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Cold rescue of the thermolabile tailspike intermediate at the junction between productive folding and off-pathway aggregation

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Abstract

Off-pathway intermolecular interactions between partially folded polypeptide chains often compete with correct intramolecular interactions, resulting in self-association of folding intermediates into the inclusion body state. Intermediates for both productive folding and off-pathway aggregation of the parallel β-coil tailspike trimer of phage P22 have been identified in vivo and in vitro using native gel electrophoresis in the cold. Aggregation of folding intermediates was suppressed when refolding was initiated and allowed to proceed for a short period at 0 °C prior to warming to 20 °C. Yields of refolded tailspike trimers exceeding 80% were obtained using this temperature-shift procedure, first described by Xie and Wetlaufer (1996, *Protein Sci* 5:517–523). We interpret this as due to stabilization of the thermolabile monomeric intermediate at the junction between productive folding and off-pathway aggregation. Partially folded monomers, a newly identified dimer, and the protrimer folding intermediates were populated in the cold. These species were electrophoretically distinguished from the multimeric intermediates populated on the aggregation pathway. The productive protrimer intermediate is disulfide bonded (Robinson AS, King J, 1997, *Nat Struct Biol* 4:450–455), while the multimeric aggregation intermediates are not disulfide bonded. The partially folded dimer appears to be a precursor to the disulfide-bonded protrimer. The results support a model in which the junctional partially folded monomeric intermediate acquires resistance to aggregation in the cold by folding further to a conformation that is activated for correct recognition and subunit assembly.

Keywords: bacteriophage P22; endorhamnosidase; inclusion body; non-native; polymerization; protein aggregation; protein folding

An important aspect of the protein folding problem is the process of protein misfolding and the generation of aggregated states. Protein aggregation under nondenaturing conditions in vitro is driven by the specific off-pathway self-association of partially folded polypeptide chains (Mitraki et al., 1987, 1991b; Brems, 1988; Mitraki & King, 1989; Oberg et al., 1994; Speed et al., 1996). A similar polymerization process involving specific off-pathway interactions may be involved in the aggregation of proteins implicated in the amyloid diseases and in the formation of inclusion bodies frequently observed during the overexpression of recombinant proteins in *Escherichia coli* (Marston, 1986; Wetzel, 1994; Betts et al., 1997).

The fate of conformational intermediates at branch points to productive and nonproductive pathways is sensitive to environmental conditions and to changes in the primary amino acid sequence. Single amino acid substitutions in the P22 tailspike protein, interleukin-1β, and other proteins can dramatically shift folding intermediates from the productive pathway to the competing, inclusion body pathway (Haase-Pettingell & King, 1988; Wetzel, 1994; King et al., 1996; Betts et al., 1997). Similarly, destabilizing mutations in transthyretin, α1-antitrypsin, and other proteins whose aggregation is associated with human diseases can promote partial unfolding of native proteins to intermediate conformations that are susceptible to off-pathway polymerization (Kelly, 1996; Carrell & Lomas, 1997).

The P22 tailspike protein is a homotrimeric parallel β-coil protein with an endorhamnosidase activity that cleaves the O-antigen present on the surface of *Salmonella typhimurium* (Steinbacher et al., 1994, 1996). The wild-type tailspike polypeptide is 666 amino acids in length and is encoded by gene 9 of phage P22 (Sauer et al., 1982). The crystal structure of a mutant trimer lacking the first 108 amino acids is shown in Figure 1. Between residues 143 and 540, the tailspike subunit forms a coil of 13 complete turns; each turn of the coil contributes one β-strand to each of three parallel β-sheets, thus forming the higher-order β-coil motif

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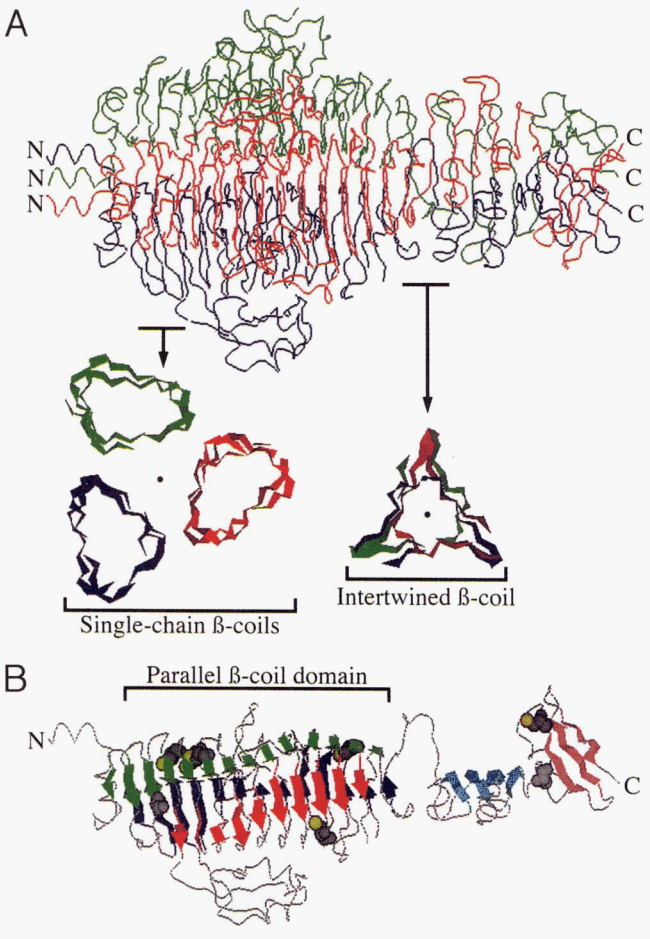

