Potential of *Escherichia coli* GTP Cyclohydrolase II for Hydrolyzing 8-Oxo-dGTP, a Mutagenic Substrate for DNA Synthesis*

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Organisms are always exposed to attacks of active oxygen species that are generated not only by exogenous environmental factors such as ionizing radiation and redox-cycling chemicals but also through endogenous oxygen metabolism (1, 2). Active oxygen species are able to produce modifications in proteins, lipids, carbohydrates, and nucleotides (3). Among the various types of DNA damage caused by active oxygen species, 8-oxo-7,8-dihydroguanine (8-oG)\(^1\) may be responsible for a significant portion of spontaneous mutations, which would lead to induction of cancer as well as other age-related disorders (4). The 8-oG residue present in DNA efficiently induces G:C \(\rightarrow\) T:A transversion both *in vitro* and *in vivo* (5, 6), and two genes of *Escherichia coli*, mutM and mutY, are involved in the repair process of this lesion (7–10). The protein encoded by the mutM gene (MutM or Fpg protein) possesses a DNA glycosylase activity that specifically removes 8-oG from DNA, whereas the MutY protein has an enzymatic activity that removes an adenine base from an A:8-oG pair as efficiently as from an A:G pair in DNA.

Oxidation of guanine also proceeds in the form of free nucleotides, and an oxidized form of dGTP, 8-oxo-dGTP, is a potent mutagenic substrate for DNA synthesis (11). In contrast to the consequence of 8-oG arising in DNA, 8-oxo-dGTP can induce A:T \(\rightarrow\) C:G as well as G:C \(\rightarrow\) T:A transversion (9). In *E. coli*, 8-oxo-dGTP can be eliminated from the nucleotide pool by MutT protein, the product of the mutT gene, which hydrolyzes the mutagenic nucleotide to 8-oxo-dGMP (11). 8-Oxo-dGMP is further hydrolyzed by other enzymes and finally eliminated from the cellular nucleotide pools (12). Lack of the mutT gene increases the occurrence of A:T \(\rightarrow\) C:G transversion but not G:C \(\rightarrow\) T:A (13). In the mutT mutant, 8-oG misincorporated opposite the C residue of the template may be removed by the MutM protein before the next round of DNA replication. MutT homologues have been found in human, mouse, and rat (14–16). Thus, 8-oxo-dGTP hydrolysis by MutT and related proteins may play a crucial role in reducing spontaneous mutation frequency in a wide range of organisms.

In view of the importance of preserving genetic material, it is not surprising that organisms possess multiple pathways for removing oxygen-induced lesions. For example, oxidative pyrimidine residues, such as thymine glycol, in DNA can be removed by both endonuclease III and endonuclease VIII glycosylases in *E. coli* (17, 18). In mammalian cells, N-methylpurine-DNA glycosylase functions to repair 8-oG in DNA, in addition to 8-oxoguanine DNA glycosylase, a mammalian MutM homologue glycosylase (19, 20). Then, we have pursued a possibility that *E. coli* may possess a backup system for MutT, which plays a crucial role in high fidelity of DNA replication. By phenotypic rescue, we were able to clone a second *E. coli* gene that suppresses increased mutation frequency of mutT mutants. The gene was subsequently shown to be the ribA, which encodes GTP cyclohydrolase II (GCHII) that catalyzes the first step of riboflavin biosynthesis (21–23). We have demonstrated that GCHII can indeed hydrolyze 8-oxo-dGTP and its ribonucleotide counterpart, at a rate higher than that for GTP, the substrate for riboflavin biosynthesis.

