Research article

Insights into the role of Val45 and Gln182 of Escherichia coli MutY in DNA substrate binding and specificity

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Abstract

Background: Escherichia coli MutY (EcMutY) reduces mutagenesis by removing adenines paired with guanines or 7,8-dihydro-8-oxo-guanines (8-oxoG). V45 and Q182 of EcMutY are considered to be the key determinants of adenine specificity. Both residues are spatially close to each other in the active site and are conserved in MutY family proteins but not in Methanobacterium thermoautotrophicum Mig.MthI T/G mismatch DNA glycosylase (A50 and L187 at the corresponding respective positions).

Results: Targeted mutagenesis study was performed to determine the substrate specificities of V45A, Q182L, and V45A/Q182L double mutant of EcMutY. All three mutants had significantly lower binding and glycosylase activities for A/G and A/8-oxoG mismatches than the wild-type enzyme. The double mutant exhibited an additive reduction in binding to both the A/G and A/GO in comparison to the single mutants. These mutants were also tested for binding and glycosylase activities with T/G- or T/8-oxoG-containing DNA. Both V45A and Q182L mutants had substantially increased affinities towards T/G, however, they did not exhibit any T/G or T/8-oxoG glycosylase activity. Surprisingly, the V45A/Q182L double mutant had similar binding affinities to T/G as the wild-type EcMutY. V45A, Q182L, and V45A/Q182L EcMutY mutants could not reduce the G:C to T:A mutation frequency of a mutY mutant. Expression of the V45A mutant protein caused a dominant negative phenotype with an increased G:C to A:T mutation frequency.

Conclusion: The substrate specificities are altered in V45A, Q182L, and V45A/Q182L EcMutY mutants. V45A and Q182L mutants had reduced binding and glycosylase activities for A/G and A/8-oxoG mismatches and increased affinities towards T/G mismatch. However, in contrast to a previous report that Mig.MthI thymine DNA glycosylase can be converted to a MutY-like adenine glycosylase by replacing two residues (A50V and L187Q), both V45A and Q182L EcMutY mutants did not exhibit any T/G or T/8-oxoG glycosylase activity. The dominant negative phenotype of V45A EcMutY mutant protein is probably caused by its increased binding affinity to T/G mismatch and thus inhibiting other repair pathways.
Background

Reactive oxygen species are generated by endogenous processes such as mitochondrial oxidative phosphorylation as well as exogenously following exposure to ionizing radiation and chemicals [1]. Oxidative DNA damage including strand breaks and oxidative base lesions are specifically repaired by base excision repair pathways [2]. The first step in this pathway is carried out by a lesion-specific DNA glycosylase, which cleaves the N-glycosidic bond between the base and deoxyribose sugar [3]. The most abundant and highly mutagenic oxidative DNA damage lesion is 8-oxo-7,8-dihydroguanine (8-oxo-G or GO) that can form base-pair with adenine or cytosine during DNA replication to produce a G:C to T:A transversion [4,5]. In *Escherichia coli*, MutE, MutM (Fpg), MutY, MutS, and Nei (End VIII) are involved in defending against the mutagenic effects of 8-oxoG lesions [reviewed in [6] and [4]]. The MutT protein has pyrophosphohydrolase activity, which eliminates 8-oxo-dGTP from the nucleotide pool. MutM glycosylase (Fpg protein) removes both mutagenic GO adducts and ring-opened purine lesions paired with cytosines. MutS and MutY increase replication fidelity by removing the adenines misincorporated opposite GO or G [5,7,8], and thus reduce G:C to T:A transversions. Nei can excise GO, when GO is paired opposite a cytosine or adenine and can serve as a backup pathway to repair 8-oxoG in the absence of MutM and MutY [6,9].

Unlike most DNA glycosylases, MutY does not target a damaged base, but a normal base, e.g., removing A mispaired with GO, C, and 5-hydroxymethyluracil [reviewed in [4]]. In addition, MutY can excise guanines from G/GO mismatches [5]. MutY also binds tightly to DNA containing T/GO and C/GO, however, it exhibits no catalytic activity on them [10]. The N-terminal domain of EcMutY retains the catalytic activity [10-14] while the C-terminal domain of MutY plays an important role in the recognition of GO lesions [10,11,14,15]. The X-ray crystal structures of EcMutY catalytic domain with bound adenine [16] and intact *Bacillus steathermotrophus* MutY (Bs-MutY) bound to DNA [17] show that MutY distorts the bound DNA substrate and the mismatched A is flipped out of the helix. In the active site pocket of EcMutY, seven amino acids (R19, E37, L40, N140, Q182, M185, and D186) are involved in adenine binding (filled circles in Figure 1) [16]. V45 and Q182 of EcMutY are proposed to be key residues in determining substrate specificity. V45 is considered to be important for glycosylase activity because V45N is inactive [16] and is conserved among MutY family members (Figure 1). Q182 forms a hydrogen bond with adenine at the domain interface of EcMutY, but is less conserved [18].

