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Isolation and Characterization of the Bacteriophage T4 Tail-Associated Lysozyme

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Direct evidence has been obtained that the tail-associated lysozyme of bacteriophage T4 (tail-lysozyme) is gp5, which is a protein component of the hub of the baseplate. Tails were treated with 3 M guanidine hydrochloride containing 1% Triton X-100, and the tail-lysozyme was separated from other tail components by preparative isoelectric focusing electrophoresis as a peak with a pI of 8.4. The molecular weight as determined from sodium dodecyl sulfate electrophoresis was 42,000. The tail-lysozyme was unambiguously identified as gp5 when the position of the lysozyme was compared with that of gp5 of tube-baseplates from 5*ts*1/23*am*H11/eL1ainfected *Escherichia coli* cells by two-dimensional gel electrophoresis. The tail-lysozyme has *N*-acetylmuramidase activity and the same substrate specificity as gene *e* lysozyme; the optimum pH is around 5.8, about 1 pH unit lower than for the e lysozyme. We assume that the tail-lysozyme plays an essential role in locally digesting the peptidoglycan layer to let the tube penetrate into the periplasmic space. The tail-lysozyme is presumably also responsible for "lysis from without."

After recognition of its host bacterium and adsorption to it, T4 bacteriophage undergoes a series of irreversible morphological changes. First, the "hexagon"-shaped baseplate changes its shape to that of a star (21). Simultaneously, gp12, which has a compact shape and is located beneath baseplates, extends itself to form short tail fibers (2, 11). The short tail fibers thus formed tightly connect the phage particle to the bacterium. The conformational change of the baseplate triggers the contraction of the tail sheath. The change in arrangement of the sheath subunits is transmitted like a wave from the baseplate-proximal end to the distal end of the tail (17). Contraction of the sheath is a spontaneous process and does not require energy. As a result, the tube of the tail protrudes from the bottom of the baseplate and penetrates into the outer membrane of the cell. The phage DNA folded in the head is then injected into the bacterium through the tube. This process requires that the tube or DNA pass through the peptidoglycan layer. In this connection, "lysis from without" has long implied that the tail has a lytic activity (10). Initially, it was suspected that the T4 lysozyme, the gene *e* product, was responsible for it. However, since e^- phage can cause lysis from without, a lytic enzyme other then gpe was considered to be present in the tail (15). In 1980, Kao and McClain presented indirect evidence that the lytic activity was due to gp5 by isolating a temperature-sensitive (*ts*) mutant, 5*ts*1e^- (9).

In this paper, we present direct evidence that the tail-lysozyme is gp5. Furthermore, our experiments, which utilized 5hs mutants, suggested that gp5 plays an essential role in locally digesting peptidoglycan so that the tube can penetrate into the periplasmic space. DNA can then be ejected through the tube.

MATERIALS AND METHODS

Reagents. Ampholines (pH 5 to 8, 7 to 9, 9 to 11) were purchased from LKB Co. Ltd. Hen egg white lysozyme (EC 3.2.1.17; six times crystallized) was a product of Seikagaku Kogyo Co. *N*-Acetylmuramic acid, *N*-acetylglucosamine, and *meso*-diaminopimelic acid were purchased from Sigma Chemical Co. All reagents used were reagent grade.

Phage and bacteria. Wild-type T4D and 23*am*H11 are from our collection. 5*ts*1eL1a was supplied by W. McClain (University of Wisconsin-Madison). 5hsY204 and 5hsY213 were from M. Yamamoto (University of Tokyo). *Escherichia coli* BE was used as a nonpermissive host for amber mutant phages, and *E. coli* CR63 was the permissive host. *Pseudomonas aeruginosa* P14 was from our collection.

Media and buffer. M9A medium was used to grow *E. coli*. M9A medium contained, per liter of water, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 1 g of NH_4Cl, 4 g of glucose, and 10 g of Casamino Acids (Difco Laboratories). *P. aeruginosa* P14 was grown in G medium. G medium contained, per liter of water, 20 g of sodium glutamate, 5.6 g of $Na_2HPO_4 \cdot 12H_2O$, 0.25 g of KH_2PO_4, 1 g of yeast extract (Difco), 5 g of glucose, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 0.014 g of $Ca(NO_3)_2 \cdot 4H_2O$. Phages were stored in B^+ buffer which contained, per liter of water, 7 g of Na_2HPO_4, 4 g of NaCl, and 3 g of KH_2PO_4 supplemented with 1 mM $MgSO_4$ for use. Tris-hydrochloride buffer (pH 8.0 to 9.0) was made 0.1 ionic strength (16).

Preparation of chloroform-killed cells and peptidoglycan. Chloroform-killed *P. aeruginosa* P14 cells used as a substrate of tail-lysozyme were prepared essentially as described by Black and Hogness (1) and were stored at −80°C until use. *P. aeruginosa* cells were used instead of *E. coli* BE because of their less autolytic property. Chloroform-killed *P. aeruginosa* cells are severalfold more sensitive than lyophilized *E. coli* BE cells. Peptidoglycan was prepared from *E. coli* BE cells based on the method of Yanai et al. (29).

