Head Proteins from T-Even Bacteriophage

I. Molecular Weight Characterization¹

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T-even bacteriophage capsid proteins were separated on 6% agarose columns by use of 6 M guanidine hydrochloride containing 5 mM dithiothreitol both to dissociate and to elute the proteins. The head capsids of T2H, T4B, T4B01, T4D, and T6r⁺ contained at least three structural proteins with molecular weights of 40,000, 18,000, and 11,000 daltons, amounting to 76, 2, and 8%, respectively, of the total capsid protein. On the other hand, T2L head capsids contained only two structural proteins with molecular weights of 40,000 and 18,000 daltons (81 and 2.5%, respectively, of the total protein). A discussion of the possible role of these structural head proteins and a T-even phage head model suggesting a structural arrangement of the 40,000 dalton subunit are presented.

Much work has been done in characterizing the protein components in the heads of T-even bacteriophage. It was first thought that there was a single protein with a molecular weight of 80,000 daltons (26). Later work by Cummings (6) showed that the major structural head protein of T2L had a molecular weight of 40,000 daltons, and that the molecular weight obtained for the subunit was a function of the method used for dissociating the head. Recently, Kellenberger (17), using polyacrylamide gel electrophoresis, found that there was one major head protein, M, and possibly two minor components, k and l. In addition, L. Larcom and I. Bendet (Abstr. Biophys. Soc., MI5, p. A-66, 1968) and L. Larcom, I. Bendet, and S. Mumma (Abstr. Biophys. Soc., SAM-J2, p. A-258, 1969) studied heads obtained from a T4D amber mutant and reported that there were two proteins with molecular weights of 46,000 and 10,600 daltons.

There were two major problems associated with characterizing head proteins. (i) Previously, purified heads could only be obtained from T4D amber mutants grown in a nonpermissive host. (ii) The methods of analyzing structural head proteins (6, 26; Larcom and Bendet, 1968; Larcom et al., 1969) relied on statistical measurements because methods were not available to isolate and purify these proteins. Consequently, those proteins which were present in very limited quantities were not detected until Kellenberger (17) used polyacrylamide gels. The polyacryl-

amide gel technique for analyzing head proteins has the disadvantage of not separating sufficient quantities of protein for physical and chemical analyses. We have developed methods (8) for disrupting bacteriophage into their component substructures; thus, we can obtain purified heads from any of the T-even bacteriophage. Davison (12) has shown that a variety of proteins can be separated according to their molecular weights on agarose columns with 5 M guanidine hydrochloride as the eluant. In our system, the guanidine hydrochloride also dissociates the bacteriophage heads into their component proteins and allows each component to be separated according to its molecular weight.

This paper confirms the finding that the major head protein in T-even wild-type bacteriophage has a molecular weight of approximately 40,000 daltons. We have also shown that the heads of wild-type T-even bacteriophage contain a second protein component with a molecular weight of approximately 18,000 daltons. All of the wild type T-even bacteriophage heads examined contained an 11,000 dalton protein, except for T2L. T2L heads contained the major 40,000 dalton protein and the 18,000 dalton protein.

MATERIALS AND METHODS

Phage growth. *Escherichia coli* B was grown overnight in a tris(hydroxymethyl)aminomethane-glycerol-Casamino Acids medium (19). The overnight culture was diluted 200-fold into fresh media at 37°C with vigorous aeration. At a density of 2×10^8 to 3×10^8 cells/ml, the bacteria were infected with a multiplicity

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of one phage per bacterium. Aeration was continued for an additional 5 hr, after which time chloroform (1.0 ml/liter), deoxyribonuclease (1 μ g/ml), and ribonuclease (1 μ g/ml) were added to the lysate.

The phage particles were harvested by differential centrifugation or by a modified two-phase polymer method (2).

Differential centrifugation. The lysate was stored at 4 C overnight and then was filtered through Celite to remove bacterial debris. The phage were collected by centrifugation at $28,000 \times g$ for 1.5 hr in the batch rotor of an International B20 centrifuge. The phage pellets were resuspended in deoxyribonuclease solution (6) for 1.5 hr at room temperature. The phage particles were centrifuged at $7,000 \times g$ for 15 min to remove debris and then again at $28,000 \times g$ for 1.5 hr. The pellet was resuspended in approximately 10 ml of saline stock solution (0.15 M NaCl, 1 mM $MgCl_2$, 1 mM PO_4 , pH 7.5).

