Structure of the Lipid-Containing Bacteriophage PRD1: Disruption of Wild-Type and Nonsense Mutant Phage Particles with Guanidine Hydrochloride

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The lipid-containing bacteriophage PRD1 was disrupted, and the subviral particles were studied. Guanidine treatment released two phage proteins (P3 and P5). These proteins form the polyhedral capsid. The remaining phage proteins were associated with the phage membrane vesicle. The vesicle was capable of forming a tubular structure. The isolated phage membrane vesicles aggregated readily. We found that aggregation and tube formation were associated with specific phage proteins (P11 and P18, respectively) by using protease treatment and an analysis of nonsense mutant phage particles. In addition, the possibility that free vesicles might be precursors to empty virions was studied.

PRD1 belongs to a group containing several similar phages (PR3, PR4, PR5, L17, and PR722), which can be differentiated only by structural protein and genome restriction fragment analysis (1). Thus, the results obtained with one member are true also for the others.

PRD1 is a lipid-containing phage having a double-stranded DNA genome (10). The lipids are structural components of the virion, as shown for PR4 and PR5 (11, 14). This phage can infect a variety of gram-negative bacteria that harbor an appropriate plasmid (3). Among the efficient hosts are *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*.

In this study phage PRD1 was disrupted by controlled guanidine hydrochloride treatment, and subsequently subviral particles were isolated in sucrose gradients. The functions of some of the viral proteins were revealed by using deficient particles produced by nonsense mutants of this virus.

MATERIALS AND METHODS

Bacteria and bacteriophages. Nonsuppressor strains, *S. typhimurium* LT2(pLM2) and *P. aeruginosa* PAO1(pLM2) and amber suppressor strain *S. typhimurium* PSA (7, 8) were used as bacteriophage hosts in this study. Phage PRD1 was obtained from R. H. Olsen. The phage nonsense mutants used in this study are listed in Table 1 (6, 7).

Preparation of purified labeled phage particles. For [³⁵S]methionine labeling the cells were grown in M8 medium (12) to a density of 3×10^8 to 5×10^8 cells per ml and infected at a multiplicity of about 20. At 35 to 40 min postinfection 10 μ Ci of [³⁵S]methionine (1,360 Ci/mmol; Amersham Corp.) per ml was added. The

cells were incubated at 37°C with aeration until lysis occurred. For ³²P labeling the cells were grown in low-phosphate medium (9) to a density of about 2×10^8 cells per ml and infected at a multiplicity of 20. At the time of infection, 15 μ Ci of ³²PO₄ per ml and cold phosphate (final concentration, 0.05 mM) were added. The culture was incubated at 37°C with aeration until lysis occurred.

The liberated particles were concentrated with polyethylene glycol 6000 (15) and purified through a 5 to 20% (wt/vol) linear sucrose gradient in phage buffer (0.01 M phosphate, pH 7.2, 1 mM Mg²⁺); this was followed by concentration of the purified particles by sedimentation. The final phage pellets were suspended in phage buffer.

Assay for PRD1 disruption and membrane aggregation. Wild-type and mutant phage particles (designated A in the phage listing) were treated with guanidine hydrochloride for 30 min at 37°C, diluted 1:1 with phage buffer, layered on top of a linear 10 to 35% (wt/vol) sucrose gradient with an 85% (wt/vol) sucrose cushion in phage buffer, and centrifuged at 30,000 rpm for 45 min at 20°C by using an SW41 rotor. The fractions were collected by pumping the gradient from the tube with a capillary. Phage membranes isolated in guanidine were also used in this assay (see below). Protease treatments of phage particles, disrupted phages in 2 M guanidine, and isolated phage membranes in 2 M guanidine were carried out in phage buffer at 37°C for different times (see below). The trypsin treatment was stopped at different times by adding a 10-fold excess of soybean trypsin inhibitor, followed by at least 15 min of incubation at 37°C.