Fig. 1. Crystal structure of the P22 tailspike protein at 2 Å resolution (Steinbacher et al., 1994). Crystallographic coordinates are available in the Protein Data Bank (PDB code 1tsp) (Bernstein et al., 1977; Abola et al., 1987). **A:** Tailspike trimer of subunits lacking residues 1–108. The three subunits are colored. Top: side view. Bottom: axial views showing the first three coils of each subunit (residues 143–197) and the hybrid β-coil (residues 538–564). The threefold symmetry axis is indicated by the central dot. **B:** Subunit from tailspike trimer showing locations of β-sheets (colored ribbons) and cysteine residues (thiols in yellow).

(Fig. 1B). The β-coil domain of each subunit is in direct contact along its full length with the β-coil of each of the other two subunits, but the domains remain independent (Fig. 1A). The lateral surface of the β-coil domain binds the O-antigen lipopolysaccharide of *Salmonella* (Steinbacher et al., 1996). In contrast to the independent β-coil domains, the C-terminal domains of the three subunits intertwine to form a single hybrid β-coil (Fig. 1A).

Studies of tailspike refolding and aggregation both in vivo and in vitro have implicated a partially folded single-chain intermediate as the precursor to inclusion body formation (Danner & Seckler, 1993; Speed et al., 1995). The refolding pathway outlined in Scheme 1 shows both productive intermediates to the native tailspike protein and off-pathway intermediates to aggregates of tailspike polypeptides.

$$\begin{array}{l} U \rightarrow\rightarrow [I] \rightarrow [I_b] \rightarrow\rightarrow [Pt] \rightarrow N \\ \qquad\quad \downarrow\uparrow \\ \qquad\quad [I^*] \rightarrow [2I^*] \rightarrow [3I^*] \rightarrow\rightarrow\rightarrow [nI^*]. \end{array} \qquad (1)$$

The monomeric species $[I]$ is the junctional folding intermediate (King et al., 1996). A later, aggregation-resistant monomeric intermediate, $[I_b]$, associates into the protrimer species, denoted $[Pt]$ (Goldenberg et al., 1983; Fuchs et al., 1991). The three polypeptide chains in the protrimer are still not fully folded and contain transient interchain disulfide bonds (Robinson & King, 1997). Disulfide reduction with further folding leads to the very stable native state, N.

At elevated temperatures, $[I]$ rearranges to an off-pathway conformation, denoted $[I^*]$ (Mitraki et al., 1991a; Danner & Seckler, 1993). Self-association of $[I^*]$ species depletes productive intermediates, resulting in decreased yields of native tailspike. Tailspike polypeptides synthesized at high temperatures that are restrictive for productive folding can be rescued from the aggregation pathway following a shift to lower temperatures, indicating the reversibility of the $[I] \rightarrow [I^*]$ transition (Haase-Pettingell & King, 1988, 1997). The temperature downshift must be applied before chains occupying the $[I^*]$ conformation associate irreversibly to form multimeric aggregation intermediates ($[nI^*]$). These off-pathway multimeric species are not disulfide bonded (Speed et al., 1995). Temperature-sensitive folding (tsf) mutations destabilize $[I]$ and thus mimic the effect of increased temperature by shifting the $[I] \leftrightarrow [I^*]$ equilibrium toward $[I^*]$. Global suppressor (su) mutations inhibit the self-association reaction by stabilizing $[I]$ with respect to $[I^*]$ or by interfering with the polymerization reaction (Mitraki et al., 1991a; Beißinger et al., 1995).

Spectroscopic and hydrodynamic techniques have been used to monitor tailspike refolding in vitro. Seckler and co-workers have presented evidence for the existence of a structured monomeric folding intermediate ($[I_b]$ in Scheme 1) that is both resistant to aggregation and assembly competent (Fuchs et al., 1991; Danner & Seckler, 1993). Xie and Wetlaufer (1996) described the cold accumulation of a partially folded intermediate to bovine pancreatic carbonic anhydrase that exhibited the same property of aggregation resistance.

A trimeric tailspike folding intermediate, the protrimer ($[Pt]$ in Scheme 1), was first identified by native gel electrophoresis as a sharp band with decreased electrophoretic mobility relative to the native protein (Goldenberg & King, 1982). The protrimer forms from structured monomers, as proposed by Fuchs et al. (1991). However, structured monomers cannot be in a fully native conformation because formation of the hybrid β-coil shown in Figure 1A can only occur after subunit association (King et al., 1996).

Speed et al. (1995) reported a methodology using native gel electrophoresis for trapping and fractionating multiple intermediates on both folding and aggregation pathways. For example, analysis of tailspike refolding in vitro revealed that the off-pathway trimer $[3I^*]$ migrated as a broad band between the relatively sharp bands of native trimers and protrimers. At least two forms of both monomeric and dimeric intermediates were also resolved by native gel electrophoresis, but assignment of these species to a specific pathway was not possible. Here we report conditions that allow the selective accumulation or trapping of either on-pathway folding intermediates containing one to three polypeptide chains or multimeric off-pathway aggregation intermediates.

