Characterization of an *Escherichia coli* Mutant MutY with a Cysteine to Alanine Mutation at the Iron−Sulfur Cluster Domain†

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ABSTRACT: *Escherichia coli* MutY is an adenine and a weak guanine DNA glycosylase involved in reducing mutagenic effects of 7,8-dihydro-8-oxoguanine (8-oxoG). The [4Fe-4S] cluster of MutY is ligated by four conserved cysteine residues and has been shown to be important in substrate recognition. Here, we show that the C199A mutant MutY is very insoluble and can be denatured and renatured to regain activity only if iron and sulfur are present in the renaturation steps. The solubility of C199A-MutY can be improved substantially as a fusion protein containing streptococcal protein G (GB1 domain) at its N-terminus. Here, we describe the first biochemical characterization of the purified GB1-C199A-MutY protein which contains a [3Fe-4S] cluster. The apparent dissociation constant (K_d) values of GB1-C199A-MutY with both A/G and A/8-oxoG mismatches are slightly higher than that of the wild-type protein. The DNA glycosylase activity of GB1-C199A-MutY is comparable to that of the wild-type enzyme. Interestingly, the major difference between the C199A-MutY and wild-type proteins is their trapping activities (formation of Schiff base intermediates). The GB1-C199A-MutY mutant has a weaker trapping activity than the wild-type enzyme. Importantly, highly expressed GB1-C199A-MutY and untagged C199A-MutY can complement *mutY* mutants; however, GB1-C199A-MutY and untagged C199A-MutY cannot complement *mutY* mutants in vivo when both proteins are poorly expressed. Therefore, an intact [4Fe-4S] cluster domain is critical for MutY stability and activity.

Reactive oxygen species (ROS) are mutagens produced during cellular metabolism as well as exogenous stimuli such as ionizing radiation and various chemical oxidants (*1*). 7,8-Dihydro-8-oxoguanine (8-oxoG or GO)[1] is one of the most stable products of oxidative DNA damage and has the most deleterious effects because it can mispair with adenine (*2*, *3*). In *Escherichia coli*, MutY, MutM, and MutT are involved in defending against the mutagenic effects of GO lesions (*2*, *3*). The short patch MutY base excision repair pathway specifically repairs A/GO and A/G to C/GO and C·G, respectively, and corrects A/C to G·C and G/GO to C/GO mismatches at a much lower rate (*4*−*12*). The major function of MutY is to reduce mutation frequency caused by GO lesions (*5*). Adenines are frequently incorporated opposite GO bases during DNA replication (*13*, *14*) that subsequently lead to G·C to T·A transversions (*14*−*17*). Thus, MutY provides the defense by removing adenines misincorporated opposite GO or G following DNA replication (*2*, *7*, *18*).

The N-terminal domain of the MutY protein shares structural similarity with endonuclease III (endo III), AlkA, and human 8-oxoG glycosylase (OGG1) (*11*, *19*−*23*). This similarity includes the helix−hairpin−helix (HhH) and Gly/Pro...Asp loop motifs. Both MutY and endo III contain an iron−sulfur cluster $[4Fe\text{-}4S]^{2+}$ (*7*, *11*, *22*, *24*, *25*). The iron−sulfur cluster of MutY is ligated by four conserved cysteines at positions 192, 199, 202, and 208 that are spaced as C-X_6-C-X_2-C-X_5-C. Attempts to alter the conserved cysteines of MutY with other amino acids are hampered by the instability of some mutant proteins (*26*, *27*). Golinelli et al. (*26*) tried to replace the four conserved Cys individually with His, Ser, and Ala and found that only the C192H, C192S, C199H, and C199S mutants were expressed at significant levels. They showed that substitutions of Cys to Ala at positions 192 and 199 resulted in very low expression of the mutant proteins. The X-ray crystal structures of MutY revealed that the region of Ile191−Cys199 forms a surface-exposed loop, referred to as the iron−sulfur cluster loop (FCL) motif (*22*). The residues spacing the conserved cysteines are dominated by positively charged amino acids and are important for DNA recognition (*28*).