Two-phase polymer method. At the end of the growth period, chloroform (1.0 ml/liter) and a small quantity of deoxyribonuclease and ribonuclease were added to the lysate. The lysate was placed on a magnetic stirrer, and as soon as a vortex was formed the following materials were added in the order: sodium chloride, 17 g/liter; polyethylene glycol 6000 (Chemical Sales Co., Denver, Colo.), 71 g/liter; and sodium dextran sulfate 500 (Pharmacia, Uppsala, Sweden), 2.3 g/liter. Each compound was dissolved completely before the next was added. Then the lysate was placed in a cold room at 4 C for at least 15 hr. The upper phase containing the polyethylene glycol was removed and discarded. The whitish dextran layer was collected and diluted with saline stock solution to 480 ml for each 8 liters of lysate. This solution was placed in a cold room overnight. The supernatant fluid from the diluted dextran phase was collected and centrifuged at $5,000 \times g$ for 15 min. The dextran pellets were discarded, and the supernatant fluid containing the phage particles was centrifuged at $28,000 \times g$ for 1.5 hr. The phage pellets were resuspended in saline stock solution and repurified by centrifugation as before.

Capsid preparation. Bacteriophage particles devoid of deoxyribonucleic acid (DNA), i.e., phage capsids, were prepared by use of osmotic shock methods (15). The DNA was removed by deoxyribonuclease digestion for 2 hr at room temperature (6), and the empty capsids were centrifuged at $45,000 \times g$ for 3 hr. The pellets were resuspended in saline stock solution and purified by banding in a $CsCl$ - D_2O step gradient.

Bacteriophage characterization. The bacteriophage and their capsids were characterized according to sedimentation velocity (7, 9) and cofactor requirements (1). For further identification of the particular bacteriophage, the DNA of the phage was extracted by the phenol method (22) and analyzed in a Cs_2SO_4 gradient (11, 13).

Labeling with 3H -amino acids. An overnight culture of *E. coli* B was diluted 150-fold into 100 ml of C^+ minimal medium (10; Na_2HPO_4 , 8.0 g; KH_2PO_4 , 2.0 g; NaCl, 3.0 g; NH_4Cl , 3.0 g; gelatin, 0.2 g; 3×10^{-4} M $MgSO_4$; 8×10^{-5} M $CaCl_2$; glucose, 10 g; 1 liter of demineralized water; pH 7.2; 200 mg of L-tryptophan per liter for T4B) and incubated with

aeration at 37 C. When the bacteria reached a concentration of 2×10^8 to 3×10^8 /ml, they were infected with a 5:1 multiplicity of bacteriophage. At 5 min after infection, tritiated amino acids [250 μ Ci 3H (G) mix, lot # 368-165; New England Nuclear Corp., Boston, Mass.] plus 10 mg of Casamino Acids were added. Incorporation of tritiated amino acids was monitored by taking 1.0-ml samples and precipitating the protein with 10% trichloroacetic acid. The precipitate was collected and washed on a membrane filter (Millipore Corp., Bedford, Mass.), dried under an infrared light, and counted in toluene scintillation fluid (5 g of 2,5-diphenyloxazole and 5 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene, obtained from Amersham-Searle Co., Des Plaines, Ill., in 1 liter of toluene) in a Beckman CPM-100 liquid scintillation counter. Incorporation appeared to be complete 45 min after infection. After 2.5 hr, chloroform (2 ml), deoxyribonuclease, and ribonuclease were added to the lysate. The bacteriophage were purified by differential centrifugation. Capsids were prepared as previously mentioned.

Bacteriophage head capsids. T4D am H21 (gene 54) was obtained from R. S. Edgar. Phage stocks were grown in the permissive host *E. coli* CR63. Heads (and tail plates) were prepared by growth in the non-permissive host *E. coli* B. The parts were purified by a two-phase polymer method (sodium chloride, 30 g/liter; polyethylene glycol, 91.5 g/liter; sodium dextran sulfate, 2.3 g/liter). The purified parts were separated in a Spinco SW25 rotor by centrifugation through a 5 to 25% sucrose gradient with a D_2O - $CsCl$ cushion at the bottom of the tube.

T6 heads were prepared by treatment with 67% dimethyl sulfoxide (8) and purified as above.