Preparation of e lysozyme. e lysozyme was prepared according to Tsugita and Inouye (25) except that *E. coli* BE in a 25-liter culture was infected with T4D at multiplicities of infection of 4, 3, and 3 (t = 0, 7, and 11 min) and the cells were harvested at t = 60 min by using a Sharples continuous-flow supercentrifuge. The cells were then suspended in 60 ml of B^+ buffer and lysed with chloroform.

Assay for lytic activity. Frozen chloroform-killed cells were thawed and diluted with 0.05 M phosphate buffer, pH

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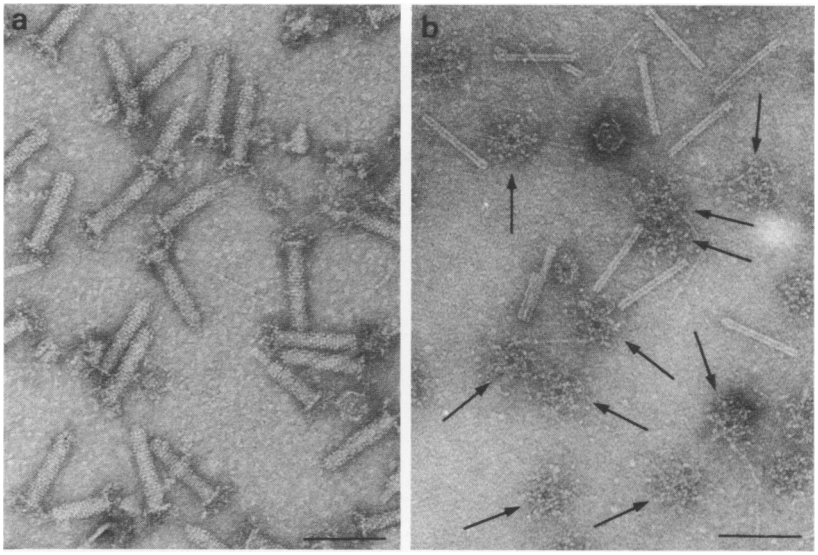

FIG. 1. Electron micrographs of T4 phage tails before (a) and after (b) treatment with 3 M Gu · HCl containing 1% Triton X-100. After the tails were placed on a grid, a drop of the Gu · HCl solution was charged on the grid for 10 s. The grid was then washed with water twice and dried. Arrows indicate baseplates in star form. Bars, 100 nm.

6.0. A portion (2.0 ml) of the resulting suspension (turbidity of 0.3 absorbance unit of 660 nm) was preincubated at 37°C for 2 min and was mixed with 0.05 ml of enzyme solution. The decrease in turbidity was measured at 37°C with a Hitachi 181 spectrophotometer. Enzyme activity, in units, was defined as the decrease in turbidity in 1 min with this assay mixture (2.05 ml).

Preparation of tails and tube-baseplates. Tails were obtained from lysates of 23*am*H11-infected *E. coli* BE cells according to Tschopp et al. (24) except that the lysate was precipitated by ammonium sulfate of 40% saturation (0.224 g/ml) to remove ribosomes and membrane fragments. The ammonium sulfate precipitation was repeated three times, and the resultant suspension was dialyzed against 0.1 M phosphate buffer, pH 7.2, before ultracentrifugation.

To obtain tube-baseplates for two-dimensional electrophoresis, tails from T4D wild type and the triple mutant 5*ts*l/23*am*H11/eL1a were dialyzed against 1 mM phosphate buffer, pH 7.2, at 4°C for 2 days to dissociate sheath protein. The dialyzed solution was centrifuged at 140,000 × *g* for 90 min, and the pellet was resuspended in a small volume of 1 mM phosphate buffer, pH 7.2. The concentrated tube-base-