Thus far, it has not been possible to characterize C192A- and C199A-MutY mutants in vitro because they are unstable for homogeneous purification. Here, we have overproduced and purified the C199A-MutY mutant protein by attaching 62 residues of the B1 immunoglobin binding domain of streptococcal protein G (GB1) at its N-terminus. The solubility of the fusion protein is much improved over the nonfusion protein. In this paper, we describe the first biochemical characterization of a mutant protein containing an iron−sulfur cluster ligated by three cysteines. The purified GB1-C199A-MutY protein contains a [3Fe-4S] cluster and has a weaker activity to form a Schiff base intermediate than

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[1] Abbreviations: AP, apurinic/apyrimidinic; C199A, a mutant MutY with Ala at position 199; endo III, endonuclease III; FCL, iron−sulfur cluster loop; GB1, B1 immunoglobin binding domain of streptococcal protein G; GO, 8-oxoG or 7,8-dihydro-8-oxoguanine; HhH, helix−hairpin−helix; K_2, rate constant; K_d, apparent dissociation constant; LB broth; Luria−Bertani broth; OD, optical density; OGG1, 8-oxoG glycosylase; PCR, polymerase chain reaction; Rif, rifampicin; SDS, sodium dodecyl sulfate.

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the wild-type enzyme. Moreover, GB1-C199A-MutY and untagged C199A-MutY cannot complement *mutY* mutants in vivo when both proteins are poorly expressed. Therefore, an intact [4Fe-4S] cluster domain is critical for MutY stability and activity.

MATERIALS AND METHODS

Bacteria. The *E. coli* strains PR8 (Su- *lacZ X74 galU galK* Sm^r) and *mutY* mutant PR70 (like PR8 but *micA68*::Tn*10*kan) were obtained from M. S. Fox. The strain CC104 containing a *lac*Z mutation at residue 461 of β-galactosidase and its derivative CC104 *mutM*::mini-kan *mutY*::mini-Tn*10* were generous gifts from J. H. Miller. Strains with λDE3 lysogen were constructed according to the procedures described by Invitrogen.

Plasmids. The cloning of pJTW10-12 and pMYW-1 containing the entire *mutY* gene in pKK223-2 (Amersham Parmarcia) and pET11a (Novagen) expression vectors, respectively, have been described (*11*, *29*).

Mutant C199A-MutY was constructed by the PCR splicing overlap extension method (*30*). Plasmid pMYW-1 was used as template. The oligonucleotide pairs containing Chang 199A1 (5′CGGACAGAGCGAAGCTTTCGGTTTCGAGCG3′) and Chang 222 (5′GCGACGCATATGCAAGCGTCGCAATTTTC3′) were used as primers to amplify the N-terminal region of the *mutY* gene. The oligonucleotide pairs containing Chang 199A2 (5′CGCTCGAAACCGAAAGCTTCGCTCTGTCCG3′) and Chang 90 (5′GCCGGAGGATCCCTAAACCGGCGCGCCAGTGC3′) were used as primers to amplify the C-terminal region of the *mutY* gene. Both purified PCR products were then mixed in a 1:1 ratio and used as templates for another PCR reaction containing Chang 222 and Chang 90 primers. The PCR product was digested with *Nde*I and *Bam*HI and ligated into the *Nde*I−*Bam*HI-digested pET11a vector. A mutant pET-C199A clone was first screened for the generation of a *Hin*dIII site and then confirmed by DNA sequencing.