Capsid subunits. The capsids were dissociated in 6 M guanidine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), 0.05 M LiCl, 0.01 M ethylenediaminetetraacetate (EDTA), and 5 mM dithiothreitol (Calbiochem, Los Angeles, Calif.) at 37 C for various lengths of time (3 to 12 hr).

Molecular weight determinations. Molecular weights were determined from the partition coefficients of the proteins on a 6% agarose column (12). Agarose (Bio Gel A-5m, 100 to 200 mesh, lot # 6090; Bio-Rad Laboratories, Richmond, Calif.) was equilibrated with 5 M guanidine hydrochloride, 0.05 M LiCl, 0.01 M EDTA, and 5 mM dithiothreitol, and was poured into a column (2 $\times$ 95 cm). A flow rate of 7 ml per hr was used, and 5-ml samples were collected. Dextran blue and dinitrophenyl-alanine were used to determine the void volume and the internal volume, respectively. Molecular weight standards were denatured in 6 M guanidine hydrochloride, 0.05 M LiCl, 0.001 M EDTA, and 5 mM dithiothreitol, and were chromatographed on a 6% agarose column. The results (Fig. 1) showed that a linear relationship exists between the partition coefficient [$K = (V_e - V_o)/(V_i - V_o)$, where V_e is the elution volume, V_i the internal volume, and V_o the void volume] and the log of the molecular weight. Samples were analyzed in an Aminco-Bowman Spectrophotofluorometer (excitation, 280 nm; emission, 340 nm). Portions (0.9 ml) were dissolved in scintillation fluid (2 parts toluene and 1 part Triton X100)

FIG. 1. Molecular weight standards on a 6% agarose column. Bovine serum albumin, 69,000 daltons; pepsin, 35,500 daltons; trypsin, 24,000 daltons; and hemoglobin, 15,500 daltons (25) were dissociated with 6 M guanidine hydrochloride containing 5 mM dithiothreitol and were chromatographed on a 6% agarose column.

and were counted in a Beckman CPM-100 liquid scintillation counter.

Electron microscopy. Electron micrographs of the phage head capsids were made with the use of the phosphotungstic acid-negative staining method (4). The electron microscope was an RCA-EMU 4 fitted with a modified high-intensity re-entry type grid cap to enhance contrast (21). A 5-ml condensing aperture and an 18- μ m objective aperture were used.

Results were recorded on Dupont Cronar Ortho 708 Litho A film at a magnification of about 15,000 times and were photographically enlarged 3 times.

RESULTS

Three proteins with molecular weights of 40,000, 18,000, and 11,000 daltons were isolated from purified head capsids of wild-type T6r⁺ and T4D am H21, gene 54 (Fig. 2).

The heads were purified as described, and Fig. 3 shows an electron micrograph of the purified heads from T6r⁺ and T4D am H21. Head capsids were dissociated in 6 M guanidine hydrochloride and chromatographed on a 6% agarose column. In both cases, the purified heads contained three proteins with molecular weights of

40,000, 18,000, and 11,000 daltons corresponding to peaks 3, 4, and 5 (Fig. 2). The dip in the base line is the position of the void volume marker, dextran blue.

Further analyses of the complete phage capsids revealed that T2L and T2L_{RII} head capsids contained the 40,000 and 18,000 dalton proteins but the 11,000 dalton protein (Fig. 4D) was absent. T4B01, T4B, and T2H (Fig. 4A, B, and C) contained, in their head capsids, three proteins of the same molecular weights as did T6r⁺ and T4D (Fig. 4). Since it was possible that the T2L capsids lost the 11,000 dalton protein during preparation, the supernatant solution obtained from the osmotic shock procedure was concentrated and analyzed on a 6% agarose column. A very small amount of protein (less than 0.2% of the total phage protein) did elute in the region of 10,000 daltons. This was thought to be subunits from another phage protein component which appear to elute in this region (*unpublished data*).

Figure 4 also shows that T-even phage capsids contain a protein with a molecular weight of 84,000 daltons (peak 2) and also a protein or proteins that elute in the void volume (peak 1). The 84,000 dalton protein eluted near the void volume, and its position and amount were variable on different agarose columns; therefore, it was not possible to be certain whether this protein was present in the head capsids of T6r⁺ or T4D (Fig. 2). T6r⁺ phage yielded very pure head preparations with little sheath contamination (Fig. 3A) after dimethyl sulfoxide treatment. The other T-even phages yielded head capsids with variable amounts of purity, depending upon the extent of purification. Since the void volume peak decreased with a corresponding decrease in sheath contamination, and since the void volume contained protein(s) from the complete phage capsids (Fig. 4) and not from T-even head capsids (Fig. 2), it was concluded that the void volume peak was comprised of tail structure components.

