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Isolation of the Prohead Core of Bacteriophage T4 After Cross-Linking and Determination of Protein Composition

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The naked core of bacteriophage T4 was isolated ex vivo after cross-linking with either glutaraldehyde or dithiobis(succinimidyl propionate). The isolated particles appeared to be morphologically identical to the cores found in thin sections, to those demonstrated in in situ lysis preparations, and to core structures assembled in vitro. Treatment with glutaraldehyde provided core particles which were morphologically well preserved, whereas dithiobis(succinimidyl propionate)-induced cross-linking was reversible and allowed analysis of the protein composition of the isolated particles. The identity of the reversibly cross-linked particles with those obtained after irreversible cross-linking was suggested by their morphology and their similar sedimentation behavior. Immunolabeling confirmed the structural presence of the main core protein in both structures. Gel electrophoresis of reversibly cross-linked cores revealed the essential head proteins gp22, gp67, and gp21, the three internal proteins IPI, IPII, and IPIII, and a 17K protein.

Head formation of bacteriophage T4 proceeds by assembly of a prohead which consists of a shell around a proteinaceous core, but does not yet contain DNA. T4 prohead assembly takes place at the bacterial inner membrane, from where it detaches during conversion into the mature capsid in a series of interconnected processes (see, e.g., reference 24). These processes include cleavage and partial release of the core proteins, cleavage and rearrangement of the shell proteins, and DNA packaging.

The prohead has a defined size and shape, suggesting that form determination must be achieved at the prohead assembly stage. Since the shell protein does not have full morphogenetic competence, the hypothesis of a "morphopoietic core" was established, and such a function was ascribed to the highly organized structure seen within proheads (2, 3). Although direct experimental access to the prohead core turned out to be rather difficult, its possible role as the nucleation site for shell assembly was inferred from genetic and biochemical data (15). Its nature as an independent head-related particle and its involvement in size determination became evident when Van Driel and Couture (21) obtained structures resembling the prohead core in their in vitro assembly experiments of prohead proteins. The accompanying paper (18), describing prohead core formation in vivo, confirms that core assembly is independent of shell assembly and suggests the equivalence of the naked core and the core inside the prohead core by genetic and structural data.

This work describes the isolation of these extremely fragile particles and gives results of an analysis of their protein composition. In a broader context, this study is a further step towards understanding how proteins interact to establish a product of defined architecture and defined size such as the naked prohead core.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. *Escherichia coli* B was used as the nonpermissive strain and *E. coli* CR63 was used as the permissive strain for amber mutants. The cores were isolated from T4D 10(*amB*255) · 18(*amE*18) · 20(*amE*481) · 23(*amH*11)-infected cells, whereas the isolation of proheads was done with T4D 10(*amB*255) · 18(*amE*18) · 21(*tsN*8)-infected cells. The mutant T4D 10(*amB*255) · 18(*amE*18) · 22(*amE*209) · 23(*amH*11) was used for control experiments. All multiple mutants were constructed from single mutants from our collection. The nomenclature of proteins and the abbreviations used are described in the accompanying paper (18).

Bacterial growth and phage infection. Fresh overnight *E. coli* B cultures were diluted 1/100 into 500 ml of M9 medium supplemented with Casamino Acids (Difco Laboratories), 1% final concentration (5), and were shaken at 37°C until a density of 2×10^8 cells per ml was reached. The cells were infected and superinfected 6 min later with an average of five phages per cell. Immediately after infection the temperature was reduced to 30°C (cores seem to be less stable at high temperature as described in the accompanying paper [18]). After 90 min the infected cells were centrifuged at 5,000 × *g* in a IEC DPR 6000 centrifuge at room temperature for 5 min and then resuspended in 1 ml of extraction buffer (150 mM potassium phosphate [pH 7.6], 60 mM NaCl, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 100 μg of DNase per ml [10, with modifications]). The experiment was only continued when no lysis occurred at this stage.

