Amino Acid Residues Involved in Substrate Recognition of the *Escherichia coli* Orf135 Protein†

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ABSTRACT: The *Escherichia coli* Orf135 protein, a MutT-type enzyme, hydrolyzes mutagenic 2-hydroxy-dATP (2-OH-dATP) and 8-hydroxy-dGTP, in addition to dCTP and 5-methyl-dCTP, and its deficiency causes increases in both the spontaneous and H_2O_2-induced mutation frequencies. To identify the amino acid residues that interact with these nucleotides, the Glu-33, Arg-72, Arg-77, and Asp-118 residues of Orf135, which are candidates for residues interacting with the base, were substituted, and the enzymatic activities of these mutant proteins were examined. The mutant proteins with a substitution at the 33rd, 72nd, and 118th amino acid residues displayed activities affected to various degrees for each substrate, suggesting the involvement of these residues in substrate binding. On the other hand, the mutant protein with a substitution at the 77th Arg residue had activitiy similar to that of the wild-type protein, excluding the possibility that this Arg side chain is involved in base recognition. In addition, the expression of some Orf135 mutants in *orf135*$^-$ *E. coli* reduced the level of formation of *rpoB* mutants elicited by H_2O_2. These results reveal the residues involved in the substrate binding of the *E. coli* Orf135 protein.

Organisms are equipped with mechanisms to prevent mutations caused by the incorporation of oxidized deoxyribonucleotides (*1*, *2*). The *Escherichia coli* MutT and its mammalian counterpart (MTH1) proteins hydrolyze 8-OH-dGTP[1] to the monophosphate derivative (*3*, *4*). Deficiencies in these proteins result in increased mutation frequency and enhanced tumor formation (*5*, *6*). Thus, these types of enzymes are quite important for nucleotide pool sanitization.

The *E. coli* Orf135 protein, another MutT-type enzyme, hydrolyzes 2-OH-dATP and 8-OH-dGTP, in addition to 5-Me-dCTP and dCTP (*7*, *8*). Its deficiency causes increases in both the spontaneous and H_2O_2-induced mutation frequencies (*9*). The 2-OH-dATP hydrolyzing activity of Orf135 may be a crucial mechanism for preventing mutations induced by this damaged DNA precursor, because 2-OH-dATP is mutagenic in *E. coli* and mutations are induced more frequently in an *orf135*$^-$ strain than in the isogenic *orf135*$^+$ strain (*9*, *10*). The importance of 2-OH-dATP is also supported by the observation that the expression of MTH1 harboring the D119A mutation, which is unable to hydrolyze 2-OH-dATP, only partially suppresses the cell dysfunction and delayed cell death of MTH1-null mouse embryo fibroblast cells, while the expression of wt MTH1 effectively suppresses these phenomena (*11*).

The overall structures of the *E. coli* MutT and mammalian MTH1 proteins, which both hydrolyze 8-OH-dGTP, resemble each other, although the level of sequence identity between the MTH1 and MutT proteins outside of the "phosphohydrolase module" or "MutT signature" is low (*12*). A model structure of the Orf135 protein was recently built, using the coordinates of the MutT protein as a template, and a nucleotide binding pocket was proposed by comparison with the structure of the human MTH1 protein and NMR titration experiments (see the Supporting Information). This model suggests that the Glu-33, Arg-72, Arg-77, and Asp-118 residues of Orf135 are exposed on the surface of the base-binding pocket, and thus are possible candidates for residues interacting with the base moieties of substrates.

In this study, to clarify the significance of these Orf135 amino acid residues in nucleotide binding, we carried out a site-directed mutagenesis study. The in vitro activities of the mutant proteins were examined by their abilities to hydrolyze 2-OH-dATP and 8-OH-dGTP, as well as 5-Me-dCTP and dCTP. Their in vivo activities were studied by determining whether the expression of the mutant proteins restored the mutator phenotype observed for the *orf135*$^-$ strain. The results obtained in this study revealed amino acid residues involved in the nucleotide binding of the *E. coli* Orf135 protein.

EXPERIMENTAL PROCEDURES

Materials. E. coli strain JD22899, an *orf135*$^-$ strain [*lacI*^{q}, *lacZ*Δ*M15-gal*$^-$, *F*$^-$, *orf135::*mini Tn10 (*kan*)] (T. Miki et

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[1] Abbreviations: ROS, reactive oxygen species; 8-OH-dGTP, 8-hydroxy-2′-deoxyguanosine 5′-triphosphate; 8-OH-dGDP, 8-hydroxy-2′-deoxyguanosine 5′-diphosphate; 2-OH-dATP, 2-hydroxy-2′-deoxyadenosine 5′-triphosphate; 5-Me-dCTP, 5-methyl-2′-deoxycytidine 5′-triphosphate; wt, wild-type; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; LB, Luria−Bertani; IPTG, isopropyl β-D-thiogalactopyranoside; amp, ampicillin.