The N-terminal domain of MutY shares similar structure, including the helix-hairpin-helix (HhH) and Gly/Pro Asp loop motifs (Figure 1), with AlkA, EndoIII, and OGG1. The G/T mismatch glycosylase Mig.MthI of *Methanobacterium thermoautotrophicum* is a member of the HhH superfamily [19] and is involved in reducing spontaneous deaminated bases from 5-methyl-cytosine residues. The substrate specificity of Mig.MthI is in the order: U/G > T/G > G/G > T/C = U/C > A/G. In the binding pocket for the target base, Mig.MthI shares six out of seven residues of EcMutY’s adenine binding (R19, E37, L40, N140, Q182, M185, and D186). The only non-conserved residue in this binding pocket is Q182 of EcMutY, which corresponds to L187 of Mig.MthI (Figure 1). Interestingly, Fondufe-Mittendorf et al. [20] could convert Mig.MthI into a MutY-like glycosylase with altered substrate preference of Mig.MthI from T/G to A/G by replacing two residues (A50V and L187Q) in the substrate binding pocket. Their data point to the potential importance of V45 and Q182 in EcMutY substrate recognition.

In this study, we have investigated the role of V45 and Q182 of EcMutY by targeted mutagenesis. V45A, Q182L, and V45A/Q182L mutant proteins have reduced binding affinity and glycosylase activity towards T/G or T/GO. Therefore, V45 and Q182 are considered as key residues in determining substrate specificity of EcMutY.

Results and discussion

Selection of EcMutY mutation

The study by Fondufe-Mittendorf et al. [20] indicates that the substrate preference of Mig.MthI can be altered from T/G to A/G by replacing two residues (A50V and L187Q) in the substrate binding pocket. Their results are in agreement that V45 and Q182 of EcMutY are the key determinants of adenine specificity [16]. According to the adenine soaked EcMutY structure (Figure 2A) [16], V45 is located in the minor-groove reading α2–α3 motif (37-EVM-LQQTQY-45) and Q182 is located in the adenine recognition α10 motif (182-QAMMD-186). Interestingly, the proximity of V45 and Q182 to adenine in EcMutY structure closely correlates with the BsMutY structure with the DNA product and cleaved adenine [17]. However, the residues corresponding to Val45 and Gln182 in D144N BsMutY with A/GO-containing DNA (so called the lesion recognition complex) [17] do not directly make contacts with the adenine of the A/GO mismatch. In the later structure, the Val seems to be positioned below the adenine, but the Glu188 of BsMutY (corresponding to Glu182 of EcMutY) is far away from adenine. The fact that there are differences in adenine recognition among various MutY structures raises a question whether these residues really
are involved directly in adenine recognition. To determine whether these two residues are important for adenine recognition and excision, we carried out targeted mutagenesis study to determine the effect of EcMutY V45A, Q182L, and double mutant on repair of A/G- and A/GO-containing DNA. We also further determined whether their substrate specificity is altered in terms of binding or catalysis of T/G or T/GO mismatches.

A/G and A/GO binding and glycosylase activity

We compared the binding affinities of V45A, Q182L, and V45A/Q182L double mutants with wild-type EcMutY to DNA substrates containing A/G and A/GO mismatches. First, we determined the active site concentrations of these proteins by performing A/GO binding with DNA concentrations which are at least 17-fold higher than their estimated dissociation constants ($K_d$). Figure 3 shows the average results from two independent experiments with 8 nM of A/GO-DNA. The increase in wild-type (WT) EcMutY binding reached a maximum at a concentration about 25 nM of MutY and 5.44 nM DNA was bound (68% of 8 nM). This horizontal line intercepts with the slope line of initial rate at 8 nM of MutY. Thus, the WT EcMutY preparation is 68% active. Similar analyses showed that V45A, Q182L, and V45A/Q182L were 48%, 25%, and 40% active, respectively. Dissociation constant value for each mutant was determined with nine enzyme concentrations by at least three independent experiments (Table 1). Because MutY displays a high affinity for its products [21], the $K_d$ values measure its affinities to a mixture of the substrate and the product. Compared to wild-type EcMutY of which the $K_d$ to A/G is $1.3 \pm 0.11$ nM, the V45A and Q182L mutants had $K_d$ values of $6.1 \pm 1.7$ and $8.9 \pm 5.0$ nM, respectively. The $K_d$ value (15 ± 2 nM) of V45A/Q182L double mutant was higher than the single mutants. Thus, the affinities to A/G of V45A, Q182L, and V45A/Q182L are weakened by 5-, 7-, and 12-fold, respectively. For A/GO binding, V45A and Q182L mutations resulted in a 94- and 11-fold increase in $K_d$ values, respectively. The V45A/Q182L double mutation resulted in a 94- and 11-fold increase in $K_d$ values, respectively.

