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Identification of the dITP- and XTP-Hydrolyzing Protein from *Escherichia coli*

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A hypothetical 21.0 kDa protein (ORF O197) from *Escherichia coli* K-12 was cloned, purified, and characterized. The protein sequence of ORF O197 (termed *Ec*O197) shares a 33.5% identity with that of a novel NTPase from *Methanococcus jannaschii*. The *Ec*O197 protein was purified using Ni-NTA affinity chromatography, protease digestion, and gel filtration column. It hydrolyzed nucleoside triphosphates with an O6 atom-containing purine base to nucleoside monophosphate and pyrophosphate. The *Ec*O197 protein had a strong preference for deoxyinosine triphosphate (dITP) and xanthosine triphosphate (XTP), while it had little activity in the standard nucleoside triphosphates (dATP, dCTP, dGTP, and dTTP). These aberrant nucleotides can be produced by oxidative deamination from purine nucleotides in cells; they are potentially mutagenic. The mutation protection mechanisms are caused by the incorporation into DNA of unwelcome nucleotides that are formed spontaneously. The *Ec*O197 protein may function to eliminate specifically damaged purine nucleotide that contains the 6-keto group. This protein appears to be the first eubacterial dITP- and XTP-hydrolyzing enzyme that has been identified.

Keywords: Deoxyinosine triphosphate, *Ec*O197, Mutation, Nucleotide hydrolysis, Xanthosine triphosphate

Introduction

Chemical mutagens and oxidative damages to DNA can result in mutagenesis in organisms, but cells have their own repair mechanisms by DNA repair proteins. These include DNA glycosylases, hydrolyses, and many others to damaged nucleotides or DNA strands (Friedberg *et al*., 1995; Wood, 1996; Lindahl *et al*., 1997; Petit and Sancar, 1999, Lee, 2001). Although the repair proteins, which can remove inosine or xanthosine in the DNA strand, have been identified in *E. coli* (Yao *et al*., 1994; Schouten and Weiss, 1999; He *et al*., 2000) and eukaryotic systems (Asaeda *et al*., 2000; Saparbaev *et al*., 2000), relatively little is known about the proteins that intercept damaged nucleotides. These include dITP or XTP at the level of the free nucleoside triphosphate before it forms a phosphodiester linkage with the nascent polynucleotide chain. Previously, Hwang *et al.* (1993) reported a novel NTPase from an archaeon, *Methanococcus jannaschii*, which hydrolyzes some damaged nucleotides. This protein has pyrophosphate-releasing activity by the hydrolysis of dITP, ITP, and XTP.

The enzymes, which hydrolyze nucleotide triphosphates to their monophosphate form and PPi, have very important roles in cells. These proteins function as a DNA repair protein that sanitizes the damaged nucleotides. Typical enzymes that can hydrolyze (d)NTP to (d)NMP and PPi are MutT (Maki and Sekiguchi, 1992; Bridges, 1997) and dUTPase (McIntosh *et al*., 1992; Larsson *et al*., 1996), eliminating 8-oxo-dGTP and dUTP from the nucleotide precursor pools, respectively. The importance of these DNA repair enzymes in bacteria and eukaryotes has been well established (Maki and Sekiguchi, 1992; McIntosh *et al*., 1992; Larsson *et al*., 1996; Bridges, 1997). However, repair proteins that deal with the damaged nucleoside triphosphates that are produced by oxidative deamination, such as dITP or XTP, have been unknown until now.

The ORF O197 (accession number P52061), a hypothetical 21.0 kDa protein, in the GSHB-ANSB intergenic region from *E. coli* K-12 showed a high-sequence similarity with a novel NTPase of *M. jannaschii*. We hypothesized that the hypothetical protein that is encoded from ORF O197, which has been named *Ec*O197, functions as a hydrolyzing enzyme for damaged nucleoside triphosphates. Here, we purified the

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recombinant *Ec*O197 protein and identified its biochemical function. This study is the first report on the eubacterial dITP- and XTP-hydrolyzing enzyme. Investigating the characteristics of this enzyme could serve as the basis to understand the repair mechanism to DNA damage in eubacteria.