Isolation of irreversibly cross-linked cores. For simultaneous lysis and cross-linking, the resuspended bacteria (1 ml) were added to a precooled mixture of 0.5% (50 mM) glutaraldehyde (Fluka, EM grade), 1% polydisperse octyl-oligooxyethylene (octyl-POE [12]; kindly provided by J. P. Rosenbusch), and 0.5% acrolein (Serva, research grade) in 1 ml of extraction buffer. Lysis was controlled by light microscopy. The cross-linking reaction was stopped after 45 min at 0°C by the addition of 50 mM alanine. The lysed cells were centrifuged at 17,300 × *g* in a Sorvall SS34 rotor at 15°C for 10 min. The supernatant (0.8 ml) was applied to a 12-ml continuous 15 to 40% glycerol gradient or sometimes to a sucrose step gradient of four steps of 10, 20, 30, 40% sucrose in the extraction buffer. The gradients were run at 160,000 × *g* in an SB 283 rotor of an IEC centrifuge at 15°C for 90 min; 0.6-ml fractions were collected and samples were immediately prepared for electron microscopic observation.

Isolation of reversibly cross-linked cores. For lysis and

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reversible cross-linking, the resuspended bacteria were added to a mixture prepared as follows: 2.02 mg of dithiobis(succinimidyl propionate) (DTSP [6]; Pierce Chemical Co.) in 100 μl of dimethyl formamide (E. Merck AG) was added to 750 μl of prewarmed (40°C) extraction buffer to give a final concentration of 2.5 mM DTSP. After addition of 50 μl of 20% octyl-POE and 2 μl of acrolein, the mixture was completed by the addition of 100 μl of chloroform saturated with DTSP. The cross-linking reaction was performed at 40°C for 15 min and stopped by addition of alanine. A precipitate sometimes formed after addition of the chloroform-DTSP mixture, but dissolved quickly at 40°C.

Prohead isolation. Proheads were isolated according to Onorato et al. (10) except that in some cases the pH of the extraction buffer was adjusted to 7.6 instead of 6.0.

Radioactively labeled cores. The bacteria were grown in M9a (M9 supplemented with Difco Casamino Acids at a final concentration of 0.1%) and labeled at 10 and 45 min after infection with 100 μCi of ^{3}H (1 mCi/ml)- or ^{14}C (50 μCi/ml)-labeled amino acid mixtures (Amersham International Ltd.). The cores were then isolated as described above.

Gel electrophoresis and silver staining. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Laemmli (4). The gels were stained with silver according to the method of Wray et al. (25), with the modification that the gels were washed twice for 5 min with water after being stained in silver solution. DTSP-cross-linked structures were cleaved by adding 5% mercaptoethanol for 30 min at 37°C before electrophoresis. Protein band identification was based on comparison with the prohead protein profile as a standard (10) and on comparison with reference gels of different amber mutants kindly provided by T. Grütter from our laboratory.

Immunoelectron microscopy. Protein A-gold complex was prepared according to Roth et al. (14). The diameter of gold particles was approximately 10 nm. A drop of sample containing isolated cores or T7 phages was adsorbed to a glow-discharged copper grid (Balzer; 200 mesh) covered with carbon-coated collodion. Subsequent immunolabeling with protein A-gold essentially followed the technique described by Roth (13).

The grids were first placed for 3 min on a drop of a solution containing 10% fetal calf serum and 1% ovalbumin (Serva) in phosphate-buffered saline to prevent unspecific binding of the antibodies. Subsequently, they were incubated on a drop containing anti-gp22 for 1 h (room temperature) or, as a control, antitubulin in a fetal calf serum–ovalbumin–phosphate-buffered saline solution. After thorough rinsing with phosphate-buffered saline, the grids were applied to a drop of protein A-gold in phosphate-buffered saline for 0.5 h at room temperature. Careful washing in phosphate-buffered saline preceded negative staining with a 5% sodium phosphotungstate solution.

Quantitative evaluation of immunolabeling was performed on a Zeiss EM 10 electron microscope at 80 kV.

Immunoblotting. Electrophoretic transfer of proteins from gels to nitrocellulose sheets and immunological detection of proteins were carried out according to a modified version of the technique of Towbin et al. (17) described by Murray et al. (8). This version uses ^{125}I-labeled protein A to detect bound antibodies.

Antibodies were gifts from M. K. Showe (anti-gp22, anti-gp67, anti-gp23) and J. Caldentey (anti-gp20). In control experiments with prohead proteins, they reacted only with their specific antigen.

