Photodynamic Inactivation of Antigenic Determinants of Single-Stranded DNA Bacteriophage ϕ X174

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Bacteriophage ϕ X174 when photodynamically inactivated (i.e., when rendered unable to produce plaques as a result of exposure to visible light in air in the presence of proflavine) progressively lost their capacity to bind efficiently with homologous antiserum. Such loss of serum-blocking power was evident with heat-inactivated but not with UV-irradiated phage. The ability of the phages to adsorb to host cells, however, remained practically unaltered even after photodynamic inactivation. It thus appears that photodynamic damages in the so-called "jacket" component of the ϕ X174 coat proteins are partly responsible for the loss of plaque-forming ability, whereas the "spikes" are either poor antigens or insensitive to photodynamic treatment.

The minute single-stranded DNA bacteriophage ϕ X174 (like many other organisms) undergoes inactivation, i.e., it loses its plaque-forming ability when exposed to visible light in the presence of molecular oxygen and photosensitizing agents, such as acridine dyes. This phenomenon is known as "photodynamic" inactivation.

It appears from the literature that viruses thus inactivated may have their nucleic acid or the protein component damaged in this process. Fraser and Mahler (7) interpreted the kinetics of photodynamic inactivation of ϕ X174 in terms of a reversible complex between the photosensitizing dye and the phage DNA. Earlier results from our laboratory also indicated that photodynamic inactivation of ϕ X174 caused intracellular formation of aberrant double-stranded replicative-form molecules in subsequently infected host cells (3) and that the photodynamic damages in these phages can be repaired in a manner somewhat analogous to the UV-reactivation phenomenon (1) by *rec*⁺ host cells pre-irradiated with UV light. On the other hand, Cramer and Uretz (4) and Kadish, Fisher, and Pardee (8) suggested the possibility of changes in the protein coat of bacteriophage T2 and T4 as a result of photodynamic inactivation. It is well known that antigenicity of many proteins and their ability to react with their corresponding antibodies are destroyed by photodynamic treatment (see reference 10).

In the present paper we describe a few experiments designed to study the effect of this kind

of inactivation on the properties associated with the coat proteins of bacteriophage ϕ X174, namely, the serum-blocking power (SBP) or the ability to bind with specific antiviral antibodies and the capacity to adsorb to host cells. Comparative data for the SBP of heat-inactivated and UV-irradiated ϕ X174 have also been included.

MATERIALS AND METHODS

Bacterial and phage strains. Bacterial strains of *Escherichia coli* C and HF 4714 and bacteriophage ϕ X174 wild type (*wt*) as well as its amber mutant strain *am*₃ were gifts of R. L. Sinsheimer, California Institute of Technology. *E. coli* C was the nonpermissive host and HF 4714 was the permissive host for ϕ Xam₃. Bacteriophage ϕ Xwt grew on both strains.

Preparation of phage stock (cold and ³²P-labeled). *E. coli* C was allowed to grow with aeration in Tris-glucose medium containing 0.1% Casamino Acids (5) at 35°C until the cell density reached 1 to 2 × 10⁸ per ml and then was infected with phages at a multiplicity of infection of 5. Vigorous aeration was continued until lysis in the case of ϕ Xwt, or for 3 to 4 h in the case of ϕ Xam₃. In the former case, cell debris was discarded after centrifugation at 5,000 rpm for 10 min. The supernatant was brought to 0°C, made stepwise 0.5 M in NaCl and 2% in polyethylene glycol (molecular weight 6,000), allowed to stand at 0°C for 90 to 120 min, and finally centrifuged at 11,000 rpm for 10 min. The sediment was again discarded, and the supernatant was now made 6% in polyethylene glycol, allowed to stand at 0°C for 90 to 120 min as before, and was then spun at 20,000 rpm for 30 min. The phage pellet was finally suspended in 0.01 M phosphate buffer, pH 7.4. When ϕ Xam₃ was used, the infected cells were collected by centrifugation at 5,000 rpm for 15 min, washed with 0.01 M borate buffer, pH 9.0, and lysed by freezing and thawing after EDTA lysozyme

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treatment (5). Subsequent procedures were same as above.

For ^{32}P -labeling of phages, *E. coli* C was grown and infected with unlabeled ϕXam_3 as before. Carrier-free radioactive orthophosphate (^{32}P) was added at 200 μCi per ml at the time of infection. Subsequent procedures were as described earlier. The final phage suspension contained ^{32}P -labeled ϕXam_3 , which showed specific activity of about 10^{-6} radioactivity count per min per PFU. When a portion of this phage preparation was run on a 5 to 20% linear neutral sucrose gradient in an SW39 rotor at 25,000 rpm for 45 min and the fractions were collected dropwise from the bottom, the two profiles corresponding to radioactivity counts and the plaque-forming units were practically identical.

