Monoclonal Antibody Epitope Mapping Describes Tailspike β-Helix Folding and Aggregation Intermediates*§

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Madhulika Jain‡§, Michael S. Evans†, Jonathan King‡, and Patricia L. Clark¶

From the †Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556 and
‡Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

There is growing interest in understanding how the cellular environment affects protein folding mechanisms, but most spectroscopic methods for monitoring folding in vitro are unsuitable for experiments in vivo or in other complex mixtures. Monoclonal antibody binding represents a sensitive structural probe that can be detected against the background of other cellular components. A panel of antibodies has been raised against Salmonella typhimurium phage P22 tailspike. In this report, nine α-tailspike antibody binding epitopes were characterized by measuring the binding of these monoclonal antibodies to tailspike variants bearing surface point mutations. These results reveal that the antibody epitopes are distributed throughout the tailspike structure, with several clustered in the central parallel β-helix domain. The ability of each antibody to distinguish between tailspike conformational states was assessed by measuring antibody binding to tailspike in vitro refolding intermediates. Interestingly, the binding of all but one of the nine antibodies is sensitive to the tailspike conformational state. Whereas several antibodies bind preferentially to the tailspike native structure, the structural features that comprise the binding epitopes form with different rates. In addition, two antibodies preferentially recognize early refolding intermediates. Combined with the epitope mapping, these results indicate portions of the β-helix form early during refolding, perhaps serving as a scaffold for the formation of additional structural features. Finally, three of the antibodies show enhanced binding to non-native, potentially aggregation-prone tailspike conformations. The refolding results indicate these non-native conformations form early during the refolding reaction, long before the appearance of native tailspike.

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¶ Present address: Dept. of Biochemistry and Biophysics, Box 22900, University of California at San Francisco, San Francisco, CA 94143-2200.

† Supported by National Institutes of Health Postdoctoral Fellowship GM19715 at Massachusetts Institute of Technology and by an award from the Clare Boothe Luce Program of the Luce Foundation at the University of Notre Dame. To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, University of Notre Dame, 251 Nieuwland Science Hall, Notre Dame, IN 46556. Tel.: 574-631-8353; Fax: 574-631-6652; E-mail: pclark1@nd.edu.

‡ Present address: Dept. of Biochemistry and Biophysics, Box 22900, University of California at San Francisco, San Francisco, CA 94143-2200.

1 The abbreviations used are: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBST, phosphate-buffered saline + Tween.

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The formation and stabilization of protein structure during folding involve the development of numerous weak, non-cova-
monella typhimurium phage P22. Tailspike is a homotrimeric protein; each subunit chain is 666 amino acid residues long, with a native structure dominated by a central 13-rung parallel \( \beta \)-helix domain (18) (Fig. 1A). The \( \beta \)-helix structural motif is typically found in proteins involved in bacterial and viral pathogenesis (19, 20) and has been proposed by Perutz et al. (21) and others (22) as a model for the \( \beta \)-strand arrangement of amyloid fibrils. Other structural features of native tailspike include an N-terminal domain that binds to the phage capsid, a “dorsal fin” subdomain that occurs as an extended loop between two consecutive rungs of the \( \beta \)-helix domain, and a C-terminal domain that includes an interdigititation region, where the three monomer chains wrap around one another to form a modified parallel \( \beta \)-prism structure (23, 24) (Fig. 1A).

Tailspike folds much more slowly than many of the small globular proteins that have been the subjects of detailed folding studies. Refolding in vitro occurs with a half-time of \( \sim 12 \) h at 10 °C (25); folding in vivo at 30 °C occurs with a half-time of \( \sim 5 \) min (26). In addition, chilling tailspike polypeptide chains to 0–4 °C arrests folding, both in vitro (27) and in vivo (26). Tailspike refolding kinetics exhibit Arrhenius-like behavior; changes in temperature therefore do not alter the tailspike refolding pathway (27, 28). The slow rate of refolding and the stability of tailspike refolding intermediates at lower temperatures mean that tailspike refolding intermediates can be monitored using experimental techniques with relatively slow time resolution. In particular, non-denaturing gel electrophoresis and high pressure liquid chromatography size-exclusion chromatography at 4 °C have successfully resolved assembly intermediates populated during the tailspike folding and aggregation pathways (25, 29–31). These studies have revealed a pathway consisting of polypeptide chain folding and assembly, followed by additional trimeric folding steps (Fig. 1D). The intertwined structure of the trimer fold develops during the post-trimerization folding steps (23, 31). The low concentration dependence of the final kinetic phase of tailspike refolding (28) indicates that the post-trimerization folding process determines the rate-limiting step for overall refolding.

Anti-tailspike mAbs were raised against native trimeric tailspike but exhibit a wide range of specificities for tailspike conformational states (12, 17, 32). ELISA competition tests have shown that whereas some anti-tailspike antibodies exhibit tight binding to the native protein (anti-N), others bind preferentially to non-native states of the protein (17). These non-native states include either denatured tailspike or tailspike refolding intermediates (anti-D and -I). In addition, an initial characterization of the epitopes for these antibodies indicated that they consist of sequence elements from a variety of locations along the polypeptide chain, including several within the \( \beta \)-helix domain (17). Additional work with three of these antibodies demonstrated that when they are added to tailspike refolding reactions cold-trapped at various times, there is differential recognition of tailspike folding intermediates (12). The antibodies also exhibit specific recognition of nascent tailspike chains tethered to Salmonella ribosomes (32).

