Temperature-sensitive Mutations in the Phage P22 Coat Protein Which Interfere with Polypeptide Chain Folding*

Carl L. Gordon$ and Jonathan King§

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Temperature-sensitive mutations in the coat protein of phage P22 severely restrict formation of infectious particles at restrictive temperature. A set of 25 temperature-sensitive strains, which had been localized to regions of the coat gene (Casjens, S., Eppler, K., Sampson, L., Parr, R., and Wyckoff, E. (1991) Genetics 127, 637–647), define 17 sites of single amino acid substitutions by DNA sequencing. Particles assembled from the mutant proteins at permissive temperature were not thermolabile at restrictive temperature, nor defective in the infectious process. At restrictive temperature, ts mutant polypeptide chains were synthesized at near wild-type rates. These inactive chains were not degraded within the cells. The ts chains failed to interact with scaffolding proteins to form the procapsid precursor shell, and they did not polymerize with each to form aberrant shells. Rather, the mutant coat protein accumulated as insoluble aggregates, with the amorphous morphology of inclusion bodies. The results indicate that the chains fail to reach the conformation needed for subunit-subunit or subunit-scaffolding interaction. These mutations appear to be of the class of temperature-sensitive folding mutations, which destabilize an intermediate in the intracellular folding pathway.

The capsids of viruses are constructed as polymers of one or a small number of major capsid proteins (Caspar and Klug, 1962). In the mature viruses, these subunits make intimate contact with their neighbors, forming stable protective coats for their nucleic acids packaged within. In many double-stranded DNA-containing viruses, including the bacteriophages T4, A, P22, T7, and Qβ (Hendrix, 1985), herpesviruses (Newcomb and Brown, 1991), and adeno-viruses (Horwitz, 1991), the mature capsid is not formed directly from the polymerization of viral coat proteins. The coat subunits first polymerize with scaffolding proteins to form a precursor shell, called the procapsid, which does not contain DNA. Upon removal of the scaffolding subunits, the DNA is driven into the shell, and the procapsid lattice transforms into the mature virion particle.

Coat protein molecules which comprise icosahedral viral shells often assume several different conformations. Crystal structures of RNA plant viruses (Harrison, 1984) and SV40 (Liddington et al., 1991) demonstrate that coat protein molecules with the same covalent structure have multiple conformations in the shell. For P22, multiple bonding interactions within the lattice of the procapsid precursor shell and a different set of multiple interactions within the lattice of the mature expanded shell have been observed (Prasad et al., 1993). The coat protein of phage P22 is not covalently modified during the folding and assembly process, so that these states are believed to be encoded in the primary amino acid sequence.

The P22 coat protein, encoded by gene 5 of phage P22, contains 430 amino acids and has a predicted molecular weight of 47,000 (Eppler et al., 1991). In the wild-type P22 assembly pathway (Fig. 1), 420 molecules of coat protein, approximately 300 molecules of scaffolding protein, and minor proteins polymerize into a procapsid (King and Casjens, 1974). Upon DNA packaging, the scaffolding molecules exit and the shell expands (Earnshaw et al., 1976). Other minor proteins are added to seal the DNA in the shell (Strauss and King, 1984).

Temperature-sensitive mutations have been isolated in genes of many organisms, including viruses (Edgar and Liebaluisis, 1964; Schaeffer et al., 1978), bacteria (Hubscher and Kornberg, 1980), yeast (Game, 1976) and Drosophila (Suzuki, 1970). Of those viral coat ts mutations which have been studied in detail, some have been shown to perturb the viral assembly process while others seem to prevent assembly from initiating (Ginsberg, 1979; Behm et al., 1988). This latter class of mutations is not well understood.

A group of well-characterized temperature-sensitive mutations in phage T4 lysozyme have been shown to act by destabilizing the native state of the protein (Hawkes et al., 1984). In contrast, a group of temperature-sensitive mutants of D-lactate dehydrogenase (Truong et al., 1991), luciferase (Sugihara and Baldwin, 1988), and phage P22 tailspike protein (Goldenberg et al., 1983) act by impairing the folding of polypeptide chains carrying these mutations at restrictive temperature, but not the stability of the native folded molecule at restrictive temperature. These mutants, in which folding intermediates are destabilized, have therefore been termed temperature-sensitive folding mutants (Sturtevant et al., 1989; King et al., 1990).

We have been particularly interested in the relation of the folding of coat protein molecules to their assembly into structures in which they assume multiple conformations. For a number of multimeric proteins whose folding and subunit assembly pathways have been well characterized (Goldenberg and King, 1982; Jaenicke, 1987), the monomers are not completely folded and are not in the conformation found in the final multimer.

We describe here the nature of the intracellular defect associated with a set of temperature-sensitive mutations of
the P22 coat protein. The results indicate that the ts mutations prevent the polypeptide from assembling into the pro-"capsid at restrictive temperature. The assembly-competent conformation of coat protein is not stably formed in these infections. The coat protein then misfolds and accumulates in the misfolded inclusion body state, which is consistent with a defect in protein folding (Mitraki and King, 1989).

