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Conformation of P22 tailspike folding and aggregation intermediates probed by monoclonal antibodies

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Abstract

The partitioning of partially folded polypeptide chains between correctly folded native states and off-pathway inclusion bodies is a critical reaction in biotechnology. Multimeric partially folded intermediates, representing early stages of the aggregation pathway for the P22 tailspike protein, have been trapped in the cold and isolated by nondenaturing polyacrylamide gel electrophoresis (PAGE) (Speed MA, Wang DIC, King J. 1995. *Protein Sci* 4:900–908). Monoclonal antibodies against tailspike chains discriminate between folding intermediates and native states (Friguet B, Djavadi-Ohaniance L, King J, Goldberg ME. 1994. *J Biol Chem* 269:15945–15949). Here we describe a nondenaturing Western blot procedure to probe the conformation of productive folding intermediates and off-pathway aggregation intermediates. The aggregation intermediates displayed epitopes in common with productive folding intermediates but were not recognized by antibodies against native epitopes. The nonnative epitope on the folding and aggregation intermediates was located on the partially folded N-terminus, indicating that the N-terminus remained accessible and nonnative in the aggregated state. Antibodies against native epitopes blocked folding, but the monoclonal directed against the N-terminal epitope did not, indicating that the conformation of the N-terminus is not a key determinant of the productive folding and chain association pathway.

Keywords: aggregation; bacteriophage P22; epitope; folding intermediates; in vitro refolding; inclusion body; nonnative; protein folding; tailspike protein

The formation of inclusion bodies in vivo or insoluble aggregates in vitro occurs by the polymerization of folding intermediates (Mittraki & King, 1989; DeBernardes-Clark & Georgiou, 1991; Wetzel, 1994). At the junction between the productive folding and aggregation pathways, the folding reaction is in kinetic competition with the aggregation of the partially folded species. During folding, certain sites on the surface of the folding intermediates may cause the polypeptide chains to be susceptible to self-association (Zettlmeissl et al., 1979; Brems et al., 1986; Cleland & Wang, 1991).

It has been difficult to obtain structural data on insoluble aggregated states of polypeptide chains. For solid phase aggregates, FTIR analysis of interleukin-1 β inclusion bodies indicated nativelike struc-

ture (Oberg et al., 1994), whereas β -lactamase inclusion bodies contained an increased amount of nonnative β -sheet structure and less α -helical content (Przybycien et al., 1994). X-ray scattering and solid-state NMR on β -amyloid fibrils support β -sheet models for chain conformation in the amyloid filaments (Sun et al., 1994).

Although little structural analysis has been performed previously on soluble aggregation intermediates, antibodies have been used to probe the structure of productive folding intermediates formed either in vitro or in vivo. Antibodies directed against specific domains of native bovine serum albumin were used to determine that refolding occurred progressively from the C-terminus to the N-terminus (Chavez & Benjamin, 1978). Monoclonal antibodies (Mabs) against the β_2 subunit of native tryptophan synthase were used to identify nativelike epitopes on early folding intermediates during in vitro refolding (Blond & Goldberg, 1987). In vivo folding and trimerization of influenza hemagglutinin within the endoplasmic reticulum was followed by similar immunoreactivity experiments using Mabs which distinguished various folding intermediates (Braakman et al., 1991). Likewise, sets of monoclonal antibodies against native and nonnative P22 tailspike chains have been used to monitor the folding of tailspike chains both in vitro and in vivo (Friguet et al., 1990; Friguet et al., 1994).

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Abbreviations: anti-I, anti-nonnative (“intermediate”) tailspike antibodies; anti-N, anti-native tailspike antibodies; ELISA, enzyme-linked immunosorbent assay; MAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TBS, tris buffered saline.

The presence of such MAbs in the refolding buffer can also affect the progress of refolding and aggregation reactions. Addition of MAbs against the native form of the S-protein of RNase enhanced the refolding yield of reduced S-protein by 3.6-fold (Carlson & Yarmush, 1992). Likewise, MAbs against native carboxypeptidase A assisted in the refolding of partially heat-denatured carboxypeptidase A in a manner analogous to molecular chaperone interactions (Solomon & Schwartz, 1995). The presence of MAbs raised against certain fragments of the β -amyloid peptide (residues 8–17 and 1–28) inhibited amyloid fibril formation in vitro (Solomon et al., 1996). In these studies, it is unknown whether the antibody binds to the soluble native peptide or an alternate amyloidogenic intermediate.

The folding and aggregation pathway of P22 tailspike endorhamnosidase has been extensively studied both *in vivo* and *in vitro* (Goldenberg et al., 1983; Haase-Pettingell & King, 1988; Danner & Seckler, 1993). The trimeric native protein consists of five structural domains (Fig. 1A, B). The N-terminus is the binding site to the phage head, but the tailspike protein that was crystallized had this domain deleted (residues 1–108). The main body of the tailspike (residues 143–540) is a parallel β -coil, with intramolecular β -sheet contacts forming between successive rungs of the 13-stranded coil (Steinbacher et al., 1994). The dorsal fin domain (residues 197–259) shields the side of the β -coil. The beta-sheet regions adjacent to the β -coil (residues 541–619) interdigitate and form intermolecular β -sheet contacts. The chains separate (residues 620–666) to form C-terminal β -sheets which are not tightly packed against one another. The conformation of the partially folded intermediates is not known, but is likely to have substantial β -sheet content. The monomer depicted in Figure 1A is the monomeric polypeptide chain within the native trimer and does not represent a proposed structure of the folding intermediate.

Along the productive folding pathway within cells (Fig. 1C), the monomeric polypeptide chain forms a single-chain folding intermediate which then associates to form a trimeric folding intermediate followed by formation of the thermostable native trimer (Goldenberg & King, 1982). At higher temperatures the monomeric folding intermediate associates into the aggregated inclusion body state (Haase-Pettingell & King, 1988).

Seckler et al. (1989) described conditions for the *in vitro* refolding of denatured tailspike chains. Fuchs et al. (1991) have followed the *in vitro* refolding of the polypeptide chains to monomeric folding intermediates by monitoring tryptophan fluorescence and circular dichroism, and the later stages by HPLC and gel electrophoresis. The *in vitro* folding pathway of the tailspike protein corresponds to the *in vivo* folding reaction, having similar folding intermediates and folding kinetics (Fuchs et al., 1991; Danner & Seckler, 1993). The final stage of refolding is a unimolecular reaction representing the conversion of the protrimer folding intermediate to the native trimer (Danner & Seckler, 1993). At higher chain and residual urea concentrations the aggregation pathway becomes predominant *in vitro* (Mitraki et al., 1993).

