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Inactivation of T4D Bacteriophage by Antiserum Against Bacteriophage Dihydrofolate Reductase

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Antiserum was prepared against highly purified T4D bacteriophage-induced dihydrofolate reductase (DFR). This serum not only inactivated the enzyme but also inactivated all strains of T4D examined. T6 was inactivated to a lesser extent, and T2L, T2H, and T5 were unaffected by the antiserum. The phage-killing power of the serum could be blocked by prior incubation with partially purified T4D dfr obtained from host cells unable to make phage structural proteins. These observations confirm earlier results that the phage dfr is a structural component of the phage particle, and they offer new evidence on the manner in which this enzyme in incorporated into the tail structure.

In 1970 Kozloff and co-workers (7) presented evidence that the phage-induced dihydrofolate reductase (DFR) was a structural component of the tail plate of T-even bacteriophages. This conclusion was supported by Mathews (10), who showed that changes in the phage dfr gene resulted in changes in physical properties of the phages which were formed. Progress has been made in determining the biological role of the phage tail-plate DFR in phage infection (9), but little is known of the structural details of incorporation of this enzyme into the tail structure. It was found earlier (7) that antisera prepared against highly purified T4 tail plates inhibited the activity of T4D phage DFR. However, these experiments only supported the conclusion that enzyme was a tail plate component and did not show how the enzyme was integrated into the tail plate. With the recent purification of the phage enzyme to homogeneity by use of affinity chromatography (4), it became possible to prepare highly specific antiserum to DFR and to use this antiserum to investigate the structural role of this enzyme in the phage tail.

MATERIALS AND METHODS

All of the phage strains and biological methods have been previously described, including the T4 strain carrying the T6 gene for dfr designated T4dfr^{T6} (1, 7, 10). The original designation of phage mutants carrying an altered dfr gene as "wh" mutants (for the "white halo" given on special plating media [5]) has been changed to "dfr" to clearly indicate the nature of the changed gene product. For example, the T4Dwh1 strain of Hall (5) is now referred to as T4Ddfr1. Phage inactivation by antisera was performed as described by Adams (1).

DFR was assayed as described by Mathews and Sutherland (11) except that the assay volume was 1.0 ml, and the concentrations of reaction components were: phosphate buffer, 50 mM; 2-mercaptoethanol, 5 mM; dihydrofolate, 0.05 mM; and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), 0.1 mM.

Homogeneous T4 dfr was prepared as described by Erickson and Mathews (4). A solution of 90 μg of enzyme per ml in 0.004 M potassium phosphate buffer, pH 7.0, was mixed with an equal volume of Freund complete adjuvant. Two New Zealand white rabbits were injected in the hind foot pads with 2 ml each of the antigen-adjuvant homogenate. One month later a second injection of 1 ml per rabbit of the same antigen-adjuvant complex was given subcutaneously. The rabbits were then bled periodically to obtain antisera.

The DFR induced after infection of *Escherichia coli* B with T4D defective in gene 32 (amA453 [3], obtained from R. Edgar) was used for serum-blocking experiments. A sample (1.5 liters) of *E. coli* B was grown to 5×10^8/ml in glucose-salts medium, infected with four phage per bacterium and incubated for 15 min at 37 C. The enzyme was extracted and partially purified as described earlier (4). After ammonium sulfate precipitation and dialysis against 0.05 M phosphate buffer at pH 7.5, the entire solution (17 ml) was layered on top of a $CaHPO_4 \cdot 2H_2O$ column (2.1 by 28 cm; Brushite, Bio-Rad Laboratories) which had been pre-equilibrated with 0.05 M potassium phosphate buffer, pH 6.5. The material on the column was eluted with a linear gradient consisting of 200 ml of 0.05 M potassium phosphate, pH 6.5, in the mixing chamber and 200 ml of the same buffer at 0.5 M in the

reservoir. Approximately 3.8-ml fractions were collected and assayed for protein and enzyme activity. The double peak of enzyme activity (see Fig. 4) is characteristic of this enzyme and may reflect enzyme molecules containing bound cofactors (12).

For examining the serum-blocking power of the various fractions, 4 μliters of each fraction obtained above was added to 50 μliters of a 1:41 dilution of the antiserum in tryptone broth (pH 6.8), and the mixture was incubated for 4 h at room temperature. T4D (10 μliters at 10^7/ml) was then added, and the suspension was incubated for 60 min at 37 C and then assayed for viable phage.

