JOURNAL OF VIROLOGY, Dec. 1975, p. 1401-1408
Vol. 16, No. 6

Printed in U.S.A.

Bacteriophage T4 Baseplate Components

II. Binding and Location of Bacteriophage-Induced Dihydrofolate Reductase

LLOYD M. KOZLOFF,* LOUISE K. CROSBY, MURL LUTE, AND DWIGHT H. HALL

Department of Microbiology, University of Colorado Medical School, Denver, Colorado 80220, and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710*

Received for publication 17 April 1975

The location of T4D phage-induced dihydrofolate reductase (*dfr*) has been determined in intact and incomplete phage particles. It has been found that phage mutants inducing a temperature-sensitive *dfr* (*dfr*^{ts}) produce heat-labile phage particles. The structural *dfr* produced by these ts mutants was shown to assume different configurations depending on the temperature at which the phage is assembled. Morphogenesis of incomplete phage particles lacking the gene 11 protein on their baseplates was found to be inhibited by reagents binding to *dfr*, such as antibodies to *dfr*. Further, cofactor molecules for *dfr*, such as reduced nicotinamide adenine dinucleotide phosphate and reduced nicotinamide adenine dinucleotide, also inhibited the step in morphogenesis involving the addition of gene 11 product. On the other hand, inhibitors of *dfr*, such as adenosine diphosphoribose, stimulated the addition of the gene 11 protein. It has been concluded that the phage-induced *dfr* is a baseplate component which is partially covered by the gene 11 protein. The properties of phage particles produced after infection of the nonpermissive host with the one known T4D mutant containing a nonsense mutation in its *dfr* gene suggested that these progeny particles contained a partial polypeptide, which was large enough to serve as a structural element.

The original report (10) that the T4D phage-induced dihydrofolate reductase (*dfr*) was a tail plate component indicated that (i) the *dfr* enzymatic activity of phage ghosts could be entirely accounted for by the activity of purified baseplates, (ii) the activity could only be demonstrated after treatment to disrupt the phage structure, and (iii) the intact phage particle could react with cofactors of *dfr*, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH). Later, the preparation and use of antiserum to purified *dfr* to inactivate phage (13), as well as the experiments in which the *dfr* gene (also called the *frd* gene [6]) was transferred to related phage strains (12), all supported the conclusion that this viral-induced early enzyme played a structural role in the phage tail. A possible role for the baseplate *dfr* in invasion was described by Male and Kozloff (11) which did not involve any enzymatic functions for the *dfr*. Dawes and Goldberg (1, 2) independently proposed that *dfr* did not function enzymatically, and whereas it might play a role in the early stages of invasion it was not necessary for later stages of infections.

Experiments also indicated that the baseplate was the most heat-sensitive element of the phage structure and that changes in baseplate components, such as in the *dfr* incorporated, caused changes in heat sensitivity (10). Recently, Yamamoto and Uchida (16) also observed that the tail plate is the most heat-sensitive structure of the phage particle. These observations on the lability of the tail plate were also noted earlier by To et al. (14).

This report deals with the alternative conformations that *dfr* can assume during phage assembly, the effect of various normal cell constituents on the conformation, and the location of the enzyme within the baseplate. These conformational changes not only cause changes in heat sensitivity of the virus particle but also affect the rate of phage assembly. In addition, evidence is presented indicating that even a partial polypeptide of *dfr* can be used for structural purposes provided it has binding sites for the dihydrofolate and nicotinamide adenine nucleotide (NAD) (7).

MATERIALS AND METHODS

Preparation and purification of bacteriophage stocks and substructures. The procedures described in the accompanying paper (8) were also

used in this work. Three of the *folate-analogue-resistant* T4D mutants (*far* mutants) described by Johnson and Hall (6), P1, C1, and C31, were grown on *Escherichia coli* B at either 28 or 42 C and purified in the usual manner. These mutants were spontaneous mutants found in $T4D_0$ stocks, the T4D strain used by D.H.H., and are presumed to be otherwise isogenic with $T4D_0$. The P1 mutant, which was extensively used, was checked for its resistance to pyrimethamine, an inhibitor of phage *dfr*, both by the plating techniques of Johnson and Hall (6) and by its enzymological properties.