Both productive folding intermediates and off-pathway aggregation intermediates that form during *in vitro* refolding can be trapped and resolved by nondenaturing gel electrophoresis, as shown in Figure 2 (Speed et al., 1995). The native tailspike trimer migrates as a well-defined band on a nondenaturing gel. A ladder of aggregation intermediates is also resolved as a sequential set of broad bands. The native trimer migrates slower than the dimer aggregate, and the protrimer folding intermediate migrates to a position at the trailing edge of the trimer aggregate.

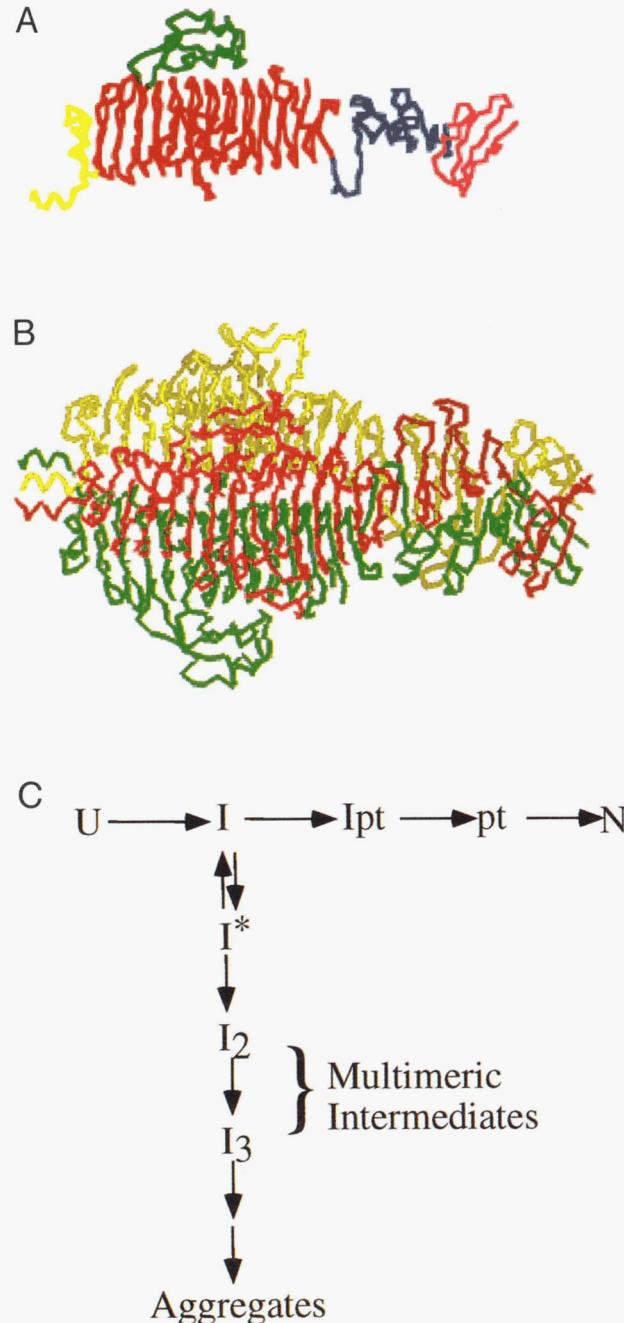


Fig. 1. **A:** Ribbon diagram of monomeric subunit within native tailspike trimer, with the N-terminus (residues 113–142) shown in yellow, the β -coil domain (residues 143–540) in red, the dorsal fin (residues 197–259) in green, the caudal region (541–619) in blue, and the C-terminus (residues 620–666) in magenta. **B:** Ribbon diagram of native trimer with each monomeric subunit represented by a different color. **C:** In vitro refolding and aggregation pathways for P22 tailspike chains. Unfolded polypeptide chains (U), partially folded intermediate (I), protrimer intermediate (I^{pt}), protrimer (pt), and native trimer (N). The off-pathway intermediate (I^*) and multimeric aggregation intermediates along the aggregation pathway lead to insoluble aggregates.

Aggregation intermediates of P22 tailspike polypeptide chains are nonnative in their biophysical properties, such as thermolability and sensitivity to SDS and protease (Speed et al., 1995). These aggregation intermediates form by the noncovalent association of

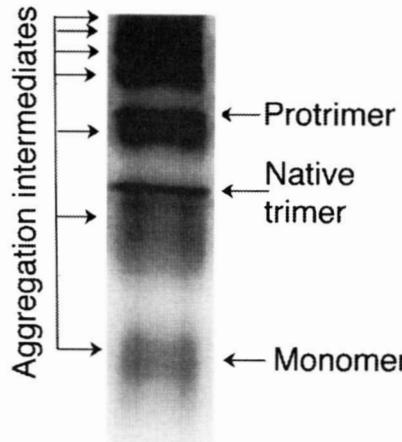


Fig. 2. Nondenaturing gel electrophoresis used to isolate multimers formed during in vitro refolding and aggregation. Purified native P22 tailspike was denatured in 5 M urea, pH 3, for ≥45 min at room temperature. Refolding was performed by 20× dilution to final conditions of 100 µg/mL protein, 20°C, and 0.8 M urea in 40 mM sodium phosphate buffer, pH 7.6. After 15 min of refolding, a sample was withdrawn and combined with cold sample buffer on ice. 1.3 µg of protein was loaded onto a 9% acrylamide nondenaturing gel and electrophoresed for 4 h at 4°C. The protein bands were visualized by silver staining.

a monomeric folding intermediate rather than by aggregation of nativelike trimers. In this paper, we further characterize the conformation of tailspike aggregates and folding intermediates and determine whether these intermediates display nativelike structural epitopes.

Over thirty temperature sensitive folding (tsf) mutations in phage gene 9 have been isolated and characterized which interfere with the in vivo and in vitro folding of the P22 tailspike protein (King et al., 1996). These single amino acid substitutions cause an early folding intermediate to shift from the productive folding pathway onto the aggregation pathway at restrictive temperatures of 38–42°C (Goldenberg & King, 1981). Global suppressor mutations at two sites in the center of the chain (Val331Ala and Ala334Val) correct the tsf folding defects by inhibiting the aggregation reaction of the tailspike intermediates both in vivo and in vitro (Mitraki et al., 1991; Lee et al., 1991; Mitraki et al., 1993; Beißinger et al., 1995). All of the tsf mutations occur in the center of gene 9 between residues 150 and 500 in the β -coil domain (Villafane & King, 1988; Haase-Pettingell & King, 1997). Structural analysis of the native tailspike suggests that tsf mutations may cause the misfolding of the β -coil domain in the monomeric folding intermediates (King et al., 1996).

