MUTATIONAL ALTERATION OF BACILLUS SUBTILIS DNA POLYMERASE III TO HYDROXYPHENYLAZOPYRIMIDINE RESISTANCE: POLYMERASE III IS NECESSARY FOR DNA REPLICATION

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SUMMARY: A spontaneous mutant of Bacillus subtilis resistant to killing by two hydroxyphenylazopyrimidines has been isolated. The DNA polymerase III of this mutant is resistant to inhibition by these drugs. The K_i for 6-(p-hydroxyphenylazo)-uracil (HPUra) is 20 μM, about 40 times higher than the K_i of the wild-type enzyme. The mutant and wild-type polymerases behave similarly during purification, are sensitive to N-ethylmaleimide and to 0.1 M KCl, and have the same K_m for dGTP (0.5 μM). The HPUra inhibition of both enzymes is attenuated competitively by dGTP. We conclude that polymerase III is the target for hydroxyphenylazopyrimidines in vivo, and since the drugs specifically inhibit replicative DNA synthesis, polymerase III is necessary for DNA replication.

Our previous work has demonstrated that Bacillus subtilis, like Escherichia coli, contains three distinct DNA polymerases (1-3). Polymerase I acts in the repair of chromosomal damage (1, 4). We concluded that B. subtilis polymerase III is necessary for DNA replication, since HPUra, an inhibitor of replicative DNA synthesis (5), selectively inhibits polymerase III of B. subtilis (3). The inhibition by HPUra is reversed competitively by dGTP while the inhibition by a related drug, 6-(p-hydroxyphenylazo)-isocytosine (HPIso), is reversed by dATP. Both drugs must first be reduced chemically in order to act as inhibitors. Others have also shown an effect of arylazopyrimidines on a DNA polymerase fraction of B. subtilis (6, 7). We report here the isolation of a mutant resistant to HPUra and HPIso whose polymerase III activity is resistant to these drugs, thereby showing that polymerase III is indeed the target for HPUra and HPIso in vivo and confirming the role of this enzyme in DNA replication.

METHODS AND MATERIALS

The mutants were obtained from strain F2, a derivative of HA101(59)F (ref. 1) which

lacks DNA polymerase I and has an additional mutation causing partial resistance to HPUra in vivo but not in vitro. Since most HPUra-resistant mutants are still HPIso sensitive, we attempted to minimize this trivial class of mutants by simultaneous selection with both HPUra and HPIso. About 10^8 cells of F2 were spread on CHT-50 plates (8) supplemented with 3 μg/ml HPUra and 2 μg/ml HPIso. After two days at 30°, the resistant colonies (10-100 per plate) were isolated. Toluene-treated cells (9) of one of the 19 mutants analyzed, F22, are resistant to both drugs.

The details of the polymerase assay have been described (2). Except where indicated otherwise, the assay mixture contained in a total volume of 0.1 ml, 15 mM potassium phosphate, pH 7.4, 6.5 mM $MgCl_2$, 10 μM each of dATP, dCTP, dGTP, and [^{3}H] dTTP, 3 mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 15% glycerol, 0.35 mM activated salmon testes DNA, and enzyme. The reactions were performed at 30° for 15 min. One unit of enzyme catalyzes the incorporation of 10 nanomoles of total nucleotide into an acid-insoluble product in 30 min. The 210-fold purification of wild-type B. subtilis polymerase III has been detailed previously (2). Polymerase III from F22 was purified similarly. Before use in enzyme assays, both HPUra and HPIso were reduced to the active form (probably the hydrazine derivative) by dithiothreitol (3).

RESULTS

F22 is a spontaneous mutant of F2 resistant to HPUra and HPIso. After toluene treatment, 5 μM HPUra inhibited DNA synthesis in F2 by 58% but had no effect on F22, and 50 μM HPUra inhibited F22 DNA synthesis by only 26%. The inhibition of DNA synthesis in crude extracts of F22 and F2 is detailed in Table I. Since both F2 and F22 lack polymerase I, the DNA synthesis is the result of polymerases II and III. N-ethylmaleimide (NEM), an inhibitor of polymerase III but not of polymerase II (2), reduced the synthetic activity of both extracts similarly; therefore, the activity of both polymerases II and III is about the same in F22 and F2. However, whereas 10 μM HPUra or 10 μM HPIso reduced the activity of F2 extracts by about one-half, this level of drug did not inhibit F22. At 200 μM HPUra, the

TABLE I

Drug sensitivity of DNA polymerase activity of mutant and wild-type extracts

Addition to assay	Polymerase specific activity (units/mg protein)	
	Wild type	Mutant
None	0.92	0.99
10 μM HPUra	0.56	1.04
10 μM HPIso	0.41	0.99
100 μM HPUra	0.23	0.79
200 μM HPUra	-	0.74
5 mM NEM	0.22	0.29

Sonic extracts of F2 and F22 were prepared as described previously (1).

polymerase III activity in F22 was inhibited only about 35%. The degree of inhibition of wild-type polymerase III was not altered by the presence of mutant extract.

