Folding and Stability of Mutant Scaffolding Proteins Defective in P22 Capsid Assembly*

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Bacteriophage P22 scaffolding subunits are elongated molecules that interact through their C termini with coat subunits to direct icosahedral capsid assembly. The soluble state of the subunit exhibits a partially folded intermediate during equilibrium unfolding experiments, whose C-terminal domain is unfolded (Greene, B., and King, J. (1999) J. Biol. Chem. 274, 16135–16140). Four mutant scaffolding proteins exhibiting temperature-sensitive defects in different stages of particle assembly were purified. The purified mutant proteins adopted a similar conformation to wild type, but all were destabilized with respect to wild type. Analysis of the thermal melting transitions showed that the mutants S242F and Y214W further destabilized the C-terminal domain, whereas substitutions near the N terminus either destabilized a different domain or affected interactions between domains. Two mutant proteins carried an additional cysteine residue, which formed disulfide cross-links but did not affect the denaturation transition. These mutants differed both from temperature-sensitive folding mutants found in other P22 structural proteins and from the thermolabile temperature-sensitive mutants described for T4 lysozyme. The results suggest that the defects in these mutants are due to destabilization of domains affecting the weak subunit-subunit interactions important in the assembly and function of the virus precursor shell.

Although the scaffolding proteins of bacteriophage P22 participate in polymerization of the viral coat monomers into a stable icosahedral capsid lattice, the spherical complexes of 200–300 scaffolding subunits assembled within the precursor shells are metastable structures, dissociating in the cell as a prerequisite to DNA packaging and dissociating in the test tube under very mild conditions (1–3). The assembled state of the scaffolding subunits lacks icosahedral symmetry (4) and, although closely packed, may be only loosely associated. The dissociated subunits are not covalently modified with respect to their assembled state and participate in subsequent rounds of procapsid assembly.

Its biological role in coupled assembly/dissociation cycles suggests that the scaffolding protein would need to be a conformationally flexible molecule. Although the three-dimensional structure is not known, Parker et al. (5) estimated dimensions of 232 Å long by 22 Å wide from analytical ultracentrifugation data. This elongated molecule does not appear to have a tightly packed hydrophobic core like that of a typical globular protein, because no protected core was detected by deuterium exchange (6), and it binds many molecules of 1–1–bi (4-anilino)naphthalene-5-sulfonic acid (bisANS), a probe for exposed hydrophobic surfaces on proteins (7). Denaturation and proteolysis experiments indicated that the C-terminal end of the molecule was a distinct easily melted domain that interacts with the coat subunits (8). Low stability of this domain may reflect its need to undergo conformational transitions during assembly within the cell.

Despite many efforts at their isolation, mutations in the scaffolding protein gene with physiological defects in folding or assembly have been recovered at very low frequency, considering the recovery of mutations in other P22 genes (9, 10). Only four strains of independent origin defective in scaffolding protein function have been isolated over a period spanning two decades (11, 12). This represents a property of the protein, rather than the gene, because nonsense mutations generating stop codons occur at expected frequencies (10, 13).

By comparison some 70 sites of temperature-sensitive folding (tsf) mutants have been characterized in the tailspike gene. In the adjacent gene for the coat protein, some 17 sites of tsf mutations have been isolated (14). The distinctive feature of these mutants is that they do not act by destabilizing the native state of the mutant protein; mutant proteins formed at low temperature are as thermostable as wild type when incubated at restrictive temperature. This is true both for tailspike tsf mutants (15–17) and coat tsf mutants (18). In both cases the ts amino acid substitutions act by destabilizing an already thermolabile partially folded intermediate in the folding pathway. At restrictive temperatures these species associate to form inclusion bodies in vivo and high molecular weight aggregates in vitro (19, 20).

In contrast, no evidence of competing off pathway aggregation reactions have been detected during scaffolding protein folding either in vivo or in vitro, even during thermal denaturation (8, 12). The mutant scaffolding proteins expressed by the four ts strains folded and assembled into procapsid-like structures within infected cells at both permissive and restrictive temperatures (12). However, the procapsids formed from the mutant proteins at restrictive temperatures were not competent for DNA packaging or other steps in virus maturation (12). Two of the mutant strains carry single amino acid substitutions in their scaffolding protein, S242F and Y214W. The other two mutants carry more than one substitution: R74C/L177I and S45A/R74C/Q149W/A199V. The double mutation V45A/A199V, however, is silent.2 The distribution of the substitutions is shown in Fig. 1.