Materials and Methods

Materials Restriction endonuclease and T4 DNA ligase were obtained from Promega (Madison, USA). *Taq* DNA polymerase was a product of Takara (Shiga, Japan). BioSynthesis, Inc. (Lewisville, USA) synthesized primers for the polymerase chain reaction (PCR). Amersham Pharmacia Biotech (Uppsala, Sweden) supplied all of the instruments and columns for FPLC. The [γ-^{32}P]GTP and [^{32}P] pyrophosphates were purchased from NEN (Boston, USA). Xanthosine triphosphate (XTP), 7-methyl-GTP, 8-bromo-dATP (8-Br-dATP), 5-Br-dCTP and 5-Br-dUTP were purchased from Sigma (St. Louis, USA). Deoxyinosine triphosphate (dITP), ITP, dUTP and 7-deaza-dGTP were from Roche (Mannheim, Germany). The 8-oxo-dGTP, dATP, dTTP, dGTP and dCTP were from Amersham Pharmacia Biotech (Uppsala, Sweden). Purine deoxyribose triphosphate and N6-etheno-ATP were obtained from Biolog (Bremen, Germany). The 2-Aminopurine deoxyribose triphosphate, 2,6-diaminopurine deoxyribose triphosphate, 6-chloropurine deoxyribose triphosphate, 8-Br-dGTP were purchased from Trilink Biotech (San Diego, USA). K2-cellulose TLC plates were purchased from Whatman (Maidstone, UK). The plasmid DNA purification kit and Ni-NTA agarose resin were from Qiagen (Hilden, Germany). The *E. coli* strains DH5α and BL21(DE3) were used for molecular cloning and protein expression.

Cloning of *Ec*O197 A hypothetical 21.0 kDa protein (ORF O197) in the GSHB-ANSB intergenic region (accession number P52061) was identified in the complete genome sequence of *E. coli* K-12 during searches for genes that encode the protein sequence that is homologous to an NTPase (accession number Q57679) of *M. jannaschii* using the program BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997). The genomic DNA of *E. coli* K-12 was prepared using a Genomic DNA Midi Kit (Qiagen, Hilden, Germany). The ORF O197 (termed *Ec*O197), the hypothetical gene in *E. coli*, was amplified by 25 cycles of a polymerase chain reaction (PCR) using the GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, USA). PCR was performed in 100 μl reaction mixtures that contained 600 ng of genomic DNA as a template, 200 μM dNTPs, 2 units of *Taq* DNA polymerase, 10 μl of 10X reaction buffer, and 1 pmol of each primer. The *Ec*O197 fragment was PCR-amplified using the oligonucleotides 5'-TCACGTT CACATATGCAAAAAGTTGTCCTC-3' (upstream primer, the underlining indicates the *Nde*I site) and 5'-GCTAGGCATGGATCC TTAACCATTACGTAAAGC-3' (downstream primer, the underlining indicates the *Bam*HI site). The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and digested with *Nde*I and *Bam*HI. The digested fragment was subcloned into a pET28a expression vector (Novagen, Madison, USA). The pET-*Ec*O197 plasmid was used to transform the *E. coli* DH5α cells for propagation. The DNA sequence of the *Ec*O197 gene in the pET28a vector was confirmed using an ABI 373 DNA automated sequencer.