Measurements of heat sensitivity were carried out as described earlier (9). Phage stocks in saline (or phage lysates) were diluted at least 200-fold in 0.1 M phosphate buffer, pH 7.0, and then a small volume (10 to 100 μl) was added to a much larger volume (1.0 to 2.0 ml) of preheated buffer in a rapidly stirred water bath at 60 C. All phage stocks were treated with pancreatic DNase to remove any DNA which might affect stability. The Mg^{2+} concentration was always diluted to less than 10^{-6} M. All comparisons of relative heat stabilities between different phage preparations were made on the same day using the same water bath and buffer solutions. Variations in temperature ($\pm$0.2 C) from day to day or decreases in the ionic strength of the suspending buffer caused apparent large changes in heat sensitivities. Most commercial laboratory water baths do not have the thermoregulators that regulate reproducibly at 60 C from day to day. In some early experiments, a water bath without an adequate regulator was used. These early results qualitatively agreed with later observations. No claim is made that the heat inactivation rates are precise. In some experiments (see Fig. 1 for example) the heat sensitivities of two different T4D phage preparations were identical within the limits of the phage assays. A further indication of the reliability of heat inactivation studies is shown for a series of 10 different phage stocks all heated at the same time (Table 2). The rate constants had a standard deviation of $\pm$10%.

The preparation of T4D particles lacking the 11 and 12 proteins and the procedures for carrying out complementation reactions were as described (8). The antiserum to T4D-induced *dfr* was a gift of C. Mathews (4).

Selection and characterization of trimethoprim-resistant E. coli mutants. *E. coli* B cells were grown to a titer of 2×10^9/ml in nutrient broth (18 g/liter). Agar tubes (2 ml) (0.7% purified agar in nutrient broth to which has been added 2 μg of trimethoprim per ml) were seeded with 0.1 ml of the cell culture and overlayed on plates (50 mm in diameter) containing 10 ml of 1.5% purified agar in nutrient broth plus 2 μg of trimethoprim per ml. Plates were incubated at 37 C overnight. Resistant colonies were picked, and the bacteria were suspended in broth and then replated and again picked from trimethoprim plates. The strains were maintained on nutrient broth-agar slants containing trimethoprim (2 μg/ml). All bacteria were tested for susceptibility to adsorption and infection by T4D. Those mutants which grew very slowly or which were resistant to T4D were discarded. Nutrient broth was the "Wellcotest" brand, obtained from Wellcome Research Laboratories. Purified agar and tryptone broth were Difco products. "Syraprim" brand trimethoprim was a gift from Burroughs-Wellcome Co.

Preparation of enzyme extracts for *dfr* assay. Cultures (40 ml) of each bacterial mutant and of *E. coli* B were grown in tryptone broth to a titer of 4×10^8 at 37 C. Cells were centrifuged, directly resuspended in 3 ml of 0.05 M potassium phosphate buffer, pH 7.4, and then disrupted with a Branson sonifier with 30-s pulses in the cold for 3 min. The sonicated extracts were clarified at 20,000 $\times$ *g* for 20 min in the cold and dialyzed against the assay buffer overnight in the cold. Fluorometric assays of *dfr* activity were as described earlier (10). The enzyme was inactivated by heat in a 40 C water bath, the inactivation being halted at given times by immersion of the sample in ice water.

RESULTS

Properties of T4D far mutants. Johnson and Hall (6) reported that three of the *far* mutants they isolated (P1, C1, and C31) from their strain of T4D, called $T4D_0$, induced the formation of a *dfr* that was physically different from the parental *dfr*. The *dfr*'s produced by these three mutants were more heat labile than the *dfr* induced by the parental $T4D_0$. These mutants behave in plating tests (see reference 7) as the dfr^{ts} mutants. In view of the evidence that *dfr* is a baseplate constituent, the heat sensitivity of these three mutants was examined. Phage stocks were grown at both 30 C, a permissive temperature for the plating test, and at 42 C, a nonpermissive temperature for the plating test, on *E. coli* B and then heat inactivated. The results shown in Fig. 1 indicate that changes in the *dfr* gene product caused changes in the physical properties of the phage particles. It should be emphasized (Fig. 1) that parental $T4D_0$ has the same heat sensitivity (and other properties) irrespective of the temperature of assembly. In three separate experiments, it was found that all three mutant phage particles, when assembled at 42 C, were both more heat labile than parental T4D and more labile than mutant phage assembled at 30 C. *far* mutant T4D P1 phage particles assembled at 30 C were slightly more heat resistant than the parental T4D, whereas C1 and C31 particles assembled at 30 C were either more sensitive or quite similar to T4D particles. The characteristic heat sensitivity of phage mutant particles grown at different temperatures did not change when these phage stocks were purified and stored for some months in the cold. It can be concluded that the *far* mutations affect a viral constituent, *dfr*, determining stability to heat,

