Biochemical Studies on Polyamine and its Analogues

Part II. Mechanism of Phage Inactivation by Enzymatically Oxidized Spermine (I)

By Toshikazu Oki,† Haruhiko Kawasaki, Koichi Ogata, Hideaki Yamada,*† Ichiro Tomida,** Tetsuo Morino** and Hiroshi Fukami**

Department of Agricultural Chemistry, Kyoto University, Kyoto
*Research Institute for Food Science, Kyoto University, Kyoto
**Pesticide Research Institute, Kyoto University, Kyoto

Received November 4, 1968

Preincubation with spermine, of λ, T7 and P 465 phages which were sensitive to oxidized spermine, resulted in a decrease of their susceptibility to the action of oxidized spermine. Phages resistant to oxidized spermine such as T4 and φX 174 became susceptible to this agent after dialysis.

The mechanism of phagocidal action of oxidized spermine was examined with 32P-labelled λ phage. Oxidized spermine interfered neither with the absorption of λ phage, nor with the injection of its DNA into the host cells. The injected DNA, however, did not lead to the formation of mature phage.

The interaction of oxidized spermine with the DNA of phages T4 and T7 was investigated by thermal denaturation studies. DNA treated with oxidized spermine showed the same Tm as untreated DNA. However, the treated DNA was decreased in its hyperchromicity and was renatured to a great extent, even after rapid cooling. These facts are explained by the formation of cross-links which prevents the separation of complementary DNA strands.

The naturally occurring polyamine, spermine NH2(CH2)3NH(CH2)4NH(CH2)3NH2, is oxidized by purified plasma amine oxidase to the corresponding formyldiamine OHC-(CH2)2NH(CH2)4NH(CH2)2CHO.1) Oxidized spermine has been shown to be toxic for bacteria,1,2 bacteriophages,3 plant viruses4) and Ehrlich ascites cells.5) It has been reported that oxidized spermine forms a complex with the DNA of phage T5, and that this complex is injected into the host cells during infection. The injected complex does not give rise to the complementary RNA.6)

In the previous paper,7,8 we have reported

† Present address: Central Research Laboratories, Sanraku-Ocean Co., Ltd., Fujisawa.
* To whom requests for reprint should be addressed.
the inhibitory action of oxidized spermine and its analogues against a large variety of bacteriophages. The present work deals with the mechanism of inactivation of phage by oxidized spermine. It will be shown by using 32P-labelled λ phage that the phages, inactivated by oxidized spermine, are normally adsorbed to their bacterial host and inject their DNA. However, this injected DNA does not induce the formation of mature phages. The protective effect of spermine on the phagocidal action of oxidized spermine and the interaction of oxidized spermine with phagal DNA will also be described.

EXPERIMENTAL

Materials and methods

Cultures and chemicals. The following phages and their host bacteria were used; T4 and T7 phages (E. coli B), λ phage (E. coli K12 C600), φX174 phage (E. coli C) and P465 phage (Brevibacterium lactofermentum).10) Phage λ was obtained by induction with mitomycin C (Mitomycin Kyowa-S, Kyowa Hak'ko Kogyo Co.) from E. coli K12 W3350 (λ).11)coli phages and their host bacteria were kindly provided by Dr. H. Ozeki, National Institute of Health, Tokyo. The plaque forming units (PFU) of phage suspensions were assayed by the Adams' double layer method12) using the nutrient agar. Radioactivity was determined in a windowless gas flow counter (Aloca), after the samples were dried on stainless steel planchets.

Spermine tetrahydrochloride was a product of Sigma Chemical Company. Highly purified amine oxidase was prepared from beef plasma by the method of Yamada and Yasunobu.13) Oxidized spermine was prepared at 30°C in a Warburg manometer as described previously.9) The reaction was usually completed within 3 hr, with the consumption of the theoretical amount of oxygen.

Preparation of 32P-labelled λ phage. 32P-labelled λ phage was prepared according to Hershey and Chase.14) E. coli K12 W3350 (λ) was grown in TCG minus phosphate medium at 37°C for 20 hr. The TCG minus phosphate medium consisted of 0.1 M Tris buffer, pH 7.5, containing 0.08 M NaCl, 0.02 M KCl, 0.02 M NH4Cl, 0.02 M FeCl2, 0.16 mM Na2SO4, 1 mM MgSO4, 0.1 mM CaCl2, 1% glucose and 0.5% casamino acid. Ten milliliters of the seed culture were inoculated into 200 ml of the medium supplemented with 3 mc of 32P-H3PO4, and the incubation was carried out with aeration at 37°C. When cells were grown to approximately 4×10⁸ cells per ml, 500 μg of mitomycin C was added to the culture for the induction of λ phage. After 5 hr, the 32P-labelled phages were harvested and purified by the differential centrifugation, by the successive treatments with DNase, RNase and trypsin, and by the CsCl density gradient centrifugation. The purified λ phage was suspended in a dilution buffer (0.01 M Tris buffer, pH 7.5, containing 0.5% NaCl, 0.01% MgSO4, 7 mM O2, 0.005% CaCl2·2H2O and 0.01% casein) to the concentration of 2×10¹¹ PFU per ml and 163,000 cpm per ml.