Both wild-type and C199A mutant *mutY* genes were transferred to pGEV1 (*31*) (obtained from M. Clore) to express MutY as fusion proteins containing streptococcal protein G (GB1 domain) at their N-termini. PCR reactions with pMYW-1 and pET-C199A as templates and Chang 348 (5′GCGACGGCTAGCATGCAAGCGTCGCAATTTTC3′) and Chang 349 (5′GCCGGACTCGAGCTAAACCGGCGCGCCAGTGC3′) as primers were performed. The PCR products were digested with *Nhe*I and *Xho*I and ligated into the *Nhe*I−*Xho*I-digested pGEV1 vector (*31*), a derivative from pET21a (Novagen). The sequences of pGEV-MutY (containing the wild-type *mutY* gene) and pGEV-C199A (containing the C199A mutant *mutY* gene) were confirmed by DNA sequencing. The *mutY* gene and its derivatives in the plasmid pET11a and pGEV1 were under the control of the T7 promoter.

The C199A mutant *mutY* gene was transferred to pKK223-−3 (Amersham Pharmacia) for expression under the *tac* promoter. PCR amplification with pET-C199A as template and Chang 191 (5′GCCGAATTCGGAGATGCTATGCAAGCGTCGCAATTTTC3′) and Chang 96 (5′GCCGGAGAATTCCTAAACCGGCGCGCCAGTGC3′) as primers was performed. The PCR product was digested with *Eco*RI and ligated into the *Eco*RI-digested pKK223-3 vector. One of the clones with the correct orientation, pKK-C199A, was confirmed by DNA sequencing.

Western Blot Analysis. Proteins were resolved on a SDS−10% polyacrylamide gel and transferred to a nitrocellulose membrane (*32*). The membrane was subjected to the enhanced chemiluminescence analysis system from Amersham Pharmacia International according to the manufacturer's protocol. The anti-MutY antibodies were affinity purified by reaction with the membrane-bound MutY protein (*33*).

Measurement of Mutation Frequency. Independent overnight cultures (0.1 mL) of each strain were plated onto LB agar plates containing 0.1 mg/mL rifampicin. The cell titer of each culture was determined by plating 0.1 mL of a 10^{-6} dilution onto LB agar plates. For each measurement, four independent cultures were plated, and the experiments were repeated three times. The ratio of Rif^R cells to total cells was the mutation frequency.

Protein Expression and Purification. E. coli strains PR70/DE3 harboring the expression plasmid pET-C199A, pGEV-MutY, or pGEV-C199A were grown in LB broth containing 50 μg/mL ampicillin at 37 °C. The expression of proteins was induced at an OD_{600} of 0.6 by the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.2 mM to the culture at 20 °C. The cells were harvested 16 h later.

The GB1-C199A-MutY was purified from *E. coli* PR70/DE3 cells harboring the overproduction plasmid pGEV-C199A, similar to the method used with the wild-type MutY enzyme (*11*). All column chromatography steps were conducted in a Waters 650E FPLC system at 4 °C, all buffer solutions were flushed with helium gas, and centrifugation was done at 15000*g* for 30 min. Cells (25 g of cell paste) were resuspended in 120 mL of buffer T (50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride) and disrupted with a bead beater (Biospec Products, Bartlesville, OK) using 0.1 mm glass beads. After the cell debris was removed by centrifugation, the supernatant was then treated with 5% streptomycin sulfate. After being stirred for 45 min, the solution was centrifuged, and the supernatant was collected as fraction I (160 mL). Ammonium sulfate (64 g) was added to fraction I to a final concentration of 65%, the solution was stirred for 45 min, and the protein was precipitated overnight. After centrifugation, the protein pellets were resuspended in 15 mL of buffer T and dialyzed against two changes of 1 L of the same buffer for 1.5 h each. The dialyzed protein sample was centrifuged, and the supernatant was diluted to 150 mL (fraction II) with buffer A (20 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride) containing 25 mM KCl. Fraction II was loaded onto a 30 mL phosphocellulose column, which had been equilibrated with buffer A containing 25 mM KCl. After being washed with 75 mL of equilibration buffer, proteins were eluted with a 300 mL linear gradient of KCl (0.025−0.55 M) in buffer A. Fractions eluted at about 0.25 mM KCl were pooled (fraction III, 39 mL). Fraction III was loaded onto an 18 mL hydroxylapatite column equilibrated with buffer B (0.01 M potassium phosphate, pH 7.4, 10 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride). The flow-through and early elution fractions were pooled (43 mL) and diluted to 93 mL with TEG buffer (0.05 M Tris-HCl, pH 7.6, 0.1 mM EDTA, 10%

