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Process of Infection with Bacteriophage φX174

XXXIV. Kinetics of the Attachment and Eclipse Steps of the Infection

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The products of φX cistrons II, III, and VII are demonstrated to affect the attachment of the phage to its host *Escherichia coli* C; therefore, by inference, these cistrons influence, directly or indirectly, the structure of proteins in the virus particle. Two of the mutations which alter attachment kinetics, *ts*79 in cistron III and *h* in cistron VII, also affect the electrophoretic mobility of the virus and emphasize the role of charge in the attachment interaction with the host. The kinetics for attached phage to go into "eclipse" are first-order and biphasic; about 85% of the phage eclipse at one rate (k_e = 0.86 min^{-1}) and the remainder do so at a distinctly lower rate (k_e = 0.21 min^{-1}). No φX cistrons yet identified affect the eclipse process. The lowest temperature at which eclipse is detected is 19 C. The Arrhenius activation energy for phage eclipse has the high value of 36.6 kcal/mole, indicating the cooperative nature of the event.

In a previous publication in this series, three distinct stages were described in the process whereby φX174 invades the host cell (9). These early stages were denoted attachment, eclipse (loss of recoverable phage infectivity), and deoxyribonucleic acid (DNA) penetration. This report presents further data on the attachment and eclipse steps and the influence of viral mutation on these processes.

MATERIALS AND METHODS

Escherichia coli strains. The strains used here were described previously (8, 9).

φX174 strains. The mutant strains are described in Table 1; with the exception of *ts*41D, all were isolated by Clyde A. Hutchison III. Strain *ts*41D was isolated by C. Dowell.

Media. KC broth contains 10 g of tryptone (Difco), 5 g of KCl, and 1.0 ml of 1 M $CaCl_2$ per liter of distilled water.

SVB (starvation buffer) contains 5 g of KCl, 1 g of NaCl, 1.14 g of Trizma HCl (Sigma Chemical Co.), 0.33 g of Trizma Base (Sigma Chemical Co.), 0.1 g of $MgSO_4$, and 1.0 ml of 1 M $CaCl_2$ per liter of distilled water. This is essentially the recipe described by Denhardt and Sinsheimer (2).

Biological assays. The plating procedures and the preparation of genetically pure mutant phage stocks have been described (7).

Radioactive phage. The preparation of *am*3 labeled either with ^{32}P or ^{3}H-*l*-histidine has been described (9).

^{32}P-labeled 110 S *am*3*ts*4 defective particles were prepared by a slight modification of the same procedure. The *E. coli* C cells were infected at 40 C, and the culture was incubated at that temperature for 3 hr. The culture was then poured into 3 volumes of SVB at 0 C, and the cells were harvested by centrifugation at 2 C. The cell pellet was resuspended in cold 0.05 M borate and lysed at 0 C by the addition of lysozyme and ethylenediaminetetraacetate (EDTA) and subsequent freezing and thawing. The ^{32}P-labeled 110 S species was purified by two cycles of sucrose gradient centrifugation. The purified 110 S species contained 0.1 ^{32}P atom per particle; the specific infectivity of the preparation, calculated from the radioactivity and the titer of plaque-forming units, was 2 × 10^{-4} plaque formers per particle; i.e., the level of contamination by infective 114 S phage particles was, at most, 0.02%.

Attachment kinetics. *E. coli* C cells were grown in KC broth to 5 × 10^7 cells/ml. The cells were collected by centrifugation, washed once with 1 volume of SVB, and then resuspended in 2 volumes of SVB, i.e., cells were at 2.5 × 10^7 cells/ml. The phage were diluted to 10^8/ml in SVB. Portions (0.5 ml) of each phage preparation were set at 37 C, and 9.0 ml of the cells, prewarmed to 37 C, was added to each to initiate the attachment reaction. Attachment was monitored after 0, 0.5, 1, 2, 4, 8, 15, and 30 min by removing a 0.1-ml sample from the reaction mixture and diluting it 100-fold into KC broth at 0 C and again 10-fold into a centrifuge tube containing 10^9 *E. coli* K12W6 cells (as nonadsorbing carrier) also in KC broth at 0 C (the 10^3-fold dilution is sufficient to stop further attachment). When all of the samples were prepared, they were centrifuged at 2 C to pellet the cells, and the supernatant fluids were titered for residual phage.

