Inhibition of a DNA Polymerase from Bacillus subtilis by Hydroxyphenylazopyrimidines (base-pairing/antimicrobials/DNA replication)

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ABSTRACT The antimicrobial agent, 6-(p-hydroxyphenylazo)-uracil (HPUra), specifically inhibits DNA polymerase III of Bacillus subtilis with a Kᵦ of less than 1 μM. The inhibition requires prior reduction of the drug, is reversible, and is competitive with dGTP. High amounts of dATP prevent inhibition by the closely related drug, 6-(p-hydroxyphenylazo)-isocytosine. A model is presented in which the inhibitors base-pair with the template while part of a ternary complex with the enzyme. These results imply that DNA polymerase III of B. subtilis is necessary for chromosomal replication.

The antimicrobial agent, 6-(p-hydroxyphenylazo)-uracil (HPUra), specifically inhibits DNA synthesis in several Gram-positive bacteria (1). In Bacillus subtilis, the inhibition is limited to replicative synthesis, while repair synthesis and DNA synthesis directed by several bacteriophages is unimpaired (2, 3). Semiconservative DNA synthesis in tolune-treated B. subtilis cells is inhibited by HPUra in the presence of reducing agents (4).

Recently, we described a mutant of B. subtilis lacking the DNA polymerase isolated by Okazaki and Kornberg (polymerase I) (5, 6). In these polA– cells, the residual DNA-polymerizing activity has markedly different properties from polymerase I (5). During the analysis of this residual activity we have isolated two additional DNA polymerases that we call polymerases II and III, in order of discovery. The three B. subtilis polymerases are similar to the correspondingly numbered Escherichia coli enzymes (7–10), but there are clear differences (11). In this report, we show that HPURa specifically inhibits DNA polymerase III of B. subtilis by an unexpected mechanism.

MATERIALS AND METHODS

Bacteria. Strain BC26 (phe–, argA–) was obtained from Dr. J. Copeland. The polA69 locus in HA101(59)F (5) was introduced into BC26 by transduction with bacteriophage PBS1 to form BC26(P). Among the phe+, argA+ transductants, the frequency of polA– bacteria is 95%.

Nucleotides and Nucleic Acids. Unlabeled nucleotides, poly(dA), poly(dT), and (dT)₉₉ were purchased from P. L. Biochemicals, poly[d(A-T)] from Miles Laboratories, and salmon testes DNA from Worthington Biochemical Corp. The deoxynucleotides were purified before use (5). Labeled nucleotides were purchased from Schwarz BioResearch. Activation of salmon-testes DNA for use as a template-primer for DNA polymerase used limited digestion by pancreatic DNase (4% acid-soluble A₂₆₀) (11). The concentration of nucleic acids is expressed as nucleotide equivalents.

Drugs. HPURa and 6-(p-hydroxyphenylazo)-2-amino-4-hydroxypyrimidine (HPIso) were generously provided by Dr. Bernard Langley of Imperial Chemical Industries, Ltd., Macclesfield, England. The removal of minor contaminants from HPURa by paper or column chromatography (1) did not alter the inhibitory activity of the drug. To convert the drug to an active inhibitor, 2.0 mM HPURa was treated with 25 mM dithiothreitol in 50 mM Tris·HCl (pH 7.5) at 37° for 21 min; longer incubation times result in a gradual loss of inhibitory activity. During the activation reaction the red color of the drug is lost at a rate that is directly proportional to the concentration of both HPURa and dithiothreitol; the second-order rate constants are 4.4 and 6.5 M⁻¹·min⁻¹ at 30° and 37°, respectively. Although the activated form (absorption maximum at 260–265 nm) can be readily purified from dithiothreitol and inactive drug by DEAE-cellulose chromatography, it rapidly reoxidizes in air to the colored form. Thus, for the experiments in this report, the activated drug was used without purification, and its concentration was assumed to be equal to the input inactive form.

Enzymes. Deoxyribonuclease I was purchased from Worthington Biochemical Corp. E. coli DNA polymerase I, Fraction 7 (12), and T4 DNA polymerase, Fraction VII (13), were gifts of Dr. P. T. Englund. B. subtilis polymerase I was purified through the phosphocellulose chromatography step by the published method (6). Purification and properties of polymerases II and III of B. subtilis are detailed elsewhere (11). The specific activity of the polymerases II and III preparations used here were increased 2700-fold and 210-fold, respectively, over that of the crude extract of BC26(P) used in the purification. The preparations of each of three B. subtilis DNA polymerases are free of cross contamination by the other two, and polymerases I and II contain negligible nuclease activity. Polymerase III, unlike the other two B. subtilis enzymes, is inhibited (>90%) by 2.0 mM N-ethyl maleimide and 100 μM HPURa, and by raising the assay temperature to 56°. It is also the most salt-sensitive (95% inhibition at 0.20 M KCl). Both polymerases II and III, unlike polymerase I, are inhibited by p-chloromercuri phenyl sulfonic acid, single-
The standard determination volume e-0.003-0.02 enzyme phosphate (ara-CTP).