Robinson and King (1997) reported that the protrimer intermediate is stabilized by inter-subunit disulfide bonds, which are absent in the native trimer. This result was consistent with an earlier experiment in which assembly of newly synthesized tailspike polypeptides was blocked in vivo following exposure to sulfhydryl modifying reagents (Sather & King, 1994); the same result was

observed during in vitro refolding (Robinson & King, 1997). These observations suggest that exposure of essential cysteinyl thiols, perhaps buried in an earlier intermediate, may be an essential step in the formation of productive monomeric intermediates.

Results

Early stages of off-pathway aggregation

After dilution from urea into buffer at 20 °C, native tailspikes were detected within 5 min and continued to accumulate for several hours. The yield of refolded tailspikes was 33 ± 3%, with a half-time of 62 min. In contrast, off-pathway multimers containing four or more chains appeared within seconds after dilution (Speed et al., 1995). To examine these early stages more carefully, native wild-type tailspike protein was denatured in 5 M urea at pH 3. Refolding was initiated by 20-fold dilution of denatured chains to 100 μg/mL and 0.8 M urea at pH 7.6. The reaction temperature was maintained at 20 °C. Samples were withdrawn at various times and chilled to slow the reaction.

Figure 2 shows the trapped intermediates resolved by native gel electrophoresis in the cold. The formation of the native trimer is clearly visible in the central region of the gel. A ladder of multimeric intermediates appeared in the earliest sample taken during the first minute of the refolding reaction (lane 1). The indicated stoichiometry of these intermediates was determined previously (Speed et al., 1995). The lower band in lane 1 contains partially folded monomers, the second band from the bottom contains partially folded dimers, the third band contains partially folded trimers, etc. Multimeric species containing more than 8–10 chains are trapped at the top of the gel.

As the reaction proceeded, monomeric, dimeric, and trimeric species were depleted. These early, transient species include precursors both to the native trimer and to the large aggregates that predominated in later samples. The band above the native trimer is a mixture of at least two species, a partially folded trimer, [3*I**], that is on the aggregation pathway and the productive protrimer intermediate (Goldenberg & King, 1982; Speed et al., 1995). The protrimer is evident as a sharp band on the trailing edge of the [3*I**] distribution.

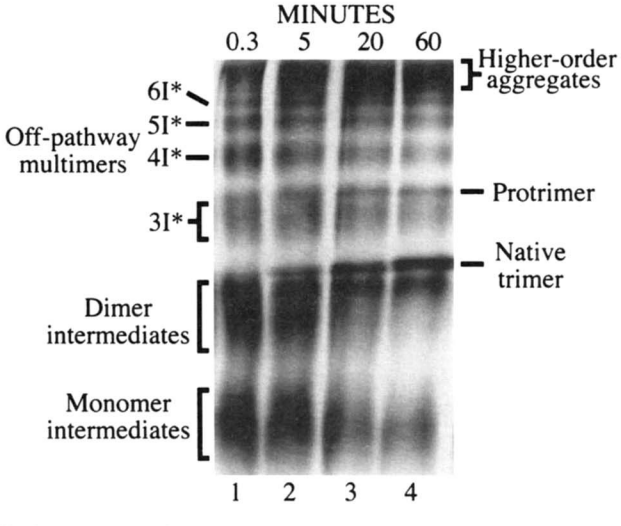

Fig. 2. Tailspike refolding and aggregation analyzed by native gel electrophoresis. Denatured tailspike polypeptides were diluted 20-fold to 100 μg/mL and 0.8 M urea at pH 7.6 and 20 °C. Samples were quenched on ice without DTT. Polypeptides were visualized by silver staining.

Multimers containing four or more chains are assigned to the aggregation pathway. The presence of off-pathway multimers in the earliest sample (Fig. 2, lane 1) demonstrates that partially folded tailspike polypeptide chains can become committed to the aggregation pathway within seconds.

Inhibition of aggregation by initiating refolding in the cold

Both in vivo and in vitro, the partitioning between off-pathway aggregation and productive folding is very sensitive to temperature (Haase-Pettingell & King, 1988). This is believed to be the basis of the high frequency of sites of temperature-sensitive folding mutations in the tailspike chain (King et al., 1996; Haase-Pettingell & King, 1997). Danner and Seckler (1993) examined the thermolability of the monomeric tailspike folding intermediate during the in vitro refolding reaction. They identified by spectroscopy structured monomeric folding intermediates, which, once formed at permissive temperature, were stable and aggregation resistant when shifted to nonpermissive temperatures.

These results indicated that the aggregating species were partially melted monomeric intermediates ([*I**]) generated at higher temperatures. We therefore attempted to suppress aggregation of tailspike polypeptides by first accumulating aggregation-resistant folding intermediates during refolding on ice. Samples were incubated on ice for various lengths of time and then shifted to 20 °C for up to 24 h. Trapped intermediates were fractionated by native gel electrophoresis and stained with Coomassie blue for quantitative analysis.

Figure 3A shows the formation of native tailspike trimers in samples shifted from 0 to 20 °C at different times. No native trimer was detected in samples maintained in the cold (dashed line). Samples diluted from 0 °C directly into buffer at 20 °C gave a 20% yield after 2 h and reached a maximum of 45% after 24 h (dotted line). In contrast, native trimers accumulated to above 40% after 2 h in the three samples incubated in the cold and shifted to 20 °C. When refolding was initiated at 0 °C for just 1 min, the 24-h yield increased from 45 to 67% following a shift to 20 °C (Fig. 3A, squares). Cold incubation for 20 min or longer resulted in yields exceeding 70% after 24 h and were higher than any previously observed under conditions that are in all other respects more favorable for refolding.