Phage were not inactivated in a 30-min period under

TABLE 1. *Mutant phage strains*

Mutants	φX cistron	Cistron function
*am*3	I	Lysis
*ts*4	II	Spike protein
tsγ, *ts*79, *ts*41	III	Spike protein
*ts*9	IV	Spike protein[a]
h, *ts*41D	VII	Capsid protein[a]

[a] The assignment of these gene functions is at present only tentative.

the conditions of the attachment reaction in the absence of cells.

Attachment of capsids and spikes. The preparation of these subviral fractions was described previously (4). The cells were grown in KC broth to 2×10^8 cells/ml. A 5-ml amount of these cells was set at 37 C in a centrifuge tube, and the radioactive capsids or spikes were added in 0.1 ml or less. The mixture was incubated at 37 C for 10 min. A 2-ml portion was removed for assay of total radioactivity, and the remaining 3 ml was centrifuged to pellet the cells. The supernatant fluid was removed and the pellet was resuspended in 3.0 ml of fresh KC broth. The supernatant fraction was added to a previously prepared pellet containing the original number of uninfected *E. coli* C cells, namely, 6×10^8 cells; this pellet was resuspended.

Both pellet and supernatant fractions were sampled (2 ml) for assay of radioactivity. The three 2-ml samples (total, supernatant fluid and pellet) were mixed with an equal volume of cold 12% trichloroacetic acid and allowed to stand at 0 C for 1 hr. The precipitate was collected by filtration through a Whatman glass filter disc, rinsed with 10 ml of cold 2% trichloroacetic acid, dried, and counted. The filter discs were placed into 10 ml of a toluene-liquifluor scintillator (New England Nuclear Corp.) and counted in a Beckman liquid scintillation counter. (The addition of the cells to the supernatant fraction is necessary to obtain equal quenching of radioactivity in the three samples.)

Eclipse kinetics. *E. coli* C cells were grown in KC broth to 2×10^8 cells/ml. The cells were collected by centrifugation and washed once with an equal volume of SVB. The cells were then resuspended in 0.1 volume of SVB (cells are about 2×10^9 cells/ml) and set at 15 C. Phage were added to a multiplicity of about one, and a 30-min period was allowed for phage attachment at 15 C. The cell-attached phage complexes were collected by centrifugation and resuspended in fresh SVB at 0 C. A 0.1-ml amount of this culture was diluted into 10 ml of SVB, which was equilibrated to the desired temperature; this initiates the eclipse process. Eclipse was monitored by cooling 0.1-ml samples of the reaction mixture to 0 C by dilution into 5.0 ml of 0.05 M sodium tetraborate, 6 mM EDTA, saturated with chloroform at 0 C.

Further eclipse is prevented by the low temperature (9). The uneclipsed phage are eluted from the cells by the borate-EDTA (9); the chloroform and the borate-EDTA kill the infected cells. The titer of phage in the borate-EDTA solution thus represents the residual, uneclipsed phage.

Under the conditions of the eclipse experiments, no inactivation was found for free phage, i.e., no inactivation occurred in a 30-min period at 37 C in SVB.

RESULTS

Kinetics of phage attachment to cell. The time course of phage attachment to *E. coli* C cells exhibited first-order kinetics with respect to phage concentration, and the rate constant (k_e) for φX *wt* was 8.2×10^{-9} ml per min per bacterium. Under these same conditions, the lysis defective mutant *am*3 demonstrated identical attachment kinetics as the *wt*.