Disruption of the phage particles with guanidine hydrochloride, isolation of subviral particles, and assay for vesicle formation by mutants unable to form particles. The purified wild-type and class IX mutant *sus*/phages were mixed with guanidine hydrochloride (final concentration, 2 M). The mixture was incubated for 30

TABLE 1. Phages used

Class	Mutant	Virus protein missing	Use in this study ^a	
II	<i>sus198</i>	P2	A,	EM
III	<i>sus14</i>	P3	V	
	<i>sus24</i>	Fragment of P3	V	
— ^b	<i>sus218</i>	P7	A,	EM
IX	<i>sus1</i>	P9	D, V,	EM
X	<i>sus13</i>	P10 ^c	V	
XI	<i>sus44</i>	P11	A, V,	EM
	<i>sus64</i>		A, V,	EM
XIV	<i>sus234</i>	P14	A,	EM
XVII	<i>sus151</i>	P17 ^c	V	
XVIII	<i>sus25</i>	P18		EM
	<i>sus148</i>		A,	EM
—	<i>sus116</i>	P21, mobility changed	A,	EM
XXII	<i>sus42</i>	P22		EM

^a D, Guanidine disruption; A, membrane aggregation; V, vesicle formation; EM, electron microscopy.

^b —, Class not determined.

^c Nonstructural protein.

min at 37°C and layered on top of a linear 7.3 to 45% (wt/vol) sucrose gradient in phage buffer containing 2 M guanidine hydrochloride and a 65% (wt/vol) sucrose cushion. Either a Beckman Spinco SW41 rotor (30,000 rpm) or an SW50.1 rotor (35,000 rpm) was used at 20°C as described below. The gradients were fractionated, and the radioactivities of the fractions were measured by counting samples with a Beckman scintillation counter.

Lipid extraction of the guanidine gradient fractions was done by the method of Bligh and Dyer (2). Both chloroform and methanol-water fractions were dried under nitrogen and counted.

The density of the membrane material was determined by centrifuging the isolated membrane peaks in a linear 20 to 60% sucrose gradient in phage buffer (SW41 rotor, 35,000 rpm, 19 h, 20°C). The refractive indices of the fractions containing the radioactivity were measured.

To assay vesicle formation by mutants that did not form particles (7), *P. aeruginosa* PAO1(pLM2) in M8 medium (density, 5×10^8 cells per ml) was infected at a multiplicity of infection of 20 with wild-type phage and the mutants designated V (Table 1). At 40 min postinfection 10 μ Ci of [³⁵S]methionine per ml was added. After cell lysis, guanidine was added to the lysates to obtain a final concentration of 2 M, and the lysates were incubated for 30 min at 37°C. Samples (0.2 ml) were centrifuged in a linear 7.5 to 45% (wt/vol) sucrose gradient with a 65% sucrose cushion in phage buffer containing 2 M guanidine (SW50.1 rotor, 35,000 rpm, 2.83 h, 20°C). After centrifugation the gradients were fractionated and counted.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membrane samples from the guanidine gradients were diluted with phage buffer and sedimented by centrifugation in a Beckman Ti50 rotor for 4 h at 35,000 rpm and 5°C. The pellets were suspended in

sample buffer (13) and boiled for 3 min. The capsid protein samples from a guanidine gradient were dialyzed against phage buffer, and sample buffer was added before boiling. Trypsin-treated membrane samples were sedimented after trypsin inhibitor addition and processed like the membrane samples from the guanidine gradients. The proteins were analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as previously described (1), except that the pH of the separating gel was 8.5 and the acrylamide concentration 16.5%.

Electron microscopy. The purified virus particles were stained on carbon-coated grids with 1% phosphotungstic acid (PTA), pH 6.5. The guanidine-containing specimens were first adsorbed to carbon-coated grids for 5 min, and the grids were thoroughly rinsed with water before staining. The micrographs were taken with either a JEM model 100B or a Philips model 300 electron microscope. The phages used in this study (in addition to the wild type) are designated EM in the phage listing (Table 1).

RESULTS

Guanidine hydrochloride disruption of virions.