Protein was determined by either the biuret reaction of Layne (8) or by its fluorescence when excited at 280 nm.

RESULTS

Inactivation of DFR and various phages by antiserum to purified T4D DFR. The effect of the antiserum on the enzyme activities of T4D-induced DFR and host *E. coli* DFR is shown in Fig. 1. The antiserum specifically inactivated the T4D-induced enzyme. In contrast to this inactivation, host *E. coli* enzyme was unaffected by incubation with the same antiserum. Serum from unimmunized rabbits had no effect on the enzyme activity of either the T4D-induced enzyme or the host enzyme. The inhibition of enzyme activity indicated that the serum contained antibody molecules able to react with the enzyme so as to inhibit or block the active site.

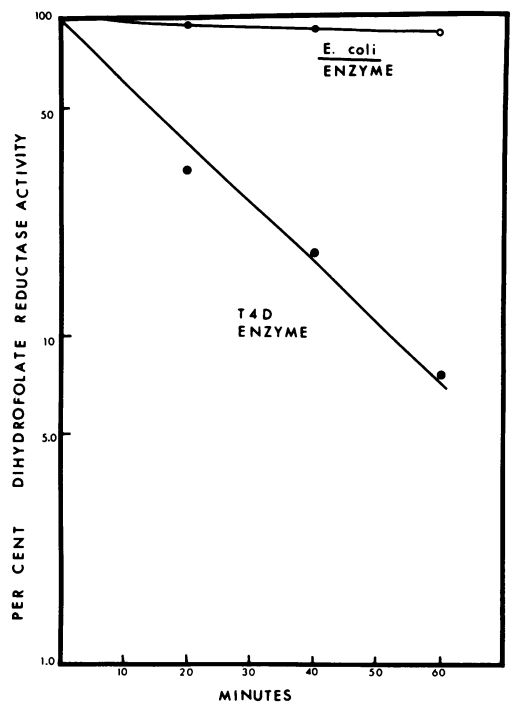

FIG. 1. *Effect of antiserum to T4D dihydrofolate reductase (DFR) on DFR activities of T4-induced enzyme and Escherichia coli enzyme. Enzyme preparations used were undialyzed ammonium sulfate fractions (11). Enzyme was incubated with an equal volume of antiserum at 30 C for the indicated time intervals before assay.*

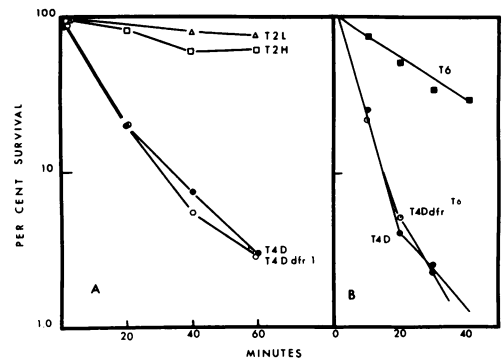

FIG. 2. *Inactivation of various phages by antiserum to T4D dihydrofolate reductase. In Experiment A, temperature was 37 C, suspending media was tryptone broth, and the serum was diluted 1:60. In experiment B, pH was 7.0, suspending media was 0.1 M phosphate buffer, temperature was 37 C, initial phage concentration was 10^8/ml, and serum was diluted 1:50.*

The effect of this antiserum on various phages is shown in Fig. 2 and in Table 1. All strains of T4D examined were inactivated by this serum to the same extent, T6 was inactivated to a lesser extent, and T2L and T2H were mostly unaffected. T6 DFR presumably cross-reacts with the T4 antibodies. Although the T2 strains have not been extensively studied, it seems likely that they do contain DFR (unpublished data) which does not cross-react with this T4 antiserum. T5 is not believed to contain DFR as a structural component and was not inactivated by the antiserum. Although the rate constants for T4 inactivation by this antisera are considerably below those normally found for serum prepared against whole T4 phage (where the inactivation rate constants may be several hundred), these results support the view that a T4-induced protein with the antigenic characteristics of DFR is an integral part of the T4 structure.

Serum-blocking power of T4D DFR. Because it was conceivable that a trace of some other T4D structural protein was present in the highly purified enzyme preparation used to inoculate the rabbits, the ability of a separate preparation of partially purified enzyme to block the serum inactivation was examined.