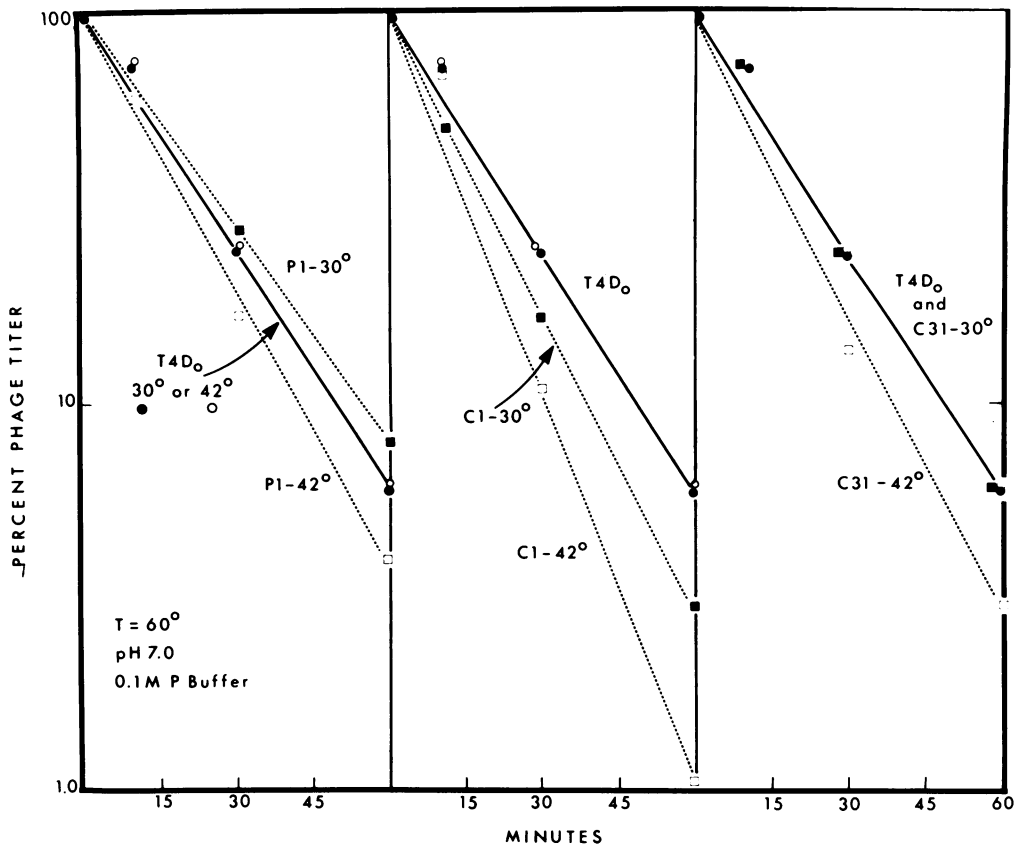

FIG. 1. *Heat sensitivity of various far mutants (6) as compared to the original T4D$_0$ phage. The far mutant stocks (and the parent T4D$_0$ stock) were grown at either 30 or 42 C in broth on E. coli B and examined for their heat sensitivity.*

and that this protein can be assembled in different configurations which result in phenotypic differences.

When these three mutants were examined in three separate experiments for their ability to react with antiserum made against parental T4D *dfr* (13), again noticeable phenotypic differences were observed, as shown typically in Fig. 2. These differences, in most cases, reflected their heat sensitivity. For example, stocks assembled at 42 C, which were more heat sensitive than parental T4D$_0$, were also more sensitive to antiserum inactivation. Parental T4D$_0$ particles grown at either temperature were equally susceptible to the antiserum.

These two experiments support the conclusion that *dfr* is a tail plate component. The properties of phage assembled at different temperatures also showed that there are some as yet undefined permissible variations in the conformation of the *dfr* used for assembly which are then reflected in properties of the final phage structure.