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Table 1: PCR Primers for Orf135 Mutants[a]

primer	sequence (5′ → 3′)[b]
E33A (+)	GATTATGGG**C**GTTTGCCGG
E33Q (+)	GGATTATGG**CA**GTTTGCCGG
E33D (+)	GATTATGGG**AC**TTTGCCGGT
R72A (+)	AGCCATCAG**GC**AGAAGTTTC
R72K (+)	AGCCATCAG**AAA**GAAGTTTCG
R77A (+)	TTTCGGGGG**CG**ATTATCCAT
D118A (+)	CCCTGCTG**C**CATTCCATTAT
D118E (+)	CCCTGCT**GAG**ATTCCATTATT
D118N (+)	GCCCCTGCT**A**ACATTCCATT

[a] Primers corresponding to the sense strand are shown. Their complementary oligodeoxyribonucleotides were also used in the mutagenic PCR. [b] The targeted codons are underlined. The mutated positions are shown in bold.

al., unpublished results), was kindly provided by Y. Yamamoto and T. Miki. dATP, dGTP, dCTP, and pGEX-6P-3 DNA, containing the GST gene, were from Amersham Biosciences (Piscataway, NJ). 5-Me-dCTP was from F. Hoffmann-La Roche (Basel, Switzerland) and was purified by high-performance liquid chromatography. Oligodeoxyribonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified forms.

Preparation of Damaged Nucleotides. 8-OH-dGTP and 2-OH-dATP were prepared as previously described (*13*, *14*). These purified nucleotides were eluted as a single peak in both reverse-phase and anion-exchange HPLC (data not shown). They were stable under the assay conditions in the absence of Orf135 (data not shown).

Mutant Plasmid Construction. Mutant *orf135* genes were prepared by site-directed PCR mutagenesis using the wt *orf135* plasmid (*7*) as the template, mutagenic primers (Table 1), and high-fidelity Pyrobest DNA polymerase (Takara, Otsu, Japan). The mutant *orf135* gene was then inserted into the pGEX-6P-3 plasmid, as described previously (*7*). This manipulation generated the gene encoding the GST−Orf135 fusion protein. The nucleotide sequence of the gene was confirmed by sequencing, using a BigDye Terminator Cycle Sequencing Kit (Applera, Norwalk, CT) and an ABI model 377 DNA sequencer (Applera).

Purification of the GST−Orf135 Fusion Protein and Orf135. BL21(DE3) cells with the *GST-orf135* gene were cultured in 15 mL of LB medium containing amp at 37 °C until the turbidity at 610 nm reached 0.6. IPTG was added to a final concentration of 670 μM, and the *E. coli* culture was incubated at 37 °C for a further 2 h. The GST−Orf135 protein was purified as described previously (*15*). The purified proteins were analyzed by SDS−PAGE followed by Coomassie brilliant blue staining. They were visualized as single bands (data not shown).

The Orf135 protein without the GST tag was purified as described previously (*15*). The purified proteins were analyzed by SDS−PAGE, followed by Coomassie brilliant blue staining. Their purities and concentrations were determined by analysis of SDS−polyacrylamide gels using NIH Image, with bovine serum albumin as a protein for standard curves. Their purities were ≥94%.

Enzyme Assays. Enzymatic assays of the Orf135 protein was carried out as described previously (*7*, *16*). Detection was performed with UV absorbance at 272 nm (dCTP), 277 nm (5-Me-dCTP), or 292 nm (2-OH-dATP and 8-OH-dGTP). The Michaelis constant (K_m) and the catalytic constant (k_{cat}) were obtained from Lineweaver−Burk plots of the kinetic data (*17*). All reaction rates were linear during the course of the reaction.

Calculation of the Mutant Frequency. A single colony of JD22899, harboring the gene for either the GST or GST−Orf135 fusion protein, was taken from an LB agar plate with kanamycin (10 μg/mL), amp (50 μg/mL), and IPTG (2 μM), and was inoculated into 7 mL of LB medium containing kanamycin, amp, and IPTG. The *E. coli* culture was incubated at 37 °C until the turbidity at 570 nm reached 0.8, and then was diluted 10-fold with prewarmed LB medium containing amp and IPTG. When the turbidity at 570 nm reached 0.25, H_2O_2 was added to a final concentration of 2 mM, and the culture was incubated at 37 °C for 30 min. The culture was centrifuged at 2150*g* for 15 min at room temperature. The pellet thus obtained was resuspended in the same volume of prewarmed LB medium containing amp and IPTG, and then was diluted 2-fold with prewarmed LB medium containing amp and IPTG. The *E. coli* culture was incubated at 37 °C until the turbidity at 570 nm reached 0.85, and then was placed on ice for 10 min. A portion of the suspension was diluted with ice-cold LB medium, transferred onto an LB agar plate containing amp (a titer plate), and incubated at 37 °C for 12 h. Another portion of the suspension was transferred onto an LB agar plate containing rifampicin (100 μg/mL) and amp (a selection plate) and was incubated at 37 °C for 20 h. The mutant frequency was calculated according to the number of colonies on the titer and selection plates.

