Mechanism of Inhibition of Bacillus subtilis DNA Polymerase III by the Arlyhydrazinopyrimidine Antimicrobial Agents

(ternary complex/exonuclease/DNA replication/base pairing/polyamine)

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Communicated by Albert Dorfman, May 30, 1974

ABSTRACT Arlyhydrazinopyrimidines inhibit DNA synthesis in Bacillus subtilis by promoting formation of a specific, long-lived ternary complex with DNA polymerase III and the template-primer DNA. DNA polymerase III contains an associated, single-strand-selective exonuclease which generates 5’-mononucleotides. Drug inhibition of the nuclease similarly proceeds through formation of the ternary complex. The ternary complex was isolated by agarose chromatography. Like inhibition of the nuclease, optimum formation of the complex requires duplex DNA with single-stranded regions such as bacteriophage λ DNA (purely single- and double-stranded DNA are ineffective) and is antagonized by specific deoxyribonucleoside triphosphates. Formation of the ternary complex requires a di- or polyvalent cation and is inhibited by sulfhydryl reagents and high ionic strength. The complex dissociates with a half-life of the order of minutes at 4°C. The ternary complex dissociates slowly and the enzyme is thus effectively trapped in an inactive state.

Several lines of evidence led to this postulated mechanism (3). First, OHPh(NH)2Ura inhibition was selectively and competitively antagonized by dGTP and dTTP. Since presumably the polymerase will bind only dGTP or dTTP when cytosine is the next base to be copied, the specificity of attenuation is explained. Attenuation of OHPh(NH)2Iso inhibition was specific for dATP. Second, polymerization directed by an adenine-containing template was inhibited by neither drug, while that directed by an adenine- and thymine-containing template was inhibited only by OHPh(NH)2Iso. Third, the individual incorporation of all four deoxyribonucleoside triphosphates was inhibited. Fourth, while the oligo(dT)-poly(dA)-directed incorporation of dTTP was insensitive to high concentrations of the drug, the addition of a minute amount of gapped DNA permitted full inhibition. Thus, the formation of the ternary complex efficiently scavenged free enzyme in the presence of a large excess of potential priming sites. In light of this effect, the model will be referred to as ternary scavenging.

This report describes the drug inhibition of an exonuclease intrinsic to DNA polymerase III and the isolation of the inhibitory ternary complex. The study reveals the requirements for ternary complex formation and provides strong support for the inhibition scheme.

MATERIALS AND METHODS

Unless otherwise specified, materials and experimental procedures were as described previously (3, 7).

Nucleotides and Nucleic Acids. Poly(U) was purchased from Miles Laboratories and 5'- and 3'-dTMP from P. L. Biochemicals. Salmon testes DNA digested with DNase I until 2% of the material absorbing at 260 nm became acid-soluble (7), which contains nicks and gaps of various lengths, is referred to as “gapped DNA.” The gapped DNA was separated from low molecular weight material by Sepharose 2B filtration. Bacteriophage DNA was obtained by phenol extraction of purified virions. Phage λ c5λ DNA was provided by R. Gayda. T7, λ, and N4 (8) DNA contained less than one single-strand break per molecule as judged by analytical sedimentation in alkali (9). External 3'-hydroxyl terminated gaps were introduced into N4 DNA by digestion with exonuclease III (10) to 3% solubility in acid. DNase I, from bovine pancreas, was used to generate 3'-hydroxyl-terminated nicks in T7 DNA. [3H]Thymidine-labeled E. coli DNA was isolated by the method of Marmur (11) and after denaturation had an average molecular weight of 5 × 106. Single-
strand, 3'-phosphoryl-terminated DNA was produced by micrococcal nuclease treatment of *E. coli* [3H]DNA to 9% acid solubility followed by heating at 100° for 2 min. A portion was treated with bacterial alkaline phosphatase and heated again to provide a dephosphorylated control. Sonicated *E. coli* [3H]DNA was prepared by eight 30-sec sonic bursts at 1.25 kamps with an MSE sonicator. Native *E. coli* [3H]DNA was separated from single-stranded DNA by chromatography with benzoylated naphthoylated DEAE-cellulose (Gerhard Schlesinger Co.) by the procedure of Schlegel et al. (12). The concentration of nucleic acids is expressed as nucleotide equivalents.