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\(^1\) The abbreviations used are: 8-oG, 7,8-dihydro-8-oxoguanine; 8-oG-dGTP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate; 8-oG-dGMP, 8-oxo-7,8-dihydroguanosine 5′-monophosphate; GCHII, GTP cyclohydrolase II; 8-oxo-GTP, 8-oxo-7,8-dihydroguanosine 5′-triphosphate; LB, Luria-Bertani broth; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; 8-oxo-GMP, 8-oxo-7,8-dihydroguanosine 5′-monophosphate; Fapy, (formamidopirimidine) 2,6-diamino-4-hydroxy-5-(N-methylformamido)-pyrimidine; kbp, kilobase pair; HPLC, high pressure liquid chromatography.
**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The bacterial strains used in this study were all derivatives of _E. coli_ K12. Strain SY11 is a _mutT_Cm derivative of SY5, which is an _F_ factor-negative derivative of JM107 (24). Strain T-198 has the _mutT1_ allele (25). T-198R is a _ribA_Km derivative of T-198. The _mutT:Cm_ and _ribA:Km_ alleles were made by the gene replacement method using the _JCT762_ (recBC _shb_ _str_ _tra_ _X_-strain) (26, 27). An episome from strain CC101, derived from P90C (28), was transferred to SY11, yielding strain SY11F. The _lacZ_ gene of the episome carries _G → T_ transversion mutation at position 461 of the gene, and thus, only A:T to C:G transversion would revert the strain to the wild-type phenotype (28). XL1-Blue MRF (Stratagene) was used as the host of the M13 phage. Plasmid pMT11, carrying a _mutT:Cm_ allele, is a derivative of pBR322 (29). Plasmid pBST was constructed by inserting a DNA fragment carrying the _mutT_ gene from pSK6 into _pBluescript_ II. Plasmid pGRa, carrying the _E. coli ribA_ gene, is a derivative of _pBluescript_ II. Plasmid pBS29 possesses the _BgII-EcoRI_ fragment carrying the _ribA_ gene. A His-tag fusion vector, pQE9, was purchased from Qiagen. The 0.9-kbp DNA fragment encoding the full-length GCHII was amplified by polymerase chain reaction (PCR) using pBS29 as a template, using primers A (5'-CATCGACGTGAAAACTGTTGGCA-3', corresponding to sequence 1 to +21 of the _ribA_ gene) and B (5'-TGGAAACGACGGCGATT-3', which is an M13 universal primer). The PCR fragment was cloned into pKlenow polymerase, then _SacI_ and ligated to the Klenow polymerase-treated BamHI site and SalI site of vector pQE9, yielding pH29. Vector plasmids, pJKKmn(–), pT718R, pT721R, and _pBluescript_ II, were from Stratagene (La Jolla, CA).

**Reagents and Media**—The _Luria-Bertani_ (LB) broth, LB plates, _M9_ medium, and phosphate buffer used were described (30). The minimal glucose (or lactose) agar medium is composed of M56 salt plus 0.35% glucose (or lactose), 1.5% agar and supplemented with 1 mM sodium acetate. Glucose (or lactose) agar medium is composed of M56 salt plus 0.35% glucose (or lactose), 1.5% agar and supplemented with 1 mM sodium acetate.

**Purification of GCHII**—To purify GCHII from _E. coli_ XL1-Blue MRF, carrying pH29 at 37 °C for 16 h. The cells (5.5 g) were suspended in 15 ml of a buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl) and disrupted by sonication. The supernatant was loaded onto a _Ni-NTA_ agarose (Qiagen) column. After the column was washed with 200 ml of 200 mM Tris-HCl, pH 8.0, 7.5 mM MgCl2, and 20 mM EDTA, containing 1 M NaCl, the enzyme was eluted with a gradient of 0 to 0.5 M imidazole in 150 ml of the buffer. Samples were used for analysis by SDS-polyacrylamide gel electrophoresis and for enzyme assay.