TABLE 1. *Inactivation of various phages by antiserum to T4D dihydrofolate reductase (dfr)*

Phage	Inactivation rate constants			
	Expt A (pH 5.0)	Expt B (pH 6.8)	Expt C (pH 7.7)	Expt D (pH 8.0)
T4D	7.2	4.3	3.4	2.0
T4Ddfr11 (CR63)	7.1	4.0	3.0	1.4
T4Ddfr11 (B)	7.4	4.2	2.5	2.0
T4Ddfr1	7.2	4.2		
T4Ddfr7	6.4	4.5		
T4Ddfr15	8.4	4.4		
T2L		0.2		
T2H		0.3		
T5		0		

T4D-induced DFR produced in cells infected with T4D defective in gene 32 was purified as described in Materials and Methods. This phage mutant was chosen because it is DNA-negative and does not produce the late proteins which are phage structural components (3). The phage-induced enzyme was extracted from these infected cells, separated from host enzyme by ammonium sulfate precipitation, and then chromatographed on a $CaHPO_4$ column. Fractions were examined for their protein content, enzyme activity, and ability to protect T4D from inactivation by the antiserum to T4D DFR. The serum-blocking activity of the material richest in enzyme activity was found to be roughly proportional to the volume added to the test system (Fig. 3 and 4). When 4 μliters of each of the various fractions were examined for their serum-blocking power, the profile of the serum-blocking activity was identical to that for the enzyme activity. In view of the failure of the T4D gene 32 mutant to produce any known capsid proteins (other than DFR) and the similarity of the chromatographic behavior of the enzyme and serum-blocking activities, it can be concluded that T4D particles possess antigenic determinants identical to those of phage DFR.

Properties of the reaction between the phage particles and antiserum to DFR. The pH of the suspending media had a considerable effect on the inactivation rate of T4D by the antiserum (Table 1). At pH 5 the inactivation rate was almost twice that at pH 6.8. Normally phage inactivation by antisera is independent of pH from 5 to 7.2 (6). In view of the increased ability of phage to react with NADPH at pH 5.0 (7) as compared with the reaction rate at pH 7.0, it seems likely that there is a conformational change in the tail plate which increases the exposure of the phage enzyme antigens to the antibodies.

The effect of heating the antiserum on its subsequent ability to inactivate phage was also examined. Antiserum, heated to 70 C for 15 min, was just as active as unheated antiserum. It can be concluded that complement is not involved in phage inactivation.

The ability of two compounds, (which bind to the NADPH binding site of the phage structural DFR [9]), to alter the rate of antiserum inactivation was examined. Neither 0.02 M adenosine diphosphoribose nor 0.02 M nicotinamide altered the rate of phage inactivation by antiserum. This binding site is not identical then to the antigenic site reacting with the antibody.

Configurational stages of the phage tail enzyme. Two observations indicate that the incorporation of DFR into the phage tail structure alters the tertiary structure of the enzyme, especially with regard to the antigenic sites exposed. Phage T6 induces DFR with heat and urea sensitivities quite different from those induced by T4D infection (10). However, T6 particles are susceptible to inactivation by antiserum to T4 enzyme (Fig. 2) even though the rate constant for T6 inactivation is one-fourth that for T4 inactivation. However, when the gene for the T6 dfr is genetically crossed into T4D to produce T4Ddfr^{T6}, the phage particles produced are identical to T4D with regard to inactivation by the antiserum. It would appear that incorporation of the T6 enzyme in the T4D tail plate alters the conformation of the T6 enzyme so that a site antigenically identical to that in T4D is exposed.

The structure of T4D particles containing the dfr11 mutation (Table 1) also is relevant to the problem of conformational changes. The dfr11 mutant has been characterized as an amber

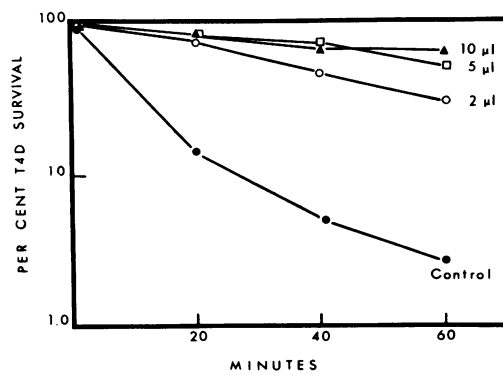

FIG. 3. *Protection of T4D from inactivation by antiserum by use of various amounts of partially purified T4D-induced dihydrofolate reductase (DFR). The DFR present in fraction 19 (Fig. 4) was used to block phage inactivation.*