The 45% refolding yield of the 20 °C control was significantly higher than the average refolding yield of 33 ± 3% at this temperature. This enhancement can be attributed to the equilibration of denatured chains to 0 °C. In the standard procedure, samples are equilibrated to 20 °C before dilution. The value of 67% obtained after a cold incubation of just 1 min thus represents a doubling of the average refolding yield.

The formation of off-pathway multimers is shown in Figure 3B. Off-pathway multimers did not accumulate to detectable levels in the control maintained at 0 °C (dashed line). Multimerization was most extensive in the control sample diluted at 20 °C (dotted line). Incubation at 0 °C reduced the formation of multimers in all three samples shifted to 20 °C (solid lines). The extent of suppression was proportional to the time of incubation in the cold.

Monomeric and dimeric species persisted at 0 °C and were depleted after warming to 20 °C (Fig. 3C,D). The levels of non-

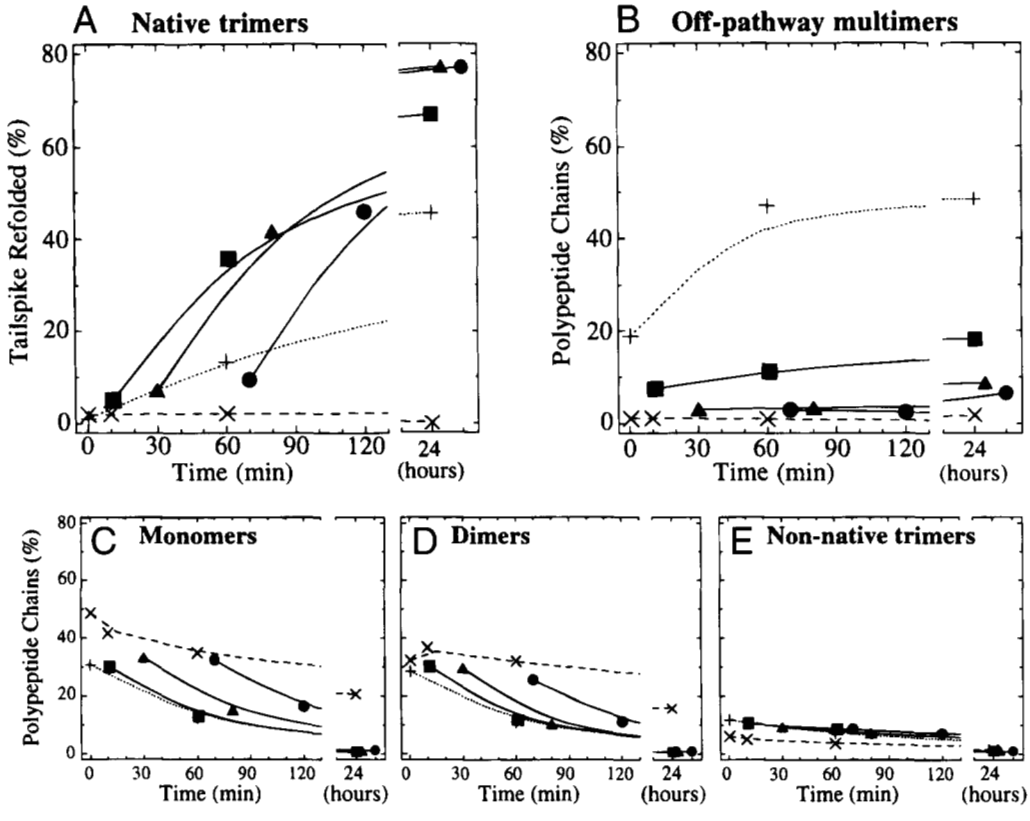

Fig. 3. Initiation of refolding in the cold suppresses aggregation. Denatured tailspike chains were equilibrated to 0 °C and refolding was initiated by dilution. Dashed line, 0–4 °C control; dotted line, 20 °C control. Samples diluted at 0 °C were shifted to 20 °C after 1 min (■), 20 min (▲), and 60 min (●). The reactions were quenched without DTT present and fractionated by native gel electrophoresis. **A:** Native tailspike trimers. **B:** Off-pathway multimers in the 9% resolving gel containing four or more polypeptide chains. **C:** Productive and off-pathway monomers. **D:** Productive and off-pathway dimers. **E:** Protrimers and off-pathway trimers.

native trimeric intermediates, including both the protrimer and $[3I^*]$, were relatively unaffected by dilution and incubation in the cold (Fig. 3E). These results suggest that under our standard refolding conditions most tailspike polypeptides become committed to either productive folding or off-pathway aggregation on a time scale of seconds.

According to the model in Scheme 1, the above results can be explained by stabilization of the thermolabile intermediate, $[I]$, at low temperature. Folding at low temperature would favor accumulation of the partially folded monomer $[I]$ over the aggregation-prone monomer $[I^*]$. These experiments do not, however, distinguish between a temperature effect on the equilibrium between $[I]$ and $[I^*]$ and destabilization of the $[I^*]$–$[I^*]$ binding interaction in the cold.

