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Analysis of T4 Phage Proteins, I. Conversion of Precursor Proteins into Lower Molecular Weight Peptides during Normal Capsid Formation*

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Abstract. Radioisotopically labeled T4-proteins extracted from purified capsids and phage and from infected cells were separated by gel electrophoresis in the presence of sodium dodecyl sulfate and a reducing reagent. They were identified by autoradiography and by counting of the fractionated gels.

Four major protein bands (F, A, D, and E) were detected in capsid and phage. These accounted for more than 90% of the total capsid protein and 70% of the phage protein (60% of the total capsid protein was in A-band). Co-electrophoresis of [^{14}C]proteins from capsids and [^{3}H]proteins from phage-infected cells indicated that the protein coded by gene 23 (P23) was a peptide chain approximately 25% longer than A-protein. Pulse-chase experiments combined with differential extraction indicated that conversion of P23 into A-protein and alteration of the protein coded by gene 22 (P22) appeared to be vital steps in formation of normal capsids. Mutations in several other genes known to prevent normal capsid formation inhibited conversion of P23 to A-protein and alteration of P22.

Introduction. We have previously analyzed and reported the precursor-product relationship between subunits and larger components in T4 capsids.[1] The products of genes 23 and 22 (P23 and P22) behaved as T4-capsid precursor proteins. As Kellenberger[2] and Baylor and Roslansky[3] demonstrated, both the major protein extracted from purified capsids and the 23-protein extracted from phage-infected cells migrated approximately the same distance in acrylamide-urea gels. Electrophoretic analyses of capsid extracts failed to reveal a protein band corresponding to the product of gene 22, which suggests that 22-protein may be altered during incorporation into capsids.[1]

Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and Cleland's reagent was used to analyze proteins extracted from purified capsids and phage and from phage-infected cells. This system had two advantages over the urea-gel system: (1) many protein complexes were more easily dissociated into individual polypeptide chains; and (2) polypeptides separated on the basis of size and approximate molecular weights of components could be estimated from relative positions in the gels.[4,5] Such molecular weight differences indicated that shortening of P-23 peptide occurred when capsid membranes formed.

Autoradiography of dried gels prior to fractionation provided excellent resolution of individual [^{14}C] and [^{35}S]labeled protein components. Using [^{3}H]-labeled proteins as internal standards, subsequent fractionation allowed quantitative analysis and molecular weight determination of labeled proteins.

Materials. Phage and bacterial strains used in this work were reported previously.[1,6] Media used were M9[1] and M9-low sulfate ($MgSO_4$ replaced by $MgCl_2$ and Na_2SO_4 added to a final concentration of 2×10^{-4} M). Other materials used were [^{14}C]leucine (NEC-297) from New England Nuclear; [^{14}C]amino acid mixture (3122-10), and [^{3}H]amino acid mixture (3130-09) from Schwarz BioResearch; Cleland's reagent (DTT) from Calbiochem; Liquifluor and NCS from Nuclear Chicago; chemicals for electrophoresis from Canalco; molecular weight standard proteins (kit no. 8109 A) from Mann Research Lab., Inc.; Sephadex from Pharmacia.

Methods. (*a*) **Preparation of labeled cells:** Methods of infection, preparation, and purification of pulse-labeled cells were the same as those described for radioactive lysates at 37°C[1]; 1 μCi/ml of [^{14}C]leucine, 2 μCi/ml of [^{14}C]amino acid mixture, or 20 μCi/ml of [^{3}H]amino acid mixture was used. In chase experiments, L-leucine (100 μg/ml) was used to dilute [^{14}C]leucine. Labeled cells collected by centrifugation were resuspended ($2–4 \times 10^{10}$ cells/ml) and frozen in 0.005 M Tris-HCl, 0.001 M $MgCl_2$, and 100 μg/ml each of DNase and RNase, pH 7.4.