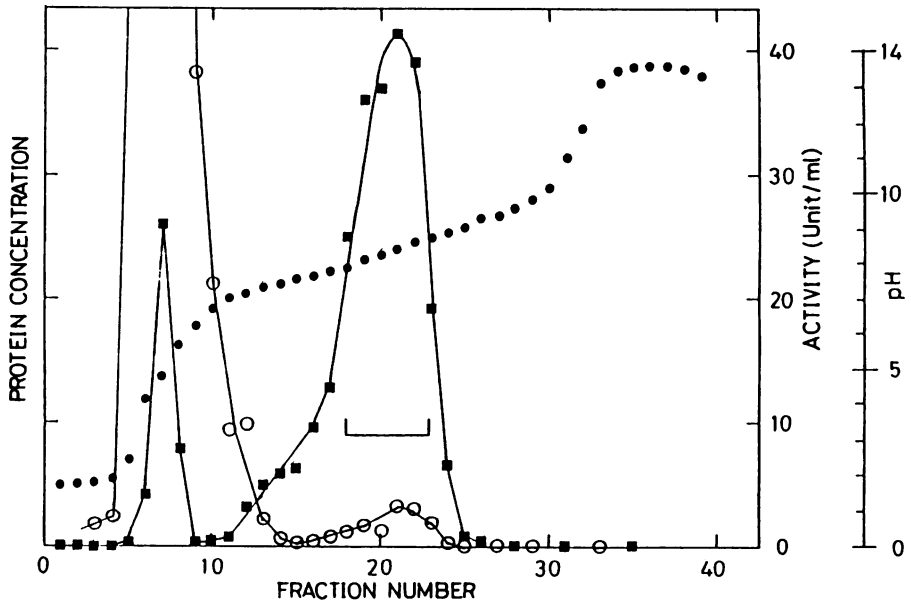

FIG. 2. Preparative isoelectric focusing of tails after treatment with 3 M Gu · HCl containing 1% Triton X-100. Tails were treated with 3 M Gu · HCl containing 1% Triton X-100 at 25°C for 1 h and then dialyzed against 1 mM phosphate buffer, pH 7.2, containing 1% Triton X-100. The dialyzed solution was then loaded on a preparative isoelectric focusing column. The fractions indicated (around pH 8.4) were pooled as tail-lysozyme. Symbols: ●, pH; ■, activity; ○, protein concentration. (Because of the interference from Triton X-100 and ampholine present in the solution, relative protein concentrations were estimated by scanning SDS polyacrylamide gels after staining with Coomassie brilliant blue R-250.)

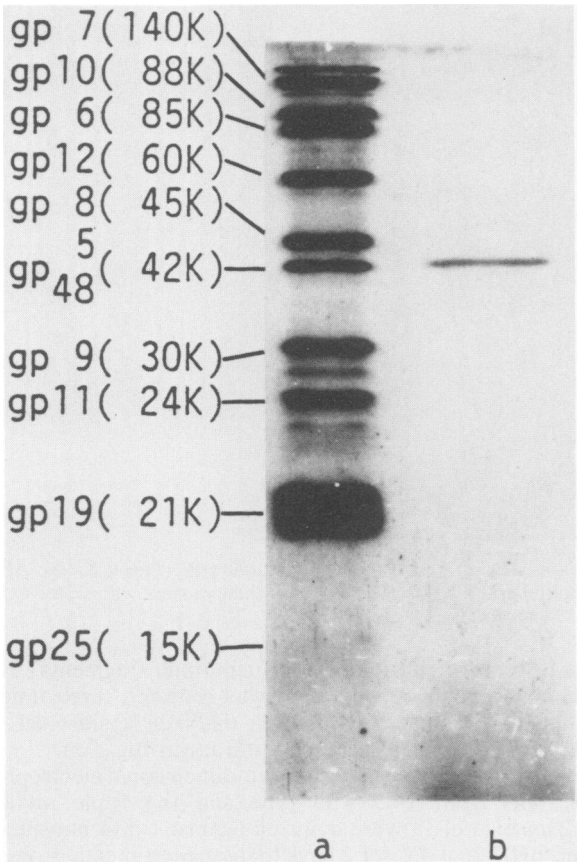

FIG. 3. SDS gel electrophoresis of tail-lysozyme as compared with that of the tube-baseplates. Polyacrylamide gel electrophoresis in the the presence of SDS was carried out according to Laemmli (13). The gel was stained with Coomassie brilliant blue R-250. The polyacrylamide concentration was 14%. (a) Tube-baseplates; (b) tail-lysozyme purified in a preparative isoelectric focusing column as shown in Fig. 2.

plate solution was then layered on a 10 to 30% linear sucrose gradient containing 0.1 M phosphate buffer, pH 7.2, and centrifuged for 2.5 h at 200,000 × *g*. The tube-baseplate peak was detected by sodium dodecyl sulfate (SDS) gel electrophoresis (see below), and the peak fractions were collected and dialyzed against 1 mM phosphate buffer, pH 7.2, to remove sucrose.

SDS gel electrophoresis. SDS gel electrophoresis was carried out according to Laemmli (13) in a vertical mini-slab gel (9 by 7.5 cm) and stained with Coomassie brilliant blue R-250 according to Wyckoff et al. (27), which is a simplified version of Fairbanks et al. (4), or silver stained according to Oakley et al. (18).

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was carried out according to O'Farrell et al. (19) as adapted to mini-slab gels. Isoelectric focusing in the first dimension was performed in a glass cylinder (diameter, 1 mm; length, 10 cm) made from a 0.1-ml disposable serological pipette (Kimble). The cylindrical gel was then directly loaded onto a mini-slab gel for SDS gel electrophoresis (see above).

Preparative isoelectric focusing. Preparative isoelectric focusing was done in an LKB 8100 ampholine electrofocusing column (110 ml). Ampholines of the pH ranges 5 to 8, 7 to 9, and 9 to 11 were mixed in a ratio of 1:4:2 and used. To avoid contamination by precipitation which occurs at pH 5 to 6 (lower part), the column solution was fractionated from the upper part of the column.