Monoclonal antibodies against tailspike polypeptide chains have been used to study the folding pathway both in vivo and in vitro (Friguet et al., 1994). These monoclonals were originally isolated from hybridomas derived from mice immunized with native tailspike. Upon screening the purified monoclonals by ELISA competition assays, a set of 7 antibodies reacted with native tailspike protein and 4 antibodies reacted with thermally denatured tailspike (Friguet et al., 1990). Friguet et al. (1994) showed that monoclonal antibodies against nonnative chains reacted with early folding intermediates, and the anti-native MAbs reacted with the protrimer intermediate and native protein formed during in vitro refolding of purified tailspike chains. As a probe for the in vivo folding path-

way, refolding reactions performed in crude extracts revealed that the anti-nonnative MAbs recognized structured folding intermediates still bound to the ribosome, suggesting that the initial steps of in vivo folding occur while the polypeptide chain is ribosome-bound (Friguet et al., 1994). In this study, we used these monoclonal antibodies to examine the relationship between intermediates along the productive folding pathway and those species associating along the aggregation pathway of P22 tailspike polypeptide chains. This involved refining the Western blotting procedure to avoid denaturation during transfer.

Results

A nondenaturing Western blot procedure

In the standard Western blotting procedure, the conditions used to transfer polypeptide chains from the separating gel to the surface of the membrane are denaturing for many proteins. A nondenaturing Western blot procedure was developed to ensure that the folding and aggregation intermediates did not undergo a conformational change during the procedure. The PVDF transfer membrane was pre-wet with methanol in order to enable macromolecules in the aqueous phase to bind to the hydrophobic surface of the membrane. The membrane was then rinsed repeatedly with tris-glycine buffer to remove all the methanol before use. The electrotransfer solution was changed to a 20 mM Tris, 150 mM glycine buffer without the standard 20% methanol and 0.1% SDS. In addition, both the electrotransfer and Western blot incubation steps were performed at 4°C to maintain the conformation of the aggregation intermediates. Bands were visualized by enhanced chemiluminescence.

Though nondenaturing buffers and low temperatures used in the gel electrophoresis and Western blotting procedures were chosen to minimize denaturation of the folding and aggregation intermediates, minor conformational changes could occur during these procedures. However, the results of the Western blotting studies were consistent with additional data involving refolding tailspike in the presence of antibodies, as described below.

Probing the conformation of aggregation intermediates

To characterize the epitopes of aggregation intermediates, the reactivity of the aggregates with a set of monoclonal antibodies was screened by nondenaturing Western blotting. The seven anti-native tailspike MAbs, abbreviated as "anti-N," were designated 33, 51, 54, 155, 175, 219, 236 in the original MAb characterization studies (Friguet et al., 1990). The four anti-nonnative tailspike MAbs were designated 70, 92, 105, and 124. We refer to these as "anti-I" MAbs which recognize nonnative intermediates. These antibodies were tested for reactivity with native tailspike and refolding tailspike (100 µg/mL protein, 20°C, 0.8 M urea, 40 mM sodium phosphate buffer, pH 7.6, 0.5 min after dilution). The refolding conditions were chosen to generate multimeric aggregation intermediates and a minor fraction of protrimer and native protein. The samples of purified native protein and refolding tailspike were loaded in triplicate onto a nondenaturing gel (9% acrylamide) and electrophoresed for 4 h in the cold.

The silver stained gel (Fig. 3A) showed a distribution of folding and aggregation intermediates. Since the sample was taken immediately after dilution, a minimal fraction of tailspike had refolded into the native trimer. The majority of polypeptide chains were in the monomer, dimer, and trimer association states, with some higher ordered aggregates seen faintly at the top of the gel.

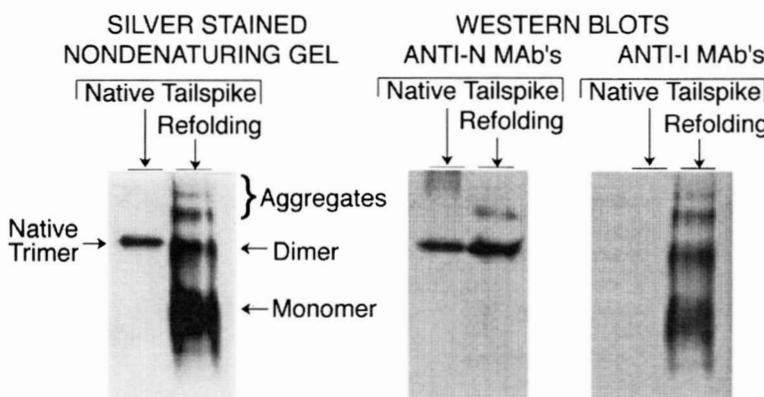


Fig. 3. Screening of MAbs for reactivity against native tailspike and in vitro refolding tailspike. Refolding was performed at 100 µg/mL, 0.8 M urea, in 40 mM phosphate buffer, pH 7.6, at 20 °C, and aliquots of sample were taken 0.5 min after dilution. 0.3 µg of purified native protein and 2 µg of refolding and aggregating tailspike were loaded onto a 9% acrylamide nondenaturing gel and electrophoresed for 4 h at 4 °C. Bands were visualized by silver staining (A) and Western blot using MAbs against native tailspike (anti-N MAbs, panel B) and against nonnative tailspike chains (anti-I MAbs, panel C).

The reactivity of anti-native tailspike MAbs with the folding and aggregation intermediates and purified native protein is shown in Figure 3B. Anti-native tailspike MAbs (anti-N) recognized the purified native tailspike and the fraction of tailspike chains which refolded into the native conformation. The anti-N MAbs did not bind to the ladder of aggregation intermediates. In addition, the protrimer folding intermediate, migrating with an electrophoretic mobility similar to the trimer aggregate, was detected by anti-N MAbs. This result indicated that the protrimer intermediate displayed the nativelike epitope recognized by the anti-N MAbs.

The reactivity of anti-intermediate (anti-I) tailspike MAbs is shown in Figure 3C. The anti-I MAbs reacted with the aggregates and not with the native species. The distribution of multimeric intermediates was similar to that of the silver stained bands. The higher ordered multimers at the top of the gel appeared to display

antigenic reactivity similar to the early multimeric intermediate precursors. Since there was no apparent cross reactivity of the anti-I MAbs with the native tailspike protein, the multimeric aggregation intermediates displayed epitopes which were nonnative. This suggests that an early folding intermediate lacking native epitopes was susceptible to aggregation rather than the protrimer folding intermediate.

