Structural Aberrations in T-Even Bacteriophage

V. Effects of Canavanine on the Maturation and Utilization of Specific Gene Products

REX W. BOLIN AND DONALD J. CUMMINGS

Department of Microbiology, University of Colorado Medical Center, Denver, Colorado 80220

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Previous results have shown that when a T-even bacteriophage-infected cell was exposed to L-canavanine followed by an exposure to L-arginine, a monster phage particle, termed a lollipop, was formed. L-Canavanine was necessary for the induction event but L-arginine was required for the maturation of the particle. We now describe the effects of canavanine on the maturation of certain T4 proteins and their role in the induction of lollipops. The cleavage reactions of the head proteins P22, P23, P24, and IPIII are prevented by L-canavanine as shown by the accumulation of the precursor proteins and the failure of the cleaved products to appear. L-Canavanine also prevents the appearance of P12 (tailplate protein) and P20 (head protein) indicating that these proteins may undergo a proteolytic cleavage during normal assembly. The formation of P10 (tailplate protein) and P18 (tail sheath protein) is also affected by L-canavanine. The data suggest that P23 in conjunction with P20 plays a major role in the determination of the length of the phage head.

Results from our laboratory have shown that L-canavanine, an analogue of L-arginine, interferes with the growth of T-even bacteriophages (3, 8). L-Canavanine induces the formation of large numbers of polyheads which are similar to those produced by amber mutants in gene 20 in that both types of polyheads which are similar to those produced by amber mutants in gene 20 in that both types of polyheads consist primarily of the uncleaved major head protein, P23 (molecular weight 55,000) (7, 11, 13, 17). L-Canavanine also results in a marked decrease in T4 DNA synthesis although net RNA and protein synthesis is unaffected (1, 4). The addition of L-arginine results in the stimulation of DNA synthesis and the appearance of viable monster phage particles, termed lollipops (1, 6). The lollipops contain the cleaved major head protein (6), P23* (molecular weight 45,000) (5, 9, 11). In the companion paper, we showed that the formation of the lollipop appears to involve a two-step process (1). The induction step involves a late phage function and under favorable conditions can require only a 3-min exposure to L-canavanine which results in the accumulation of an undefined precursor substance. L-Arginine is required for the second step which leads to the actual formation of the lollipop.

We now describe the effects of L-canavanine on the maturation of certain T4 proteins. (In

this paper, the term maturation is used to describe the known and postulated cleavage reactions of proteins which are required for normal phage assembly. The term is also used to describe the assembly of the phage.) L-Canavanine prevents all of the known cleavage reactions involved in head assembly (9, 11, 13, 17) and it also prevents the appearance of P12, a tail plate protein and P20, a head protein which has been reported not to undergo cleavage (17). Other proteins of the tail and baseplate are also affected. As a possible consequence of these effects on specific phage proteins, tail assembly as well as head assembly is inhibited. Our results also indicate that although most proteins synthesized in the presence of canavanine are not used in phage or lollipop assembly, certain proteins are selectively incorporated into the lollipop structures.

MATERIALS AND METHODS

Bacteria and bacteriophage. *Escherichia coli* B was used in all experiments. *E. coli* CR63 was used as the permissive strain for the growth of the amber mutant stocks. T4B was used in experiments in which lollipops were isolated since T4B produces a greater number of lollipops than does T4D (6). T4D was used in all other experiments. The following amber mutants were supplied by W. Wood: 10⁻ (amB255); 12⁻ (amN69); 20⁻ (amN50); 23⁻ (amB17); X4E which is

defective in genes 34, 35, 37, and 38; and XF18 which is defective in genes 5, 6, 7, 8, 9, and 10. Amber mutants in genes 17 (amBU33), 18 (amE18), and 19 (amN6524) were supplied by W. Saurbier.

Growth conditions. The cells were grown with aeration at 37°C in minimal medium to 2×10^8 cells/ml (3). The minimal medium was usually supplemented with a mixture of amino acids unless the culture was to be labeled with ^{14}C -labeled amino acids after infection. When the label was to be [^{14}C]-leucine, leucine was omitted from the mixture. The cells were infected with T4 at a multiplicity of infection of 5 phage/bacterium and then superinfected with the same amount of virus 7 to 9 min later. L-Canavanine sulfate (Calbiochem) and L-arginine (will be referred to as canavanine and arginine) were added as noted in the text.

