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Structure and Functions of the Bacteriophage P22 Tail Protein

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The product of gene *9* (gp9) of *Salmonella typhimurium* bacteriophage P22 is a multifunctional structural protein. This protein is both a specific glycosidase which imparts the adsorption characteristics of the phage for its host and a protein which participates in a specific assembly reaction during phage morphogenesis. We have begun a detailed biochemical and genetic analysis of this gene product. A relatively straightforward purification of this protein has been devised, and various physical parameters of the protein have been determined. The protein has an $s_{20,w}$ of 9.3S, a $D_{20,w}$ of 4.3×10^{-7} cm^2/s, and a molecular weight, as determined by sedimentation equilibrium, of 173,000. The purified protein appears as a prolate ellipsoid upon electron microscopic examination, with an axial ratio of 4:1, which is similar to the observed shape when it is attached to the phage particle. The molecular weight is consistent with the tail protein being a dimer of gp9 and each phage containing six of these dimers. An altered form of the tail protein has been purified from *supF* cells infected with a phage strain carrying an amber mutation in gene *9*. Phage "tailed" with this altered form of gp9 adsorb to susceptible cells but form infectious centers with a severely reduced efficiency (ca. 1%). Biochemical analysis of the purified wild-type and genetically altered tail proteins suggests that loss of infectivity correlates with a loss in the glycosidase activity of the protein (2.5% residual activity). From these results we propose that the glycosidic activity of the P22 tail protein is not essential for phage assembly or adsorption of the phage to its host but is required for subsequent steps in the process of infection.

The first step in a bacteriophage infection is the recognition of host cell receptors by the adsorption organelles of the phage. In the case of phage P22, which infects *Salmonella* serotypes A, B, and D, the cellular receptor is well characterized; it is the repeating structure of the O-antigen lipopolysaccharide (12).

The interaction between phage P22 and susceptible bacteria results in the specific enzymatic cleavage of a particular rhamnosyl-1,3-galactose linkage in the O-antigen (12). Highly purified preparations of a particular phage structural protein, the "tail protein," can adsorb reversibly to P22-sensitive strains of *Salmonella* (11). They contain a glycosidic activity normally associated with the phage particle which is capable of hydrolyzing the O-antigen (7, 13). P22 heads which lack only the tail protein due to a mutation in gene *9*, the structural gene for the tail protein, neither infect nor adsorb to cells (4, 10). These particles also lack the phage-specific rhamnosidase activity (unpublished data).

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The tail protein is also involved in the P22 life cycle at another point. The addition of the tail protein to heads is the last step in P22 morphogenesis (16). The attachment of P22 tails to heads proceeds very efficiently in vitro and was one of the first phage assembly reactions to be studied in vitro (10). Therefore, not only does the tail protein of P22 function during the infection process as a supramolecular enzyme recognizing specific sugars in the polysaccharide receptor, but it also participates during phage morphogenesis by recognizing specific attachment sites on other phage structural proteins.

We have begun to examine the multifunctional aspects of the P22 tail protein by using genetic, physical, and biochemical techniques to study its structure and identify its functions. In this paper we describe a physical characterization of the tail protein and an analysis of the defects of an altered form of the protein isolated from a mutant phage strain.

MATERIALS AND METHODS

Media and buffers. LB contains 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 ml of 1 N NaOH per liter. Super broth contains 32 g of tryptone, 20 g of yeast extract, 5 g of NaCl, and 0.2 g of NaOH per liter.

M9 salts contains 5.8 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of NaCl, and 1.0 g of NH_4Cl per liter, and is made 1 mM $MgSO_4$ after autoclaving. BS is 0.85% NaCl (wt/vol) in 66 mM potassium phosphate buffer (pH 7.1). Buffer B consists of 50 mM Tris-hydrochloride (pH 7.4)-10% (vol/vol) glycerol-2 mM EDTA-3 mM 2-mercaptoethanol, with different concentrations of NaCl, expressed in millimoles, indicated by subscripts. Lambda agar contains 10 g of tryptone, 2.5 g of NaCl, and 11 g of agar per liter. Soft agar contains 8 g of nutrient broth, 5 g of NaCl, and 7 g of agar per liter. Lambda plates and soft-agar overlays are routinely used for P22 plating.

Strains. All bacterial strains used are derivatives of *Salmonella typhimurium* LT2. DB7000 (*leuA am*414) and DB7004 (*leuA am*414 *supE*) are restrictive and permissive for P22 amber mutants, respectively. The series of strains carrying amber suppressors was isolated and generously supplied by Fred Winston. The following strains were used: DB7136 (*leuA am*414, *hisC am*527), DB7154 (*leuA am*414, *hisC am*527, *supD*), DB7155 (*leuA am*414, *hisC am*527, *supE*), and DB7156 (*leuA am*414, *hisC am*527, *supF*) (22).

The isolation and characterization of phage P22 mutants is described by Botstein et al. (3). All phage used in this study are from the collection of David Botstein. P22 (*c2 am*8, *mnt*-1, 5^- *am*N114, 8^- *am*H202, 13^- *am*H101) was used for the production of wild-type tail protein. P22 (*c1*-7, 3^- *ts*3.1, 9^- *am*H1014, 13^- *am*H101) was constructed for production of the mutant protein described in this paper.