Screening each of the MAbs individually gave consistent results (Fig. 4). Certain MAbs displayed a relatively high binding affinity, including anti-I MAb 92 and anti-N MAb 51. Two of the anti-N MAbs (33, 54) failed to detect the tailspike bands by Western blotting. Previous studies reported that MAb 33 had a low binding affinity ($K_D = 3 \times 10^{-9}$ M, Friguet et al., 1990), and MAb 54 (IgG₂ class) reacted poorly with the secondary Ab (Amersham horseradish peroxidase labeled goat anti-mouse IgG₁). The pres-

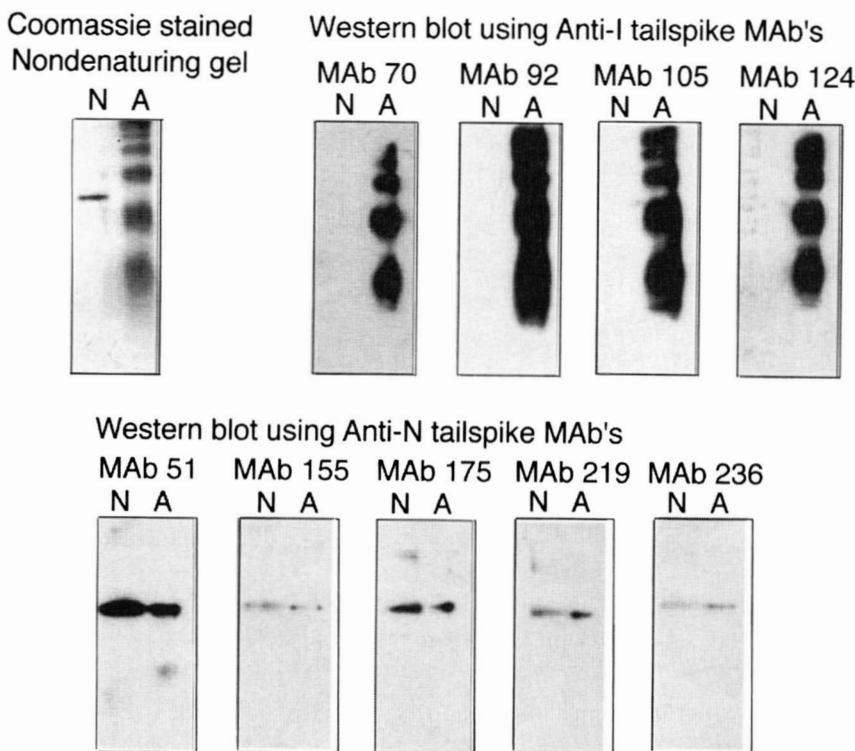


Fig. 4. Screening of individual MAbs for reactivity with native tailspike (N) and in vitro aggregation intermediates (A). Samples were obtained as described in Figure 2 and electrophoresed through a nondenaturing gel. Bands were visualized by Coomassie staining and Western blot using MAbs against nonnative tailspike chains (anti-I: 70, 92, 105, 124) and native tailspike (anti-N: 51, 155, 175, 219, 236). Numbers refer to identification code used during the original ELISA screening of the monoclonal antibodies, as described (Friguet et al., 1990).

ence of the protrimer band, which was detected by anti-N MAbs as shown in Figure 3, was not apparent in these exposure levels of the film in Figure 4.

Characterization of the nonnative epitopes

After electrotransfer using the standard denaturing buffer containing SDS and methanol, partial renaturation during the initial steps of the Western blot is often required for the chains to reacquire the structural epitopes that the antibodies recognize in the Western blot. Under standard Western blot conditions, the incubation of the blots in powdered milk and TBS solutions at room temperature allows the polypeptide chains to renature and aggregate on the surface of the membrane.

If the nonnative tailspike epitope recognized by the anti-I MAbs is expressed on denatured polypeptide chains, then renaturation on the membrane would not be necessary. To determine whether anti-I MAbs recognize denatured polypeptide chains, native and thermally denatured monomeric tailspike chains were electrophoresed through an SDS gel, and the protein bands were electrotransferred onto the PVDF membrane using the standard transfer buffer containing SDS and methanol. Western blots were then performed under standard and denaturing conditions, with 0.1% SDS in the blocking and primary MAb solutions (Fig. 5).

Under standard Western blotting conditions without SDS in the blotting solutions, anti-N reacted with the purified native tailspike trimer. Anti-I MAbs bound to the monomeric tailspike species but did not recognize the fully folded native trimer. Without the addition of SDS, the chains in this monomer band had presumably undergone aggregation during the standard Western blot procedure.

Using the modified denaturing Western blot procedure with 0.1% SDS in the blotting solutions, the tailspike polypeptide chains were maintained in the monomeric denatured state. Native tailspike protein, which is SDS resistant, displayed the native epitopes recognized by the anti-N MAbs (Fig. 5). However, the anti-I monoclonals did not efficiently recognize denatured polypeptide chains in the monomeric tailspike band. This is not merely due to a lower binding affinity of the anti-I MAbs or the SDS causing a reduced affinity. Without SDS present, the intensity of the Western blot signal

of anti-N MAbs recognizing native tailspike was comparable to the signal of the anti-I MAbs recognizing the tailspike monomer band. Any reduction in the affinity of antibodies in the presence of 0.1% SDS should be similar for both anti-N and anti-I MAbs. Therefore, the results suggest that the aggregation intermediates display unique nonnative structural epitopes recognized by anti-I MAbs.

Originally the anti-I MAbs were designated "anti-denatured" tailspike MAbs because they were screened for reactivity against thermally denatured tailspike by ELISA (Friguet et al., 1990). This screening method may have inadvertently selected for MAbs against aggregates rather than truly denatured polypeptide chains.

Location of the nonnative epitopes

Previous studies showed that 3 anti-I MAbs (92, 105, 124) disrupted binding of the tailspike to the capsid head of phage 22, which involves the N-terminus of the protein (Friguet et al., 1990). In addition, anti-I MAbs could not recognize fully folded native tailspike trimer but could bind to tailspike with a partially unfolded N-terminus. This species, which forms upon heating, electrophoreses slightly faster than the folded tailspike trimer on an SDS gel (Chen & King, 1991). Removal of the partially denatured N-terminal domain (residues 1–107) leaves the remaining molecule trimeric and detergent resistant, similar to the full-length native tailspike. In fact, the tailspike protein that was crystallized lacked residues 1–108 of the N-terminus (Steinbacher et al., 1994).

To pinpoint the location of the structural epitopes recognized by the MAbs, the antibodies against native and nonnative tailspike were screened for reactivity with the tailspike protein, truncated tailspike protein, and peptide fragments prepared by S. Miller and R. Seckler at the University of Regensburg. The tailspike species that were tested were the full length wild type protein, the N-terminal peptide fragment (residues 1–124 with a 6 His tag on the C-terminus for purification purposes), and 3 forms of truncated tailspike (residues 109–666: wt, Asp392Asn, and Asp395Asn). A fraction of these samples was boiled for 3 min to obtain monomeric polypeptide chains. The samples were electrophoresed through an SDS gel, the protein was electrotransferred to a membrane, and a standard Western blot was performed using anti-I and anti-N MAbs (Fig. 6).