SDS-polyacrylamide gel electrophoresis and autoradiography. Extracts were labeled with either [^{14}C]-leucine (>270 mCi/mmol, New England Nuclear Corp., $0.67 \mu\text{Ci/ml}$ final activity) or ^{14}C -labeled amino acid protein hydrolysate (Schwartz/Mann, $0.5 \mu\text{Ci/ml}$ final activity). The electrophoresis was performed by using a modification (28) of the procedures for thin slabs (27). The gel was then dried and Kodak Brand Blue film was exposed from 5 to 30 days. Densitometer tracings were made of autoradiograms by using a Beckman densitometer.

Isolation of phage structures. The method of King and Laemmli was used for the isolation of tail structures (15), and the lollipop and phage particles were isolated as previously described by Cummings et al. (6).

Electron microscopy. Specimens were negatively stained with phosphotungstic acid and examined as previously described (3).

Terminology. Known gene products are referred to by the respective gene number preceded by "P" (i.e., P23). Gene products produced as the result of the cleavage reaction are denoted with an asterisk (i.e., P23*). Unknown gene products are identified by using a letter.

RESULTS

Effects of canavanine on the maturation of T4 proteins. We reported that the induction of lollipops involves a T4 late function and that canavanine results in the accumulation of a precursor substance which influences the head length of the lollipop upon maturation. To investigate this process, the effect of canavanine on T4 late proteins was examined by using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. At 10 min postinfection (PI), $130 \mu\text{g}$ of canavanine per ml was added and at 15 min PI [^{14}C]-leucine was added, and a sample was taken 1.5 min later (termed a pulse sample). Cold leucine ($200 \mu\text{g/ml}$, final concentration) was added to the remainder, and a sample was removed at 18 min PI (termed a chase sample). Canavanine had a dramatic

effect on the maturation of T4 proteins (Fig. 1). The cleavages of P22 to acid-soluble fragments, P23 to P23*, P24 to P24*, and IPIII to IPIII* were clearly inhibited, indicating that canavanine interfered with proper head assembly. Two additional proteins, P20 (a head protein) and P12 (a base plate protein), were missing from canavanine-treated extracts. In addition, new protein bands (X and Y) appeared slightly above P10 and P18. A third band, Z, was frequently absent in extracts not treated with canavanine. However, in the presence of canavanine, it was always present and it was usually intensified severalfold relative to the amounts in untreated extracts. A fourth protein band, A, is missing from the canavanine-treated extracts in Fig. 1. The band probably represents an early protein as it labels more intensely in samples labeled earlier in the infection (unpublished results). When the label is added late in infection, the presence of band A is not consistent, regardless of whether canavanine is present. It should be noted that each and every effect of canavanine was not always elicited to the same degree. This is illustrated in Fig. 1; however, most of the described effects in Fig. 1 can be further documented by examining the gels to follow.

The effects of three other amino acid analogues which affect T-even phage maturation (8) were examined to determine if the effects of canavanine on T4 proteins were unique. 1, 2, 4-Triazole-3-alanine (TRA), a histidine analogue which results in large numbers of small-headed phage; para-fluorophenylalanine (PFPA), an analogue of phenylalanine and tyrosine which results in a large percentage of empty capsids, and L-azetidine-2-carboxylic acid (ACA), a proline analogue which gives rise to polytail tube formation, were examined. The same general protocol was followed as was described above for canavanine except for ACA in which [^{14}C]-leucine was allowed to label continuously from 14 to 18 min PI. In each experiment, the L-form of the analogue was present at $130 \mu\text{g/ml}$ which is sufficient to produce the above described effects. As is shown in Fig. 1, PFPA and ACA inhibited the cleavage of P22, P23, and P24 as did canavanine. Very little of the label was chased, indicating an inhibition of proper head assembly. PFPA, like canavanine, also depressed the level of P20 although ACA did not. Note that P20 appears to intensify in the chase samples of the untreated and TRA extracts. Interestingly, ACA, which did not lower the level of P20, did not completely inhibit the cleavage of IPIII as did PFPA and canavanine.

FIG. 1. Autoradiogram of an SDS-10% polyacrylamide slab gel showing the effects of several amino acid analogues on the maturation of T4 proteins. The proteins were pulse-chased (except for ACA which was labeled continuously) in the presence of 130 μ g of the L-form of the analogue per ml as described in the text. P, Pulse sample; C, chase sample; CANAV, canavanine; TRA, 1,2,4-triazole-3-alanine; PFPA, parafluorophenylalanine; ACA, L-azetidine-2-carboxylic acid. The numbers and letters identify bands described in the text. The ACA sample is from a separate gel run and as a result, some of the bands do not exactly match the bands in the remainder of the gel. The band identifications correspond to bands in the first eight samples and in some cases the ACA bands are slightly shifted. P24 is the band slightly below P23* and P24* is the top band of the doublet seen clearly in the untreated and TRA samples. Defective lysate profiles can be seen in references 3, 16-18, and in figures to follow.