To determine the effect of guanidine on PRD1 virions, purified [³⁵S]methionine-labeled virus was treated with guanidine at 37°C for 30 min. Concentrations of guanidine between 0.5 and 3 M were tested. When the treated virions were sedimented through a 10 to 35% sucrose gradient, we found that the virus peak disappeared at a guanidine concentration of 1.5 M (data not shown). Between 1.5 and 3 M guanidine, material was found on the cushion and on top of the gradient (Fig. 1B shows the 2 M treatment). An electron microscopic examination showed that the peak on the cushion consisted of aggregated membranous material (Fig. 2B) and that the top of the gradient contained capsomers (Fig. 2F).

To isolate nonaggregated phage membranes, phage treated with 2M guanidine was centrifuged in a 7.5 to 45% sucrose gradient containing 2 M guanidine. When [³⁵S]methionine and ³²P-labeled phages were mixed, it was possible to separate different subviral particles in the gradient. After 5 h of centrifugation in an SW41 rotor at 30,000 rpm, three separate [³⁵S]methionine peaks were observed. Two of these peaks also contained chloroform-extractable ³²P radioactivity (Fig. 3A). Water-soluble ³²P was found in the fastest-moving peak, as well as in a separate peak in the upper one-third of the gradient (Fig. 3A). These results indicated that lipids were associated with the two fastest-moving peaks. DNA was associated with the fastest-moving peak and also with a peak that was free of protein and lipid (fractions 15 through 17). The uppermost peak (fractions 18 and 19) contained only proteins. To confirm that the fastest-moving peak in this 5-h gradient was a DNA-containing lipid-protein complex, *sus1*, a PRD1 nonsense mutant that lacks protein P9 and makes

empty particles (7), was included in this study (Fig. 3B). This mutant formed only the slowly moving lipid-protein complex without DNA plus the protein peak at the top of the gradient.

The ratio of DNA-containing complexes to empty lipid-protein complexes varied with the guanidine treatment. Guanidine concentrations somewhat lower than 2 M yielded more DNA-containing complexes, whereas concentrations greater than 2 M yielded less. However, even at the disruption concentration of the virus (approximately 1.7 M guanidine), both types of complexes were found (data not shown).

When the morphology of the different subviral particles was studied by using PTA-stained preparations, we found that the DNA-containing lipid-protein complexes were mainly spherical membrane vesicles with a diameter of about 54 nm. The stain did not penetrate these vesicles. A minority of empty round vesicles was observed in this fraction (Fig. 2E). The non-DNA-containing lipid-protein complexes were empty pleomorphic membrane vesicles with an average diameter of 47 nm. These vesicles had a very characteristic tubular structure, with an average length of 60 to 65 nm and a radius of about 7 nm, associated with them (Fig. 2C). The *susI* membrane fraction had the same morphology as the wild-type empty membrane vesicles (Fig. 2D). The soluble top fraction containing only protein showed 7 to 8-nm protein complexes (Fig. 2F) that appeared to be capsomeres.

A protein analysis of the fractions revealed that the soluble top fraction contained proteins P3 and P5 (Fig. 4) (for protein nomenclature, see reference 6). Both vesicle fractions contained the rest of the virion proteins (Fig. 4).

When the membranes from a guanidine gradient were centrifuged to equilibrium in a sucrose gradient (under these conditions the membranes aggregated [see below]), the approximate density of the DNA-containing vesicles was 1.17 g/cm³, and that of the empty vesicles was 1.14 g/cm³.

Membrane aggregation. Several approaches (such as urea, high-salt, ethylene glycol, Triton X-100, and glycerol treatments) were tried in addition to guanidine treatment to keep the phage membrane vesicles from aggregating, but no satisfactory results were obtained. To identify the aggregation factor in the membranes, virus in 2 M guanidine was treated with 100 μ g of trypsin per ml for 30 min, and this was followed by soybean trypsin inhibitor treatment. When the material was centrifuged in a sucrose gradient in which the membranes normally were found aggregated on the cushion (Fig. 1B and 2B), no such aggregate was obtained. In addition to the coat protein peak at the top of the gradient, a smooth peak around fractions 12

FIG. 1. PRD1 membrane aggregation assay. The material tested was applied on top of a linear 10 to 35% sucrose gradient with a 0.6-ml 85% (wt/vol) sucrose cushion. The gradients were centrifuged at 30,000 rpm for 45 min at 20°C in an SW41 rotor. (A) Untreated wild-type viruses. (B) Wild-type viruses treated with 2 M guanidine for 30 min at 37°C. (C) Wild-type viruses treated with guanidine and with 100 μ g of trypsin per ml for 30 min at 37°C. (D) Nonsense mutant *sus64* lacking protein P11 treated with guanidine. (E through H). Isolated empty wild-type PRD1 membrane vesicles in 2 M guanidine (see Fig. 3A) treated with 60 μ g of trypsin per ml at 37°C for 5, 15, 30, and 60 min, respectively.

through 14 was found (Fig. 1C). The approximate sedimentation value for this peak was 150 to 300S.