Properties of T4D particles lacking gene 11 and 12 proteins. In view of the evidence that the gene 11 protein covers the pteridine group of the phage folic acid (8) and the likelihood that dihydrofolic acid is bound to the *dfr* (10), the exposure of the *dfr* in particles lacking the gene 11 and gene 12 proteins was examined. Purified T4D 11$^-$ particles were prepared as described earlier (8). These particles were found to be highly sensitive to antiserum to T4D *dfr*. The minimum inactivation rate constant (K) for the effect of serum on these incomplete particles was more than 20, whereas for intact phage particles the same serum had a K value of about 5. Precise K values for the inactivation of incomplete particles are difficult to measure, since the final phage particle is also able to react with the antiserum. Further, the extract containing the gene 11 and gene 12 proteins

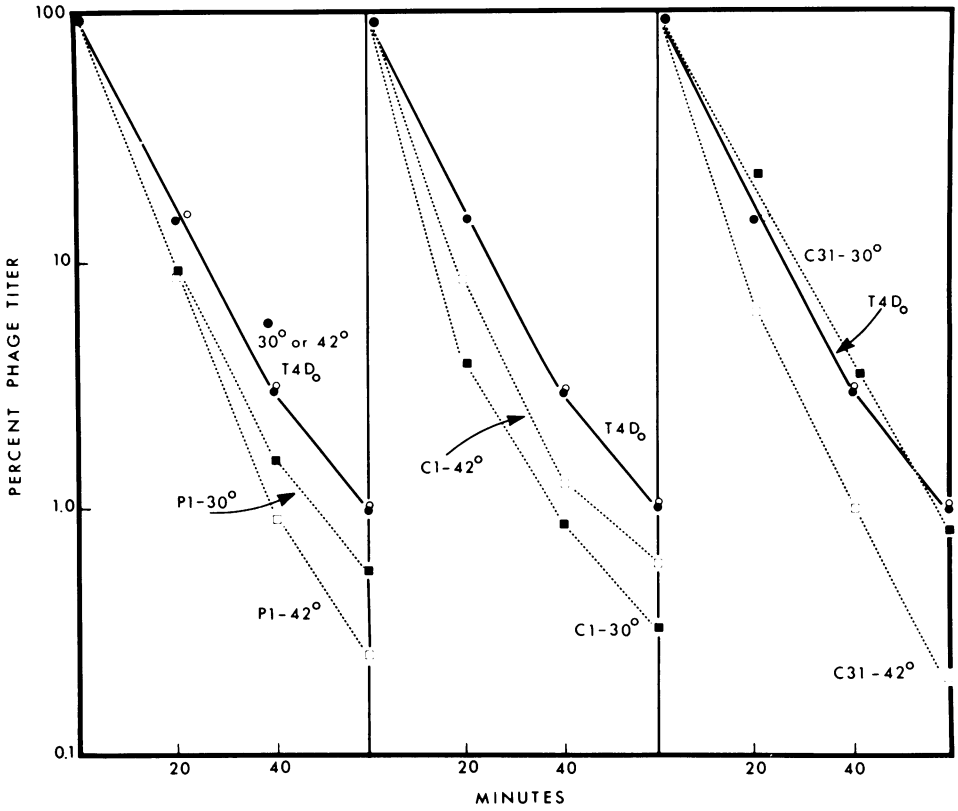

FIG. 2. *Inactivation of the T4D far mutants by antiserum to dfr (13). Phage stocks and antiserum were diluted in tryptone broth adjusted to pH 5.0. The reaction mixture was incubated at 37 C. The initial phage concentration was 2 × 10⁸/ml, and the serum was used at a dilution of 1:13. The reaction was stopped by a 1:101 dilution in cold broth.*

contains the T4D-induced *dfr*, which reacts with the serum. However, these results not only support the earlier direct evidence that the phage-induced *dfr* is a baseplate component but also indicate that the pteridine portion of the folic acid and the *dfr* are near each other, since both are at least partially covered by the gene 11 protein.

Effect of *dfr* coenzymes and inhibitors on the addition of the 11 and 12 proteins to incomplete T4D particles. Various small coenzyme molecules for *dfr*, such as NADPH, and inhibitors of *dfr*, such as adenosine diphosphoribose (ADPR) (11), have been shown to react with phage particles and to either inhibit adsorption and injection or to stimulate these initial stages of infection. These earlier experiments supported the view that these small molecules reacted with the *dfr* and changed its conformation. Since the conformation of the *dfr* might well affect the addition of the 11 protein to the baseplate, the role of these compounds on this step of phage morphogenesis was examined.