**Figure 1**

Sequence Alignment. The protein sequences of *B. stearothermophilus* MutY (1VRLA, BsMutY), *E. coli* MutY (AP 003518, EcMutY), *T. thermophilus* MutY (YP 145164, TthMutY), and *M. thermoautotrophicum* Mig. MthI (P 29588, Mig) were aligned using ClustalW algorithm. The open and shadowed boxes represent conserved and identical residues respectively. The two residues subjected to mutation in this study are shaded in black. The highly conserved HhH-GPD motif is indicated. Filled circles indicate EcMutY residues involved in adenine binding and stars indicate Mig.MthI residues important for thymine recognition. The hollow circle and triangle mark the base flipper and the conserved catalytic Asp, respectively.

**Table 1**

| Protein    | $K_d$ (nM) |
|------------|------------|
| V45A       | 6.1 ± 1.7  |
| Q182L      | 8.9 ± 5.0  |
| V45A/Q182L | 15 ± 2     |

are involved directly in adenine recognition. To determine whether these two residues are important for adenine recognition and excision, we carried out targeted mutagenesis study to determine the effect of EcMutY V45A, Q182L, and double mutant on repair of A/G- and A/GO-containing DNA. We also further determined whether their substrate specificity is altered in terms of binding or catalysis of T/G or T/GO mismatches.

A/G and A/GO binding and glycosylase activity

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102-fold increase in $K_d$ value (i.e. 102-fold weaker binding affinity). Thus, V45A is more defective in A/GO binding than A/G binding as compared to wild-type MutY. It is interesting to note that the double mutant exhibited an additive reduction in binding to both the A/G and A/GO as for the single mutants. These results confirm that V45 and Q182 play important but independent roles in adenine binding.

Because V45A and Q182L EcMutY mutants showed weakened A/G and A/GO affinities, we examined their glycosylase activities on A/G and A/GO (Figure 4). V45A mutant showed decrease in A/G glycosylase activity at 0.48 and 4.8 nM concentrations (Figure 4A, lanes 4–5) while Q182L mutant showed no activity at 0.25 nM and decrease in activity at 2.5, and 12.5 nM (Figure 4A, lanes 7–9), as compared to wild-type MutY. The glycosylase activity of V45A/Q182L double mutant on A/G was nearly undetectable at 20 nM (Figure 4A, lane 12). With A/GO substrate, both V45A and Q182L mutants showed decrease in activity at 0.48 and 0.25 nM, respectively (Figure 4B, lanes 4 and 7), whereas the V45A/Q182L double mutant
showed no activity at 0.4 nM and decreased activity at 4, and 20 nM (Figure 4B, lane 10–12). The glycosylase activity assayed at the indicated active site concentrations is based on the active site titration results upon binding with A/GO-DNA. Since the mutants’ glycosylase activities are affected differently from DNA binding, thus, the active site concentrations measured by DNA binding may be different from those measured by DNA glycosylase activity.

We also used single-turnover glycosylase kinetics with enzyme in excess over DNA (time-course study) to compare the activities of wild-type and mutant EcMutY proteins. As shown in Figure 5, the rates of cleavage on both A/G- and A/GO-containing DNA by MutY-V45A were only slightly lower than those of the wild-type EcMutY protein. However, the rates of cleavage of both A/G- and A/GO-containing DNA were much lower for Q182L and V45A/Q182L mutants than those of the wild-type EcMutY (Figure 5). The rate constants \(k_2\) of Q182L and V45A/Q182L mutants with A/G mismatch at 37°C were 0.025 ± 0.016 Min\(^{-1}\) and 0.032 ± 0.026 Min\(^{-1}\), respectively, and were about 10-fold lower that of the wild-type EcMutY (with \(k_2\) of 0.24 ± 0.03 Min\(^{-1}\)) (Table 2). The \(k_2\) of Q182L and V45A/Q182L mutants with A/G mismatch at 4°C were 0.16 ± 0.02 Min\(^{-1}\) and 0.37 ± 0.05 Min\(^{-1}\), respectively, and were about 10-fold lower that of the wild-type EcMutY (with \(k_2\) of 2.4 ± 0.3 Min\(^{-1}\)) (Table 2). These results indicate that the overall repairing ability is reduced by Q182L and V45A/Q182L mutants but not by the V45A mutant. The above results demonstrate that V45A and Q182L mutations have significant effect on binding and glycosylase activities to A/G and A/GO mismatches. A catalytic domain of EcMutY with a V45N mutation has been shown to be inactive [16]. These results indicate that V45 and Q182 are significant for the adenine-specific activity of EcMutY.