Drugs. Dr. Bernard Langley generously supplied 6-(p-hydroxyphenylazo)uracil and 6-(p-hydroxyphenylazo)isocytosine, which were converted to the active hydrazino derivatives immediately before use (3).

Enzymes. *B. subtilis* DNA polymerase III was purified by a modification of the previously published procedure (7) that will be described elsewhere. 5'-nucleotidase from *Crotaulus adamanteus* venom was purchased from Sigma Chemical Co. *E. coli* exonuclease III was a gift from Dr. P. Englund.

Enzyme Assays. The polymerase III assay has been described (7). The reaction mixture for the polymerase-associated polynucleotide contained in a total volume of 50 µl, 30 mM Tris-HCl (pH 7.5), 6.5 mM MgCl₂, 3 mM 2-mercaptoethanol, 10% glycerol, 0.1 mg/ml of bovine-serum albumin, 20 µM denatured (100°, 2 min) *E. coli* [3H]DNA (3.6 × 10⁶ cpm/µmol), and 0.02-0.05 unit of enzyme. After 20 min at 30°, the radioactivity soluble in 1.0 M perchloric acid was measured. A unit of activity catalyzes the release of 10 mmol of nucleotide in 30 min. Endonuclease determinations for double- and single-strand specific activities employed the exonuclease assay mixture except the substrate was either native or irreversibly-denatured (13) duplex minicircular [3H]DNA from *E. coli* 15 (14), respectively. After a 60 min, 30° incubation with 0.25 unit of polymerase III, the introduction of strand breaks was monitored by sedimentation through alkaline sucrose gradients.

Isolation of the Ternary Complex. A preincubation mixture (175 µl) of 50 mM potassium phosphate (pH 7.4), 5 mM MgCl₂, 5 mM glutathione, 25 µM OHPh(NH₂)Ur, 0.1 mg/ml bovine-serum albumin, 17% glycerol, 0.04 mM gapped DNA, and 0.1-0.5 unit of polymerase III was incubated 3 min at 30° and filtered through a 0.8 × 10-cm Sepharose 4B column equilibrated at 4° with the preincubation mixture without DNA and enzyme and a glycerol concentration of 10%. Some experiments included an internal reference of 0.5 µM [γ-32P]ATP. The flow rate averaged 0.2 ml/min and 0.25-ml fractions were collected and assayed for polymerase and nuclease in the presence of 250 µM dGTP to overcome drug inhibition. Recovery of enzyme activity averaged 40%.

Other Methods. Kinetic plots were fitted by a least squares method. The solvents for paper (Whatman 3 MM) chromatography were: (I) isobutyric acid: 1 M NH₄OH : 0.1 M ethylene-diaminetetraacetate (EDTA), 100:60:1.6 and (II) 1-propanol: concentrated ammonia:H₂O, 60:30:10.

RESULTS

DNA Polymerase III Has an Associated Exonuclease. Extensive purification of *B. subtilis* DNA polymerase III revealed an associated nuclease activity which co-chromatographed with polymerase activity on hydroxylapatite—the final step in the purification. The enzyme purified by hydroxylapatite was used in all subsequent experiments. Under optimal conditions for each activity the polymerase is about six times more active than the nuclease. Like polymerase III activity (7), the nuclease is inhibited more than 95% by 8 mM N-ethylmaleimide, has a pH optimum near pH 7.5, and has sharply reduced activity at high ionic strength; 100 mM KCl inhibits 90%. Both activities require Mg⁺⁺⁺⁺ in the absence of the metal ion, with or without 2 mM EDTA, there is little or no enzymatic activity.