To determine whether the disaggregation of membranes also correlated with the disappearance of certain proteins during trypsin treatment, isolated wild-type virus membranes in 2 M guanidine were treated with 60 μ g of trypsin per ml for 5, 15, 30, and 60 min. The reaction was stopped by adding a 10-fold excess of trypsin inhibitor. One part of the samples was diluted 1:20, pelleted by centrifugation, and prepared for protein gels. Another part was run in a cushioned 10 to 35% sucrose gradient. We found (Fig. 1E through H) that membrane aggregation was gradually decreased by increasing the length of the trypsin treatment. After 30 min of treatment a broad peak was formed around fraction 7 (500 to 600S). After 60 min, a peak was found at the same position as after treatment with 100 μ g of trypsin per ml.

When the protein composition of the trypsin-treated vesicles was analyzed, we found that 5- and 15-min treatments did not have any signifi-

FIG. 2. Electron microscopy of PTA-stained subviral particles obtained by guanidine disruption of virions. (A) Untreated starting material (wild type). (B) Aggregating PRD1 membrane material obtained on the cushion of a sucrose gradient after guanidine treatment of the virus (see Fig. 1B). (C) Empty PRD1 membrane vesicles obtained from a sucrose gradient containing 2 M guanidine after guanidine treatment (see Fig. 3A). (D) Empty PRD1 membrane vesicles released by *susI* nonsense mutant (see Fig. 3B). (E) DNA-containing vesicles formed by wild-type virus (see Fig. 3A). (F) Protein complexes obtained from the top of the guanidine-containing sucrose gradient after guanidine disruption of the wild-type virus. Bar = 100 nm.

cant effect on the membrane protein pattern (Fig. 4). After 30 and 60 min, bands P7, P9, P11, and P16 gradually weakened. If the trypsin treatment was not stopped by trypsin inhibitor and the reaction time was prolonged to approximately 2 h, all of the bands except protein P6 changed mobility or disappeared. Since the treated vesicles were diluted and centrifuged to collect the material, the cleaved proteins observed in Fig. 4, lane h, were sedimentable and thus still in the vesicles.

Since aggregation seemed to be released by cleavage of proteins P7, P9, P11, and P16 with trypsin, several nonsense mutant particles lacking specific proteins were studied to determine whether lack of a particular protein would prevent aggregation. The mutant particles were treated with guanidine and centrifuged in a sucrose gradient as described above. Mutants lacking proteins P2, P9, P14, P18, and P21 behaved like wild-type viruses in this assay (Fig. 1B). However, class XI mutants, which lacks membrane protein P11, behaved like trypsin-treated viruses (Fig. 1D). Two independent isolates of this mutant class (*sus64* and *sus44*) were treated, and they behaved similarly. This identified protein P11 as the aggregation factor.

When whole virus particles were treated with proteases (trypsin, chymotrypsin, pronase, and

FIG. 3. Purified virions treated with 2 M guanidine at 37°C for 30 min were run in a 7.5 to 45% (wt/vol) sucrose gradient containing 2 M guanidine (SW41 rotor, 30,000 rpm, 5 h, 20°C). Symbols: ●, ³⁵S; ○, ³²P-labeled chloroform preparation; △, water-methanol ³²P radioactivity. (A) Wild-type virus. (B) Nonsense mutant *sus1* forming particles without DNA.