EXPERIMENTAL PROCEDURES

Bacteria—All strains were derivatives of Salmonella typhimurium LT2. The suppressor minus host DB7136 (Leu4, arg4, his3, C225am, supE) have been described (Winston et al., 1979).

Bacteriophage—The strains carrying mutations in the coat protein gene are described in Table I. Most of these temperature-sensitive mutations were isolated by mutagenizing phage and screening phage for temperature-sensitive growth. A second group was isolated by screening apparent revertants of cold-sensitive mutations in gene 1 for temperature sensitivity (Jarvik and Botstein, 1975). A third group was isolated by screening apparent revertants of a gene 1 amber mutation growing on cells which inserted an amino acid not yielding functional gene 1 protein for temperature sensitivity (Jarvik and Botstein, 1975). A third group was isolated by screening apparent revertants of a gene 1 amber mutation growing on cells which inserted an amino acid not yielding functional gene 1 protein for temperature sensitivity (Jarvik and Botstein, 1975). A third group was isolated by screening apparent revertants of a gene 1 amber mutation growing on cells which inserted an amino acid not yielding functional gene 1 protein for temperature sensitivity (Jarvik and Botstein, 1975).

Media—Luria Broth was used to support bacterial growth for phage experiments and preparation of phage stocks. M9 medium was 0.6% NaHPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.01% NH₄Cl. M9/Mg²⁺ medium was M9 plus 0.002 mg MgsO₄. Minimal medium was M9/Mg²⁺ plus 0.4% glucose, 0.005% histidine, 0.008% leucine, 0.04% methionine, 0.002 mg FeCl₃, 10⁻⁵ M CaCl₂.

Preparation of Phage Stocks—Young plaques picked with a glass capillary tube were added to 30 ml of LB, about 5 x 10⁹ log-phase cells and aerated by shaking at 30 °C until lysis. Cell debris was pelleted (10,000 rpm for 10 min in a Sorvall SS34). The pellets were drained and resuspended in 4 ml of M9/Mg²⁺. Residual debris was pelleted by a 90-min centrifugation at 15,000 rpm in the SS34 rotor. The pellets were drained and resuspended in 4 ml of M9/Mg²⁺. Residual debris was pelleted by a 90-min centrifugation at 15,000 rpm.

Phage Crosses—Exponentially growing DB7135 cells in broth at a concentration of 2 x 10⁹/ml were infected with phage strains carrying desired alleles, each at a multiplicity of infection of 5. Progeny phage were screened for desired alleles. All mutants discussed in this work were crossed into a cl-7 background.

DNA Sequencing—Eppler et al. (1991) have sequenced the P22 DNA packing genes, which include gene 5 (encoding the coat protein) and surrounding DNA. Following their notation, gene 5 extends from nucleotide 4804 to 6993. Casjens et al. (1991) have mapped most of the coat protein mutations discussed here by deletion mapping to intervals of gene 5, which they call EE, FF, GG, HH, and JJ. We explored this information in the design of oligonucleotide primers and choice of gene 5 regions to be sequenced. In short, symmetric PCR was performed on phage DNA, generating double-stranded DNA spanning regions of interest. An asymmetric PCR reaction was designed.

**Table I**

| Mutant name | Mutagenesis | Origin |
|-------------|-------------|--------|
| ts7         | N           | A      |
| ts22        | N           | A      |
| tsN105      | N           | A      |
| tsN107      | N           | A      |
| tsrH58       | N           | A      |
| ts3         | N           | A      |
| ts13         | N           | A      |
| ts13.1       | N           | A      |
| ts15.1       | N           | A      |
| ts15         | N           | A      |
| ts15.2       | N           | A      |
| ts5          | N           | A      |
| ts6          | N           | A      |
| ts10         | N           | A      |
| tsN26       | N           | A      |
| ts26.1       | N           | A      |
| tsrH137      | N           | A      |
| tsrH137C     | N           | A      |
| tsrH137D     | N           | A      |
| tsrH58G      | N           | A      |
| ts11         | N           | A      |
| ts34         | N           | A      |
| tsrU221      | N           | A      |
| tsrH58H      | N           | A      |