(*b*) **Purification of radioactive phage and capsids:** Phage-infected *E. coli* B in M9 were labeled with [^{14}C]amino acid mixture (2–5 μCi/ml) or [^{3}H]amino acid mixture (15–30 μCi/ml) added 2 or 3 times starting 4 min after infection. [^{35}S]sulfate (50–70 μCi/ml) was added to culture in M9-low sulfate medium 4 min after infection. Cultures were lysed by chloroform and cell debris was removed by low speed centrifugation. Whole phage were purified from lysates of *E. coli* B infected with wild-type T4D by high-speed centrifugation and subsequent banding in previously formed CsCl gradients[2] and equilibrium CsCl gradients. Capsids were purified from lysates of *E. coli* B infected with a tailless mutant, B255(10), whose capsid readily loses DNA during purification. When infected cells were lysed in the presence of EDTA, the purified capsid had no DNA (empty head). When the lysate was treated with pancreatic DNase (20 μg/ml) at 37°C, the purified capsid retained a piece of DNA approximately 10% the length of phage DNA (partially filled heads).[7,8] Capsids were banded in previously formed CsCl gradients followed by equilibrium CsCl centrifugation (empty heads density = 1.290–1.300 and partially filled heads density = 1.315–1.320), and passed through a Sephadex column (G75, $0.9 \times 7–11$ cm) to remove CsCl. They were then purified by centrifugation in 5–20% linear sucrose gradients for 25 min in a Spinco SW 39 rotor at 30,000 rpm.

(*c*) **Extraction of proteins:** Three methods of extracting labeled proteins from samples of purified phage, capsids, or infected cells were compared. (1) Hot SDS:[9] The sample in 0.4% DTT, 1% SDS, and 0.1 M Na_2CO_3 (final pH 10.6) was incubated at 80°C for 10 min. (2) Cold SDS:[4,5] The sample in 0.4% DTT and 1% SDS (final pH 7.4) was incubated at 37°C for 60 min. (3) Freeze-thaw: the cold SDS mixture was quick frozen and thawed several times before 60-min incubation at 37°C.

(*d*) **Electrophoresis:** Gels were formed from solutions containing acrylamide (5 or 7.5%) and *NN'*-bismethyleneacrylamide (0.4%), *N,N,N',N'*-tetramethylene diamine (0.1%), and ammonium persulfate (0.07%), in gel buffer (2% SDS, 0.04 M Tris-acetate, pH 7.5, and 0.002 M EDTA).[10] The lower electrode buffer had the same composition and concentration as the gel buffer. The upper electrode buffer was one-fifth the concentration of the lower buffer. Protein extracts were mixed with 0.2 vol 54% sucrose and 1 μl of bromophenol blue (tracking dye) per gel. This mixture (0.02-0.1 ml) was applied to the gel (10×0.6 cm) and electrophoresed at 5 mA/tube.

(*e*) **Protein fixation of SDS-acrylamide gels (fixed gels):** Gels were immersed at 42°C in 15% trichloroacetic acid for 18 hr and then in 7.5% acetic acid for 24 hr. The position of the tracking dye was denoted by tattooing with stamp ink.

(*f*) **Identification of labeled proteins in gels:** Three methods were used to identify

labeled protein bands: (1) Gels were frozen and transversely sliced into 1-mm thick discs with a gel slicer (Diversified Scientific Instruments, Inc., San Leandro, Calif.). Each slice was incubated 6 hr at 50°C in either 0.3 ml of 30% H_2O_2[11] or 0.5 ml NCS reagent. (2) Gels were sliced longitudinally and dried. Autoradiograms of dried gels were taken for analysis of [^{14}C] or [^{35}S]labeled proteins.[12] (3) Dried gels were fractionated transversely into 1-mm slices and each section was incubated overnight in 0.3 ml of 30% H_2O_2 at 50°C. [^{3}H]labeled proteins which had not been detected autoradiographically were detected after extraction. Procedures similar to those of Weber and Osborn[5] were used to determine molecular weights (Fig. 1*b*).