TRA did not noticeably affect any of the head proteins. TRA, PFPA, and ACA did not affect the appearance of P12 nor did they induce the appearance of bands X or Y.

In the companion paper, we demonstrated that inhibition of DNA replication, reduction of phage yield, and induction of lollipops all occurred at the same concentration of canavanine, about 15 μ g/ml. Consequently, an experiment was performed to determine whether all of the canavanine-induced effects on specific phage proteins occurred at the same concentration of canavanine. At 10 min PI, canavanine was added to a T4 infection at concentrations from 5 to 200 μ g/ml. [14 C]leucine was added at 15 min PI, and the cultures were sampled at 20 min PI. As is shown in Fig. 2, all of the described effects

of canavanine occurred between 10 and 60 μ g of canavanine per ml. Head assembly appeared to be inhibited by about 20 μ g of canavanine per ml. Note that band Z intensified with increasing concentrations of canavanine.

It is possible that one or more of the proteins affected by canavanine are responsible for the induction of lollipops. For one of the uncleaved precursor proteins to take part in the assembly of lollipops, it would have to undergo cleavage after the addition of arginine. An experiment was performed to determine if any of the uncleaved proteins which accumulate in the presence of canavanine are cleaved upon the addition of arginine. Canavanine at 130 μ g/ml was added at 11 min PI, [14 C]leucine was added at 15 min PI, and the arginine and leucine

chases were added at 28 min PI. The untreated and canavanine-treated samples were taken at 24 min PI and the arginine chase samples were taken at 38 and 60 min PI. As is shown in Fig. 3A-D, the majority of the proteins did not chase after the addition of arginine. However, the chase samples taken at 38 and 60 min PI show that a small percentage of P23 may have been cleaved to P23* (see later Results).

Identification of the X and Y protein bands. As described above, canavanine induces or intensifies at least three protein bands. Protein bands X and Y always appear just above P10 and P18, respectively, and protein band Z is located between P10 and P18. Canavanine has been shown to affect the maturation of viral proteins by interfering with cleavage reactions (3, 6, 12, 29) and it has been shown to inhibit proteolysis in uninfected *E. coli* (23). This suggests that the appearance of the X, Y, and Z protein bands may be the result of inhibited cleavage reactions.

To identify the protein bands X and Y, cells infected with a variety of amber mutants were examined in the presence of canavanine. The infected cells were treated with 200 μ g of canavanine per ml at 10 min PI and were labeled from 15 to 19 min PI. The results clearly indicate that the proteins of bands X and Y are products of genes 10 (a baseplate protein) and 18 (sheath protein), respectively (Fig. 4). However, in each case the normal protein bands for P10 and P18 were still present indicating that if P10 and P18 were cleaved during maturation, canavanine resulted only in a partial inhibition of cleavage. During assembly of the tail, P18 polymerizes around the tail tube (P19) (14, 16). An amber mutant in gene 19 was examined to determine if P19 was required for the proper maturation of P18. However, as is shown in Fig. 4, P19 was not required. This result is not unexpected since polysheaths do form in the absence of proper tail assembly (16). An amber mutant in gene 17 was also examined and in the

FIG. 2. Autoradiogram of an SDS-10% polyacrylamide slab gel showing the effect of increasing concentrations of canavanine on the maturation of T4 proteins. Canavanine was added at 10 min PI, and [14 C]leucine was added at 15 min PI.

FIG. 3. Autoradiogram of an SDS-10% polyacrylamide slab gel. A, B, C, and D show the effect of arginine on cleavages previously inhibited by canavanine. A represents a normal lysate not treated with canavanine and sampled at 24 min PI; B represents a lysate treated with canavanine at 11 min PI and sampled at 24 min PI; C and D represent lysates treated with canavanine at 11 min PI, followed by arginine at 28 min PI and sampled at 38 and 60 min PI, respectively. The label was added at 15 min PI, and was chased prior to the addition of the arginine. The P23/P23* ratios are A, 0.4; B, 5.3; C, 4.0; and D, 4.1. This indicates that the amount of protein at the P23* position increased approximately 20% after the arginine chase.

presence of canavanine, the Y band had an apparent molecular weight slightly greater than that normally seen in the presence of canavanine. This result was somewhat surprising as P17 is normally required for the proper filling of the phage head with DNA and since there is no evidence that it is involved in tail assembly. However, it has been noted that P18 may have a slightly greater molecular weight in an infection with an amber mutant in gene 17 than in the wild-type phage infection (K. Hercules, personal communication).