RESULTS

Amino Acid Residues of the Orf135 Protein Possibly Involved in Nucleotide Binding. A certain degree of sequence homology has been noted between the *E. coli* MutT and mammalian MTH1 proteins in a homologous region, the phosphohydrolase module or MutT signature (*18*, *19*). However, the level of sequence identity between the two proteins outside of this region is as low as 9.3%. Nevertheless, the overall folds of the MutT and MTH1 proteins resemble each other (*12*). The level of sequence identity between the MutT and Orf135 proteins is relatively high (31%), and their structures may be similar. A homology model of the Orf135 protein was built, based on the structure of the MutT protein as a template, using MODELLER (*20*). Distance restraints derived from the coordinates of MutT and energy minimization using a CHARMM force field were implemented for the modeling. Its nucleotide binding pocket was proposed by comparison with the structure of the human MTH1 protein (*12*) and by substrate titration experiments monitoring the HSQC spectrum using heteronuclear NMR (see the Supporting Information). The modeled structure was consistent with the preliminary NOE data obtained from NMR measurements. This model suggests that the Glu-33, Arg-72, Arg-77, and Asp-118 residues of Orf135 are exposed on the surface of the putative base-binding pocket, and are possible candidates for residues interacting with the base (Figure 1). We thus planned to substitute these amino acid residues.

These Glu-33, Arg-72, Arg-77, and Asp-118 residues of Orf135 were replaced with Ala, and some amino acids were substituted with their related ones. These mutant genes were

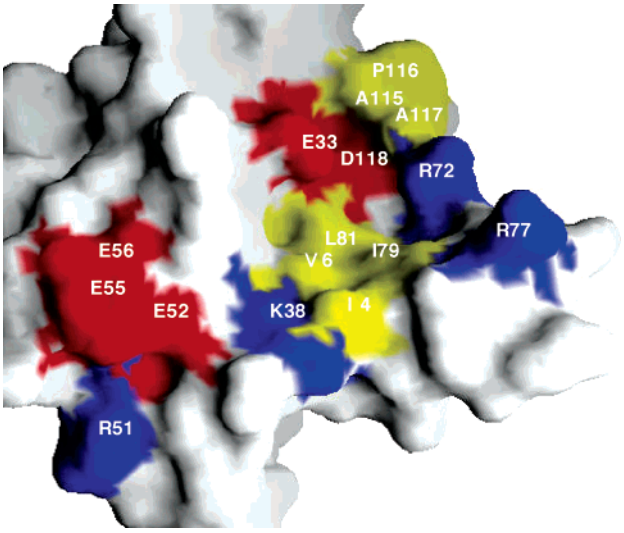

FIGURE 1: Model of the substrate-binding pocket of Orf135. This model was built based on the coordinates of the MutT protein, as described in the text. Acidic and basic residues are colored red and blue, respectively. Hydrophobic residues are colored yellow. The K38, R51, E52, E55, and E56 residues may be involved in phosphate binding, and thus were mutagenesis targets in the previous paper (*15*).

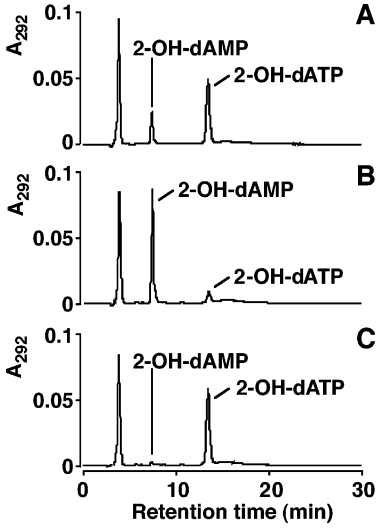

FIGURE 2: Hydrolysis of 2-OH-dATP by the GST−Orf135 protein, monitored by anion-exchange HPLC. 2-OH-dATP (20 μM) was incubated with 150 nM GST−Orf135 protein at 37 °C for 10 min, and analyzed as described in Experimental Procedures: (A) wt, (B) E33A, and (C) R72A. 2-OH-dAMP is 2-hydroxy-2′-deoxyadenosine 5′-monophosphate.

constructed by site-directed mutagenesis with mutagenic PCR primers (Table 1). The presence of the desired mutation was confirmed by sequencing.

Enzymatic Activities of the GST−Orf135 Protein Mutants. The *E. coli* Orf135 protein hydrolyzes 5-Me-dCTP and dCTP as well as 2-OH-dATP and 8-OH-dGTP (*7*, *8*), and the significance of the activities for the former two nucleotides is unknown. The 2-OH-dATPase activity of the Orf135 protein is particularly important, because a deficiency in the Orf135 protein affects the degree of mutation induction only in the case of 2-OH-dATP (*9*). First, various Orf135 mutants were tested for their abilities to hydrolyze these four deoxyribonucleotides. We used GST-fused Orf135 mutants purified by small-scale affinity chromatography in these screening experiments.