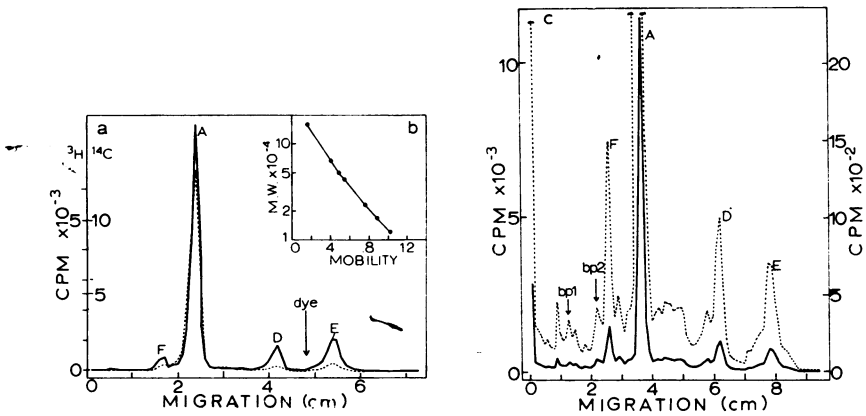


FIG. 1.—Proteins of purified empty heads and purified phage, extracted by hot SDS, separated in 7.5% gels, and extracted from unfixed gels by method (f)1. (*a*) - - -, [^{35}S]labeled empty head proteins; ——, [^{3}H]labeled empty head proteins. (*c*) —, [^{14}C]amino acid labeled phage proteins; (- - -, expanded phage proteins); (*b*) Mobilities of standard proteins relative to tracking dye in order of decreasing molecular weight: γ-globulin, serum albumin, γ-globulin heavy chain, ovalbumin, γ-globulin light chain, tobacco mosaic virus protein (supplied by Dr. S. Mandeles), and cytochrome *c*.

(*g*) **Isotope counting:** Isotopes were counted in a Mark I scintillation counter (Nuclear Chicago). Samples extracted with H_2O_2 were counted in 6 ml 2-methoxyethanol + 10 ml toluene-liquifluor mixture and those extracted with NCS were counted in 10 ml toluene-liquifluor mixture.

Results and Discussion. Capsid proteins: Four major protein bands (F, A, D, and E) were extracted from empty and partially filled heads (Fig. 1*a*). These bands accounted for about 90% of the total capsid protein and 70% of the total phage protein (Table 1). More than 60% of the capsid protein (80% of [^{35}S]label), corresponding to 47% of the total phage protein, consisted of A-protein (48,000 daltons) extractable by hot SDS. The combination of freeze-thaw and cold SDS failed to extract A-protein subunits while it did extract F, D, and E. 5% of the proteins from capsids and 7% of those from whole phage were F-protein (82,000 daltons). This difference could be caused by partial loss of F during capsid purification, or to the presence of another phage protein (Table 1). 9% of the total capsid protein was D-protein (18,000 daltons) and 13% was E-protein (11,000 daltons or less). Some radioactive material, probably acid-soluble peptides, was lost from E-band after fixation of gels. Ac-

TABLE 1. *Summary of capsid proteins.*

Protein	Approx.† molecular weight	Content (% of Total Recovered Count)*						Average Content			
		Empty Heads		Partially Filled Heads			Phage	Empty and Partially Filled Heads		Phage	Approx. no.‡ of molecules per phage
		^{3}H	^{35}S	^{3}H	^{14}C	^{35}S	^{14}C	^{3}H, ^{14}C	^{35}S	^{14}C	
F	81,000	4.3	3.4	4.8	5.7	5.5	7.0	4.7	...	7.1	130 (70)§
A	48,000	60.0	73.0	58.2	53.3	62.3	79.1	45.8	47.8	61.4	79.4	46.8	1500
D	18,000	7.5	6.4	2.6	9.4	10.5	10.5	3.0	6.6	6.6	8.9	2.8	6.6	550
E	≤11,000**	14.5	10.1	4.9	14.5	13.7	14.0	5.0	8.6	8.6	13.4	5.0	8.6	≥1300
F + A + D + E		86.4	93.5	90.2	86.9	83.3	92.3	91.0	68.0	72.0	88.5	90.6	70.0	

* Average recovery of all isotopes from the gels was 92% of the input.
† Average of 14 hot SDS extracts, 7.5% unfixed gels.
‡ [^{35}S] data not included in calculation; protein-part of phage is assumed to be 150×10^6 daltons.
§ Value in parentheses is assuming F-content of capsid is 4.7%.
** Fraction E contains acid-soluble peptides.

curate measurement of the molecular weight of E-band was difficult because of limitations of this SDS-gel system.[4,5]

The molecular weight of each major protein was calculated from the distance it migrated during gel electrophoresis relative to tracking dye. Assuming a particle weight of 150×10^6 daltons for total phage protein,[13] the numbers of molecules of each major component per capsid were calculated (Table 1) from that component's molecular weight and its percentage per phage (total capsid = 76% of total phage).

Autoradiograms of very highly labeled [^{35}S]capsid preparations showed more than 10 minor head proteins. The sum of these did not exceed 10% of the total capsid protein content. Individually, each would be less than 1% of the total.

In addition to the four major bands, extracts from whole phage yielded more minor bands than did extracts from capsids (Figs. 1*c* and 5*i*). These accounted for a maximum of 24% of the total phage protein (calculated from the ratio of A-protein in phage to that in capsid) and probably came from tails, tail-fibers, and internal proteins.[14] Two of these minor bands (bp 1 and bp 2) were related to base plate assembly (unpublished data).