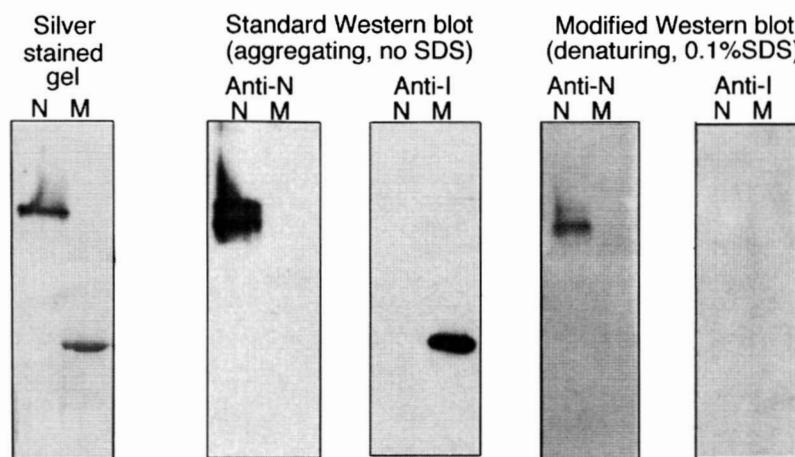


Fig. 5. Determining whether anti-I MAbs recognize denatured polypeptide chains (0.1% SDS) or aggregated tailspike. Native tailspike (N) and thermally denatured monomeric tailspike (M, boiled for 3 min) were electrophoresed through an SDS gel. Bands were visualized by silver staining and by Western blotting with MAbs against native tailspike (anti-N) and nonnative tailspike chains (anti-I) using either the standard Western blotting procedure or modified protocol with 0.1% SDS in the blocking and MAb solutions.

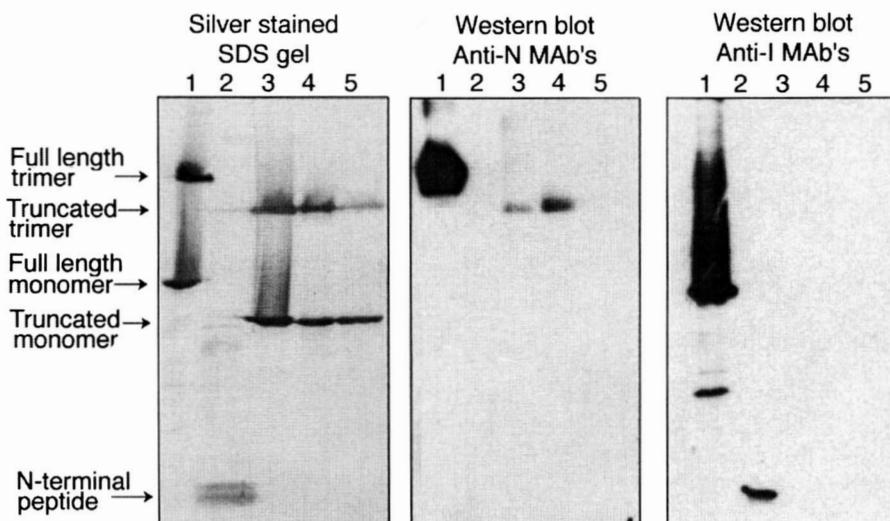


Fig. 6. Locating the binding site of anti-I MAbs to the N-terminus of tailspike peptide chains. Native tailspike (N) and thermally denatured monomeric tailspike (M, boiled for 3 min) were electrophoresed through an SDS gel. The samples were full chain wildtype (wt) tailspike (lane 1), the N-terminal peptide fragment (residues 1–124 with 6 His tag; lane 2), truncated tailspike protein (residues 109–666) having the wt sequence (lane 3), truncated mutant Asp392Asn (lane 4), and truncated Asp395Asn (lane 5). Bands were visualized by silver staining and standard Western blotting using MAbs against native tailspike (anti-N) and nonnative tailspike chains (anti-I).

The anti-N MAbs recognized all of the native tailspike proteins that contain the main body of the β -coil (lanes 1, 3, 4, 5). This suggested that the nativelike epitope was located on the β -coil domain of the protein. However, since the reactivity of the anti-N MAbs for the truncated tailspike protein (lanes 3, 4, 5) was significantly reduced, the binding of some of the antibodies was inhibited by structural changes upon removal of the N-terminus. The N-terminal peptide fragment (residues 1–124), which was missing the β -coil, did not react with the anti-N MAbs (lane 2).

In the last panel of Figure 6, anti-I MAbs were screened for reactivity with the set of tailspike species. Anti-I MAbs recognized the monomeric (boiled) full length wt tailspike chains (lane 1) and the N-terminal peptide fragment (lane 2). However, these antibodies did not bind to the truncated tailspike chains missing residues 1–108 on the N-terminus (lane 3, 4, 5). Thus, the nonnative epitope recognized by anti-I MAbs was located in the N-terminus. This section of the tailspike protein was absent from the crystals used for structure determination (Steinbacher et al., 1994).

Nonnative epitopes on productive folding intermediates

If anti-I MAbs recognized epitopes of a productive folding intermediate, the binding of the MAbs to the folding intermediate might interfere with productive folding. To test the interaction of MAbs with productive folding intermediates during in vitro refolding, a refolding experiment was performed in the presence of the anti-tailspike antibodies. The protocol had three incubation periods for each of the three reactions taking place: incubation at 10°C for 5 min for binding the MAbs to folding intermediates, incubation at 20°C for 0.5 min to form aggregation intermediates (100 μ g/mL tailspike, 0.8 M urea, 40 mM sodium phosphate buffer, pH 7.6), and 5 \times dilution to productive folding conditions for 30 min (20 μ g/mL tailspike, 0.16 M urea, 40 mM sodium phosphate buffer, pH 7.6). The overall yield of native protein after the refolding period provided a measure of the amount of productive folding intermediates that were competent for folding. Similar results were obtained using variants of this protocol, including omission of the MAb binding period at 10°C and diluting the denatured protein directly to productive folding conditions of 20 μ g/mL tailspike. The samples were electrophoresed through both nondenaturing

and SDS gels, and bands were visualized by silver staining. A higher percentage of acrylamide (12%) was used for the separating gel to achieve sufficient separation between the native trimer and the immunoglobulin bands.

Under productive folding conditions, the anti-N monoclonals blocked native trimerization (Fig. 7a). No native trimer was seen on the SDS gel for the sample after the refolding period with anti-N present (lane 7). This suggests that anti-N MAbs bind to a productive folding intermediate and block folding. Incubation with anti-I MAbs did not block folding, as determined by the presence of the native trimer band on the SDS gel (lane 6). Since the binding of these antibodies did not interfere with productive folding, the nonnative structural epitopes recognized by the anti-I MAbs were located on either off-pathway aggregates or on a domain of productive intermediates that was not sterically prevented from folding.