Purification of P22 heads. Purified P22 heads (9^- particles) are prepared from extracts of DB7000 (su^-) infected with P22 (*c1*-7, 9^- *am*N110, 13^- *am*H101). Cells are grown at 37°C in LB broth to 2×10^8 per ml and infected at a multiplicity of 5 phage per cell. After 90 min of continued aeration, the cells are harvested by centrifugation (5,000 rpm, 20 min, Sorval GSA). The infected cells from 250 ml of culture are suspended 50-fold, concentrated in M9 salts, and lysed by agitation in the presence of a minimal amount of $CHCl_3$ by heating to 37°C and cooling to 0°C twice. The resulting extract is centrifuged at 10,000 rpm for 10 min (Sorval SS34) to remove debris. The supernatant fraction is diluted to 9 ml and then centrifuged into a CsCl block gradient (in a Beckman SW27.1 tube: 2 ml of 1.7 g/cm^3, 2 ml of 1.5 g/cm^3, 4 ml of 1.3 g/cm^3 in 10 mM Tris-hydrochloride [pH 7.6]-5 mM $MgSO_4$ [TM]) for 90 min at 25,000 rpm at 20°C. The opalescent band of phage heads in the 1.5 g/cm^3 layer is removed with a 20-gauge needle and syringe through the side of the tube. The phage head solution is dialyzed against TM and repurified on a second CsCl block gradient in an SW50.1 tube (2 ml of 1.3 g/cm^3, 1 ml of 1.5 g/cm^3, 1 ml of 1.7 g/cm^3). A 1-ml amount of sample is layered onto this gradient and centrifuged at 45,000 rpm for 60 min at 20°C. The phage head band is removed and dialyzed as described above.

At least 10^{13} heads are recovered from such a preparation. The fraction of contaminating phage is usually less than 10^{-5}. Heads are diluted to 10^{11}/ml and stored in M9 salts or BS. Quantitation of heads is accomplished by serially diluting a head preparation in M9 salts and incubating with either crude or purified P22 tails. After 2 h of incubation at 37°C, the reactions are further diluted and plated on DB7004. The number of heads per milliliter is calculated from that portion of the dilution curve in which tails are in excess (see Fig. 2 in reference 10) and in which the reaction has gone to completion (no further increase in titer upon prolonged incubation).

Purification of P22 tails. DB7000 is grown in super broth at 37°C to a density of 5×10^8 cells/ml. These cells are infected at a multiplicity of 5 with P22 (*c2 am*8, *mnt*-1, 5^- *am*N114, 8^- *am*H202, 13^- *am*H101). After infection, the culture is aerated vigorously for 3 h and the cells are harvested by centrifugation. The cell pellet from 12 liters of culture (53 g) is frozen at −80°C after the addition of 20 ml of B_{25}. The pellet is thawed at 4°C and is passed through a French press at 1,300 kg/cm^2. An additional 40 ml of B_{25} is added to the suspension, which is then centrifuged for 90 min at 49,000 rpm in a Beckman Ti 50 rotor. The supernatant fraction (58 ml) is decanted, diluted to 275 ml with B_{25}, and brought to 20% $(NH_4)_2SO_4$ saturation by the addition of 29.1 g of solid ammonium sulfate. After dissolving the ammonium sulfate the solution is left at 4°C for 1 h and then clarified by centrifugation in a Sorval GSA rotor at 10,000 rpm for 10 min. The supernatant fraction (350 ml) is decanted from a very small pellet and brought to 35% saturation by the addition of 37.1 g of solid ammonium sulfate. After stirring overnight at 4°C, the cloudy solution is clarified by centrifugation in the Sorval GSA rotor at 12,000 rpm for 2.5 h. The resulting precipitate is suspended in 160 ml of B_{25} and dialyzed against several changes of this buffer.

The dialyzed material is applied to a DEAE-cellulose column (35 by 5 cm, Whatman DE-52) equilibrated with B_{25}. The column is washed with 1 liter of B_{25} and eluted with a 5-liter linear gradient made from equal volumes of B_{25} and B_{220}. The majority of the P22 tailing activity elutes between 50 and 75 mM NaCl. The peak fractions are pooled and concentrated by the addition of solid ammonium sulfate to 50% saturation. The ammonium sulfate precipitate is suspended in B_{25}, dialyzed against B_{25}, and passed over a BioGel A 0.5-m (200 to 400-mesh) column (2.6 by 85 cm) in two 6-ml portions. Peak fractions from the sequential runs are pooled. The yield is 58 ml, with 6.3×10^{14} phage equivalents of tail protein per ml.

Tail protein is assayed during the purification by a rapid qualitative assay. Serial 10-fold dilutions of fractions suspected of containing tail protein are spotted onto plates seeded with approximately 2×10^7 purified P22 heads and 2×10^8 DB7004 bacteria. These plates are incubated for 4 h or longer at 39°C. On these plates, P22 heads become tailed where sufficient concentrations of tail protein exist and subsequently infect the bacterial lawn. Continued growth of phage is maintained by the (su^+) bacteria, leading to zones of clearing of the bacterial lawn. Relative quantitation of tail protein can be estimated from the time of appearance of the cleared zone and the extent of clearing. This assay can detect the presence of as few as 5×10^8 phage equivalents of tail protein per ml.

^{14}C-labeled P22 heads. DB7000 bacteria are grown at 37°C in M9 minimal medium plus leucine at 20 μg/ml. At 2×10^8 cells/ml P22 (*c1*-7, 9^- *am*N110, 13^- *am*H101) phage are added at 5 phage per cell. The

culture is aerated for 35 min and labeled for 10 min with the 1-μCi/ml ^{14}C-labeled amino acid mixture (no. NEC445; New England Nuclear Corp.). After the labeling period, the culture is made 0.2% Casamino Acids and incubated an additional 10 min. The cells are harvested by centrifugation at 5,000 rpm for 10 min (Sorval SS34). The labeled heads from 30 ml of culture are purified as described above for unlabeled heads, using two consecutive SW50.1 CsCl gradients.

Rhamnosidase assays. The release of radioactivity from ^{14}C-labeled, acid-killed bacteria is used to quantitate the rhamnosidase activity of P22 tail protein (12, 14). ^{14}C-labeled cells are prepared by growing DB7000 in LB broth at 37°C to 3×10^8 to 4×10^8 cells/ml. To 20 ml of culture is added 200 μCi of [^{14}C]glucose (240 mCi/mmol; Schwarz/Mann), and growth is continued for 40 min. At that time, 4 ml of 3 N H_2SO_4 is added, and incubation is continued for 10 min. The labeled cells are harvested, washed twice in BS, and stored 10-fold concentrated. The substrate is relatively stable to storage at 4°C for 1 month. The cells were usually washed once in BS before use after prolonged storage. Each assay contains 20,000 cpm of labeled cells and tail protein in 100 μl of BS or M9 salts. The reactions are terminated by the addition of 1 ml of 1 M acetic acid and chilling the reactions on ice. The reactions are centrifuged at 5,000 rpm for 10 min (Sorval SS34). The radioactivity in 0.5 ml of the supernatant fraction is measured by counting in 10 ml of Biofluor (New England Nuclear).