Cell extract. Amber mutants in gene 23 (B17 and H36) did not synthesize P23, and mutants in gene 22 (B270 and N98) did not synthesize P22 (Figs. 2 and 3). H36 and B270 synthesized amber peptides, but B17 and N98 did not produce any detectable peptides. This confirmed our previous observation,[1] established genetically by H. Makala (personal communication), that the amber mutation is near the C-terminal in B270 and near the N-terminal in N98.

The molecular weights of P23 and P22 were 61,000 and 36,000 respectively, and their amber peptides, H36 and B270, were 30,000 and 35,000 respectively. P23 had

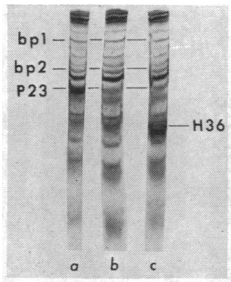


FIG. 2.—Identification of P23. Proteins from T4-infected cells, [^{14}C]amino acid labeled from 12 to 14 min after infection, extracted by cold SDS; 7.5% gels, fixed. (*a*) N50(20), (*b*) B17(23), and (*c*) H36(23).

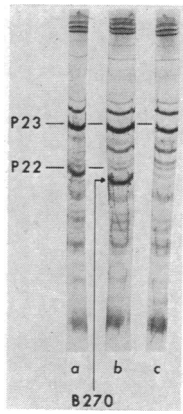


FIG. 3.—Identification of P22. Proteins from T4-infected cells, [^{14}C]leucine labeled from 12 to 14 min after infection, extracted by cold SDS; in 7.5% gels, unfixed. (*a*) N65(24), (*b*) B270(22), and (*c*) N98(22).

a longer peptide chain than did A-protein with a difference in molecular weight of approximately 13,000 daltons (Figs. 4 and 5).

In normal capsid formation, P23 is apparently the precursor of A-protein. Wild type phage-infected cells pulse-labeled for 2 min with [^{14}C]leucine (Fig. 6) contained: (1) a quantity of P23 and P22 extractable by either hot or cold SDS; (2) A-protein which must have come from capsid, extractable only by hot SDS; and (3) a considerable amount of radioactive protein extractable by cold SDS and appearing at the same position as A-band (denoted by A*). P23 disappeared when chased for 2 min. Coinciding with the disappearance of P23, the amount of A-protein extractable by hot SDS increased. After chasing, the increased counts found in A-protein equaled approximately 60% of the counts lost from P23. These findings agreed with previous peptide analyses which identified gene 23 as coding the major capsid protein.[15] A-protein was not extractable by cold SDS from chased cells. Thus the A*-protein extractable by cold SDS from pulse-

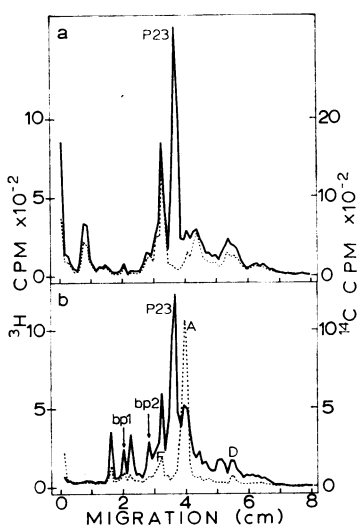
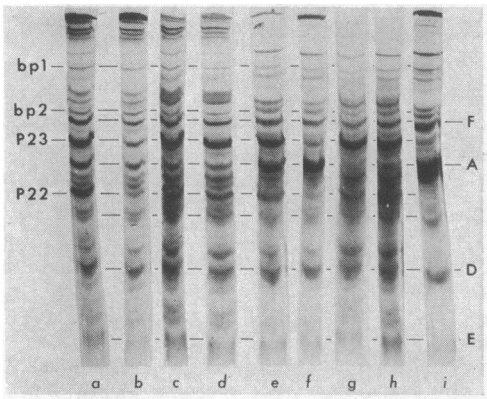


FIG. 4.—Identification of P23 and comparison with phage protein. [^{14}C]amino acid labeled - - -, [^{3}H] amino acid labeled —; (*a*) proteins from mixture of [^{3}H]N90(21) infected cells and [^{14}C]B17(23) infected cells, extracted by cold SDS; 5% gels, fixed, and dried; (*b*) Proteins from mixture of [^{3}H]N90 infected cells and [^{14}C]phage, extracted by hot SDS; 5% gels, fixed, and dried.