Assembly of a viable procapsid requires the incorporation of

1 The abbreviations used are: tsf, temperature-sensitive for folding; ts, temperature-sensitive; DTT, dithiothreitol.
2 B. Greene, unpublished observations.
a dodecameric ring of the gene 1 portal protein at one vertex of the shell to form a DNA packaging channel (21). During particle assembly scaffolding subunits presumably interact with coat subunits, with each other, and with portal subunits. The S242F and Y214W mutations in the C-terminal third of the scaffolding sequence are defective in insertion of the DNA packaging portal into the procapsid structure (12, 21). The mutant subunits carrying the R74C substitutions were defective in scaffolding protein release from the procapsid (12).

We report here the solution properties of the mutant scaffolding proteins and their behavior during equilibrium unfolding experiments. To be able to relate the properties of the purified proteins to the actual defects expressed within cells, it was necessary to characterize the purified proteins carrying the multiple substitutions because the severity of the phenotype is affected by the interaction of the substitutions.

The results reported here with the purified mutant proteins indicate that unlike the tsf mutations in the coat and tailspike subunits, these mutations do destabilize the folded conformation of the subunit. However, unlike thermolabile ts mutations, of T4 lysozyme for example, which are predominantly buried (22), these substitutions appear to be on the subunit surface. They are most likely to be affecting the subunit-subunit interactions involved in the cycle of assembly and dissociation needed for capsid assembly by destabilizing local functional regions.

**EXPERIMENTAL PROCEDURES**

**P22 Phage Strains**—The phage strains used in the preparation of wild type proteins carried the c1-7 mutation to ensure entry into the lytic pathway, 13amH101, which delays lysis, and either 2amH202 or 3amN6, which block DNA packaging. The mutant strains used for the purification of the scaffolding proteins are listed in Table I. The isolation of these mutants is described by Greene and King (12). Column 1 shows the amino acid substitutions originally identified from sequencing regions of gene 8. Subsequent characterization of these mutants has revealed additional mutations. These are shown in column 2 of Table I.

**Folding of ts Scaffolding Proteins**—Phage strains carrying the 8ts mutations were used for the preparation of procapsids containing mutant scaffolding proteins. Infections with these strains were carried out at 30 °C. Apart from this the mutant procapsids were prepared by the same protocol as for wild type (3). Procapsids were obtained from mutant-infected cells in amounts comparable with preparations of wild type scaffolding protein. Mutant scaffolding proteins were purified from procapsids using the protocol previously described for wild type (3), although a higher concentration of guanidine HCl (0.9 M instead of 0.5 M) was used to extract scaffolding protein from R74C/L177I and R74C/Q149W procapsids. The yield of R74C/L177I mutant scaffolding protein was somewhat less than that of the others because it was not possible to remove all the R74C/L177I scaffolding protein from procapsids even after three extractions with guanidine HCl.

Wild type and mutant scaffolding proteins were dialyzed into phosphate buffer (20 mM K2HPO4, 25 mM NaCl, pH 7.6, with HCl) before use. The concentration was checked by absorbance at 280 nm, based on an extinction coefficient of 1.61 × 10^4 liters mol^-1 cm^-1 for wild type, R74C/L177I, and S242F; 2.08 × 10^4 liter mol^-1 cm^-1 for Y214W, and 2.21 × 10^4 liter mol^-1 cm^-1 for R74C/Q149W. The extinction coefficienct of the wild type and mutant proteins were calculated using the method of Johnson (23).

**Fluorescence and Circular Dichroism Spectra**—Fluorescence spectra were recorded of wild type or mutant scaffolding proteins at 100 μg/ml (0.3 μM) at constant temperature, with the excitation wavelength set to 280 nm, and the emission scanned from 300 to 400 nm. The excitation and emission slit widths were 2.5 and 5 nm, and the PMT voltage was set to 700 V. Circular dichroism spectra were recorded from 200 to 250 nm at constant temperature in a 0.5-cm-pathlength cell. The spectral bandwidth was 1.5 nm, the step size was 1 nm, the averaging time was 0.4 s, and five scans were recorded and averaged for each sample.

**Fluorescence Quenching**—KI quenching experiments were performed in phosphate/NaCl buffer with 10 mM Na2S2O5 added to keep the iodide reduced (24). The final concentration of wild type scaffolding protein was 1 μg/ml (0.3 μM); that of the two mutant proteins Y214W and R74C/Q149W was 50 μg/ml because the fluorescence of these mutants was approximately twice that of the wild type protein. To avoid possible effects of ionic strength changes on protein conformation, the ionic strength was held constant by the addition of KCl to give a total concentration of KI and KCl of 0.25 M. These concentrations of KI are sufficiently low so as to produce no inner filter effect (25). Samples were excited at 280 nm, and the emission was monitored at 330 nm. Excitation and emission slit widths were 2.5 and 5 nm, the PMT voltage was 700, and the temperature was 3 °C.