Quantitative tailing assays. Purified P22 heads (0.99 ml at 10^{10}/ml in M9 salts) are mixed with 10-μl portions of serial dilutions of tail protein, incubated for 2 h at 37°C, and titrated for viable phage on DB7004. The number of PFU in each reaction is plotted versus the log of the relative concentration of tail protein (10) (see Fig. 4). Extrapolation of that portion of the plot where heads are in excess to the plateau where tail protein is in excess gives the dilution factor required to reach head-tail protein equivalence. Tail protein concentrations are calculated from this dilution factor and the final titer of the reaction and expressed as phage equivalents.

Spot tests for phage growth. Phage are diluted in M9 salts to approximately 10^9/ml. Portions of 10 μl are transferred to a freshly plated soft agar overlay containing 2×10^8 log-phase plating bacteria of the genotype to be tested. The plates are incubated at the required temperature and scored after 12 to 18 h.

Analytical ultracentrifugation. Samples used in ultracentrifugation experiments are dialyzed against 10 mM Tris-hydrochloride (pH 7.5)–100 mM NaCl. The dialysate is used as a blank sample in all runs. Both the sedimentation velocity and equilibrium experiments are performed at three concentrations, corresponding to 0.25, 0.50, and 0.75 mg/ml. In general, no dependence upon concentration is observed, and the results presented are the average values for the three concentrations used. The centrifuge was a Spinco model E equipped with a UV Scanner/Multiplexer and monochrometer. The velocity experiments are performed in a type AN-F rotor, and the equilibrium experiments are performed in a type AN-J rotor. Both rotors are equipped with 12-mm, double-sector Kel-F centerpieces and sapphire windows. Scans of the cells are performed at 280 nm at a scan rate of 3.02 cm/min. Scans are recorded on chart paper at 5 mm/s. FC-43 silicone oil is used as a lower boundary in the equilibrium cells. The ultracentrifuge facility was provided by Paul Doty, Harvard University Biology Laboratories.

Amino acid analysis. Samples to be analyzed for amino acid content are exhaustively dialyzed against water and lyophilized to dryness. For our particular analysis, 1 mg of purified tail protein is suspended in 1 ml of 5.7 N HCl–1% (vol/vol) phenol. The sample is sealed in vacuo, incubated for 24 h at 108°C, and lyophilized to dryness. Amino acid analysis was performed on a Beckman 121M amino acid analyzer.

Protein assays. Protein is assayed by the colorimetric method of Lowry et al. (17). All samples are precipitated with 5% cold trichloroacetic acid and suspended in 0.1 M NaOH before assaying. Bovine serum albumin is used as a standard protein.

RESULTS

Purification of P22 tails. We have devised a convenient way to purify large amounts of highly active P22 tail protein. Cells infected with P22 mutants which are either defective in DNA encapsulation or which produce an inactive immunity I repressor (*mnt*$^-$) or which fail to lyse the cells at the normal time after infection overproduce the tail protein (S. Casjens, G. Weinstock, and R. Griffin Shea, unpublished data). The mechanisms involved in the overproduction are unknown. The phage mutant that was used to produce P22 tail protein was constructed to take advantage of all of these overproducing phenomena.

The purification scheme outlined in the Materials and Methods section resulted in a yield of approximately 210 mg of tail protein from 53 g of infected cells. Recovery was approximately 50% of the activity in the high-speed supernatant, with most of the loss occurring in the ammonium sulfate fractionation (data not shown). Subsequent experience has indicated that higher recoveries are possible with precipitation at higher ammonium sulfate concentrations. The purified tail protein migrates as a polypeptide with the mobility of the gp9 component of P22 phage particles on a sodium dodecyl sulfate-polyacrylamide gel (Fig. 1).

Physical characterization of P22 tail protein. A series of analytical ultracentrifuge experiments was performed to determine the size of the active tailing unit. P22 tail protein amino acid composition (moles percent) was as follows: Asx, 12.9; Thr, 7.0; Ser, 7.4; Glx, 7.3; Pro, 4.6; Gly, 11.3; Ala, 7.8; Val, 7.4; Met, 1.2; Ile, 7.1; Leu, 8.2; Tyr, 4.4; Phe, 2.4; His, 1.6; Lys, 5.6; Arg, 3.8; Trp, not determined; and Cys, not determined. From these data, a partial specific volume of 0.73 cm^3/g was calculated (5). Sedimentation

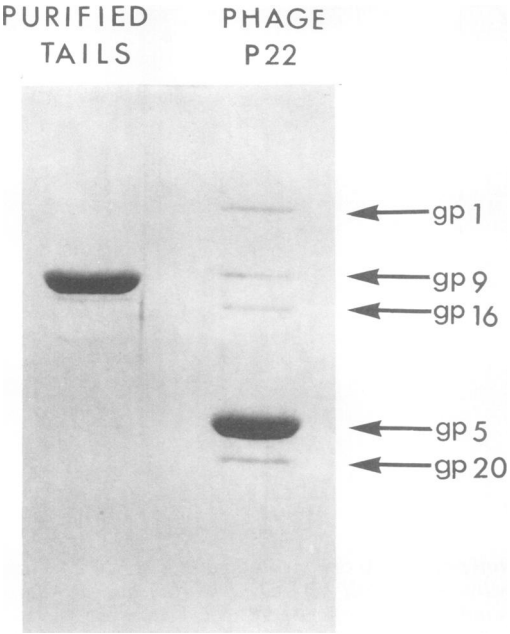

FIG. 1. *Sodium dodecyl sulfate gel electrophoresis of purified P22 tail protein. Samples containing equal amounts of protein were prepared for electrophoresis as previously described (2). Purification of tail protein and P22 phage are described in the text. The acrylamide concentration in the gel was 10%. Gels were stained with Coomassie brilliant blue. Five of the nine polypeptides which comprise P22 phage are identified on this gel. The remaining four polypeptides are of smaller molecular weight and have migrated below the limits of this figure. The identification of the polypeptides (16) is according to the nomenclature of Kikuchi and King (15) and indicates the protein product of a known structural gene; i.e., gp1 refers to the product of gene 1 of phage P22. The purified tail protein sample, although severely overloaded, is identified as the gp9 component of P22 phage. The sample of tail protein was greater than 95% homogeneous by this gel analysis.*