A genetic screen has identified more than 60 tailspike mutations that confer a temperature-sensitive folding (\( tsf \)) phenotype (33). These mutations are located exclusively in the \( \beta \)-helix domain, largely on the surface of the native tailspike structure, and do not diminish the activity or stability of the native folded protein (34). The effects of the mutations are limited exclusively to the destabilization of folding intermediates at high temperature (33). Two mutations that globally suppress (\( suv \)) the \( tsf \) phenotype have also been identified (35). Hence, the genetic screen has provided a set of tailspike mutant proteins, each preserving the native state characteristics of the wild type protein, but with subtle, local changes to the surface of the folded structure. In addition, Cys–Ser mutations have been made at each of the eight wild type cysteine locations, and all eight of these mutant proteins form functional, native tailspike trimers (36).

In this report, nine anti-tailspike mAbs have been screened for binding to 15 tailspike \( tsf \), \( suv \), and site-directed mutant proteins, in order to more narrowly define the binding epitopes of these antibodies. We have also used the sensitive solution-phase ELISA competition test in order to evaluate mAb recognition of tailspike in vitro refolding intermediates. Identification of mAb binding epitopes provides a set of conformational probes specific for native and non-native structure formation within each of the tailspike structural domains. Our results indicate that the N- and C-terminal portions of the \( \beta \)-helix form early during folding and could serve as a scaffold for the formation of the final \( \beta \)-helix structure. In addition, mAb binding to non-native states plateaus very quickly in the refolding reaction, suggesting non-native conformations that lead to aggregation form early and remain unchanged as aggregation proceeds. Taken together, these results establish the utility of mAb binding to follow the folding, assembly, and aggregation of a complex protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wild type P22 tailspike and K163E, G177R, W292Q, D238S, and V331A tailspike \( tsf \) mutant proteins were produced and purified from phage-infected \( S. \) typhimurium strain DB7136 as previously described (37). Wild type tailspike was also produced, as were the G244R and E405K \( tsf \) mutant proteins, as cell lysate supernatants from cultures of \( E. \) coli overexpressing tailspike from an inducible promoter (38). Additionally, recombinant wild type tailspike and the eight Cys–Ser tailspike mutant proteins (C169S, C267S, C287S, C298S, C237S, C448S, C468S, C638S) were overexpressed in \( E. \) coli and purified as previously described (36).

Tailspike protein concentrations were determined using one of the following two methods. For purified proteins, concentrations were determined based on an absorbance of 1.0 at 278 nm for a 1 mg/ml solution (38). For tailspike proteins in cell lysate supernatants, SDS-PAGE analysis was used to quantify the amount of tailspike present in the cell lysate supernatants by comparing the density of the lysate tailspike band with bands for serial dilutions of purified native tailspike of known concentrations.

Monoclonal antibodies specific for native tailspike (anti-N: 33-2, 51-2, 155-3, 175-3, 219-2, and 236-3) and non-native tailspike chains (anti-D1: 70-5, 92-3, and 124-5) were a generous gift from Prof. Michel Goldberg (Institut Pasteur). Antibody numbering refers to the identification codes used during the original screening of the monoclonal antibodies (17). An additional anti-D antibody, 105, was not included in the current study because the large dissociation constant (6 \( \times 10^{-7} \) M) would have required prohibitively large quantities of the tailspike proteins for the binding assays (39).

**ELISA Competition Test**—Antibody binding was determined using the ELISA competition test (39, 40). The final concentration of each antibody was chosen as described previously (39, 41). The antigen (wild type or a mutant tailspike protein) was serially diluted 1:1 in PBST buffer (137 mM NaCl, 1.5 mM KH\(_2\)PO\(_4\), 7.8 mM Na\(_2\)HPO\(_4\), 2.7 mM KCl, and 0.05% (w/v) Tween 20) to obtain 10 concentrations of antigen from 20 \( \mu \)g/ml to 0.0391 \( \mu \)g/ml. Anti-tailspike mAb (250 \( \mu \)l) was added to 250 \( \mu \)l of each antigen sample and incubated at 4 °C overnight. Aliquots (100 \( \mu \)l each) of each of the competition mixtures were added in triplicate to empty wells of a 96-well plate previously coated with 1 \( \mu \)g/ml native wild type tailspike and incubated at room temperature for 30 min. The 30-min incubation time allowed a portion of the free antibody to bind to the tailspike coating the plate but did not significantly disrupt the equilibrium between bound and unbound antibody in solution (39).

After exactly 30 min of incubation in each well, the competition solutions were removed by aspiration, and the plate was washed three times with PBST buffer.

Alkaline phosphatase-linked goat anti-mouse IgG (Southern Biotechnology Associates) (100 \( \mu \)l) was added to each well at a concentration of 0.5 \( \mu \)g/ml and incubated at room temperature for 30 min. The plate was then washed three times with PBST buffer. The amount of plate-bound anti-tailspike antibody was determined by the conversion of 100 \( \mu \)l of
β-Helix Folding Monitored by Antibody Binding

p-nitrophenyl phosphate disodium hexahydrate (Sigma-Aldrich) (2 mg/ml in p-nitrophenyl phosphate buffer (1.0 M diethanolamine, 1.0 mM MgSO₄, pH 9.8)) to p-nitrophenol, as measured by the increase in absorbance at 405 nm.