**T/G and T/GO binding and glycosylase activity**

We next measured the T/G and T/GO binding affinities of these mutants. Wild-type EcMutY showed a comparatively high \(K_d\) (14 ± 1 nM) with a T/G mismatch (Table 1), however, this value is much lower than that of EcMutY with C:G homoduplex (315 ± 49 nM) [22]. The \(K_d\) values of V45A and Q182L mutants to T/G drastically decreased to 0.011 ± 0.004 nM and 0.043 ± 0.014 nM, representing a 1273 and 326-fold increase in binding affinity, respectively (Table 1). Surprisingly, V45A/Q182L double mutant showed a \(K_d\) value of 8.4 ± 0.7 nM, which is close to that of wild-type EcMutY. The geometry or architecture of the substrate binding pocket in the V45A/Q182L double mutant may not favor thymine binding. For T/GO binding, V45A, Q182L, and V45A/Q182L mutants showed similar \(K_d\) values as wild-type EcMutY (Table 1), indicating that the T/GO binding affinity of EcMutY does not involve V45 and Q182. This result is consistent with the findings of Li et al. [10] that tight T/GO binding is controlled by the C-terminal domain of EcMutY.

**Table 1: Apparent dissociation constants (\(K_d\)) of MutY mutants.**

| DNA    | WT       | V45A     | Q182L   | V45A/Q182L |
|--------|----------|----------|---------|------------|
| A/G    | 1.3 ± 0.11\(^a\) | 6.1 ± 1.7 (0.21)\(^b\) | 8.9 ± 5.0 (0.15) | 15 ± 2 (0.09) |
| A/GO   | 0.0048 ± 0.0029 | 0.45 ± 0.06 (0.01) | 0.051 ± 0.009 (0.09) | 0.59 ± 0.12 (0.01) |
| T/G    | 14 ± 1 | 0.011 ± 0.004 (1273) | 0.043 ± 0.014 (326) | 8.4 ± 0.7 (1.7) |
| T/GO   | 0.036 ± 0.013 | 0.024 ± 0.009 (1.5) | 0.088 ± 0.061 (0.41) | 0.013 ± 0.008 (2.77) |

\(^a\) \(K_d\) values (nM) are mean ± standard deviation for more than three experiments using nine protein concentrations.  
\(^b\) The numbers in parenthesis are the folds of binding affinity relative to wild-type (WT) MutY.
Because Mig.MthI T/G glycosylase can be converted to an A/G glycosylase by replacing two residues (A50V and L187Q) [20], we examined the glycosylase activities of Vl45A, Q182L, and V45A/Q182L on T/G and T/GO. None of the EcMutY mutants showed any T/G and T/GO glycosylase activity at enzyme concentration up to 1 μM under different buffers and temperatures (data not shown). Therefore, recognition and glycosylase of EcMutY mutants to T/G and T/GO are controlled by different mechanisms.

The previous success to mutate Mig.MthI into MutY-like enzyme [20] is in contrast to our failure to covert EcMutY to thymine glycosylase. It is possible that it is easier to catalyze adenine removal than thymine excision. It is interesting to point out that wild-type Mig.MthI is a weak adenine glycosylase and Mig.MthI with A50V/L187Q mutation becomes a stronger adenine glycosylase.
ever, wild-type EcMutY has no thymine glycosylase activity and V45A/Q182L mutant may be harder to gain this activity. In addition, the active site of Mig.MthI is more relaxed for both purine and pyrimidine excision while MutY is more strict to excise purines. The difference in the base removal by these two enzymes may be contributed by other residues surrounding the active site.