This intrinsic hydrolytic activity is exonucleolytic. The enzyme preparation is free of endonuclease activity as measured by the absence of rupture of native and irreversibly denatured, duplex circular DNA. Chromatography with solvent I of the hydrolysis products of [3H]thymidine-labeled *E. coli* DNA revealed only [3H]dTMP; no (<2%) labeled oligonucleotides or thymidine were detected. The [3H]dTMP product was identified as the 3'-isomer on the basis of its quantitative conversion to thymidine by 5'-nucleotidase (15) and its coincidence with 5'-dTMP after chromatography with Solvent II, which resolves 3'- and 5'-dTMP.

The nuclease preferentially attacks single-stranded nucleic acids. Native *E. coli* [3H]DNA purified by benzoylated naphthoylated DEAE-cellulose chromatography was hydrolyzed 100 times slower than denatured *E. coli* [3H]DNA. RNA may also be a substrate, since 43 µM poly(U) inhibited the digestion of single-stranded DNA by 84%. The nuclease prefers small chains, since sonication or DNase I digestion (25%), acid-solubility) of *E. coli* DNA followed by heat denaturation caused a 3- to 4-fold increase in activity as compared to saturating concentrations of untreated, denatured *E. coli* DNA. In contrast, negligible activity was seen with short 3'-phosphoryl-terminated single-stranded DNA. After removal of the terminal phosphates by bacterial alkaline phosphatase the activity increased to 7-fold that obtained with control, phosphatase-treated *E. coli* DNA. Reactions containing a mixture of 3'-phosphoryl- and 3'-hydroxyl-terminated chains indicated that 3'-phosphoryl termini are strongly inhibitory.

Drug Inhibition of the Polymerase III Associated Nuclease. The intrinsic nuclease permitted a critical test of the ternary scavenging scheme for drug action. Single-stranded DNA is an
effective substrate for the nuclease but not for the polymerase. With this substrate, even a high concentration of OHPh(NH)2Ura (120 μM) caused only a minimal inhibition of the nuclease (<10%). Since the nuclease reaction mixture contained polymerase III, template cytosine residues, and drug, this result suggested that the ternary complex formation also requires a primer terminus (see Fig. 1). Indeed, if unlabeled gapped DNA is included with 120 μM OHPh(NH)2Ura in the nuclease reaction mixture, substantial inhibition (>90%) ensues. Half-maximal inhibition required 1 μM gapped DNA (Fig. 2A), which is 1/50 the concentration of the nuclease substrate. The drug dose dependence for nuclease inhibition at two concentrations of scavenging unlabeled DNA is shown in Fig. 2B. The linear relationship between reciprocal velocity and OHPh(NH)2Ura concentration implies a single binding site for the inhibitor. The proportionality, within experimental error, of the slope of these lines to the concentration of trapping DNA indicates the cooperative requirement for inhibition expected for a mechanism involving a ternary complex.

Like the inhibition of polymerase (3), the inhibition of the nuclease is attenuated specifically by dGTP. 200 μM dGTP reduced inhibition by 60 μM OHPh(NH)2Ura from 70% to 10%; even a 5-fold excess of dTTP, dCTP, and dATP over drug did not alter inhibition. OHPh(NH)2Is also inhibits the nuclease in the presence of trapping DNA and this effect is reversed specifically by dATP.

To define more precisely the DNA requirement for ternary complex formation, we employed homogeneous preparations of DNA from several bacteriophages (Table 1). Intact duplex N4 and T7 DNA, even at 170 μM, did not promote inhibition of the nuclease. However, terminally gapped N4 DNA generated by exonuclease III treatment and, even more impressively, phage λ DNA, which contains natural 3'-hydroxyl-terminated ends and a template cytosine residue adjacent to one primer terminus (16), were quite effective. T5 DNA, containing about five naturally occurring nicks (17), and T7 DNA containing about four DNase-I-generated nicks were less effective than λ DNA; but at higher nicked DNA concentrations inhibition ensued, perhaps after fraying or nucleolytic enlargement of the nicks. Thus, double-stranded DNA, single-stranded DNA, and double-stranded ends of DNA do not permit ternary complex formation; 3'-hydroxyl-terminated breaks are required.