Effect of canavanine on tail formation. Protein bands X and Y appear to be related to P10 and P18, respectively. These proteins are structural proteins of the baseplate and tail

sheath, and therefore, an inhibition of their proper maturation would be expected to affect tail assembly. To examine the effects of canavanine on tail formation, a culture was infected with an amber mutant in gene 23 so as to eliminate any head structures, and at 10 min PI, 200 μ g of canavanine per ml was added followed by 14 C-labeled amino acids at 15 min PI. Tail structures were isolated (15) at 20 min PI. The infected cells were concentrated, treated with CHCl_3 and DNase, and followed by treatment with EDTA and RNase, and the samples were placed on 5 to 20% sucrose gradients. Two peaks were consistently observed in the untreated samples but only the faster sedimenting peak was observed after treatment with canavanine (Fig. 5). Electron microscope examination showed that the fast-sedimenting peak contained primarily debris although a few sheathed tail structures were occasionally present in the untreated samples. The slow-sedimenting peak contained large numbers of non-sheathed tails (tridents) (Fig. 6), but only a rare trident was observed in the corresponding fractions in canavanine-treated samples. After a 24-h treatment with CHCl_3 to insure release of the tail structures from the cellular debris, only about 10% of the total proteins synthesized from 15 to 20 min PI in the untreated samples were still bound to the debris (Table 1). However, in the canavanine-treated samples, approximately 50% of the proteins were still bound to the debris. No additional tail structures were released after the extended treatment. Treatment with SDS disrupted the debris, releasing the remaining proteins. These results suggest that the bound proteins were attached to membranous material in an unassembled state. Analysis of the respective samples on gels showed that certain proteins from the canavanine-treated cells were selectively bound to the debris (not shown). Among the proteins selectively bound were P34 and P37 (tail fiber proteins), P7 (tail plate protein), P18 (tail sheath protein), and P19 (tail tube protein).

Are P12 and P20 cleaved? Canavanine clearly prevents the appearance of P12 and P20. Canavanine must therefore be inhibiting either the synthesis or the proper cleavage of the proteins. To identify possible precursor proteins, the effect of canavanine on the maturation of proteins in pulse-chase experiments using amber mutants in genes 12 and 20 was examined (Fig. 7A). Canavanine was added at 10 min PI, and [14 C]leucine was added at 15 min PI. Except for the wild-type lysate not treated with canavanine, all pulse samples were re-

FIG. 4. Autoradiogram of an SDS-10% polyacrylamide slab gel showing the effect of a variety of amber mutations on the appearance of bands X and Y after an exposure to canavanine. Canavanine was added at 10 min PI, and the infected cells were labeled from 15 to 19 PI.

moved after an additional 1.5 min. The wild-type lysate without canavanine was pulsed for 0.7 min followed by a 1.5-min chase. Using a 1.5-min pulse, it was not possible to demonstrate the disappearance of a possible precursor protein with the concurrent appearance of either P12 or P20, although it is clear that P20 did intensify in the appropriate chase samples. However, using a 0.7-min pulse it was possible to demonstrate that the band Z did chase into the P20 position. It should be pointed out that this result was not always obtained since it was difficult to label the Z band protein by using short pulses of [14 C]leucine. As stated earlier, band Z was not always present in untreated lysates, although it was always intensified in lysates treated with canavanine. There is a further complication to this result. Band Z was present in the extracts from the amber mutant in gene 20. This would suggest that it could not represent the P20 precursor unless the gene 20 mutant used (N50) had a mutation very close

to the carboxy terminal end of the protein such that the amber fragment had essentially the same molecular weight as the precursor, although it would be unable to undergo cleavage. A second amber mutant in gene 20 (am38) was examined and it too had the band present. It is not known where these mutants map within gene 20 (W. Wood, personal communication).

We have shown that two amino acid analogues, canavanine and PFPA, reduce the amounts of P20 in extracts. Assuming these analogues prevent the appearance of P20 by interfering with the cleavage of a P20 precursor, the effect should be mimicked by a mutation which prevents head assembly. Figure 7B shows the effect of an amber mutation in gene 23 on the appearance of P20. In this experiment, 130 μ g of canavanine per ml was added at 10 min PI, [14 C]leucine was added at 15 min PI, and the lysates were sampled at 20 min PI. It is clear that P20 was reduced to a level that is approximately as low as the level observed in the

FIG. 5. The effect of canavanine on the formation of T4 tails. Samples were prepared as described in the text and then placed on 5 to 20% sucrose gradients and centrifuged at 35,000 rpm for 1 h at 25°C by using an SW39 rotor. Symbols: ●, untreated sample; ○, sample treated with canavanine at 10 min PI. In untreated samples the debris peak occasionally contained sheathed tails.

presence of canavanine in the normal lysate, suggesting that the appearance of the active form of P20 requires an association with P23.