Surprisingly, when the samples were electrophoresed through a nondenaturating gel, there was no native trimer band in the sample that was refolded in the presence of anti-I MAbs (lane 6, Fig. 7B). This was not due to anti-I blocking the folding reaction because native trimer was detected by SDS gel electrophoresis (lane 6, Fig. 7A). Apparently, anti-I bound to a productive folding intermediate and was incorporated into a native-like trimer, changing its electrophoretic mobility. This indicated that the aggregation intermediates contained structural epitopes in common with productive folding intermediate. The region on which the nonnative epitope is located was nonessential for folding, and data suggested that the N-terminus was the target for binding.

To confirm that the antibodies were binding to productive folding intermediates, an immunoprecipitation experiment was performed on tailspike chains after in vitro tailspike refolding in the presence of MAbs (anti-I and anti-N). MAb binding was initiated by diluting the denatured tailspike protein with MAbs in excess (20 μ g tailspike/mL, 0.8 M urea, 40 mM phosphate buffer, pH 7.6, at 10°C for 10 min). The solution was transferred to refolding conditions (20°C), and samples were taken after refolding for 30 minutes.

Immunoprecipitation of the tailspike-MAb complex confirmed that the antibodies recognized productive folding intermediates (Fig. 8). Compared to the control lane of refolding tailspike without the presence of monoclonal antibodies (lane 2), the presence of

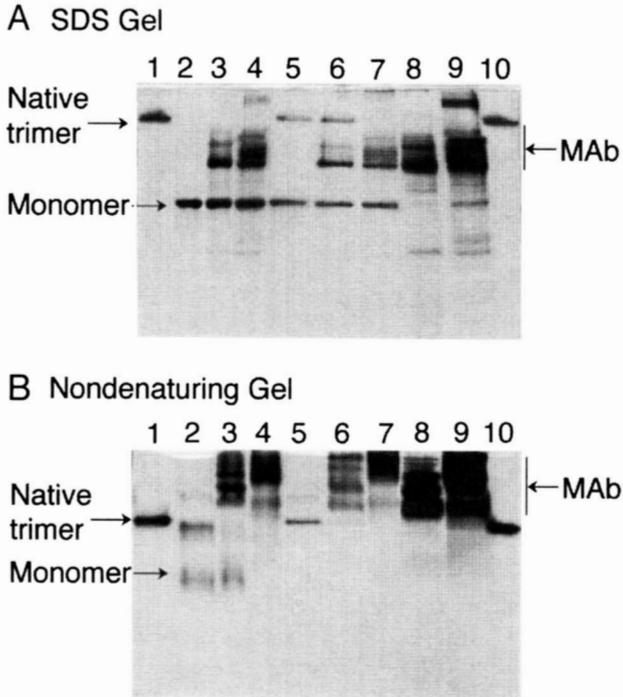


Fig. 7. In vitro refolding and aggregation in the presence of tailspike MAbs (anti-I and anti-N). MAb binding was initiated by diluting the denatured tailspike protein with MAbs in excess (100 µg tailspike/mL, 0.8 M urea, 40 mM phosphate buffer, pH 7.6) at 10°C. After 5 min, the solution was transferred to aggregating conditions at 20°C for 0.5 min, and sample #1 was taken. The solution was then diluted 5× to productive refolding conditions (20 µg tailspike/mL, 20°C, 40 mM phosphate buffer, 0.16 M urea), and sample #2 was taken at 30 min. Samples were electrophoresed through (A) SDS and (B) nondenaturing gels, and bands were visualized by silver staining. Native tailspike (lanes 1 and 10); sample 1 of aggregating tailspike without MAbs (lane 2), with anti-I MAbs (lane 3), or with anti-N MAbs (lane 4). Sample 2 after refolding period without MAbs (lane 5), with anti-I MAbs (lane 6), or with anti-N MAbs (lane 7). Controls of anti-I MAbs (lane 8) and anti-N MAbs (lane 9).

anti-I MAbs did not affect the yield of native protein, as seen on the SDS gel (lane 3). Anti-N MAbs blocked the formation of native trimer, as determined by the absence of the trimer band in lane 4. In the immunoprecipitation of refolding tailspike in the presence of anti-N MAbs, nonnative tailspike chains were found in the supernatant (lane 6) and the pellet (lane 8) in equal amounts. These fractions represent aggregation intermediates that are not recognized by anti-N MAbs in the supernatant and the productive folding intermediates in the pellet. Likewise, both the supernatant (lane 5) and pellet (lane 7) fractions of tailspike refolding with anti-I MAbs contained native and monomeric chains. However, a greater fraction of native tailspike is in the pellet, which is about equivalent to the amount of folded protein for this sample. The immunoprecipitation of native trimer which formed in the presence of anti-I MAbs suggests that these antibodies were incorporated into the native trimer. The nonnative epitope that anti-I MAbs recognized was found on productive folding intermediate as well as aggregates.

Discussion

The multimeric intermediates on the aggregation pathway contained nonnative epitopes in common with the folding interme-

diates from which they formed. These epitopes were not found on denatured polypeptide chains nor on the fully folded native tailspike. The existence of an epitope of aggregation intermediates in common with productive folding intermediates suggests that the partially folded intermediates are the precursors to the multimeric aggregation intermediates.

The model describing the anti-tailspike antibodies binding to epitopes of productive folding and aggregation intermediates is shown in Figure 9. Anti-nonnative tailspike MAbs recognize early productive folding intermediates before the junction between the folding and aggregation pathways. These nonnative epitopes are also present and exposed on the aggregation intermediates. Since anti-I MAbs do not recognize the native trimer, either the nonnative epitope is not present on the folded protein or the site is buried. During tailspike refolding, anti-I MAbs can become incorporated into the native trimer, which suggests that the epitope is located in a region that is relatively flexible. Anti-native tailspike MAbs recognize a nativelike conformational epitope on a folding intermediate apparently after the junction with the aggregation pathway (Ipt). Binding of antibodies to the nativelike epitope prevented the formation of the protrimer intermediate or native tailspike trimer.

Domains nonessential for folding

The binding of antibodies to an N-terminal epitope in a productive folding intermediate without interference with the folding reaction was unexpected. This result indicates that the N-terminus is not critical in the chain folding pathway. The N-terminal domain is not required for native trimerization (Danner et al., 1993), and may be flexible enough to accommodate the antibody complex.

Most of the MAbs against native tailspike do not react with temperature sensitive folding mutants Gly244Arg and/or Gly323Asp (Friguet et al., 1990). These mutations are located on the dorsal fin region of the main β -coil, suggesting that some of the anti-N MAbs bind to that domain. Since anti-native tailspike MAbs blocked productive folding, the nativelike epitope is a critical step in folding. This suggests that formation of the β -coil precursor is an essential folding event. Perhaps binding of the anti-N MAbs to the interface of β -coil precursor sterically hinders native trimerization.

