Bacteriophage Tail Components

IV. Pteroyl Polyglutamate Synthesis in T4D-Infected Escherichia coli B

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The nature of pteroyl polyglutamates in uninfected and T4D bacteriophage-infected Escherichia coli B has been examined. ^H-p-aminobenzoic acid has been used to label the folate compounds and gel permeation chromatography on glass beads to separate the folate compound by molecular size. It has been found that, although the major folate compound in uninfected bacteria is pteroyl triglutamate, E. coli B cells also contain folate compounds having as many as six glutamate residues. Infection with T4Dstimulated the addition of glutamate residues to the lower-molecular-weight host pteroyl compounds, resulting in the conversion of the host compounds into the hexaglutamate form. This viral-induced conversion is chloramphenicol sensitive and appears to be due to a late phage gene product. The phage gene responsible for this conversion has not been identified. In cells infected with a T4Dmutant defective in gene 28, there was an apparent production of the large pteroyl polyglutamates equivalent in size to pte(glu)\textsubscript{12}. These high-molecular-weight forms were converted into pte(glu)\textsubscript{6} by incubation with bacterial extracts made after infection with T4D 28\textsuperscript{+}. Apparently, the product of T4D gene 28\textsuperscript{+} is capable of specifically cleaving the high-molecular-weight polyglutamates to the form necessary for phage tail assembly.

The observation that T4D bacteriophage tail plates have dihydropteroyl hexaglutamate as a structural component (5) has prompted an examination of the synthesis of pteroyl polyglutamates in both uninfected Escherichia coli and infected E. coli. In an earlier analysis of folates of uninfected and infected E. coli, it was reported that all of the extracted bacterial folates could be accounted for as pteroyl triglutamates or smaller molecules (13). However, these earlier cell extracts were prepared by procedures which did not include protective agents such as ascorbate, and all reduced folates would have been broken down.

Studies on the larger pteroyl polyglutamates also have been hampered because of the difficulty in separating them for analysis. Conventional gel permeation chromatography using the usual supporting substrates does not give good resolution because of adsorption of folates to the supporting matrix. High-voltage paper electrophoresis (2) also suffers from the disadvantage that electrophoretic mobility is influenced by pteridine substituents as well as by the number of glutamate residues. A permeation chromatography method has now been developed to separate pteroyl polyglutamates solely on the basis of size by using controlled-pore-size, glass beads coated with polyethylene glycol. Standard folate compounds containing up to seven glutamate residues have been shown to separate as expected and the adsorption problem has been overcome.

Radioactive p-aminobenzoic acid (PAB) has also been used to label the folate compounds in both uninfected and infected host cells. Bacteriophage T4D infection has been found to stimulate conversion of host cell folates into a PAB-containing compound having a molecular weight corresponding to pte(glu)\textsubscript{6}. In cells infected with T4D containing an amber mutation in gene 28\textsuperscript{+}, there is the formation of still larger pteroyl compounds. The T4D gene 28 product is thought to be an enzyme which cleaves the larger PAB-containing compounds to the pteroyl hexaglutamate compound needed for tail assembly. In an accompanying paper, biological evidence is presented that pteroyl hexaglutamate stimulates in vitro formation of phage particles in extracts of E. coli infected with T4D 28\textsuperscript{−}. A preliminary report of this work has been presented earlier (L. M. Kozloff, Fed. Proc., 30:1263, 1971).

MATERIALS AND METHODS

Most of the biological materials and methods were
identical to those used earlier for the growth, purification, and assay of the E. coli bacteriophages (4-6). Hog kidney conjugase, an exopeptidase which hydrolyses the terminal γ-glutamyl residues from pteroyl polyglutamate, was purified from fresh hog kidney as described elsewhere (3). Microbiological assay for folate was carried out using Lactobacillus casei (ATTC 7469) (3).