Apparently, P20, like P22, P23, P24, and IPIII, is cleaved during head maturation. However, the identity of the P20 precursor remains obscure. A P12 precursor was also not identified. However, there is a possibility that a P12 or P20 precursor protein could have the same molecular weight as another T4 protein, thus making its detection difficult. This possibility was examined by treating the multiple amber mutants X4E (defective in genes 34, 35, 37, and 38) and XF18 (defective in genes 5, 6, 7, 8, 9, and 10) with canavanine. The extracts were then examined for the appearance of new proteins. The results in Fig. 8 show that no additional protein was induced in the presence of canavanine, although very little P12 was made in XF18, possibly because of polarity effects. Note that band Z was not present in the absence of canavanine. The presence of band Z in XF18 also indicates that it does not represent P6 which has approximately the same molecular weight. This result was verified by using an amber mutant defective in only gene 6.

Role of structural proteins in capsid length determination. We have demonstrated that the induction of lollipops results from an exposure to canavanine which induces the formation of a precursor substance(s) which influences the

head length of the lollipop upon maturation (1). To identify a structural protein(s) which may play a role in determining the length of the phage head, proteins were labeled in the presence of canavanine to determine if any were selectively incorporated into or deleted from the lollipops. Cells were grown in normal C-salt medium except that the amino acid concentrations were reduced from 50 µg/ml to 2 µg/ml to facilitate incorporation of ^{14}C -labeled amino acids. The culture was infected with T4B (T4B produces a higher yield of lollipops than T4D [6]) and after removal of a portion of the culture to serve as a control, 200 µg of canavanine per ml was added at 10 min PI. The control culture received 0.4 µCi of ^{14}C -labeled amino acids per ml at 15 min PI, and the canavanine-treated culture received an identical amount of labeled amino acids at 18 min PI. The labeled amino acids were incorporated for 7 min and then they were chased with 1.5% Casamino Acids and 50 µg/ml of each of the amino acids contained in the radioactive mixture. An additional 150 µg of arginine per ml was added to chase the canavanine, and the infection was continued for 2.5 h. The normal phage and lollipops present in this lysate were then purified, and samples were examined by using gel electrophoresis and autoradiography (Fig. 9).

To interpret this experiment, it is necessary to compare the protein bands detected by staining (Fig. 9 A-C) with those detected by autoradiography (Fig. 9 D-F). Only those proteins synthesized in the presence of canavanine should contain label. Each sample was applied to the gel such that the amount of P23* was approximately the same. As is shown in Fig. 9 A and B, the overall protein composition of the untreated phage and the phage isolated after treatment with canavanine was essentially the same. However, certain structural proteins, which were affected by the lengthening of the phage head were reduced relative to P23* in the lollipop sample. (It was shown previously that P24* and the internal proteins were present in the same proportion relative to P23* in both the normal phage and in the lollipops [6].) The stained gel (Fig. 9C) shows that although the amount of P24* may be reduced in the lollipops relative to P23*, a second band is present in compensating proportions at the P24 position. The autoradiogram in Fig. 9 is in marked contrast to the stained gel. The results indicate that very few of the proteins synthesized in the presence of canavanine were assembled into the mature phage structures, but that several proteins in the lollipop structures (P23*, IPIII*, the band 18 and 19 proteins) were labeled to a

FIG. 6. Electron micrograph of the tridents obtained from the trident peak in Fig. 5.

TABLE 1. *Effect of canavanine on the release of phage proteins from cellular debris^a*

Fraction	Percent of total protein	
	Untreated cells	Treated cells
Supernatant I ^b	49.1	33.0
Supernatant II ^c	40.8	16.4
Debris ^d	10.2	50.6

^a Canavanine (200 μ g/ml) was added to T4-infected cells at 10 min PI, label was added at 15 min PI, and the cells were harvested at 20 min PI.

^b Supernatant I was obtained by treating the cell extracts as described by King and Laemmli (15).

^c Supernatant II was obtained by resuspending the pellet obtained from supernatant I in additional buffer plus CHCl_3 and lysozyme for 24 h. Lysozyme could be omitted without significantly changing these results.