To determine the relative amounts of protein in each peak and to determine again whether T2L head capsids contained a small amount of the 11,000 dalton protein, T2L and T4B were uniformly labeled with tritiated amino acids. The distribution of label was plotted, and the peaks were cut out and weighed to determine the relative areas in each peak. The void volume (peak 1) contained 11% in T4B and 14% in T2L of the total capsid proteins. This amount of protein corresponds to the percentage of protein contained in the tail structure of T-even bacteriophage (5, 24). Peak 2, the 84,000 dalton protein, amounted to 1% of the total protein

FIG. 2. $T6r^+$ and $T4D$ am H21 head capsid proteins. Purified head capsids were dissociated in 6 M guanidine with 5 mM dithiothreitol and were chromatographed on a 6% agarose column. The absence of peak 1 indicated that most of the tail structure proteins elute in the void volume. This also demonstrates that peaks 3 (40K), 4 (18K), and 5 (11K) were components of the head structure. (See Fig. 4 for numbering of peaks.)

in T4B and 2.4% in T2L. Peak 3, the major structural head protein with a 40,000 dalton molecular weight, accounted for 76% of the total protein in T4B and 81% in T2L. Peak 4, the 18,000 dalton head protein, amounted to 2% of the total protein in T4B and 2.5% in T2L. Peak 5, the 11,000 dalton head protein, amounted to 8% of the total capsid protein in T4B (Fig. 5A) and was absent from T2L (Fig. 5B). The 11,000 dalton protein accounted for 8% of the total protein in the labeling experiment; however, on the basis of fluorescence measurements obtained from at least three separate analyses on each of the phages examined, this value may range from 8 to 15% of the total capsid protein.

DISCUSSION

It is now possible to isolate large quantities of wild type T-even phage head capsid protein by use of 6 M guanidine hydrochloride and 6% agarose gel columns. The results showed that T2H, T4B, T4B01, T4D, and $T6r^+$ head capsids contain at least three structural proteins with molecular weights of 40,000, 18,000, and 11,000 daltons, amounting to approximately 76, 2,

and 8%, respectively, of the total phage capsid protein. The tritium-labeled phage profile presented in Fig. 5 confirms that the 11,000 dalton protein was absent from T2L. If there was an 11,000 dalton protein in the T2L capsids, it amounted to less than 0.2%. It is interesting to note that the sedimentation coefficient of T2L dimethyl sulfoxide heads is lower than the sedimentation coefficients of the other T-even dimethyl sulfoxide heads [see (9)]. Comparing the fast forms of the T-even phage dimethyl sulfoxide heads to T2L dimethyl sulfoxide heads, one obtains a difference of approximately 10% in the sedimentation coefficient. Since the 11,000 dalton protein amounted to about 8% of the total phage proteins, the difference in sedimentation coefficients could reflect the absence of this protein in T2L.

The 84,000 dalton protein (peak 2) may be a dimer of the 40,000 dalton subunit. It has been reported that the 40,000 dalton subunit will form dimers under certain conditions (6; Larcom et al., Abstr. Biophys. Soc., 1969). This protein appeared to be missing from the isolated heads of T4D am H21 and $T6r^+$ (Fig. 6), but we cannot be certain of its absence.

FIG. 3. Electron micrograph of T6r⁺ and T4D am H2I purified heads. (A) T6r⁺ capsids were disrupted with 67% dimethylsulfoxide and the heads were purified on a glycerol-D₂O-CsCl gradient (unpublished data). (B) T4D am H2I was grown on the nonpermissive host *E. coli* B and the heads were separated from tail plates on a similar gradient.

FIG. 4. *T*-even phage capsid proteins. Phage capsids were dissociated in 6 *M* guanidine hydrochloride containing 5 *mM* dithiothreitol and were chromatographed on a 6% agarose column. All of the phage capsids yielded five protein peaks except T2L, where (D) the 11,000 dalton protein was absent (peak 5). The arrow indicates the position where the 11,000 dalton protein would elute. The chromatograms were not aligned because of sample volume differences which varied from 2.5 to 5 ml, although the sample volume was constant for each particular analysis.