The following labeled compounds were used: ^3H-p-aminobenzoic acid, 500 mCi/mmole, Nuclear-Chicago Corp.; ^3H-folic acid, 236 mCi/mmole, Nuclear-Chicago Corp.; ^32P, 200 mCi/mmole, Radiochemical Centre, Amersham; and ^14C-pteroyl (glu)3, a gift from K. Kumdieck (7). Most other chemicals were obtained from commercial sources: blue dextran, Pharmacia; glucagon and insulin, Sigma Chemical Co.; TPNH, PLB, Inc.; polyethylene glycol 1000, J. T. Baker, Chemical Co.; dithiothreitol, Calbiochem, 2,4-dinitrophenol-glycine, (DNP) Sigma; and Aquasol, New England Nuclear Corp. Synthetic pteroyl pentaglutamate, pteroyl hexaglutamate, and pteroyl heptaglutamate were gifts of Charles Baugh and were reduced to the dihydro or to the tetrahydro form by the procedures of Silverman and Noronha (8).

For the separation of the different pteroyl polyglutamates, a permeation chromatography procedure was developed with controlled-pore-size beads. The glass beads (Sigma Chemical Co., stock no. 6-75-50) had nominal pore sizes with diameters of 7.5 nm (range of ±10%). One volume of glass beads, mesh size 120 to 200, was suspended, with stirring, in 5 vol of 1% polyethylene glycol 1000 for 10 min and then deaerated twice in a desiccator for 10 min. The supernatant fluid was decanted, and the beads were washed three times with 10 vol of water. The beads were never exposed to air drying and were then used to pack a dimethyl dichlorosilane-treated glass column 94 cm long by 1.4 cm i.d. The column was equilibrated with 0.2 M Tris, pH 7.4, containing 0.6% polyethylene glycol 1000 and 0.2% dithiothreitol. Usually 0.3- to 0.5-ml samples were placed on top of the column and eluted with the Tris-polyethylene glycol-dithiothreitol solution at a rate of 1.0 ml/min. The column was washed with 3 vol of water after each run and used for a total of 10 runs, after which the internal volume appeared to decrease.

E. coli pteroyl compounds were labeled with radioactive PAB by growing the cells with 2 μg of labeled compound per ml in synthetic M-9 glucose medium (1). In various experiments the radioactive compound was chased by adding nonradioactive PAB to give a final concentration of 200 μg/ml. Infected cells, at 4 x 10^5 ml, were concentrated 20- to 50-fold by centrifugation in the cold without washing; the pellet was dissolved in a solution containing 8 M urea, 0.2 M Tris (pH 7.4), and 0.1% dithiothreitol. It was necessary to sonically treat uninected cells that did not lyse in the urea solution for 5 min with a Brannson sonic oscillator. All extracts were clarified at 17,000 x g for 30 min in the cold and then either stored at 20 C in the dark or chromatographed. Up to 1.0 ml of the eluted fractions obtained were mixed with 10 ml of Aquasol, a scintillation solvent, and the radioactivity of the fractions was measured in the Beckman CPM-100 liquid scintillation counter.

RESULTS

Separation of pteroyl polyglutamates by gel permeation chromatography. Conventional gel permeation chromatography with polyacrylamide or dextran beads was not satisfactory because of adsorption effects. Controlled-pore-size, glass beads with a pore diameter of 7.5 nm have recently become available and were tested for their ability to separate pteroyl polyglutamates. It was found that the large glass surface, even at high salt concentrations and alkaline pH values, adsorbed folate compounds. Following the suggestion of the Corning Glass Co., that polyethylene glycol 20,000 abolished or reduced protein adsorption effects by coating the glass-bead surface, the use of smaller molecules of polyethylene glycol was examined. It was found that coating the glass beads with polyethylene glycol of average mol wt 1000 abolished the adsorption of folate compounds to the glass surface and allowed the separation of compounds of molecular size 5000 to 300 solely on the basis of molecular size.