Accumulation of productive folding intermediates in the cold

Because refolding in the cold suppressed aggregation, we attempted to identify bands representing productive monomeric and dimeric intermediates by characterizing the electrophoretic species formed at low temperature. A tailspike refolding reaction was maintained at 0–4 °C for 24 h to selectively accumulate productive intermediates. One portion was quenched on ice and another portion was shifted to 20 °C for 1 h and then quenched on ice. Duplicate samples were quenched with and without DTT. Samples were analyzed by native gel electrophoresis.

The electrophoretic profile of intermediates formed in the cold is shown in Figure 4, lane 1. The sample was quenched under nonreducing conditions to trap disulfide-bonded species. Multimeric aggregation intermediates were not present, and very little native tailspike was formed. The protrimer is clearly visible at the top of the gel. Partially folded monomers migrated as a diffuse and weakly staining band (lane 1, lower band). This band is a candidate for $[I]$, $[I_b]$, or a mixture of both. At the dimer position, a single, sharp band was observed migrating just below the native tailspikes. This slow-migrating species appears to be the productive dimeric folding intermediate, not previously resolved.

In the sample shifted to 20 °C for 1 h, tailspike refolding and assembly proceeded in the absence of any detectable accumulation of off-pathway multimeric species (Fig. 4, lane 2). At the same time the levels of monomeric species, $[2I]$ dimers, and protrimers decreased, consistent with their identification as productive intermediates.

Strikingly different electrophoretic profiles were observed when refolding was quenched under reducing conditions. The effect of DTT on the apparent distribution of cold-accumulated intermediates can be seen in Figure 4, lane 3. A strong native trimer band appeared, and the productive $[2I]$ dimer and protrimer were absent. Monomers migrated as a well-resolved band (species $[I_b^{SH}]$). The sharpness of this band indicates that the chains trapped under these permissive conditions were uniformly structured. During incuba-

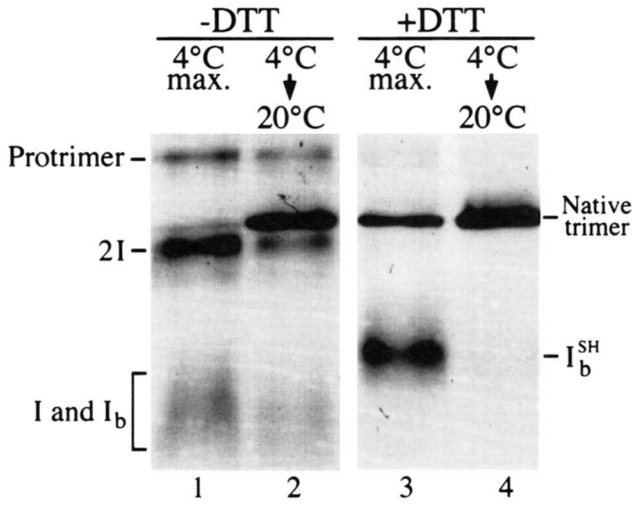

Fig. 4. Native gel electrophoresis of intermediates accumulated in the cold. Tailspike polypeptides were refolded at 0–4 °C for 24 h, and then shifted to 20 °C for an additional hour. Duplicate samples were quenched on ice with or without DTT, as indicated. Polypeptides (1.3 μg/lane) were visualized by Coomassie staining. All four lanes are from the same gel.

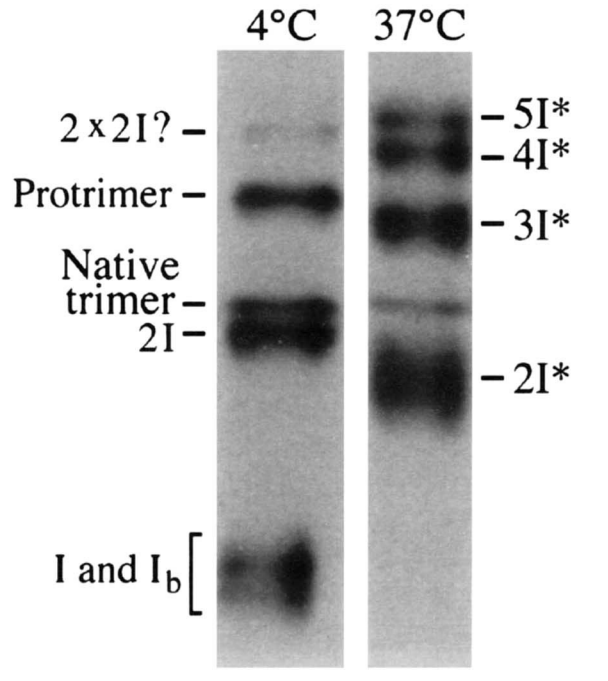

Fig. 5. Western blot analysis of intermediates generated at 0–4 °C and 37 °C. The sample diluted at 0 °C was incubated overnight at 4 °C before quenching. Refolding at 37 °C was quenched after 15–20 s and stored at 4 °C overnight. The two lanes shown are from the same gel and blot, but the images were taken from different exposures. The film on the left was exposed for 30 s and that on the right for 30 min.

tion at 20 °C, unassembled chains were converted into native trimers (lane 4). This result indicates that the monomeric band represents a productive, structured monomer [I_b] that does not act as a precursor to off-pathway aggregation (Fuchs et al., 1991).