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Enzyme Assay. In the assay for B. subtilis DNA polymerase III, the reaction mixture contained in a total volume of 0.1 ml, 65 mM Tris·HCl (pH 7.5), 6.5 mM MgCl₂, 3 mM 2-mercaptoethanol, 40 μM each of dATP, dCTP, and dGTP, 10 μM [3H]dTTP, 300 μM activated DNA, 10% glycerol, and 0.003-0.02 units of enzyme in 10 μl of diluent. A unit of enzyme activity catalyzes the incorporation of 10 nmol of total nucleotide into an acid-precipitable product in 30 min at 37°C; the enzyme diluent contains 50 mM Tris·HCl (pH 7.5), 10 mM 2-mercaptoethanol, 0.15 M KCl, 1.0 mg/ml of bovine-serum albumin, and 20% glycerol. The assay procedure is the same as described previously (5).

RESULTS

Activation of HPuR. The effect of HPuR on the time-course of a reaction of B. subtilis DNA polymerase III is shown in Fig. 1. The degree of inhibition by the drug increases with time. Preincubation experiments with various components of the reaction mixture showed that the lag in inhibition is eliminated only when the preincubation mix contained HPuR and dithiothreitol. With preincubated drug, the onset of inhibition is immediate and the incorporation rate is linear with time (see Fig. 5), even when the drug is added after the polymerization reaction has begun. Therefore, dithiothreitol chemically converts the drug to an active form. The activation is essential; no inhibition is obtained with unactivated drug even at 500-times the concentration, that gives 50% inhibition with the activated form. During activation the drug becomes colorless and a new absorption peak in the UV appears. The decoloration is a second-order reaction, but at the high dithiothreitol to HPuR ratios used in the activation reactions, pseudo first-order kinetics are observed. As shown in Fig. 2, the conversion of HPuR to an active inhibitor is also a first-order reaction. Like the rate of color loss, the rate of formation of active inhibitor is augmented by an increase in the dithiothreitol concentration. For unknown reasons, the calculated rate of activation is about twice that of color loss. Several other reducing agents such as reduced glutathione, cysteine, TPNH, and ascorbic acid both activate and reduce the drug, although all do so more slowly than dithiothreitol. Oxidized glutathione neither activates nor decolorizes HPuR. Fig. 3 shows a dose–response curve for inhibition by activated HPuR; the linear relationship between reciprocal velocity and HPuR concentration implies that the reaction of drug with the enzyme is uncomplicated (14).

Enzyme Specificity of HPuR. With the activated drug in hand, we tested the effect of HPuR on four other DNA polymerases. An activated DNA template was used in all cases. Even at 100- to 1000-times the HPuR concentration that gives 50% inhibition of B. subtilis polymerase III, there was no inhibition of B. subtilis polymerase I, E. coli polymerase I, and T4 polymerase (the unactivated drug was also impotent). B. subtilis polymerase II was slightly inhibited; however, it was at least 500-fold less sensitive than B. subtilis polymerase III. Therefore, HPuR is a highly specific inhibitor of B. subtilis polymerase III.

Site of Action of HPuR. Since HPuR does not inhibit several DNA polymerases, it is unlikely that it affects the template and not the enzyme, as some other DNA polymerase inhibitors (15). Indeed, preincubation of the drug and template did not alter the degree of inhibition, nor did the addition of more template to an inhibited reaction. Increasing the template concentration from 0.3 mM, already a saturating level, to 1.0 mM also did not diminish inhibition. Therefore, it seems likely that HPuR interacts directly with DNA polymerase III.
Competitive Attenuation of Inhibition by dGTP. An unexpected finding of the study of toluene-treated cells was that HPUra inhibition is abolished by high amounts of dGTP (16). The effect is the same on polymerase III. In Fig. 4, double-reciprocal plots of reaction velocity as a function of dGTP concentration at two concentrations of activated HPUra are shown. HPUra inhibition is competitive with dGTP concentration since the maximum velocity is not altered by the presence of inhibitor. From these and other data the $K_m$ for dGTP is calculated to be 6$ \times 10^{-7}$ M and the $K_i$ for HPUra to be 3 to 6$ \times 10^{-7}$ M. The following nucleotides at 0.2 mM did not alter the degree of inhibition by 20 mM HPUra: rGTP, dGDP, dGMP, dATP, dTTP, and dCTP; in the same experiment 0.2 mM dGTP abolished inhibition. The only nucleotide, other than dGTP, that we have found to attenuate inhibition is dITP, which, like dGTP, base-pairs with C residues. Maximal activity requires a higher concentration of dITP than dGTP, and thus a higher concentration of dITP is needed to diminish inhibition. With no dGTP in the assay mixture, the inhibition by 3 $\mu$M drug was 86, 63, 25, and 9% at 0, 4.0, 90, and 490 $\mu$M dITP, respectively.