The fraction of bound antibody, f, in each of the competition solutions was calculated using:

\[ f = \frac{A_f - A_0}{A_f} \]  (Eq. 1)

where \( A_0 \) is the absorbance at 405 nm of a control well incubated with antibody alone (no antigen), and \( A_f \) is the absorbance of a well incubated with an equilibrated antibody:antigen mixture (39).

The fraction of bound antibody in solution was plotted versus tailspike antigen concentration to yield a binding curve. This curve was fit to the following equation, to yield the dissociation constant (\( K_d \)) between the antibody and the antigen:

\[ f = \frac{x}{x + K_d} \]  (Eq. 2)

where \( x \) is the concentration of antigen in the competition solution.

Experiments were performed in triplicate; the S.D. was <10%.

Efficient Screening of Tailspike Mutant Proteins by Anti-tailspike mAbs—The competition ELISA test was used to screen nine anti-tailspike monoclonal antibodies for binding to 15 tailspike mutant proteins. In order to screen efficiently, a high throughput version of the assay was developed, which permitted determination of dissociation constants using only five concentrations of antigen. The high throughput assay resulted in dissociation constants that were equivalent to the values determined with 10 or more antigen concentration points (Supplementary Fig. S1, A) and permitted four antigen:antibody combinations to be assayed in triplicate on a single 96-well plate. Dissociation constants were also determined for the binding of anti-tailspike mAbs to wild type tailspike in crude cell lysate supernatants. No significant differences were observed from the dissociation constants determined using purified tailspike (data not shown). As a result, some dissociation constants were determined using tailspike tsf mutants in crude cell lysate supernatants.

In Vitro Refolding—Tailspike refolding in vitro was performed using conditions described previously (25). Acetone was used to unfold the tailspike protein; refolding was initiated by 50-fold dilution into refolding buffer at 10 °C. The final protein concentration was 20 μg/ml. Tailspike refolding was initiated at \( t = 0 \), 0.25, 0.5, 1, 1.5, 2, 3, 6, and 24 h, and all samples were allowed to refold until \( t = 24 \) h. Additional time points were used for some antibodies to ensure a complete description of the antibody binding profile during refolding. The staggered initiation of refolding eliminated the need to quench the refolding process for short refolding time points. All refolding samples were then transferred to an ice-water bath, diluted with ice-cold PBST buffer, and subjected to an ELISA competition test at 4 °C, with a 10-min incubation: antigen incubation step (12). Varying the duration of the antibody: antigen incubation time between 10 min and 1 h did not alter results significantly (Supplementary Fig. S1, B), indicating there was minimal rearrangement of the folding/aggregation conformations during the incubation time. Previous studies have shown that the progression of tailspike folding essentially halts at 4 °C (27), with only minimal rearrangement of the folding and assembly intermediates (25). A control ELISA competition test was also performed using an equivalent concentration of native wild type tailspike; this level of binding was set to 100% (see Fig. 3). The concentration of tailspike for each mAb binding experiment was chosen such that antibody binding to the native tailspike control was >80%.

Gel Electrophoresis—Non-denaturing polyacrylamide gel electrophoresis of tailspike refolding samples was performed at 4 °C as previously described (30). SDS-PAGE of identical samples was also performed as previously described (30), except that gels were electrophoresed at room temperature using 7.5% acrylamide. Gels were silver-stained as previously described (42).

RESULTS

Antibody Binding to Mutant Tailspike Proteins

All of the tailspike mutations used in this study have previously been shown to have no effect on the thermodynamic stability, phase head binding, or cell killing properties of native tailspike (34, 36). Thus the mutant proteins are functionally identical to wild type tailspike, correctly folded, and presumably exhibit only subtle structural differences in the vicinity of the mutation site. Altering these surface-exposed amino acid residues is therefore expected to perturb the binding of an antibody only if it has an overlapping or adjacent epitope. The seven tsf and su (tsf suppressor) mutations (G244R, E405K, V331A, K163E, G177R, W202Q, and D238S) are located throughout the β-helix domain, at positions largely exposed on the surface of the native tailspike crystal structure (Figs. 1 and 2). Lys163 is the most buried residue and closest to the trimeric interface of the three polypeptide chains (Fig. 1C). The eight cysteine to serine (Cys→Ser) mutations provided additional conservative mutations in regions of the tailspike structure other than the β-helix (Figs. 1 and 2).

Competition ELISA tests were performed for each antibody: antigen pair. The ratio \( K_d, w/t/K_d, m/t \) provides a measure of the affinity of a given antibody for a mutant protein as compared with the affinity for wild type tailspike. Values of \( K_d, w/t/K_d, m/t \) are listed in Table I. Italicized values indicate antibody binding to the mutant protein was significantly weaker than binding to wild type tailspike (\( K_d, w/t/K_d, m/t < 0.5 \)), whereas bold values indicate binding to the mutant protein was significantly stronger than binding to wild type tailspike (\( K_d, w/t/K_d, m/t > 3 \)).