^d The debris is the pellet from the centrifugation that gave rise to supernatant II.

degree similar to that in the untreated phage. A measurement of the total incorporation of the proteins synthesized in the presence of canavanine substantiates these results (Table 2). In comparison to untreated phage, only about 20% of the proteins which were synthesized in the presence of canavanine were assembled

into phage with normal head lengths, whereas about 85% of the proteins in lollipop structures were synthesized in the presence of canavanine. Table 3 shows the contribution of each gene product to the total incorporation of protein synthesized in the presence of canavanine. (The incorporation of total labeled proteins into lollipop structures as compared to untreated phage shown in Table 2 does not agree quantitatively with the data in Table 3 as we were unable to accurately measure the contribution of many of the minor proteins in the lollipops to the total protein content.) Possibly of the greatest significance, very little of the P23* (band 10) which was synthesized in the presence of canavanine was used in the assembly of phage with normal size heads, although the same P23* was selectively incorporated into lollipops at approximately normal levels as compared to the untreated phage. A second major point is that the protein which may be the uncleaved product of gene 24 (band 11a) and appeared only in the lollipops was synthesized during the presence of canavanine while the cleaved product, P24*, was both synthesized and assembled into the phage structure after the arginine chase. Relative to P23*, labeled IPHIII* was assembled into the untreated phage and the lollipops to a similar

FIG. 7. (A) Autoradiogram of an SDS-10% polyacrylamide slab gel showing the effect of canavanine on the maturation of T4 proteins in pulse-chase experiments by using amber mutants in genes 12 and 20. Note that in the untreated wild-type extract, band Z chases to the P20 position. (B) Autoradiogram of an SDS-10% polyacrylamide slab gel showing the effect of an amber mutation in gene 23 on the appearance of P20. Note that as a result of the mutation, P20 is reduced to a level that is approximately as low as the level observed in the presence of canavanine.

degree. IPIII* assembled into the treated phage to a greater extent than most proteins. The labeled band 9 protein was assembled into the untreated and treated phage to approximately the same extent, but it was not detected in the lollipops, possibly because of its abundance relative to P23*. Band 9 represents a phage protein which is not present in either isolated head or tail structures (16), thus implying it could be weakly bound between the head and the tail. The proteins represented by bands 13 and 19 also appear to have been selectively assembled into lollipops implying that they are distributed throughout the phage head. P12 and P20, which are not matured in the presence of canavanine, were synthesized and assembled into the treated phage and lollipops after the arginine chase was added. Thus, there are four proteins which are selectively assembled into lollipop particles as opposed to phage of normal head length which mature in the same lysate. These are the major head protein (P23*), the band 11a protein (possibly P24), the band 13

protein, and the band 19 protein. IPIII* made in the presence of canavanine is also assembled into lollipops with near normal efficiency and it is assembled into treated phage of normal head length with an above average efficiency. Proteins with molecular weights approximately equal to those represented by bands 11a and 13 are present in tau particles isolated from cells infected with T4 ts 21⁻ phage (19). The result that P23, the band 11a protein, the band 13 protein, and the band 19 protein are selectively incorporated into lollipops as opposed to phage of normal head length, which indicates that these proteins exist in some compartmentalized form, probably as a head precursor which eventually matures to form the lollipop head.

DISCUSSION

We have described the effects of canavanine on the maturation of certain T4 proteins. Not only does canavanine inhibit all of the known cleavage reactions involved in head assembly,

FIG. 8. Autoradiogram of an SDS-10% polyacrylamide slab gel showing that the P12 and P20 precursor proteins are not comigrating with other high molecular weight T4 proteins. The multiple amber mutants X4E (defective in genes 34, 35, 37, and 38) and XF18 (defective in genes 5-9, and 10) were examined in the presence of canavanine for the appearance of new proteins in the high molecular weight region of the gel.

FIG. 9. Photograph of a stained SDS-10% polyacrylamide slab gel (A, B, and C) and an autoradiogram of the identical gel (D, E, and F) showing the effect of canavanine on the utilization of T4 proteins in phage assembly. A and D represent untreated, purified phage; B and E represent phage obtained from a lysate exposed to canavanine from 10 to 25 min PI; C and F represent lollipops isolated from the identical treated lysate. The bands in the stained gel represent the entire protein composition of the particles and the bands in the autoradiogram represent only the proteins synthesized in the presence of canavanine. The DNase (A, B, and C) was added to digest the phage DNA in the preparation of the sample for electrophoresis.