velocity measurements indicated an $s_{20,w}$ of 9.3S. One such set of measurements is shown in Fig. 2A. Figure 2B shows a plot of one sedimentation equilibrium distribution of the tail protein; from this and other plots, an average molecular weight of 173,000 ± 2,000 was calculated. It is clear from the plot in Fig. 2B that the tail protein preparation is monodisperse and shows no tendency to self-aggregate.

The diffusion constant of the purified tail protein was determined in the laboratory of Victor Bloomfield, University of Minnesota, using laser-beat spectroscopy. An average $D_{20,w}$ obtained from two measurements at 14 and 4.2 mg/ml was 4.4 (± 0.1) × 10^{-7} cm^2/s.

Examination of the P22 tail protein by electron microscopy. Purified P22 phage and 9^- tailless particles mixed in approximately equal amounts were examined in the electron microscope after negative staining with 2% uranyl acetate. The resulting micrograph (Fig. 3A) shows the characteristic morphology of P22 phage and of 9^- particles (1, 4, 10, 21). The isometric phage head is about 58 nm in diameter, as previously determined by solution X-ray scattering (6). The baseplate structure is 20 nm across and appears to have lateral, spikelike protrusions. These protrusions are distinct from the long spike or fiber which emanates from the center of the baseplate. The 9^- particles lack the lateral protrusions but retain a "neck" structure and the central spike or fiber. The image of the negatively stained preparation of purified tail protein (Fig. 3B) indicates that the active tailing unit resembles the lateral protrusions which are missing in 9^- particles but seems slightly larger than the attached tail protein. The tail protein appears to be a prolate ellipsoid, approximately 25 by 7.5 nm, which agrees reasonably well with previous observations (10). Thus, the baseplate structure of P22 is comprised of a "neck" structure around which are attached several molecules of the tail protein.

Genetically altered tail protein. One method for analyzing the functions of a multifunctional protein is to modify it in specific ways and observe the accompanying change in its activities. We attempted to find variants of the P22 tail protein altered by genetic mutation in such a way as to be partially functional. We obtained 10 strains of P22 carrying amber mutations in gene *9*, the structural gene for the tail protein. These mutants were tested for growth on several host cell strains carrying different suppressor mutations. As described in Materials and Methods, the bacteria tested insert serine (*supD*), glutamine (*supE*), or tyrosine (*supF*) into the nonsense codon site. Growth on these strains was compared with growth on the su^- parent strain. There were, among the 10 phage strains tested, many which showed variations in growth at different temperatures depending upon which bacterial strain was used as a host. Some phage strains failed to grow at 20°C and were thus characterized as cold sensitive (*cs*). Others failed to grow at high temperatures (40°C) on certain suppressors and were thus characterized as temperature sensitive (*ts*). Still others failed to grow at any temperature on a particular suppressor and were thus absolute defective (*abs*). The remainder of this paper describes our characterization of the biological defects of one of these *abs* mutants and the molecular defects of the resulting mutant tail protein.

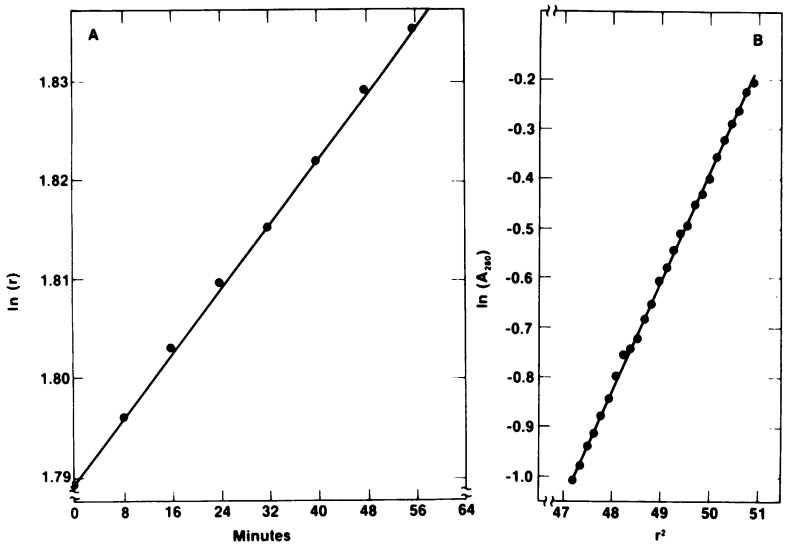

FIG. 2. *Analytical ultracentrifugation analysis of P22 tail protein. (A) Sedimentation velocity. Purified tail protein at a concentration of 0.25 mg/ml in 10 mM Tris-hydrochloride (pH 7.5)-100 mM NaCl was centrifuged at 37,020 rpm at 20°C in a type AN-F rotor. Time points indicated are after rotor achieved the set velocity. Absorbance scans across the entire cell at 280 nm were made every 8 min. The "r" values are determined from the midpoint of the rise in absorbance at 280 nm at the sedimenting protein-buffer boundary. No UV-absorbing material was detected remaining in the upper portion of the cell after the run. (B) Low-speed sedimentation equilibrium. Purified tail protein at a concentration of 0.5 mg/ml in the same buffer described above was centrifuged at 4,609 rpm in a type AN-J rotor at 20°C. Apparent equilibrium was achieved after 24 h; however, the final scan was taken at 48 h. After the final scan, the rotor was accelerated to 16,200 rpm, and the protein-buffer boundary was observed to sediment leaving no residual UV-absorbing material in the upper part of the cell.*

