Cleavage of T4-Induced Proteins During Phage Morphogenesis: Characterization of Peptides

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(Accepted 16 December 1975)

SUMMARY

Polypeptides of low mol. wt. have been extracted from T4 coliphages and from Escherichia coli B cells infected with a wild type and various amber mutants of bacteriophage T4. Six peptides were fractionated by chromatography on phosphocellulose: three of them were cleaved from proteins synthesized late in infection and related to phage head. The remaining three peptides have been shown to arise from early-labelled phage-induced proteins. Two of these six small peptide fragments were found in the head of the T4 phage.

INTRODUCTION

Coliphage T4 is a complex structure containing different proteins controlled by at least fifty genes. Some of these proteins are synthesized as high-mol. wt. precursors which are cleaved by specific peptidases during phage assembly. Thus, normal phage head formation involves the cleavage of the products of genes 22, 23 and 24 (Dickson, Barnes & Eiserling, 1970; Hosoda & Cone, 1970; Kellenberger & Kellenberger-van der Kamp, 1970; Laemmli, 1970; Laemmli & Johnson, 1973; Luftig & Lundh, 1973; Showe & Black, 1973). Internal proteins, which are associated with phage DNA, comprise another group of proteins which are cleaved during phage morphogenesis. It has been shown that precursors of internal proteins and head proteins are not cleaved in Escherichia coli B cells infected with amN90, defective in gene 21 (Laemmli, 1970; Bachrach & Benchetrit, 1974a). It has been suggested that gene 21 controls the activity of a specific peptidase which cleaves precursor proteins, 14 to 15 min post-infection. Three acid-soluble peptides were detected in cells infected with bacteriophage T4D (Hershey, 1955; Eddleman & Champe, 1966; Champe & Eddleman, 1967). It has been suggested (Sternberg & Champe, 1969) that these peptides are cleaved from their respective precursors during phage morphogenesis. Since more than three proteins are cleaved in phage-infected bacteria, more than three peptides could be encountered.

In this report, we show that early-labelled phage proteins yield three peptides upon cleavage whereas six radioactive peptides are found in infected cells after cleavage of proteins associated with head assembly.
METHODS

**Bacterial and bacteriophage strains.** A wild type of T4 coliphage was grown on *Escherichia coli* B. Strain CR63 was used as permissive host for phage amber mutants. The following amber mutants were used: N9o mutation in gene 21; B27o in gene 22; H11 in gene 23; 65 in gene 24.

**Culture medium and buffers.** M9 minimal medium contained 0.1 % NH4Cl, 0.3 % KH2PO4, 0.6 % Na2HPO4, 1 mM-MgSO4, 0.4 % glucose and was supplemented with 200 μg/ml of L-tryptophane (Adams, 1959). TMC buffer was 0.1 M-tris-HCl (pH 7.2), 10 mM in MgSO4 and contained 30 μg/ml of chloramphenicol.

**Preparation of radioactive cell-free extracts.** Cultures of *Escherichia coli* B (2 × 10⁸ cells/ml), in 15 ml quantities of M9 medium, were infected with the various phages at a multiplicity of 2 phages/cell and incubated at 37 °C with [14C]-aspartic acid (0.5 μCi/ml; sp. act. 180 μCi/μmol; The Radiochemical Centre, Amersham). Eight min after infection, the cells were superinfected (when necessary) with the same phage and the same multiplicity to ensure lysis inhibition. The incorporation of radioactive amino acid was stopped at desired times by quickly filtering the culture on membrane filters (HA type, 0.45 μm pore size, 47 mm diam., Gelman, U.S.A.) previously soaked in TMC buffer. Infected bacteria were washed with cold TMC buffer, suspended in 3 ml quantities of water and immediately frozen at −70 °C. The cells were then sonicated for 3 min at maximum output in a MSE ultrasonic disintegrator. Residual intact cells and cell debris were removed by centrifuging at 8000 g for 15 min. The supernatant fluids thus obtained served as the source of radioactive substrates or peptides (Bachrach & Benchetrit, 1974a). In pulse-chase experiments, radioisotope was chased 3 min after infection by the addition of a 100-fold excess of unlabelled amino acid. Cells were recovered by filtration 20 min after infection and sonicated as described above.

**Preparation of crude bacterial extracts.** Exponentially growing *Escherichia coli* B cells were infected with T4 phages, superinfected and filtered as above. Washed bacteria were sonicated and the unfractionated extract served as a crude cleaving enzyme preparation.

