designation	Phenotype, properties of protein, remarks	References[a]
G244R	*tsH304*	*tsf*, wild-type T_m (DSC), turn peptide model, aggregation and suppression studies	1-6
D238S	*tsU2*	*tsf*, more severe than G244R	6-8
G435E	*tsU38*	Severe *tsf*, T_m reduced by >10 °C (Raman)	1, 9
T235I	*tsH300*	*tsf*, predicted turn, internal sequence repeat, wild-type T_m (DSC)	1, 2, 6
Y232X	*tsR(am51)-e*	Extremely severe *tsf*, X probably serine	6, 10
A334V	*su334*	Global suppressor of *tsf* phenotype	3, 6, 10
V331A	*su331*	Global suppressor of *tsf* phenotype	3, 6, 10

[a] References: [1]Yu and King (1984); [2]Sturtevant et al. (1989); [3]Mitraki et al. (1991); [4]Stroup and Gierasch (1990); [5]Haase-Pettingell and King (1988); [6]Fane et al. (1991); [7]Villafane and King (1988); [8]Yu and King (1988); [9]Sargent et al. (1988); [10]Fane and King (1991).

V331A), is depicted in Figure 2A. Although all three sets of data could be fitted equally well by single exponentials, both the resulting rate and the amplitude were decreased by the *tsf* substitution relative to the wild type. For the *tsf/su* double mutant, intermediate rates and amplitudes were obtained.

The results of a large number of fluorescence kinetics measured with a variety of mutants are documented in Table 2 and Figure 2B. The rates and amplitudes determined for the wild type and the mutants carrying *su* substitutions in the wild-type background were identical

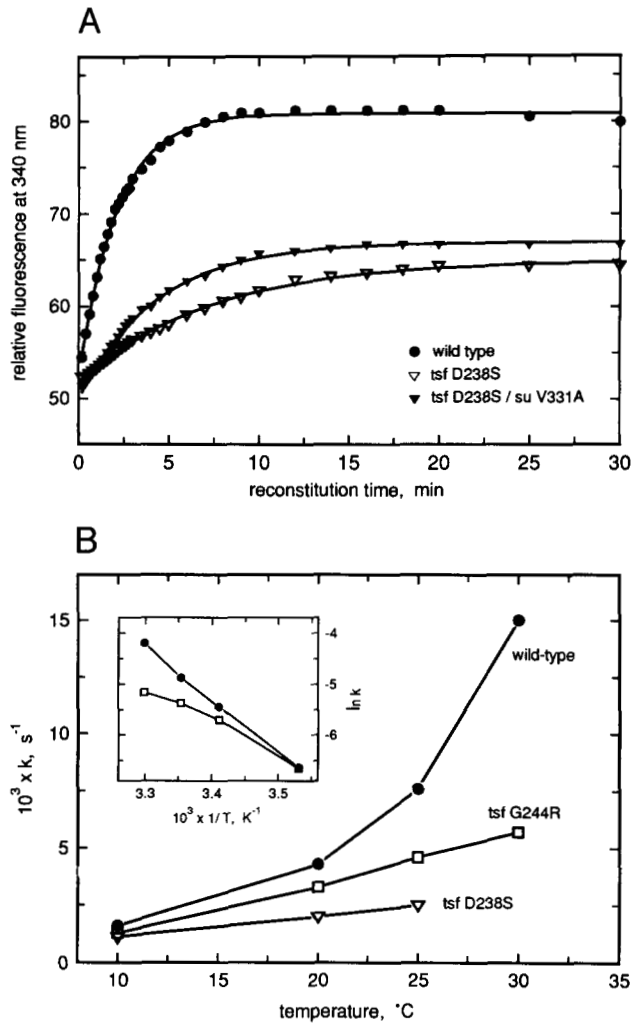

Fig. 2. Subunit folding kinetics observed by fluorescence spectroscopy at 25 °C. **A:** Time course of fluorescence change at 340 nm relative to a native control. Solid line represents a single exponential fit ($f(t) = a - be^{-kt}$) to the data (symbols). **B:** Temperature dependence of the apparent rate constants (k) derived from the fits for the wild type and two *tsf* mutants.

Table 2. *Subunit folding kinetics measured by the increase in fluorescence*

	$10^3 \times k^a$ (s^{-1})			b^b (%$[F_N - F_U]$)		
Protein	10 °C	20 °C	25 °C	10 °C	20 °C	25 °C
Wild type	1.6 ± 0.1[c]	4.3 ± 0.2	7.6 ± 0.4	43.0 ± 1.3	39.8 ± 2.6	32.5 ± 1.2
tsf G244R	1.3 ± 0.0	3.3 ± 0.3	4.6 ± 0.1	39.9 ± 1.0	31.8 ± 0.8	21.0 ± 0.7
su A334V	1.5 ± 0.1	4.4 ± 0.1	7.5 ± 0.2	44.8 ± 2.8	33.0 ± 2.0	27.0 ± 1.4
tsf G244R/*su* A334V	1.1 ± 0.0	3.5 ± 0.0	5.4 ± 0.1	31.3 ± 3.5	26.5 ± 0.5	19.0 ± 2.1
tsf D238S	1.1 ± 0.1	2.0 ± 0.0	2.5 ± 0.2	31.1 ± 1.3	25.6 ± 0.6	15.6 ± 0.7
su V331A	1.6 ± 0.0	4.7 ± 0.2	7.8 ± 0.6	46.1 ± 1.1	31.7 ± 5.4	33.8 ± 1.3
tsf D238S/*su* V331A	1.1 ± 0.0	3.1 ± 0.1	4.1 ± 0.3	41.6 ± 4.1	26.8 ± 0.5	19.4 ± 1.9
tsf D238S/*su* A334V	1.1 ± 0.0	2.8 ± 0.1	3.8 ± 0.6	38.0 ± 1.3	26.8 ± 2.7	20.3 ± 0.8