Electron microscopy. Samples were applied to carbon-coated collodion grids which had been rendered hydrophilic by glow discharge. Specimens were negatively stained with 1% uranyl acetate and examined in a Hitachi HU12A electron microscope.

Determination of substrate specificity. Peptidoglycan (1.5 mg) from *E. coli* BE was dispersed in 1.7 ml of 0.02 M sodium 2-(*N*-morpholino)ethanesulfonate (MES-Na), pH 5.8, with a Branson W185 Sonifier (40 W, 30 s) and mixed with 0.1 ml of an enzyme solution (1.8 U). The determination of reducing groups in the reaction mixture was carried out according to the Park-Johnson procedure (20), with *N*-acetylmuramic acid as a standard, ranging from 10 to 50 nmol.

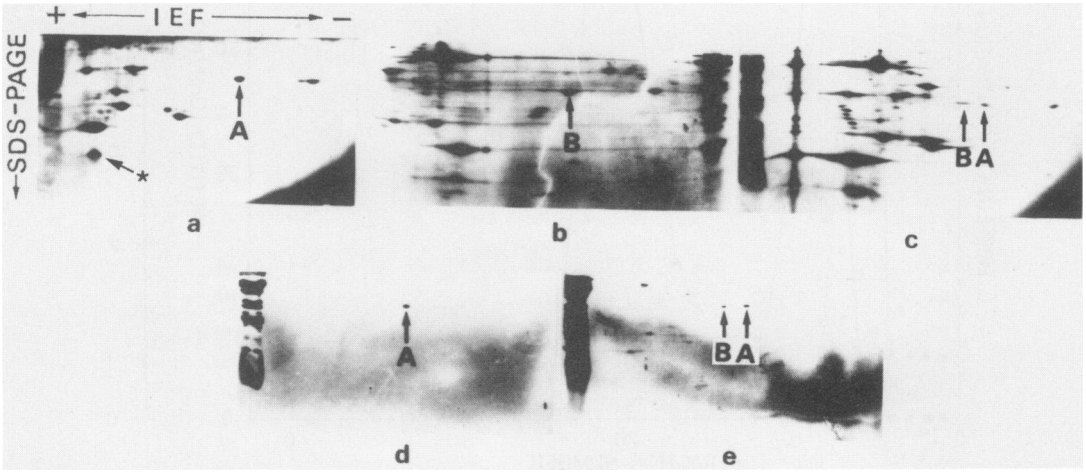

FIG. 4. Two-dimensional gel electrophoreses of tail-lysozyme as compared with those of tube-baseplates which contain wild-type or mutant gp5. The gels were silver stained according to Oakley et al. (18). (a) Tube-baseplates isolated from wild-type tails. A spot marked with * is due to gp25. (b) 5*ts*1 tube-baseplates containing mutant gp5. (c) (a) + (b). (d) Tail-lysozyme purified as in the legend to Fig. 3b. (e) (b) + (d). A and B indicates wild-type and mutant gp5, respectively. The pH range in the first dimension was from approximately 4 to 10. The polyacrylamide concentration in the second dimension was 15%. Tube-baseplates were added on the second dimension as molecular weight markers to the left of each panel [except (b)]. IEF, Isoelectric focusing; PAGE, polyacrylamide gel electrophoresis.

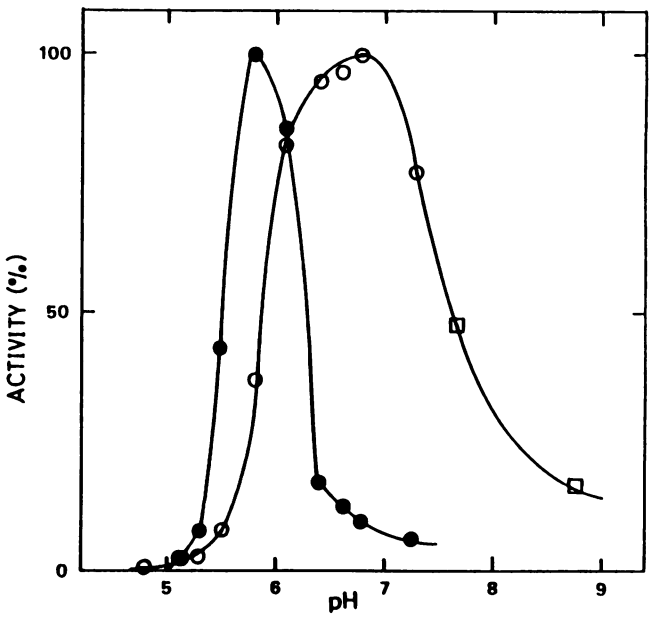

FIG. 5. Optimum pH for activity of tail-lysozyme and e lysozyme. Activity is expressed as percentage of the maximum activity. Symbols: ●, tail-lysozyme; ○,□, e lysozyme; circles, 0.1 M MES-Na buffer; squares, Tris-hydrochloride buffer (I = 0.1).