The partition coefficient of the eluted compounds was determined by using blue dextran to mark the void volume (partition coefficient = 0) and ^32P, (partition coefficient = 1.0) to mark the total internal volume. The void volume of the column used was about 55 ml, and the internal volume was about 33 ml. Two (I and II) typical standard runs made 1 year apart with different batches of glass beads are shown in Fig. 1. Insulin was oxidized with

![Fig. 1. Chromatographic behavior of various compounds on a polyethylene-glycol-coated, controlled-pore-size, glass-bead column. Runs I and II were made 1 year apart on different batches of glass beads. Further details on the preparation and operation of the column are given in Materials and Methods.](http://jvi.asm.org/Downloaded from http://jvi.asm.org/)
performic acid to give the separate oxidized A and B chains, and the two polypeptides were measured fluorometrically in the collected fractions. H$_3$ pte(glu)$_7$ and H$_4$ pte(glu)$_8$ were measured fluorometrically. H$_3$ pte(glu)$_7$ was used in some experiments and its chromatographic behavior was identical to H$_4$ pte(glu)$_8$. The pte(glu)$_8$ was labeled with $^3$H, and the pte(glu)$_4$ was labeled with $^3$H. As shown later (Fig. 2 and 3), the pte(glu)$_7$, extracted from uninfected cells, which is a mixture of folates in various oxidation states containing a variety of "1 carbon" additions, also behaved as expected. In addition to $^{32}$P, $^3$H-PAB and DNP-glycine were suitable markers to determine the internal volume. Although the compounds used to standardize the column gave peak elution values as indicated, their elution curves were quite broad, especially for compounds of lower molecular weight.

The molecular size of pteroyl polyglutamates in uninfected E. coli. Labeled PAB, a folate constituent, was added to a synthetic glucose-salt medium (M-9) at a level of 2 $\mu$g/ml. The labeled compound was taken up by the bacteria and appeared to be converted solely to folate compounds. The conclusion that the PAB labeled only the folate compounds of the cell is based on the following observations. (i) It is known that added PAB can be incorporated into folate compounds in E. coli (10). (ii) No label was found in proteins or other components of the cell, and the addition of various aromatic amino acids such as phenylalanine, tyrosine or tryptophan, or purines or pyrimidines did not affect the incorporation of PAB label into low-molecular-weight compounds. (iii) In uninfected cells the major PAB-labeled compound had a molecular size equivalent to pte(glu)$_3$, which is the major folate component of bacteria. (iv) Microbiological assays with L. casei for folic acid indicated microbiological activity corresponding to the labeled pte(glu)$_7$, peak. (v) Treatment of PAB-labeled compounds from E. coli cell extracts by hog kidney conjugase, an exopeptidase specific for the $\gamma$-glutamyl bonds of pteroyl glutamyl compounds, converted labeled compounds having sizes equivalent to pte(glu)$_{5,6}$ into a compound with a size equivalent to pte(glu)$_4$. (vi) Only 0.6 to 0.7% of the added labeled PAB was taken up by the cells. Assuming that E. coli contains about 5 $\times$ 10$^8$ folate molecules (3), it can be calculated that only 10 to 15% of the cell folates contain labeled PAB. It is clear that no excess of radioactivity is taken up by the cells and that excess exogenous PAB does not prevent considerable endogenous folate biosynthesis. (vii) T4D infection caused an increase in the average molecular size of the PAB compounds corresponding to the apparent production of pte(glu)$_1$, a known phage component.

The size distribution of $^3$H-PAB-containing
compounds from uninfected E. coli B is shown in Fig. 2A. In this experiment 3H-PAB, 2 μg/ml, was added to the synthetic medium, and the cells were grown to a concentration of 4 x 10^9/ml. At this time unlabeled PAB (at a final concentration of 200 μg/ml) was added, and the cells were incubated for 20 min more at 30 C. Approximately 25 ml of the cell suspension was chilled, centrifuged, and resuspended in 0.8 ml of a solution containing 8 M urea, 0.2 M Tris (pH 7.4), and 0.1% dithiothreitol. The suspension was sonically treated for 3 min and clarified at 17,000 x g for 20 min. One-half of the total solution plus marker compounds were then chromatographed.

The major peak of radioactivity was in compounds having a size equivalent to pte(glu)_4, but the size distribution was much larger than that for known marker pte(glu)_4. It is not surprising that the peak would be broad due to the presence of precursor pte(glu)_5 and pte(glu)_6, and some free PAB, but the broadness at the larger-molecular-size ranges was unexpected. When a sample of the extract was treated with hog kidney conjugase, the 3H activity appeared in a single peak corresponding to pte(glu)_4. These results suggest that uninfected E. coli cells normally contain small amounts of folate compounds having sizes corresponding to at least pte(glu)_6 as well as pte(glu)_5, pte(glu)_4, pte(glu)_3, and pte(glu)_1.