Preparation of rabbit anti- ϕX174 antiserum. A 2-ml portion of sterile physiological saline containing 10^{10} ϕXwt per ml was injected into a healthy rabbit twice a week for 3 consecutive weeks. After a further interval of 4 weeks, blood was collected in silicone-coated glass tubes by splitting the marginal ear vein of the rabbit. The blood was allowed to clot at 37 C for 1 h and then at 15 C for 18 h. The tubes were then centrifuged at low speed, and the supernatant, consisting of clear yellowish serum, was decanted off and incubated at 56 C for 30 min to inactivate the complement. The antiserum inactivated both ϕXwt and ϕXam_3 at the same rate, the rate constants K , about 450 min^{-1} at 37 C (at the time of preparation), whereas bacteriophage T4 was completely immune to the antiserum (unpublished data). It could be stored frozen in the freezing chamber of a refrigerator without any significant loss of activity for a period of 2 to 3 months.

Photodynamic treatment. Suspensions of normal ϕXam_3 in physiological saline containing 3×10^{10} to 5×10^{10} PFU per ml were made $1.45 \times 10^{-5} \text{ M}$ in neutral proflavine hemisulfate (ICI, England). These were then incubated at 37 C for 45 min, chilled to ice temperature, and finally exposed for the desired time intervals to visible light from two 40-W Osram daylight fluorescent lamps at a distance of 15 cm. These as well as all subsequent operations were carried out in dim yellow light. The dye was always freshly dissolved in 0.01 M phosphate buffer, pH 7.4, and its concentration was determined from the absorbance of the solution at 440 nm taking the molar extinction coefficient as $4.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

UV irradiation and heat inactivation. A 2-ml suspension of normal ϕXam_3 in starvation buffer (5) containing 10^{10} to 5×10^{10} PFU per ml was irradiated for 30 s at ice temperature with a 15-W General Electric germicidal lamp at a dose rate of about 33 ergs per mm^2 per s. For heat inactivation a 5-ml sample of the same phage stock was incubated in a water bath at 56 C for 30 min. About 0.01% of the plaque-forming units survived each of the treatments.

Measurement of SBP. Samples of normal or variously pretreated ϕXam_3 in 0.5 ml of 0.01 M phosphate buffer in graded concentrations were separately mixed with 0.5 ml of appropriate dilutions (1:100 or 1:80) of the antiserum in a series of test tubes and then allowed to react with the antiserum by incubating at 37 C for 3 h. SBP of the treated phage is

measured by the difference between the phage-inactivating capacity of the serum before and after mixing with the treated phage. To measure the SBP, a constant number (3.75×10^4) of normal ϕXwt was added as test phage to each of the test tubes. These were then incubated at the same temperature for a further period of 3 h, after which their contents were assayed for the surviving plaque-forming units of ϕXwt by using *E. coli* C as the indicator bacteria.

Measurement of phage absorption. *E. coli* C was allowed to grow in Tris-glucose medium containing 0.1% Casamino Acids with aeration at 37 C until the cell density became approximately $4 \times 10^8/\text{ml}$. CaCl_2 was then added to 10^{-3} M . ^{32}P -labeled normal or inactivated ϕXam_3 was added (gross multiplicity of infection of 0.02), and incubation was continued at 37 C. From time to time 0.8-ml samples were withdrawn and filtered through 0.45- μm membrane filters (Sartorius), washed with 2 ml of 0.01 M phosphate buffer, pH 7.4, and dried; radioactivity counts were taken with a Geiger counter. The difference between input counts and counts on the filters expressed as percentage of the input counts is shown as the percentage of unadsorbed phage.

RESULTS

Changes in SBP of variously inactivated ϕXam_3 . Results of this series of experiments have been summarized in Fig. 1. About 2×10^8 PFU of normal ϕXam_3 were able to completely neutralize the antibodies contained in 0.5 ml of 1:100 dilution of the anti- ϕX antiserum under the conditions of these experiments because 100% of the test phage (ϕXwt) survived subsequent exposure to it. As the number of neutralizing phage was reduced, more and more test phages lost their plaque-forming ability. When

FIG. 1. SBP of normal and variously treated phage ϕXam_3 . Symbols: $\circ$, normal phage; Δ , photodynamically inactivated to a survival of 0.01%; $\bullet$, UV-irradiated to a survival of 0.01%; and $\blacktriangle$, thermally inactivated to a survival of 0.01%. The titers of neutralizing phages refer to the number of plaque-forming units before inactivation treatment. Serum dilution used, 1:100.

the antiserum was treated with 2×10^6 PFU of ϕ Xam₃, only 4% of ϕ Xwt survived subsequent incubation. Behavior of ϕ Xam₃, pre-irradiated with UV light to a survival of 0.01%, was identical with that of normal phage with regard to their SBP. In contrast, when 2×10^6 to 2×10^8 PFU of ϕ Xam₃ that had subsequently been photodynamically inactivated to a survival of 0.01%, were used for neutralization of the antiserum, they seemed to have completely lost their SBP because the test phages subsequently incubated in it did not survive. (Only when larger numbers of photodynamically treated ϕ Xam₃ were used for neutralization of the antiserum, could survival of test phages ϕ Xwt be observed [see Fig. 2]). Heat-inactivated phages were also found to lose their SBP in the similar manner. These results clearly indicate that photodynamic or heat inactivation must have severely damaged antigenic determinants of the phages.