FIG. 4. Discontinuous sodium dodecyl sulfate-16.5% (wt/vol) polyacrylamide gel of PRD1 proteins after guanidine treatment and after the virus membrane was treated with trypsin (60 µg/ml). Lanes a and i, Untreated virions; lane b, protein peak at the top of the guanidine gradient (see Fig. 3A); lane c, empty PRD1 membrane vesicles from a guanidine gradient (see Fig. 3A); lanes d through g, empty membrane vesicles in 2 M guanidine digested with 60 µg of trypsin per ml for 5, 15, 30, and 60 min, respectively (the reaction was stopped with a 10-fold excess of soybean trypsin inhibitor); lane h, trypsin digestion run for 120 min and not stopped with the inhibitor.

proteinase K; 100 µg/ml, 37°C, 30 min), no proteins were digested, nor did the sedimentation of the particles change until a denaturing agent (e.g., 0.1% sodium dodecyl sulfate) was added (data not shown).

Protein associated with membrane tube formation. It was possible to see separated phage membranes on the background of P3 protein complexes when guanidine-treated particles were directly stained with PTA. To determine whether any of the mutants lacking defined proteins could not form the tubes, mutants lacking proteins P2, P7, P9, P11, P14, P18, P21, and P22 were treated with guanidine and examined under the electron microscope after PTA staining. Only one mutant class (class XVIII), which lacks protein P18, did not form any tubes associated with membranes. Spherical vesicles with diameters of about 54 nm were observed. The other mutants always formed tubes. Figure 5B

FIG. 5. Guanidine-disrupted viruses (2 M, 30 min, 37°C) stained with PTA. (A) Nonsense mutant *sus64* lacking protein P11, the aggregation protein, but forming tubes like wild-type virus. (B) Nonsense mutant *sus148* lacking protein P18 formed round membrane vesicles with an average diameter of 54 nm. No tubular structures were observed. The P3 protein complex can be seen in the background in both of these preparations. Bar = 100 nm.

shows vesicles formed by *sus148* (P18⁻), and Fig. 5A shows vesicles formed by the nonaggregating mutant *sus64* (P11⁻).

Precursor membrane vesicle formation in cells infected with PRD1. Guanidine hydrochloride treatment of virions and subsequent centrifugation in a guanidine-containing sucrose gradient led to nonaggregation and normal sedimentation of the viral membrane vesicles (Fig. 3), which was not true in normal sucrose gradients (Fig. 1B). When particle formation by different nonsense mutants of the virus was tested (7), the conditions used would not have revealed possible free precursor membrane vesicles if they had existed in cases where virions were not produced. The non-particle-forming nonsense mutants of PRD1 lacking proteins P3, P11, and P17 were analyzed in guanidine-containing gradients, as described above. None of the mutant lysates showed any hint of free membranes in the gradients (Fig. 6C). Wild-type virus and *sus1* mutant lysates (Fig. 6A and B) showed membrane vesicle patterns similar to the patterns observed for the respective virus particles under these conditions (Fig. 3A and B).

DISCUSSION

Guanidine treatment released major viral proteins P3 and P5 in a slowly sedimenting form. These two proteins, which have apparent molecular weights of 42,000 and 37,000, respectively, form the outer polyhedral coat of the virion. The image of the guanidine-released material showed complexes of uniform size, which were too large (6 to 7 nm) to be single protein subunits. Instead,

these were probably viral capsomers composed of several protein subunits. It is not known how many P3 subunits are involved per capsomer and whether protein P5 is a member of this complex. Both protein P3 and protein P5 are soluble in cells before they appear in empty virus particles (7). These proteins are known to form multimers which can be identified in sodium dodecyl sulfate-polyacrylamide gels (7).

In addition to removing the outer protein coat of PRD1, gentle guanidine treatment favored the formation of DNA-containing spherical vesicles. When the DNA is released from these vesicles by higher guanidine concentrations, the emptied vesicles have a drastically changed morphology. The vesicles are smaller, and every vesicle has a tubular structure protruding from it, the length of which varies between 60 and 65 nm. The tubes seem to have an axial hole 8 nm in diameter. When the surface areas of the DNA-containing vesicles and vesicles formed by non-tube-forming mutants (Fig. 2E and 5B) were calculated, an average of $9.5 \times 10^3 \text{ nm}^2$ was obtained for each (an average measured radius of 27.5 nm was used). The surface area of an average tube (62.5 nm long, with a radius of 7 nm) was calculated to be approximately $2.7 \times 10^3 \text{ nm}^2$. The size of the vesicles in contact with the tubes was difficult to measure accurately since the structure often was folded, but the estimated spherical radius of these vesicles was approximately 23.5 nm, thus giving a surface area of about $6.9 \times 10^3 \text{ nm}^2$. Therefore, the tube plus the vesicle in connection with it had a total surface area of about $9.6 \times 10^3 \text{ nm}^2$, which is very close to the area calculated for the DNA-