**RESULTS**

**Isolation of an _E. coli_ Gene That Suppresses the Mutator—** The mutator phenotype of strain SY11F, defective in the _mutT_ gene, is evident when colonies are produced on MacConkey agar. Numerous red papillae appeared, reflecting the frequent occurrence of A:T to C:G transversion in the _lacZ_ gene, and this was the basis for screening of a second gene that suppresses the _mutT_ mutator. DNA prepared from _E. coli_ SY11 cells was digested with EcoRI, and the resulting DNA fragments were inserted into a multi-copy plasmid vector, _pBluescript_ II. The genomic library was introduced into _SY11F_ cells, and bacterial colonies that exhibit a reduced number of papillae were isolated. Among several thousand transformants examined, one clone which showed distinctly lower mutation frequencies was isolated. The plasmid carried a 4.5-kbp insert, and further analyses revealed that a 1.4-kbp _BgII-EcoRI_ fragment possessed an ability to suppress the mutator phenotype. The DNA fragment was inserted into _pBluescript_ II, yielding plasmid pBS29.

To ascertain the extent of suppression of the mutator effect, _GCHII_ Hydrolyzes Mutagenic 8-Oxo-dGTP
more precisely, mutation frequencies of three distinct characters were determined. As shown in Table I, the mutation frequencies of SY11F cells with pBS29 were considerably lower than those of SY11F cells with the vector alone. The extent of suppression achieved with pBS29 was, however, low compared with the effect of the authentic mutT gene, located on pBST.

Identification of the Gene—The nucleotide sequence of the 1.4-kbp insert of pBS29 was determined using the dideoxy method. The sequence contained a 588-bp open reading frame, which would code for a protein with 196 amino acid residues. Computer-aided analysis revealed that it is identical to the ribA gene, encoding GCHII of E. coli (23). To see whether the gene of pBS29 can complement the ribA-defective character, strain JC7623: ribA was transformed with plasmid pBS29. The transformant was able to grow in a medium without riboflavin, indicating that the plasmid carried the ribA gene.

GCHII is one of the enzymes involved in biosynthesis of riboflavin. It catalyzes conversion of GTP to 2,5-diamino-6-hydroxy-4-(5-phosphoribosylamino)-pyrimidine, releasing formate and pyrophosphate (21). This GTP pyrophosphorylase partly mimics the reaction catalyzed by MutT protein, although there is no ring opening in the latter reaction (see Fig. 4). We have then asked whether GCHII acts on oxidized forms of guanine nucleotide.

Purification of the Gene Product—GCHII protein was overproduced in SY11F cells carrying pBS29 and purified to apparent physical homogeneity. His-tag GCHII, which carries additional amino acids residues (MRGSH-GC) at the N-terminal, was also purified from an extract of XL1-Blue MRF' cells harboring pHT29, by using a different purification procedure. On SDS-polyacrylamide gel electrophoresis, the 21.8-kDa GCHII protein moved slightly faster than did the 25.8-kDa His-tag GCHII protein (Fig. 1A). As shown in Fig. 1B, GTP cyclohydrolase activity was evidently present in the preparation of GCHII. A similar result was obtained with His-tag protein (data not shown).

Hydrolysis of Oxidized Forms of dGTP and GTP by GCHII—We examined abilities of purified preparations of GCHII and His-tag GCHII proteins to hydrolyze 8-oxo-dGTP and 8-oxo-GTP. Fig. 2 shows that both preparations can hydrolyze 8-oxo-dGTP and 8-oxo-GTP, yielding unique fast-moving products. The $R_f$ values of the fast-moving products coincided with those of 8-oxo-dGMP and 8-oxo-GMP, respectively. GTP was also hydrolyzed by the enzyme preparations under the same conditions; however, the autoradiographic profile of the fast-moving radioactive materials was smearsy, probably due to the instability of 2,5-diamino-6-hydroxy-4-(5-phosphoribosylamino)-pyrimidine (21), a product of the reaction of GTP with GCHII. dGTP was not totally hydrolyzed by GCHII or His-tag GCHII.

Fig. 3 shows the results of reactions of GCHII with various nucleotides at 20 μM. 8-Oxo-dGTP and 8-oxo-GTP were hydrolyzed efficiently at almost the same rates. GTP was cleaved at a lower rate, but dGTP was not hydrolyzed. Based on these results, we calculated the kinetic values for 8-oxo-dGTP, 8-oxo-GTP, and GTP (Table II). The apparent $K_m$ values for hydrolysis of 8-oxo-dGTP and 8-oxo-GTP were 30 and 21 μM, respectively. $K_m$ of GTP is 29 μM, consistent with the value calculated previously by Foot and Brown (21).