mutant by Hall (5). When grown in the permissive host (CR63), the mutation is suppressed and the phage particles contain active enzyme and are inactivated by the antiserum at the same rate as the wild-type T4D. However, this mutation is not lethal and viable phage particles are formed even in the nonpermissive host. Furthermore, phage assembled in the nonpermissive host cell are also inactivated by the antiserum at the same rate as wild-type T4D. It is possible that the incomplete polypeptide formed in the infected nonpermissive host has the same antigenic determinants as the complete T4D-induced enzyme and is used for phage assembly. Alternatively, as suggested earlier, in the nonpermissive host the host *E. coli* enzyme may substitute for the phage enzyme in forming the phage particle (7). Since host DFR reacts very poorly, if at all, with the antiserum, this would mean that incorporation of the host DFR into the phage tail causes a major conformational change in the host enzyme, exposing a new antigenic site to react with the antibody. While unlikely, this possibility cannot yet be ruled out.

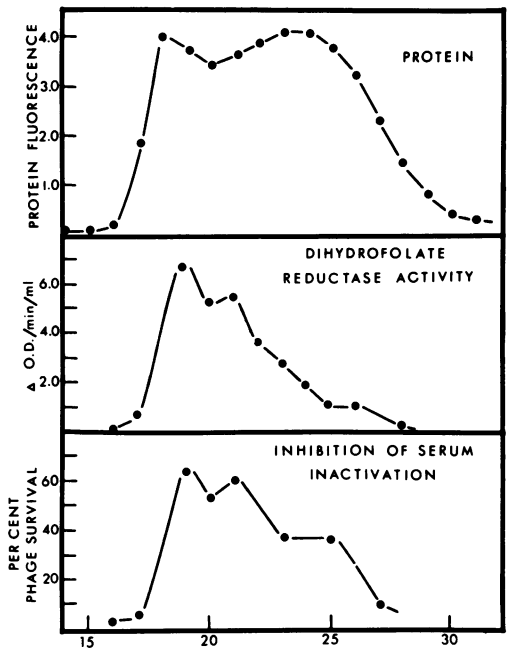

FIG. 4. *Protection of T4D from inactivation by antiserum to T4D dihydrofolate reductase. The procedure for this experiment is given in Materials and Methods; for the measurement of serum-blocking power 4 μliters of each fraction were added to the test system.*

DISCUSSION

Several lines of evidence lead to the conclusion that the inactivation of T4D bacteriophage by the antisera prepared by inoculating rabbits with DFR is due to antibodies against the antigenic determinants of this enzyme. These lines of evidence are as follows. (i) The enzyme used for antibody production was prepared by affinity chromatography which yielded a homogenous product, (ii) The enzyme used for preparing the antiserum was obtained from a culture infected with T4D amN82 (gene 44$^-$), which produces neither DNA nor late phage structural proteins (3). (iii) Enzyme fractions obtained during the purification of DFR from *E. coli* infected with a different T4D mutant (gene 32$^-$, also defective in producing late phage structural components) had serum-blocking power correlating with their enzyme activity. (iv) All strains of T4D were inactivated to the same extent, whereas other related phages were inactivated to a lesser extent, and unrelated phages were not inactivated at all. It can be concluded that T4D phage must possess this enzyme (or a significant portion of it) as a structural component.

It is known that binding of antibody to either head or tail components such as the tail sheath does not lead to phage inactivation, whereas antibody binding to tail fibers (2, 3) or to the tail plate does lead to inactivation (7). Therefore, although the site of DFR antibody-binding on the T4D particle has not yet been determined, the observed inactivation is in agreement with the earlier finding that the enzyme is a tail plate component (7). Binding of antibody to this phage structure would interfere with its function during phage infection and would cause phage inactivation.

The unexpectedly rapid inactivation of T4D dfr^{Te} and T4Ddrf11 by the antisera might be construed as an indication that the antisera were reacting with a tail component other than DFR. This would imply that the purified DFR used was contaminated with another phage antigen. This is highly unlikely in view of the evidence above concerning the source of the enzyme used. It should be emphasized that DFR antigen was purified 6,000-fold, was homogeneous in two different electrophoretic systems (4), and was immunologically pure as judged by its behavior on Ouchterlony double-diffusion plates. It seems more likely that conformational changes during tail assembly induce the exposure of antigenic sites in these closely related strains to equal those found in normal T4D.

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