Phage carrying the amber mutation *am*H1014 fail to grow at any temperature on the tyrosine-inserting (*supF*) suppressor strain DB7156. To determine the reason for this growth defect, single-step growth experiments were performed, using three different bacterial strains as host for the infection. The results of these growth experiments, expressed as PFU produced per infected cell, are summarized in Table 1. When P22 *am*H1014 is grown on su$^-$ bacteria, less than 1 PFU/cell is produced. However, by adding purified tail protein to the lysed culture, phage are produced in vitro, indicating that several hundred heads per cell are produced during the infection. When the amber mutant is grown on glutamine-inserting (*supE*) bacteria, a normal burst of infectious phage is produced. The addition of purified tail protein results in no significant change in the titer of the culture; thus, the particles produced in these infected cells are fully tailed. When *supF* bacteria are used as the host, less than 2 PFU/cell are produced; furthermore, when purified tail protein is added to this culture, no increase in titer occurs. Thus, the phage heads which are produced in this infection seem to have interacted with altered tail protein, rendering them noninfectious.

To determine the nature of the "deleterious activity" of this altered tail protein, we purified it from crude extracts and examined its properties. The P22 (*c1*-7, 3$^-$ *ts*3.1, 9$^-$ *am*H1014, 13$^-$ *am*H101) strain was constructed to infect *supF* cells. The 13$^-$ *am*H101 mutation, like the 9$^-$ *am*H1014 mutation, is functionally poorly suppressed by the *supF* suppressor, making the phage lysis defective in the host strain employed. The 3$^-$ *ts*3.1 mutation causes a block at the DNA encapsulation step of P22 head assembly at 37°C (4), resulting in overproduction of the mutant tail protein and preventing its attachment to head structures. Preparation of infected cells and protein purification steps were as described for the wild-type protein in Materials and Methods, except that the host cell was DB7156, and, in the gel filtration chromatography step, only the leading tail protein-containing fractions were pooled. This was necessary because of a slightly smaller protein species which copurifies with the mutant tail protein through the earlier steps. The mutant tail protein has a low, residual tailing activity (see below) and thus could be detected by the assay described in Materials and Methods. Purification of the mutant tail protein was also monitored by sodium

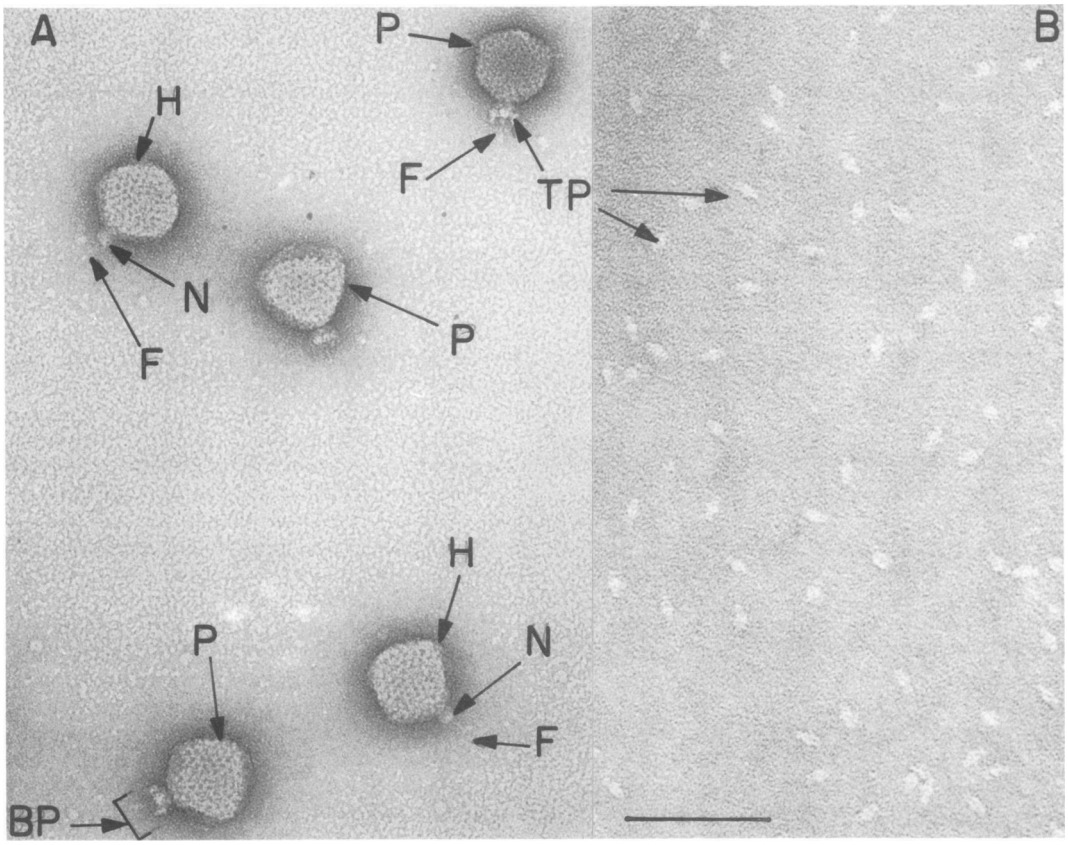

FIG. 3. *Electron micrographic comparison of P22 phage, P22 heads, and P22 tail protein. Grids for electron microscopy were prepared by depositing a thin film of carbon over a Parlodion film and then dissolving the plastic film with amyl acetate vapors. Morphologically identifiable structures are indicated with arrows: H, Head (9⁻ particle); P, phage particle; N, neck structure; F, central fiber; TP, tail protein; BP, baseplate structure (neck + fiber + tail protein). The bar represents 100 nm. (A) Purified P22 phage and heads. Equal numbers of P22 phage and 9⁻ particles were mixed and diluted in TM, and a drop was applied to carbon-coated grids. The grid was washed with water and then washed with 2% uranyl acetate. The stain was blotted off, and the grids were allowed to dry. Grids were examined in a Jeol 100B electron microscope operating at 80 kV accelerating voltage. (B) Purified tail protein. Purified tail protein was serially diluted in TM and applied to grids and stained as above. This particular micrograph was chosen because individual protein molecules were well resolved. Operating voltage was 100 kV.*

dodecyl sulfate-polyacrylamide gel electrophoresis. Thus, we obtained a homogeneous preparation of tail protein with a tyrosine residue inserted at the position of the *am*H1014 mutation.