as is evidenced by the accumulation of precursor proteins and the absence of the cleaved products, but canavanine also prevents the appearance of two additional proteins, P12 and P20. It is well documented that canavanine is able to inhibit a variety of cleavage reactions but we know of no instance where canavanine actually prevents the synthesis of a polypeptide. P12 and P20 may require a proteolytic cleavage for proper maturation. Although the P20 precursor protein has not been conclusively identified, band Z, which is intensified in the absence of proper head assembly, has been shown to chase to the P20 position by using short pulses of [^{14}C]leucine. P20 is also reduced in lysates of amber mutants in gene 23 suggesting that normal maturation of P20 depends on its proper association with P23. Laemmli and Johnson have recently demonstrated the presence of an unidentified protein in tau particles which has a molecular weight similar to that of band Z (19). This unidentified protein is present when P22, P23, P24, and IP III are uncleaved but it is not

present after the cleavage of these proteins. Band Z may well be cleaved along with the other head proteins to become P20*. Interestingly, it was originally demonstrated that one of the acid-soluble fragments found in T4 heads appeared as a result of a cleavage from either P20 or P21 (26). However, it has since been reported that the fragments arise from the cleavage of P22 (25). We were not as successful in finding a possible precursor protein for P12. Recently, it has been shown that P12 exists as a polymerized form in the mature virus (22), and it may be that cleavage is required for this polymerization step in assembly. The uncleaved precursor protein may have an increased susceptibility to proteolysis.

Canavanine resulted in the synthesis of two distinct forms of P10 and P18. Earlier results have suggested the existence of two forms of P18 (10, 24) although King (16) was unable to detect an alternate form of P18, even in situations where assembly was not favored. We were not able to mimic the effects of canavanine or either

TABLE 2. Total incorporation of proteins synthesized in the presence of canavanine into phage particles^a

Determination	Untreated phage ^b	Treated phage ^b	Lollipops ^b
Protein (μ g)	28.4	46.0	24.5
¹⁴ C counts/min	60,212	19,585	44,100
Counts per min per μ g of protein (normalized)	1.0	0.20	0.85

^a Proteins were labeled only during the exposure to canavanine, as described in the text. The phage and lollipops were purified and the total counts per minute per microgram of protein was determined. The Lowry protein assay was used to determine total protein (21).

^b Untreated phage are phage from a lysate not treated with canavanine. Treated phage are phage obtained from a lysate exposed to canavanine from 10 to 25 min PI. The lollipops were purified from the same lysate as the treated phage.

P10 or P18 by using amber mutants. However, an amber mutation in gene 17 did result in an increase in the molecular weight of the canavanine-induced form of P18 and, in separate experiments, P18 has been shown to have a slightly increased molecular weight in infections with an amber 17 mutant (K. Hercules; personal communication). Figure 9E shows that the induced form of P18 is not assembled into phage structures. The results do suggest that canavanine is preventing the cleavage of a protein fragment from P10 and P18 although it is not clear whether this cleavage has any assembly function. It may be that there is a secondary site for either the initiation or the termination of the synthesis of P10 and P18 and a certain degree of either preinitiation or faulty termination may occur. During normal conditions this extra segment would be rapidly removed, but in the presence of canavanine this cleavage is prevented. An amber mutation in gene 17 could

TABLE 3. Incorporation of proteins synthesized in the presence of canavanine into phage particles

Band no.	Gene no.	Structure	Untreated phage ^a		Treated phage ^a		Lollipops ^a	
			Amt protein ^b	% of total	Amt protein ^b	% of total	Amt protein ^b	% of total
1	34	Fiber	12.0	3.8	1.0	1.7	Neg	
2	7	Plate	5.5	1.7	Neg		Neg	
3	37	Fiber	13.0	4.1	1.5	2.5	Neg	
4	10	Plate	8.0	2.5	1.5	2.5	Neg	
5	6	Plate	11.0	3.5	3.5	5.8	Neg	
6	18	Sheath	31.5	10.0	5.3	8.8	3.5	2.3
7	20	Head	19.0	6.0	Neg		Neg	
8	12	Plate	6.5	2.1	Neg		Neg	
9			8.5	2.7	5.8	9.6	Neg	
10	23	Head	99.0	31.4	17.8	29.4	107.0	71.5
11a	(24) ^c		Neg		Neg		5.0	3.3
11b	24	Head	10.0	3.2	2.0	3.3	Neg	
12			6.5	2.1	Neg		Neg	
13			5.0	1.6	0.7	1.2	2.2	1.5
14			3.5	1.1	0.6	1.0	Neg	
15			5.0	1.6	Neg		Neg	
16	IPIII	Internal	15.3	4.9	5.0	8.3	11.0	7.3
17	19	Protein	19.0	6.0	4.8	7.9	Neg	
18		Tube	Trace		Neg		Neg	
19			Trace		Neg		1.0	0.7
20 ^d			37.0	11.7	11.0	18.2	20.0	13.4

^a Untreated phage are phage from a lysate not treated with canavanine. Treated phage are phage obtained from a lysate exposed to canavanine from 10 to 25 min PI. The lollipops were purified from the same lysate as the treated phage.