Electron microscopy. Specimens for electron microscopy were prepared by adsorbing a drop of the sample to a copper grid covered with a carbon-coated collodion film. The preparations were negatively stained with 2% sodium phosphotungstate. In experiments with DTSP as cross-linker, the samples were treated with 1% glutaraldehyde before electron microscopy preparation. Micrographs were taken with a Zeiss EM 10 electron microscope on Kodalith film at 80 kV.

RESULTS

Isolation of naked prohead cores. Intracellularly assembled cores could only be isolated if lysis of bacteria and cross-linking of proteins were simultaneous. In a synergistic way the two components acrolein and octyl-POE (an anionic detergent [12]) ensured a rapid and complete lysis of T4-infected cells. The concentrations of acrolein and glutaraldehyde were critical. Every change in the conditions described in Materials and Methods resulted in either incomplete lysis or bad preservation of cores and decreased the already low yield of structurally preserved cores.

After the cross-linking reaction and separation from bacterial debris, the particles were purified on glycerol gradients. The isolation procedure based on glutaraldehyde (25 mM) cross-linking yielded core particles as a band on a glycerol gradient (fractions 10 to 16) as determined by electron microscopic examination. Isolated core particles were indistinguishable in size (about 60 by 80 nm) and morphology from the naked cores reported in the accompanying paper (18). Figure 1a shows examples of cores cross-linked with glutaraldehyde. Note the neck structure of the core. A distinct stain-excluding region characterizes the central part of the particles. This probably reflects the presence of gp21, as suggested by Van Driel et al. (22). Reversible cross-linking was achieved by using DTSP dissolved in dimethylformamide to increase solubility. DTSP-saturated chloroform was added to increase the final concentration of DTSP. Although isolated core particles cross-linked with DTSP were found at the same position in the gradient as irreversibly cross-linked cores, DTSP-treated particles appeared to be less well preserved. The length of ill-defined particles varied from 80 to 150 nm. Some particles probably represented core pieces. However, a small fraction of intact cores was always present. These cores were morphologically identical to those obtained after glutaraldehyde cross-linking (Fig. 1b).

Comparison of reversibly and irreversibly cross-linked cores by sedimentation behavior. To substantiate the identity of the DTSP-cross-linked structures with the morphologically well-characterized cores cross-linked by glutaraldehyde, the sedimentation behaviors of both types of structures were compared. ^{3}H- or ^{14}C-labeled cores cross-linked by either DTSP or glutaraldehyde were isolated by sedimentation in glycerol. Fraction 13 (Fig. 2a) of each gradient was dialyzed against extraction buffer. About equal amounts of radioactivity were loaded on a subsequent glycerol gradient. Figure 2b shows the result of the sedimentation analysis. The two peaks almost coincided and were found at the position of the gradient where electron microscopy had revealed the presence of cores. DTSP-treated particles with a slower sedimentation characteristic appeared to be morphologically less well defined.

Antibodies against the main core protein bind specifically to isolated cores. Core particles obtained after reversible and irreversible cross-linking were incubated with anti-gp22 and then treated with a solution containing protein A-gold complex. This treatment improves electron microscopic visualization of the antigen-antibody binding, since protein A attaches preferentially to the Fc portion of antibodies (13).