Tailing activity of the *am*H1014(*supF*) tail protein. Quantitative tailing assays were performed by mixing purified phage heads with serial dilutions of both the wild-type and mutant tail protein preparations. The results of these assays are plotted in Fig. 4. As increasing amounts of wild-type protein are added to a constant number of heads, the final titer of the reaction increases until a plateau is reached when all heads are saturated. The slope of the log PFU versus log tail protein concentration is approximately 3.3, as originally described by Israel et al. (10). The same slope is obtained when the mutant tail protein is assayed. However, the final plateau value of infectious particles is 100-fold lower than the value of the wild-type protein reactions. Portions of the mutant tail protein reactions were mixed with an excess of wild-type protein and reincubated to determine the number of tailable heads remaining after the first incubation. The results from this two-step reaction (Fig. 4) indicate that the plateau value reached in the mutant protein assay represents complete tailing of all available heads with mutant protein. The data in Fig. 4 also indicate that the concentration of mutant protein required to saturate the heads is about four

TABLE 1. *Growth pattern of 9⁻ amH1014*[a]

Host	Phage/cell	Heads/cell
DB7136 (su⁻)	0.2	400
DB7155 (*supE*)	330	288
DB7156(*supF*)	1.7	2.0

[a] Mid-log phase cells at 2×10^8/ml in LB were infected at a multiplicity of 7 phage per cell. These infected cells were incubated with vigorous aeration for 90 min, and $CHCl_3$ was added to assist cell lysis. Phage titers were determined by plating appropriate dilutions directly onto DB7004 bacteria. Head titers were determined by incubating a portion of the lysate with 2×10^{11} phage equivalents of pure tail protein for 2 h at 37°C before diluting and plating.

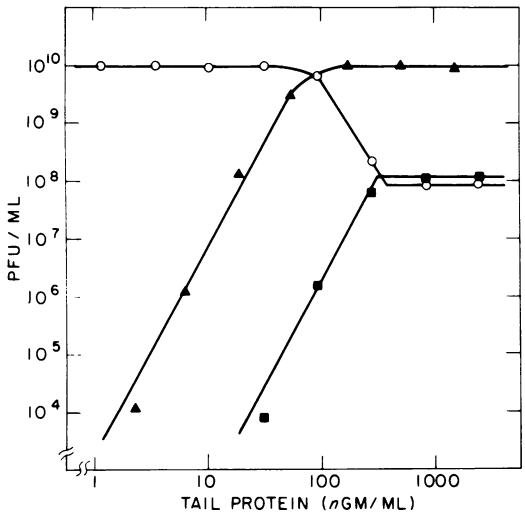

FIG. 4. *Quantitative tailing assay of wild-type and amH1014(supF) tail protein. Serial threefold dilutions of purified wild-type and amH1014(supF) tail protein were made in M9 salts. A 10-μl portion of each dilution was added to 0.99 ml of purified phage heads at 1×10^{10}/ml and incubated at 37°C. After 60 min, 0.15 ml was removed from each reaction and diluted 100-fold to stop the in vitro assembly reaction. To the remainder of those reactions containing amH1014(supF) tail protein was added 8.5 μl containing 1,250 ng of pure wild-type tail protein. These reactions were incubated an additional 60 min and diluted as described above to stop the reaction. The titer of viable phage in each reaction tube was determined by plating an appropriate dilution on DB7004 bacteria. Wild-type tail protein (▲); amH1014(supF) tail protein (■); two-step reaction (○).*

times that required for wild-type protein, as if only 25% of the purified mutant protein is active in the assembly reaction.

Rhamnosidase activity of the mutant protein. What is the molecular basis for the reduced efficiency of plating of the mutant tail protein-containing particles? One easily measured activity associated with the tail protein of P22 phage is its O-antigen hydrolyzing activity which has been shown to be due to a specific endorhamnosidase (12, 13). This activity is thought to be responsible for the adsorption of phage to the O-antigen polysaccharide layer surrounding the cell and for the subsequent penetration of this layer by the phage, thus bringing it to the cell surface.

We measured the rhamnosidase activity of both the wild-type and mutant tail protein by using previously established assay conditions (13, 14) which measure the release of radioactive O-antigen components from acid-killed *Salmonella typhimurium* which had been labeled in a rich medium with [^{14}C]glucose. Up to 40% of the radioactivity incorporated into these cells during the labeling period can be found in carbohydrate components. At least 10% of this incorporated label can be released specifically during incubation of these cells with phage or purified tail protein; furthermore, most of the released material has been shown by chemical analysis to be derived from O-antigen (13, 14).