Interestingly, both mAb 70 and mAb 92 bound more tightly to K163E than to wild type tailspike (Table I). In addition, K163E disrupts the binding of all but one (mAb 155) of the remaining antibodies, indicating that this mutation has a greater than expected effect on the surrounding tailspike structure. K163E represents a non-conservative substitution of a glutamate for a lysine, and charge change mutations such as this have been shown to cause subtle yet long-range perturbations in native protein structures (41). Moreover, Lys163 is located at a partially buried position at the interface between the tailspike monomer chains (Fig. 1C), and this core location may contribute to its unusual effects on antibody binding. Apart from K163E, however, no significantly lower dissociation constants were observed for binding to a mutant tailspike protein (Table I).

Mapping the Binding Epitopes of Anti-tailspike Monoclonal Antibodies

None of the eight Cys→Ser mutations significantly affected the binding of any of the nine mAbs. This perhaps not surprising; whereas these mutations are spread throughout the native structure (Fig. 1A), they are all at least partially buried from the surface of the protein (Fig. 2), which may mask the effects of these mutations on neighboring surface epitopes. In contrast, the tsf and su mutations are more solvent-exposed (Figs. 1C and 2), and all but one altered the binding of at least one mAb.

mAb 51 Epitope Includes the 400–407 Disordered Loop—If each tailspike tsf or su mutation altered the binding of one and only one anti-tailspike antibody, identifying the binding epitopes would be entirely straightforward. However, this situation was observed only for the E405K mutation, which exclusively alters the binding of mAb 51 (Table I). This result suggests that Glu405 is located within the binding epitope of mAb 51. Glu405 is found in a disordered surface loop within the β-helix domain of the tailspike crystal structure (18), and this loop may adopt a more disordered, peptide-like native environment than the other mutation sites, all of which are visible in the tailspike crystal structure. This may explain why E405K is the only mutation that affects the binding of only one mAb; as described below for the other mutations, the “perturbation area” (see below) of E405K is predicted to be quite small.

mAbs 70, 92, and 155 Bind to Tailspike Terminator—For antibodies 70, 92, and 155, binding to tailspike was not disrupted
by any of the mutations used, including the G244R mutation that severely disrupts the binding of five other antibodies (Table I; \(K_{d, \text{mut}} / K_{d, \text{wt}} \approx 0.1\)). This binding pattern suggests that antibodies 70, 92, and 155 bind to one of the extreme ends of the tailspike, as opposed to the central \(\beta\)-helix region, where the \(tsf\), \(su\), and six of the eight Cys\(\rightarrow\)Ser mutations are located. Previous studies have demonstrated that mAb 70 binds strongly to a 14-kDa N-terminal fragment of tailspike (17). Thus the insensitivity of mAb 70 to the \(\beta\)-helix mutations reinforces the assignment of the mAb 70 epitope within the tailspike N-terminal domain (Fig. 2). Likewise, mAb 92 binding is known to disrupt the ability of tailspike to bind to the P22 phage capsid (17). Because the N-terminal domain is crucial for capsid binding (43, 44), the mAb 92 epitope is most likely located within the N-terminal domain as well.

Conversely, mAb 155 binding does not prevent tailspike attachment to the phage capsid but instead prevents \textit{Salmonella} cell killing (17). Both the \(\beta\)-helix and C-terminal regions of tailspike are involved in cell killing. Because mAb 155 binding is not affected by any of the \(\beta\)-helix tsf mutations (Table I), this antibody is likely to bind to the C-terminal caudal/tail fin region of tailspike, although not in close proximity to Cys\(^{613}\) or Cys\(^{635}\) (Fig. 2).

\textbf{mAb 124 Binds to the N Terminus of the \(\beta\)-Helix Domain—}

As mentioned above, with the exception of E405K, the tailspike tsf and su mutations that cause a change in binding for one anti-
body also produced changes in binding for other antibodies (Table I). This is consistent with other studies, which have indicated that even subtle, conservative mutations with no apparent effect on protein stability or function can produce long-range changes in mAb binding to a distant epitope (41, 45). Each of these mutations can therefore be regarded as influencing the fine details of the tailspike surface in a defined area of variable size (a perturbation area), and a given antibody binding epitope may or may not be located within this area. Conversely, it is also possible to evaluate the inability of a mutation to alter the binding of an antibody; these mutations are not likely proximal to the binding epitope. The differences in binding for the entire set of mutants and antibodies were therefore examined in order to develop a coherent model for the location of anti-tailspike antibody binding epitopes.