Free amino groups were determined by dinitrophenylation, with L-alanine as a standard, ranging from 10 to 50 nmol (6). Another mixture was prepared as above and was incubated at 37°C for 4 h. The enzyme digest (1.5 mg of peptidoglycan) was reduced with 2 mg of $NaBH_4$ (0.1 ml of a 20-mg/ml aqueous solution) at 37°C for 48 h after the addition of 10 mg of Na_2CO_3 (0.1 ml of a 100-mg/ml solution), evaporated to dryness, and then hydrolyzed with 1 ml of 6 M HCl at 105°C for 24 h. The components having amino groups in the hydrolysate were analyzed with a Hitachi KLA-3B amino acid analyzer.

RESULTS

Isolation of T4 phage tail-lysozyme. Tails were used as the starting material for the isolation of tail-lysozyme (see Materials and Methods). Freshly isolated tails did not show any lytic activity. However, lytic activity appeared when the tails were treated with 3 M guanidine · HCl (Gu · HCl) containing 1% Triton X-100. Figure 1 shows the morphological change of tails after this treatment. Baseplates are mostly "star"-shaped and tubes are disconnected from tube-baseplates after treatment as has been described by Duda and Eiserling (3). Apparently, at least part of the tail-lysozyme activity was solubilized by Gu · HCl and Triton X-100. In fact, when the Gu · HCl-treated tails were subjected to sucrose density gradient centrifugation after removal of Gu · HCl, all lysozyme activity remained at the top of the gradient (data not shown). Tails in 3 M Gu · HCl containing 1% Triton X-100 were incubated at 25°C for 1 h and then dialyzed against 1 mM phosphate buffer, pH 7.2, containing 1% Triton X-100. Tails thus treated were loaded on a preparative isoelectric focusing electrophoresis column, where tail-lysozyme was separated from other tail components as a peak with a pI of 8.4 (Fig. 2). The shoulder to the left of the pH 8.4 peak is contaminated with some other proteins. There was another peak of lytic activity at around pH 5. We have not yet looked at this fraction closely (see Discussion for a possibility about this fraction). Active fractions at around pH 8.4 were collected and dialyzed against phosphate buffer, pH 7.2, to remove sucrose and ampholine and examined by SDS-polyacrylamide gel electrophoresis. A band with the molecular weight of 42,000 was

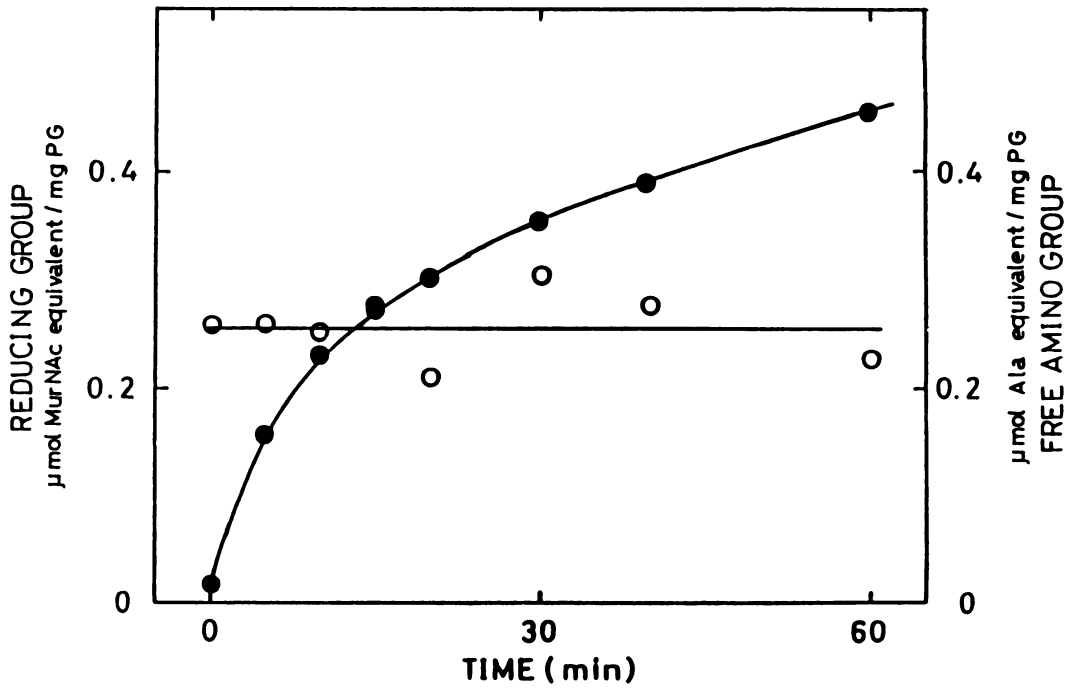

FIG. 6. Mode of action of tail-lysozyme. Peptidoglycan (PG) from *E. coli* BE was mixed with tail-lysozyme and the reaction was monitored by measurement of reducing (●) and free amino (○) groups. Reducing groups increase as the reaction proceeds, whereas the concentration of free amino groups stays constant.