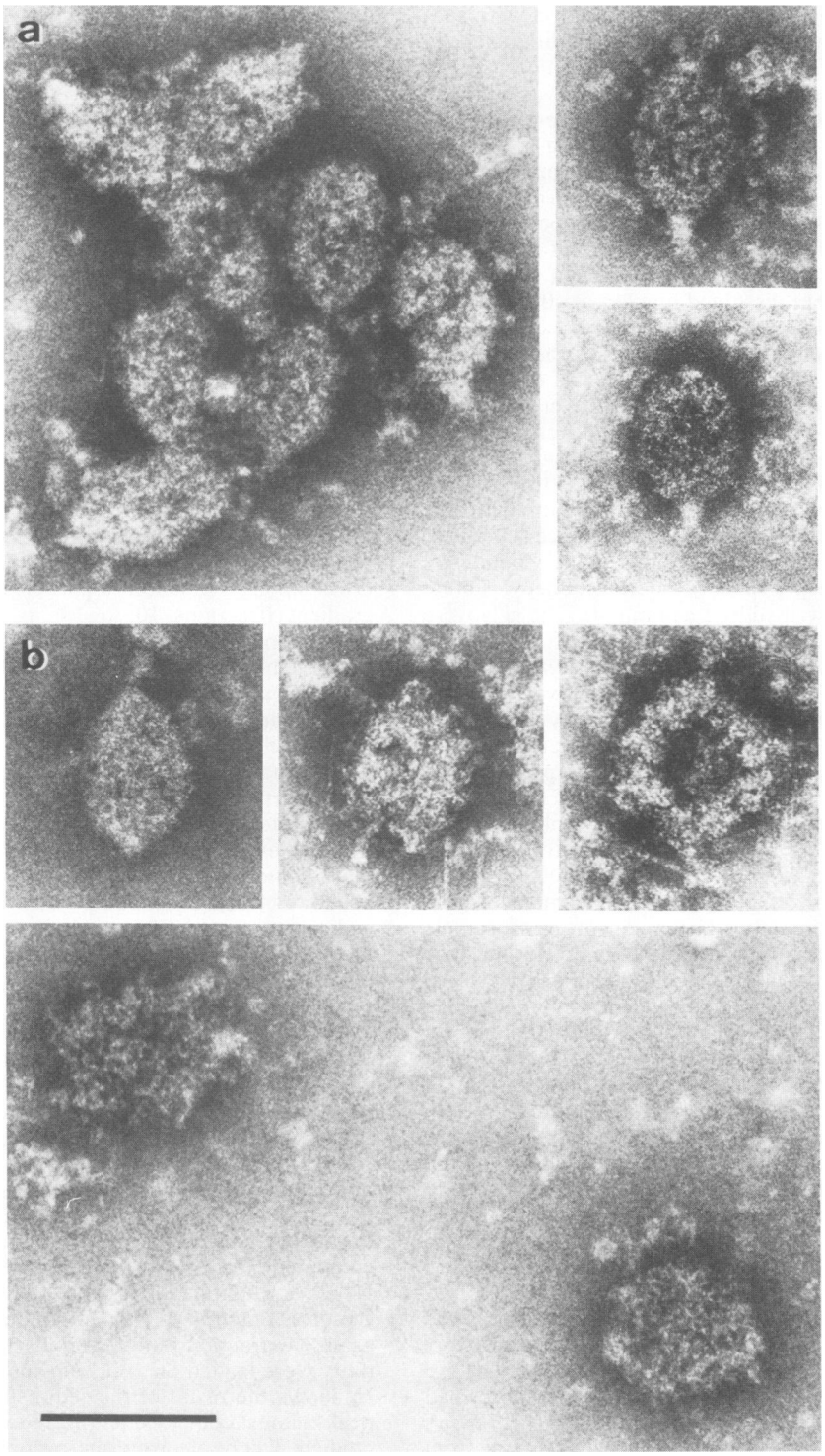

FIG. 1. Electron micrographs of negatively stained isolated naked prohead cores. (a) Cores irreversibly cross-linked with glutaraldehyde. (b) Cores reversibly cross-linked with DTSP. Note the neck structure which is sometimes visible. Bar, 100 nm.

The quantitative estimates of the number of gold particles which decorated the isolated core particles and thus indicated antibody-antigen binding are given in Table 1. The number of gold particles bound to cores was significantly higher with anti-gp22 pretreatment of the cores compared with the controls where antitubulin pretreatment was used or antibody incubation was omitted (Table 1). In a further control the level of unspecific binding of anti-gp22 was tested, using T7 phage, which is similar in size to the T4 prohead core, as a target structure. Although neither T7 nor