**Cleavage in vitro.** Proteins labelled during the first 3 min of infection were purified by filtration through Sephadex G-25 columns (1.5 × 30 cm) packed to a height of 25 cm with a pressure differential of 20 cm of TMC buffer. Dextran Blue (0.5 %, Pharmacia) was used to measure the void volume. Eluates from the column were assayed for proteins by counting the radioactivity in a toluene-Triton scintillation fluid (0.4 % PPO, 0.3 % POPOP and 33 % Triton X-100 in toluene). The high mol. wt. labelled protein fractions (eluted in the void volume of the column) served as the substrate for the cleaving enzyme. The activity of the latter was assayed by incubating extracts from phage-infected bacteria (prepared 20 min after infection) with the radioactive substrates. After 3 h of incubation at 37 °C, cellular debris was discarded after centrifuging at 8000 g for 15 min.

**Extraction of peptides.** Radioactive proteins from the supernatant fluids of sonicated cells or those obtained after cleavage in vitro, were precipitated by trichloroacetic acid (TCA-final concentration 20 %) at 4 °C. TCA-soluble cleavage products (peptides) were separated from the precipitated proteins by filtration through Millipore filters. TCA was removed by repeated (4 to 5) extraction with ether which was, in turn, removed by bubbling air through the solution.

**Phosphocellulose chromatography.** Peptides were made 0.025 N in phosphoric acid (starting buffer, pH 2.5) and applied on to a phosphocellulose column (1.2 × 2.5 cm, treated according to Pollock, 1966) and equilibrated with H₃PO₄ (Chin & Wold, 1972). The column
was washed with 50 ml of starting buffer, followed by three linear KCl gradients (50 ml each; pH 2.5); the first between 0.1 and 0.16 M; the second between 0.16 and 0.25; the third between 0.25 and 1.0 M. Fractions, 1 ml each, were collected and assayed for radioactivity as above.

Internal peptides of phages. T4 bacteriophages were labelled with [14C]-aspartic acid, concentrated by the 2-phase separation method and purified by centrifugation in sucrose gradients as previously described (Bachrach & Benchetrit, 1974a). Radioactive phages were made 5% in trichloroacetic acid, heated at 80°C for 10 min and immediately chilled in ice water. Acid was removed from the TCA-soluble material by gel filtration using a Sephadex G-25 column equilibrated in twice distilled water at 4°C (Bachrach & Benchetrit, 1974a). The low mol. wt. peptide fractions were pooled, acidified with H3PO4 as above and peptides were separated by chromatography on phosphocellulose.

Large scale preparation of peptides. Escherichia coli B was grown in a fermentor (Microferm, New Brunswick) containing 10 litres of M9 medium. The impeller was operated at 500 rev/min and the temperature maintained at 37°C. Air was sparged in the jar at 0.5 vol./vol./min. An overnight culture of 1 litre was used as inoculum. When the suspension reached the density corresponding to 5 x 10^5 cells/ml the culture was infected and later on superinfected as above. Twenty min after infection, cells were collected by centrifuging for 10 min at 5000 rev/min (at 4°C) and washed twice with TMC buffer. They were resuspended in water (final volume 40 ml) and pressed through a Power Laboratory Press (American Instrument Co.) under a pressure of 20000 p.s.i. in the 1” diam. piston. DNase (20 µg/ml), 1 mM-MgSO4 and RNase (50 µg/ml) were added and the bacterial extract incubated at 37°C for 2 h. Cellular debris was discarded after centrifugation and TCA added to the supernatant fluid to a final concentration of 20%. Peptides were separated from proteins of higher mol. wt. by filtration through Gelman filters and TCA removed from the peptide material as above. This preparation (approx. 4 mg) was made 0.025 N in H3PO4 (pH 2.5), loaded onto a phosphocellulose column (1.5 x 25 cm, capacity 0.80 mEq/gm) and washed. Three gradients of KCl (200 ml each; pH 2.5) were run in the above given molarities. Two ml fractions were collected and the peptide concentrations were determined by the method of Lowry et al. (1951) using hypertensin (mol. wt. 800, Ciba) as a standard.

Amino acid composition. Peptides were concentrated by lyophilization, dissolved in 6 N-HCl (0.5 to 1.0 mg amounts) and hydrolysed in an oil bath for 24 h in vacuum-sealed ampoules at 105°C. The hydrolysed samples were dried over phosphorus pentoxide and analysed in a Beckman model 120 C amino acid analyser. The nearest integers were used to estimate the peptide size.