[a] Apparent rate constant (k) determined by nonlinear regression according to $f(t) = a - be^{-kt}$.
[b] Amplitude (b) of the observed fluorescence change expressed as a fraction of the difference in fluorescence signal between the native (F_N) and fully unfolded (F_U) form, the latter comprising 20% of F_N.
[c] The data represent the average of two to five determinations ±1 SD (protein concentration: 10 μg/mL).

within experimental error over the whole temperature range. Their temperature dependence was that expected for a single rate-limiting reaction with a constant activation energy resulting in a linear Arrhenius plot (insert in Fig. 2B). For all mutants, rates were similar to those of the wild type at low temperature (10 °C). With increasing temperature, there was a progressively larger difference in rates and amplitudes between *tsf* mutants and wild type, resulting in a deviation of the mutant rates from the normal Arrhenius behavior observed with the wild type. In the *tsf/su* double mutants, the *su* substitutions increased the apparent subunit folding rates at elevated temperature and thus partially alleviated the *tsf* effects. The amplitudes of the fluorescence kinetics, when normalized by the fluorescence of a native control, were somewhat variable and strongly dependent on the efficiency of mixing during dilution from the denaturant solution, but decreased continuously with increasing protein concentration (data not shown). However, the observed rates were highly reproducible and essentially independent of the protein concentration (10–20% increase between 1.5 and 20 μg/mL; Fig. 1A).

Temperature resistance of wild-type and mutant folding intermediates

As outlined in detail below (see Discussion), the differential effects of *tsf* and *su* mutations on the apparent rate constants of subunit folding can be explained by the *tsf* mutations destabilizing and the *su* mutations stabilizing an essential subunit-folding intermediate. To assess more directly the thermal stabilities of wild-type and mutant folding intermediates, the development of temperature resistance during refolding was probed by temperature-shift experiments. Refolding was initiated at 25 °C and, at varied times of refolding, the temperature was increased rapidly by transferring an aliquot of the sample into a reaction vessel prewarmed at 40 °C, where the reaction was allowed to come to completion. Refolding yields determined by SDS-gel electrophoresis and densitometry of trimer bands were normalized with respect to a control shifted to 40 °C after completion of refolding at 25 °C. Pairwise comparisons of the wild type with the *tsf* D238S mutant, with the *su* V331A mutant, and with the respective *tsf/su* double mutant are depicted in Figure 3A–C. Similar results, but with less pronounced effects, were obtained with the *tsf* G244R and *su* A334V set of mutants.

As is apparent from the traces obtained with the wild type, resistance to a 25 → 40 °C temperature shift was not attained in a single phase of tailspike folding. Rather, the kinetics were multiphasic, indicating that the thermal stability of folding intermediates increases gradually along the folding pathway. Full resistance to the temperature shift was only attained more than 1 h after initiation of refolding, i.e., when tailspike trimers had matured into the native, SDS-resistant conformation (see Fig. 1). The most distinct difference between wild-type and mutant traces was observed in the time range, where subunit folding occurs (see Table 2). Compared to the wild type, the kinetics for the *tsf* D238S mutant (Fig. 3A) exhibited a distinct lag of about 5 min, indicating that subunit folding intermediates are highly susceptible to temperature shift. A similar amount of wild-type and *tsf*-mutant intermediates (near 15%) was resistant to the shift after 3 min and 10 min, respectively, i.e., after two half-times of subunit folding. In contrast, mutant tailspike polypeptides carrying the *su* V331A substitution in the wild-type background (Fig. 3B) were partly resistant to the temperature shift even 30 s after initiation of refolding. Traces