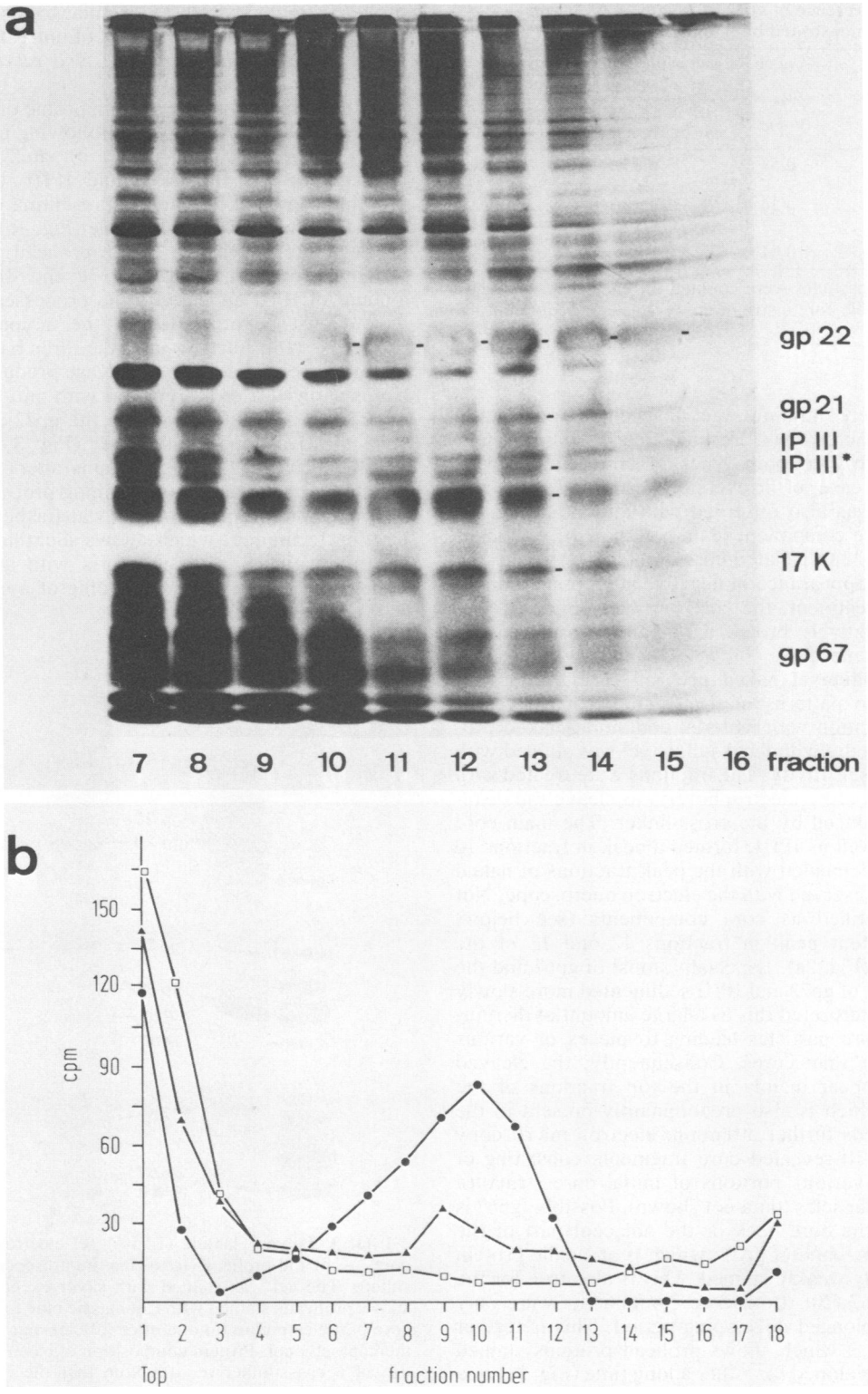

FIG. 2. Isolation of naked prohead cores by sedimentation in glycerol. A crude lysate containing prohead cores was layered on a continuous 15 to 40% glycerol gradient (in extraction buffer). After centrifugation the gradient was divided into 20 fractions. Samples (20 μl) of each fraction were treated with mercaptoethanol to cleave the cross-linker and loaded on a polyacrylamide gel for electrophoresis. The gel (12.5% acrylamide) was stained with silver and developed for a long time to render gp22 visible (a). Samples (0.5 ml) of fraction 13 containing cross-linked cores were dialyzed against extraction buffer and layered on a second glycerol gradient. To compare the sedimentation behavior, ^{3}H-labeled cores cross-linked with DTSP (●) and ^{14}C-labeled cores cross-linked with glutaraldehyde (▲) were loaded on the same gradient. After centrifugation the radioactivity of each fraction was examined (b). For a control the profile of a T4 $10^{-} \cdot 18^{-} \cdot 22^{-} \cdot 23^{-}$-infected lysate containing no cores was cross-linked with glutaraldehyde (□).