glycerol, 1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride) containing 25 mM KCl (fraction IV). Fraction IV was loaded onto a 6 mL heparin column equilibrated with TEG buffer containing 25 mM KCl. After being washed with 12 mL of equilibration buffer, the column was developed with a 60 mL linear gradient of KCl (0.025−0.65 M) in TEG buffer. Fractions containing the GB1-C199A-MutY, which eluted at about 0.3 M KCl, were pooled (15 mL) and diluted to 75 mL with TEG buffer containing 25 mM KCl to yield fraction V. Fraction V was then applied to a 1 mL MonoS column (Amersham Pharmacia Biotech) that had been equilibrated in TEG buffer containing 25 mM KCl. After being washed with 10 mL of equilibration buffer, the protein(s) was/were eluted with a 20 mL linear gradient of KCl (0.025−0.5 M) in TEG buffer. Fractions containing the GB1-C199A-MutY protein, which eluted at about 0.2 M KCl, were pooled (fraction VI, 2.5 mL), divided into small aliquots, and stored at −80 °C.

The GB1-MutY was purified from *E. coli* PR70/DE3 cells harboring the overproduction plasmid pGEV-MutY, similar to the method described above for the GB1-C199A-MutY enzyme except that the MonoS column was omitted. Protein concentration was determined by the Bradford method (*34*).

Denaturation and Renaturation of Untagged C199A-MutY. The denaturation and renaturation of the C199A-MutY protein were performed according to the procedures described by Tsai-Wu et al. (*11*). PR70/DE3 cells expressing C199A-MutY were expressed and lysed as above. The cell debris (11 g) was dissolved in 20 mL of 6 M guanidine hydrochloride in dialysis buffer (0.05 M Tris-HCl, pH 7.6, 0.15 M NaCl, 2% glycerol, and 2 mM β-mercaptoethanol). The solution was left rotating for 2 h at 4 °C, and the undissolved material was removed by centrifugation. The supernatant containing denatured proteins was renatured by dialysis against 2 L of dialysis buffer containing 0.2 mM ammonium sulfide and 0.2 mM ferrous ammonium sulfate for 16 h, followed by a second dialysis against 2 L of dialysis buffer containing 0.1 mM ammonium sulfide and 0.1 mM ferrous ammonium sulfate for 8 h. The dialyzed protein sample was centrifuged, and the supernatant was divided into small aliquots and stored at −80 °C.

Iron Assay. Iron analysis was measured by the method of Kennedy et al. (*35*). The iron standard was purchased from Sigma-Aldrich Chemical Co. The protein samples (25−50 μg) and iron standards (25−400 ng) were diluted with water to 0.1 mL and mixed with 0.1 mL of reagent A (4.5% SDS in 1.5% saturated sodium acetate) and 0.1 mL of reagent B (4.5% ascorbic acid and 0.15% sodium metabisulfite in 6.7% saturated sodium acetate). The mixtures were incubated at 30 °C for 15 min and supplemented with 5 μL of reagent C (1.8% Ferene). The absorption at 593 nm was measured.

Oligonucleotide Substrates. The nucleotide sequences of the mismatch-containing heteroduplexes used in this study were as follows:

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19-mer 5′ CCGAGGAATTAGCCTTCTG     3′
       3′  GCTCCTTAAXCGGAAGACG    5′

40-mer 5′ AATTGGGCTCCTCGAGGAATTAGCCTTCTGCAGGCATGCC     3′
       3′     CCCGAGGAGCTCCTTAAXCGGAAGACGTCCGTACGGGGCC 5′
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where X = G or GO. The top strand is referred to as the mismatched adenine-containing strand (A-strand), and the bottom strand is referred to as the mismatched G- or GO-containing strand (G- or GO-strand). Both the 19-mers and 40-mer heteroduplexes were labeled at the 3′ end with [α-^{32}P]-dCTP on the A-strand and were converted to 20-mers and 44-mer, respectively, after the sticky ends were filled in with the Klenow fragment of DNA polymerase I as described by Lu et al. (*18*).