Preparation of inactivated λ phage. Ten milliliters of the labelled λ phage (2×10¹⁰ PFU per ml) were incubated at 37°C for 3 hr with 10 μmoles of oxidized spermine. The excess of oxidized spermine in the treated phage suspension was removed by dialysis overnight against 1 liter of 0.01 M Tris buffer, pH 7.5, containing 0.1 M NaCl and 5 mM MgSO4. The titer of phages after the treatment with oxidized spermine decreased to approximately 10⁶ PFU per ml.

Adsorption of 32P-labelled λ phage to host bacteria. Cells of E. coli C600 grown in the nutrient broth were harvested by centrifugation and suspended in 16 ml of TMC buffer (10 mM Tris buffer, pH 7.1, containing 1 mM MgSO4 and 10 mM CaCl2) and starved by standing them at 25°C for 4 to 5 hr. To this suspension, 4 ml of the 32P-labelled λ phages were added (multiplicity of infection was 0.1 or 0.4). After incubation for 10 min at 37°C, the mixture was cooled in ice water and the unadsorbed phages were removed by centrifugation at 10,000 rpm for 10 min.

Injection of DNA of $^{32}$P-labelled $\lambda$ phage into host bacteria. The blender experiment was carried out according to the method described by Hershey and Chase. The precipitate containing the bacterial cells and adsorbed phages, as described above, was suspended in 10 ml of cold TMC buffer. The suspension was cooled in ice water and spun in a Waring blender at 18,000 rpm for 5 min to cut off phages from the infected bacteria. The stripped phages and phage ghosts were separated from the bacterial cells by centrifugation at 10,000 rpm for 10 min, and the supernatant and precipitate were assayed for radioactivity. The precipitate containing the DNA-injected cells was resuspended in 20 ml of the peptone glucose medium. The suspension was incubated at 37°C for 1 hr, and the mature phages were separated from bacterial cells by centrifugation. The radioactivities of both mature phage and intracellular phage remaining in bacterial cells were measured.

Preparation of phage DNA and its thermal transition. Phage DNA was isolated by the phenol method of Kaiser and Hogness. The purified phage suspension (10$^{13}$ PFU/ml) was gently shaken with an equal volume of freshly distilled phenol, which had been saturated with saline-citrate buffer (0.15 M NaCl plus 0.015 M Na-citrate, pH 7.0; hereafter referred to SSC). The mixture was centrifuged at 2,500 rpm for 10 min and the phenol layer was discarded. The aqueous DNA layer was re-extracted twice with equal volumes of SSC-saturated phenol. The final aqueous DNA layer was dialyzed at 4°C against SSC, until phenol was completely removed. Relative absorbance of the preparations at 260, 280 and 230 nm indicated high degree of purity.

The thermal denaturation profiles were determined by the methods of Marmur and Doty and Doty et al. The DNA samples were diluted in SSC to give the final concentration of approximately 20 µg per ml. The diluted DNA solution (3 ml) was heated in a glass-stoppered quartz cuvette with a 1-cm light path in a Shimazu Multipurpose Spectrophotometer Model MP-50. Heating was carried out at the rate of approximately 1°C per min, which was measured by a Tosiba Thermistor Thermometer TT-2D, by circulating heated water around the wall of cell compartment. DNA denaturation was measured by the increase in the absorbancy at 260 m.$\mu$.