RESULTS

Peptides in T4 phages and in Escherichia coli B infected with T4

Exponentially growing Escherichia coli B cells were infected with T4 phages and proteins were immediately labelled for the first 3 min by the addition of [14C]-aspartic acid. Bacteria were harvested, washed and sonicated as described under Methods and TCA-soluble material was then fractionated by chromatography on phosphocellulose columns. No appreciable amounts of peptides were detected when phage-induced proteins were labelled for the first 3 min of the infectious cycle. On the other hand, 6 distinct peptides were eluted from the column when phage proteins were labelled with [14C]-aspartic acid for 20 min (Fig. 1a). Only 3 peptides were isolated (Fig. 1b) when a phage-infected culture was labelled for 3 min and subsequently treated with a crude cleaving enzyme obtained from a phage-infected culture (20 min after infection). A similar elution pattern was observed when
Fig. 1. Chromatographic separation of the acid-soluble polypeptides isolated from T4-infected cells. Cultures of *Escherichia coli* B (15 ml) were infected with phage and labelled with [14C]-aspartic acid for 20 min (a) or for 3 min then cleaved *in vitro* (b). Phage-induced proteins were also labelled for 3 min then chased for 17 min (c). Non-infected cells were incubated with radioactive amino acid and extract prepared after 20 min (d). Radioactivity applied on the columns: (a) 50000 ct/min; (b), (c) 30000 ct/min.

Phage-infected bacteria were pulse-labelled with [14C]-aspartic acid for the first 3 min after infection and then chased for another 17 min with unlabelled aspartic acid (Fig. 1c). None of these components was derived from the bacterial host because non-infected cells labelled with [14C]-aspartic acid for 20 min did not yield detectable radioactive polypeptides (Fig. 1d). Proteins labelled in these cells have not been incubated with a crude enzyme preparation.

In order to demonstrate that the late peptides (A, D, and F) are cleavage products and to exclude the possibility that TCA may favour the isolation of acidic peptides as TCA-soluble material, a culture of *Escherichia coli* B was incubated with [14C]-aspartic acid 18 min after infection and superinfection with T4 phages. Two min later, infected bacteria were harvested, sonicated and centrifuged as described under Methods. The supernatant fluid was applied onto Sephadex G-25 columns and fractionated as described elsewhere (Bachrach & Benchetrit, 1974a). The peptide fractions were pooled, acidified with phosphoric acid and loaded onto a phosphocellulose column. Fractionation of peptides was carried out as described under Methods. Fig. 2a shows that no more than 6 peptides (A to F) were formed (as the result of the cleavage process) when the radioactive amino acid was administered.
Peptides in infected cells

at late times of infection. This indicates the appearance of newly synthesized phage-induced proteins which were subsequently cleaved to yield peptides (Bachrach & Benchetrit, 1974a).

On the other hand, only 2 TCA-soluble peptides could be extracted from T4 phages labelled with [14C]-aspartic acid. These peptides correspond to peptides A and E when fractionated by chromatography on phosphocellulose columns (Fig. 2b).

Peptides formed in Escherichia coli B cells infected with amber mutants

To correlate the formation of specific peptides with the various head proteins, exponentially growing Escherichia coli B cells were infected with various amber mutants and peptides fractionated by chromatography on phosphocellulose after precipitating proteins with TCA. It may be seen (Fig. 3a) that mutants defective in gene 22, gave rise to two peptides only. Practically identical elution patterns were obtained when peptides were isolated from bacteria infected with amber mutants defective in gene 23 (Fig. 3b) or in gene 24 (Fig. 3d). In these experiments the infected cultures were labelled with [14C]-aspartic acid for the first 20 min of the infectious cycle. When Escherichia coli B was infected with amN90 defective in gene 21, and labelled with [14C]-aspartic acid for 20 min, no detectable amounts of peptides were formed (Fig. 3c). However, 6 distinct peptides were eluted from the phosphocellulose column when radioactive proteins from an amN90-infected culture were cleaved in vitro by incubating with a crude cleaving enzyme obtained from phage-infected bacteria (Fig. 3c). It may also be noticed that the peptides obtained after cleaving amN90 proteins in vitro, resembled the peptides produced in Escherichia coli cells infected with a wild type of T4 and labelled with [14C]-aspartic acid for 20 min (cf. Fig. 1a and Fig. 3c). It may be inferred from this experiment that precursors are formed in amN90-infected bacteria, but cleavage did not take place.
In order to identify *in vitro* formed cleavage products with those formed *in vivo* a double-label was employed as follows: Cultures of *Escherichia coli* B cells were infected with either T4 or *am* N90 coliphages and respectively labelled with [14C]-aspartic acid or [3H]-lysine for 20 min. Labelled proteins formed in cells infected with *am* N90 were purified by gel filtration as described under Methods and incubated with a crude bacterial extract obtained 20 min after infection. At the end of the incubation period the TCA-soluble cleavage products were mixed with acid-soluble products extracted from T4-infected cells. The sample was then chromatographed on phosphocellulose as described under Methods. It may be