Expression and purification of *Ec*O197 The recombinant plasmid (pET-*Ec*O197) was introduced into *E. coli* BL21(DE3), which carried in its genome the gene that encodes T7 RNA polymerase under the control of an IPTG inducible promoter for protein overexpression. *E. coli* BL21(DE3) transformants with pET-*Ec*O197 were grown at 37°C in 1 L of a Luria-Bertani broth that contained 35 μg/mL of kanamycin. When the culture grew to an absorbance of 0.5 at 600 nm, a final concentration of 0.5 mM IPTG was added in order to induce the *Ec*O197 protein expression. After another 4 h of growth, the cells were harvested and resuspended in 50 ml of buffer A (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 1 mM PMSF). The resuspended cells were lysed by ultrasonication and centrifuged at 16,000 rpm for 30 min at 4°C in order to pellet any insoluble material. The supernatant was loaded onto a Ni-NTA affinity column (Qiagen, Hilden, Germany) and washed with buffer A that contained 20 mM imidazole. The His-tagged *Ec*O197 was eluted with a linear gradient from 20 mM to 1 M imidazole in buffer A. Then additional amino acids that contained hexahistidine in His-tagged *Ec*O197 N-terminus were removed by thrombin digestion at 4°C for 12 h. The *Ec*O197 protein was purified by gel filtration chromatography using a Superdex 75 FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden) that was pre-equilibrated with buffer A. The proteins were separated on a 12.5% SDS polyacrylamide gel and visualized using Coomassie blue staining in order to estimate the yield and purity. The purified *Ec*O197 protein was concentrated to 1 mg/ml and stored at −80°C. The protein concentration was determined by Bradfords method (Bradford, 1976) using bovine serum albumin as a standard.

Enzyme assay The thin layer chromatography (TLC) method was used for the primary assay for the nucleoside triphosphate-hydrolyzing activity of the *Ec*O197 protein. The reaction mixtures (reaction volume 20 μl) that contained 25 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 1 μCi of [γ-^{32}P]GTP, and *Ec*O197 protein (0.110 pmol) were incubated at 37°C for 20 min. Reaction mixtures were spotted on a K2-cellulose TLC plate (Whatman, Maidstone, UK), then spot-separating experiments were performed in a small chamber with a solvent system that was composed of acetone, acetic acid, NH_4OH, and water (10 : 4 : 1.2 : 8.8, v/v). The individual spots on the TLC plate were detected by autoradiography using X-ray film. To assay the optimal reaction condition and substrate specificity of the *Ec*O197 protein, the high-performance liquid chromatography (HPLC) method (Hwang *et al*., 1999) was used. The standard reaction mixture (final volume 50 μl) contained a 25 mM CAPS buffer, pH 10.0, 5 mM $MgCl_2$, 1 mM substrate, and 2 pmol *Ec*O197 protein. The reaction mixtures were incubated at 37°C for 10 min and terminated by the addition of 10 μl of 12% trichloracetic acid. After centrifugation, the products were quantified using a Hypersil SAX 5 μ HPLC column (ThermoHypersil, Runcorn, UK) with a linear gradient from 5 mM $NH_4H_2PO_4$ (pH 2.8) to 750 mM $NH_4H_2PO_4$ (pH 3.7), and with UV absorbance. Peaks were identified with the aid of standards, and quantified by area integration.

Fig. 1. Sequence alignment of *Ec*O197 with *M. jannaschii* NTPase. The shaded amino acids represent identical residues. The nucleotide-binding residues are boxed. Alignment was performed with the ClustalX program.

Results and Discussion

Cloning and purification of *Ec*O197 We identified the hypothetical gene ORF O197 (accession number P52061), named *Ec*O197, from *E. coli*. It showed a sequence homology to an NTPase from *M. jannaschii* by GenBank database searches using the BLAST program at NCBI (Altschul *et al*., 1997). The *Ec*O197 protein was composed of 197 amino acids with a predicted molecular mass of 21,039 Da and pI 5.9. *Ec*O197 has a 55.3% overall amino acid sequence similarity (33.5% identity) with the *M. jannaschii* NTPase sequence. Using the ClustalX program (Heringa, 1999), the homology between *Ec*O197 and the *M. jannaschii* NTPase appeared throughout the whole amino acid sequence (Fig. 1). From an analysis of the sequence alignment, it appears that several regions are highly conserved. Notably, *Ec*O197 possessed nucleotide-binding sites that are identical to those of *M. jannaschii* NTPase (Hwang *et al*., 1999) that was determined with the structure of the protein-nucleotide complex.