RESULTS AND DISCUSSION

Susceptibility of spermine-treated or fully dialyzed phage to oxidized spermine

Effect of spermine on the $\lambda$ phage inactivation with oxidized spermine was investigated. The $\lambda$ phage (4 x 10$^9$ PFU/ml) in the dilution buffer was preincubated with various concentrations of spermine from 0.05 mM to 10 mM for 3 hr at 37°C. This preincubation did not cause any significant decrease of plaque-forming ability and 3.8 x 10$^9$ PFU/ml in the

**FIG. 1.** Effect of Spermine Concentration on Inactivation of $\lambda$ Phage with Oxidized Spermine.

Phage $\lambda$ was preincubated with spermine, at the concentration of: 0 (I, control); 0.05 (II); 0.1 (III); 0.5 (IV); 1 (V); 5 (VI) or 10 mM (VII); for 3 hr at 37°C. The spermine-treated phages were incubated with 0.6 mM oxidized spermine and the number of survivors was assayed.
TABLE I. EFFECTS OF SPERMINE-TREATMENT AND DIALYSIS ON PHAGE INACTIVATION WITH OXIDIZED SPERMINE

<table>
<thead>
<tr>
<th>Phage treated</th>
<th>Plaque formed (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ</td>
</tr>
<tr>
<td>Native phage (control)</td>
<td>3.9 x 10⁴</td>
</tr>
<tr>
<td>Spermine-treated phage</td>
<td>3.8 x 10⁴</td>
</tr>
<tr>
<td>Spermine-treated and dialyzed phage</td>
<td>3.0 x 10⁴</td>
</tr>
<tr>
<td>Dialyzed phage</td>
<td>~</td>
</tr>
</tbody>
</table>

**Note:** Spermine-treated phages were prepared by incubation with 2 mM spermine for 3 hr at 30°C. Dialyzed phages were prepared by dialysis for 3 days at 4°C against 0.002 M phosphate buffer (pH 6.0), as described in the text. The phages were incubated with oxidized spermine (OSP) at a concentration of 0.6 mM for 60 min (30 min) at 30°C. The number of survivors was assayed by plating on the agar double layer.

Phage suspension was observed at the end of preincubation. To this spermine-treated λ phage suspensions, which contained various concentrations of spermine, oxidized spermine was added up to a final concentration of 0.6 mM, and the incubation was continued at 37°C. As a control experiment, the susceptibility of spermine-untreated phage to oxidized spermine was examined similarly. As shown in Fig. 1, the susceptibility of λ phage to oxidized spermine markedly decreased with the increase in spermine concentration. Phages T 7 and P 465 treated with spermine behaved similarly to oxidized spermine. The susceptibility of these spermine-treated phages was considerably lower than that of the untreated phages, which were potently inactivated by oxidized spermine. When, however, spermine was removed from the spermine-treated λ phage suspension by extensive dialysis, its susceptibility to oxidized spermine was restored to some extent, but was lower than that of the untreated phage.

It has been reported that the T-even phages contain high concentrations of polyamines (putrescine and spermidine). Then, for the purpose of removing polyamine from T 4 and φX 174 phages, the dialysis was carried out. Two milliliters of phage suspension (3 x 10⁸ PFU/ml) were dialyzed for 48 hr at 4°C against 2 changes of 1 liter of 0.002 M phosphate buffer, pH 6.0, containing 0.02 M MgSO₄, 0.02 M CaCl₂ and 0.3 M NaCl and for further 8 hr against 1 liter of the same buffer without NaCl. Any significant decrease of PFU was not observed during these dialysis. The fully dialyzed phages were incubated at 37°C for 60 min with 0.6 mM oxidized spermine, and the number of survivors was assayed by plating. The data presented in Table I show that both T 4 and φX 174 get changed to be susceptible to oxidized spermine after dialysis, and 20- to 100-fold reduction in the infective titer was obtained with the dialyzed phages, although the intact phages were not susceptible at this concentration of oxidized spermine at all.

Adsorption and injection of DNA of inactivated λ phage to host bacteria

Bachrach et al. carried out a blender ex-
TABLE II. ADSORPTION AND INJECTION OF DNA OF INACTIVATED \( \lambda \) PHAGE TO \( E. coli \) C 600

<table>
<thead>
<tr>
<th>Adsorption temp. ((^\circ C))</th>
<th>Phage</th>
<th>Adsorbed phage (Blendor mixture) (cpm)</th>
<th>Adsorption (%)</th>
<th>Precipitate (Injected DNA) (cpm)</th>
<th>Supernatant (Uninjected DNA) (cpm)</th>
<th>Injected DNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>xp. 1. 37</td>
<td>Native</td>
<td>68,200</td>
<td>29,600</td>
<td>43</td>
<td>15,795</td>
<td>13,950</td>
</tr>
<tr>
<td></td>
<td>OSP-treated</td>
<td>57,500</td>
<td>47,300</td>
<td>84</td>
<td>27,120</td>
<td>19,450</td>
</tr>
<tr>
<td>xp. 2. 37</td>
<td>Native</td>
<td>122,800</td>
<td>75,300</td>
<td>60</td>
<td>23,480</td>
<td>52,000</td>
</tr>
<tr>
<td></td>
<td>OSP-treated</td>
<td>105,200</td>
<td>85,300</td>
<td>85</td>
<td>38,700</td>
<td>43,200</td>
</tr>
<tr>
<td>xp. 3. 0</td>
<td>Native</td>
<td>34,800</td>
<td>13,630</td>
<td>40</td>
<td>1,693</td>
<td>12,200</td>
</tr>
<tr>
<td></td>
<td>OSP-treated</td>
<td>28,500</td>
<td>19,940</td>
<td>75</td>
<td>13,540</td>
<td>6,000</td>
</tr>
</tbody>
</table>