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**Fig. 3.** Chromatographic separation of the peptides isolated from amber mutant-infected cells. Cultures (15 ml) were labelled for 20 min with [14C]-aspartic acid, cells infected with amber mutants (a) B270; (b) H11; (d) N65; (e) N90. Proteins labelled during infection with N90 were cleaved *in vitro* to yield peptides (c). Radioactivity applied on the columns: (a), (b), (d) 15000 ct/min; (c) 50000 ct/min.
Peptides in infected cells

Table 1. Amino acid composition and mol. wt. of the peptides

<table>
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<th>Peptides*</th>
<th>Peptides†</th>
<th>Peptides‡</th>
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<tr>
<td>Total residues</td>
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<td>Mol. wt.</td>
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</table>

* The numbers of residues of each amino acid for each peptide were normalized to gly = 1.00. The nearest integers are shown and were used to estimate the minimal mol. wt. of the peptides. The elution pattern of the peptides from the phosphocellulose column is that given in Fig. 1(a).
† Peptides were obtained from phage particles.
‡ Peptides were obtained from T4-infected cells.

seen (Fig. 4a) that peaks of peptides formed in vivo and labelled with 14C-aspartic acid coincided with those of peptides formed in vitro using 3H-lysine labelled proteins.

Amino acid analyses of peptides

In order to analyse the various acid-soluble peptides, Escherichia coli was grown in 10 litre quantities and infected with a wild type of T4 coliphage. Bacteria were harvested, disintegrated and peptides isolated by chromatography on phosphocellulose columns as described under Methods. Peptides from the various fractions were concentrated by lyophilization and their amino acid content determined. Table 1 shows that all the peptides were rich in acidic amino acids (glutamic and aspartic acid) and contained variable amounts of lysine. Peptides D, E and F contained valine, while alanine was detected in peptides C and E. It is noteworthy that in all peptides, the amount of glycine was practically constant. It may be seen that the composition of the components obtained from phage particles (Fig. 2b) is similar to that of peptides A and E found in infected cells (Fig. 1a).

Even though the analysis did not distinguish between glutamic acid and glutamine and between aspartic acid and asparagine, it appears that all the material consists of glutamic and aspartic acids since the amounts of ammonia found were very low.

The nearest integral residues (for each amino acid present) were used to calculate the minimum mol. wt. of the 6 peptides (Table 1). The results are consistent with a previous report on gel filtration of these peptides through a Sephadex G-25 column (Bachrach & Benchetrit, 1974a).

The relative percentage of the peptides derived from infected bacteria and T4 phages is shown in Table 2. It may be seen that the ratio of peptides B and C was very similar in all preparations. Moreover, the ratio of peptides A and E derived from phage-infected bacteria was 0.16 and 0.17. A similar ratio (0.17) was calculated for the peptides A and E, extracted from T4 phages (Table 2).

Amino acid analyses showed that peptide A was the only proline-containing peptide. This fact was used to demonstrate that peptide A formed in T4-infected cells (Fig. 1a) was
Table 2. Relative percentage of each peptide in infected cells and in phages

<table>
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<tr>
<th>Host</th>
<th>Fig. 1 (a)</th>
<th>Fig. 1 (b)</th>
<th>Fig. 1 (c)</th>
<th>Fig. 2 (a)</th>
<th>B270</th>
<th>H11</th>
<th>N90</th>
<th>N65</th>
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<td><strong>Escherichia coli B</strong>*</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
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<tr>
<td>A</td>
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<td>B:C</td>
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<tr>
<td>A:E</td>
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* The areas under the peaks were integrated for each peptide from the elution patterns given in Fig. 1, 2 and 3.