*The mutant nomenclature follows Casjens et al. (1991).*

The abbreviation used is: PCR, polymerase chain reaction.
TABLE III

Nucleotide sequence of temperature-sensitive mutations in the coat protein gene

| Mutant       | Nucleotides sequenced\(^a\) | Nucleotide substitution | Amino acid substitution\(^b\) | Local amino acid sequence\(^c\) |
|--------------|-----------------------------|-------------------------|-----------------------------|-------------------------------|
| tsrU221      | 1-1293                      | TG142:43CA              | Trp\(^{14}\) → Gln          | SSNTIWMPVEQ                  |
| ts7          | 300-480                     | C323T                   | Ala\(^{33}\) → Val          | RIQSAAARKLAN                 |
| ts22, ts15.1 | 470-755                     | G520A                   | Asp\(^{15}\) → Asn           | FNFQPQDKKAG                  |
| tsrH58E      | 470-755                     | A521G                   | Asp\(^{15}\) → Gly\(^*\)     | FNNPQDKKAG                   |
|              |                             | G583A                   | Ala\(^{33}\) → Thr           | RIPEEAYRGDT                  |
| tsN105, tsN107, tsN13, ts13.1 | 470-755 | C698T                   | Ser\(^{20}\) → Phe           | PVLTKSTATGI                  |
| ts3          | 470-755                     | G695A                   | Gly\(^{29}\) → Asp           | GITVSGAQSKF                  |
| ts8          | 470-755                     | C712T                   | Pro\(^{36}\) → Ser           | AQSFKPVAWQL                  |
| tsN26, ts26.1| 745-995                     | T860C                   | Val\(^{89}\) → Ala           | DATFSVVRVVD                  |
| tsN53        | 745-995                     | T889C                   | Val\(^{89}\) → Ala\(^*\)     | FSVVRVVDGT                   |
|              |                             | T877C                   | Thr\(^{28}\) → Ile           | DNRFATVLTSA                  |
| ts6          | 745-995                     | A905G                   | Asp\(^{62}\) → Gly           | VVRVDGTVE                    |
| tsrH137D     | 745-995                     | C928G                   | Pro\(^{30}\) → Ala\(^*\)     | HVEITPKPVAL                  |
|              |                             | G801T                   | Met\(^{37}\) → Ile           | SATTMKRGDK                   |
| tsrH58G      | 985-1293                    | T1057C                  | Phe\(^{52}\) → Leu           | ARTNVFWADD                   |
| ts10         | 1-1293                      | G1208A                  | Gly\(^{40}\) → Asp           | ISTLSGLCRIA                  |
| tsrH58H      | 985-1293                    | T1231C                  | Tyr\(^{13}\) → His           | RIALWYYGVNA                  |
| ts11, ts34   | 985-1293                    | C1252T                  | Pro\(^{68}\) → Ser           | VNATRPEAIGV                  |

\(^a\) Nucleotide sequence coding for the coat protein extends from nucleotide 1 to 1293.

\(^b\) Where more than one amino acid substitution is present, the one marked with an asterisk is believed to be responsible for the ts phenotype.

\(^c\) Wild-type amino acid which is substituted is shown in **bold**.

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**FIG. 2. Proteins synthesized in cells infected with temperature-sensitive mutants.** Infected cells were prepared and labeled as described under “Experimental Procedures.” A, lysates of infected cells incubated with radioactive amino acids at low temperature. B, lysates of infected cells incubated with radioactive amino acids at high temperature. All phage carried the cl-7 allele, which promotes lysis. Phage in lanes 1-18 and lane 20 carried gene 3 amber and gene 13 amber alleles. Lane 1, wild-type coat protein; lane 2, scaffolding amberN123; lane 3, S223F; lane 4, P310A; lane 5, T294I; lane 6, W48Q; lane 7, F335L; lane 8, Y411H; lane 9, V300A; lane 10, P418S; lane 11, G232D; lane 12, D302C; lane 13, A108V; lane 14, P298S; lane 15, G403D; lane 16, S262F; lane 17, G282D; lane 18, D174N, lane 19, uninfected cells; lane 20, cost amberN114; lane 21, D174G; lane 22, V297A.
utilizing one primer was then performed to generate single-stranded DNA for sequencing.

In more detail, 100-μl PCR reaction vessels for symmetric PCR contained 5 μl of phage stock, 10 μl of 10 × PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂), 0.2 mM concentration each of ATP, CTP, GTP, TTP (Perkin-Elmer Cetus Instruments), 0.5 μl of AmpliTaq polymerase (Perkin-Elmer), and 100 pmol of each primer (all oligonucleotide primers were obtained from the MIT macromolecular synthesis facility). Reaction mixtures were typically exposed to 2 min at 94 °C, 30 cycles of 2 min at 94 °C, 2 min at 72 °C, and 4 min at 72 °C in a Perkin-Elmer Cetus DNA Thermal Cycler 480. One primer was synthesized to be complementary to nucleotides 6193–6169 (primer 1); the other primer used was the same as nucleotides 5199–5223 (primer 2) or 4675–4699 (primer 3). For primer descriptions, the first number represents the 5’ end of the primer; the last number represents the 3’ end of the primer.