**FIG. 2.** Model for the location of anti-tailspike antibody binding epitopes. Space-filling version of ribbon diagram in Fig. 1; head domain probably sits down a bit tighter on β-helix domain. The black circles show approximate locations for the epitope of each monoclonal antibody. Only one epitope is shown; however, because tailspike is a homotrimeric protein, there may be as many as three epitopes per trimer. Colored circles indicate the approximate perturbation area of the tsf and su mutations on tailspike local surface structure: green, K163E, V331A, and W202Q, and red, G244R. Antibody binding within one of these regions is affected by the listed tsf/su mutations. For example, the binding of antibody (Ab) 124 is affected by mutations K163E, V331A, and W202Q, but not by G244R.

| Mutation | Ab' 51 | Ab 219 | Ab 175 | Ab 236 | Ab 33 | Ab 155 | Ab 124 | Ab 70 | Ab 92 |
|----------|--------|--------|--------|--------|-------|--------|--------|-------|-------|
| C169S    | 1.1    | 2.5    | 1.6    | 1.2    | 0.5   | 1.0    | 1.0    | 0.5   | 0.6   |
| C267S    | 1.2    | 2.8    | 1.6    | 1.4    | 0.7   | 1.0    | 1.0    | 0.5   | 0.6   |
| C279S    | 1.0    | 0.8    | 2.5    | 1.2    | 1.6   | 2.0    | 1.0    | 1.5   | 1.0   |
| C290S    | 1.1    | 2.0    | 2.5    | 1.6    | 1.6   | 2.0    | 1.4    | 3.0   | 1.5   |
| C458S    | 1.4    | 2.2    | 1.6    | 1.6    | 0.5   | 1.1    | 0.7    | 0.5   | 1.0   |
| C496S    | 1.2    | 2.0    | 1.2    | 1.4    | 0.7   | 1.0    | 0.7    | 0.5   | 1.0   |
| C612S    | 1.2    | 2.0    | 1.2    | 2.0    | 1.0   | 0.5    | 1.5    | 1.0   | 0.6   |
| C568S    | 0.8    | 1.1    | 1.2    | 1.2    | 1.6   | 2.0    | 1.0    | 1.5   | 1.5   |
| K163E    | 0.04   | 0.02   | 0.05   | 0.03   | 0.1   | 1.0    | 0.1    | 5.0   | 7.5   |
| G177R    | 0.8    | 1.1    | 1.2    | 1.1    | 2.0   | 2.0    | 1.1    | 1.0   | 1.0   |
| W202Q    | 0.2    | 0.4    | 0.25   | 0.3    | 0.1   | 0.6    | 0.2    | 1.0   | 1.0   |
| V331A    | 0.08   | 0.08   | 0.05   | 0.1    | 0.25  | 0.5    | 0.1    | 1.0   | 1.0   |
| D209S    | 1.0    | 0.5    | 0.4    | 0.4    | 2.2   | 0.6    | 0.7    | 1.0   | 0.8   |
| G244R    | d      | 0      | 0      | 0      | 0.07  | 1.0    | 1.5    | 2.5   | 2.0   |
| E405K    | 0.2    | 1.0    | 0.7    | 1.0    | 0.7   | 1.0    | 1.0    | 1.0   | 1.0   |

*a Tsf mutations are listed in order of appearance in the tailspike crystal structure, from N to C terminus. Cys→Ser site-directed mutations are listed similarly (see Figs. 1A and 2).
*b Dissociation constants determined for wild type tailspike were similar to published values (17). Values shown represent the average of at least three measurements for both the mutant protein and wild type tailspike (S.D. < 10%). Values in lightface black type denote antibody binding to the mutant protein is similar to binding to wild type tailspike (Kd/\(K_d\_{\text{mut}}\) < 0.5), whereas values in italic type indicate antibody binding to the mutant protein was significantly weaker than binding to wild type tailspike (Kd/\(K_d\_{\text{mut}}\) > 3). Values in boldface type indicate binding to the mutant protein was significantly stronger than binding to wild type tailspike (Kd/\(K_d\_{\text{mut}}\) > 3).
*c Ab, antibody.
*d A value of 0 indicates antibody binding to the mutant protein was too weak to measure using these assay conditions.
antibody epitope locations that is consistent with the known locations of the mutations.

Close examination of the pattern of mAb binding disruption and mutation location reveals several interesting correlations. For example, the mutations K163E, W202Q, G244R, and V331A disrupt the binding of antibodies 51, 219, 175, 236, and 33 (Table I). Yet these mutations are located throughout the β-helix, rather than in one epitope-sized area (Figs. 1A and 2). These mutations must therefore have a large, overlapping effect on the tailspike surface, a large perturbation area. Similarly, the binding of mAb 124 is affected by mutations K163E, W202Q, and V331A, but not G244R; mAb 124 binding to G244R is indistinguishable from binding to wild type tailspike (Table I). This suggests that mAb 124 binds within the perturbation area of K163E, W202Q, and V331A, but not within the perturbation area of G244R. From Fig. 1, it is clear that Gly244 is located more toward the C-terminal end of the β-helix than K163E, W202Q, or V331A. Thus mAb 124 most likely binds within the β-helix, N-terminal to G244R, yet still within the perturbation area of K163E, W202Q, and V331A (Fig. 2). However, the mAb 124 epitope is C-terminal to the epitopes for mAbs 70 and 92 because the binding of mAbs 70 and 92 are not perturbed by any of the mutations used here. Also, the mAb 124 epitope is probably not in the immediate vicinity of Gly244 because the non-conservative G177R mutation has no effect on mAb 124 binding (Table I). The binding of mAb 124 to tailspike also affects the phage head binding property of tailspike (17), and this is consistent with a binding epitope located toward the N-terminal end of tailspike β-helix.