**Thermal Denaturation**—Wild type and mutant scaffolding proteins were diluted to 100 μg/ml (0.3 μM) in phosphate/NaCl buffer before use. The samples were incubated for an hour at 4 °C before starting the experiment. Fresh samples were prepared for each experiment. The samples were heated from 3 to 90 °C, and denaturation was monitored by either fluorescence intensity at 330 nm with excitation at 280 nm, or circular dichroism ellipticity at 222 nm as described in the accompanying paper (8).

**RESULTS**

**Structure of the Mutant Scaffolding Proteins**—The fluorescence and CD spectra of the mutant proteins were first compared with that of wild type to determine whether they folded to similar conformations. Fig. 2 shows the CD spectra of the wild type and mutant scaffolding proteins at several temperatures. As expected, the mutant proteins were, like wild type, predominantly α-helical. Even at 3 °C, however, at which point all the proteins should be maximally folded, the mutant proteins all had different CD spectra than wild type.

The secondary structure compositions of the folded proteins were estimated using the standards of Chang, Wu, and Yang (26). Analysis of the wild type CD spectrum suggested a composition of 30% α-helix, 10% β-sheet, 18% turn, and 42% random coil, in excellent agreement with the composition determined by Teschke et al. (7). The spectrum did not display the high θ222/θ208 ratio typical of a coiled-coil but was characteristic of simple α-helices (27).

The spectra of the four mutant proteins at 3 °C were similar to each other and were composed of 25% α-helix, 10% β-sheet, 20% turn, and 45% random coil. The pattern of secondary structure alteration for the mutants resembled that observed for the wild type protein at higher temperatures. The spectrum of wild type scaffolding protein at 25 °C could be fit as 27% α-helix, 10% β-sheet, 19% turn, and 44% random coil. These results suggested that the mutants were in a similar conformation at 3 °C to that adopted by the wild type protein at

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3 B. Greene, P. E. Prevelige, Jr., and J. King, unpublished observations.
higher temperatures. By 90 °C the spectra of the wild type and the four mutant proteins were superimposable and predominantly random coil, demonstrating that all had reached the same unfolded state.

The fluorescence spectra of the two mutant proteins without extra tryptophans were similar to wild type over a range of temperatures; R74C/L177I was almost identical, whereas the fluorescence of S242F was slightly less, perhaps indicating a slightly less compact structure (Fig. 3). The fluorescence of both the mutant proteins containing extra tryptophans, Y214W and R74C/Q149W, was approximately twice that of wild type, as expected. The spectra of these two mutants appeared slightly red-shifted with respect to wild type, suggesting that the new tryptophans might be in more exposed environments than the wild type tryptophan.

Tryptophan Exposure—The degree of tryptophan exposure can be assessed by collisional quenching of fluorescence using iodide ion. Because iodide is an ionic molecule it cannot penetrate into protein interiors, so more deeply buried tryptophans are less effectively quenched. This experiment was carried out at 3 °C, at which point the proteins would be maximally folded.

A Stern-Volmer plot (24) was used to determine the degree of fluorescence quenching by iodide of wild type and the two mutant scaffolding proteins with extra tryptophan residues (Fig. 4). The slope of a Stern-Volmer plot indicates the degree of quenching; the steeper the slope, the greater the degree of quenching. The slope for the wild type tryptophan was about 1, which indicates relatively little quenching, as expected for a tryptophan mostly protected from solvent. The Stern-Volmer plots for the mutant proteins are not linear, because two tryptophans of different accessibility contribute to the data. The initial slopes, which primarily reflect the influence of the more readily quenched tryptophan, were 1.6 for R74C/Q149W and 2.7 for Y214W as compared with a slope of 3.3 obtained for the completely denatured P22 coat protein (25). The tryptophan at 149 is thus more exposed than that at 134 but may still be partially buried, whereas that at 214 is probably on or near the surface of the protein.