Mixtures of *am*3 with each of four different φX *ts* mutants were constructed, and the relative attachment kinetics of the two phage types in each mixture were determined by selective plating for the two phenotypes. [The *am* mutants were assyed on *E. coli* HF4714 cells at 40 C; *ts* mutants were plated on *E. coli* C at 30 C (Table 2)]. The mutants *tsγ* and *ts*9 had rate constants not significantly different from those of *wt* and *am*3. However, *ts*79 attached more slowly to *E. coli* C than did *am*3, whereas *tsγh* attached more rapidly. Since *tsγ* attached at the wild-type rate, the more rapid attachment of *tsγ*h can be ascribed to the *h* (extended host range) mutation.

The φX mutants *ts*79 and *h* have been previously assigned to φX cistrons III and VII, respectively (Hutchison, Ph.D. Thesis, California Institute of Technology, Pasadena, 1969); both of these cistrons code for proteins present in the mature phage (6).

Defective attachment of 110 S ts4 particles. The defective 110 S phage particle made in cells infected with *ts*4 at 40 C was reported earlier to be defective in adsorption (11). At that time,

TABLE 2. *Rate constant (k_e) for the stable attachment of φX strains to Escherichia coli C*

Phage strain	Rate constant for attachment (ml per min per bacterium)
*am*3	9.2×10^{-9}
tsγ	8.5×10^{-9}
*am*3	8.2×10^{-9}
*ts*9	8.2×10^{-9}
*am*3	9.0×10^{-9}
*ts*79	5.1×10^{-9}
*am*3	8.0×10^{-9}
tsγh	11.3×10^{-9}

the term "adsorption" was used rather loosely and it often encompassed all of the events in the invasion of the host.

A preparation of ^{32}P-labeled 110 S *am3ts4* defective phage was found to be essentially incapable of stable attachment to *E. coli* C. Ten minutes was allowed for attachment to the cells (2×10^8 cells/ml) in KC broth at 37 C. The mixture was then centrifuged to pellet the cells, and the supernatant fluid and pellet fractions were assayed for radioactivity. Only 3% of the input ^{32}P was found associated with the cells, whereas 102% remained free in the supernatant fluid. Under these same conditions, at least 80% of a preparation of ^{32}P-labeled (114 S) *am3* attached stably to the cells and pelleted with them.

The mutant *ts4* is assigned to φX cistron II (12), which specifies a structural protein of the phage (4). Two of the three cistrons which have been shown here to affect the attachment of phage to the host, namely, φX cistrons II and III, are known to code for proteins which are present in the 12 "spikes" of the mature phage (4). The location of the protein specified by cistron VII is uncertain. If the spikes are viral organelles for the specific attachment of the phage to the receptor sites on the host, it might be expected that capsids devoid of spikes would be incapable of specific attachment to cells and further that "free" spikes might retain that property.

To test this possibility, capsids devoid of spikes and free spikes were isolated from a preparation of *am3* and labeled with ^{3}H-*l*-histidine by the procedure previously described (4), and the ability of these labeled subviral components to attach to cells was determined. Capsids attached to *E. coli* C as efficiently as did intact phage; however, capsids also attached efficiently to *E. coli* C/φX, although they did not attach to *E. coli* K12W6 (intact phage does not attach to either of these strains). This attachment of capsids devoid of spikes to cells appears, however, to be unrelated to normal φX attachment. Prior reaction of the capsids with anti-φX serum did not block their subsequent attachment to cells; prior infection of the cells with saturating levels of nonradioactive virus (50,000 particles/cell) also did not impair spikeless capsid attachment. Either of these reactions inhibits attachment of whole phage to cells.

The free spikes also attached to cells at a low rate but displayed no host-range specificity; they attached to both W6 and C/φX as well as to *E. coli* C. It is concluded that the attachment of free spikes and capsids to cells is not closely related to attachment of intact phage.