The *Ec*O197 protein was expressed in *E. coli* BL21(DE3) as a hexahistidine-tagged recombinant protein. The recombinant plasmid pET-*Ec*O197 was introduced into *E. coli* BL21(DE3) for the protein overexpression, then the recombinant protein was induced by the addition of IPTG to the cells that harbored the recombinant plasmid. The His-tagged *Ec*O197 protein was eluted from the Ni-NTA affinity column with 150-200 mM imidazole, while the other proteins flowed through. After cleavage of the N-terminus of the His-tagged *Ec*O197 with thrombin, the *Ec*O197 protein was purified with Superdex 75 FPLC. The *Ec*O197 protein was purified with >95% homogeneity on SDS-PAGE (Fig. 2A). The final yield of the *Ec*O197 protein after purification ranged up to 15 mg/liter per culture. The molecular mass of the native *Ec*O197 protein was about 40 kDa, which was determined by gel filtration chromatography using a Superdex 75 FPLC column and marker proteins (Fig. 2B). This *Ec*O197 protein could be a dimer that consists of two identical subunits. The native protein of the NTPase from *M. jannaschii*, which is homologous to *Ec*O197, is also a dimer (Hwang *et al*., 1999).

The dITP- and XTP-hydrolyzing activity of *Ec*O197 and its biological implication In an initial attempt to delineate the potential activity of the *Ec*O197 protein, nucleotide hydrolysis activity was examined by measuring the release of

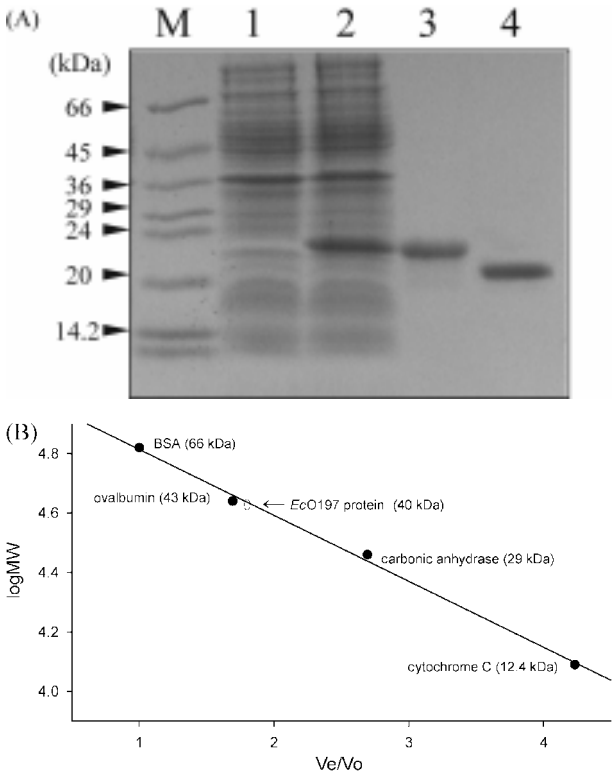

Fig. 2. Purification of the *Ec*O197 protein and determination of its native molecular mass. (A) Protein purification of the *Ec*O197 protein showing the SDS-PAGE gel that was stained with Coomassie brilliant blue. Lane M, protein molecular makers; lane 1, control (crude extract of cells harboring plasmid without insert); lane 2, crude extract of cells harboring plasmid-inserted *Ec*O197; lane 3, sample eluted from Ni-NTA column; lane 4, purified sample from gel filtration after thrombin treatment. (B) Determination of molecular mass of the native *Ec*O197 protein by Superdex 75 gel filtration chromatography.

PPi from [γ-^{32}P]GTP using TLC, described in Methods. The purified *Ec*O197 protein was able to hydrolyze the nucleoside triphosphate. Figure 3 demonstrates how the *Ec*O197 protein can hydrolyze the nucleoside triphosphate in the presence of magnesium ions, releasing pyrophosphate. Its activity was inhibited by the EDTA addition.