FIGS. 5 and 6.—Conversion of P23 to A-protein. Wild type phage or N50(20) infected cells pulse labeled with [^{14}C]-leucine 14-16 min after infection ± chase with cold leucine 16-18 min after infection were mixed with N50 infected cells pulse labeled with [^{3}H]amino acids 14-20 min after infection (used as internal standard). Proteins were extracted by cold or hot SDS; separated in 7.5% gels, fixed, sliced longitudinally, and dried. Outside slices were fractionated transversely (Fig. 6). Center slices were autoradiographed (Fig. 5).

FIG. 5.—(*a* and *e*) wild type (pulse), (*b* and *f*) wild type (chase), (*c* and *g*) N50(20) (pulse), (*d* and *h*) N50(20) (chase), (*i*) proteins from purified [^{14}C]amino acid labeled phage; (*a–d*) cold SDS extraction, (*e-i*) hot SDS extraction.

labeled cells may have represented an intermediate conversion product of P23 which had not yet been incorporated into the capsid or another chasable protein. A mutant in gene 24 had very little A*, suggesting that part of the A* peak was protein coded by gene 24 (P24). It is not clear from our experiments whether the severed part of P23 is intact or becomes short peptides, such as found by Eddleman and Champe.[16] In either case, if it remains in the phage it would most likely be found in the E-band, judging from the number of molecules of A and E in the phage and the molecular weight of the E-band. P22 also disappeared after chasing, but our analyses could not discern a conversion product.

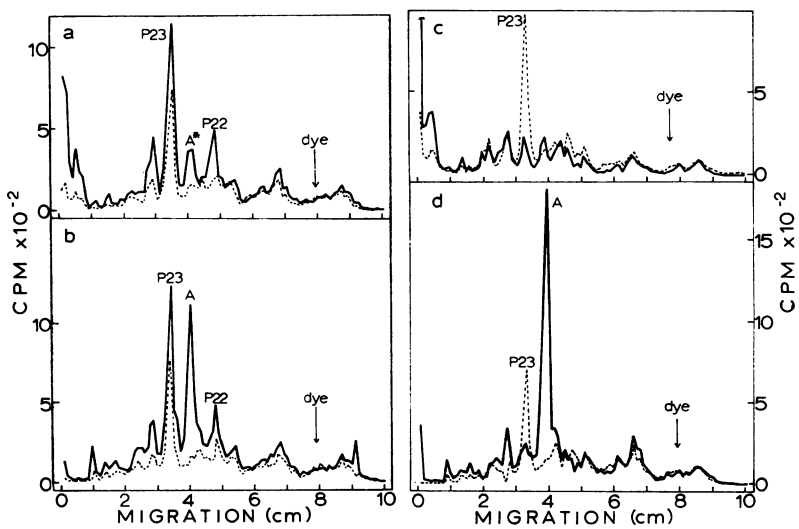
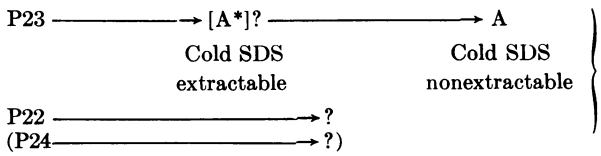


FIG. 6.—Wild type (*a* and *b*) pulse, (*c* and *d*) chase; (*a* and *c*) cold SDS, (*b* and *d*) hot SDS. Counts of four gels were calibrated relative to the internal standard. —, [^{14}C]proteins; - - -, [^{3}H]proteins.

These conversions appear to be vital processes in normal capsid formation. Schematically they are:



Amber mutations in any of the following genes (20, 21, 22, 23, 24, and 31) strongly inhibited the above processes. Degrees of inhibition varied among the mutants used. Although some mutants accumulated structures recognizable by electron microscopy (polyheads by 20, τ-particles by 21, and large lumps by 31),[6,17] none of them produced normal capsids.

More precise kinetic studies of the above processes in which differences between wild type phage and mutants in genes 20, 21, 22, 23, 24, and 31 are compared may reveal in more detail the order of normal capsid formation in T4.

Conversion of P23 to shorter peptides was also found by other investigators.[18] Cleavage of polypeptide chains was observed in animal virus capsid formation.[19]

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