Eclipse kinetics for attached phage. It was reported earlier that at 15 C φX can attach stably to cells but does not go into eclipse (9); i.e., the phage recovered from such complexes has the same specific infectivity and physical properties as the input virus. The eclipse event which occurs at higher temperatures is defined by a loss of recoverable phage infectivity and a conformational change of the phage (9). The kinetics of the eclipse event were determined by isolating cell-phage complexes at 15 C, raising the temperature, and monitoring the decrease of recoverable phage infectivity.

Figure 1 shows the eclipse kinetics for *am3* at 37.5 C following the protocol described above (*am3* shows the same eclipse kinetics as *wt*). The kinetics of the eclipse process were first-order with respect to phage concentration, about 85% of the virus particles eclipsed at one rate (k_e = 0.86 min^{-1}), and the remainder did so at a distinctly lesser rate (k_e = 0.21 min^{-1}).

Figure 2 documents the temperature dependence of the eclipse process (for its faster component). Eclipse was not detected at 16 C or below; it was detected at 19.0 C. The slope of the $ln\ k_e$ versus the inverse of the absolute temperature plot corresponds to an Arrhenius activation energy of 36.6 kcal/mole.

Eclipse kinetics for φX ts mutants. Mixtures of *am3* and each of the following φX *ts* mutants were constructed: *ts4* (cistron II), *ts41* (cistron III), *ts79* (cistron III), *tsγ* (cistron III), *ts9* (cistron IV), *ts41D* (cistron VII) and *tsγh* (cistrons III and VII, respectively). By selective plating for the *am* or *ts* phenotype, the initial eclipse kinetics of both the *ts* mutant and the

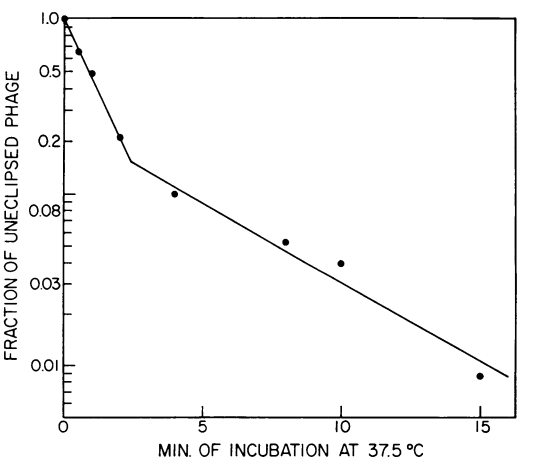

FIG. 1. *Kinetics for the eclipse at 37.5 C of φXam3 previously attached to Escherichia coli C cells.*

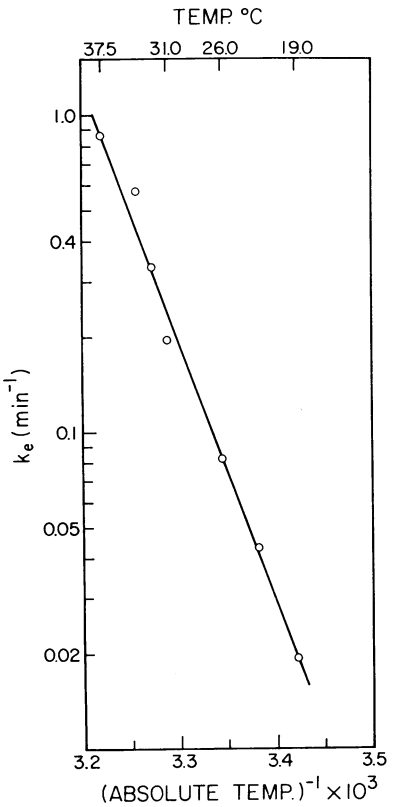

FIG. 2. *Temperature dependence of φXam3 eclipse. The Arrhenius plot of the logarithm of the initial rate constant for phage eclipse versus the inverse of the absolute temperature.*

reference *am*3 were monitored in each phage mixture at 37 C. No significant differences in the initial rate of eclipse could be detected among the mutants tested.