Correlation of loss of SBP with extent of photodynamic inactivation. A sample of phage ϕ Xam₃ was first photodynamically inactivated to a desired survival value and then distributed after proper dilution in 0.5-ml portions such that the total number of phage (normal plus inactivated) in a sample varied within a certain range. This procedure was repeated for different survival values ranging from 0.01 to 100% (control, uninactivated). SBP of each of the above portions was then measured as described in Materials and Methods. An analysis of these results, shown in Fig. 2, clearly reveals that to obtain a given percentage survival of test phages, the amount of neutralizing phages had to be increased continuously as the fraction of photodynamically inactivated phages in them increased. Moreover, at any particular photodynamic exposure, the survival of test phages increased monotonously with the number of irradiated phages used for serum neutralization. These results thus indicate that there is a positive correlation between the loss of SBP and extent of photodynamic inactivation.

Effect of photoinactivation on phage adsorption. Both normal and inactivated 32 P-labeled ϕ X174 am₃ were allowed to infect log-phase *E. coli* C cells. The kinetics of adsorption of normal phages and that of the phages photodynamically inactivated to a survival of 0.01% were almost identical, about 80% of labeled phages being adsorbed in 5 min in both cases (Fig. 3). This finding is similar to that obtained previously in the case of ϕ Xwt (3).

DISCUSSION

The major component of the coat proteins of phage ϕ X174, which may be called "jacket"

FIG. 2. Correlation between SBP and photodynamic treatment. Symbols: ○, normal ϕ Xam₃ (uninactivated); ●, photodynamically inactivated to a survival of 0.01%; △, photodynamically inactivated to a survival of 0.2%; and ▲, photodynamically inactivated to a survival of 20.0%. The titers of neutralizing phages refer to the number of plaque-forming units before inactivation treatment. The serum used in these experiments was the same as that used for experiments of Fig. 1 but had a slightly diminished activity. Hence, increased concentration of serum (dilution 1:80) was used.

FIG. 3. Adsorption of normal and photodynamically inactivated 32 P-labeled phage ϕ Xam₃. Symbols: ○, normally untreated ϕ Xam₃; ●, photodynamically inactivated (survival 0.01%). Experimental details are described in the text.

protein, is believed to contribute 60 molecules per phage, each having a molecular weight of about 48,000 arranged in the form of an icosahedron (2). The minor component, consisting of three molecular species, which can easily be removed upon treatment with 4 M urea, com-

prises "spikes" (so called because of their electron micrograph appearance). Spikes constitute 12 projections, each attached to a vertex of the icosahedron, and have been shown to be essentially required for adsorption to host cells (6). By using Ouchterlony plate methods, Rolfe and Sinsheimer (9) suggested that the rabbit anti- ϕ X antiserum contained antibodies specific for three antigenic sites of phage ϕ X174, on the basis of the number of precipitating zones in the agar gel plates, with the third site being exposed only after heating the virus. Whether these sites corresponded to separate polypeptide chains or only to separate configurations of the same chain is not known at present.

Results of the experiments described in this report show that among the functions associated with coat proteins of phage ϕ X174, photodynamic inactivation left the capacity of adsorption to host cells practically unaltered, whereas the antigenic determinants were severely damaged. One plausible interpretation of our results is that the anti- ϕ X antiserum contained very few, if any, antibodies against the spike proteins; most of the antibodies were specific against the jacket protein that is damaged as a result of this type of inactivation. It may be that the spike proteins of the phage are poor antigens. Alternatively, spike proteins might be highly resistant to photodynamic inactivation. Whichever interpretation is true it must be emphasized that unlike UV irradiation, photodynamic treatment causes conspicuous damage in the protein coat of phage ϕ X174 and that such damage, like the damage of the phage nucleic acids demonstrated earlier (1, 3) is positively correlated with the loss of plaque-forming ability of the phages (Fig. 2). Drastic alteration of the jacket could possibly cause DNA-protein cross-links in ϕ X174 and thereby interfere with the injection process, ultimately leading to loss of plaque-forming ability. Disturbance with the injection process has also been implicated in the photodynamic inactivation

of bacteriophage T4, although a different kind of experimental approach was employed (8). Our own unpublished data indicate that it becomes more and more difficult to extract single-strand DNA from free ϕ X174 as the photodynamic damage is increased.

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