The 5-Me-dCTPase activities of these mutant proteins were examined with 50 μM 5-Me-dCTP and 50 nM proteins at 37 °C for 1 min. The other enzymatic activities were examined with a single deoxyribonucleotide (20 μM) and 150 nM proteins at 37 °C for 10 min. The product and the remaining substrate were quantitated by anion-exchange HPLC, as described previously (*7*, *16*). Interestingly, an increased rate of 2-OH-dATP hydrolysis was observed with the E33A, E33Q, and D118E mutant proteins (Figures 2 and 3). On the other hand, the R72A, D118A, and D118N mutants exhibited highly impaired 2-OH-dATPase activities. Likewise, the amino acid substitutions altered the hydrolyses of the other substrates to various degrees (Figure 3).

Interestingly, the replacement of Glu-33 with Ala enhanced the 2-OH-dATPase activity and suppressed the other three activities. The E33Q mutant displayed 2-OH-dATPase activity that was lower than that of E33A but higher than that of the wt protein. The 2-OH-dATPase activity of E33D was lower than that of the wt protein. The other three activities of these E33 mutant proteins were decreased, as compared to those of the wt protein. In particular, the dCTPase and 8-OH-dGTPase activities of these mutants were very low. The R72A protein exhibited suppressed 2-OH-dATPase and enhanced dCTPase activities, and the R72K protein had enzymatic activities similar to those of the wt protein. The substitution of Arg-77 with Ala only slightly affected the hydrolyzing activity of the Orf135 protein, thus excluding the possibility that the side chain of this Arg residue is important in base recognition. The D118A protein exhibited enhanced 8-OH-dGTPase activity, while the other activities were decreased. On the other hand, D118E lost the 8-OH-dGTPase activity, but its 2-OH-dATPase activity was higher than that of the wt protein. The D118N protein lost the 2-OH-dATPase activity but retained the other three activities. Interestingly, 8-OH-dGDP was produced by D118A and D118N. These results suggest that Glu-33, Arg-72, and Asp-118 are involved in base recognition, and the four substrates interacted with these residues in various ways.

The E33A, R72A, D118A, D118E, and D118N mutants were selected at this stage for further analyses of their activities in vitro.

Detailed Analyses of Mutant Orf135 Protein Activities toward Various Deoxyribonucleotides. The five Orf135 mutant proteins thus selected were purified after removal of the GST moiety. They were incubated with 2-OH-dATP and 8-OH-dGTP, as well as 5-Me-dCTP and dCTP (Figure 4). The 5-Me-dCTPase activity was measured with 100 μM 5-Me-dCTP and 15 nM protein, and the reaction mixtures were incubated at 37 °C for 2 min. The other enzymatic activities were examined with a single deoxyribonucleotide (20 μM) and 150 nM proteins at 37 °C for 10 min. Overall, as expected, the results obtained with the mutant proteins without the GST tag were similar to those obtained with the proteins containing the GST tag. The E33A mutant exhibited enhanced 2-OH-dATPase and drastically reduced 5-Me-dCTPase activities. Enhanced dCTPase and reduced 2-OH-dATPase activities were observed with the R72A protein. The D118A, D118E, and D118N mutant proteins displayed 2-OH-dATPase and 8-OH-dGTPase activities that were highly characteristic of the individual mutants. In the case