Thermal Denaturation of Mutant Scaffolding Proteins—Thermal melts of all the mutant proteins were performed as for wild type. All the scaffolding proteins were analyzed at 100 μg/ml, a concentration at which the scaffolding proteins are almost entirely monomeric (5), so that the effects of self-association should not affect the observed unfolding process. The CD spectra demonstrated that the mutant proteins all began with slightly different structures at the initial temperature (Fig. 2). The thermal melts, as monitored by CD at 222 nm, showed that all the mutant proteins were also at least partially destabilized with respect to wild type (Fig. 5). Cooling of the proteins back to 3 °C resulted in some loss of the original signal (approximately 20%), but this effect did not appear to be due to aggregation and did not vary significantly between the wild type and mutant proteins.

The thermal denaturations of the mutant proteins S242F and Y214W had similar characteristics. Although these proteins were initially less structured than wild type, they appeared to be stably folded at low temperatures. The unfolding transition for both these mutants began earlier than for wild type. Unlike wild type, the data for these mutants were not well fit by a two-state model, indicating that one or more domains was more strongly affected than others. This effect was more visible for Y214W, for which the early part of the denaturation curve was shifted to lower temperature, whereas the later half of the curve corresponded closely to the wild type.

4 P. E. Prevelige, Jr., unpublished observations.
curve. The mutant S242F appeared more uniformly destabilized.

The R74C/Q149W and R74C/L177I mutations produced more dramatic destabilizations. The thermal melts of these mutant proteins were clearly not two-state but showed a sharp break between two processes at about 40 °C. The first region, accounting for approximately half of the total CD signal, was extremely unstable, because it began to denature at the lowest temperature. The melting of this region appeared very noncooperative, with the transition being almost flat rather than sigmoidal. Indeed, this phase resembled the melting of a molten globule more than a folded protein (28). The rest of the molecule, however, was as stable as wild type in the case of R74C/Q149W and only slightly destabilized for R74C/L177I.

Unfolding of New Domains Observed by Fluorescence of Extra Tryptophans—The thermal melts of the mutant proteins were also observed by fluorescence. Two mutant proteins, S242F and R74C/L177I, contained only the single wild type tryptophan residue. As observed by fluorescence (Fig. 6), the thermal melts of these two proteins were similar in shape to that of wild type and could also be fit by a simple two-state transition model. Both proteins, however, were somewhat destabilized with respect to wild type, with Tm values of 54 and 55 °C as compared with a Tm of 58 °C calculated for the wild type protein (Table II). The fluorescence data confirmed that the more stable tryptophan-containing region was not seriously affected by the R74C/L177I substitutions, despite the severe destabilization of secondary structure observed by CD.

The substitutions that introduced extra tryptophans would be expected to reveal new phases in unfolding if the tryptophans were in different domains than the wild type tryptophan residue. The melting curve for R74C/Q149W was not significantly different from wild type and had a Tm of 55 °C. The thermal melt of Y214W, however, was clearly not two-state. These data could be well fit by two transitions, with Tm values of 54 and 29 °C. Thus it appears that the presence of an additional tryptophan residue reveals the denaturation of a second, less stable domain. It is possible that this domain is somewhat destabilized by the effects of the mutation, as the differences between the wild type and Y214W denaturation monitored by CD would also suggest. However, the noncoincidence of the CD and fluorescence data for the wild type protein (8) demonstrates early denaturation of some portions of helical structure that is not picked up by monitoring fluorescence of Trp194 alone. We therefore think it is likely that the region containing Tyr214 unfolds at lower temperatures than that containing Trp194 in the wild type protein as well as the Y214W mutant. This would be consistent with previous results demonstrating that the C-terminal half of the protein is less stable (8).

The early unfolding event seen by CD in the thermal melt of R74C/Q149W was not observed by fluorescence. This may indicate that the destabilization of this mutant protein is primarily due to the R74C mutation, whereas the region around Q149W is not affected by this substitution. The same may be true for the L177I mutation.

Under the conditions used for these experiments, these two mutant proteins were disulfide-linked dimers (5).3 These cysteines must be on the surface of the native conformation. To distinguish the effects of dimerization from that of the mutation itself, thermal melts were redone in the presence of 2 mM DTT for these two mutants and wild type protein (Fig. 7). Thermal melts monitored by fluorescence showed no difference from wild type or with results in the absence of DTT, suggesting that dimerization does not alter stability of the region containing Trp194 or that at 149 in the Q149W mutant strain. By CD, the monomeric proteins were slightly less stable, but the thermal denaturation curves had the same characteristic two phase appearance as in the absence of DTT. Thus it appears that the mutations are intrinsically destabilizing due to loss of the arginine residue, with dimerization not exerting a significant effect on stability.