In order to investigate the optimal reaction condition and specific substrate of the *Ec*O197 protein, nucleotide hydrolysis activities were measured by the HPLC procedure

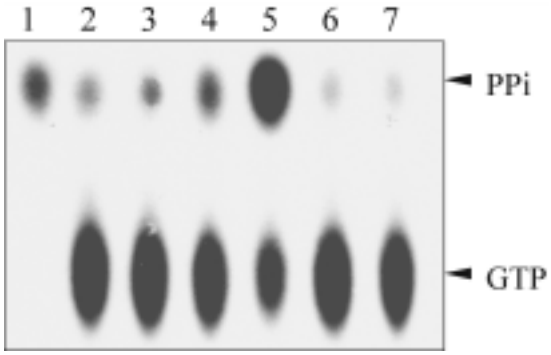

Fig. 3. Pyrophosphate-releasing activity of the *Ec*O197 protein. The *Ec*O197 protein (0.110 pmol) was incubated with 1 μCi of [γ-^{32}P]GTP at 37°C for 20 min in 25 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$. The aliquots were spotted on a K2-cellulose TLC plate and separated. Lane 1, control pyrophosphate; lane 2, control GTP; lane 3, GTP + *Ec*O197 (0.1 pmol); lane 4, GTP + *Ec*O197 (1 pmol); lane 5, GTP + *Ec*O197 (10 pmol); lane 6, GTP + *Ec*O197 (10 pmol) + EDTA (10 mM); lane 7, GTP + BSA (10 pmol).

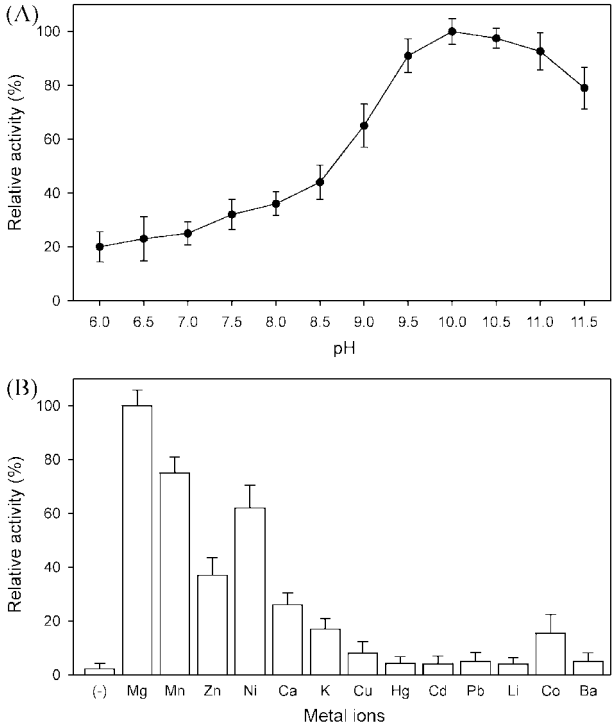

Fig. 4. Effect of pH and metal ions on the nucleotide hydrolysis activity of the *Ec*O197 protein. (A) The activity assay in various pH buffers was performed at 37°C. The following buffers (25 mM) were used: MES buffer, pH 6.0-6.5; HEPES buffer, pH 7.0-7.5; Tris-Cl buffer, pH 8.0-8.5; CHES buffer, pH 9.0-9.5; and CAPS buffer, pH 10.0-11.5. (B) The effect of metal ions was assayed at 37°C with each metal ion (5 mM) in a 25 mM CAPS buffer, pH 10.0.