An important test of the molecular basis for the HPUra resistance of F22 employed purification of the F22 polymerase III essentially by the procedure developed for the wild-type enzyme (2). The second DEAE-cellulose column resolves two DNA polymerase activities (Fig. 1): polymerase II is eluted by the 0.1 M phosphate wash and polymerase III, which is NEM-sensitive, by the phosphate gradient at about 0.3 M. This is the same chromatographic pattern observed during purification of the wild-type enzyme (2). The polymerase III was purified further by phosphocellulose chromatography and the drug sensitivity of this preparation was characterized (Fig. 2). The mutant polymerase III is 40 times less sensitive to HPUra than the wild-type enzyme in the presence (Fig. 2A) or absence of dGTP (Fig. 2B); these differences are similar to those found with crude extracts. Therefore, the polymerase III

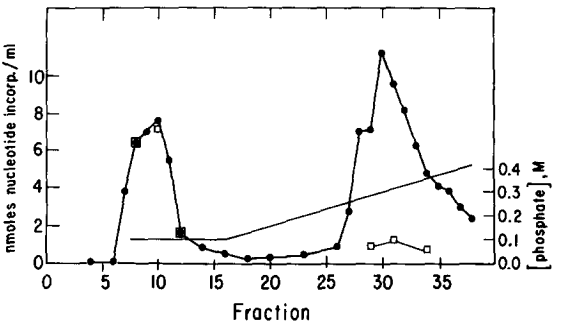

Fig. 1. Resolution of F22 polymerases II and III by DEAE-cellulose chromatography. The enzyme from the first DEAE-cellulose column (ref. 2) was applied to a 20 x 1.5 cm DEAE-cellulose column equilibrated with 0.05 M potassium phosphate, pH 7.4, 0.05 M 2-mercaptoethanol, 2 mM EDTA, and 20% glycerol. The column was washed with two volumes of 0.1 M potassium phosphate, pH 7.4, 0.05 M 2-mercaptoethanol, 2 mM EDTA, and 10% glycerol. A 310 ml 0.1 to 0.5 M potassium phosphate (pH 7.4) gradient containing 5 mM glutathione, 2 mM EDTA, and 10% glycerol was applied, and 8.0 ml fractions were collected. ●—●, standard polymerase assay; □—□, same except for addition of 5 mM NEM.

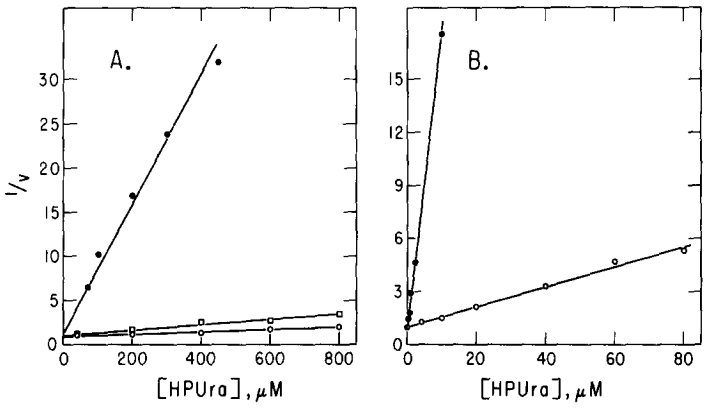

Fig. 2. Response of purified mutant and wild-type polymerases to HPUra. The reaction mixtures contained 10 μM each of dATP, dCTP, and [^{3}H] dTTP, the indicated amounts of dGTP and activated HPUra, and either mutant or wild-type polymerase III. The velocity (v) of the reaction in the absence of inhibitor was set equal to 1.0. A. O—O, mutant enzyme and 10 μM dGTP; □—□, mutant enzyme and 4 μM dGTP; ●—●, wild-type enzyme and 10 μM dGTP. B. O—O, mutant enzyme and no dGTP; ●—●, wild-type enzyme and no dGTP.