By aligning MutY and Mig.MthI sequences (Figure 1) and inspecting the structures of Mig.MthI [23], EcMutY catalytic domain [16] with docked thymine (Figure 2), we predicted that several residues may contribute to their difference in activities. The thymine docked into the EcMutY active site preserves the proposed edge-on hydrogen bonding interaction with the conserved glutamate (E37, E42 in Mig.MthI) and can support a potential hydrogen bond with a tyrosine in a potential S120Y mutant (Figure 2A). It is clear from Figs. 2C and 2D that the MutY active site poorly accommodates the methyl group of thymine relative to the Mig.MthI active site. In the present study, one important mutation (Q182L) has been created. Based on our observations and the models shown in Figure 2, the subsequent mutations most likely to promote the conversion of MutY into a thymine glycosylase activity supporting that Y126 of Mig.MthI is absolutely required for T/G or T/GO glycosylase activity. This suggests that F27 and R47 of Mig.MthI may not be the only key residues, which correspond to F27 and R47 of Mig.MthI, respectively, are also conserved in EcMutY and BsMutY. This suggests that F27 and R47 of Mig.MthI may not be absolutely required for T/G or T/GO glycosylase activity. Interestingly, Y112S TthMutY mutant lost the thymine glycosylase activity supporting that Y126 of Mig.MthI may be important for T/G catalytic activity. It remains to be tested whether S120Y, Q25R, and L22F mutants of EcMutY or in combination with V45A and Q182L can gain T/G glycosylase activity.

### Table 2: Rate constants ($K_2$) of MutY mutants.

| DNA | WT | V45A | Q182L | V45A/Q182L |
|-----|----|------|-------|------------|
| A/G | 0.24 ± 0.03<sup>a</sup> | 0.19 ± 0.03 (0.80)<sup>b</sup> | 0.025 ± 0.016 (0.11) | 0.032 ± 0.026 (0.14) |
| A/GO | 2.4 ± 0.3 | 1.3 ± 0.1 (0.54) | 0.16 ± 0.02 (0.07) | 0.37 ± 0.05 (0.15) |

<sup>a</sup>$K_2$ values (Min$^{-1}$) are means ± standard deviation for more than three experiments. 
<sup>b</sup>The numbers in parenthesis are the folds of rate relative to wild-type (WT) MutY.

CC104 strain was designed to screen G:C→T:A transitions at an essential residue in the active site of β-galactosidase encoded by lacZ gene, thus it allowed us to measure this type of mutation in EcMutY-V45A, EcMutY-Q182L and EcMutY-V45A/Q182L mutants. Similar to the results that have been shown to have strong A/GO and G/GO activities, comparatively weak T/GO and A/G activities, and no T/G glycosylase activity [24]. Unlike Mig.MthI, TthMutY and EcMutY contain extra C-terminal domains which facilitate their recognition of the mispaired GO base. V42 and Q170 of TthMutY are identical with the corresponding residues in EcMutY (V45 and Q182) but different from A50 and L187 of Mig.MthI (Figure 1). Although TthMutY has weak T/GO glycosylase activity, its L19 and Q39 residues, which correspond to F27 and R47 of Mig.MthI, respectively, are also conserved in EcMutY and BsMutY. This suggests that F27 and R47 of Mig.MthI may not be absolutely required for T/G or T/GO glycosylase activity. Interestingly, Y112S TthMutY mutant lost the thymine glycosylase activity supporting that Y126 of Mig.MthI may be important for T/G catalytic activity. It remains to be tested whether S120Y, Q25R, and L22F mutants of EcMutY or in combination with V45A and Q182L can gain T/G glycosylase activity.
of rifampicin forward assay, the mutation frequency of the
mutMmutY double mutant increased 400-fold over the
wild-type cells (Table 3, right panel, lines 1 and 2). The
EcMutY-V45A, EcMutY-Q182L and EcMutY-V45A/Q182L
mutants showed partial defect in preventing G:C→T:A
transversions as compared to the wild-type EcmutY.
Strains expressing V45A, Q182L and V45A/Q182L
mutants, respectively, showed 5, 8, and 17-fold higher
mutation frequencies than CC104 wild-type (Table 3,
right panel, lines 5–7). These increased G:C→T:A mutation
frequencies are in agreement with the reduced glyco-
sylase activities with A/G and A/GO mismatches in these
mutants.