Exp. 1 and 3: M.O.I.=0.1, Exp. 2: M.O.I.=0.4 (M.O.I.;Multiplicity of infection)

Experiment with \( ^{32}P \)-labelled T 5 phage for the examination of the mechanism of phage inactivation by oxidized spermine. In the present paper, \( ^{32}P \)-labelled \( \lambda \) phage was subjected to a blender experiment. The \( ^{32}P \)-labelled \( \lambda \) phage inactivated by oxidized spermine was incubated with cells of \( E. coli \) C 12 C 600 at 37°C or 0°C at the multiplicity of infection of 0.1 or 0.4. After 10 min, the bacterial cells and adsorbed phages were removed by centrifugation, and the percentage of adsorbed phages was calculated by determining the radioactivities of the precipitate and supernatant fluid. The total number of counts in both fractions was approximately equal to that in the original adsorption mixture.

Table II shows that the inactivated phages were adsorbed to the host cells at greater extent than the untreated phages, both at the multiplicity of infection of 0.1 and 0.4. Approximately 85% of the inactivated phages were adsorbed to the host cells, despite a decrease of 7 logarithms in their titer. The oxidized spermine-treated phages similarly behaved at 0°C as at 37°C. In view of the finding that the inactivated \( \lambda \) phage is normally adsorbed on the host cells, the ability of the adsorbed phage to inject its DNA into the bacterial cell was tested by the blender experiment of Hershey and Chase. Table II shows that the oxidized spermine-treated \( \lambda \) phage injects its DNA into the host cells similarly to the untreated phage. In Exp. 1 of Table II, 58% of \( ^{32}P \)-labelled DNA of the inactivated phage were injected into the host cells while 54% of that of the untreated phage were injected. Exp. 2 also shows that the inactivated phage injects its DNA into the host cells to a greater extent than the untreated phage.

It has been known that injection of phage \( \lambda \)-DNA is repressed markedly at 0°C. In the present experiments, \( \lambda \) phage injected only 12% of its DNA to the host cells at 0°C. On the other hand, 68% of DNA of the adsorbed phage which was inactivated by oxidized spermine, remained in the host cells even after application of strong shearing forces to a suspension of infected cells. These data suggest that the oxidized spermine-treated phage may make abnormal injection which is independent of temperature.

Maturation of phage treated with oxidized spermine

The \( ^{32}P-\lambda \) phage infected bacteria were agitated in the Waring blender and centrifuged to remove the extracellular \( ^{32}P \). The cells were resuspended in the peptone-glucose medium and aerated for 60 min at 37°C. The suspension was then centrifuged and

---

TABLE III. MATURATION OF $\lambda$ PHAGE
IN E. coli C 600

<table>
<thead>
<tr>
<th>Phage</th>
<th>Precipitate (Intracellular DNA) (cpm) (%)</th>
<th>Supernatant (Mature phage) (cpm) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1. Native</td>
<td>15,795 3,940 25 11,000 70</td>
<td></td>
</tr>
<tr>
<td>OSP-treated</td>
<td>27,120 20,720 76 6,000 22</td>
<td></td>
</tr>
<tr>
<td>Exp. 2. Native</td>
<td>23,480 8,700 37 14,050 60</td>
<td></td>
</tr>
<tr>
<td>OSP-treated</td>
<td>38,700 31,230 81 6,700 17</td>
<td></td>
</tr>
</tbody>
</table>

The yield of phages from the infected cells was assayed directly for radioactivity of each fraction. The results presented in Table III show that approximately 70% of $^{32}$P of the injected DNA are released from the normal phage-infected cells as the mature phages in the supernatant. On the contrary, the cells which were infected with the oxidized spermine-treated and inactivated phages released only 20% of the initial injected $^{32}$P, and nearly 80% of $^{32}$P remained in the cells. These results thus indicate that the $\lambda$ phage inactivated by oxidized spermine adsorbs to the bacterial cells and injects its DNA into the cells, but that this injected DNA does not lead to the formation of mature phages.