TABLE 1. Substrate specificity of tail-lysozyme

Compound	Molar ratio[a]			
	Without enzyme	Tail-lysozyme	Egg white lysozyme	e lysozyme
Muramic acid	1.2	0.45	0.20	0.41
Muramicitol	0	1.1	1.1	1.1
Glucosamine	1.2	1.2	1.0	1.0
Glucosaminitol	0	0	0	0
Glutamic acid	1.0	1.0	1.0	1.0
Alanine	1.7	1.7	1.7	1.4
Diaminopimelic acid	1.0	0.98	0.98	0.80

[a] Expressed in terms of molar ratios on the basis of glutamic acid as 1.0.

observed, which coincided with the band of gp5 and gp48 (Fig. 3).

Identification of the tail-lysozyme as gp5. To further identify the gene product, two-dimensional gel electrophoresis was carried out according to O'Farrell et al. (19). Identification of the gene product was based on the fact that gp5 of a mutant, 5*ts*1, has a slightly more acidic isoelectric point than that of the wild-type gp5 (9). For that purpose a triple mutant 5*ts*1/23*am*H11/eL1a was constructed and the tube-baseplates were isolated as described under Materials and Methods. A single spot was obtained when the tail-lysozyme fraction was subjected to two-dimensional gel electrophoresis (Fig. 4d). The position of that spot corresponds to that of the molecular weight of gp5 and gp48. When tube-baseplates from 23*am*H11 (Fig. 4a) and those from triple mutant 5*ts*1/23*am*H11/eL1a (Fig. 4b) were mixed and examined in two-dimensional gel electrophoresis, two closely located spots (A and B) appeared. Comparison of the two-dimensional gels revealed that the spots designated A and B are from wild-type and mutant gp5, respectively, and that the other more basic spot (to the right) with the same molecular weight is from gp48. In the same way, when a mixture of tail-lysozyme (Fig. 4d) and the mutant tube-baseplates (Fig. 4b) was examined, a spot which is close to spot B appeared. The above observations have demonstrated that the tail-lysozyme is gp5.

Characterization of the tail-lysozyme. (i) Optimum pH for activity. Lysozyme activity at various pH values was measured in 0.1 M MES-Na buffer (pH 5.1 to 7.3) and Tris-hydrochloride buffer (I = 0.1; pH 8.0 to 9.0). The optimum pH for enzyme activity, with chloroform-killed *P. aeruginosa* cells, was around pH 5.8 as compared with 6.8 to 7.0 for e lysozyme (Fig. 5).

(ii) Substrate specificity. Peptidoglycan prepared from *E. coli* BE was digested with the tail-lysozyme. Figure 6 shows the time course of the reaction as monitored by measuring reducing groups by the Park-Johnson method and free amino groups by the dinitrophenylation method (see Materials and Methods). Whereas free amino groups stayed constant, reducing groups increased as the reaction proceeded. This result indicated that the tail-lysozyme had a glycosidase activity. To determine which glycoside bond, either MurNAc-GlcNAc or GlcNAc-MurNAc, is cleaved by the enzyme, peptidoglycan prepared from *E. coli* BE was extensively digested, and the digest was reduced with $NaBH_4$, hydrolyzed with 6 M HCl, and analyzed with an amino acid analyzer. The results are listed in Table 1 as molar ratios to glutamic acid. Muramic acid was replaced by muramicitol. From this result we conclude that the tail-lysozyme is an *N*-acetylmuramidase, the same specificity as e lysozyme.

Lysis from without by 5hs mutants of T4 phage. What would happen if phage particles which have lost their tail-lysozyme activity infect *E. coli* cells? Since gene 5 is an essential structural gene, and furthermore the lytic activity of the tail-lysozyme is most likely essential, it would not be possible to construct a mutant which has defective gp5. Another possibility for that purpose would be to specifically inhibit the tail-lysozyme activity and let the phage infect the bacterium, but the specific inhibitor for this lysozyme is not in our hands. From that viewpoint, 5hs mutants isolated by Yamamoto and Uchida (28) were considered to be useful. There are two 5hs mutants, Y204 and Y213, both of which lose their viability after incubation at 55°C for 30 min, but the tail sheaths remain extended. They noted that the tail sheath of the resultant phage could contract when the phage particles were adsorbed to the bacterium. Based on their observations, we examined the lysis from without activity of these 5hs mutants. The results are shown in Fig. 7. As expected, the heat-inactivated phage particles lost the lysis from without activity. This agrees with our assumption that the lysis from without is due to gp5 and that the activity is lost in heat-treated 5hs particles. After heat treatment of 5hs particles, they were mixed with *E. coli* cells and examined by electron microscopy (Fig. 8). Almost all tail sheaths of the