Mutant or wild-type protein was mixed with labeled cells and incubated at 35°C. The amount of non-sedimentable radioactivity produced was measured as a function of tail protein concentration. It is clear from the results presented in Fig. 5 that the mutant protein has severely reduced enzymatic activity. (Note the two different abscissa scales.) From the linear portion of the curves shown, we estimate the specific enzy-

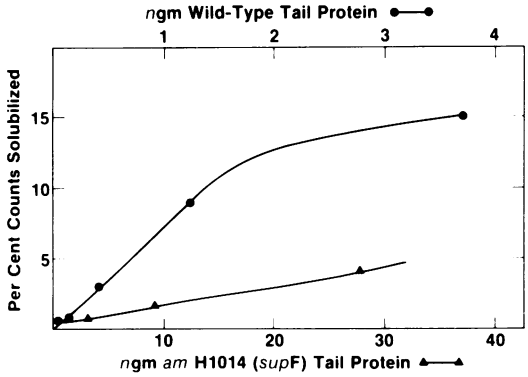

FIG. 5. *O-antigen-releasing activity of purified wild-type and amH1014(supF) tail protein. Serial threefold dilutions of purified tail proteins were made in M9 salts. Portions (90 μl) were mixed with 10 μl of ^{14}C-labeled, acid-killed S. typhimurium. Incubation was at 37°C for 13 min. Samples were processed as described in the text. Total input counts per minute per assay was 24,000. Of the counts, 6% are released by incubation of the cells alone; this background has been subtracted from the data points presented in the figure. Wild-type protein (●); amH1014(supF) tail protein (▲). Note the difference between the two abscissa scales.*

matic activity of the mutant protein to be no more than 2.5% of that of the wild-type protein.

Adsorption properties of the mutant tail protein. The reduced rhamnosidase activity of the mutant protein could be due to poor O-antigen binding or to a low rate of hydrolysis. We attempted to examine binding and hydrolysis separately in the following experiment. Phage heads labeled with radioactive amino acids were mixed with an excess of either wild-type or mutant tail protein. The tailed particles produced after suitable incubation were repurified by differential centrifugation. These two kinds of tailed particles were incubated with susceptible cells under standard conditions where phage adsorption should occur. Portions of these reactions were then mixed with various amounts of purified wild-type tail protein to cause different degrees of hydrolysis of the remaining O-antigen of the cells. The radioactivity remaining in the supernatant fractions after centrifugation to remove bacteria is plotted as a function of the tail protein concentration in the second incubation (Fig. 6). The results indicate that phage heads tailed with either tail protein adsorb to cells, but those tailed with mutant protein are far more susceptible to removal by hydrolysis of the cell O-antigen than are phage heads tailed with wild-type protein. Thus, phage particles containing mutant tail protein are incapable of reaching a stage of adsorption where they no longer depend on O-antigen for binding during a period of time when wild-type particles become irreversibly adsorbed.

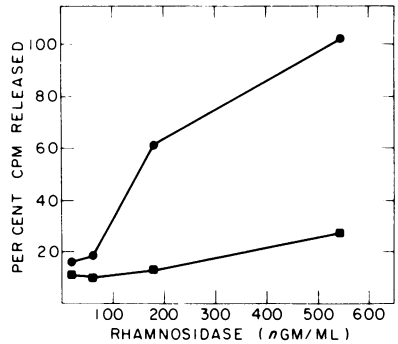

FIG. 6. *Release of adsorbed wild-type and amH1014(supF) tailed phage by enzymatic removal of O-antigen. A 0.1-ml amount of ^{14}C-labeled P22 heads (136,000 cpm, 10^{12}/ml) was mixed with 50 μl of either wild-type or amH1014(supF) tail protein at approximately 8×10^{12} phage equivalents per ml. The tailing reaction was allowed to proceed for 45 min at 35°C. These two-tailed phage preparations were separated from free tail protein by pelleting the phage at 15,000 rpm (Sorval SS34) for 90 min. The phage pellets were suspended in 1 ml of LB. Recovery was about 30%. A 0.9-ml portion of each phage preparation was mixed separately with 8.1 ml of mid-log phase DB7004 cells which had been concentrated to 10^9/ml by centrifugation. Adsorption was allowed to proceed at room temperature for 25 min. Portions (1 ml each) of these adsorption reactions were distributed to tubes containing the indicated amounts of wild-type tail protein. Enzymatic O-antigen release was allowed to proceed for 15 min at room temperature. Each reaction tube was centrifuged at 5,000 rpm (Sorval SS-34) for 10 min, and 0.5 ml of the supernatant fraction was counted in 10 ml of Biofluor. Input counts per minute per final reaction was 1,260 for wild-type tailed phage (■) and 1,940 for amH1014(supF) tailed phage (●).*

DISCUSSION

Size of the P22 tail protein. The P22 tail protein has a partial specific volume of 0.73 cm^3/g, estimated from its amino acid composition listed in Results. Its molecular weight appears to be 173,000 by sedimentation equilibrium. The sedimentation constant is 9.3S, and the diffusion constant is 4.4×10^{-7} cm^2/s. The sedimentation and diffusion constants together permit a second, independent calculation of the molecular weight, according to the equation $S/D = M(1 - \bar{v}\rho)/RT$. The molecular weight thus calculated is 191,000, in modest agreement with the sedimentation equilibrium value.

The sedimentation coefficient obtained in our experiments is in agreement with that previously reported by Israel et al. (10) of 9.5S, and it is slightly larger than that reported by Iwashita and Kanegasaki (13) of 8.3S.

There are several reports of the molecular weight of the P22 tail protein in the literature. Israel et al. (10) obtained a range of 160,000 to 240,000, into which our value falls. Iwashita and Kanegasaki (13) measured a molecular weight of 320,000 by sedimentation equilibrium. Eriksson and Lindberg (7) obtained a value of 240,000.

We believed that our value is correct for the following reasons: the data presented by Iwashita and Kanegasaki (Fig. 7 in reference 13) actually indicate a molecular weight of 189,000, in reasonably good agreement with our value. These authors have reevaluated their data and agree with this revision (S. Kanegasaki, personal communication). The molecular weight estimate of 240,000 by Eriksson and Lindberg is derived from electrophoretic mobility in a nondenaturing gel, which is not a rigorous indicator of molecular weight. The P22 tail protein has an elongated shape (see below) and thus may migrate more slowly in a nondenaturing gel than a globular protein of the same mass.