Typically, 15 μl of these reactions (now containing several μg/μl DNA of expected size) were electrophoresed through a 1% agarose gel. The band containing the amplified DNA was cut from the gel, precipitated with GeneClean (Bio101), and resuspended in 10 μl of 10 mM Tris, 0.1 mM EDTA, pH 8.0 (TE buffer). A PCR reaction designed to generate single-stranded DNA was then performed using this double-stranded DNA and one of the aforementioned three primers under conditions as above. This reaction mixture was precipitated with GeneClean and resuspended in 15 μl of TE buffer. Typically, 7 μl of this DNA was sequenced with Sequenase (U. S. Biochemical Corp.) using 5–10 ng of primer, with choice of primers described next.

Primer 3 was used for sequencing regions EE and FF (single-stranded DNA generated with primer 1). Primer 4 (corresponding to nucleotides 5000–5016) was used to sequence region GG (single-stranded DNA generated with primer 2 or 3). Primer 1 was used to sequence regions JJ and KK (single-stranded DNA generated with primer 2 or 3).

Gel purifying the double-stranded DNA generated in the first reaction was essential for obtaining consistently readable sequencing gels.

Pulse-Chase Experiments—A 1:100 dilution of an overnight culture of DB 7136 cells was grown at 30 °C in minimal media to a concentration of about 10⁹/ml. Cells were pelleted, resuspended at a concentration of 4 × 10⁹/ml, and put on ice. 0.2 ml of phage at a titer of 2.8 × 10⁹/ml was placed at 39 °C for 90 min, at which time 0.2 ml of cells were added. 12 min after infection, a portion of the culture was placed at 28 °C. The low temperature portion of the infection was labeled at 60 min with 2 μCi/ml 14C-amino acids (Du Pont-New England Nuclear), chased with casamino acids at a final concentration of 2% at 64 min, and icd at 94 min. The high temperature portion of the infection was labeled with 3 μCi/ml of 14C-amino acids at 45 min after infection, chased with casamino acids at 49 min after infection, and put on ice 74 min after infection.

Samples were mixed 1:2 with 3 × SDS sample buffer (0.1875 M Tris, 6% SDS, 15% β-mercaptoethanol, 30% glycerol), frozen at −20 °C, thawed, and electrophoresed through 10% or 7.5% SDS gels. Autoradiographs were prepared from the dried gels.

Pellet/Supernatant Separation—Samples labeled in the above pulse-chase experiment were frozen at −20 °C, thawed, then frozen again in dry ice/ethanol, and thawed. 50-μl samples were centrifuged for 3 min in a Microfuge. The supernatants were removed and the pellets were washed in 50 μl of M9/Mg²⁺ and recenterifuged. These supernatants were removed and combined with the corresponding supernatants from the first centrifugation. The pellet was resuspended in 100 μl of M9/Mg²⁺. Samples were mixed 1:2 with 3 × SDS sample buffer and electrophoresed through SDS gels. Bands were quantified by exposing gels to PhosphorImager screens and using the ImageQuant software (Molecular Dynamics).

Sucrose Gradients—5 ml of exponentially growing cells in minimal media at a concentration of 4 × 10⁹/ml were added to 5 ml of phage at 39 °C, at a multiplicity of infection of 7. This infected high temperature culture was labeled 45 min after infection with 14C-amino acids at 2 μCi/ml and chased for 49 min after infection. The pellets were resuspended in 100 μl of M9/Mg²⁺. Samples were mixed 1:2 with 3 × SDS sample buffer and electrophoresed through SDS gels. Bands were quantified by exposing gels to PhosphorImager screens and using the ImageQuant software (Molecular Dynamics).

Thin Section Electron Microscopy—10 ml of exponentially growing DB7136 cells in LB at a concentration of 4 × 10⁹/ml were added to 10 ml of phage, at a multiplicity of infection of 7, at either 38 °C or 28 °C. The 28 °C infections were placed on ice 2 h after infection; the 38 °C infections were placed on ice 90 min after infection. Fixation, embedding, and sectioning of phage-infected cells was performed essentially as described in Lenk et al. (1975). Electron microscopy was performed with a JEOL 1200 operating at 80 kV.

RESULTS

Twenty-five temperature-sensitive phage strains carrying mutations in the coat protein gene were analyzed. These strains were able to form plaques at 30 °C and were restricted for growth at 39 °C. Twenty-two strains gave very low titers at 39 °C, in the range of reversion frequencies seen for single point mutations (Table II). Three of the strains (tsRU221, tsRH58H, and ts10) formed tiny plaques at 39 °C. The frequency of large plaques formed at 39 °C by tsRU221, tsRH58H, and ts10 was also in the range expected for reversion of a single point mutation.

Sites and Amino Acid Substitutions of the Temperature-sensitive Coat Protein Mutations—These mutations had been mapped to regions of the coat protein gene by Casjens and co-workers (Casjens et al., 1991). To determine the nucleotide changes in the coat protein gene which caused the temperature...
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sensitivity, we sequenced these regions of the coat protein gene, or in some cases the whole gene, using a PCR-based protocol described under "Experimental Procedures." Wild-type DNA was sequenced in parallel. For one strain of the set, ts10, we saw no nucleotide changes by DNA sequencing in the region of the coat protein gene to which the whole phage had been mapped (Casjens et al., 1991). Sequencing the whole coat protein gene revealed a single nucleotide change outside this region, whose deduced amino acid alteration we infer is responsible for the temperature sensitivity. Twenty-one of the strains had single nucleotide replacements corresponding to single amino acid substitutions; three strains had two nucleotide replacements corresponding to double amino acid substitutions; once strain had a triple nucleotide replacement corresponding to a triple amino acid substitution.