Inhibition by HPURa Is Reversible. Addition of dGTP or dilution of the reaction mix reduced the inhibition by activated HPURa to the level expected of a reversible inhibitor (Fig. 5). Thus, polymerase III is not irreversibly inactivated by HPURa.

Inhibition by HPIso Is Reversed by dATP. HPIso is an analogue of HPURa in which the pyrimidine 2-keto group in HPURa is replaced by an amino moiety. Like HPURa, HPIso must be reduced to be an inhibitor of B. subtilis polymerase III. Unlike HPURa, the inhibition is attenuated by dATP and not dGTP, as also found in toluene-treated B. subtilis cells (17). Thus, an increase in the concentration of dATP from 5 $\mu$M to 200 $\mu$M in an assay reduced the inhibition by 20 $\mu$M HPIso from 87% to 17%, while addition of dAMP, dGTP, dTTP, or dCTP to 200 $\mu$M was without effect.

Template Specificity of Inhibition by Hydroxypyphenylasopyrimidines. The data presented thus far can be explained by postulating that HPURa and HPIso are simple competitive inhibitors of the reaction of dGTP and dATP, respectively, with B. subtilis polymerase III. Polymerase III catalyzes an extensive end-addition reaction in the absence of a full triphosphate complement (11). Under these conditions, it is clear that HPURa inhibits the incorporation of each of the four triphosphates. HPURa (100 $\mu$M) inhibited the incorporation of 10 $\mu$M dATP, dTTP, dCTP, and dGTP alone by 92, 96, 85, and 77%, respectively. A dose–response curve for the inhibition of synthesis with dGTP missing from the full triphosphate complement is plotted in Fig. 6. The $K_i$ is 6$ \times 10^{-7}$ M or about the same as that obtained in the presence of dGTP. Thus, HPURa prevents the incorporation of all four triphosphates while only dGTP attenuates inhibition.

A simple model consistent with the data postulates that HPURa binds to an enzyme–template replicative complex only when C is the base to be copied (T for HPISO); the HPURa hydrogen-bonds to the C residue. This ternary complex, enzyme–template–inhibitor, decreases the amount of enzyme available for polymerization. Since only dGTP is accepted by a polymerase copying a C residue, only dGTP competes with HPURa.

One test of the model is provided by the experiments reported in Table 1. Synthetic polymers were used to limit the template residues to A alone for (dT)$_{10}$-poly(dA) and to A
The homopolymer experiments used 83 \mu M (dT)\textsubscript{10}, 165 \mu M poly(dA), 50 \mu M \[^{3}H\]dTTP, and 0.025 unit of enzyme in a total volume of 0.1 ml. The reaction was done at 25\(^\circ\) for 15 min. Exp. 4 contained 0.28 mM poly(dA-T), 7 \mu M \[^{3}H\]dTTP, and 0.025 unit of enzyme. The reaction was at 37\(^\circ\) for 15 min. (Note that poly[d(A-T)] is a poor template for polymerase III.) The drug concentration was 200 \mu M in Exp. 4 and 100 \mu M in the others.

and T residues for poly[d(A-T)]. The incorporation of dTTP into 0.23 mM oligo(dT)·poly(dA) and 0.28 mM poly[d(A-T)] was not inhibited by HPUs. However, the addition of only 0.004 mM activated DNA, an amount that by itself promoted negligible synthesis, caused the incorporation into the homopolymers to be inhibited 85\% by 100 \mu M HPUs. Even at one-tenth this amount of DNA the activity is halved by HPUs. HPIs, however, markedly inhibited poly[d(A-T)] but not oligo(dT)-poly(dA)-primed synthesis. We interpret these results as indicating that the activated DNA provides a C residue for the HPUs ternary complex and the poly[d(A-T)] provides the T residue for the HPIs complex; evidently these complexes scavenge free enzyme efficiently.