Isolation of the Ternary Complex by Gel Filtration. A prediction of the inhibition mechanism depicted in Fig. 1 is that OHPh(NH)2Ura and OHPh(NH)2Is will potentiate the binding of DNA polymerase III to its DNA substrate. To test this prediction, we preincubated the enzyme with 40 μM gapped DNA and 5 mM MgCl₂ in the presence and absence of 25 μM OHPh(NH)2Ura, and then passed it over a Sepharose 4B column. Without drug, the enzyme was included into the gel, eluting in a rather broad zone but well separated from the void volume where the gapped DNA eluted (Fig. 3). However, with OHPh(NH)2Ura in the column and preincubation buffers, the enzyme as measured both by its polymerase and its exonuclease activity was now excluded by the gel in a sharp band (Fig. 3). At a high gapped DNA concentration (400 μM), the polymerase is largely excluded even without the drug. This binding is not seen with intact phage DNA. As documented below, the drugs appear to potentiate the binding of enzyme to DNA that is the prerequisite for polymerization.

Requirements for Ternary Complex Formation. The requirements for formation of the ternary complex were determined by the agarose column procedure. Essentially the same chromatographic profile was obtained from 10 to 100 μM OHPh(NH)2Ura; however, when the drug concentration was lowered to 1 μM in the preincubation and column buffers, about one-half of the enzyme was excluded. Therefore, only a low concentration of drug, of the same magnitude as the kinetically determined Kᵢ (3), is needed for complex formation. Omitting OHPh(NH)2Ura or DNA from the preincubation mixture abolished complex formation, as did replacement of OHPh(NH)2Ura by the azo form of the drug. OHPh(NH)2Is can substitute for OHPh(NH)2Ura.

The amount of complex recovered is somewhat sensitive to high ionic strength but less so than polymerase and nuclease

### Table 1. Sequestering DNA requirement for OHPh(NH)₂Ura inhibition of the nuclease

| Addition of unlabeled DNA | Concentration of unlabeled DNA (μM) | Inhibition by OHPh(NH)₂Ura (%)
<table>
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<tr>
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<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>Gapped salmon testes DNA</td>
<td>10</td>
<td>92</td>
</tr>
<tr>
<td>Native N4 DNA</td>
<td>170</td>
<td>8</td>
</tr>
<tr>
<td>Native T7 DNA</td>
<td>170</td>
<td>12</td>
</tr>
<tr>
<td>Exonuclease III-treated N4 DNA</td>
<td>35</td>
<td>27</td>
</tr>
<tr>
<td>λ DNA</td>
<td>30</td>
<td>92</td>
</tr>
<tr>
<td>T5 DNA</td>
<td>35</td>
<td>9</td>
</tr>
<tr>
<td>Nicked T7 DNA (4 nicks per molecule)</td>
<td>160</td>
<td>24</td>
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The standard nuclease reaction mixtures contained 7 μM heated-denatured E. coli [³H]DNA, the indicated unlabeled DNA, and 0.065 unit of enzyme. The degree of inhibition was calculated from activity in the presence and absence of 160 μM drug with each DNA tested; at the concentrations indicated, the DNA caused essentially no inhibition without drug.
activity. The amount of enzyme eluting by fraction 10 (see Fig. 3) was the measure of void volume activity. While about 85% of the enzyme was in the presence of 35–60 mM potassium phosphate and 5 mM MgCl₂, at 100 mM phosphate only about half was eluted. With 50 mM phosphate and 5 mM MgCl₂, 100 and 200 mM KCl reduced the proportion of enzyme recovered as ternary complex to 60 and 30%, respectively.