DISCUSSION

The rate constant for the stable attachment of φX *wt* to *E. coli* C cells in SVB at 37 C presented in this report, 8.2 × 10^{-9} ml per min per bacterium, is in good agreement with the value 6.0 × 10^{-9} ml per min per bacterium, determined by Fujimura and Kaesberg (5) to be the optimal rate in simple salt solution (0.1 M $CaCl_2$).

The mutations *ts*4, *ts*79, and *h* in φX cistrons II, III, and VII, respectively, are demonstrated to affect the attachment reaction. These three cistrons code for structural proteins of the mature virus (Hutchison, Ph.D. Thesis, California Institute of Technology, Pasadena, 1969). At present, four distinct polypeptides are recognized to be present in the mature virus (1, 4). Three polypeptides are found in the phage spikes, and only one is found in the capsid (4). φX cistrons II and III code for spike proteins, whereas cistron VII is thought to specify the capsid protein (1, 4). The remaining structural (spike) protein is tentatively assigned to φX cistron IV.

The defective 110 S particle produced in *ts*4-infected cells at 40 C has been shown to lack one spike protein completely (4). The cistron II protein might be directly involved in the attachment of the phage to the bacterial receptor site, in which case the inability of the *ts*4 defective particle to attach to cells is readily understood. Alternatively, the inability of the defective particle to attach to cells might be a more indirect effect caused by altered charge or shape of the particles.

The two other "attachment mutants," *ts*79 and *h*, are distinguished from *wt* by altered electrophoretic mobilities as well (6). The more positively charged the virus, the more rapidly it attaches to the host. (Under the usual conditions of infection, both the phage and the host are negatively charged.) In fact, as shown in Table 3, the product of the attachment rate constant and the R_F value [taken from the electrophoresis data of Hutchison, Edgell, and Sinsheimer (7)] is approximately constant for the different phage mutants. These data indicate that the total electrostatic charge on the virus determines, to a large extent, the rate at which stable phage-cell complexes are formed.

The biphasic kinetics for the eclipse of attached phage particles (Fig. 1) were described in an earlier report (10); as proposed in that report, the phenomenon might reflect heterogeneity among the receptor sites of the host. The activation energy for the eclipse process, 36.6 kcal/mole, is quite high. Most chemical and enzymatic reactions have activation energies in the range of 11 to 18 kcal/mole. The high value indicates that the eclipse reaction is cooperative in nature. The eclipse event has already been

TABLE 3. *Product of the attachment rate constant and the electrophoretic mobility for some of the φX strains*[a]

Phage strain	Rate constant for attachment, k_e (ml per min per bacterium)	Electrophoretic mobility (R_F)	Product ($k_e \times R_F$)
wt	8.2 × 10^{-9}	0.20	1.64
tsγ	8.5 × 10^{-9}	0.20	1.70
tsγh	11.3 × 10^{-9}	0.14	1.58
*ts*79	5.1 × 10^{-9}	0.31	1.58

[a] The electrophoresis data on whole phage are taken from reference 7.

documented to involve a conformational change in the phage protein coat (9).

The ϕX mutant *cs*70 was demonstrated by Dowell to have an abnormal eclipse pattern. This cold-sensitive mutant could attach to the host, but not eclipse, at temperatures of normal eclipse for *wt* (3). The mutant *cs*70 remains at present unassigned to a particular ϕX cistron. Analyses of a large sample of the available ϕX *ts* mutants of known cistron assignment for aberrant eclipse kinetics yielded no additional examples. As this sample included the electrophoretic mobility mutants *ts*79 and *ts*γh, charge may not play an important role in the eclipse event.

It is of interest that the low-temperature threshold for phage eclipse, about 17 C, is the same threshold as that determined for the maturation of infectious progeny phage and for single-stranded DNA synthesis (8). It is conceivable that the cooperative rearrangement of the same viral protein or group of proteins is involved in all three processes.

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