Implications for chain conformation

The nonnative epitopes found on both the productive folding intermediates and the multimeric aggregation intermediates may represent domains involved in intermolecular association. Certain classes of aggregation epitopes may indicate sites of intermolecular helix-helix docking (Brems et al., 1986), cross- β -sheet formation (Lansbury, 1992), hydrophobic patches (Ptitsyn et al., 1990), or other intermolecular structural motifs that are similar to the nativelike intramolecular interactions (Bennett et al., 1994). Since the anti-I monoclonals reacted with the tailspike chains by nondenaturing Western blotting, the nonnative epitopes on the N-terminus of the tailspike polypeptide chain are solvent accessible and probably do not represent aggregation sites involved in interchain association.

The monoclonal reactivity of the aggregation and folding intermediates suggests that the β -coil structure is involved in aggregation. One model is that unimolecular misfolding of the β -coil and misaligned β -sheet formation within a single polypeptide chain creates sites susceptible to incorrect beta strand association. The

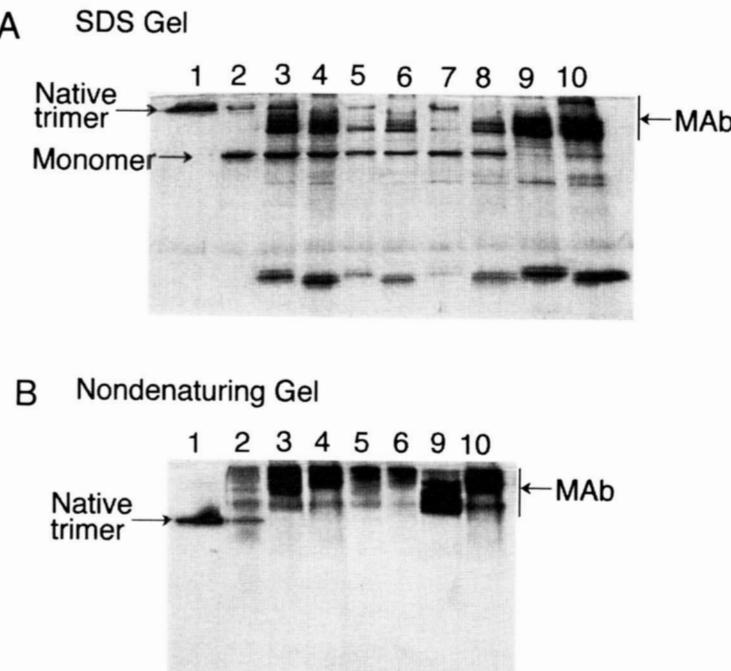


Fig. 8. Immunoprecipitation of tailspike chains after in vitro tailspike refolding in the presence of MAbs (anti-I and anti-N). MAb binding was initiated by diluting the denatured tailspike protein with MAbs in excess (20 µg tailspike/mL, 0.8 M urea, 40 mM phosphate buffer, pH 7.6, at 10°C for 10 min), and the solution was transferred to refolding conditions (20°C, 30 min). The solution was incubated with Protein-G beads at 4°C for 1.5 h while rocking gently. A sample was taken, and the remaining solution was centrifuged with a brief microcentrifuge pulse. The supernatant was saved (sup. #1), and the pellet was resuspended with SDS sample buffer. A sample (designated "pellet") was taken after another brief microcentrifuge pulse to remove the immunoprecipitated beads. Samples were electrophoresed through (**A**) SDS and (**B**) nondenaturing gels, and bands were visualized by silver staining. Native tailspike (lane 1). Refolded tailspike without MAbs (lane 2), with anti-I MAbs (lane 3), or with anti-N MAbs (lane 4). Immunoprecipitation supernatant for refolded tailspike solution with anti-I MAbs (lane 5) or with anti-N MAbs (lane 6). Immunoprecipitation pellet for refolded tailspike solution with anti-I MAbs (lane 7) or with anti-N MAbs (lane 8). Controls of anti-I MAbs (lane 9) and anti-N MAbs (lane 10). Note that the immunoprecipitation pellet samples of lanes 7 and 8 were not loaded onto the nondenaturing gel because they were resuspended in sample buffer containing SDS.

early single chain folding intermediate in the wild type pathway shows significant beta-sheet structure (Danner & Seckler, 1993). Temperature-sensitive mutations may destabilize a critical folding intermediate or slow down the correct alignment and zipping up of the β -coil, thereby affecting aggregation kinetically. Structural analysis of the suppressor mutations also suggests that the mutations may stabilize the monomeric folding intermediate (BeiBinger et al., 1995). With unimolecular misfolding as the rate limiting step, the

first order kinetics would be consistent with the weak concentration dependence of misfolding under certain refolding conditions (Danner & Seckler, 1993). An alternative model is that interdigitated β -coils form with natively aligned strand alignment. This cross- β -sheet structure would be extremely stable and consistent with the documented properties of inclusion bodies. Identifying the actual conformations involved in the off-pathway reaction will require more detailed structural studies on the associated states of the chains.

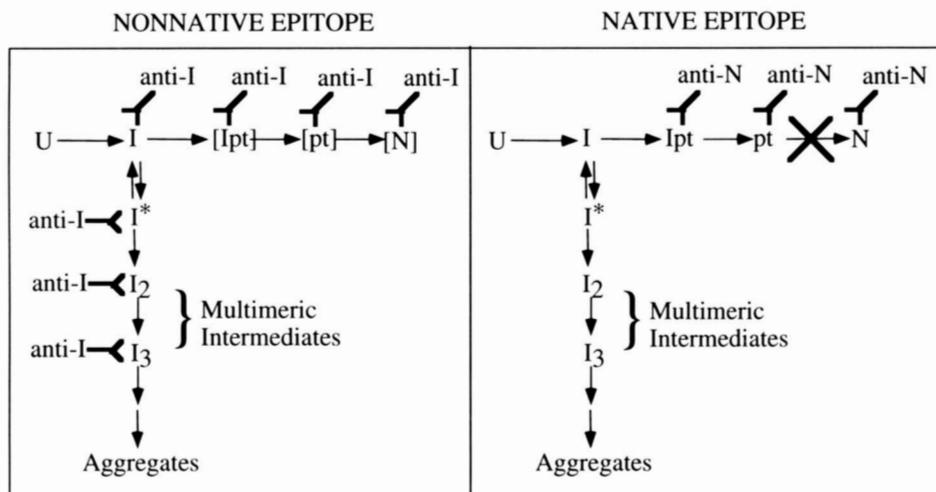


Fig. 9. Model describing the anti-tailspike antibodies binding to structural epitopes of productive folding and aggregation intermediates. Anti-nonnative tailspike MAbs (anti-I) recognize aggregation intermediates and early productive folding intermediates (I). During tailspike refolding, anti-I MAbs can become incorporated into the native trimer by binding to the productive folding intermediates. Note that the brackets represent tailspike intermediates ([Ipt], [pt], [N]) with a potentially modified conformation when complexed with the antibodies when refolding in the presence of antibodies. Anti-native tailspike MAbs (anti-N) recognize a nativelike structural epitope on a folding intermediate after the junction with the aggregation pathway (Ipt), as well as on the protrimer (pt) and native trimer (N). When refolding in the presence of anti-N, the binding of antibodies to the nativelike epitope prevents the formation of the protrimer intermediate or native tailspike trimer.