The possibility was considered that the 18,000 dalton protein was not a head component, but was a tail component or an internal protein (20). The only tail component present in sufficient amounts to account for 2 to 3% of the total protein is the sheath protein. Chemical analysis (*unpublished data*) and the chromatograms on purified heads (Fig. 2) indicate that the 18,000 dalton protein is distinct from the sheath protein. The internal protein was ruled out for the following reasons. (i) It was not preferentially released into the medium during the osmotic shock procedure. (ii) The same amount of protein was found in purified heads from the amber mutant T4D am H21 which had not been subjected to osmotic shock.

In assessing the role of these proteins in the phage head, it is necessary to consider the dimensions of the head and the protein shell as well as the physical properties of the subunits themselves. Little evidence is available on the morphology of *T*-even heads to specify their exact structure. Moody (23) suggested that the heads could have icosahedral symmetry. However, the *T*-even heads do not conform to some aspects of icosahedral symmetry; this has been

discussed at length in a forthcoming review (D. J. Cummings and N. L. Couse, *Advan. Virus Res.*, *in press*). Based on the morphological appearance of the heads, Anderson et al. (3) suggested that they were bipyramidal hexagons. Figure 6 is a schematic representation of such a structure. This structure offers the advantage that the head protein subunits can be readily accommodated. The 40,000 dalton protein is the only protein which has been shown to be the product of a gene (gene 23) defined by amber mutants (16, 17), and it is the major structural protein subunit of all the *T*-even phage heads. In agreement with previous results, this protein amounts to about 1,600 to 2,000 subunits per phage head, assuming a particle weight for the head of 80×10^6 to 95×10^6 daltons (Cummings and Couse, *in press*). From its dimensions, 1.9×27.0 nm (6), this protein can fit end to end in the faces of the bipyramidal hexagon (Fig. 6). By offsetting these subunits in successive rows, much room is allowed (within the 11.4 nm depth of the shell) for possible conformational changes (9).

It is clear from this presentation that the 40,000 dalton subunit plays a necessary and important

FIG. 5. *T4B* and *T2L* ^3H -labeled capsid proteins. The phage capsids were labeled with tritiated amino acids, dissociated in 6 M guanidine hydrochloride with 5 mM dithiothreitol, and chromatographed on a 6% agarose column. The figures indicate the percentage of the total protein that each peak contains. (A) *T4B* labeled capsid proteins chromatographed with unlabeled *T4B* proteins. The ^3H -labeled peaks corresponded to the fluorescence peaks. (B) *T2L* labeled capsid proteins chromatographed with unlabeled *T4B* proteins. Peak 5, the 11,000 dalton protein, was absent from *T2L* capsids.

role in the structure of T-even bacteriophage heads. We do not know what role the other two proteins play in this structure. Since the 11,000 dalton protein is absent from *T2L*, it is not likely that this subunit plays a crucial role in the head structure of the other T-even bacteriophage. There are roughly 1,000 subunits of the 11,000 dalton protein and about 100 subunits of the 18,000 dalton protein. These proteins may be in the

spaces between the 40,000 dalton proteins and thus obscure the subunit arrangement within the head. Alternatively, they may play a special role at the apices or at the attachment site of the head to the tail. Kellenberger (17) reported that k and l proteins could be extracted from the capsids upon heating in urea without apparent loss of the M protein. This may suggest that the k and l proteins are surface proteins, and they may corre-

FIG. 6. *T*-even bacteriophage head subunit model. This schematic diagram illustrates the dimensions of a *T*-even phage head (3, 6, 18), and represents a possible way in which the 40,000 dalton subunits with dimensions of 1.9×27.0 nm (6) could fit into the head giving a uniform shell thickness of 11.4 nm. Each of the six rectangular faces is depicted in two sections, and each section contains seven rows of subunits (bottom set) in a close packed array giving a shell thickness of 11.4 nm. The triangular face subunits (top set) have more room between the subunits, allowing both for extensive overlap and also for head closure. In this model, there would be a total of (6×168) and (12×52) or 1,632 of the 40,000 dalton subunits.

spond to the 18,000 and 11,000 dalton proteins, respectively. On the other hand, Kellenberger (17) reported that the *k* and *l* proteins appear to be missing from an aberrant substructure, polyheads. Since polyheads show variability in length, width, and morphological structure (14, 18), this suggests that the 18,000 and 11,000 dalton proteins may play some role in regulating the size and shape of the head. It is clear that much additional information is necessary before we can understand the exact role of these two proteins in the bacteriophage head.

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