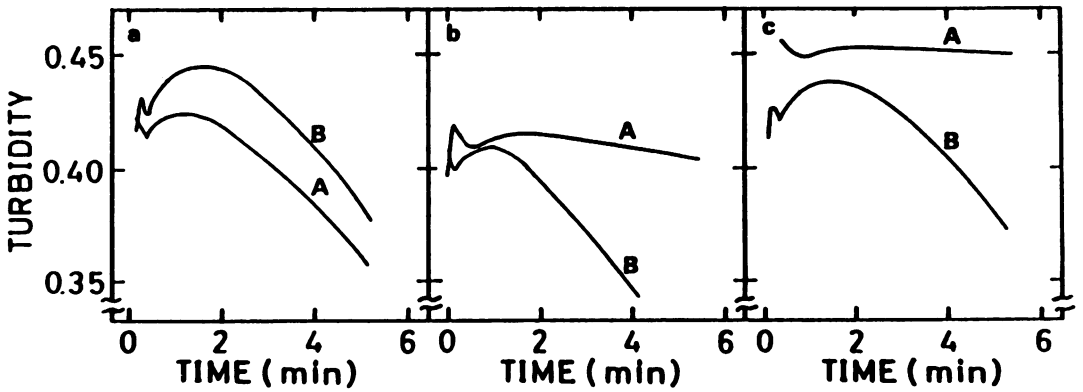

FIG. 7. Lysis from without by 5hs mutants of phage T4. Lysis-from-without activity of two 5hs mutants, 5hsY204 and Y213, isolated by Yamamoto and Uchida (28), and the wild-type T4D was measured by monitoring decrease in optical density at 660 nm before and after heat treatment. (a) Wild-type T4D; (b) 5hsY204; (c) 5hsY213. *E. coli* BE cells were infected by each strain of the phage at a multiplicity of infection of 100. A, Heat treated (55°C, 30 min); B, untreated.