Wild-type cells expressing the V45A mutant protein have
an increased G:C to A:T mutation frequency
For the strains expressing V45A MutY protein, the
G:C→T:A mutation frequency measured by Lac+ reversion
assay was much lower than that measured by the rifampicin
forward-mutation assay (154-fold increase in rifampicin
forward-mutation assay vs. 5.3-fold increase in the
Lac+reversion assay) (Table 3, compare lines 5 in right
and left panels). Because V45A mutant exhibits milder
defect in adenine glycosylase activity than the other two
mutants, we suspected that the V45A mutant with a 1273-
defect in adenine glycosylase activity than the other two
mutants, we suspected that the V45A mutant with a 1273-
fold increased T/G affinity (Table 1) might prevent T/G
mismatches from being repaired by other DNA repair
pathways. To test this idea, we isolated genomic DNA
from rifampicin resistant colonies from CC102 (mutM
mutY) cells expressing EcMutY-V45A and identified base-
base mutations of rpoB gene [25]. A significant increase
in G:C→A:T transition was found in cells expressing
EcMutY-V45A as compared to cells with vector alone
(Table 4). For unknown reason, the mutation frequency
of A:T to T:A was quite high in CC102 harboring vector
and EcMutY-V45A. CC102 strain was designed to screen
G:C→A:T transitions in lacZ gene. Thus, we further mea-
sured the G:C→A:T transition frequency by Lac+ reversion
assay in CC102 (mutM+ mutY) strain expressing EcMutY-
V45A. The EcMutY-V45A expressing strain showed a 3.6-
fold higher Lac+ reversion frequency. A′T-test of two sets of
data of rows 1 and 3 in Table 5 shows that this increase is
statistically significant with $P = 0.001$ (Table 5). These
data are consistent with our postulation that EcmutY-
V45A protein acts as a dominant-inhibitor of other DNA
repair pathways.

Conclusion
The substrate specificity of DNA glycosylases is very subtle
to the change of amino acids located in the DNA binding
pocket, and a single amino acid alteration may signifi-
cantly alter its enzyme activity. Our results show that V45
and Q182 of EcmutY are important for the binding affinity
and glycosylase activity to DNA containing A/G or A/
GO mismatches. The V45A/Q182L double mutant exhib-
it an additive reduction in binding to both the A/G and
A/GO as for the single mutants. Our unexpected results
that EcMutY-V45A and EcMutY-Q182L show increased T/
G binding affinity without gaining T/G glycosylase indi-
cate the complicated nature of the DNA-enzyme interac-
tion.

Methods
Bacteria strains
Escherichia coli DH5α (F−, $\lambda$-dlacZΔM15, endA1, recA1,
hsdR1, ($rK_mK^+$), supE44, thi-1, gryA96(NalR), relA1, Δ (lac-
ZYA-argF)Δ (U169) was purchased from Invitrogen. PR70
(Su smVLacX74 gaiU gaiK miA68::Tn10kan) was obtained
from M. S. Fox. The miA68::Tn10kan allele in PR70 con-
tains a transposon at the mutY gene at nucleotide 747 and

Table 3: Mutation frequencies of $\lambda$DE3-containing E. coli mutM mutY mutant strains.

| Strain | Mutation Frequency (Rif+108 cells) | Fold | Mutation Frequency (Lac+/108 cells) | Fold |
|--------|------------------------------------|------|------------------------------------|------|
| 1. CC104 (WT) | 4.1 ± 1.3 | 1 | 1.2 ± 1.0 | 1 |
| 2. CC104 mutMmutY | 1464 ± 314 | 357 | 486 ± 65 | 408 |
| 3. CC104M+Y + pET11a | 2055 ± 704 | 501 | 156 ± 102 | 130 |
| 4. CC104M+Y + pET-MutYb | 8.8 ± 4.4 | 2 | 0.9 ± 0.8 | 0.8 |
| 5. CC104M+Y + pET-V45Ac | 630 ± 177 | 154 | 6.3 ± 2.1 | 5.3 |
| 6. CC104M+Y + pET-Q182Ld | 181 ± 74 | 44 | 102 ± 8.7 | 8.5 |
| 7. CC104M+Y + pET-V45A/Q182Le | 728 ± 358 | 178 | 20 ± 13 | 16.7 |

Table 4: Mutation distribution of rpoB in $\lambda$DE3-containing E. coli CC102 (mutM+mutY) harboring pET11a and pET11a-V45A.

| Plasmid | No. of clones with rpoB mutation (%) |
|---------|-------------------------------------|
| pET11a  |                                    |
| pET11a-V45A |                              |
| A:T → T:A | 30 (81.3) | 19 (61.3) |
| G:C → A:T | 5 (13.3)  | 12 (38.7) |
| G:C → T:A | 1 (2.7)   | 0 (0)     |
| A:T → G:C | 1 (2.7)   | 0 (0)     |
| Total    | 37        | 31        |