Effect of oxidized spermine on the thermal transition of phage DNA

The interaction of oxidized spermine with phage DNA was investigated by thermal denaturation. The DNA of T7 phage (3 ml, 60 µg in SSC) was incubated for 2 hr at 37°C with 0.1 mM spermine or 0.01 mM oxidized spermine, and thermal denaturation profiles were determined in the presence of spermine or oxidized spermine. As shown in Fig. 2, the T7 DNA treated with spermine as well as oxidized spermine showed the same Tm, the temperature at the midpoint of the transition, as the untreated DNA. However, the hyperchromicity of the oxidized spermine-treated DNA was definitely lower than those of the untreated and the spermine-treated DNA. The hyperchromicities determined were 43, 41 and 35% with the untreated, the spermine-

FIG. 2. Effect of Oxidized Spermine on Thermal Transition of T7 DNA.

T7 DNA (○—○) was incubated for 2 hr at 37°C in 0.15 M NaCl plus 0.015 M Na-citrate with 0.1 mM spermine (×—×), 0.01 mM oxidized spermine (△—△), or 0.1 mM spermine plus 0.01 mM oxidized spermine (○—○). Thermal denaturation profiles were determined as described in the text.

TABLE IV. CHANGES IN RELATIVE ABSORBANCE AT 260 mµ ON THERMAL DENATURATION AND RENATURATION OF T4 DNA IN THE PRESENCE OF SPERMINE OR OXIDIZED SPERMINE*

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Before heating at 98°C</th>
<th>Heating at 98°C</th>
<th>Rapid cooling to 0°C</th>
<th>Slow cooling to 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 DNA (control)</td>
<td>1.00</td>
<td>1.47</td>
<td>1.24</td>
<td>1.10</td>
</tr>
<tr>
<td>$\sim$ +0.1 mM spermine</td>
<td>1.00</td>
<td>1.52</td>
<td>1.23</td>
<td>1.00</td>
</tr>
<tr>
<td>$\sim$ +0.01 mM spermine</td>
<td>1.00</td>
<td>1.36</td>
<td>1.06</td>
<td>1.00</td>
</tr>
<tr>
<td>$\sim$ +0.001 mM spermine</td>
<td>1.00</td>
<td>1.42</td>
<td>1.12</td>
<td>1.11</td>
</tr>
<tr>
<td>$\sim$ +0.1 mM OSP</td>
<td>1.00</td>
<td>1.47</td>
<td>1.27</td>
<td>1.11</td>
</tr>
<tr>
<td>$\sim$ +0.01 mM OSP</td>
<td>1.00</td>
<td>1.47</td>
<td>1.27</td>
<td>1.11</td>
</tr>
</tbody>
</table>

* Experimental conditions were described in text.
treated and the oxidized spermine-treated DNA, respectively. When the T7 DNA was incubated with both 0.1 mM spermine and 0.01 mM oxidized spermine, the treated DNA showed the same Tm and hyperchromicity as the untreated DNA. Similar results were obtained with the DNA isolated from T4 phage which was less susceptible to oxidized spermine.

The thermal denaturation and renaturation of spermine- or oxidized spermine-treated T4 DNA have been studied. The DNA (3 ml, in SSC) was incubated for 2 hr at 37°C with 0.1 mM spermine or 0.01 to 0.001 mM oxidized spermine. These DNA preparations were heated for 15 min at 98°C, in the presence of spermine or oxidized spermine. A half of each solution was rapidly cooled in an ice-bath and the other half was slowly cooled to room temperature (20°C) at the rate of −1°C/min, then change in absorbancy at 260 mμ was determined.

Table IV shows that the DNA treated with oxidized spermine is renatured to a great extent not only at slow cooling, but also at rapid cooling, showing hyperchromicity around 10%, while the untreated (control) and the spermine-treated DNA do not return to the native-like state at rapid cooling, showing still 23~24% hyperchromicity. Changes in the hyperchromicity on thermal denaturation and renaturation could be explained by the formation of cross-links between DNA and oxidized spermine which prevented the separation of the complementary DNA strands.

Acknowledgement. The authors wish to express their sincere thanks to Prof. T. Hata, Prof. M. Nakajima, Prof. Y. Morita, Kyoto University, Kyoto, for their interest and suggestion during the course of this work.