Ribonucleoside and Deoxyribonucleoside Triphosphate Pools during 2-Aminopurine Mutagenesis in T4 Mutator-, Wild Type-, and Antimutator-infected *Escherichia coli*

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Ribonucleoside and deoxyribonucleoside triphosphate pools have been measured in *Escherichia coli* infected with bacteriophage T4 DNA polymerase mutator, wild type, and antimutator alleles during mutagenesis by the base analogue 2-aminopurine. ATP and GTP pools expand significantly during mutagenesis, while CTP and UTP pools contract slightly. The DNA polymerase (gene 43) alleles and an rII lesion perturb normal dNTP pools more than does the presence of 2-aminopurine. We find no evidence that 2-aminopurine induces mutations indirectly by causing an imbalance in normal dNTP pools. Rather, it seems likely that, by forming base mispairs with thymine and with cytosine, 2-aminopurine is involved directly in causing bidirectional A-T ⇔ G-C transitions. The ratios for 2-aminopurine deoxyribonucleoside triphosphate/dATP pools are 5–9% for tsL56 mutator and 1–5% for tsL141 antimutator and 43* alleles. We conclude that the significant differences observed in the frequencies of induced transition mutations in the three alleles can be attributed primarily to the properties of the DNA polymerases with their associated 3'-exonuclease activities in controlling the frequency of 2-aminopurine-cytosine base mispairs.

Base substitution mutation frequencies are affected by replication and repair enzymes (1–7), base context surrounding a mutated site (8–17), and both relative (13, 14, 18–24) and absolute (13, 21) concentrations of deoxyribonucleoside triphosphate pools. AmPur, a base analogue of adenine, induces bidirectional A-T ⇔ G-C and G-C ⇔ A-T base substitution transition mutations in *Escherichia coli* and bacteriophage T4 at frequencies substantially above spontaneous backgrounds (for a review, see Ref. 25). The molecular basis for AmPur's mutagenicity is its ability to form relatively stable base mispairs with Thy and with Cyt when present either as a template base on DNA (15) or as a deoxyribonucleoside triphosphate substrate, dAmPurTP (21, 26, 27).

In this paper, we report measurements of deoxyribonucleotide triphosphate pools during AmPur mutagenesis in T4 tsL56-UV199 mutator, 43*-UV199-, and tsL141-UV199 antimutator T4-infected *E. coli*. These pool size determinations serve three primary purposes with regard to mutagenesis. First, knowledge of relative magnitudes of dAmPurTP/dGTP ratio relates to AmPur's direct mutagenic potential in the G-C ⇔ A-T pathway where dAmPurTP is inserted opposite template Cyt in competition with dGTP. Second, a measurement of dAmPurTP/dGTP pools for each of the three T4 gene 43 alleles will allow us to determine if the widely different AmPur-induced mutagenic rates observed in mutator, wild type, and antimutator backgrounds (see e.g. Ref. 28) might be attributed to differences in the metabolism of the analogue in the three genetic backgrounds. Alternatively, differences in AmPur mutagenesis in the T4 gene 43 alleles may be caused primarily by differences in the insertion and proofreading properties of the mutator, wild type, and antimutator DNA polymerases. Finally, the pool size measurements should allow us to determine whether AmPur exerts an indirect effect on mutagenesis by perturbing pools of the four common dNTPs.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

A measurement of the pool size of 2-aminopurine deoxyribonucleoside triphosphate allows one to estimate the mutagenic potential of the base analogue, provided that the mutations occur as a direct result of AmPur incorporation into DNA. The first step in the induction of an A-T ⇔ G-C transition by 2-aminopurine presumably requires the replacement of Ade by AmPur opposite a template Thy site. In general, the rates of insertion into DNA for any two competing nucleotide substrates should be in proportion to their relative dNTP pools. A multienzyme deoxyribonucleotide biosynthetic complex encoded by T4 (see e.g. Ref. 31) may act to concentrate dNTPs at the replication fork. Although replication fidelity can be influenced by absolute dNTP concentrations (13, 21), it is the ratio of competing dNTP substrates...
which is expected to play a dominant role in mutagenesis (21).

For the special case of AmPur and Ade deoxyribonucleo- tidies competing for insertion opposite Thy,}

$$\frac{I(\text{AmPur})}{I(\text{Ade})} = \frac{[\text{dAmPurTP}]}{[\text{dATP}]} e^{-\frac{\Delta G}{RT}} \tag{1}$$

where $I(\text{AmPur})/I(\text{Ade})$ is the ratio of inserting dAmPurTP and dATP into DNA, $[\text{dAmPurTP}]/[\text{dATP}]$ is the ratio of the pool concentrations for the two competing substrates, and $\Delta G$ is a measure of the average free energy difference between A·T and AmPur·T base pairs in DNA. Estimates of $\Delta G$ during DNA replication are in the range 1-1.3 kcal/mol (21, 32).