We next analyzed the reaction products by HPLC. The retention volume on HPLC of the product of hydrolysis of 8-oxo-dGTP by GCHII was 11 ml, which corresponds to that of an authentic 8-oxo-dGMP sample (data not shown). The retention volume of the product of 8-oxo-GTP hydrolysis by GCHII was also the same as that for 8-oxo-GMP (data not shown). It is evident, therefore, that GCHII possesses a pyrophosphatase activity for 8-oxo-dGTP and 8-oxo-GTP, in addition to the previously demonstrated cyclohydrolase/pyrophosphatase activity for GTP.

Characteristics of ribA Mutants—Spontaneous mutation fre-
GCHII Hydrolyzes Mutagenic 8-Oxo-dGTP

**FIG. 2.** Hydrolysis of various guanine nucleotides by GCHII and His-tag GCHII preparations. The reaction was performed at 30 °C for 30 min in a mixture containing 20 μM each of GTP (lanes 1, 5, and 9), 8-oxo-GTP (lanes 2, 6, and 10), dGTP (lanes 3, 7, and 11), and 8-oxo-dGTP (lanes 4, 8, and 12). As enzymes, 40 ng each of native GCHII (lanes 5–8) and His-tag GCHII (lanes 9–12) were added. The reaction mixtures were analyzed by thin layer chromatography, and positions of triphosphate and monophosphate forms are indicated.

Frequency of ribA− strain (JC7623 ribA−) is as low as that of wild-type strain, JC7623 (Table III). Thus, under the mutT+ background, RibA exerts no significant effect on mutation frequency. However, when bacteria lack MutT protein, an effect of ribA− deficiency was observed. Spontaneous mutation frequencies of ribA− mutT+ strain were 2.5 to 4 times higher than those of mutT− strain, with two different characters. This result may be taken as evidence that the GCHII protein can substitute the MutT function in vivo, although its efficiency is relatively low.

**DISCUSSION**

8-OxoG is produced in DNA by reactive oxygen species generated in cell as by-products of aerobic metabolism, and this production is enforced by oxidative stress (32, 33). This modified base can pair with cytosine and adenine with an almost equal efficiency and thus has the potential to induce G:C → T:A transversion (5, 6). Oxidation of guanine also occurs in the cellular nucleotide pool, and 8-oxo-dGTP and 8-oxo-GTP thus formed can be incorporated in DNA and RNA, respectively.

MutT protein of *E. coli* degrades 8-oxo-dGTP and 8-oxo-GTP to the corresponding nucleoside monophosphates (11, 34), thereby preventing occurrence of spontaneous mutation in transcription errors.

Considering the significant roles of MutT protein in DNA replication as well as transcription, we think that a cell might be equipped with a mechanism that can substitute the MutT function. With this in mind, we initiated screening of an *E. coli* genomic DNA library, prepared from mutT− deficient strain to clone a gene with an ability to suppress the mutT mutation. The gene isolated turned out to be the ribA gene, encoding GTP cyclohydrolase II, which functions at the first step of riboflavin biosynthesis. The RibA protein indeed has an ability to hydrolyze 8-oxo-dGTP and 8-oxo-GTP to the corresponding nucleoside monophosphates, providing the biochemical basis for this unexpected finding. It should be stressed that the RibA protein does not hydrolyze dGTP under the conditions tested (Figs. 2 and 3). The RibA protein, therefore, can specifically degrade the potent mutagenic substrate, 8-oxo-dGTP.