that detects nucleoside monophosphate that is hydrolyzed from nucleoside triphosphate, described in Methods. After the enzyme reaction, hydrolysate nucleoside monophosphate and substrate nucleoside triphosphates were separated by ion-exchange chromatography, and product amounts were quantified from the chromatographic peaks. The *Ec*O197 protein exhibited optimum enzyme activities at pH 10.0-10.5, and reaction rates under neutral conditions were <40% of the maximum (Fig. 4A). The nucleotide hydrolysis activity by the *Ec*O197 protein also required divalent cations. Maximum activity was obtained with magnesium ion, and exhibited with manganese and nickel ions that supported 60-75% of the maximum rate (Fig. 4B)

Different substrates, including the four common deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), were tested in the standard assay. The best substrates for the *Ec*O197 protein were dITP (ITP) and XTP (Fig. 5A). The *Ec*O197 protein showed very low activity toward canonical nucleoside triphosphates; dATP, dCTP, and dTTP were hydrolyzed at less than 1% of the rate of XTP hydrolysis. On the other hand, dGTP and dUTP showed relative activities 10-12% lower than those of XTP. However, several other nucleotides, (8-oxo-dGTP, purine deoxyribose triphosphate, 2-aminopurine deoxyribose triphosphate and 2,6-diaminopurine deoxyribose triphosphate) showed extremely low activities by this protein (Fig. 5A). The results of the kinetic analysis of the major substrates of the *Ec*O197 protein were presented as Lineweaver-Burk plots in Figure 5B. From the kinetic plots, the *Km* values of XTP, dITP and ITP were 0.33 mM, 0.36 mM and 0.41 mM, respectively. Their *Vmax* values were also calculated to be 463.3 μM/min (XTP), 448.2 μM/min (dITP) and 432.6 μM/min (ITP). The approximate *Km* and *Vmax* value for dGTP was 3.1 mM and 36.8 μM/min. Besides the substrates in Fig. 5A, other modified or damaged nucleotides (6-chloropurine deoxyribose triphosphate, 8-Br-dGTP, 5-Br-dCTP, 5-Br-dUTP, 7-deaza-dGTP, 7-methyl-GTP, N6-etheno-ATP, NAD, NADH, FAD, ADP-ribose, UDP-glucose, AppA and ApppA) were also examined as substrates for the *Ec*O197 protein; however, they were not hydrolyzed by this protein (data not shown). When XDP and XMP were tested (none were hydrolyzed), the *Ec*O197 protein was only reactive to the nucleoside triphosphate form, and it was absolutely specific for dITP (ITP) and XTP. This result, which showed that dITP and XTP were hydrolyzed by the *Ec*O197 protein, suggests an insight about the recognition of this protein for the substrate. A common property of these specific substrates is the purine nucleotide that contains the O6 atom in their base. The NTPase protein of *M. jannaschii* revealed the amino acid residues that interact with nucleoside triphosphate (Hwang *et al.*, 1999). The *Ec*O197 protein also possessed amino acid residues. This included Asn10 and Phe154 that are identical to Asn17, as well as Phe149 of *M. jannaschii* NTPase that may bind to the nucleotide base (Fig. 1). This suggests that the O6 atom of these nucleotides is critical for the reactivity of