The conversion of the disulfide-bonded protrimer to the native trimer is expected under reducing conditions (Fig. 4, compare lanes 1 and 3). The absence of the productive [2*I*] dimer from reduced samples suggests that this species is also disulfide bonded. The simplest explanation for the appearance of structured monomers under reducing conditions is that they were released from [2*I*] dimers upon reduction of one or more interchain disulfide bonds.

Identification of both productive folding and aggregation intermediates by Western blot analysis

Having identified the productive folding intermediates, we shifted conditions to re-examine the off-pathway intermediates. A tailspike refolding reaction was initiated at the nonpermissive temperature of 37 °C to populate and trap off-pathway aggregation intermediates. Samples were quenched on ice by mixing with electrophoresis sample buffer without DTT and analyzed by native gel electrophoresis. Multimerization proceeded rapidly at 37 °C and, within seconds, resulted in depletion of off-pathway multimers to levels below the sensitivity of detection by silver staining.

To increase sensitivity, a nondenaturing Western blot protocol developed by Speed et al. (1997) was used in conjunction with enhanced chemiluminescence. Tailspike polypeptides were immunoblotted with monoclonal antibodies that recognize intermediates on both folding and aggregation pathways (Friguet et al., 1990). When Tween-20 is omitted from the solution used to wash the membranes, as was done here, the monoclonal antibodies also bind to the native tailspike, which provides a convenient internal standard.

A control refolding reaction was incubated at 0–4 °C to selectively accumulate productive folding intermediates for comparison. Partially folded monomers, the [2*I*] dimer, the protrimer, and the native trimer were all recognized by the antibodies (Fig. 5, left). An additional minor multimeric species was detected at the top of this lane, and may correspond to a tetramer of two [2*I*] dimers (2 × [2*I*]).

The same sample was diluted at 37 °C and quenched within 20 s by chilling (Fig. 5, right). The aggregation intermediates predominated under these restrictive conditions. No monomeric polypeptide chains were trapped, indicating that monomeric aggregation precursors self-associated rapidly. The productive partially folded [2*I*] dimer and protrimer were absent as expected. The off-pathway dimer, [2*I**], migrated faster than the productive dimer in the 4 °C control. The off-pathway trimer, [3*I**], also migrated faster than its productive counterpart, the protrimer. The off-pathway multimers, [4*I**] and [5*I**], were resolved at the top of the blot. Multimers containing six or more polypeptide chains did not transfer efficiently in the nondenaturing blotting procedure.

The trapping of high-mobility conformations of dimeric and trimeric species following dilution at restrictive conditions (37 °C, 0.8 M urea) supports their identification as aggregation intermediates. The additional presence of off-pathway tetramers and pentamers is consistent with a model in which fast-migrating dimers and trimers act as precursors to larger multimers on the same pathway.

Discussion

Suppression of aggregation in the cold

Off-pathway multimerization of tailspike polypeptides was suppressed by a transient incubation in ice-cold buffer prior to further incubation at 20 °C. Incubation for 20 min on ice was sufficient to

achieve maximum suppression, resulting in a refolding yield at 20 °C approaching 80%. Xie and Wetlaufer (1996) first described the temperature-shift procedure as a method to suppress aggregation of bovine pancreatic carbonic anhydrase during in vitro refolding at high protein concentrations. One implication of these findings is that high refolding yields of recombinant proteins may be possible without adding undesirable excipients to facilitate folding and without diluting to impractically low protein concentrations. Prolonged refolding at low temperatures may also be unnecessary in many cases.

The first folding intermediate indicated in Scheme I is the thermolabile partially folded monomer [I]. Our data are consistent with earlier models that proposed that as temperature increases [I] converts to the off-pathway aggregation precursor [I^*] (Haase-Pettingell & King, 1988; Mitraki et al., 1991a; Brunschier et al., 1993; Speed et al., 1996). During refolding in the cold, the [I] $\leftrightarrow$ [I^*] equilibrium shifts strongly to the left.

Productive folding in the cold

Incubation at 0 °C suppressed off-pathway association of tailspike polypeptide chains but did not block early stages of productive folding. The folding intermediates that accumulated in the cold assembled efficiently into native trimers upon warming. Thus, the tailspike polypeptide chain proceeded down the folding pathway in the cold to a conformation past the thermolabile intermediate [I] to an assembly-competent monomer [I_b]. This assembly-competent monomer did not act as a precursor to off-pathway aggregation and exhibited a strong tendency to form dimers.

In vivo, the bacterial chaperonin GroEL/ES protects the partially folded forms of many proteins against aggregation without blocking folding (Ellis & Hartl, 1996; Fenton & Horwich, 1997). The temperature-shift procedure used here may suppress the formation of off-pathway species that display nonnative surfaces of the type recognized by GroEL (Mendoza et al., 1992; Brazil et al., 1997). This is consistent with results showing that GroEL binds tailspike monomers at high temperatures but not in the cold (Brunschier et al., 1993).

Seckler and co-workers (Fuchs et al., 1991; Brunschier et al., 1993; Danner & Seckler, 1993) have characterized the multiphasic refolding of tailspike polypeptide chains in vitro. A slow phase detected by circular dichroism and fluorescence spectroscopy was interpreted as corresponding to folding of monomeric chains to a structured conformation both resistant to aggregation and competent for assembly. This structured monomeric intermediate was designated [M] and has been referred to here as [I_b]. The results in Figure 4 indicate that [I_b] accumulates on ice and rapidly assembles into native trimers upon warming. Xie and Wetlaufer (1996) proposed the existence of an analogous aggregation-resistant intermediate to explain the suppression of carbonic anhydrase aggregation using a similar temperature-shift procedure.