TABLE 1. Presence of gp22 in isolated core particles demonstrated by immunolabeling

Cores	Avg no. of gold particles attached to a core or T7 phage[a]		
	Anti-gp22	No antibodies	Antitubulin
Irreversibly cross-linked	0.54	0.15	0.08
Reversibly cross-linked	0.49	0.14	0.18
Control (T7 phage)	0.01	ND[b]	ND

[a] More than 100 particles were counted for each case. Only grid areas with comparable core distribution were selected for counting.
[b] ND, Not done.

gold particles were limiting, the unspecific binding was below 2% for each. These results clearly indicate that binding of anti-gp22 to both types of core samples was specific. In the case of irreversibly cross-linked cores, specific binding was also observed with antibodies against gp67, another core component (data not shown). Abundant binding of anti-gp22 to isolated cores was further suggested by the change of appearance in negative stain preparations. After antibody treatment, the core particles appeared surrounded by a relatively broad, dark halo not seen in the controls (cf. reference 1).

Protein composition of naked prohead cores. Figure 2a shows the protein patterns of the fractions of a glycerol gradient centrifugation with material containing naked prohead cores. The sodium dodecyl sulfate gel was stained with silver to improve sensitivity. The fractions were treated with mercaptoethanol before electrophoresis to cleave the disulfide bridges introduced by the cross-linker. The main core protein gp22, as well as IPIII, formed a peak in fractions 10 to 15. This peak coincided with the peak fractions of naked core particles as observed with the electron microscope. Not all proteins identified as core components (see below) showed a consistent peak in fractions 12 and 13 of the glycerol gradient (Fig. 2a). Especially, most of gp67 and the cleavage products of gp22 and IPIII sedimented more slowly than cores. We interpreted this as a large amount of disrupted or digested core particles leading to pieces of various sizes, but smaller than cores. Consequently, the cleaved gene products appear mainly in the top fractions of the gradient. gp67, which is also predominantly present in the top fractions, needs further attention: electron microscopy of fractions 6 to 10 revealed core fragments consisting of core necks with various portions of initial core structure as very frequent particles (data not shown). Possibly, gp67 is a constituent of the core neck or the adjacent part of the core. Note that the band of gp22, which is an acidic protein (19), was relatively weakly stained. This is also true for the acidic protein gp67 (20). Both core components were only visible after a prolonged developing period. This is further illustrated in Fig. 3 which shows prohead proteins stained with silver and developed for either a long time (Fig. 3e) or a short time (Fig. 3f), in comparison with Coomassie blue staining (Fig. 3d). Note that the long developing period resulted in a broadening of the bands for proteins of low molecular weights (Fig. 2a).

A few bands of high-molecular-weight proteins in Fig. 2a must represent complexes of core proteins which are due to incomplete cleavage of the cross-links, as suggested by the following two observations. First, in the protein pattern of proheads treated with DTSP similar bands appeared, whereas these were missing on the gel of untreated proheads (data not shown). Second, anti-gp22 also reacted with some of these bands (Fig. 4a).

A comparison with the protein profile of proheads used as reference (Fig. 3c) reveals the following proteins present in naked cores (Figs 3b, 4a and b): gp22, gp67, the three internal proteins IPI, IPII, and IPIII, gp21, and a 17K protein. The band probably representing gp21 was strongly stained in the core protein profile (Fig. 3b), but only weakly in the reference pattern of the prohead, regardless of the staining procedure used (Fig. 3c and d). A considerable amount of IPIII appeared in its processed form IPIII* (4), which must be attributed to the action of the prohead proteinase (16). Furthermore, the three bands in brackets in Fig. 3b were probably also cleavage products of gp22. These bands showed a cross-reaction with anti-gp22 (Fig. 4a). In contrast to the native protein, the gp22-derived fragments were clearly stained with silver (Fig. 3, lane b; Fig. 2a). Possibly, cleavage of the proteins alters its conformation, resulting in a change of the staining properties.