^b The autoradiogram shown in Fig. D, E, and F was scanned by using a densitometer and the relative amount of labeled protein represented by each band was determined by weighing each peak in the tracing.

^c Band 11a may represent uncleaved P24 although it is not certain.

^d Band 20 represents all those proteins which ran with the front. Band numbers in italics signify labeled proteins which were assembled into treated phage and/or lollipop phage to a different degree relative to untreated phage. See text. Neg, Negligible; Trace, faint band detected.

allow an earlier pre-initiation on gene 18 and thus, it would explain the reason for the increase in the molecular weight of the induced form of P18. Alternatively, cleavage of the high molecular weight forms of P10 and P18 to the low molecular weight forms may be required for proper assembly of the tail plate and the sheath. This would account for the inhibition of tail assembly in the presence of canavanine. It has been shown that the cleavage of a tail protein is required in T5 assembly (29).

Laemmli and Favre have recently shown that there are several intermediates in T4 head assembly (18). The earliest detectable intermediate is termed prohead I and it is comprised of P23 as well as P20, IPIII, and P22. Upon cleavage of P23 to P23* the structure is termed prohead II. Our data indicate that there may be an earlier precursor head form in T4 assembly and that it may be at this earlier level that the length of the phage head is determined. We reported in the companion paper that the induction of lollipops results from an exposure to canavanine which influences the formation of a precursor substance which determines the length of the lollipop head (1). Our data strongly suggest that the precursor substance is an early prohead intermediate in which the head length has not yet been determined. Our model is described as follows. Active P20 is not matured in the presence of canavanine, probably as the result of an inhibition of the cleavage of P20 to P20*. (The active form of P20 will be denoted as P20*.) The absence of P20* does not inhibit the formation of the lollipop proheads which consist primarily of P23, the proteins represented by bands 11a, 13 and 19 (Fig. 9), and probably P22. In the presence of canavanine, the absence of P20* leads to the elongation of the prohead although there is probably a limit to the size of this structure due to its fragile nature and the dimensions of the host cell. After the addition of arginine, additional P20 is synthesized and incorporated into the prohead. With the cleavage of P20 to P20*, the precursor head structure becomes prohead I (as defined, prohead I contains P20* [18]) and the elongation of the structure is stopped. Shortly thereafter, P23 is cleaved to P23* and prohead II is formed. Our data suggest that P24 (Fig. 9, P24; Table 3, band 11a) assembled into the head precursor during the exposure to canavanine, but that P24 made in the presence of canavanine is not cleaved. P24 synthesized after the addition of arginine is cleaved (Fig. 9, P24*; Table 3, band 11b). Since P24 is distributed throughout the phage head, it may act to

stabilize the intermediates in head formation so as to allow the cleavage reactions to occur.

In all likelihood, head length determination involves an interaction of P20* with specific regions located on P23. P23 synthesized in the presence of canavanine is clearly utilized in the assembly of the lollipop head but P20 synthesized in the same period has not been detected in the assembled particles. This may indicate that canavanine is incorporated into P20 in such a manner that it alters the conformation of the protein, thus preventing its proper association with determinants on P23. (Our unpublished results indicate that unincorporated canavanine has little if any effect on the maturation of proteins.) Our model suggests that the cleavage of P20 may be the event which triggers the other cleavage reactions involved in head assembly. Therefore, until arginine is added to chase the canavanine, usable P20 is not available and the remaining head proteins remain uncleaved. Our finding that PFPA (which also inhibits the appearance of P20*, possibly because of a similar conformational change in P20) results in the formation of a rare lollipop supports this contention (unpublished results). ACA which does not result in the formation of lollipops also does not inhibit the cleavage of P20, but it does inhibit the cleavage of P23. This may indicate that ACA does not affect the association between P20 and P23 which does allow P20 to cleave. However, the conformation of P23 is sufficiently altered such that its cleavage cannot occur. TRA which results in the formation of a high percentage of small-headed phage may alter the conformation of P20 and P23 such that the length controlling interaction acquires an increased sensitivity thus allowing premature P20 cleavage and the termination of head elongation. The results that canavanine affects the maturation of a variety of other proteins such as P12, P10, and P18 but that other analogues do not, suggest that canavanine is incorporated near the cleavage site, whereas other analogues are not.

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