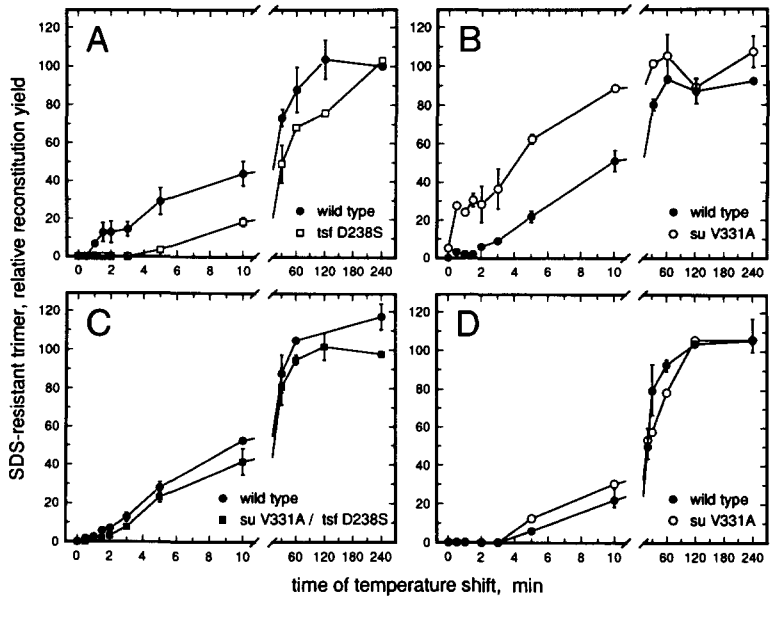

Fig. 3. Temperature resistance of wild-type and mutant folding intermediates. Pairwise reconstitution of wild type and one mutant was initiated by diluting 20 μL acid-urea denatured tailspike protein (0.5 mg/mL) into 980 μL buffer (50 mM sodium phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.0) thermostatted at 25 °C. At the times indicated, 75-μL aliquots were transferred to Eppendorf tubes preincubated at 40 °C (**A–C**) or 45 °C (**D**). After 270 min, reconstitution was quenched by adding 50 μL SDS sample buffer and samples were subjected to SDS-gel electrophoresis, silver staining, and densitometry. The trimer-band intensity of an aliquot reconstituted at 25 °C for 270 min served as a control for the final reconstitution yield at this temperature (see Fig. 1C). Error bars indicate differences between two determinations.

for the *tsf* D238S/*su* V331A double mutant closely resembled those observed with the wild type (Fig. 3C).

Although the temperature shifts confirmed that both *tsf* and *su* mutants primarily act at the level of subunit folding intermediates, they also revealed less pronounced but significant differences in the stability of later intermediates on the folding pathway. This is most evident in the case of the *su* V331A mutant (Fig. 3B). Although only 50 ± 10% of the control yield was observed with the wild type upon a temperature shift after 10 min, corresponding to seven half-times of subunit folding, the *su* V331A mutant had acquired essentially complete resistance to the shift at this time. All tailspike variants, including *su* V331A, acquired temperature resistance much later during refolding, when the target temperature of the shift was increased to 45 °C (Fig. 3D). This again indicates that thermostability is acquired gradually by the successive intermediates along the tailspike folding pathway.

Mutations result in altered rates of irreversible off-pathway reaction at high temperature

If altered thermal stabilities of mutant folding intermediates are the cause of the redistribution of tailspike polypeptides between the productive folding pathway and an unproductive off-pathway reaction, then the effect of the mutations should also become manifest in the overall rate of the irreversible side reaction at high temperature. This was tested by temperature down-shifts during refolding (Fig. 4). Refolding was initiated at 40 °C, a temperature restrictive for refolding of the wild-type and of all *tsf*-mutant tailspike proteins. At varied times, aliquots of the samples were cooled rapidly on ice, and refolding was allowed to proceed to completion at 20 °C. Under these conditions, a large fraction of the wild-type chains aggregated or misfolded irreversibly during the first seconds at 40 °C, but a significant proportion (around 30% of the 20 °C control) was recovered for productive folding, when the temperature was reduced 10 s after the onset of refolding. During the next few minutes at 40 °C, the fraction of wild-type chains rescued by the shift decreased further with an approximate half-time of 25 s. Compared to the wild type, the *su* V331A mutation not only increased the fraction of chains rescued after 10 s, but also prolonged the half-life of recoverable intermediates significantly. Again, the *su* A334V mutation had similar but less pronounced effects. In similar experiments with *tsf* mutants (*tsf* G244R and *tsf* D238S), no rescue was observed even at 10 s after initiation of refolding.

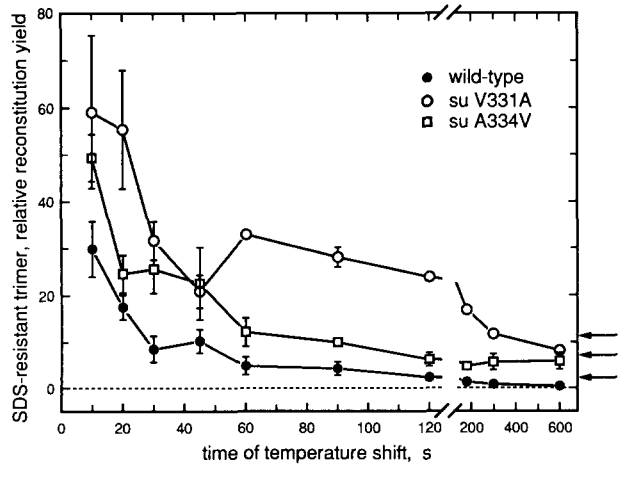

Fig. 4. Temperature down-shifts during refolding. Refolding was initiated at 40 °C and, after the times indicated, aliquots were rapidly cooled and reconstitution was allowed to come to completion at 20 °C overnight. Reconstitution yields were normalized relative to a control refolded at 20 °C. Arrows in the margin indicate the final yields at 40 °C. The error bars comprise the standard deviations of six (wild type), four (*su* A334V), or two (*su* V331A) determinations.

Unfolding of wild-type and mutant tailspikes induced by GdmCl

The temperature-shift experiments had suggested that *tsf* and *su* mutations affected not only early folding intermediates but also the stability of later intermediates on the folding and association pathway; thus, the effects of mutations on unfolding of the final native structure were examined. Because thermal unfolding of the tailspike protein is irreversible (Sturtevant et al., 1989) and because the profiles of guanidine-induced unfolding/refolding transitions exhibit apparent hysteresis (Fuchs et al., 1991), unfolding free energies cannot be determined. However, the unfolding kinetics of mutant and wild-type tailspikes should also reflect alterations in the native structure, unless these persist into the transition state of unfolding. When kinetics of unfolding in the presence of 6 M GdmCl at 25 °C were determined by fluorescence spectroscopy, the traces observed with the wild type and with most mutants investigated were monophasic and could be described by a first-order reaction with half-times of 8–11 min (Fig. 5A; Table 3). Mutants carrying the *tsf* G244R substitution, however, deviated significantly from monophasic behavior (Fig. 5B), and a double-exponential decay had to be used to arrive at a satisfactory fit (Fig. 5C). This was true for the *tsf* G244R substitution in both the wild-type and the *su* A334V backgrounds and was observed with several independent batches of protein. For both mutants, about 24% of the total fluorescence change during unfolding occurred in an additional fast phase. The rate constants describing the slower phase, however, were comparable to those determined for the other tailspike species. The fit results are summarized in Table 3.