The effect of NADs on the addition of 11 and 12 proteins to T4D 11⁻ particles is shown in Fig. 3. The reduced dinucleotides were tested at concentrations and at a pH (7.4) where their inactivation of phage is quite small (10, 11). NADPH and, to a lesser extent, reduced NAD both inhibited the step in morphogenesis involving 11 protein addition, whereas $NADP^+$ and NAD^+ either slightly stimulated or had no effect on the addition of the 11 (and 12) protein. The reduced pyridine nucleotides are known to react with phage particles, whereas the oxidized forms have little effect on phage particles. The effect of four adenine nucleotides on this step in morphogenesis is shown in Fig. 4. Unexpectedly, three adenine nucleotides, AMP, ADP, and ADPR, markedly stimulated this reaction, so that not only was the rate of phage formation increased but the total amount of particles formed was twice (200%) that formed

in the absence of these three nucleotides. A fourth adenine nucleotide, ATP, caused no stimulation or inhibition.

The three stimulating compounds are all analogues of NAD or NADP. One of these compounds, ADPR, has been shown to bind to *dfr* and inhibit its enzymatic activity. The likely explanation for increased total phage formation is that the T4D 11⁻ (B) preparation contained many apparently inactive particles which could be activated by these nucleotides so that they could then later bind the 11 (and 12) protein.

These results indicate that the conformation of the *dfr* is critical for the assembly and functioning of the phage. Binding of compounds such as NADPH which can favor injection (11) in the presence of certain inhibitors must lead to a conformational change which must be avoided during assembly. The binding of compounds such as ADPR to the *dfr*, which inhibits injection, must favor a conformational state favorable for assembly. The nature of these conformational changes and the location of the site on the *dfr* which reacts with these nucleotides will be considered later (7).

Properties of phage particles produced by infection with a T4D containing a nonsense mutation in its dfr gene. T4D *dfr*11 (old terminology was *wh*11) was shown by Hall (5) to have a nonsense (amber) mutation in its *dfr* gene that was not lethal. It was originally suggested that T4D could still be formed in nonpermissive host cells if the host *dfr* were used as a structural element (10). Dawes and Goldberg (1) later predicted that an alternative possibility was that a structurally competent nonfunctional fragment of the *dfr* enzyme could be used to form the phage particles. Table 1 shows properties of T4D *dfr*11 grown at 28 or 42 C on either the permissive (CR63) or nonpermissive

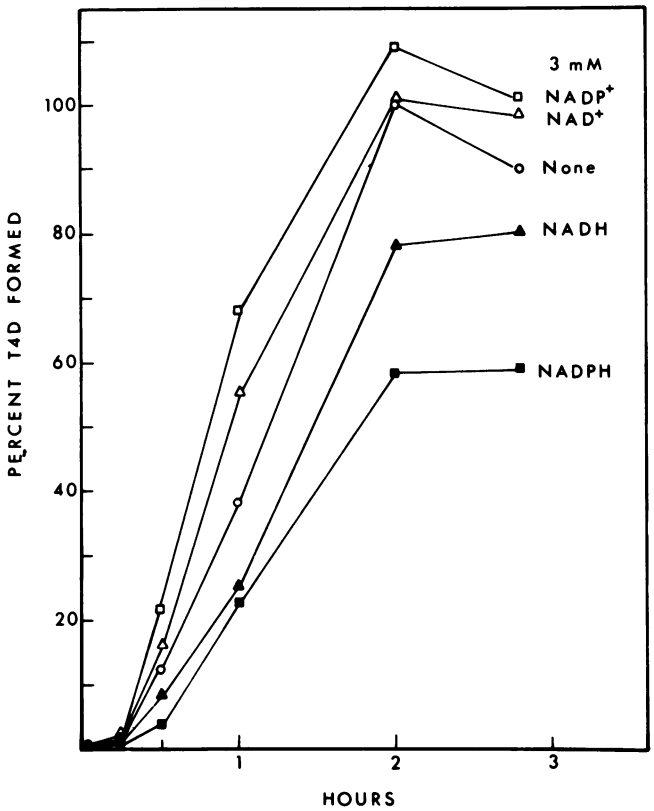

FIG. 3. *Effect of various NADs on the addition of P11 and P12 to T4D 11⁻ particles. The initial titer of the T4D 11⁻ stock was 3.4 × 10⁷/ml in the reaction mixture, and in the absence of nucleotide the final titer was 2.5 × 10¹⁰/ml (100% T4D formed). The nucleotides were dissolved in 0.1 M phosphate buffer, pH 7.0, at an initial concentration of 0.012 M and then added to the reaction mixture so that their final concentration was 3 mM. The T4D 11⁻ preparations were suspended in 0.9% NaCl containing 1 mM $MgSO_4$. Usually 20 μl of T4D 11⁻ was added to 25 μl of nucleotide. Additional buffer was added, and the reaction was started by the final addition of 20 μl of 23I27 extract.*