The Central β-Helix Epitopes of mAbs 33, 175, 219, and 236—The interpretations above have focused on the epitope regions of antibodies 51, 70, 92, 155, and 33 (Table I). These mutations must therefore have a large, overlapping effect on the tailspike surface, a large perturbation area. Similarly, the binding of mAb 124 is affected by mutations K163E, W202Q, and V331A, but not G244R; mAb 124 binding to G244R is indistinguishable from binding to wild type tailspike (Table I). This suggests that mAb 124 binds within the perturbation area of K163E, W202Q, and V331A, but not within the perturbation area of G244R. From Fig. 1, it is clear that Gly244 is located more toward the C-terminal end of the β-helix than K163E, W202Q, or V331A. Thus mAb 124 most likely binds within the β-helix, N-terminal to G244R, yet still within the perturbation area of K163E, W202Q, and V331A (Fig. 2). However, the mAb 124 epitope is C-terminal to the epitopes for mAbs 70 and 92 because the binding of mAbs 70 and 92 are not perturbed by any of the mutations used here. Also, the mAb 124 epitope is probably not in the immediate vicinity of Gly244 because the non-conservative G177R mutation has no effect on mAb 124 binding (Table I). The binding of mAb 124 to tailspike also affects the phage head binding property of tailspike (17), and this is consistent with a binding epitope located toward the N-terminal end of tailspike β-helix.

In Vitro Refolding Reveals Additional Epitope Features

To further define the recognition motifs of the anti-tailspike antibodies, we assessed the binding of each antibody to wild type tailspike in vitro refolding and aggregation intermediates. This analysis builds on previous studies that have shown that the anti-tailspike antibodies exhibit differential recognition of tailspike refolding intermediates in vitro and ribosome-bound tailspike nascent chains (12, 32).

Wild type tailspike was unfolded in acid urea buffer, and refolding was initiated by 50-fold dilution into refolding buffer at 10 °C (see “Experimental Procedures” for details); under these conditions, specific activity assays, tryptophan fluorescence measurements, and SDS-PAGE have shown that 60–80% of tailspike chains refold to native trimers after 48 h (46). Binding of the anti-tailspike monoclonal antibodies to wild type tailspike refolding intermediates was measured using the ELISA competition test. The percentage of recognition at each refolding time point was normalized to the binding to a sample of native wild type tailspike (100% normalized binding), permitting comparison of the binding of antibodies with different dissociation constants (12, 17). The binding of antibodies 175, 219, 236, 51, 124, 70, 92, and 33 to tailspike refolding and aggregation time points is shown in Fig. 3A. An equivalent procedure was used to analyze the binding of antibodies 33, 236, and 155 in a previous study (12). Our results for the binding of antibodies 33 and 236 (Fig. 3A) are indistinguishable from the results of this earlier study. Moreover, the binding of antibody 155 (12) closely resembles the binding profile of mAb 175 (this study). Additionally, refolding was monitored for very long times (up to 12 days), and quantification of monomer and trimer bands from SDS-PAGE showed native tailspike approaching the 80% maximum refolding yield (Fig. 3C). This gradual increase was also reflected in the mAb 175 recognition profile for the same refolding samples, indicating that the binding of the anti-N antibodies did indeed reflect the appearance of the native tailspike structure.

It should be noted that fully native (i.e. SDS-resistant) tailspike trimers are formed more slowly than the appearance of the band migrating at the position of native tailspike on non-denaturing polyacrylamide gels (Nt versus Nt*, Fig. 3, B and C). Some final conformational rearrangements therefore take place between the formation of the trimer-sized, SDS-sensitive species (Fig. 3B, Nt*) and the fully active SDS-resistant trimers (Fig. 3C, Nt; see also Ref. 46). This extremely slow formation of native tailspike likely explains why, after 24 h of refolding, the binding of several anti-tailspike antibodies still exhibits time-dependent changes in binding.

The anti-tailspike antibodies exhibit differential binding to the tailspike refolding time points. In addition, recognition by the three anti-D/I antibodies used in this study (antibodies 70, 92, and 124) exceeded the recognition of the native tailspike control (Fig. 3A, 100%). This is most likely due to the ability of the anti-D/I antibodies to recognize both native and non-native tailspike conformations and particularly aggregation conformations (14, 17); under the refolding conditions used here, even after 12 days of refolding, ~20% of tailspike chains remain non-native (Fig. 3C).

Conversely, there is no clear trend for the recognition of the earliest folding/aggregation conformations by the anti-tailspike antibodies (Fig. 3A). Two of the anti-D/I antibodies (antibodies 70 and 124) exhibited limited recognition of the earliest tailspike refolding/aggregation conformations. This result indicates that mAb 70, despite its ability to recognize a 14-kDa N-terminal fragment of the tailspike sequence (17), does indeed recognize a structurally, discontinuous epitope. This result is surprising because the conformation of the 14-kDa N-terminal fragment is presumably not equivalent to the conformation of this sequence in the native structure; the stabilizing C-terminal interdigitiation region is not present (Fig. 1) (23). Interestingly, mAb 92 shows full recognition of the earliest tailspike refolding/aggregation conformations (Fig. 3A), despite its inability to recognize tailspike N-terminal fragments of <52 kDa (17).