*The number in parenthesis are the percentages of each mutation type.
produces a truncated MutY protein [10]. CC102 [ara Δ
(lac-proB)314 thi F-lacI378 lacZ461 proA+ B+ ] [26] and
CC104, which is identical to CC102 except for the muta-
tion at residue 461 of MutY, which is identical to CC102 except for the muta-
tion at residue 461 of MutY, which is identical to CC102 except for the muta-
tion at residue 461 of MutY, which is identical to CC102 except for the muta-
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Table 5: Lac+ reversion rate of λDE3-containing E. coli CC102
(mutM+mutY+) strains

| Mutation frequency (Lac+)/10^6 cells | Fold |
|------------------------------------|------|
| 1. CC102 + pET11a                  | 0.45 ± 0.12 | 1  |
| 2. CC102 + pET-MutY                | 0.51 ± 0.16 | 1.1 |
| 3. CC102 + pET-V45A                | 1.60 ± 0.36 | 3.6 |

Construction of E. coli mutY mutants

The mutant mutY genes encoding proteins containing V45A, Q182L and V45A/Q182L were constructed by the QuickChange Site-Directed Mutagenesis Kit (Strategene). The mutagenesis PCR primers for the desired amino acid substitutions are listed below.

Chang564 5'-ATGTTGCAACAAACTCAAGCTGCGACCGTTATCCCC-3'
Chang565 5'-GGGATAACGGTGCTGCTGTTAGTTGTTGCAACAT-3'
Chang566 5'-GTGGAACGGTTAATCTGGCCATGATGGATTGGGTGCG-3'
Chang567 5'-CGCACACCAATCCATCATGCGGCCAGATTAACCGTTCCAC-3'

Primers Chang564 and Chang567, are designed for the V45A mutant and Chang566 and Chang567 are designed for the Q182L mutant using plasmid pMYW-1 [27] as the template. The plasmids containing the mutY mutant genes with V45A or Q182L mutation were named pMY-V45A and pMY-Q182L. The double mutant was derived from pMY-V45A using primers Chang566 and Chang 567. After the PCR reaction, the template plasmids were digested with DpnI restriction and transformed into XL1-Blue supercompetent cells (Strategene). The correct clones were confirmed by DNA sequencing.

Measurement of mutation frequency

Overnight cultures (0.1 ml) of each strain were plated on LB agar plates containing 0.1 mg/ml rifampicin. The cell titer of each culture was determined by plating 0.1 ml of and 10^6 dilution onto LB agar plates. For each measurement, four independent cultures were plated, and the experiments were repeated at least three times. The mutation frequency was determined by calculating the ratio of Rif+ cells to total cells. For LacZ+ reversion mutation assay, overnight cultures (0.2 ml) of each strain were plated on M9 agar plates containing 0.2% lactose and colonies were scored after three days. The ratio of LacZ+ cells to total CC104 cells was calculated to be the G:C→T:A transversion frequency. The ratio of LacZ+ cells to total CC102 cells was calculated to be the G:C→A:T transition frequency.

EcMutY protein expression and purification

E. coli strains PR70/DE3 harboring expression plasmids pMY-V45A, pMY-Q182L, and pMY-V45A/Q182L were grown in LB broth containing 50 μg/ml ampicillin at 37°C. At OD600 of 0.6, isopropyl β-D-thiogalactoside (IPTG) was added to the culture to a final concentration of 0.2 mM, and the culture was incubated at 20°C for 16 hrs. The EcMutY mutant proteins were purified by ammonium sulfate precipitation, phosphocellulose, hydroxylapatite, heparin, Hitrap-S column chromatographies as previously described for the wild-type MutY [21]. The purified proteins were divided into small aliquots and stored at -80°C. Protein concentration was determined by Bradford Method.

Oligonucleotide substrates

The nucleotide sequences of 40-mer DNA substrates containing mismatches used in this study were:

5'-AATGGGGCTTCTGCAGAAATTXGCTTCTGCGAGCATGCC-3'
3'-CCCAGGGAGCTTCCATAYCGGAAGACGTCGTAAG-5'

(where X = A or T and Y = G or GO). The X-strand was labeled by [γ-32p]ATP on the 5' end and then annealed with the Y-strand. The annealed double-stranded oligonucleotides were converted to 44-mers by filling the sticky ends on both sides with Klenow fragment [28].

EcMutY binding and glycosylase assays

The EcMutY binding and glycosylase activities were assayed as previously described by Lu et al [21]. The MutY binding reaction mixture contained 20 mM Tris-HCl, pH 7.6, 80 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 2.9% glycerol, 20 ng of poly dI/dC, and 1.8 fmol of labeled DNA (0.09 nM) in 20 μl reactions. After 30 min incubation at 30°C, 3 μl of 50% glycerol was added to the mixtures, and the samples were loaded to a 4% polyacrylamide gel in TBE buffer [50 mM Tris-borate (pH 8.3) and...
1 mM EDTA]. After electrophoresis, the gel was dried and exposed to a PhosphorImager screen. Enzyme-bound and free DNA bands were quantified on PhosphorImager and analyzed by ImageQuant (GE Health). To determine active site concentrations, binding experiments were performed with 8 nM of A/GO-containing DNA for wild-type, V45A, and Q182L EcMutY while 30 nM of the same DNA was tested for the V45A/Q182L mutant. The MutY concentrations used ranged from 2 to 400 nM as determined by the Bradford assay.