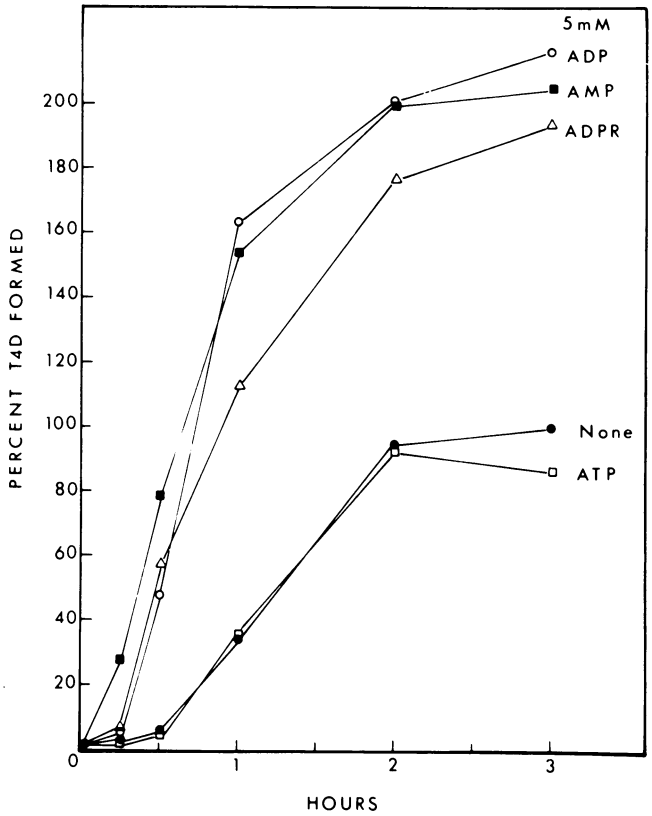

FIG. 4. *Effect of various adenine nucleotides on the addition of P11 and P12 to T4D 11⁻ particles. The initial titer was 2.4 × 10⁷, and in the control, in the absence of nucleotides, the titer increased to 1.4 × 10¹⁰ (100% T4D formed). All the nucleotides (as sodium salts) were dissolved in BUM at pH 7.4 and added to the reaction containing the T4D 11⁻ particles and the 23I27 extract as the source of P11 and P12, so that their final concentration was 5 mM.*

TABLE 1. *Properties of T4D dfr11*

T4D phage stock	Heat inactivation (rate constant)[a]	Inactivation by anti-DFR serum (*K*)
*dfr*11(B)		
28 C	0.14	1.2
42 C	0.075	2.0
*dfr*11(CR63)		
28 C	0.12	1.2
42 C	0.11	1.3

[a] Heat inactivation was carried out as described in Materials and Methods; the rate constant is the first-order reaction rate constant. This inactivation by the anti-*dfr* serum was carried out in broth using the serum at a final dilution of 1:11 and the phage at a starting titer of 10^7/ml.

(B) strain of *E. coli*. When grown in CR63-permissive host cells, the phage particles have identical properties with regard to heat inactivation (all four measured simultaneously) or inactivation by antiserum to parental *dfr*, whether they were assembled at 28 or 42 C. When grown on *E. coli* B the *dfr*11 phage particles differ in these two properties as compared to particles grown on CR63. It can be seen that the temperature of assembly affected these two properties, heat lability and sensitivity to antiserum to *dfr*. It can be concluded that when grown in the nonpermissive host the *dfr*11 particles do contain antigenic sites very similar to those of the parent T4D *dfr* and that this *dfr*11 phage component, unlike parental T4D *dfr*, can assume alternative configurations and still be a structural component. It should be emphasized that the host *dfr* is antigenically quite unlike the phage-induced *dfr* (13). The heat sensitivities and antigenic properties of *dfr*11 support the suggestion (1) that the partial peptide of the parental *dfr* is used for assembly rather than the host *dfr*.

To examine directly the possibility that host *dfr* might be used for viral assembly, mutants of *E. coli* with altered *dfr*'s were isolated. As

described above, five mutants of *E. coli* B resistant to the *dfr* inhibitor, trimethoprim, were isolated. The *dfr*'s from these mutants were found to vary greatly in their heat lability (Fig. 5). Mutant B-Trim^R-101 was not very heat sensitive, whereas B-Trim^R-106 was very heat sensitive. These five bacterial strains were then infected (at 37 C) with either parental T4D or T4D *dfr*11, and the heat sensitivities of the 10 different phage preparations were measured simultaneously (Table 2). As expected, the heat sensitivities of all parental T4D stocks were the same and were unaffected by the nature of the host cell *dfr*. Presumably the T4D-induced *dfr* was used for assembly in all these mutant cells. (This experiment also gives a clear indication of the reproducibility of measurement of heat sensitivities.)