Eighteen different patterns of substitutions were found in the 25 strains sequenced. Five nucleotide substitutions were found in more than one (Table I). Three nucleotide substitutions were duplicated in strains with similar names (tsN13 and ts13.1, tsN26 and ts26.1, ts5 and ts5.1) and probably represent stocks derived originally from the same mutant whose names have been altered. The two strains whose deduced amino acid substitution is Asp418 → Gly at 39 °C. We sequenced the region of DNA to which these nucleotide replacements corresponded to multiple amino acid substitutions: one strain had a triple nucleotide replacement corresponding to a triple amino acid substitution.

The reversion frequency data suggested that the temperature sensitivity resulted from single nucleotide substitutions. In the four cases where multiple substitutions were seen in regions to which mutations had been mapped, we attempted to determine the substitution primarily responsible for the temperature-sensitive phenotype. Apparent revertant plaques of tsN53, tsRH137B, tsRH58E, and tsRH137D were picked from restrictive plates and stocks prepared under permissive conditions. These phage did not exhibit temperature sensitivity at 39 °C. We sequenced the region of DNA to which these mutations had been mapped for several apparent revertants of each of these four mutant strains. In each case, we found at least one revertant in which one of the original substitutions had reverted to wild-type. These substitutions, which are marked with an asterisk in Table III, are presumably those responsible for the ts phenotype. The other secondary substitutions appear silent under the conditions tested. For simplicity in the subsequent discussion, we refer to these multiple change mutants by their primary substitution only.

These coat protein ts mutations, which have been gathered from a variety of screens and selections (Table I), are diverse and distributed throughout the coat protein gene, although they appear concentrated in the C-terminal half of the protein. In total, the 25 mutants sequenced have deduced amino acid changes at 17 different sites in the coat protein gene (counting only the primary substitution in the four cases with multiple substitutions). Both hydrophilic and hydrophobic sites are represented. Six of the 18 different amino acid substitutions seen (not counting secondary changes) represent changes in charge (Asp418 → Asn, Asp417 → Gly, Gly423 → Asp, Gly422 → Asp, Gly420 → Asp, and Gly420 → Asp).

The Nature of the Temperature-sensitive Defect—That these coat protein mutants propagate poorly at high temperature indicates that a function of the coat protein important for phage growth is impaired at restrictive temperature. Major stages of coat protein function which have been identified are folding, procapsid assembly, DNA packaging, transformation into stable phage particles, and infectivity. We have at-
attempted to determine the principal in vivo step in P22 growth which has been impaired by each mutation. For the five cases in which the same substitutions were found in more than one mutant, one was chosen for analysis.

The product of phage gene 3 is necessary for DNA packaging into the procapsid particle (Fig. 1). Sixteen of the eighteen ts mutants carrying different nucleotide substitutions were crossed into a gene 3 amber background in order to locate the stage of impaired coat protein function with respect to the DNA packaging event. Functional procapsid particles containing the coat protein accumulate in infections with phage carrying the gene 3 amber allele (King et al., 1973). A gene 13 amber allele, which delays premature cell lysis, was also introduced into these phage strains by genetic crossing.

Are Phage Particles Formed at Low Temperature Functional at High Temperature?—It was possible that input phage particles themselves were thermolabile. Heating phage stocks at 39 °C for 90 min, followed by titering at 30 °C, did not result in a reduction in the number of plaques formed (not shown). This result indicated that the phage particles were not thermolabile at restrictive temperature.

To measure protein synthesis, cells were infected with ts mutants of interest and exposed to radioactive amino acids to follow newly synthesized proteins. The phage were preincubated at 39 °C, and the infection was performed at 39 °C. Twelve min after infection, half the culture was shifted to 28 °C. The high and low temperature cultures were pulsed with 14C-amino acids, chased, and incubated further for 25 and 30 min, respectively. Samples of the lysates were electrophoresed through SDS gels (Fig. 2).

It was possible that the ability of some ts mutants to infect cells was impaired at restrictive temperature. Such mutants would be defective in their ability to induce late phage protein synthesis in this protocol, since we had preheated the input phage and performed the infection at restrictive temperature. The pattern of protein synthesis in uninfected cells is shown in Fig. 2, lane 19. The expected pattern of phage-specific late protein synthesis is clearly evident in all of the mutant-infected cells (King et al., 1973). This result indicates that phage particles containing mutant coat protein were not defective in stability or infectivity at restrictive temperature.