Pool concentrations of dAmPurTP appear to be similar, within a factor of 2, in T4 tsL56 mutator, 43+, and tsL141 antimutator backgrounds (Table 1). dATP concentrations also appear to be similar in 43+ and tsL141 antimutator back- grounds, but may be about 5-fold lower in tsL56 mutator-infected cells (Table 1). Although we strongly suspect that greater levels of AmPur-induced mutagenesis in L56 mutator backgrounds (see e.g. Ref. 28) are attributable primarily to a reduction in the 3’-exonuclease proofreading activity of L56 DNA polymerase in comparison to the proofreading capabilities for the active 43+ and highly active L141 polymerases (3, 21, 26), the increased $[\text{dAmPurTP}]/[\text{dATP}]$ ratio might be responsible for perhaps as much as a 5-fold greater mutation rate in tsL56-infected cells.

The second step in the A·T $\rightarrow$ G·C pathway involves the insertion of HmdCTP in place of dTTP opposite template AmPur. From Table 1 we note that the pool of HmdCTP is about 1.5-fold larger in tsL141 antimutator-infected cells compared to 43+ and about 5-fold larger in tsL141 compared to tsL56 mutator. However, these differences would not be expected to have a significant effect on AmPur mutagenesis since the frequency of HmdCTP insertion opposite AmPur should be much more dependent on the HmdCTP/dTTP ratio than on the absolute magnitude of HmdCTP concentration (21). The free energy difference, $\Delta G_2$, for inserting HmdCTP versus dTTP opposite AmPur is estimated to be about 1.8 kcal/mol (15).

The mutation frequency at each individual site on DNA depends on nearest-neighbor base-stacking partners and surrounding base composition (8-17). However, one can utilize the dNTP pool size ratios and measurements of base-pairing free energy differences to estimate the average mutation frequency, neglecting site-specific effects. This type of estimate is instructive in the case of mutagenesis by 2-aminopurine as it allows an evaluation to be made of the relative importance of proofreading in controlling the relative mutation frequencies in tsL56 mutator, 43+, and tsL141 antimutator genetic backgrounds.

We will now calculate two extreme cases to characterize AmPur-induced A·T $\rightarrow$ G·C transition rates, i.e. with and without proofreading, using Equation 1 with dNTP pool data and $\Delta G$ estimates for each step in the mutational pathway. In the absence of proofreading, the only available nucleotide discrimination step occurs during insertion. For each nucleotide insertion in the AmPur-induced A·T $\rightarrow$ G·C pathway, the formation of AmPur·T and AmPur·C mispairs, the mutation rate is given by the product of the pool ratios $[\text{dAmPurTP}]/[\text{dATP}]$ and $[\text{HmdCTP}]/[\text{dTTP}]$ multiplied by exp $-((\Delta G_1 + \Delta G_2)/RT)$ (see Equation 1). Taking the average pool size ratios $[\text{dAmPurTP}]/[\text{dATP}] = 0.02$ and $[\text{HmdCTP}]/[\text{dTTP}] = 1$ and $\Delta G_1 = 1.3$ kcal/mol and $\Delta G_2 = 1.8$ kcal/mol (15, 21, 32), we obtain an AmPur-induced A·T $\rightarrow$ G·C muta-
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RESULTS

A small aliquot (ca. 10 µl) of prepared sample was injected into reverse phase system I (Fig. 1). From this injection we quantitated nucleotide concentrations (CTP, UTP, GTP, ATP, ADP, and DADP). A shoulder often seen on the tail of CTP, distinctly broader than the other peaks, was further analyzed. CTP fractions from system I were re-injected into the same system to confirm quantitation. A quantity sufficient to double the height for each of the normal ribonucleoside triphosphates was added to a sample reaction mixture and analyzed. The tailing of the shoulder was observed to increase to twice, due to an enormous and variable quench in these large peaks.

Data Acquisition and Analysis

UV absorption data were collected simultaneously at 254 and 345 nm and later processed using Application software (Dynamic Solutions, Pasadena) in conjunction with an Apple IIc computer. This process detection and integration of each peak was then used to determine molar extinction coefficients. These were used to determine the quantity of each nucleotide, and the peak height for each normal ribonucleoside triphosphate was estimated by adding a small aliquot to the sample reaction mixture. The identity of each nucleotide was confirmed by injection of each nucleotide into reverse phase system II (with standard chromatograms, Fig. 3). The retention time and ratio of absorbance of all nucleotides in system II were compared to standard chromatograms. Calculations of molar quantity from the peak areas of UV-360, UV-395, UV-410, and UV-420 were made from large aliquots of standard solutions. A small aliquot of sample solution was also injected into reverse phase system II. The peak height was normalized to the standard solution. The ratio of absorbance at 395 nm to 410 nm was calculated. This ratio was used to calculate the concentration of each nucleotide.

Fig. 1. Representative chromatograms of A. thaliana mutagenized T4 DNA nucleotide injected into reverse phase system I at a flow rate of 1 ml/min. Ribonucleoside triphosphates are represented as follows: U, CTP; U, UTP; G, GDP; dAMP (dG, dT, dC, dAMP); T, ATP; A, ADP; dATP.