Fig. 4. Chromatographic comparison of double-labelled peptides. (a) Cultures (15 ml) were infected with T4 and labelled for 20 min with [14C]-aspartic acid (●●●). Proteins labelled with [3H]-lysine after infection with am N90 were cleaved in vitro (○○○). (b) Cultures were infected with T4 and labelled for 20 min with [14C]-proline (●●●). Phages labelled with [3H]-proline were treated with TCA (○○○). Approx. 65,000 ct/min were applied on each column in (a) and 10,000 in (b).

identical to a peptide found in phages. The TCA-soluble cleavage product from extracts of Escherichia coli B cells infected with T4 and labelled with [14C]-proline for 20 min was mixed with the internal peptide preparation from [3H]-proline labelled phages. The sample was then loaded onto a phosphocellulose column. It can be seen (Fig. 4b) that the in vivo formed component eluted with the peptide found in phage particles.
Peptides in infected cells

DISCUSSION

Hershey (1955) originally reported the presence of an acid-soluble component in T2H bacteriophage particles, which yielded amino acids on hydrolysis and was thus concluded to be a peptide. These findings were extended by Eddleman & Champe (1966) who detected and partially characterized 3 lysine-containing acid-soluble peptides in cells infected with phage T4D. Two of these, designated II and VII, were found in phage particles, from which they were released by osmotic shock or by extraction with TCA (Champe & Eddleman, 1967). The third peptide designated VI was found exclusively in infected cells (Champe & Eddleman, 1967). Amino acid analyses showed that peptide II contained proline, but no alanine, valine and isoleucine, whereas peptide VII contained 10 equivalents of glutamic acid, 6 equivalents of lysine and 1 of aspartic acid. Recently Goldstein & Champe (1974) demonstrated the cleavage of a bacteriophage protein in vitro and tentatively identified the soluble peptide as peptide II. Immunological (Showe & Black, 1973) and genetic analyses (Sternberg & Champe, 1969) strongly suggested that peptide II is derived from P22.

In the present study the formation of 6 peptides has been demonstrated in T4-infected bacteria. It appears that the resolution of the phosphocellulose column is better than that of Dowex 50, used by Champe & Eddleman (1967). The results strongly suggest that the cleavage is controlled by gene 21. Similar findings were independently reported (Goldstein & Champe, 1974; Onorato & Showe, 1972, 1975).

Amino acid analyses (Table 1) indicated that one peptide E is identical with peptide VII described by Champe & Eddleman (1967); both have practically the same amino acid composition. Peptide A (Table 1) appears to be identical with peptide II of Champe & Eddleman (1967), being the only proline-containing peptide which is devoid of alanine, valine and isoleucine. This peptide can be the degradation product of P22, whereas peptides D and F can be the cleavage products of P23 and P24. The possibility that one peptide is further cleaved to shorter molecules cannot be ruled out. Peptide E (Table 1) can be the product of IPIII, a protein formed during the early stages of the infection. This peptide, which seems to be the same as peptide VII of Champe & Eddleman (1967) is not formed in bacteria infected with phages defective in genes 22, 23, 24 (Fig. 3, and Champe & Eddleman, 1967). The mol. wt. of this peptide is 2600 (Table 1), similar to the value of 2500 calculated for the degradation product of IPIII (Laemmli, 1970). But it has to be remembered that Onorato & Showe (1975) identified this peptide as the cleavage product of P22.

It is tempting to speculate that peptides B and C (Fig. 1) are the cleavage products of IPI and IPII which are also early-labelled proteins. However, their identification as the degradation product of other early proteins has not been excluded.

Goldstein & Champe (1974), who independently studied the cleavage of phage proteins in vitro were able to detect only one peptide. It should, however, be remembered that they used denatured proteins as substrates and an extract of bacteria infected with T4 defective in gene 23 as the source of the cleaving enzyme. However, when native proteins were analysed more peptides were obtained after cleavage (Goldstein & Champe, 1974). It is of special interest that the peptides formed during cleavage have a similar composition. All of them are rich in acidic amino acids (aspartic and glutamic acid), contain one molecule of glycine and at least one molecule of lysine per peptide chain.

It is conceivable that cleavage of head proteins changes their net charges, since acidic peptides are released. This suggestion is in line with isoelectric focusing experiments (Stone & Cummings, 1970) and may explain the affinity of the cleavage product (but not of the
precursor) for viral nucleic acids (Bachrach & Benchetrit, 1974a; Bachrach, Levin & Samuni, 1974).

This study was supported by a grant from the U.S. Department of Health, Education and Welfare, (No. 06-112-1) under Public Law 480 and by the United States–Israel Binational Science Foundation.

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(Received 9 June 1975)