Is Mutant Coat Protein Present at High Levels within Cells?—As can also be seen in Fig. 2B, the levels of the ts mutant coat proteins at restrictive temperature are for the most part similar in infections with the temperature-sensitive mutants and phage-containing wild-type coat protein. This suggests that impaired synthesis of the mutant coat protein is not the source of the temperature sensitivity. In addition, it appears that newly synthesized ts coat protein was not significantly degraded during the chase period.

Does the Mutant Coat Protein Assemble into Procapsids at Restrictive Temperature?—Translation of scaffolding protein is regulated in phage-infected cells, such that the rate of scaffolding synthesis is inversely proportional to levels of soluble scaffolding protein (King et al., 1978). In gene 3 amber infections, scaffolding protein is sequestered in procapsid particles and does not participate in repression of further synthesis (King et al., 1978). Therefore, a high rate of scaffolding synthesis in coat protein ts/gene 3 amber infections would suggest that the coat protein can still associate with
scaffolding protein in procapsids. A low rate of scaffolding synthesis in these mutant infections would suggest that scaffolding protein cannot stably associate with coat protein.

In comparison to rates of scaffolding synthesis seen in the infection with wild-type coat protein at 39 °C (Fig. 2B, lane 1), scaffolding synthesis is extremely low in infections with the temperature-sensitive coat protein mutants in the gene 3 amber background (Fig. 2B, lanes 3–18). The rate of scaffolding synthesis is comparable to that seen in the coat protein amber infection (Fig. 2B, lane 20), which cannot form procapsids. A possible explanation, which we confirm below, is that the levels of procapsid formation are reduced and that the mutant coat protein is not interacting with scaffolding protein. Fig. 2A shows that for some mutants levels of scaffolding protein synthesis are also reduced at 28 °C, suggesting that the mutations are impairing procapsid production even at the lower temperature.

To determine whether the mutant coat protein was in an essentially soluble or insoluble state, we performed a pellet/supernatant separation on lysates prepared with the 18 different mutants at a high and low temperature. In this procedure, procapsids and phage particles remained in the supernatant. The supernatant and resuspended pellets were electrophoresed through SDS gels. The distribution of coat protein between pellet and supernatant at 28 °C is shown in Fig. 3A and at 39 °C is shown in Fig. 3B.

The wild-type coat protein, when assembled into procapsids in the 3am/13am background, or into phage particles in the 13am background, was not significantly pelleted at both temperatures examined. Coat protein amber fragments, which are unable to form native subunits or shell structures were recovered in the pellet, representing an aggregated state. At restrictive temperature, the coat protein from all of the ts mutant infected cells was found primarily in the low speed pellet (Fig. 3B). At low temperature, some of the mutant proteins were also recovered in the supernatant, presumably assembled into procapsids. The remaining mutants showed a significant decrease in soluble protein even at permissive temperature (Fig. 3A).

The lysate made with the N-terminal scaffolding amber mutation accumulates wild-type coat protein. Some of these molecules polymerize into aberrant shell-like species (Earnshaw and King, 1978). The percentage of ts coat protein which was pelleted was greater than the percentage of wild-type coat
Moving protein pelleted in the scaffolding amber background. This suggests that the defect exhibited by the temperature-sensitive coat protein mutants is not simply loss of the ability to interact with scaffolding protein. It appears that at restrictive temperatures the coat protein mutations give rise to conformations leading to insoluble aggregates (as seen by the low speed pelleting) in place of those necessary for coat/scaffolding or coat/coat interaction.

To analyze levels of procapsid formation with increased precision, we fractionated lysates of phage-infected cells on sucrose gradients such that fewer than 5 procapsids seen per μm² of cells. For each sample, greater than 20 cells were examined.

| Phage strain   | Temperature | Inclusion bodies % | Procapsids × | Aberrants  |
|----------------|-------------|-------------------|--------------|------------|
| Wild-type coat | 38 °C       | 1                 | +            | -          |
| Scaffolding amber | 38 °C    | 3                 | -            | +          |
| Tyr114 → His  | 38 °C       | 27                | -            | +          |
| Asp106 → Asn  | 38 °C       | 36                | -            | +          |
| Pro101 → Ser  | 38 °C       | 16                | -            | +          |
| Gly232 → Asp  | 38 °C       | 24                | -            | +          |
| Thr294 → Ile  | 38 °C       | 17                | -            | +          |
| Phe306 → Leu  | 38 °C       | 12                | -            | +          |
| Wild-type coat | 28 °C       | 0                 | +            | -          |
| Scaffolding amber | 28 °C    | 0                 | -            | +          |
| Tyr114 → His  | 28 °C       | 6                 | -            | +          |
| Asp106 → Asn  | 28 °C       | 4                 | +            | -          |
| Pro101 → Ser  | 28 °C       | 6                 | +            | -          |
| Gly232 → Asp  | 28 °C       | 7                 | +            | -          |
| Thr294 → Ile  | 28 °C       | 2                 | +            | -          |
| Phe306 → Leu  | 28 °C       | 4                 | -            | +          |

* +, greater than 40 procapsids seen per μm² of cells. -, fewer than 5 procapsids seen per μm² of cells.