### DISCUSSION

The mutant proteins studied here were isolated on the basis of defects caused in the intracellular assembly of the phage procapsid at temperatures at the high end of the physiological range (39–40 °C). Both the single amino acid substitutions and multiple amino acid substitutions acted to destabilize the conformation of the purified proteins. The fluorescence quenching experiments indicated that the two tryptophans introduced are

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**TABLE II**

| Mutant       | Tm1 | Tm2 |
|--------------|-----|-----|
| Wild type    | 58 ± 2 | 58 ± 2 |
| S242F        | 54 ± 2 | 54 ± 2 |
| Y214W        | 54 ± 3 | 29 ± 2 |
| R74C/L177I   | 54 ± 1 | 54 ± 1 |
| R74C/Q149W   | 55 ± 1 | 55 ± 1 |
at the protein surface. The cysteine substitution for arginine must also be at the protein surface. The location of the phenylalanine at the Ser242 site is unclear.

Temperature-sensitive mutations fall into at least two categories: thermolabile mutants, which destabilize the native state of the folded protein (23), and temperature-sensitive folding mutants, which do not affect the stability of the folded state but destabilize folding intermediates (29–31). ts mutants of T4 lysozyme represent the thermolabile class. The sites of the ts substitutions are buried in the protein and are thought to act by destabilizing the hydrophobic core (22). In contrast the tsf mutants of the P22 tailspike are predominantly at the protein surface (32, 33).

The scaffolding ts mutants fit neither class. At least three of them are at the protein surface, and yet they appear to destabilize the native state. We believe this reflects the distinct properties of the scaffolding protein, with an extended, flexible structure that lacks a typical hydrophobic core. Folding of such a protein is expected to be a simple process that would not involve any thermal-sensitive folding intermediates. Due to its lack of packed structure, most residues would be relatively exposed to the solvent, and mutant substitutions would have mainly local effects. The observed functional defects of the mutations examined here probably result from destabilizing the conformation of segments involved in subunit-subunit assembly and dissociation reactions.

A recently proposed model for scaffolding protein structure consisted of two elongated domains made up of helical segments (34). Interactions between the two halves of the protein appeared to be important for stabilization of the C-terminal domain, which was markedly less stable than the full-length protein when expressed as a fragment (34). This model is supported by our results, because all the mutations result in some destabilization of these regions as well.

The Portal-binding Domain—The mutations S242F and Y214W block the incorporation of the DNA packaging portal into procapsids, indicating that these mutations affect a scaffolding protein site required either for binding the portal or forming an initiation complex to which the portal is recruited. Both mutations appeared to slightly destabilize a C-terminal region, but S242F differed from Y214W in also affecting the stability of N-terminal domains as observed by CD. This difference may explain why S242F also blocks incorporation of the pilot proteins, whereas Y214W does not (12).

A Scaffolding Release Domain—The proteins carrying the R74C/Q149W and R74C/L177I substitutions had significantly decreased stability of a large portion of secondary structure. This destabilization did not seem to be affected by the formation of disulfide-linked dimers between the scaffolding protein subunits. Within cells these mutants impaired the release of the scaffolding subunits from the procapsid (12). These mutant sites seem unlikely to be part of a coat-binding region given the identification of the extreme C terminus as the essential coat-binding region (8). In addition, a cloned scaffolding fragment lacking the N-terminal 140 residues (including Arg74) could assemble into procapsids in vitro (35).

Instead, the site of mutation may be a region involved in scaffolding-scaffolding binding. This suggestion is consistent with recent studies of scaffolding proteins from herpesviruses, which have identified regions N-terminal to the coat-binding domain that mediate scaffolding-scaffolding interactions in vitro (36–38). It is striking that although the mutant proteins are severely destabilized, the phenotypic effect is tighter binding within the capsid lattice (12). Because introduction of the cysteine residues permits dimerization in vitro, this could be retarding the exit of scaffolding molecules by blocking their exit through the channels. This seems unlikely to be the case in vivo, given the reducing environment of the cytoplasm but needs further study.

Taken together these results are most consistent with the idea that the mutations alter a domain with a specific active function, for example of a scaffolding “release switch,” that it can no longer perform. The mutations may directly disrupt a release domain by destabilization. Alternatively, the mutations may disrupt interactions between domains, as indicated by the dramatic loss in unfolding cooperativity observed by CD, so that the release signal cannot be propagated from the N-terminal domain that interacts with other scaffolding protein subunits to the C-terminal domain involved in binding to the coat protein.

Acknowledgment—We thank Peter E. Prevelige, Jr. for communication of unpublished results.

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