**DISCUSSION**

In order for HPUs to inhibit DNA polymerase activity four conditions must be fulfilled. First, the drug must be chemically modified; reaction with several reducing agents including dithiothreitol, reduced glutathione, cysteine, TPNH, and ascorbic acid is sufficient. While the structure of the active form is unknown, a hydrazine derivative of HPUs is compatible with the data. The reduction of the azo linkage would interrupt the conjugated double-bond system and thereby cause a loss of the visible spectrum, and, typically, hydrazine derivatives oxidize rapidly in air (18), as does activated HPUs. Second, the enzyme must be B. subtilis polymerase III (or presumably similar polymerases from other Gram-positive bacteria); the other four DNA polymerases tested from B. subtilis, E. coli, and T4-infected cells are resistant to the drug. Third, the template probably must contain a C residue for HPUs inhibition (a T residue for HPIs inhibition). Fourth, the dGTP to HPUs ratio must not be too high (dATP for HPIs inhibition).

The inhibition model postulates that HPUs and HPIs occupy the triphosphate binding site of polymerase III, hydrogen bonding to C and T on the template, respectively. These are, of course, not the expected base pairs, but if an enol tautomer of the drug is present, then these specific pairs can be drawn. The 4-hydroxy tautomer of HPIs can make three good hydrogen bonds to T but cannot readily form two hydrogen bonds to the other bases within wobble limits (19). Since HPUs has two carbonyl groups, two monoenoic isomers are possible. With the 4-hydroxy form, two hydrogen bonds can be formed to C but two hydrogen bonds cannot be made readily to G or A within wobble limits; bonding to T brings the keto groups rather close together. It is interesting to note that another uracil analogue, 3-deazauridine, has an enolic 4-hydroxyl group (20). The 2-hydroxy derivative of HPUs can make three hydrogen bonds to C and cannot pair effectively to T and G; base pairing with A may be excluded on geometric grounds since HPUs and HPIs apparently bond like purines. These postulated base pairs do not require any significant tilting of the pyrimidine ring of the drug from the position of this ring in the purines of the Watson–Crick base pairs.

Whatever the precise structure of the base pairs*, the evidence that the drugs hydrogen-bond to the template is quite good. The binding of each of the drugs (which differ only in the pyrimidine moiety) to the template–enzyme complex is strong, but reversible, and base-specific both in its formation and its competition by triphosphates. Hydrogen bond-mediated base pairing is the most reasonable explanation of these properties. It is striking, and somewhat surprising, that the apparent affinity of the enzyme for HPUs and dGTP is the same, while dGDP and rGTP do not compete effectively with dGTP for polymerase III.

The contrast of HPUs with ara-CTP is instructive. While the effect of both drugs is enzyme-specific and reversed by a specific triphosphate for both E. coli (21) and B. subtilis DNA polymerases (11), ara-CTP has no effect on synthesis in the absence of dCTP (11). Ara-CTP acts as a simple competitive inhibitor of dCTP incorporation while HPUs prevents the incorporation of all four triphosphates.

Evidence was presented that HPUs does not alter the template nonspecifically; nor does it inhibit by binding to a triphosphate to form a toxic analogue, since raising the concentration of either dATP, dCTP, or ddCTP does not reverse inhibition, even though the incorporation of each of these triphosphates is prevented by the drug.

There are several persuasive arguments for the inhibition of polymerase III being the site of action of HPUs in vivo. First, the reaction of HPUs with B. subtilis polymerase III is highly selective. Second, the in vitro reaction, like the inhibition of toluene-treated cells, is rapid in onset, is reversible by dilution or by dGTP (dATP for HPIs), and requires a reduced drug at only very low concentrations (the \(K_{i}\), in vitro, is less than 1 \mu M). Third, the pattern of inhibition in vivo in affecting only the host, replicative DNA synthesis is clearly compatible with an action on a specific DNA polymerase. Therefore, it seems likely that B. subtilis polymerase III, like the correspondingly numbered E. coli enzyme (22), is necessary for DNA replication (see note added in proof).

**NOTE ADDED IN PROOF**

We have selected a spontaneous mutant of B. subtilis resistant to HPUs and HPIs. The polymerase III of this mutant is resistant to the drugs, thereby confirming polymerase III is necessary for replication.

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* Dr. R. Wells has suggested an alternative bonding scheme that does not postulate enolic tautomers but rather base-pairing through the N-1 hydrogen, a hydrogen from the proximal nitrogen of the presumed hydrazine link, and the 2-carbonyl of HPUs or the amino of HPIs (personal communication).
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