Many of the antibodies show a progressive increase in binding as tailspike refolds. mAbs 51 and 124, on the other hand, bind more strongly to tailspike folding and/or aggregation states present after 1.5–4.0 h of refolding than they do to native tailspike. Interestingly, at 1.5–4.0 h, the protrimer intermediate is the dominant species in the refolding/aggregation ensemble, as determined by native gel electrophoresis (Fig. 3B). We have therefore classified the conformational preference of these two antibodies as anti-Pt (Table II), although we cannot formally rule out the possibility that they preferentially bind extremely strongly to a smaller population of misfolding conformations populated during this time range. Based on these results, we have divided the monoclonal antibodies into three groups: anti-native (antibodies 33, 155, 175, 219, and 236), anti-protrimer (antibodies 51 and 124), and anti-non-native (antibodies 70 and 92) (Table II).
DISCUSSION

In this study, epitopes for nine anti-tailspike mAbs were defined by screening against 15 tailspike point mutations. Some mutations affected mAb binding only near the mutation site, whereas other mutations affected mAb binding at a site relatively distant from the residue itself. In fact, some mutations cause unexpectedly large changes to the surface of native tailspike. This may reflect the previously observed “plasticity” of the β-helix domain (47), meaning that the mutations can cause a large rearrangement of side chain packing within the β-helix domain, whereas the backbone configuration (and native protein function) remains essentially unchanged.

FIG. 3. Tailspike in vitro refolding reactions. A, anti-tailspike antibody binding as a function of tailspike refolding time. The normalized percentage of recognition of each antibody for wild type tailspike in vitro refolding and aggregation intermediates is plotted versus refolding time. Normalized percentage of recognition for each antibody is calculated versus binding to an equivalent concentration of native wild type tailspike. Lines drawn are to guide the eye. Dashed lines, anti-D/I antibodies; solid lines, anti-N antibodies. Each data point represents the average of at least three separate determinations. Black, mAb 124; red, mAb 70; green, mAb 92; blue, mAb 51; purple, mAb 236; teal, mAb 175; gray, mAb 33; orange, mAb 219. In all cases, S.D. was <10%. B, non-denaturing PAGE of refolding intermediates. [M], monomer; [D], dimer; Pt, protomer; N*, trimer-sized tailspike (see text for details). C, tailspike refolding monitored by the appearance of SDS-resistant trimers (detected by SDS-PAGE) and mAb 175 binding. Blue and orange circles are quantification of tailspike trimer and monomer bands, respectively, from an SDS-polyacrylamide gel. A total of 400 ng of refolding tailspike was loaded in each well for each time point. Teal circles represent the binding of mAb 175 at the same time points. Lines are provided to guide the eye.
Epitope mapping is often performed by scanning libraries of linear peptides (48–50). However, the tailspike secondary structure consists mostly of parallel \( \beta \)-sheets, formed from contacts between amino acids that are distant in the primary sequence. This secondary structure arrangement is unlikely to be faithfully reproduced by short peptides, and epitope localization based on peptide scanning therefore may not reflect antibody binding to the native protein. Mutational analysis has been successfully used to identify epitopes for other proteins (51, 52); nevertheless, because the mutation of one residue can perturb the surrounding protein structure (52), the assignments described here define the areas of the native structure where binding is significantly perturbed by a mutation, rather than a precisely defined, residue-specific epitope boundary.

It was known from previous work that tailspike requires \( \geq 24 \) h to refold in vitro at \( 10 \) °C. The refolding yield is 60–80%; the remainder of the refolding chains misfold and aggregate, failing to reach the native tailspike structure (46). Thus, if an antibody binds to a structural feature present only on the surface of the native protein, a slow rise in antibody binding as refolding proceeds would be expected, and the level of maximal binding would be \( \approx 80\% \) of the binding to an equivalent concentration of completely native tailspike. From Fig. 3A, it appears that anti-N antibodies 33, 175, and 236 (as well as antibody 155 from a previous study, Ref. 12) exhibit this behavior. In fact, for antibody 33, there is a lag for \( \approx 1 \) h and then a gradual increase in binding to the refolding chains. Presumably, these anti-native antibodies have no low recognition of the structural features present on early refolding conformations and only bind after the appearance of later structural features.

Preliminary characterization of the anti-tailspike mAbs identified four antibodies (three of which were used in the current study: antibodies 70, 92, and 124) that preferentially recognize non-native tailspike conformations (17). All three of these mAbs appear to have particularly tight binding to structural features on non-native misfolding chains; hence, binding reaches a maximum equivalent to \( \approx 160\% \) of the binding to the native tailspike control (Fig. 3A). These were initially described as “anti-denatured” (anti-D) (17), yet it is clear that at least two of these three anti-non-native antibodies recognize distinct structural features, rather than a continuous, peptide-like epitope. Antibodies 70 and 124 both show greatly reduced binding to early refolding/aggregation conformations. In addition, mAb 124 shows dramatically increased binding to conformations present at 1.5–4.0 h after the initiation of refolding (Fig. 3A), coincident with the formation of the protrimer intermediate (Fig. 3B). The preferential recognition of a non-native conformation by mAb 124 is similar to other mAbs that preferentially recognize non-native conformations, including the aggregated amyloid conformation of several proteins (15). Intriguingly, the results in Fig. 3A indicate that mAb binding to non-native, aggregation-prone conformations reaches a maximum long before the formation of native trimer. This result suggests that the antibody binding conformations of misfolded tailspike chains do not change as larger aggregates are formed later during refolding.