Productive and off-pathway subunit association

Two lines of evidence mentioned above indicate that formation of the parallel β-coil domain precedes assembly. First, the prevalence of tsf mutations in the β-coil suggests that the stability or formation of this domain is a prerequisite for assembly (Haase-Pettingell & King, 1997). Second, spectroscopic analysis revealed near-native levels of β-sheet structure in a monomeric tailspike intermediate (Fuchs et al., 1991). Stabilization of the elongated β-coil domain in a single-chain intermediate is consistent with the mobility shift in the direction of increased size observed for the productive dimer and protrimer (2I and Pt in Fig. 5).

The intertwined hybrid β-coil structure of the native tailspike (Fig. 1A) can only form after trimerization. Transient disulfide bond formation has been proposed as a mechanism to correctly align the three subunits in preparation for concerted formation of the mixed β-sheets (King et al., 1996; Robinson & King, 1997). The intertwining of subunits in the formation of the hybrid β-coil would be expected to result in a tightly folded and compact conformation relative to that of a nonintertwined trimeric folding intermediate. The productive intertwining of subunits in the formation of the native tailspike from the protrimer intermediate results in an overall decrease in hydrodynamic volume (Fuchs et al., 1991). This decrease in hydrodynamic volume almost certainly contributes to the increased electrophoretic mobility of the native trimer relative to the protrimer.

Under nonpermissive folding conditions, the thermolabile monomer [I] goes off-pathway in a step involving the partial melting or misfolding of the β-coil domain. The resulting misfolded monomer, [I^*], self-associates through specific chain–chain interactions, yielding nondisulfide–bonded [nI^*] multimers (Speed et al., 1995, 1996). One can imagine an off-pathway self-association mechanism by which unfinished or out-of-register β-sheets on one chain recruit the nascent or misfolded β-coil of a second chain.

The incorrect formation of interchain β-sheets or β-coils is analogous to the loop-sheet polymerization model describing the aggregation of α1-antitrypsin. According to this model, aggregation is driven by the off-pathway formation of mixed β-sheets (Lomas et al., 1992). The interchain swapping of structural elements has also been proposed to drive off-pathway multimerization of other proteins. The aggregation of bovine growth hormone during in vitro refolding involved the incorrect interchain association of α-helices (Brems, 1988). Eisenberg and co-workers have described several examples of off-pathway domain-swapping (Bennett et al., 1995). Finally, the ordered fibrils characteristic of Alzheimer's and other amyloid diseases appear to be composed of polymeric β-sheets (Blake & Serpell, 1996).

Role of transient disulfide bond formation

During folding, a subset of cysteines form interchain disulfide bonds to yield the protrimer intermediate. These are then reduced in the formation of the native trimer (Robinson & King, 1997). Analysis of tailspike refolding at different redox potentials indicated that productive folding was dependent on disulfide bond formation (Robinson & King, 1997). Evidence for the essential involvement of cysteines in tailspike folding and assembly came first from thiol modification studies (Sather & King, 1994). It is not clear at what stage or in which intermediates the Cys thiols first become competent to participate in disulfide-bond formation.

The productive dimeric species referred to as [2I] was only present under nonreducing conditions and appears to be a disulfide-bonded dimer. The [2I] dimer dissociated into structured monomers when exposed to DTT. This result demonstrates that productive monomers have limited affinity for self-association through strictly noncovalent interactions. In contrast, noncovalent chain–chain interactions in off-pathway multimers are irreversible under refolding conditions.

The data do not exclude the possibility that the [2I] dimer is a nonproductive species formed by off-pathway disulfide formation.

Whether on-pathway or not, the data show that the $[2I]$ dimer is stabilized by disulfide bonds between structured, aggregation-resistant monomers.

Productive dimeric and trimeric intermediates to the native tailspike were distinguishable from the corresponding off-pathway intermediates by three properties: decreased electrophoretic mobility, relatively sharp electrophoretic bands, and stabilization by interchain disulfide bonds. These results suggest that the productive folding intermediates are less globular, more structured, and disulfide bonded with respect to off-pathway aggregation intermediates.

Model of tailspike refolding and aggregation

The results reported here support a more detailed model for tailspike folding, partitioning, and assembly outlined in Scheme 2.

$$\begin{array}{l} [I_b] \rightarrow [I_b^{S-}] \rightarrow [I^{SS}I] \rightarrow [Pt^{SS}] \rightarrow N^{SH} \\ \quad \uparrow \text{ low temperature; su mutations} \\ U \rightarrow\rightarrow [I] \\ \quad \uparrow\downarrow \text{ high temperature; tsf mutations} \\ [I^*] \rightarrow [2I^*] \rightarrow [3I^*] \rightarrow\rightarrow\rightarrow [nI^*]. \end{array} \quad (2)$$