The presence of higher-molecular-weight forms of bacterial folates was confirmed in a pulse-chase experiment. 3H-PAB, 2 μg/ml, was added to a 5.0-ml culture of E. coli which was grown overnight. This small culture was used to inoculate 100 ml of synthetic medium containing 200 μg of unlabeled PAB per ml. Initially, the bacterial concentration in the 105 ml gave a Klett turbidity of 15, and it was incubated for several hours to a Klett turbidity of 75 (equal to 4 x 10^8 cells/ml). One-quarter of this culture was left uninfected, and the other three-quarters were used for experiments with T4D. With a fivefold increase in cell mass, there was a change in the molecular-size distribution of the labeled folates. The original 3H-PAB in uninfected cells was now in compounds having a molecular size equivalent to pte(glu)_5 (Fig. 1B). The synthesis of these large forms does not seem to be reversible, and the “chasing” of the lower-molecular-weight forms into the higher forms with further cell growth would then result in the trapping of the 3H-PAB in the higher forms. The fraction of bacterial folates having more than three glutamyl residues can be estimated only roughly from the 3H distribution, but is of the order of 5 to 15% of the total bacterial PAB-containing compounds.

Effect of T4D infection on pteroyl polyglutamate synthesis in infected E. coli. By cruder and less-sensitive methods, we reported (3) that T4D infection induced the synthesis of pteroyl hexaglutamate in E. coli. E. coli was grown to 4 x 10^9/ml in the presence of 3H-PAB at 30 C. The culture was divided into four portions, and unlabeled PAB (final concentration 200 μg/ml) was added to each. One portion remained uninfected, and the remaining three were multiply infected with T4D. Nothing additional was added to the second culture. Chloramphenicol (50 μg/ml) was added immediately before infection to the third culture and at 10 min after infection to the fourth culture. All cultures were incubated for a total of 20 min at 30 C, and then extracts were prepared as described above and chromatographed.

The results (Fig. 3) show that T4D infection induces a conversion of host cell lower-molecular-weight folates into higher-molecular-weight compounds. These phage-induced PAB-containing compounds appear to be mixtures of pteroyl compounds containing from five to possibly seven glutamate residues. They were degraded to the size of pte(glu)_5, by hog kidney conjugase treatment.

The phage-induced folate conversion was sensitive to addition of chloramphenicol at either the time of infection or even as late as 10 min after infection at 30 C. Figure 3 shows that chloramphenicol added at zero time completely prevented the appearance of higher-molecular-weight compounds. The results from the culture in which chloramphenicol was added at 10 min were superimposable to those shown for the experiment in which chloramphenicol was added at zero time. It can be concluded that a phage-induced protein, which is presumably a product of a late gene, is responsible for the production of the higher-molecular-weight folate compounds.

The three T4D phage genes thought to act catalytically in tail plate assembly, genes 26, 28, and 51 (9), were tested for their ability to induce the conversion of lower-molecular-weight compounds to higher-molecular-weight compounds. Figure 4 shows that E. coli containing 3H-labeled folate compounds infected with T4D defective in genes 26 or 28 or 51 still induced the conversion. Since in each T4D-infected culture the host cell folates were converted into higher-molecular-weight forms, it can be concluded that none of these three gene products is responsible for the production
Role of T4D gene 28 in regulating pteroyl polyglutamate concentrations. It has been shown that E. coli B extracts made after infection with T4D gene 28\(^{-}\) are apparently deficient in pteroyl hexaglutamate and will form phage in response to added synthetic pteroyl hexaglutamate (5). The experiments described above showed that the gene 28 product was not involved in the conversion of host folates to the higher-molecular-weight pteroyl polyglutamates. Furthermore, uninfected host cells apparently contain a significant amount of pteroyl hexaglutamate as shown in the pulse-chase labeling of host folates by \(^3\)H-PAB (Fig. 2B). Since it was possible to selectively label the host higher-molecular-weight pteroyl polyglutamates, the effect of phage infection on these host cell components was examined.