Thermal unfolding of wild-type and mutant tailspikes

Chen and King (1991) have shown that thermal unfolding of the tailspike protein is a biphasic process involv-

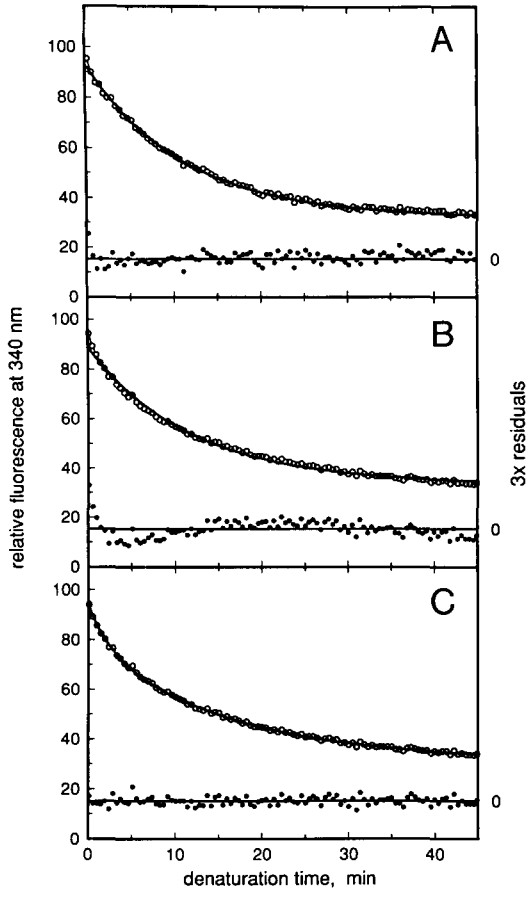

Fig. 5. Tailspike unfolding in 6 M GdmCl monitored by fluorescence spectroscopy. Native tailspike protein (25 μL, 1 mg/mL) was diluted into 2,475 μL 6 M GdmCl, 50 mM sodium phosphate, 1 mM EDTA, 1 mM dithioerythritol, pH 7.0 at 25 °C. Fluorescence amplitudes were corrected for buffer fluorescence, and the native control was set to 100%. Data were fitted by nonlinear regression using one or two exponentials, as indicated. The residuals scale (right axis) is enlarged threefold compared to the left one; ○, measured data; ——, fit; ●, residuals. **A:** Wild type, one exponential. **B:** *tsf* G244R, one exponential. **C:** *tsf* G244R, two exponentials.

ing a trimeric intermediate in which the N-termini (residues 1–107) of all three chains are accessible to proteases and detergent. They have also indicated that *tsf* mutations appear to affect the second rather than the first phase of unfolding. This was confirmed when unfolding kinetics of mutant tailspikes in the presence of 2% (w/v) SDS at 65–67 °C were assessed by gel electrophoresis, silver staining, and densitometry. In addition to the mutants studied in the above experiments (*tsf* D238S, *tsf* G244R, *su* V331A, *su* A334V, and combinations), for which the refolding yields have been characterized previously in detail (Mitraki et al., 1993), three *tsf* mutants (G435E, T235I, and Y232X) and one *tsf/su* double mutant (*tsf* Y232X/*su* V331A) were included in this part of the study. Their in vitro refolding phenotypes are illustrated in Figure 6.

Examples of the thermal unfolding results are depicted in Figure 7. Because the bands of the native trimer, the unfolding intermediate, and the unfolded monomer exhibited different sensitivities to silver staining or staining with Coomassie brilliant blue, and because rates varied strongly with the temperature of unfolding, calculation of rate constants was not attempted. Due to the strong temperature dependence, the wild-type profiles varied to some extent between individual experiments depicted in the different panels. However, mutant and wild-type unfolding kinetics were determined in pairwise comparisons at exactly identical temperature and staining conditions. Under these conditions, the observed effects of the mutations on the relative denaturation rates were highly reproducible even for mutants with less severe phenotypes. Thus, a semiquantitative evaluation was possible. Although none of the *tsf* or *su* substitutions affected the rate of formation of the trimer unfolding intermediate to any detectable degree, all *tsf* substitutions studied accelerated the second phase of unfolding significantly. In contrast, the *su* V331A substitution strongly retarded unfolding of the intermediate, both in the wild-type and in *tsf*-mutant

Table 3. *Kinetics of tailspike unfolding in guanidinium chloride observed by fluorescence*