It can also be seen that the *dfr*11 phage particles produced by these different host mutant strains all had the same heat sensitivity, although the sensitivity was about twofold higher than the T4D wild-type heat sensitivity. Since these phage stocks grown on Trim^R mutants did not reflect the changes in the host *dfr*, one can conclude that these *dfr*11 phage particles are probably all made from identical structural components and that these are viral induced. The use of a partial polypeptide produced by the T4D *dfr* gene would be in agreement with these results.

DISCUSSION

The observation that phage which produce heat-sensitive *dfr*'s the *far* mutants, produce heat-sensitive virions confirms that *dfr* is a phage component (10). Since the baseplate is the most heat-sensitive phage component, the

TABLE 2. *Heat sensitivity of T4D and T4D dfr11 particles grown on E. coli B and various mutants resistant to trimethoprim*

Host	Inactivation rate constants[a]	
	T4D	T4D *dfr*11
B-Trim^S	0.043	0.106
B-Trim^R-101	0.037	0.086
B-Trim^R-102	0.045	0.072
B-Trim^R-103	0.047	0.098
B-Trim^R-106	0.050	0.088
B-Trim^R-109	0.050	0.088
Average	0.046 ± 0.004	0.090 ± 0.01

[a] Heat inactivation was performed as described in Materials and Methods. The inactivation rate constant = 2.3/min × log (titer$_0$/titer$_t$).

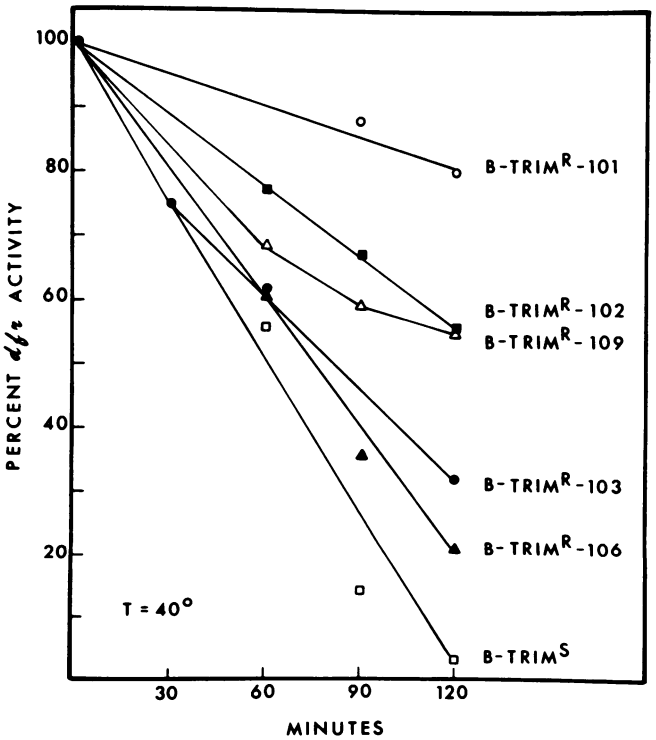

FIG. 5. *Heat sensitivity of dfr in extracts from E. coli B and various trimethoprim-resistant mutants of E. coli B.*

heat sensitivity of the *far* mutants agrees with the earlier direct demonstration of *dfr* in the baseplate. These experiments also show that the temperature of assembly does affect the conformation of *dfr* produced by the dfr^{ts} mutants. The fact that the gene 11 and gene 12 proteins cover the *dfr* also correlates with the earlier observations that mild urea or guanidine treatment was necessary to release enzyme activity from phage ghosts or baseplates.

The remarkable effects of the small molecules which bind to *dfr*, such as ADPR, which stimulates assembly and inhibits infection, suggest that this binding site on *dfr* is critical because it changes the conformation of the *dfr* between two states, favoring either completion of the structure or release of the DNA.

The evidence suggesting that the T4D *dfr*11 produces a partial peptide which can be used for a structural component is not unexpected. Earlier evidence indicated that the "*dfr*" in the phage particles produced after *dfr*11 infection of *E. coli* B contained binding sites for NADPH as well as folate and therefore was a large portion of the *dfr* molecule. Recently, preliminary mapping of the *dfr*11 mutation by one of us (D.H.H.) suggests that the amber peptide produced may be 90% of the length of the normal protein.