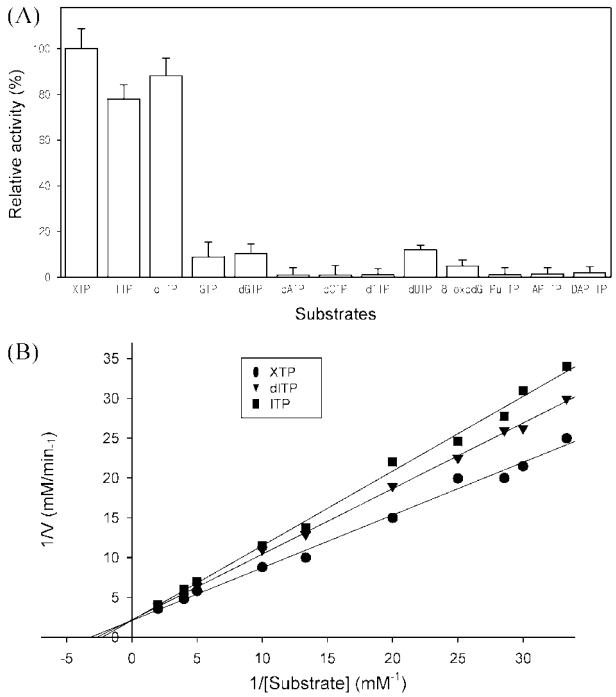

Fig. 5. Substrate specificity of the *Ec*O197 protein and the kinetics on dITP, ITP and XTP. (A) The reaction mixture that contained a 25 mM CAPS buffer, pH 10.0, 5 mM $MgCl_2$, 1 mM substrate, and 2 pmol of the *Ec*O197 protein was incubated at 37°C for 10 min. The hydrolysis of the substrates was assayed using HPLC (described under Materials and Methods). The graph values are relative to XTP. 8-oxodG, 8-oxo-dGTP; Pu-TP, purine deoxyribose triphosphate; AP-TP, 2-aminopurine deoxyribose triphosphate; DAP-TP, 2,6-diaminopurine deoxyribose triphosphate. (B) Reactions were performed in 25 mM CAPS buffer, pH 10.0, 5 mM $MgCl_2$ at 37°C. Nucleotide hydrolysis rates were determined by time-course experiments with different concentrations of the substrates. Product amounts were also measured using a Hypersil SAX 5 μ HPLC column. The kinetic parameters were calculated using the EnzFitter Program (Biosoft, Cambridge, UK).

*Ec*O197.

An intriguing question is the biological role of *Ec*O197 in *E. coli*. When adenine and guanine residues are damaged by oxidative deamination, they are converted to hypoxanthine and xanthine, respectively (Shapiro and Pohl, 1968; Lindahl 1979). As reported, dITP (ITP) can be incorporated into deoxyribo- or ribonucleic acid by polymerases (Shuman and Moss, 1988; Donlin and Johnson, 1994; Auer *et al*., 1996). Also, dITP behaves as a dGTP analogue and is incorporated opposite cytosine by both purified DNA polymerase and isolated nuclei (Thomas *et al*., 1978). The high frequency of mutation by the polymerase reaction with the mixture that contains dITP *in vitro* has also been observed (Spee *et al*., 1993). Practically, dITP has been found in the nucleotide pool of cells (Vanderheiden, 1969; Vanderheiden, 1979; van Waeg *et al*., 1988). The enzyme activities that hydrolyze dITP (ITP) and XTP were also presented for the extract of some organisms (Wang and Morris, 1974; Holmes *et al*., 1979). Variations in the mutagenesis and repair mechanism during DNA synthesis among the organisms may reflect differences in not only the nucleotide insertion fidelity of polymerases (Bebenek *et al*., 1990; Cline *et al*., 1996; Kim *et al*., 2000; Garcia-Diaz *et al*., 2002), but also the role of DNA repair proteins (De Flora *et al*., 1992; Wood, 1996). Therefore, these results suggest that the major biological role of *Ec*O197 may be to sanitize rogue nucleoside triphosphates from the nucleotide pool in cells.

This nucleotide-cleansing function has been seen for dUTPase (McIntosh *et al*., 1992; Larsson *et al*., 1996) and MutT (Bridges, 1997; Fowler and Schaaper, 1997), removing dUTP and 8-oxo-dGTP from the nucleotide pool, respectively. These enzymes catalyze the hydrolysis of the nucleotide triphosphate to its monophosphate and pyrophosphate, simultaneously removing aberrant dNTPs (dUTP or 8-oxo-dGTP) during DNA synthesis. The importance of the function of dUTPase and MutT in prokaryotic and eukaryotic systems has been firmly established (McIntosh *et al*., 1992; Larsson *et al*., 1996; Bridges, 1997; Fowler and Schaaper, 1997). It is notable that the *Ec*O197 protein also releases pyrophosphate from dITP and XTP in the same way as dUTPase and MutT. *Ec*O197 and its homologs could conceivably play an important role in the repair mechanism in cells.

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