Protein	*a*	*b*	$10^3 \times k$ (s^{-1})	*c*	$10^2 \times l$ (s^{-1})
Wild type[a]	32.4 ± 0.3[b]	60.8 ± 0.4	1.5 ± 0.1		
tsf G244R[a]	33.9 ± 0.4	54.4 ± 0.7	1.5 ± 0.1		
tsf G244R[c]	31.7 ± 0.4	47.7 ± 1.0	1.1 ± 0.1	14.7 ± 0.4	0.8 ± 0.1
su A334V[a]	29.4 ± 0.1	61.5 ± 1.6	1.1 ± 0.0		
tsf G244R/*su* A344V[a]	32.3 ± 0.5	54.5 ± 0.7	1.8 ± 0.1		
tsf G244R/*su* A344V[c]	31.1 ± 0.6	48.8 ± 0.1	1.5 ± 0.1	15.2 ± 0.9	1.5 ± 0.1
tsf D238S[a]	34.1 ± 0.6	57.8 ± 0.3	1.3 ± 0.1		
su V331A[a]	31.5 ± 0.0	62.1 ± 1.4	1.1 ± 0.0		
tsf D238S/*su* V331A[a]	31.7 ± 0.3	59.4 ± 0.7	1.2 ± 0.1		

[a] Fitted to the single exponential $f(t) = a + be^{-kt}$.
[b] All values represent the average of two to four independent determinations ±1 SD.
[c] Fitted to the double-exponential equation $f(t) = a + be^{-kt} + ce^{-lt}$. For the other proteins, no significant deviation from the monophasic fit was observed (compare Fig. 5).

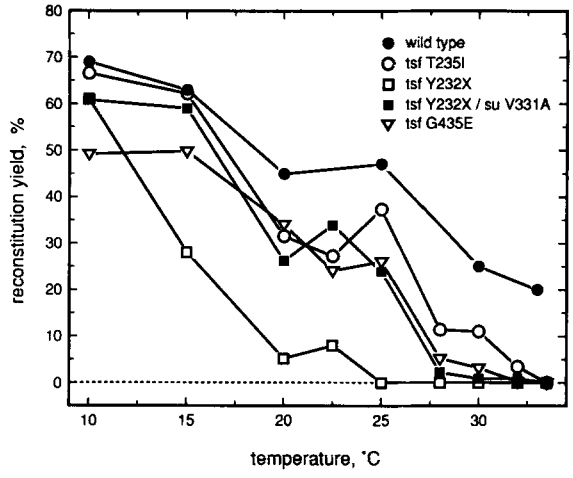

Fig. 6. Reconstitution efficiency at varied temperature. Wild-type and mutant proteins were reconstituted to completion at each temperature. Band intensities of reconstituted SDS-resistant trimers were compared to those of native controls on the same SDS gels.

backgrounds. The latter effect was not observed with the second suppressor substitution *su* A334V. Generally, the effects of the folding mutations on the stability of the thermal unfolding intermediate (*su* V331A > *su* A334V ≈ wild type > *tsf* G244R ≈ *tsf* T235I ≈ *tsf* D238S > *tsf* G435E ≈ *tsf* Y232X) paralleled their effects on refolding at elevated temperature.

Discussion

Our results show that point mutations in the tailspike gene leading to temperature sensitivity or to suppression of temperature sensitivity both exert their effects during refolding of the purified tailspike protein in vitro, in the absence of cellular factors. This confirms previous conclusions from a comparison of mutant and wild-type tailspike refolding yields at varied temperature (Mitraki et al., 1993) and allows the mechanism of the folding mutations to be analyzed in refolding experiments. Although a number of mutants were included in some aspects of the experiments described above, the detailed analysis focused on two well-characterized temperature-sensitive mutants (*tsf* G244R and *tsf* D238S), the two known global suppressor mutants (*su* V331A and *su* A334V), and the respective *tsf/su* double mutants. The effects of the mutations on tailspike refolding parallel their phenotypes observed during tailspike biosynthesis in the *Salmonella* cell, where D238S is the more defective *tsf* mutant, and V331A increases folding yields of the wild-type protein to a greater extent.

Folding kinetics of mutants and wild type were analyzed in detail at 25 °C and by temperature-shift experiments starting at 25 °C. This temperature was chosen because it is close enough to the restrictive temperature of the *tsf* mutants to expect observable effects, whereas wild-type folding is largely unaffected, and the mutant refolding yields are still high enough to follow their folding kinetics. The folding and assembly pathway of the tailspike protein at 25 °C reflects the earlier observations at 10 °C (Fuchs et al., 1991); however, at the higher temperature, the kinetic phases are somewhat less well separated. The data may be summarized as follows:

	U →→	M →→	PT →	N
$t_{1/2}$ at 10 °C	7 min	~30 min	~10 h	
$t_{1/2}$ at 25 °C	1.5 min	~10 min	~30 min	

The times at which the formation of structured subunits (M), of stably associated, but incompletely folded "protrimers" (PT), and of native, SDS-resistant trimers (N) are half completed are indicated beneath the corresponding arrows.