The inclusion bodies form at high temperature could be forming as a result of destabilization of procapsid particles. To examine the possibility that the inclusion bodies were derived from procapsids dissociating at restrictive temperature, we infected cells with PhaE50 → Leu and labeled at 39 °C and then transferred samples as a function of time to 28 °C. Samples were lysed and fractionated into low speed pellets and supernatants. As shown in Fig. 7A, labeled coat protein which remained at 39 °C for 30 min before being transferred to 28 °C was largely pelleted, indicating that inclusion body formation was irreversible. Newly synthesized coat protein appeared increasingly in the supernatant as the time of incubation at 39 °C was reduced, presumably assembled into procapsids. This suggests that some early step on the pathway leading to inclusion body formation was reversible or that at early times after labeling the newly synthesized coat protein had not yet attained the conformation leading to inclusion body formation.

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As shown in Fig. 7B, coat protein in samples incubated at low temperature, and then shifted up to 39 °C at 12 or more minutes after labeling at 28 °C, remained largely in the supernatant, suggesting that the ts mutant coat proteins were not unstable. While it is possible that procapsids formed at low temperature could have fallen apart at high temperature without irreversible coat protein aggregation, this result indicates that the inclusion bodies do not arise from this process. Coat protein derived from samples transferred to 39 °C at 4 min after labeling was largely pelleted, suggesting that some...
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**Fig. 7. Reversibility of mutant phenotype.** Exponentially growing cells were infected with F353L, in a 3am/13am/cI-7 background. After labeling and chasing at indicated times and temperatures, infected cells were lysed by freezing and thawing. A pellet/supernatant separation was then performed. Samples were electrophoresed through 7.5% SDS gels and exposed to films, which are shown. **A**, infection, labeling, and chase were at 39°C. At times indicated, portions of the samples were shifted to 28°C and incubated further for 30 min. Lane labeled none was put on ice 33 min after labeling at 39°C and was never shifted down. **B**, infection, labeling, and chase were at 28°C. At times indicated, portions of the samples were shifted to 39°C and incubated further for 30 min. Lane labeled none was put on ice 33 min after labeling at 28°C and was never shifted up.

Early step on the procapsid pathway was reversible or that the coat protein had not yet passed through the conformation leading to inclusion body formation.

Coat protein which was labeled after shift-up and maintained at high temperature aggregated, while coat protein which was labeled after shift-down and maintained at low temperature remained in the supernatant (not shown). In these temperature shift experiments, the degree to which the coat protein was pelleted depended on the time of incubation at high temperature, but not the temperature at which the labeled protein was synthesized or the prior temperature of incubation. These results establish that it is the temperature at which coat folding and assembly occur which is of primary importance in determining the phenotype observed.

Boiling any of the labeled samples shown in Fig. 2 before loading them on gels did not change the amount or mobility of coat protein detected, indicating that these inclusion bodies could be solubilized in 2% SDS without boiling (not shown). The P22 coat protein contains 1 cysteine residue. To determine whether or not the inclusion body aggregates contained intermolecular disulfide bonds, samples treated with or without the reducing agent β-mercaptoethanol were electrophoresed through SDS gels. Dimeric coat protein molecules or other aggregated species were not detected in the nonreducing gels (not shown).

**Discussion**

The coat polypeptide chain passes through a number of conformations en route to the mature virion. These include the nascent polypeptide chain, the assembly-competent monomeric conformation, the subunit polymerized with scaffold-
host cells which overexpress the GroEL/S chaperonin proteins substantially alleviate the coat protein temperature-sensitive defects. GroEL has been shown to interact with folding intermediates, thereby preventing their aggregation, but not with native proteins (Viitanen et al., 1992; Zahn and Pluckthun, 1992; van der Vies et al., 1992; Martin et al., 1991). This suggests that the coat protein species destabilized by the \( ts \) mutations is a partially folded intermediate in the folding pathway. The temperature-sensitive folding phenotype has been documented for the P22 tailspike protein, for which \( ts \) mutations act by destabilizing an intermediate in the folding pathway (Goldenberg et al., 1983; Haase-Pettingell and King, 1988; King et al., 1990).

These coat protein \( ts \) mutants appear to exhibit varying degrees of a similar phenotype. While they all lead to aggregation, differences in the extent of this aggregation at 28 °C were seen. The various amino acid substitutions may destabilize different conformations of coat protein or the same conformation but to different extents. Even though the original screens used to generate these mutants were for temperature sensitivity, many of the mutants also exhibited defects in growth at the permissive temperature. It seems that the destabilized conformation of coat protein is also important for folding at the permissive temperature.