Fig. 3. A 200 µl aliquot of samples from Fig. 1, representing 2 x 10^12 cells, was injected into reverse phase system I at a flow rate of 1 ml/min. The A254 and A360 absorbance ratios for each nucleotide were determined.
A second approach exploited the difference in elution order or retention time as a function of base moiety observed in comparison of reverse phase system I vs. system II. The positive amphoteric nature of AMP and GMP and the negative ion properties of DMP and DMP had a marked effect on the elution order of nucleotides when T-25 nm was injected as a flow rate of 0.5 ml/min into reverse phase system I. The insert indicates a portion of the 25 nm tracing taken from this same chromatogram. Ribo- and deoxyribonucleotide triphosphates are labelled as in Fig. 2. With the addition of dA, dAMP; dG, dGMP; dT, dTMP; and dU, dUMP; which are compounds not found in our extract.

Drucking concentrations of DNA and RNA precursors in E. coli-mutagenized and non-mutagenized T4-infected E. coli CB3 are shown in Table I. The pools of ATP and GTP expanded during Amphi mutagenesis in all extracts. ATP was the most abundant triphosphate in all extracts, regardless of treatment. The AMP/GMP ratio was highest during mutagenesis. While the porine deoxyribonucleotide triphosphates may expand and contract upon exposure to mutagens, data are equivocal. Moreover, the gene 43 alleles and the II11 lesion appear to be more important than Amphi mutagenesis in perturbing nucleotide pools (compare [Ref. 39 to Table II]. The daMPurTP/GTP ratio for U919-L16 is slightly higher (5 to 9%) than U919-L14 (4 or 6 to 14) and U919-LG11 (1 to 7) (Table II). Mutation rate data appear in Table III.

Table I. Nucleotide pools during Amphi mutagenesis of T4 and U919 bacteriophage. All data are expressed as micromoles/gene 43. All extracts were passed through C18 to remove bases and fractionated on an HPLC column and analyzed by reverse phase systems I and II as described in EXPERIMENTAL PROCEDURES.

| U919-L16 | U919-L142 | U919-LG141 |
|----------|----------|----------|
| average range average range average range |
| CTP 18 16-20 6.4 5.6-7.9 14.4 2.8-20 |
| UTP 14 16-16 2.25 3.5-5.0 14.6 5.0-24 |
| GTP 19 17-20 25.5 19-28 17 12-22 |
| ATP 92 77-100 95 89-101 81.5 70-93 |
| IMCTP 2.6 1.5-3 5.5 1.9-9.6 8.2 2.1-14 |
| TTP 2.5 1.5-3 2.6 2.3-2.8 13.6 11-16 |
| GTP 5.9 4.0-11 6.8 4.0-8.0 6.2 1.7-3 |
| GATP 2.2 1.7-3 8.0 4.0-12 9.5 7-12 |
| aMPT 0.12 0.06-0.2 0.2 0.06-0.2 0.24 0.16-0.32 |

The results have been expressed as nucleotide pools in DNA-defective mutant mutants of T4 using an assay involving 32P-triphosphate (30). We compared our analytical technique to that of Matthews [39] using counting 200 ml. of T-25-infected E. coli II grown in "media A." Extracts were either treated withwant or were passed through a C18 column equilibrated with 1 M triethylammonium bicarbonate buffer pH 8.5. Preliminary analysis of extracts on the SAX system with a 5-m phase system II (Ref. 39] yielded a larger pool of aMPurTP and an underestimate of the CMPurTP pool. The agreement with Matthews [39] high-treated extracts could not be analyzed analyzed or was shown by reverse phase system I. The DNA-pools were then analyzed on a reverse phase system I.

Table II. Nucleotide pools from control extracts of T4 and U919 bacteriophage. All data are expressed as micromoles/gene 43. All extracts were assayed and analyzed for Table I.

| U919-L16 | U919-LG141 |
|----------|----------|
| molecules per cell x 10^5 |
| CTP 33 5.5 20 |
| UTP 23 7.1 20 |
| GTP 12 1.6 12 |
| ATP 33 9.4 30 |
| IMCTP 37 0.5 9 |
| TTP 2.7 9 |
| GATP 6.4 3.7 3 |
| GTP 1.5 1.4 5.8 |

Table III. T1 U919 conversion frequencies in gene 43 alleles expressed as lethal on restrictive host, E. coli B.

| U919-L16 | U919-LG141 |
|----------|----------|
| Amphi-induced 2 x 10^-3 4 x 10^-5 3 x 10^-7 |
| Spontaneous 5 x 10^-5 3 x 10^-7 1 x 10^-9 |

Fig. 3. Ribo- and deoxyribonucleotide triphosphate standards. A. 0.2 ml of H2O, AMP, GMP, UMP, and GTP, and 2 ml of the other compounds are injected at a flow rate of 0.5 ml/min into reverse phase system I. The insert indicates a portion of the 25 nm tracing taken from this same chromatogram. B. The deoxyribonucleotide triphosphates are labelled as in Fig. 2.