It is evident from the fluorescence kinetics that the *tsf* mutations reduce the overall rate of subunit folding (U → M) at high, but not at low, temperature, and the *su* mutations alleviate the reduction when present in the mutant background but have no observable effect in the wild-type background at 25 °C. The data may be explained readily by a simple model based on the folding pathway outlined above, but involving a thermolabile intermediate (I), which is destabilized by the *tsf* and stabilized by the *su* mutations:

$$\begin{array}{lcccccc} & & \overset{k_f}{} & & & & \\ & & \mathrm{I} & \rightarrow & \mathrm{M} & \rightarrow\rightarrow \ \mathrm{PT} \rightarrow & \mathrm{N} \\ \mathrm{U} & \rightarrow\rightarrow & \Updownarrow & K_{eq} & & & \\ & & \mathrm{I}^* & \rightarrow & \text{misfolding/aggregation} & & \end{array}$$

If the unfolding equilibrium I ⇌ I* is attained rapidly compared to the rate-limiting folding reaction, then the overall rate of subunit folding results as the product of the concentration of the productive intermediate [I] and the intrinsic folding rate constant k_f, and the apparent rate constant of subunit folding becomes $k_{app} = k_f \cdot K_{eq}/(K_{eq} + 1)$. This holds only if the rate of the irreversible misfolding or aggregation reaction from I* is low under the reaction conditions; otherwise, an increase in the apparent rate constant and a deviation from first order would be expected (Kiefhaber et al., 1991). According to the model, thermal denaturation of I, i.e., a shift of the equilibrium toward I*, would result in a reduction of the overall rate of folding and, at the same time, reduce the yield of folding, because off-pathway reactions would be accelerated. Because the folding yields of wild-type and mutant tailspikes are only moderately dependent

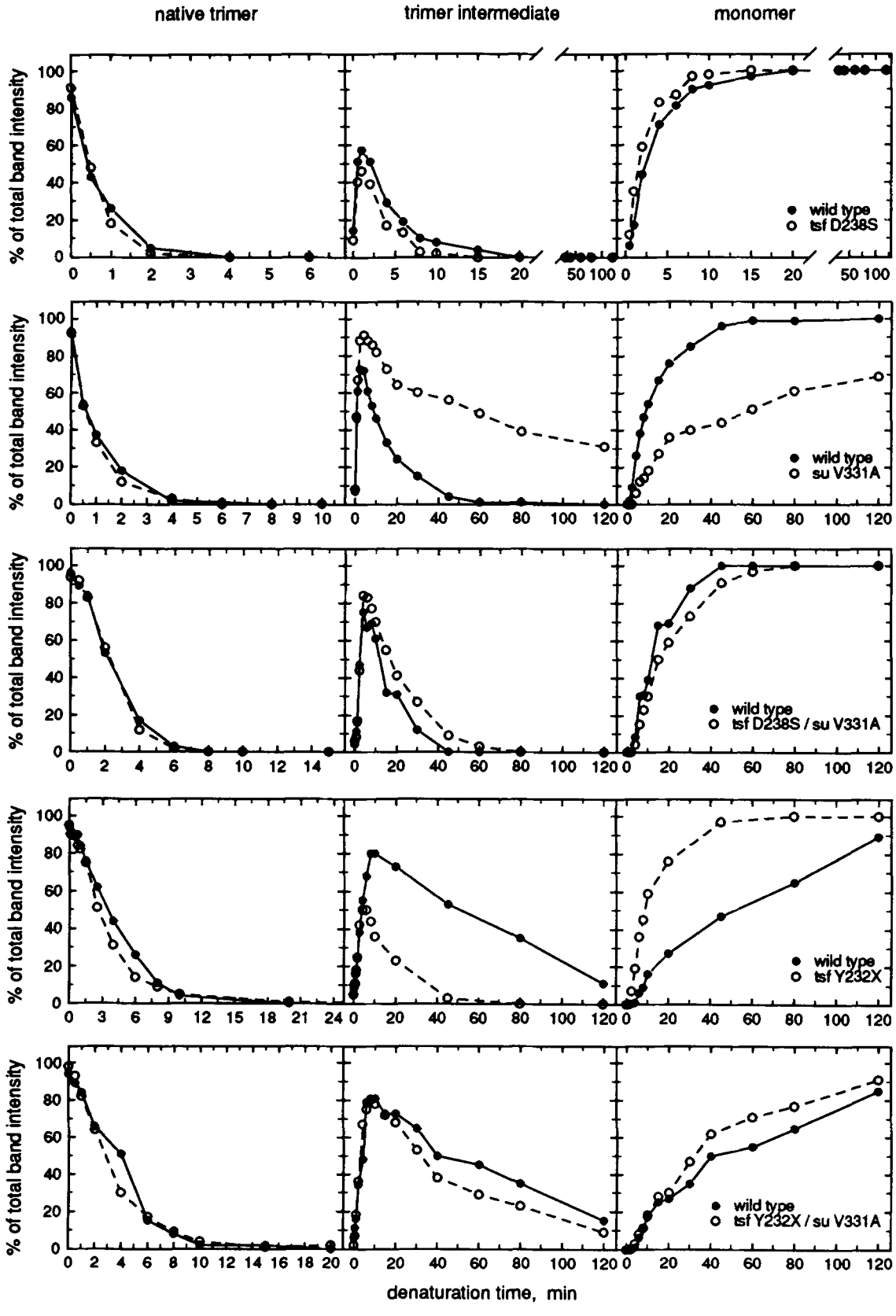

Fig. 7. Thermal unfolding kinetics of wild-type and mutant tailspikes in the presence of SDS at 65 °C or 67 °C (uppermost panels, comparison of wild type with *tsf* D238S). Left panels show the relative band intensities of the native SDS-resistant trimer. Middle panels show those of the trimer intermediate species with unfolded N-termini. Right panels show the accumulation of the monomer band with time.

on protein concentration during refolding (M. Danner, unpubl.), the rate-limiting step of the irreversible off-pathway reaction is more likely unimolecular ("misfolding") rather than bimolecular (aggregation). It should be noted that I* does not have to be an off-pathway intermediate but could as well precede I on the folding pathway.