Mg++ affects strongly the binding of DNA polymerase III to its DNA substrate. In the absence of both drug and MgCl₂, with and without 0.6 mM EDTA, the enzyme chromatographic profile was smeared into the void volume (47% elutes by fraction 10). The addition of drug caused only slightly more enzyme to be excluded, about 58%. Thus, Mg++ apparently inhibits binding of polymerase to DNA in the absence of drug but is required for ternary complex formation. This is not an ionic strength effect. In the absence of MgCl₂, OHPh(NH)₂Ura had little effect on the chromatographic profile of the enzyme at 50–75 mM phosphate buffer, and 2 mM MgCl₂ suffices in promoting formation of the ternary complex. The effect of Mg++ is not totally specific, however, since with 3 mM spermidine and 0.15 mM EDTA but no Mg++, 50 mM OHPh(NH)₂Ura increased the void volume fraction of enzyme from 25 to 65%; in the control with drug and 2 mM MgCl₂, 80% was excluded. In polymerase but not in exonuclease assays, 3 mM spermidine at low EDTA concentrations can largely replace the Mg++ requirement. Thus, maximum formation of the specific drug–enzyme–DNA complex, like polymerase activity, requires a divalent or polyvalent cation.

The formation of the ternary complex is inhibited by sulfhydryl reagents, as is polymerase and nuclease activity. With 0.1 mM p-chloromercuriphenylsulfonic acid, a reversible inhibitor, in the preincubation and column buffers, OHPh(NH)₂Ura did not promote complex formation.

Isolation of the ternary complex by agarose chromatography has the same DNA requirement as seaving of the nuclease shown in Table 1. Purely double-stranded DNA (400 µM N4 and T7 DNA) and purely single-stranded DNA (370 µM denatured N4 DNA) promoted no (<10%) detectable complex formation. However, with exonuclease III-denatured N4 DNA at 40 mM and 350 mM MgCl₂, 20% and 81% of polymerase was excluded in the presence of drug, and with 35 mM MgCl₂ 66% of the enzyme was excluded. Nicked DNA was less effective than λ DNA. With 35 and 260 µM T5 DNA, 6 and 29% of the enzyme was excluded, and with DNase-I-nicked (4 per molecule) T7 DNA at 30 and 300 µM, 30 and 66% of the enzyme was excluded.

**Attenuation and Reversal of Complex Formation.** The inhibition of polymerase III by OHPh(NH)₂Ura is competitively and selectively attenuated by dGTP (3). In the presence of 500 µM dGTP and 10 µM OHPh(NH)₂Ura only 7% of the polymerase was excluded by agaroase, while with 500 µM dTTP, DATP, dCTP, rGTP, or no nucleotide, about 85% was excluded.

The ternary complex is sufficiently stable that it can be isolated without drug in the column buffer; the yield of void volume enzyme diminished with a decreasing column flow rate. This time dependence was used to obtain an approximation of the stability of the ternary complex; dGTP was included in the column buffer to block reassociation of the ternary complex. When the time for elution of the void volume peak of enzyme was 270, 590, 730, and 1300 sec, the yield of excluded enzyme was about 70, 45, 38, and 20%, respectively. The excluded enzyme eluted sharply, as shown in Fig. 4 for an intermediate flow rate. These data obtained at 4°C can be fitted to an exponential decay curve and indicate a half-life of about 9 min. A single determination at 10°C indicated a
dissociation rate three to four times faster than that at 4º (Fig. 4). The data in Fig. 4 also show that the formation of the ternary complex is reversible, as is drug inhibition of polymerase activity (3).

An independent measurement of the stability of the ternary complex was made by means of kinetics. The relief of OHPh(NH)2Ura inhibition of polymerase activity by dGTP is not immediate (Fig. 5) but follows a lag averaging 50 sec at 10º in three determinations. The controls shown are for the addition of dGTP in the absence of drug and the addition of dATP in its presence; no effect of the nucleotides was seen. If the decay of ternary complex is first order, then this 50-sec lag corresponds to a half-life of 35 sec. The reasons for this approximately 5-fold difference in the physical and kinetic estimates of stability of the ternary complex are not known, but the conditions of the experiments and the assumptions in the calculations are quite different. However, both determinations imply that the half-life of the complex at 4º is of the order of minutes, and therefore confirm the prediction of the scavenging model that the ternary complex dissociates slowly.