A set of temperature-sensitive folding mutations has been studied in T4 lysozyme. In this case, the mutations occurred preferentially in solvent-inaccessible sites as defined by the crystallographic structure (Alber et al., 1987). Some of these mutations have been shown to destabilize the native structure (Hawkes et al., 1984). The temperature-sensitive folding mutations in the P22 tailspike are believed to occur preferentially at surface sites in the native protein (Yu and King, 1988; Villafane and King, 1988). As of yet, a high resolution structure of phage P22 or the coat protein alone, in which the location in the folded protein of residues involved in causing these \( ts \) mutations could be visualized, is unavailable.

It is clear that the location of an amino acid in the coat protein molecule, not just the amino acid substitution itself, is important for determining whether or not a substitution leads to temperature sensitivity. For example, the substitution Ala\(^{198}\) → Val is temperature-sensitive, as are the substitutions Val\(^{177}\) → Ala and Val\(^{207}\) → Ala. The substitutions Asp\(^{119}\) → Gly and Asp\(^{302}\) → Gly are temperature-sensitive, as are the substitutions Gly\(^{292}\) → Asp, Asp\(^{307}\) → Asp, and Gly\(^{403}\) → Asp. Thr\(^{368}\) → Ile appears to be a silent mutation even though Thr\(^{368}\) → Ile is temperature-sensitive. The 25 mutant strains examined here were derived from several independent screens. The observation that this diverse set of amino acid substitutions all interfere with chain folding indicates that this is the major mechanism of temperature sensitivity for the coat protein. We believe this reflects the lability of the putative folding intermediate, in comparison with the stability of its own native polymerized state.

Three of the \( ts \) mutations analyzed here (\( ts \) RH137D, \( ts \) RH137C, and \( ts \) RH137D) were isolated originally as second site suppressors of cold sensitive mutations in gene I, which encodes the portal protein (Jarvik and Botstein, 1975). These mutations cluster around amino acid 300 and may identify a site in the functional coat protein which interacts with the portal protein during assembly.

As can be seen in Fig. 2, the \( ts \) coat protein mutations perturb the accumulation of some other phage proteins. First, as discussed above, the scaffolding synthesis is reduced in these infections at restrictive temperature. Second, the minor proteins gp16 (Fig. 3) and gp20 (not shown) are barely de-

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fectable in the phage mutant lysates at restrictive temperature, a result not seen in the infection with phage carrying the coat protein amber mutation. Third, the folding of the P22 tailspike protein into trimers is impaired in the coat protein mutant infections. Native P22 tailspike protein forms a trimer resistant to SDS denaturation (without heating), while partially folded tailspike or tailspike aggregated into inclusion bodies migrates as monomers on SDS gels (Haase-Pettingell and King, 1988). As is shown in Fig. 3, the fraction of tailspike protein which forms SDS-resistant trimers is reduced by the presence of the coat protein mutations.

In general, temperature-sensitive mutants which have been isolated in coat proteins of other viruses have not resulted in the formation of thermolabile virions. Some temperature-sensitive and absolute lethal mutants isolated in other viral coat proteins are defective in the assembly process, while other mutations prevent assembly from initiating. Two temperature-sensitive mutants in the T4 coat protein gene 23 and seventeen temperature-sensitive mutants in the T4 coat protein gene 24 were isolated by Edgar and Lielausis (1964) (Edgar et al., 1964). For three of these mutants, the stability of phage particles produced at permissive temperature was examined, and they were found to show no substantial difference in their rate of heat inactivation as compared to wild-type phage. These authors then concluded that the temperature-sensitive step was acting before completion of the mature particle.

Temperature-sensitive mutants in the major coat protein of SV40 have been isolated and described (Behm et al., 1988). At nonpermissive temperature, some mutants form partially assembled virion particles, while others are deficient in the initiation of particle assembly. These defects were interpreted by assuming that each amino acid substitution caused a local perturbation in the native protein structure (Behm et al., 1988).

Ginsberg (1979) and co-workers have studied temperature-sensitive mutants in the adenovirus hexon protein, which is the major coat protein of the virus. Wild-type hexons form stable hexameric structures. One set of mutants yielded hexon protein which failed to assemble into hexamers. A second set could assemble into hexamers but was deficient in transport into the nucleus. A third set formed hexamers which were transported into the nucleus but which were deficient in their ability to assemble into capsids (Ginsberg, 1979).

Katsura has reported the isolation of a set of missense mutations in phage λ gene E, which is the major coat protein of λ (Katsura, 1980). The mutants he isolated were absolute lethals which were propagated as prophage. Of 71 such mutants studied, Katsura reported that 41 produced no head-related structures, 16 produced polyheads (tubular structures), 8 produced petit λ particles, and 7 produced structures of normal size (Katsura, 1980). The mutants which produced no head-related structures were not characterized further.

Thus, in SV40, adenovirus, and λ, mutations have been identified which prevent shell assembly from initiating. The studies reported in this work may provide an explanation for such mutations.

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