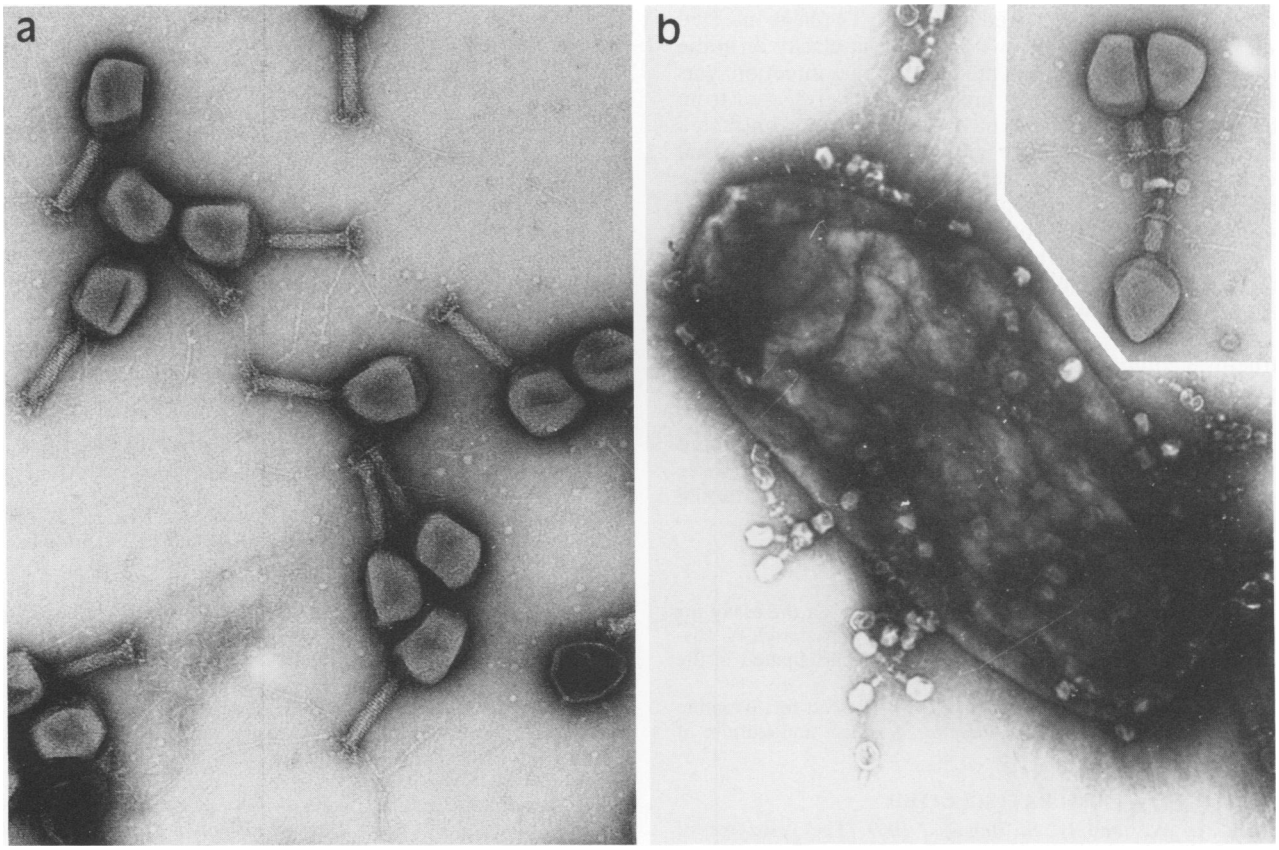

FIG. 8. Adsorption of a heat-treated 5hs mutant of phage T4 to *E. coli* BE. (a) Heat-treated 5hs phage (Y204); (b) heat-treated 5hs phage particles adsorbed to an *E. coli* BE cell. (Insert) Heat-treated 5hs particles presumably once adsorbed and then released from the cell. They have contracted sheaths and "disk-shaped" material at the distal end of the tubes.

phage particles were normally contracted, and a sizable fraction of the phage detached from the bacterial cells. Regardless of whether they were attached or detached, most of the phage particles retained DNA in the heads, and when detached, the contracted tails had a disk-shaped structure at the distal ends, which is most likely material from the outer membrane of *E. coli*.

DISCUSSION

It has been reported by Szewczyk and Skórko (22) that phage ghosts possess a lytic enzyme which is solubilized by Triton X-100 (22). To directly identify the gene which codes for the enzyme, we have purified it by using tails as the starting material. Initially, we detected some lytic activity when the tails were treated with Triton X-100. To determine to which fraction, either tube or baseplate, the enzyme associates, we treated tails with 3 M Gu · HCl, since Duda and Eiserling (3) reported that this treatment disconnected tubes from baseplates (3). It turned out that the Gu · HCl treatment also solubilized the enzyme and that the induced activity was severalfold higher than that solubilized by Triton X-100. Our final choice was to treat tails with 3 M Gu · HCl containing 1% Triton X-100. The presence of Triton X-100 was to keep the enzyme soluble after removal of Gu · HCl. We originally believed that the enzyme was identical to what Szewczyk and Skórko solubilized from ghosts with Triton X-100. Meanwhile, they have purified the T4 particle-associated lysozyme and reported that the enzyme is possibly gp25 based on the molecular weight of 15,000 (23). The tail-lysozyme which we purified was shown to be gp5 and its molecular weight was 42,000. Although the reason for the different results has not been defined, there are some indications that the fraction of lytic activity at around pI 5 in Fig. 2 is due to gp25. First, the pI of gp25 is around 5. Second, gp5 appears to be absent in that fraction based on two-dimensional gel electrophoresis. Third, we have detected a fraction of lytic activity whose major component has the molecular weight of 15,000 when we tried to isolate the enzyme from Gu · HCl-treated tails by using DEAE-Toyopearl column chromatography (data not shown). In any case, the fraction of the lytic activity due to the putative gp25 represents only 15% and most of the activity is due to gp5. We are currently working on the pI 5 fraction to settle this problem.

gp5 is a structural protein which is located in the hub of baseplates. Several species of bacteriophage have been reported to possess virion-associated glycanase or endolysin. *Salmonella* sp. phage P22 has a tail protein, gp9, which has endorhamnosidase activity (8). A number of virion-associated glycanase activities which digest capsule or exopolysaccharides are also known (14). *Pseudomonas* sp. phage PM2 and phi6 also have virion-associated endolysin (7, 26), but the enzyme specificity is not known. To our knowledge, T4 phage is the only one whose virion-associated enzyme has been determined to be *N*-acetylmuramidase.

We have observed with heat-inactivated 5hs mutants that, when these phage particles are adsorbed to host bacteria, the tail sheath contracted and a disk-shaped material was attached to the distal tip of the tubes (Fig. 8b, insert).

Zorzopoulos et al. (30) reported that when T4 phage infected *E. coli* B which had been grown in high osmolarity or in the presence of divalent heavy metal ion, the infection was unsuccessful and most of the phage particles released from the bacteria were observed to have full heads and contracted sheaths with unusual pieces of cell walls (30). The altered tail structure they presented is very similar to those which we observed in Fig. 8b, insert. In both cases, the heads are full. This is in accord with the notion that the DNA is ejected into the periplasmic space when the tip of the tube comes in contact with cytoplasmic membrane (5). Injection of the DNA into cytoplasm would then require membrane potential (12).

Structural comparison between e lysozyme and the active domain of gp5 would be of great interest from the viewpoint of evolution. A structural study of gp5 protein and its gene is under way in our laboratory. If gp25 is also a tail-associated lytic enzyme, the structural homology among these three enzymes, if any, might give us insight into the evolutional relationship among these proteins.

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