The direct demonstration that a partial polypeptide of *dfr* is used for the formation of the baseplate appears to be very difficult in view of the fact that the demonstration of protein components in phage structures has not given any support to the conclusion that *dfr* (or thymidylate synthetase) is a phage component. However, most of these studies used acrylamide gel electrophoresis to separate the viral proteins after labeling with ^{14}C-labeled amino acids 5 min after infection (3, 15). The late labeling avoids any complications due to ^{14}C in host proteins, but *dfr* is formed as an early protein and no radioactive *dfr* band is seen in these late-labeling experiments. The Coomassie blue staining of nonlabeling phage protein in gels also presents problems. In either case the presence of only a very few molecules of *dfr*, or a large partial peptide of *dfr*, presents a difficult analytical problem. Reconstitution experiments would appear to be the best approach to confirming the use of the phage-induced *dfr* for forming the baseplate. Such experiments, while also difficult, are underway.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-0336 from the National Institute of Allergy and Infectious Diseases to L.M.K., and by Public Health Service grants GM-33299 and 6M-16303 from the National Institute of General Medical Sciences to D.H.H.

LITERATURE CITED

1. Dawes, J., and E. B. Goldberg. 1973. Functions of baseplate components in bacteriophage T4 infection. I. Dihydrofolate reductase and dihydropteroyl glutamate. Virology 55:380–390.
2. Dawes, J., and E. B. Goldberg. 1973. Functions of baseplate components in bacteriophage T4 infection. II. Products of genes 5, 6, 7, 8 and 10. Virology 55:391–396.
3. Dickson, R. C., and S. L. Barnes. 1970. Structural proteins of bacteriophage T4. J. Mol. Biol. 53:461–473.
4. Erickson, J. S., and C. K. Mathews. 1971. T4 bacteriophage-specific dihydrofolate reductase: purification to homogeneity by affinity chromatography. Biochem. Biophys. Res. Commun. 43:1164–1176.
5. Hall, D. H. 1967. Mutants of bacteriphage T4 unable to induce dihydrofolate reductase activity. Proc. Natl. Acad. Sci. U.S.A. 58:584–591.
6. Johnson, J. R., and D. H. Hall. 1973. Isolation and characterization of mutants of bacteriophage T4 resistant to folate analogues. Virology 53:413–426.
7. Kozloff, L. M., L. K. Crosby, and M. Lute. 1975. Bacteriophage T4 baseplate components. III. Location and properties of the bacteriophage structural thymidylate synthetase. J. Virol. 16:1409–1419.
8. Kozloff, L. M., M. Lute, and L. K. Crosby. 1975. Bacteriophage T4 baseplate components. I. Binding and location of the folic acid. J. Virol. 16:1391–1400.
9. Kozloff, L. M., M. Lute, L. K. Crosby, N. Rao, V. A. Chapman, and S. S. DeLong. 1970. Bacteriophage tail components. I. Pteroyl polyglutamates in T-even bacteriophages. J. Virol. 5:726–739.
10. Kozloff, L. M., C. Verses, M. Lute, and L. K. Crosby. 1970. Bacteriophage tail components. II. Dihydrofolate reductase in T4D bacteriophage. J. Virol. 5:740–753.
11. Male, C. J., and L. M. Kozloff. 1973. Function of T4D structural dihydrofolate reductase in bacteriophage infection. J. Virol. 11:840–847.
12. Mathews, C. K. 1971. Identity of genes coding for soluble and structural dihydrofolate reductases in bacteriophage T4. J. Virol. 7:531–533.
13. Mathews, C. K., L. K. Crosby, and L. M. Kozloff. 1973. Inactivation of T4D bacteriophage by antiserum against bacteriophage dihydrofolate reductase. J. Virol. 12:74–78.
14. To, C. M., E. Kellenberger, and A. Eisenstark. 1969. Disassembly of T-even bacteriophage into structural parts and subunits. J. Mol. Biol. 46:493–511.
15. Vanderslice, R. W., and C. D. Yegian. 1974. The identification of late bacteriophage T4 proteins on sodium dodecyl sulfate polyacrylamide gels. Virology 60:265–275.
16. Yamamoto, M., and H. Uchida. 1973. Organization and function of bacteriophage T4 tail. I. Isolation of heat-sensitive T4 tail mutants. Virology 52:234–245.