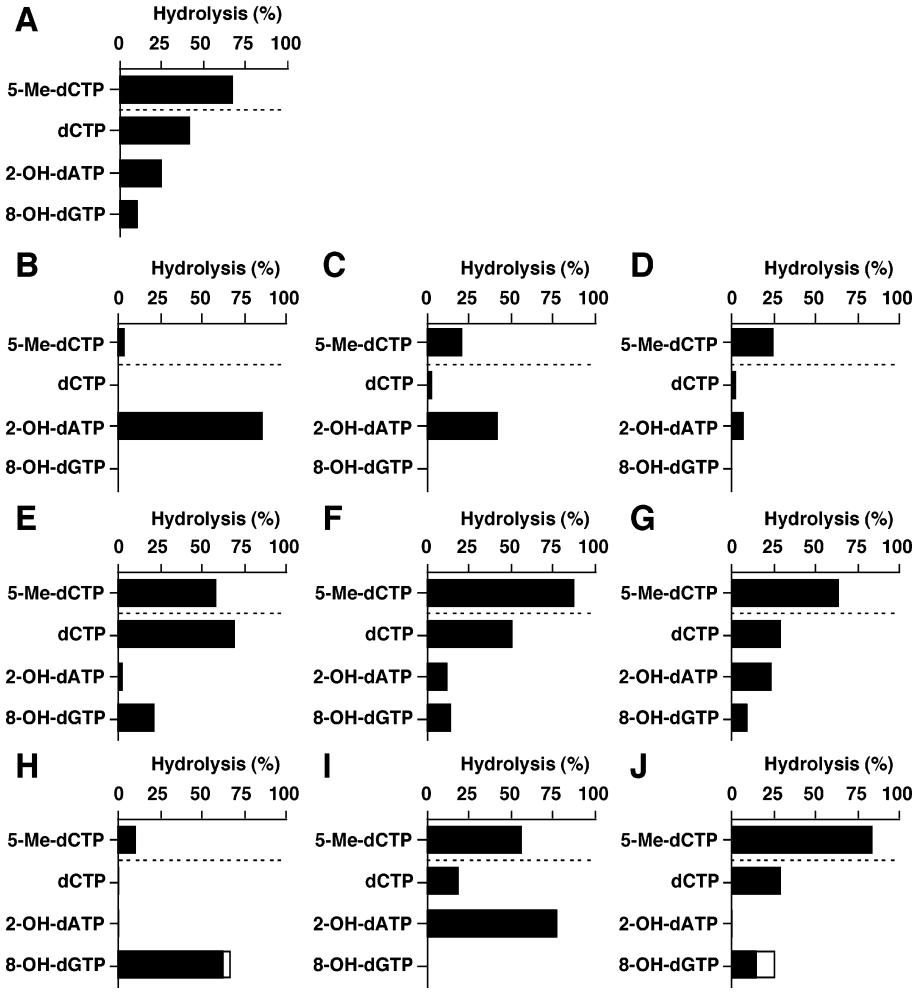

FIGURE 3: Hydrolysis of deoxyribonucleoside triphosphates by GST−Orf135 mutant proteins. 5-Me-dCTP (50 μM) was incubated with 50 nM GST−Orf135 protein at 37 °C for 1 min. dCTP, 2-OH-dATP, or 8-OH-dGTP (20 μM) was incubated with 150 nM GST−Orf135 protein at 37 °C for 10 min. The hydrolysis percentage was measured by HPLC, as described in Experimental Procedures: (A) wt, (B) E33A, (C) E33Q, (D) E33D, (E) R72A, (F) R72K, (G) R77A, (H) D118A, (I) D118E, and (J) D118N. The horizontal axis indicates the hydrolysis percentage for the total substrate added. Experiments were carried out at least in duplicate, and the mean values are represented. The empty boxes in panels H and J represent the percentage of 8-OH-dGDP.

of D118A, 8-OH-dGDP was not detected under the conditions described above (20 μM 8-OH-dGTP), in contrast to the case of D118A with the GST tag (Figures 3H and 4D). The production of 8-OH-dGDP was observed when a higher concentration of 8-OH-dGTP was used (data not shown).

The Michaelis constant (K_m) and the catalytic constant (k_{cat}) of the reactions catalyzed by the wt and mutant proteins were calculated (Table 2). Some kinetic parameters were not calculated, since the severely impaired activities made it difficult to determine the amount of product. In the case of the D118A mutant protein, 8-OH-dGDP was also produced, and the kinetic parameters were determined by considering both 8-OH-dGDP and 8-hydroxy-dGMP as the "product".

The K_m and k_{cat} values of E33A for 5-Me-dCTP were 8-fold larger and 13-fold smaller than those of the wt protein, respectively, resulting in a 100-fold reduced k_{cat}/K_m value. In contrast, the k_{cat} value of E33A for 2-OH-dATP was 8-fold larger than that of the wt protein. The K_m and k_{cat} values of D118A for 5-Me-dCTP were 9-fold larger and 4-fold smaller than those of the wt protein, respectively, resulting in a 34-fold reduced k_{cat}/K_m value. In contrast, the K_m and k_{cat} values of this mutant for 8-OH-dGTP were 2-fold smaller and 3-fold larger than those of the wt protein, respectively, resulting in the 6-fold increased k_{cat}/K_m value. The replacement of Asp-118 with Glu decreased the K_m value for 2-OH-dATP by 5-fold and increased the k_{cat} value by 3-fold. The k_{cat}/K_m value of the this mutant for 2-OH-dATP was thus 13-fold larger than that of the wt protein.

Mutation Suppression by Orf135 Mutant Proteins. A deficiency in the Orf135 protein causes an increase in the H_2O_2-induced mutation frequency (*9*). We then examined whether expression of the mutant proteins used in this study suppressed the H_2O_2-induced mutations. Plasmid DNAs containing the gene for either GST or the GST−Orf135 fusion protein were transfected into the *orf135*$^-$ strain. The expression of these genes was induced by IPTG treatment, and the protein production in the cells was confirmed by SDS−PAGE (data not shown). The amounts of GST or GST−Orf135 fusion proteins produced in cells were similar under the culture conditions that were used. These cells were treated with 2 mM H_2O_2, and their *rpoB* mutant frequencies were measured.

The expression of most of the tested mutant Orf135 proteins in the *orf135*$^-$ strain reduced the H_2O_2-induced mutant frequency, as compared with that of the GST protein (Figure 5). The expression of the E33A, E33Q, R72A, R72K,