The experiments, in which the \(^3\)H-PAB was added to the subinoculum and then unlabeled PAB was added at the time of inoculation of the main culture, were carried out as described above. Under these circumstances the labeled PAB is found mainly in the host folates which have five or six glutamate residues. The cells were infected, and extracts were prepared and chromatographed on a glass-bead column. In the first experiment (Fig. 5), in addition to the uninfected culture, extracts were prepared 12 min after infection at 37 C with either T4D\(^{+}\), T4D 26\(^{-}\), T4D 28\(^{-}\), or T4D 51\(^{-}\). The uninfected cells contained a mixture of labeled folates, the majority of which were pte(glu)\(_5\) and pte(glu)\(_6\). Infection with wild-type T4D (labeled T4D 28\(^{+}\)) caused an apparent shift of the \(^3\)H into higher-molecular-weight folate compounds. Infection with T4D 26\(^{-}\) and T4D 51\(^{-}\) gave distributions of folate compounds identical to that found with wild-type phage and are not shown in Fig. 5. Infection with T4D 28\(^{-}\) gave an even more pronounced conversion of host cell folates to higher-molecular-weight forms than did infection with wild-type phage, and significant amounts of \(^3\)H-PAB eluted in positions corresponding to pte(glu)\(_5\) to pte(glu)\(_8\).

The conversion of host folates to higher-molecular-weight forms after T4D infection was confirmed in a similar experiment (Fig. 6). In this pulse-chase experiment the \(^3\)H-PAB in the uninfected host cells was largely in pte(glu)\(_2\) rather than being a mixture of pte(glu)\(_1\) and pte(glu)\(_5\) (Fig. 5). In this latter experiment the chasing of the \(^3\)H-PAB in uninfected host cells from largely pte(glu)\(_2\) to pte(glu)\(_5\) was more efficient. Wild-type phage infection (T4D 28\(^{+}\)) did not change the size distribution of the folate compounds. However, T4D 28\(^{-}\) infection clearly caused a conversion of a portion of the host pte(glu)\(_5\) to much larger compounds including pte(glu)\(_7\) to pte(glu)\(_9\).

**Fig. 4.** Effect of infection with T4D and various T4D amber mutants on the size distribution of compounds containing \(^3\)H-PAB in E. coli extracts. Procedures are given in the text.

**Fig. 5.** Effect of infection with wild-type T4D (gene 28\(^{+}\)) and T4D gene 28\(^{-}\) on the size distribution of compounds containing \(^3\)H-PAB in E. coli extracts. The host folates were labeled with \(^3\)H-PAB in a "pulse-chase" experiment and then multiply infected with phage. Extracts were heated in the usual manner.
treating the suspension for 5 min in the cold. The suspension was clarified at 10,000 × g for
10 min, and 0.1 ml of extract was added to 0.4 ml of the high-molecular-weight folate fraction.
The final incubation mixture contained 0.02 M MgSO4, 1% ascorbate, and 0.1 M Tris, pH 7.4.
This mixture was incubated for 1 h at 37°C, heated at 95°C for 2 min, and then clarified.

Figure 7 shows that the extract from wild-type-infected cells (28+) cleaved the high-
molecular-weight folates to the size of pteroyl hexaglutamate. The crude 28- extract (which
contains a variety of compounds including free glutamate) actually increased the molecular
size of the folate compounds so that there were significant amounts of folates containing as
much as 10 to 12 glutamate residues. It can be concluded that 28- extracts possess the ability
to add glutamyl residues to folate compounds but lack the enzyme (present in T4D 28+-
infected cells) which cleaves these high forms to the size of the pteroyl hexaglutamate
necessary for phage tail assembly. These results are in agreement with those results on phage as-
sembly obtained in the companion studies (4), which also indicated that the addition of glu-
tamyl residues to folate compounds was uncontrolled in the absence of the gene 28 product.
FIG. 8. Patterns of synthesis of pteroyl polyglutamate(s) in uninfected and T4D-infected E. coli B.