Whereas most of the mAbs showed a monotonic increase in tailspike recognition during refolding, two of the antibodies show quite different binding profiles. mAbs 124 and 51 preferentially recognize tailspike refolding (and/or aggregation) intermediates populated between 1.5 and 4 h after the initiation of refolding. Antibodies 124 and 51 recognize epitopes at opposite ends of the \( \beta \)-helix domain, indicating that structural organization in this domain occurs prior to native trimer formation. Whether the decreasing recognition at later time points is due to the partial occlusion of the epitope by other structural elements or the formation of more native-like structure is unclear. CD and fluorescence spectroscopy studies have shown that tailspike secondary structure formation occurs prior to trimer assembly (25), consistent with the formation of some native-like structure at both ends of the \( \beta \)-helix domain prior to or during the formation of the protrimer. For high contact order proteins (i.e. those with tertiary structure contacts formed between residues that are distant in the primary structure, Ref. 53), formation of a partially organized yet not fully native-like structure may be important to reduce the conformational space explored by the polypeptide chain and avoid misfolding. More information is clearly needed in order to understand the details of how structurally complex proteins fold and assemble into larger complexes.

An important consideration when using mAb binding to monitor conformational changes is the effect of the antibody itself on the conformation of the antigen. It is possible that an antibody may not recognize an existing conformational epitope on an antigen but rather induces the antigen to adopt that conformation (16). However, in the results described here, for all but one mAb, tailspike binding is dependent on refolding time. Therefore, in most cases, regardless of the binding mode of the antibodies, some degree of tailspike refolding must take place before mAb binding can occur. An additional consideration is the binding stoichiometry: tailspike is a trimer, and each IgG has two antigen binding sites. Some epitopes may therefore bridge two monomer chains. At most, there could be three epitopes per native trimer if the epitope is localized to one monomer chain. In such a case, the binding of one antibody could potentially influence the binding of another copy of the antibody to an epitope on another monomer chain. This in turn could lead to an underestimation of the \( K_d \) or affect how the antibody reports on tailspike conformation. However, none of the mAbs preferentially recognized the tailspike monomer, and the monomer is unlikely to adopt a fully native conformation before trimerization and chain interdigitation, at which point multiple antibody binding could be problematic. Also, it is possible that, given one epitope per monomer chain, one antibody could recognize two epitopes on a single tailspike trimer. However, given the large size of an IgG molecule and the relative distance between equivalent sites on two monomer chains, this would require considerable flexibility in the IgG molecule.

Based on the known features of the tailspike refolding pathway (Fig. 1D), we propose a model for the conformations of refolding intermediates that are recognized by the anti-tailspike antibodies. The first conformation-sensitive mAb able to recognize refolding tailspike chains is mAb 70. Antibody 70 recognizes an epitope in the N terminus that forms (or can be

### Table II

| mAb     | Epitope location                  | Conformational preference | \( K_b \) |
|---------|-----------------------------------|---------------------------|-----------|
| 92      | N-terminal domain                 | None                      | \( 2 \times 10^{-7} \) |
| 70      | N-terminal domain                 | [M]                       | \( 6 \times 10^{-8} \) |
| 124     | \( \beta \)-Helix: N terminus     | Pt                        | \( 2 \times 10^{-5} \) |
| 236     | \( \beta \)-Helix: dorsal fin/center | Pr/NT                      | \( 1 \times 10^{-10} \) |
| 175     | \( \beta \)-Helix: center         | Nt                        | \( 1 \times 10^{-10} \) |
| 219     | \( \beta \)-Helix: center         | Nt                        | \( 5 \times 10^{-11} \) |
| 33      | \( \beta \)-Helix: center         | Nt                        | \( 3 \times 10^{-9} \) |
| 51      | \( \beta \)-Helix: C terminus     | Pt                        | \( 3 \times 10^{-10} \) |
| 155     | C-terminal domain                 | Pr/NT                      | \( 2 \times 10^{-9} \) |

* [M], structured monomer; Pt, protrimer; Nt, native trimer.

\( K_b \) was determined for binding to native tailspike in solution, using established methods (39).
induced) very early during refolding, and the level of mAb 70 binding does not change as the chains progress along either the productive folding or aggregation pathways. Because the mAb 70 epitope is recognized before multimerization occurs, it is likely that the epitope is localized within one monomer chain. The epitopes recognized by the anti-Pt mAbs (mAbs 51 and 124) are the next conformation-sensitive structure to form. The binding of these antibodies decreases as the dominant species shifts from protrimer to native trimer, suggesting that mAbs 51 and 124 recognize non-native structural epitopes or a native structure epitope that is occluded by additional late-forming structure. Interestingly, the mAb 51 and 124 epitopes are at opposite ends of the β-helix domain. This supports a previous model suggesting early organization of this domain is important for productive assembly (25). Three anti-native mAbs enable more detailed studies.

Whereas mAb binding is clearly useful as a structural probe for studying proteins in complex mixtures, in part because there are very few experimental approaches that can selectively report on the folding of the subject protein in the presence of such a high background of other proteins. Preliminary work has validated the use of antibody binding to measure tailspike folding in complex mixtures (12, 32), and the results presented here will enable more detailed studies.

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