The major core protein gp22 was further identified by the blotting technique, which allows identification of proteins separated by gel electrophoresis with antibodies (8, 17). Lane a of Fig. 4 shows the profile of a core peak fraction

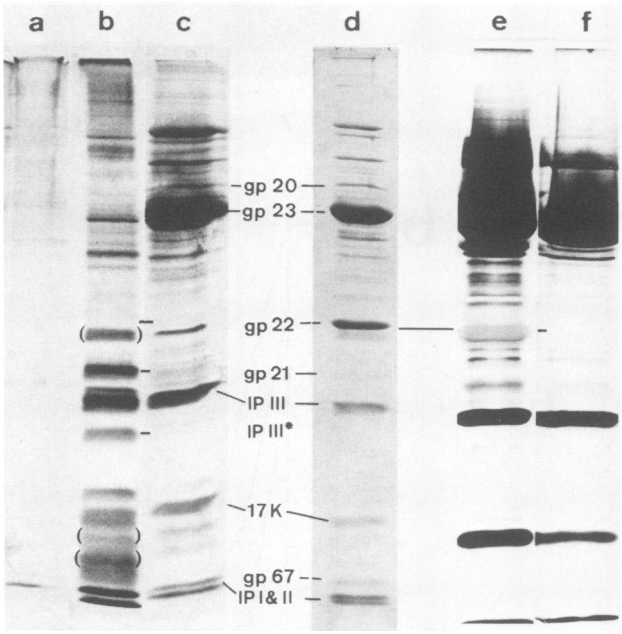

FIG. 3. Polyacrylamide (12.5%) gel electrophoresis of isolated cores (a, b) and proheads (c to f) in the presence of sodium dodecyl sulfate. The gel was stained with silver except for lane d, which shows proheads stained with Coomassie blue for reference. Isolated core particles before (a) and after (b) cleavage of DTSP bridges by mercaptoethanol. Protein composition of isolated proheads without use of a cross-linker (c, d). Note that the bands for the acidic proteins gp22 and gp67 are missing after silver staining (b, c) but are present when stained with Coomassie blue (d). Breakdown products of gp22, however, are also visible after silver staining (b). The bands in parentheses showed positive reaction with anti-gp22 (see Fig. 4). Lanes e and f further illustrate the particular staining properties of proheads isolated without use of a cross-linker: (e) proheads silver stained with long developing time and (f) silver stained with short developing time. gp22 appears only after prolonged developing on silver-stained gels.

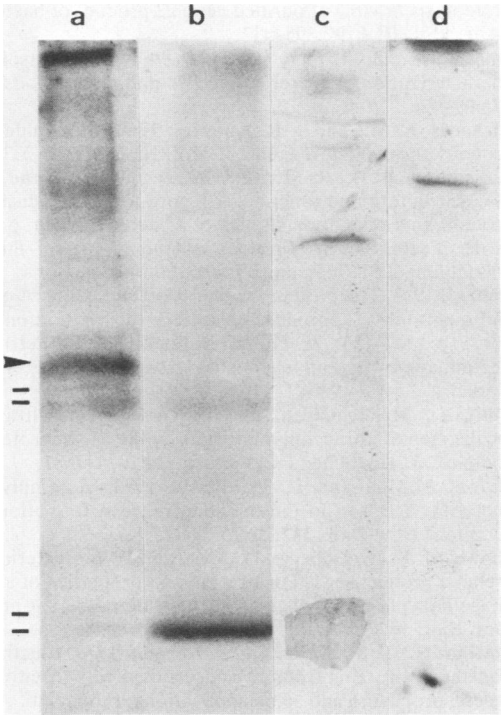

FIG. 4. Structural presence of gp22 and gp67 in isolated cores shown by the immunochemical blotting technique (8, 17). Banding pattern of separated core proteins treated with (a) anti-gp22 antiserum, (b) anti-gp67, (c) anti-gp23, and (d) anti-gp20. The arrow shows the position of native gp22; the bars mark four bands of lower molecular weights than the native gp22. These presumably represent cleavage products of gp22, whereas the bands with higher molecular weight than native gp22 protein are ascribed to complexes containing gp22 and held together by uncleaved bonds of the cross-linker (see text). Note that only trace amounts of the shell proteins gp20 and gp23 are present.

when treated with antibodies against gp22. A main band was found at the position of native gp22. Four proteins of lower molecular weight than gp22 reacted also with anti-gp22 antibodies. The two bands just below the main band of gp22 have also been found by Onorato et al. in proheads and were characterized as gp22 cleavage products (10). Lane b in Fig. 4 shows the same protein profile when treated with anti-gp67. Treatment of the same fraction with anti-gp20 and anti-gp23 revealed only trace amounts of these proteins in the core fraction (lanes c and d).