The apparent monomer molecular weight of gp9 is 76,000 (4, 13). This value is derived from electrophoretic mobility in a sodium dodecyl sulfate-polyacrylamide gel. The data presented in this paper lead us to believe that the tail

protein is a dimer of gp9. If this is the case, then the actual monomer molecular weight must be somewhat greater than 76,000. The tail protein is particularly stable to heat, acidity, chaotropic agents, and several proteases (unpublished data). It thus seems possible that gp9 might not be completely unfolded under the conditions of sodium dodecyl sulfate gel sample preparation and electrophoresis and therefore may migrate anomalously in a sodium dodecyl sulfate gel. Eriksson et al. (8) reported an apparent monomer molecular weight of 16,000 for the P22 tail protein. This value is remarkably different from those reported by others and from what we observe. These authors may actually have measured the size of a fragment of the tail protein. M. R. McConnell (Ph.D. thesis, Tufts University, Boston, Mass., 1977) has preliminary data indicating that the tail protein of the related *Salmonella* phage ϵ^{15} can be reduced in size by 12,000 daltons without affecting its rhamnosidase activity. Similarly, cleavage of a larger polypeptide may have created the enzymatically active fragment characterized by Eriksson et al. (8).

Israel et al. (10) calculated a mass difference between P22 heads and complete phage of 1.2×10^6, based on the difference in density and relative DNA and protein compositions. Taken together with our molecular weight values, this seems to indicate that complete phage contain 6.3 to 6.9 tail protein molecules. A stoichiometry of six tail proteins per phage particle is consistent with the appearance of P22 baseplate structures as seen in the electron microscope (Fig. 3) (1). Electron microscopic examinations of some of our preparations of P22 phage have revealed hexagonally symmetrical objects which seem to be complete baseplate structures released from mature phage (not shown). Such observations have also been made by other investigators (10).

Shape of the P22 tail protein. Measurement of the physical properties described above suggests that the P22 tail protein is irregularly shaped. Assuming that the shape is best approximated by a prolate ellipsoid, we have calculated an axial ratio of approximately 4 from the $D_{20,w}$ (19, 20). This is consistent with the appearance of the tail protein in electron microscopy.

Comparison of the free tail protein, phage, and heads in Fig. 3 indicates that the tail protein appears shortened after binding to the phage head-neck structure. Such an observation is subject to interpretation as an artifact of sample preparation for microscopy, but suggests that the tail protein may be attached nonorthogonally to the neck structure or may undergo a conformational change upon assembly.

The linearity of the sedimentation equilibrium data (Fig. 2b) indicates that the tail protein dimers have no tendency themselves to aggregate or to assemble into higher-order structures. Thus, although phage assembly interactions are extremely strong (as judged by the overall stability of phage particles), when the appropriate substrate for assembly (heads) are absent, no aberrant assembly reactions seem to occur.

Mutational analysis of tail protein functions. One way to study the mechanism of action of a biologically interesting macromolecule is to obtain altered forms of it. Certain types of genetic mutations are particularly useful in this kind of study because they can alter the gene product in extremely specific ways. In the genetics of bacteria and their viruses, amber mutations have had widespread use because of their drastic, conditional, polypeptide chain-terminating effect on the product of the mutated gene. Amber mutations also provide the experimenter with the ability to insert a variety of different amino acids into defined sites in the polypeptide chain through the use of specific amber suppressors. Some of these substitutions can lead to production of a functionally altered product. This kind of analysis with amber (and other nonsense) mutations has been carried out extensively in the case of the *E. coli lac i* gene by Schmitz et al. (18).

In starting a mutational analysis of the P22 tail protein, we looked for an altered form of the protein that would retain some, but not all of the activities of normal tail protein. The tail protein produced by insertion of tyrosine into the site of the 9^- *am*H1014 mutation apparently assembles onto heads, but does not function in the next round of infection, as shown by the single-step growth experiment in Table 1.

The activity of the mutant tail protein is examined in more detail in the experiments in Fig. 4 and 5. The mutant tail protein combines with P22 heads (Fig. 4). The phage thus assembled are defective, plating on permissive (*supE*) cells with an efficiency only 1% that of phage assembled with normal tail protein. The data in Fig. 4 also indicate that the mutant protein has a fourfold lowered, head-binding specific activity than wild-type tail protein. Other observations indicate that the mutant protein is generally less stable than the wild-type protein (data not shown). Thus, a significant fraction of the mutant tail protein may have been inactivated during purification. This reduced head-binding activity, however, cannot account for the defect of phage assembled with the mutant tail protein under conditions where the mutant protein is in excess and saturates the available sites on the head. In addition, the mutant protein does not appear to exchange with added wild-type pro-

tein. Incubation of mutant protein-tailed phage with either 10-fold excess of wild-type protein (Fig. 4) or even higher concentrations (data not shown) does not increase the titer of the reaction mixture. Thus, the association between the tail protein and the phage head is very strong once established.

The experiment in Fig. 5 indicates that the mutant tail protein is deficient in glycosidase activity. This result suggests that the mutant tail protein makes the phage noninfectious because it is unable to hydrolyze its way through the lipopolysaccharide layer of the host cell.

From the experiment in Fig. 6, phage assembled with the mutant tail protein do adsorb to cells. However, during a period of time sufficient for wild-type phage adsorption and infection, the mutant-tailed phages apparently remain attached to a portion of the O-antigen that is susceptible to removal by the glycosidase activity of wild-type tail protein. Wild-type phage seem irreversibly adsorbed. Our interpretation of this result, which is consistent with the published model for P22 infection (9), is that P22 binds to the outer polysaccharide of the host cell by the enzymatic site of the tail protein. Hydrolysis of the polysaccharide results in the relative movement of the phage toward the cell body. This movement ends with the phage bound to some structural component of the lipopolysaccharide of the host cell other than the O-antigen. The mutant tail protein, unable to hydrolyze the O-antigen, does not enable the phage to locate its secondary receptor site efficiently and thus renders the phage less infectious than wild type. This particular genetic lesion in gene *9* has allowed the physical separation of the cell adsorption function of the tail protein from its other functions required for successful infection of the host, among which is O-antigen cleavage.

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