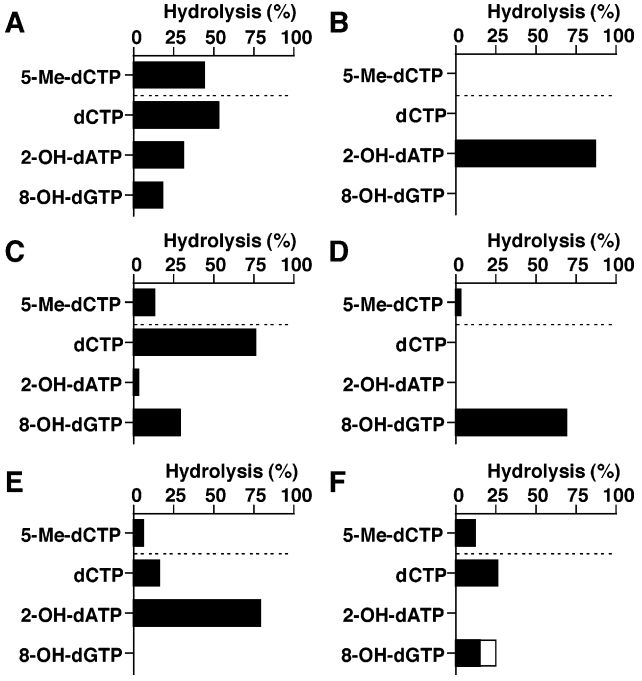

FIGURE 4: Hydrolysis of deoxyribonucleoside triphosphates by purified Orf135 mutant proteins without the GST tag. 5-Me-dCTP (100 μM) was incubated with 15 nM Orf135 at 37 °C for 2 min. dCTP, 2-OH-dATP, or 8-OH-dGTP (20 μM) was incubated with 150 nM GST−Orf135 protein at 37 °C for 10 min. The hydrolysis percentage was measured by HPLC, as described in Experimental Procedures: (A) wt, (B) E33A, (C) R72A, (D) D118A, (E) D118E, and (F) D118N. The horizontal axis indicates the hydrolysis percentage for the total substrate added. Experiments were carried out at least in duplicate, and the mean values are represented. The empty box in panel F represents the percentage of 8-OH-dGDP.

Table 2: Kinetic Parameters of Deoxyribonucleotides for the Orf135 Proteins[a]

Orf135	substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m[b] (mM^{-1} s^{-1})
wt[c]	5-Me-dCTP	0.028	35	1300 (1.00)
	dCTP	0.99	6.3	6.4 (1.00)
	2-OH-dATP	0.027	0.17	6.4 (1.00)
	8-OH-dGTP	0.41	0.89	2.2 (1.00)
E33A	5-Me-dCTP	0.22	2.8	13 (0.01)
	2-OH-dATP	0.067	1.42	21 (3.28)
R72A	5-Me-dCTP	0.015	11	780 (0.60)
	dCTP	0.65	8.8	14 (2.19)
	8-OH-dGTP	0.31	1.4	4.6 (2.09)
D118A	5-Me-dCTP	0.24	9.1	38 (0.03)
	8-OH-dGTP	0.19	2.7	14 (6.36)
D118E	5-Me-dCTP	0.0069	6.5	944 (0.73)
	dCTP	0.93	2.1	2.3 (0.36)
	2-OH-dATP	0.0055	0.45	82 (12.81)
D118N	5-Me-dCTP	0.0057	7.7	1400 (1.08)
	dCTP	0.77	2.7	3.5 (0.55)

[a] Experiments were carried out at least in duplicate, and the mean values are represented. [b] Values relative to that of wt are shown in parentheses. [c] Data from ref *15*.

and D118A mutant proteins in the *orf135*$^-$ strain seemed to reduce the H_2O_2-induced mutation frequency.

DISCUSSION

Nakabeppu and his collaborators substituted the Trp-117 and Asp-119 residues of the MTH1 protein, and were able to "separate" its 8-OH-dGTPase and 2-OH-dATPase activities (*21*). In a subsequent study, they showed that both activities were important for the suppression of cell dysfunction and the delayed cell death of MTH1-null mouse embryo fibroblast cells, using these mutant MTH1 proteins (*11*). We carried out a mutagenesis study of the Orf135 protein, which has 2-OH-dATPase, 8-OH-dGTPase, dCTPase, and 5-Me-dCTPase activities, to identify the important amino acid residues for the recognition of each deoxyribonucleotide, and to examine which activities are important for the suppression of reactive oxygen species-induced mutations.

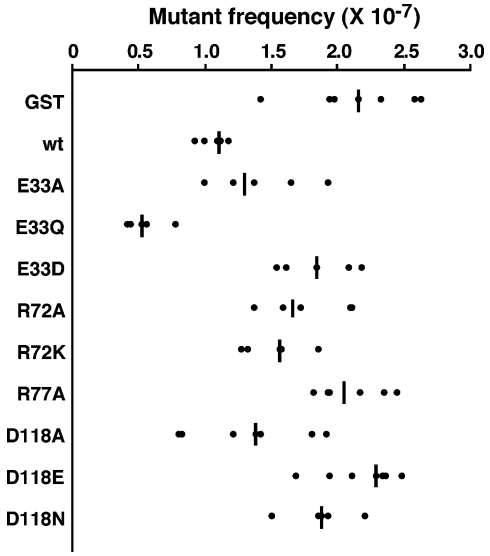

FIGURE 5: Suppression of H_2O_2-induced mutations by expression of the recombinant Orf135 protein. The *orf135*$^-$ *E. coli* strains, harboring a plasmid for GST or the GST−Orf135 protein, were treated with 2 mM H_2O_2, as described in Experimental Procedures. Experiments were carried out five to seven times. Filled circles represent each datum. Bars represent medians.