DISCUSSION

Isolation of naked core particles proved to be rather difficult. Establishing the in vitro assembly conditions of Van Driel and Couture (21) in the lysis medium did not allow isolation of these obviously extremely unstable particles, nor did the procedure previously used for prohead isolation (10). For successful isolation, a rapid lysis method for the pregnant cells had to be combined with immediate stabilization of particles by cross-linking. We think that there are two different reasons for the observed instability of cores and the low efficiency of our isolation procedure. The first relates to the self-destroying system inherent in the core structure: the prohead proteinase present in the core is activated by lysis of the pregnant cells and rapidly degrades gp22, the main component of the core (9). Cleaved products of gp22 and IPIII that appeared on the gels of isolated core particles may indicate this proteinase activity. A second reason for the fragility of the naked cores involves the presumed thermodynamic instability of this assembly structure. After opening the cells the concentration of free core proteins is drastically lowered compared with intracellular conditions. Consequently, the equilibrium between assembled and free core proteins is disturbed and now favors dissociation of the assembled structures.

The use of glutaraldehyde as cross-linker for core isolation provided perfectly preserved cores. On the other hand, the reversible cross-linker DTSP led to particles of poor structural integrity, although a minor fraction always appeared to be well preserved. This difference might be related to the low concentration of 2.5 mM DTSP which could be applied, compared with the 25 mM concentration of glutaraldehyde used. Moreover, DTSP had to be brought into the water phase by dimethylformamide, which at higher concentrations has a denaturing effect on proteins. The well-known potency of glutaraldehyde as a cross-linking agent can be explained by the fact that it polymerizes to chains of very different lengths in solution (11). Consequently, it forms cross-links between distant proteins, whereas DTSP as a unit-length cross-linker connects only neighboring proteins at a maximal distance of 1.2 nm. This prevents contaminating cross-linking of surrounding material in the crude lysate, but obviously a structure "fixed" by variable-length cross-links might be much more stable than a structure held together with short cross-links only. We therefore expect that the introduction of a reversible cross-linker which could form longer bridges, or cross-links of different lengths, and which could be used in higher concentrations would not only improve the quality of structural preservation of isolated cores, but also would substantially enhance their yield. This in turn would allow quantitative analysis of the proteins forming the core structure.

The prohead proteins gp23 and gp24 are known to represent shell constituents (7). gp20, another shell protein, seems responsible for correct shell initiation (18). Further, the proteins which are present in the prohead but are not constituents of the empty capsid (4, 7) were attributed to the transient prohead core structure. Showe and Black (15) isolated a complex of gp22, IPI, IPII, and IPIII and proposed that these proteins compose the assembly core. Two other proteins, gp67 (the former PIP [23]) and a 17K protein, were detected in isolated proheads and polyheads (10). Morphological evidence has been given that the zymogen for the prohead proteinase, gp21, is localized in the core center (22). This gene product has also been found to be essential for naked core formation in vivo (18).

We show here directly that the structures we have isolated ex vivo and which are identified by morphological criteria as naked prohead cores consist of all proteins previously described to be core components: gp22, gp21, gp67, IPI, IPII, IPIII, and a 17K protein. The structural presence of the main core protein gp22 and gp67 in intact core particles obtained with both isolation procedures could be assured with an immunolabeling technique, using protein A-gold complex as a marker for antibody-antigen binding. The other constituents were determined by direct comparison of the protein patterns of isolated cores and proheads. These results strongly suggest equivalence of the naked core and the core inside the prohead. This is in full agreement with the genetic and structural arguments presented in the foregoing work (18) and corroborates the finding that prohead core assembly

is not dependent on concomitant prohead shell formation. However, this notion does not necessarily imply that cores are normally intermediate structures in prohead assembly. But, as we will show in a future publication (F. Traub, A. Kuhn, B. Keller, and M. Maeder, manuscript in preparation), the naked core particles accumulated in vivo can be matured into active phage heads, and isolated naked cores can be complemented in vitro with shell proteins to form prohead-like particles.

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