**DISCUSSION**

Several lines of evidence suggest that B. subtilis DNA polymerase III contains an intrinsic exonuclease. The activities co-purify, co-chromatograph within experimental error on Sepharose 4B in the presence and absence of OHPh(NH)2Ura (Fig. 3), and are similarly inhibited by N-ethylmaleimide, high ionic strength, and aryldrazinopyrimidines. OHPh(NH)2Ura inhibition of both activities requires template-primer DNA and is selectively attenuated by dGTP.

The nuclease and gel filtration experiments provide strong support for the ternary complex scavenging mechanism for aryldrazinopyrimidine inhibition. First, the drugs do not act simply by starving the enzyme of a specific deoxyribonucleoside triphosphate, since inhibition of the nuclease and formation of the ternary complex as monitored by agarose chromatography occur in the absence of deoxyribonucleoside triphosphates. Second, only a template-primer for the polymerase permits isolation of the ternary complex by gel filtration and drug inhibition of the nuclease. The failure of single-stranded DNA to form the stable ternary complex is particularly revealing, since it contains cytosine residues and is therefore nuclease substrate. Therefore, OHPh(NH)2Ura strengthens only one specific mode of binding of the enzyme to DNA, namely that which leads to polymerization. If the single-stranded nuclease substrate binds like a primer strand, then OHPh(NH)2Ura bound in the deoxyribonucleoside triphosphate binding site cannot bind simultaneously to a cytosine residue as required by the ternary scavenging scheme.

Third, the proposition that the drugs bind to specific template residues is strengthened, since inhibition of the nuclease and the formation of the ternary complex are attenuated selectively by dGTP and dATP for OHPh(NH)2Ura and OHPh(NH)2Iso, respectively. The base specificity of deoxyribonucleoside triphosphate attenuation and the strict requirement for a primer terminus imply that the drug binds in the enzyme's deoxyribonucleoside triphosphate binding site and to the specific template residue adjacent to the primer terminus rather than to other sites on the enzyme and template. Fourth, the stability of the ternary complex is shown both by the slow breakdown of the complex on agarose gels (Fig. 4) and by the lag in reversal of polymerase inhibition by dGTP (Fig. 5). The longer half-life measured by the former method may be an overestimate, since it assumes that all void volume activity still retains drug and that the dissociation of the enzyme-DNA binary complex occurs instantaneously. Both methods indicate a half-life of dissociation at 4º of the order of minutes. This rate is orders of magnitude slower than our estimate for the turnover number of the polymerase. Also, the catalytic complex of enzyme, DNA, and nucleoside triphosphate must be less stable than the inhibitory ternary complex, since dGTP has no detectable effect on the agarose profile of the enzyme. The inhibition scheme suggests two explanations for the slow breakdown. First, the binding of the phenol moiety of the drug to the enzyme, an interaction not shared with deoxyribonucleoside triphosphate substrates, may be only slowly reversible. Second, perhaps nucleotide incorporation, not occurring in the ternary complex, facilitates enzyme release from the DNA.

The discussion has implicitly assumed that the chromatographic and inhibitory complexes are identical, because isolation of the polymerase in the agarose void volume has the same requirements as inhibition. The agarose complex requires only low concentrations of drug, of a similar magnitude to the Kd and both OHPh(NH)2Ura and OHPh(NH)2Iso suffice. Formation of the complex is reversible, and is attenuated by the same deoxyribonucleoside triphosphates that antagonize inhibition. The DNA structural requirements for the drug-promoted exclusion of enzyme and for inhibition are the same.

This work was supported by grants from the National Science Foundation (GB-32243) and the National Institutes of Health (GM-19425). R.L.L. was supported by Public Health Service Grant HD-00001.