One of the objectives of this study was to examine the role of the amino acid residues in the putative nucleotide binding pocket of the Orf135 protein. This nucleotide binding pocket was proposed by a comparison of an Orf135 protein model with the human MTH1 protein structure (Figure 1) (*12*). We focused on the Glu-33, Arg-72, Arg-77, and Asp-118 residues, which were suggested to be exposed on the surface of the putative base-binding pocket.

The substitution of the Glu-33 residue of the Orf135 protein affected its activities to various degrees (Figure 3). The dCTPase and 8-OH-dGTPase activities were almost completely abolished, and the 5-Me-dCTPase activity was also reduced. Thus, this Glu residue may favorably interact with these three deoxyribonucleotides. The replacement of this residue with Ala and Gln increased the 2-OH-dATPase activity, and the E33D mutant exhibited reduced 2-OH-dATPase activity. These results suggest that the Glu-33 residue unfavorably interacts with 2-OH-dATP, because of the presence of a carboxyl (COO^-) group. The corresponding residue of the MTH1 protein is Asn-33. This residue was suggested to be important for nucleotide binding, because its side chain is exposed in the binding pocket (*12*). A site-directed mutagenesis study indicated that the presence of the side chain carbonyl (C═O) group at this position is important for recognizing 2-OH-dATP (*12*). The N33A MTH1 mutant exhibited 14% of the wt 8-OH-dGTP activity, whereas the N33E mutation totally abolished the 8-OH-dGTPase activity. In this case, the carboxyl group of this Glu may unfavorably interact with 8-OH-dGTP.

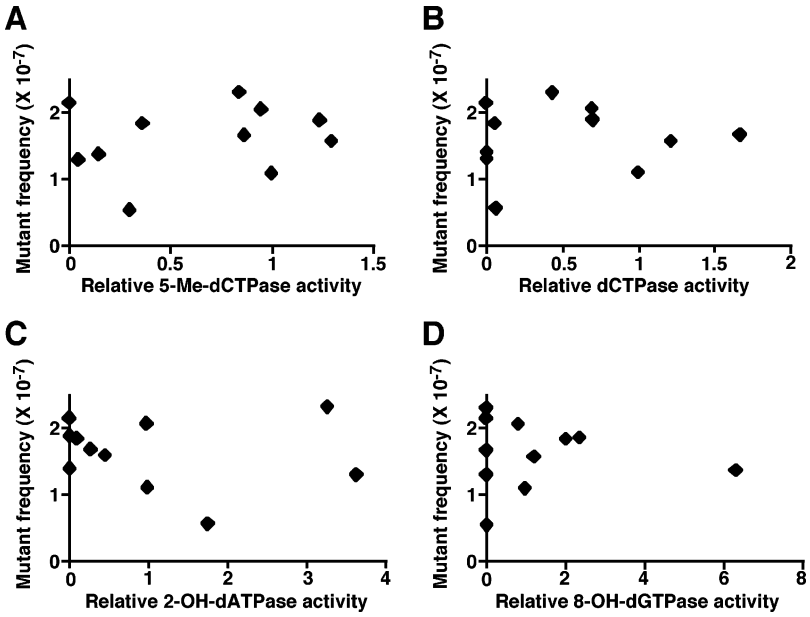

FIGURE 6: Correlation between the enzymatic activities of the GST−Orf135 and GST proteins and the *rpoB* mutant frequency. The relative activity was defined as described in the text: (A) 5-Me-dCTPase activity, (B) dCTPase activity, (C) 2-OH-dATPase activity, and (D) 8-OH-dGTPase activity.

The R72A mutant displayed increased dCTPase and reduced 2-OH-dATPase activities, suggesting favorable and unfavorable interactions, respectively, with 2-OH-dATP and dCTP. Since the R72K mutant had activities similar to those of the wt protein, these interactions may depend on the positive charge of the 72nd residue. The substitution of the Arg-77 residue affected the enzymatic activities only slightly, excluding the possibility that this Arg side chain is important in substrate binding. The Arg-78 residue of the MutT protein may interact with 8-OH-dGTP by hydrogen bonding (*22*). However, the substitution of the 72nd Arg residue of Orf135 with Ala or Lys did not affect the 8-OH-dGTPase activity drastically, indicating little, if any, interaction occurs between Arg-72 and 8-OH-dGTP.