A simple interpretation incorporated into the model is that completion of the β-coil domain marks the formation of the aggregation-resistant monomer $[I_b]$, as proposed by Fuchs et al. (1991). Aggregation is suppressed in the cold both by inhibition of the off-pathway step $[I] \rightarrow [I^*]$ and also by further folding of $[I]$ to $[I_b]$. This species then undergoes a conformational change to $[I_b^{S-}]$, where the $S-$ superscript indicates the presence of one or more side-chain thiolate anions. This single-chain species is thus competent for intermolecular disulfide bonding involving one or more Cys residues in the C-terminal sequence (Sather & King, 1994). Activation of $[I_b]$ for disulfide-mediated assembly may be coupled to β-coil formation; however, because β-coil formation and disulfide-bond formation involve separate domains, the two processes are presented as consecutive steps in the model. The resulting $[2I]$ dimer is represented in Scheme 2 as the disulfide-bonded species $[I^{SS}I]$, since its mobility was altered by exposure to DTT. Reduction of disulfides in the partially folded protrimer allows conversion to the thermostable native tailspike, N^{SH}.

Materials and methods

Tailspike expression and purification

Bacteriophage P22 ($5^-/13^-$) was propagated on *S. typhimurium* strain DB7155. Tailspike protein was obtained from cultures of *S. typhimurium* strain DB7136 grown at 30 °C and infected with P22 particles (King & Yu, 1986). These phage carry an amber mutation in P22 gene 5, which encodes the coat protein. In the absence of coat protein, the tailspike protein does not assemble into phage particles and accumulates in the cytoplasm. Tailspike protein was purified from the soluble fraction of culture lysates by ammonium sulfate precipitation (40%), anion-exchange chromatography (DE-52, Whatman), and hydroxyapatite chromatography (Bio-Gel HTP, Bio-Rad) as described (King & Yu, 1986; Fuchs et al., 1991). The purified protein was concentrated by precipitation in 40% ammonium sulfate. Residual ammonium sulfate was removed by dialysis against 50 mM Tris-HCl (pH 7.6), 25 mM NaCl, and 2 mM EDTA (buffer B). The concentration of tailspike protein was determined based on an absorbance value of 1.0 at 278 nm for a 1 mg/mL solution (Sauer et al., 1982).

Denaturation and refolding

Native tailspike was denatured in acid-urea and refolded by dilution as described (Speed et al., 1995) with modifications. Phosphate buffer was replaced with citrate and Tris buffer systems to allow uranyl acetate staining for analysis by electron microscopy (work in progress). Denaturation was initiated by addition of 32.5 μL of 8 M urea-citrate (pH 3.0) to 100 μg of native tailspike protein in 17.5 μL of buffer B. After incubation for 1 h at room temperature, samples were equilibrated to the refolding temperature in a circulating water bath for at least 10 min. Refolding was initiated by addition of 950 μL of equilibrated refolding buffer (0.58 M urea, 100 mM Tris (pH 7.6), 1 mM EDTA). Refolding was quenched by mixing two volumes of sample with one volume of ice-cold electrophoresis sample buffer (29% glycerol, 14 mM Tris, 109 mM glycine, 0.1% Bromphenol blue ±50 mM dithiothreitol).

Dithiothreitol (DTT; Cleland's reagent) was obtained from Calbiochem (La Jolla, California). Stock solutions of the reductant (1 M) were prepared in deionized water and stored in aliquots at −20 °C. The DTT solution (or deionized water) was added last to complete the electrophoresis sample buffer no longer than 90 min before use and stored on ice. Quenched samples were stored on ice prior to electrophoresis at 4 °C. The time of incubation in sample buffer (±DTT) necessarily varied for different samples. Samples in both Figures 2 and 4 were collected over time courses of 60 min and were thus incubated in the sample buffer for 20–80 min before electrophoresis. In Figure 3, samples were quenched by mixing with ice-cold sample buffer at time points up to 120 min and electrophoresis was started by 140 min. Samples in Figure 3 that were collected after 24 h incubated in sample buffer for 20–80 min before electrophoresis. Electrophoresis of the low temperature sample in Figure 5 started within 20 min after quenching, and electrophoresis of the high temperature sample in Figure 5 started 24–25 h after quenching.

Native gel electrophoresis and densitometry

Native gel electrophoresis was performed in a 4 °C cold room according to Speed et al. (1995). The resolving and stacking gels contained 9 and 4.3% acrylamide, respectively. Coomassie-stained gels were analyzed using a Molecular Dynamics Personal Densitometer and ImageQuant software. The intensity of Coomassie-stained bands containing native wild-type tailspike protein was linear. Refolded tailspike protein as well as monomeric tailspike polypeptides and non-native multimers were quantified using native tailspike standards loaded on the same gel. For convenience, the data for non-native polypeptide species are plotted in the figures as "Polypeptide Chains," with 100% corresponding to the staining intensity expected if all chains refolded into native trimers.

Western blot analysis

The procedure for nondenaturing Western blot analysis was essentially as described in Speed et al. (1997). Protein samples were fractionated by native gel electrophoresis and then transferred

to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) in Tris-Glycine buffer. Electrophoresis, transfer, and all antibody binding steps were performed at 4 °C. Blots were immunodecorated with monoclonal antibodies that recognize epitopes present only on non-native conformations of tailspike polypeptides. The selection and characterization of the four monoclonal antibodies used here (70-5, 92-3, 105-3, 124-5) are described in detail elsewhere (Friguet et al., 1990; Speed et al., 1997). The blots were developed using enhanced chemiluminescence (Amersham, Piscataway, New Jersey).

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