Materials and methods

Materials

P22 tailspike protein (full length wt) was produced by infecting *Salmonella typhimurium* with P22 phage as described (King & Yu, 1986; Fuchs et al., 1991) and then purified to approximately 95% purity. All experiments with the P22 tailspike used protein from the same preparation. The three forms of truncated tailspike protein (residues 109–666; wt, Asp392Asn, and Asp395Asn) and the N-terminal peptide fragment (residues 1–124 with a 6 His tag on the C-terminus for purification purposes) were produced and purified by Miller and Seckler. The monoclonal antibodies against nonnative tailspike chains (anti-I: 70, 92, 105, 124) and native tailspike (anti-N: 33, 51, 54, 155, 175, 219, 236) were produced and purified as described (Friguet et al., 1990). Numbers refer to the identification code used during the original ELISA screening of the monoclonal antibodies.

In vitro refolding

Native P22 tailspike protein was denatured for approximately 45 min in 5 M urea at pH 3. The refolding and aggregation reaction was initiated by rapid dilution (20-fold) with 40 mM sodium phosphate buffer and 0.58 M urea (1 mM EDTA, 1 mM β -mercaptoethanol, pH 7.6). The final refolding conditions were 100 μ g/mL protein and 0.8 M urea in 40 mM sodium phosphate buffer (pH 7.6) at 20 °C. Aliquots of sample were taken at various time points and rapidly transferred to preincubated tubes containing cold 3× sample buffer (0.015 M Tris, 0.12 M glycine, 3 mM dithiothreitol, 30% glycerol, bromophenol blue). These samples were immediately frozen in liquid nitrogen.

Gel electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed using a discontinuous buffer system (Andrews, 1986; Speed et al., 1995). The resolving gel contained 0.37 M Tris buffer (pH 8.9) with 3.8 mM TEMED, 3.0 mM ammonium persulfate, and 9% acrylamide. The stacking gel contained 0.07 M Tris buffer (pH 6.7) with 4.3% acrylamide, 7.5 mM TEMED, 2.5 mM ammonium persulfate. An aliquot of 30 μ L of protein solution in sample buffer (1.3 μ g protein) was loaded onto the gel for each sample. The gels were run at constant current (10 mA/gel) for 3–4 hours at 4 °C. SDS gel electrophoresis with a discontinuous buffer system was performed as described (King & Laemmli, 1971) using 7.5% or 12% acrylamide in the resolving gel. Bands were visualized by Coomassie staining, silver staining (Sather & King, 1994), or Western blotting, as described below.

Western blotting

Conformational studies on the folding and aggregation intermediates were performed using a set of monoclonal antibodies (MAbs) against native and nonnative tailspike polypeptide chains (Friguet et al., 1990). In vitro aggregation intermediates and purified native tailspike protein were isolated by nondenaturing gel electrophoresis, and a modified Western blot procedure was developed to preserve the conformation of the aggregation intermediates. The PVDF membrane was presoaked in methanol and washed repeatedly with transfer buffer to remove the solvent. The electrotransfer procedure was done in the cold (4 °C) in a nondenaturing buffer

(20 mM Tris, 150 mM glycine) without the standard 0.1% SDS and 20% methanol. The nondenaturing Western blot was also performed in the cold to ensure that the polypeptide chains did not change in conformation during the washes and antibody binding procedures.

To test for reactivity of the MAbs with fully denatured tailspike chains, a Western blot was run under denaturing conditions with 0.1% SDS in the washing solution and primary antibody steps. The positive control for this experiment was reactivity of anti-native tailspike MAbs with the native tailspike band under identical conditions.

Refolding in presence of antibodies

In vitro refolding and aggregation studies were performed in the presence of tailspike MAbs to determine whether these antibodies recognized epitopes on productive folding intermediates. The initial dilution of denatured protein was performed at 10 °C with the antibodies in the dilution buffer in order to allow the antibodies to bind to the tailspike polypeptide chains under conditions in which folding and aggregation were slow compared to antibody binding. The antibody concentration was 0.2 mg/mL and tailspike polypeptide chains at 100 μ g/mL, making the molar ratio of 1.1:1.0 with antibodies in slight excess. After a 5 minute incubation, the mixture was transferred to aggregating conditions at 20 °C (100 μ g tailspike/mL, 0.8 M urea, 40 mM phosphate buffer, pH 7.6) for 0.5 min, and a sample was taken. The solution was then diluted 5× to productive refolding conditions (20 μ g tailspike/mL, 20 °C, 40 mM phosphate buffer, 0.16 M urea), and a sample was taken after 30 min of refolding. This refolding step provided a sensitive measurement of the fraction of tailspike polypeptide chains that were productive folding intermediates.

Each of the samples was taken in duplicate and combined with the appropriate SDS or nondenaturing sample buffer. Samples were electrophoresed through SDS gel (12% acrylamide) and nondenaturing gel (9% acrylamide), and bands were visualized by silver staining. The bands visible on the nondenaturing gel represented the tailspike species that did not react with the antibodies, whereas missing bands represented the species that were prevented from being formed or were bound to the antibodies, migrating to the position of the antibody complex. The results were compared to SDS gels to determine the yield of native protein and to identify which tailspike species that were not visible on the nondenaturing gel.

Immunoprecipitation

Immunoprecipitation of tailspike chains after in vitro tailspike refolding in the presence of MAbs (anti-I and anti-N) was performed to determine whether MAbs bind to productive folding intermediates. MAb binding was initiated by diluting the denatured tailspike protein with MAbs in excess (20 μ g tailspike/mL, 0.8 M urea, 40 mM phosphate buffer, pH 7.6, at 10 °C for 10 min), and the solution was transferred to refolding conditions (20 °C, 30 min). 100 μ L of refolding solution was incubated with 100 μ L Protein-G beads at 4 °C for 1.5 hours while rocking gently. Samples were taken, combined with SDS or nondenaturing sample buffer, and stored on ice. The remaining solution was centrifuged with a brief microcentrifuge pulse, and the supernatant was saved. The pellet was resuspended with 75 μ L SDS sample buffer to free the species bound to the MAb-bead complex. A sample (designated “pellet”)

was taken after another brief microcentrifuge pulse to remove the immunoprecipitated beads. Samples were electrophoresed through SDS and nondenaturing gels, and bands were visualized by silver staining.

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