That the *tsf* mutations affect the stability of subunit folding intermediates, and thereby retard folding, is consistent with the in vivo observations and with results of studies on model peptides. Upon biosynthesis at restrictive temperature, *tsf* mutant tailspike polypeptides accumulate at a folding step preceding subunit association, and eventually form insoluble aggregates (Haase-Pettingell & King, 1988). Formation of aggregates is irreversible, but the accumulated aggregation-sensitive intermediate(s) can be partially recovered for the productive folding pathway by a shift to lower temperature (Smith & King, 1981; Haase-Pettingell & King, 1988). Stroup and Gierasch (1990) have studied the stability of a predicted turn in the vicinity of the *tsf* G244R site with the aid of peptide models. Using decamers and dodecamers comprising residues 240–247 and 239–248 of the tailspike polypeptide, terminated by cysteine residues at both ends, they found that the Gly → Arg substitution decreases the tendency of the peptide to assume an antiparallel strand conformation with a type-II β-turn around residues 243–244. The difference in redox potential of the peptides corresponds to a destabilization of the secondary structure by $\Delta\Delta G \approx 4$ kJ/mol. A corresponding destabilization of the essential intermediate I would be sufficient to explain, on the basis of the above model, the twofold reduction in the apparent rate of subunit folding observed at 25 °C with the *tsf* G244R mutant, if the I ⇌ I* equilibrium for the wild type was 80:20 at this temperature.

The results of the temperature down-shifts from 40 °C to 20 °C during refolding are also consistent with the above scheme. As predicted by the model, the *tsf* mutants accelerate, and the *su* mutants decelerate the irreversible off-pathway reaction at 40 °C.

That the global suppressor mutations indeed stabilize subunit folding intermediates is corroborated by the results of the temperature up-shift experiments. Although these substitutions in the wild-type background (*su* V331A, *su* A334V) do not affect subunit folding kinetics at ≤25 °C to any detectable degree, they increase the fraction of the protein reaching the native trimer state upon a temperature shift from 25 °C to 40 °C immediately after the onset of refolding. The temperature-shift experiments also demonstrate that thermolability is not limited to subunit-folding intermediates. Only a fraction of the wild-type protein reaches the native state, when the temperature is raised from 25 °C to 40 °C around 7 min after the onset of refolding. At this time, only intermediates with native-like fluorescence and circular dichroism spectra, i.e., folded subunits and the protrimer intermediate, are significantly populated. Resistance against a temperature shift to 45 °C is not acquired before the final trimer maturation reaction, which is associated with a very large activation barrier ($E_a \approx 165$ kJ/mol). Consequently, effects of the mutations remain detectable in temperature-shift experiments up to this last rate-limiting step of folding.

As suggested previously by the results of Chen and King (1991), effects of the folding mutations on the stability of the native structure have escaped detection because they are masked by the sequential unfolding pathway of the tailspike trimer. The unfolding reaction involves a trimer intermediate, in which the N-terminal domains (~110 of the 666 residues) are denatured (Chen & King, 1991), but the C-terminal major parts of the tailspike polypeptides remain stably folded in the native conformation (Danner et al., 1993). All folding mutations examined in this study (*tsf* G244R, *tsf* D238S, *tsf* T235I, *tsf* G435E, *tsf* Y232X, *su* V331A), with the exception of *su* A334V, affect the rate of thermal denaturation of the trimer unfolding intermediate in a manner correlating with their folding phenotypes. In addition, for one of the mutations (*tsf* G244R), a local unfolding reaction is indicated by an additional fast phase of fluorescence change upon dilution of the native tailspike protein into 6 M GdmCl.

In summary, tailspike folding involves a series of marginally to moderately stable intermediates, before the high resistance to thermal denaturation is acquired only in a final rate-limiting folding reaction at the trimer level. As suggested by Lecomte and Matthews (1993), the *tsf* and *su* mutations lead to small changes in stability that strongly affect marginally stable early intermediates, have smaller effects later in the pathway, and are nearly undetectable in the stably folded, native structure. In addition, the major part of the tailspike protein containing the mutations remains folded during the first step of thermal denaturation, explaining the difficulty in detecting mutant effects on the thermal stability of the native trimer.

Materials and methods

Materials

Bacterial strains and phage P22 (5^-_{am}, 13^-_{am} [Botstein et al., 1973]) stock solutions were provided by Dr. J. King (MIT, Cambridge, Massachusetts). Amino acid substitutions resulting from point mutations in the tailspike gene (P22 gene *9*) are listed in Table 1 together with short descriptions of the mutant protein properties and pertinent references. Tailspike protein was purified from *Salmonella typhimurium* LT2-DB7136 (Winston et al., 1979) infected with phage P22 essentially as described (King & Yu, 1986). The protein was stored as a suspension in 40% saturated $(NH_4)_2SO_4$, 10 mM sodium phosphate, 3 mM 2-mercaptoethanol, 1 mM EDTA, 10 mg/mL glycerol,

pH 7.5. Its concentration was determined spectroscopically using a specific absorbance of $A_{278nm}^{1mg/mL} = 1.01$ (Sauer at al., 1982). Ultrapure GdmCl and urea were obtained from USB (Cleveland, Ohio) or from ICN Biomedicals (Costa Mesa, California). Concentrations of urea and GdmCl solutions were determined by refractive index measurements (Pace, 1986). Dithioerythritol was from Roth (Karlsruhe); polyoxyethylenesorbitan monolaurate (Tween 20), SDS, and acrylamide were from Serva (Heidelberg). Other chemicals were analysis grade, and quartz-bidistilled water was used throughout.