The replacement of the Asp-118 residue generated quite interesting results (Figures 3 and 4). The D118A and D118N mutations almost completely abolished the 2-OH-dATPase activity. On the other hand, the D118E mutant displayed a 2-OH-dATPase activity higher than that of the wt protein (13-fold in the k_{cat}/K_m values, Table 2). In marked contrast, the 8-OH-dGTP hydrolyzing activity was diminished in the case of D118E, and the D118A and D118N proteins had increased 8-OH-dGTPase activity. Thus, the carboxyl (COO^-) group (negative charge) of this Asp residue appeared to contribute to 2-OH-dATP binding and to suppress 8-OH-dGTP binding. The 5-Me-dCTP and dCTPase activities were drastically impaired by the substitution of Asp-118 with Ala. The D119A mutant of MTH1 exhibited approximately half of the wt activity for 8-OH-dGTP, but had almost no activity for 2-OH-dATP, and the activities of the D119N mutant were similar to those of D119A (*21*). These results suggest that the charged carboxyl (COO^-) group of the side chain, but not the carbonyl (C═O) group, is crucial for discriminating 2-OH-dATP. Thus, Asp-118 of Orf135 and Asp-119 of MTH1 may interact with 2-OH-dATP in a very similar fashion. On the other hand, the charged carboxyl (COO^-) group of the Asp-118 residue in Orf135 unfavorably interacts with 8-OH-dGTP, although the Asp-119 residue in MTH1 does not seem to be very important for 8-OH-dGTP binding. In contrast, the Asn-119 residue of MutT seems to interact with 8-OH-dGTP by hydrogen bonding (*22*).

Intriguingly, the D118A and D118N mutants hydrolyzed 8-OH-dGTP to 8-OH-dGDP. Experiments using ^{18}O-enriched water revealed that the β-phosphorus atom of the substrate is attacked by water and the substrate Pα−Pβ bond is subsequently cleaved during the reactions by the MutT, Orf17, and MTH1 proteins (*12*, *23*, *24*). The Orf135 protein probably hydrolyzes the substrate nucleotides by the same mechanism. The emergence of 8-OH-dGDP as a product suggests the cleavage of the substrate Pβ−Pγ bond, followed by the attack of a water molecule on the β- and/or γ-phosphorus atom of the substrate during the reactions by these mutant proteins. The D118A and D118N mutants possessed 8-OH-dGDPase activities, and their total 8-OH-dGTPase activities were higher than their 8-OH-dGDPase activities (data not shown). Thus, more than half of the 8-hydroxy-dGMP produced by these mutant proteins was derived from the same mechanism as that of the wt enzyme, which possibly involves the nucleophilic attack of a water molecule on the β-phosphorus atom and the subsequent cleavage of the substrate Pα−Pβ bond. The substitution of Asp-118 with Ala or Asn might alter the local structure of the binding pocket, and the hydrolysis reaction may follow different pathways.

The other objective of this study was to examine the suppression of the mutagenesis induced by oxidatively damaged DNA precursors, by the mutant proteins with various substrate specificities. Previously, we reported that the Orf135 protein could hydrolyze deoxyribonucleotides, such as 2-OH-dATP, and that the expression of the recombinant Orf135 protein reduced the frequencies of both the H_2O_2-induced and spontaneous mutations (*7*, *9*).

The 5-Me-dCTPase, dCTPase, 2-OH-dATPase, and 8-OH-dGTPase activities of each mutant protein with the GST tag were normalized to those of the wt protein (wt = 1.0). We then examined the relationship between the H_2O_2-induced

mutant frequencies and the relative enzymatic activities of the mutant GST−Orf135 proteins in vitro (Figures 3 and 5). As shown in panels A, B, and D Figure 6, no correlation exists among the 5-Me-dCTPase, dCTPase, and 8-OH-dGTPase activities and the H_2O_2-induced mutant frequencies. Statistically significant correlation exists only between the H_2O_2-induced mutant frequencies and the 2-OH-dATPase activity (0−1.8 of relative activity, Figure 6C). The Pearson's correlation coefficient was calculated to be −0.71, which is statistically significant ($P < 0.05$). Thus, the results of these experiments may suggest that the 2-OH-dATPase activity of the Orf135 protein contributes to the suppression of the reactive oxygen species-elicited mutagenesis derived from oxidized deoxyribonucleotide(s) in *E. coli* cells. However, expression of the two mutants, E33A and D118E, did not suppress the H_2O_2-induced mutations. The actual reasons for these exceptions are unknown. These proteins may be unstable upon being treated with H_2O_2.

Tassotto and Mathews tried to measure the amount of 8-OH-dGTP in *E. coli*, and concluded that its intracellular concentration is below 0.34 μM (the limit of detection) (*25*). This failure to detect 8-OH-dGTP in extracts of *mutT* strains cast some doubt on the expected MutT function. However, the ratio of 0.34 μM 8-OH-dGTP to the estimated concentration of dGTP in the bacterium (100 μM) (*26*) is 3.4×10^{-3}, and this ratio appears to be too high when the in vivo situation is considered. Thus, their result did not deny roles of the nucleotide pool sanitization. In contrast, deficiencies in the *E. coli* MutT and Orf135 and mammalian MTH1 proteins result in increased mutation frequency and enhanced tumor formation, indicating the importance of the nucleotide pool sanitization (*5*, *6*, *9*). Thus, oxidatively damaged DNA precursors appear to be formed in cells and be involved in mutagenesis by ROS.

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SUPPORTING INFORMATION AVAILABLE

Structures of MutT, MTH1, and Orf135. This material is available free of charge via the Internet at http://pubs.acs.org.

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