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of the mutant is itself resistant to HPUra. The data in Fig. 2 imply that HPUra inhibition of the mutant polymerase III occurs by the same mechanism as inhibition of the wild-type enzyme, notwithstanding the quantitative differences, since dGTP attenuates the inhibition of both enzymes. This conclusion is confirmed by the Lineweaver-Burk plots (Fig. 3) which

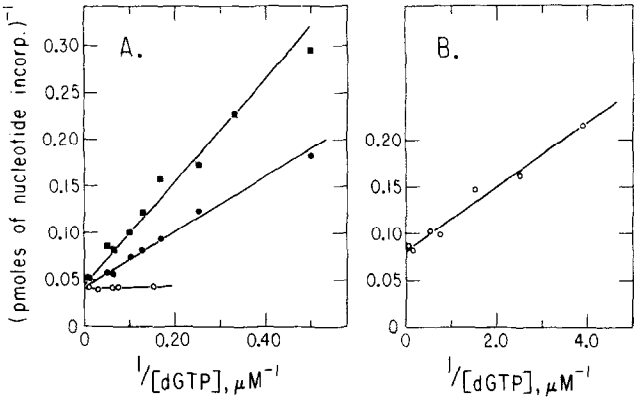

Fig. 3. Lineweaver-Burk plots of the relationship between dGTP, HPUra, and purified mutant polymerase III. A. The reaction mixtures contained 40 μM each of dATP and dCTP, 10 μM [^{3}H] dTTP, the indicated amounts of dGTP, and 0.005 unit of enzyme. The assay was at 30° for 10 min. O—O, no HPUra; ●—●, 200 μM HPUra; ■—■, 400 μM HPUra. B. To minimize substrate depletion at the low levels of dGTP used, the reaction time was decreased to 7 min.

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show that HPUra inhibition of F22 polymerase III is competitive with dGTP. High concentrations of dCTP, dATP, and dTTP do not attenuate the HPUra inhibition of either the F2 or the F22 polymerases. The F22 polymerase has a K_m for dGTP of 0.5 μM and a K_i of 20 μM (Figs. 2, 3). Wild-type polymerase III has a K_m and K_i of about 0.5 μM (3). Thus, the apparent affinity of the F22 polymerase for dGTP has remained the same while the affinity for HPUra has been reduced to one-fortieth that of the wild-type enzyme. The F22 polymerase has a similarly reduced sensitivity to HPIso.

The F22 mutation has not altered several other properties of polymerase III. NEM (2 mM) inhibits wild-type and mutant polymerases by 80%. KCl at 0.1 M inhibits both enzymes by 70% and at 0.25 M abolishes synthesis. The alternating copolymer poly (d [A-T]) promotes synthesis by both enzymes at only one-fiftieth of the rate of activated DNA. The mutant enzyme appears to be more thermolabile, since at 43° it loses activity at twice the rate of the wild-type enzyme. The alteration in polymerase III does not change the growth rate of F22 discernably, since at temperatures ranging from 30° to 52° F22 grows at the same rate as F2 in several different media.

Unlike a number of other HPUra-resistant mutants, including F2, which are not altered in polymerase III activity, F22 is still resistant to HPUra and HPIso in the presence of 0.45 M Tris-HCl, pH 7.3. Presumably, Tris facilitates penetration of the drugs (10). The use of high Tris concentrations to expedite mutant isolation is being explored.

DISCUSSION

Since HPUra specifically inhibits replicative DNA synthesis and an alteration in the polymerase III of F22 prevents killing by HPUra, polymerase III is necessary for DNA synthesis and is an important target for the drug *in vivo*. It is likely that polymerase III catalyzes the bulk synthesis of DNA.

In an earlier study of the wild-type polymerase III (3), we suggested that HPUra binds at the triphosphate binding site of the enzyme and base-pairs specifically with the template deoxycytidylate residues. The resulting non-reactive ternary complex efficiently scavenges free enzyme. The model explains the specificity of dGTP antagonism of HPUra inhibition and why the K_i is independent of the dGTP concentration. The inhibition of the mutant enzyme appears to occur by the same mechanism HPUra inhibition is attenuated specifically by dGTP in a competitive fashion and the K_i is again independent of the dGTP concentration. It is interesting, therefore, that the apparent affinity of the mutant enzyme for dGTP is unaltered by the F22 mutation, while the affinity for HPUra is reduced forty-fold; a mutation which greatly increased the K_m for dGTP would probably be lethal. The mutational alteration of the F22 polymerase must create a negative interaction with a portion of the HPUra molecule which is not shared by dGTP. Alkylation of chymotrypsin can cause an increase in the K_m or K_i for some substrates and inhibitors but not for others binding at the same active site (11).

The F22 mutation is probably in the structural gene for polymerase III, since it results in a qualitative change in the properties of the enzyme. We name the polymerase III locus altered in F22, *polC*, in order to maintain the same nomenclature as in *E. coli* (12).

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