To determine the $K_d$ values, initially, wide ranges of enzyme concentrations with 5-fold series dilution were tested and then nine different enzyme concentrations with 2-fold series dilution (four above and four below the estimated $K_d$) were used. The experiments were repeated at least three times. The $K_d$ values were determined under condition of ligand depletion by computer-fitted curve generated by Graph Pad Prism version 3.03 (Graph Pad Software, Inc) using Eq. 1. The fraction of DNA bound to protein ($f_{\text{bound}}$) was plotted as a function of total active protein concentration in each binding reaction ([P]$_{\text{active}}$). These data sets were then resolved by nonlinear regression using equation 1, which incorporates ligand depletion and returns the maximal binding fraction ($f_{\text{max}}$) and the equilibrium dissociation constant ($K_d$). Fractional binding in the absence of protein ($f_{\text{min}}$) was set to 0 and the concentration of DNA competent for binding in each reaction is given by [DNA]$_T$.

$$f_{\text{bound}} = f_{\text{min}} + (f_{\text{max}} - f_{\text{min}}) \times \left[ \frac{b - \sqrt{b^2 - 4[\text{DNA]_T}[\text{P]_{\text{active}}]}{2[\text{DNA]_T}} \right]$$

where $b = K_d + [\text{DNA]_T} + [\text{P]_{\text{active}}$.

For $K_d$ values without ligand depletion, Eq. 2 was used. Under conditions where [DNA]$_T << K_d$ (and hence [P]$_{\text{active-total}} \approx [\text{P]_{\text{active-free}}$), plots of $f_{\text{bound}}$ versus [P]$_{\text{active}}$ were then resolved by nonlinear regression using equation 2.

$$f_{\text{bound}} = f_{\text{min}} + (f_{\text{max}} - f_{\text{min}}) \times \frac{[\text{P]_{\text{active}}}{[\text{P]_{\text{active}} + K_d}$$

The glycosylase assay was carried out in a 10 μl reaction containing 1.8 fmol of DNA substrate (0.18 nM), 20 mM Tris-HCl (pH 7.6), 1 mM DTT, 1 mM EDTA, 2.9% glycerol and 50 μg/ml of bovine serum albumin. After incubation at indicated temperature for 30 min, reaction mixtures were supplemented with 1 μl of 1 M NaOH and heated at 90°C for 30 min. Five μl of formamide dye (90% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) was added to the sample and 5 μl from this mixture was loaded onto a 14% polyacrylamide sequencing gel containing 7 M urea. For time course studies, enzyme reaction was initiated at 37°C or at 4°C for A/ G or A/GO-containing DNA, respectively, and aliquot taken at different time points were immediately frozen in -70°C in the presence of 0.1 N NaOH, followed by heating at 90°C for 30 min before adding 5 μl formamide dye and loading to 14% 7 M urea sequencing gels. Bands corresponding to cleavage products and intact DNA were quantified from PhosphorImager images. Graphs and rate constants of glycosylase activities are generated by SigmaPlot for Windows Version 10.0 (Systat Software, Inc).

**Sequencing of the rpoB gene**

*E. coli* chromosomal DNA was isolated using a genomic DNA purification kit (Genta System, Minneapolis, MN). The main group of mutations (cluster II) of the rpoB gene was PCR amplified using primers Chang440 (5’-CGTCGTAATCCGTTCGGTGG-3’) and Chang441 (5’-CTCACCCGGATACATCTC GTC-3’) as described and designed previously [25]. The PCR product was purified with the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and sequenced directly with Chang442 primer (5’-CGTG TAGAGCCTGCGGTGAAA-3’).

**Abbreviations**

8-oxoG or GO: 7,8-dihydro-8-oxo-guanines; DTT: dithiothreitol; EcMutY: Escherichia coli MutY; HhH: helix-hairpin-helix; IPTG: isopropyl β-D-thiogalactoside; $k_d$: rate constants; $K_d$: dissociation constant.

**Authors’ contributions**

AL conceived the study with the participation of PC and AM in the experimental design. PC carried out most of the experiments and initial writing of the manuscript. AM performed the active site titration experiments, analyzed the dissociation constants, and edited the paper. AL revised the manuscript that is then confirmed and approved by other authors.

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