**TABLE II**

**Kinetics of hydrolysis by GCHII**

| Substrate       | $K_m$ (μM) | $V_{max}$ (μmol·M·min$^{-1}$) |
|-----------------|------------|------------------------------|
| 8-Oxo-dGTP      | 30         | 11.9                         |
| 8-Oxo-GTP       | 21         | 4.0                          |
| GTP             | 29         | 1.2                          |

**TABLE III**

**Mutation frequencies of ribA-deficient strains**

Mean values of mutation frequencies were calculated from at least five independent experiments. Mutations toward Rif$^r$ were also examined in JC7623 and JC7623 ribA−::Km. The $K_m$ for 8-oxo-dGTP of GCHII is 30 μM, a value considerably higher as compared with that of the MutT protein, being 0.48 μM (11). Since the concentration of 8-oxo-dGTP in the cellular nucleotide pool is extremely low, it seems that the role of RibA protein in control of mutation frequency in *E. coli* cells may be rather limited. Indeed, the ribA mutation afforded no increase in spontaneous mutation frequency of the mutT− cells. However, in the mutT− background the ribA deficiency causes increased frequencies in spontaneous mutation. The antimutagenic effect of RibA was further evident when the gene product was overproduced in mutT− cells. Under ordinary conditions, MutT protein exerts almost exclusively its function to degrade

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FIG. 3. *Action of GCHII on four types of guanine nucleotides.* 20 μM each of GTP, 8-oxo-GTP, dGTP, and 8-oxo-dGTP were incubated in 10 μl of reaction mixture containing 20 mM Tris-HCl, pH 7.5, 0.8 μg of bovine serum albumin, 8 mM MgCl2, 5 mM dithiothreitol, 4% glycerol with various amounts of GCHII. The reaction mixtures were analyzed as shown in Fig. 2, and the amounts of the monophosphate forms were determined by autoradiographic analysis with a molecular imager. ●, GTP; ○, 8-oxo-GTP; ×, dGTP; ■, 8-oxo-dGTP.

**TABLE IV**

**Mean values of mutant frequencies**

| Strain          | Rif$^r$/10$^8$ cells | Sm$^r$/10$^8$ cells |
|-----------------|----------------------|---------------------|
| T-198 mutT$^+$  | 16.9 ± 8.6           | 4.9 ± 2.9           |
| T-198R mutT− ribA− | 12.2 ± 6.6         | 497.2 ± 169.2       |
| JC7623          | 0.67 ± 0.25          | 497.2 ± 169.2       |
| JC7623 ribA−::Km | 0.32 ± 0.29          | 497.2 ± 169.2       |
the mutagenic substrate, 8-oxo-dGTP; however, in the absence of MutT or under specific conditions such as increased reactive oxygen species, GCHII may function to protect the genetic material. Recently, Koh et al. (35) reported that the ribA gene is inducible by redox-cycling compounds under regulation of the soxRS system of E. coli.

Médigue et al. (36) suggested that the mutT gene may be one of the exogenous genes, since its codon usage is different from other E. coli genes. The ribA gene, on the other hand, exhibits the ordinary codon usage and may be authentic. It can be inferred that the ancestral RibA protein might function to eliminate oxidized forms of guanine nucleotides during the early evolutionary phase when oxygen tension was relatively low. Once E. coli acquires a strong anti-mutator mutT to adapt to the aerobic state, the biological role of the ribA gene might be restricted, e.g. biosynthesis of riboflavin.

The amino acid sequence for the GCHII shows no similarity with that for the MutT nor has specific motif such as MutT box. The protein responsible has some sequence homology with MTH1 homologue (12, 14–16). In contrast to MutT, GCHII even though mammalian cells are devoid of an ability to recognize compound 3, 8-oxo-dGTP and 8-oxo-GTP, as well. It is highly probable that 8-oxoguanine-related mutagenesis is an important part of spontaneous mutagenesis in higher organisms and that similar systems address the threat of oxidation of guanine residues. An 8-oxo-dGTPase similar to MutT protein is present in mammalian cells, and the gene responsible was named MTH1 for mutT homolog (12, 14–16). In MTH1-deficient mouse cells, there is yet a low level of enzyme activity to degrade 8-oxo-dGTP. It is of interest to see whether the protein responsible has some sequence homology with GCHII even though mammalian cells are devoid of an ability to synthesize riboflavin.

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