Unfolding and refolding

Tailspike protein was dissociated and unfolded (Seckler et al., 1989) in 5 M urea, pH 2.9–3.0 (50–65 mM H_3PO_4), for ≥30 min at room temperature. Refolding was initiated by a 1:50 or 1:100 dilution, as indicated, of denatured protein into thermostatted reconstitution buffer (50 mM sodium phosphate, 1 mM EDTA, 1 mM dithioerythritol, pH 7.0). The refolding buffer was agitated vigorously by a small magnetic stirring bar during addition of unfolded protein in order to guarantee rapid dilution to 0.1 M or 0.05 M residual urea concentration. Eppendorf tubes pretreated by a rinse with 5% Tween 20 followed by drying were used where indicated to avoid loss of material by protein sticking to the vessel walls.

Subunit folding and tailspike unfolding observed by fluorescence spectroscopy

Increase (folding) and decrease (unfolding) of fluorescence emission upon excitation at 280 nm was recorded at the uncorrected emission maximum of the native protein, i.e., at 340 nm with a Perkin-Elmer MPF44A spectrofluorimeter connected to a digital voltmeter interfaced with a personal computer or at 342 nm with a Spex Fluoromax. To initiate refolding, 25 μL of denatured protein solution was injected rapidly into 2,475 μL of thermostatted reconstitution buffer in a 1 × 1-cm stirred quartz cell. Unfolding was monitored after dilution of 25 μL native protein solution (1 mg/mL) into 2,475 μL 6 M GdmCl, 50 mM sodium phosphate, 1 mM EDTA, 1 mM dithioerythritol, pH 7.0, at 25 °C. The fluorescence amplitudes were corrected for buffer fluorescence and normalized relative to the native control set to 100%. Data were fitted by nonlinear regression using one or two exponentials, as indicated.

Subunit association

To test for hybrid formation of polypeptide chains not yet associated, simultaneous reconstitution of wild-type tailspikes and of a mutant with altered net charge (G244R) was started in separate Tween-treated vessels, by 1:50 dilutions (final volume 1.2 mL) at 25 °C to protein concentrations of 5, 10, 15, 20, or 30 μg/mL. At varied times after the onset of reconstitution, aliquots of the mutant and wild-type samples were mixed and reconstitution was allowed to proceed to completion for ~20 h. To compensate for the different reconstitution efficiencies of wild type and the *tsf* mutant at this temperature (Mitraki et al., 1993), 60-μL aliquots of the wild-type sample were mixed with 100-μL aliquots of the mutant one. Reconstitution was quenched by adding 107 μL SDS sample buffer (163 mM Tris/HCl, 250 mg/mL glycerol, 0.25 mg/mL bromphenol blue, 5 mg/mL SDS, 5 mM dithioerythritol, pH 6.8). The SDS-gel electrophoresis (5% acrylamide), silver staining (Fuchs et al., 1991), and densitometry (Brunschier et al., 1993) were done as described.

Maturation of tailspike trimers

To follow the formation of native, SDS-resistant tailspike trimers, reconstitution of wild-type protein was initiated by pipetting 14 μL of denatured protein (0.5 mg/mL) into 686 μL thermostatted buffer. After the times indicated, aliquots of 50 μL were transferred into cups containing 33 μL SDS sample buffer to quench reconstitution. Samples were analyzed by electrophoresis, silver staining, and densitometry. Trimer band intensities were normalized relative to the sample incubated at 25 °C for 8 h, which served as a control for complete reconstitution at this temperature.

Temperature down-shift during reconstitution

Reconstitution was started at 40 °C by 1:50 dilution of 16 μL denatured tailspike solution (0.5 mg/mL) into 786 μL prewarmed buffer. After the times indicated, 50-μL aliquots of the reconstitution sample were pipetted onto brass blocks on ice covered by a thin layer of paraffin film for rapid cooling of the samples. After 5 min on ice, 45-μL aliquots were transferred to vessels preincubated at 20 °C, and reconstitution was allowed to come to completion at the permissive temperature for ≥20 h. The reference samples were reconstituted at 20 °C but were also transferred to ice for 5 min and then back to 20 °C. The samples were analyzed by electrophoresis, silver staining, and densitometry, and trimer band intensities were normalized relative to the reference for permissive conditions.

Reconstitution efficiency at varied temperature

To determine the reconstitution efficiencies at varied temperature, 10 μL each of denatured wild-type or various mutant proteins (0.5 mg/mL) were pipetted into 490 μL thermostatted, stirred buffer. Samples were incubated for 70 h at 10, 15, and 20 °C, and for ≥15 h at temperatures

above 20 °C. Because the reconstitution yields were low at temperatures higher than 20 °C, native controls were diluted twofold prior to SDS-gel electrophoresis, so that the band intensities after silver staining did not exceed the linear range.

Thermal unfolding in the presence of SDS

For each time point, 10 μL tailspike protein solution (0.4 mg/mL) was pipetted into 390 μL unfolding buffer (50 mM Tris/HCl, adjusted to pH 7.0 at room temperature and containing 150 mM 2-mercaptoethanol and 20 g/L SDS) and thermostatted at 65 °C in a water bath. At varied times, samples were rapidly cooled on ice and were later mixed with 267 μL SDS sample buffer and analyzed by SDS-gel electrophoresis. In each experiment, one mutant was compared directly to wild type in order to avoid slightly different denaturation temperatures or silver-staining conditions.

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