containing spherical vesicles. This implies that the membrane is capable of forming a tubular structure of defined length and radius. If the whole spherical membrane could form a tube, its length would be about 215 nm. Under the conditions used, no tubes longer than approximately 80 nm were observed. From studies of nonsense mutants, it became clear that the class XVIII mutant lacking protein P18 (6) is not capable of forming the tubular structure.

The reorganization of the phage membrane into a smaller vesicle with an attached tube may occur naturally during the phage life cycle. Several reports (3-5, 10, 14) have shown that a taillike structure is seen in connection with empty particles. Furthermore, when the infection of another member of this phage group, the closely related phage PR4 (5), was followed by electron microscopy, the infecting particles showed membrane contraction in the phage capsid. Therefore, it is possible that formation of a tube and contraction of the vesicle are involved in DNA injection.

Isolated membranes of the virus aggregate readily. The morphology of the aggregate shows that the tubular structures are present in the aggregate. However, mutant particles that do not form tubes also aggregate. Proteolysis of the aggregate disaggregates the vesicles. Four of the viral proteins (P7, P9, P11, and P16) were markedly digested under those conditions. Viral mutant class XI, which lacks protein P11 (6, 7) (Fig. 5A) does not form aggregates but forms tubes (Fig. 6A). Thus, the sticking and tube formation are due to independent functions in the membranes. The absence of these proteins inhibits neither the formation of virus particles (7) nor the packaging of DNA, although the yield of particles is decreased in the case of class XI mutants (7). The aggregation of the vesicles and the availability of protein P11 to the protease suggest that this protein is on the surface of the vesicle. Protein P18, which is responsible for tube formation, is not easily accessible to trypsin. During prolonged trypsin treatment all proteins except protein P6 were affected. Since the vesicles seem to be open to the outside through the tube, it is impossible to conclude on which side of the membrane these proteins are located. Many of the proteins seem to have a hydrophobic portion which remains in the membrane even if the protein is clipped (Fig. 4).

The guanidine method described here was also used to test whether mutants that did not form particles formed free precursor vesicles. Guanidine prevents membrane aggregation and should reveal aggregated vesicular precursors if they exist. No evidence of this type of intermediate structure has been obtained by using other approaches (7). Likewise, no indication of any

FIG. 6. Assay for intracellular membrane vesicle formation in *P. aeruginosa*. Strain PAO1(pLM2) was infected with different PRD1 nonsense mutants and labeled with [35 S]methionine. The cultures were allowed to lyse, treated with 2 M guanidine for 30 min at 37°C, and run in a linear 7.5 to 45% (wt/vol) sucrose gradient containing 2 M guanidine (SW50.1 rotor, 35,000 rpm, 2.83 h, 20°C). (A) Vesicles formed after wild-type infection. (B) Vesicles formed after *sus1* infection, when empty particles were produced. (C) Gradient after infection with non-particle-forming mutants *sus13* (class X), *sus14* (class III), and *sus151* (class XVII). Mutant *sus14* is shown.

type of free vesicles was observed in these experiments. The overall structure of this virus seems to be somewhat surprising. The virus coat containing two protein species surrounds a vesicle structure which contains the rest of the virion proteins. The vesicle seems to be capable of forming a tubular structure which may be used in DNA injection. Protein P2 is involved in the attachment to the host cell (7). After virus disruption this protein remains in the virus membrane vesicle, is not involved in tube formation, and is not easily accessible to proteases. Thus, these viruses have two shape-determining proteins (P3 and P5) surrounding a flexible membrane vesicle containing the viral DNA.

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