MutY Binding, Trapping, and Cleavage Assays. The MutY activity assays with labeled oligonucleotide substrates were performed as described by Lu et al. (*36*) with some modifications. The MutY enzyme was diluted with diluent (20 mM potassium phosphate, pH 7.4, 50 mM KCl, 1.5 mM dithiothreitol, 0.1 mM EDTA, 200 μg/mL bovine serum albumin, and 50% glycerol) before use. The MutY binding reaction mixture contained 20 mM Tris-HCl, pH 7.6, 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2.9% glycerol, 20 ng of poly(dI-dC), and 1.8 fmol of labeled DNA in a total volume of 20 μL. After incubation at 37 °C for 30 min, the mixtures were supplemented with 3 μL of 50% glycerol and analyzed on 8% polyacrylamide gels in 50 mM Tris−borate (pH 8.3) and 1 mM EDTA. To determine the K_d values, nine different MutY enzyme concentrations were used to bind DNA substrates, at a fixed concentration, and experiments were repeated at least three times. Bands corresponding to enzyme-bound and free DNA were quantified from PhosphorImager images, and K_d values were obtained from analyses by a computer-fitted curve generated by the Enzfitter program (*37*).

Covalent complexes of MutY with 20-mer DNA substrates (trapping assays) were formed in a 10 μL reaction containing 20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 1 mM EDTA, 2.9% glycerol, and 0.1 M $NaBH_4$. A $NaBH_4$ (1 M) stock solution was freshly prepared and was added immediately after the enzyme was added. After incubation at 37 °C for 30 min, 5× dye buffer (25% glycerol, 5% SDS, 155 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, and 0.5 mg/mL bromophenol blue) was added to the samples, which were heated at 90 °C for 2 min and separated on a 12% polyacrylamide gel in the presence of SDS according to Laemmli (*38*).

The glycosylase assays were carried out similarly to the trapping assay except no $NaBH_4$ was added. After incubation at 37 °C for various times the reaction mixtures were lyophilized, resuspended in 3 μL of formamide dye (90% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue), heated at 90 °C for 2 min, and loaded onto 14% 7 M urea sequencing gels. For time−course studies, a 75 μL reaction containing enzyme in 20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 1 mM EDTA, 2.9% glycerol, and 50 μg/mL bovine serum albumin was performed, and 10 μL of the reaction mixture was withdrawn at different time points. Samples (10 μL) were heated at 90 °C for 30 min in the presence of 0.1 N NaOH, supplemented with 5 μL of formamide dye, and heated at 90 °C for 2 min, and 5 μL of the mixture was loaded onto 14% 7 M urea sequencing gels.

RESULTS

Denaturation and Renaturation of the C199A-MutY Protein. Several attempts to purify the C199A-MutY protein were unsuccessful because the majority of the mutant protein is expressed as inclusion bodies. This is consistent with the

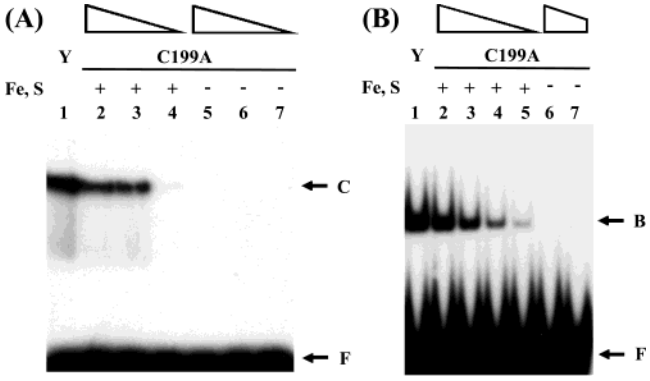

FIGURE 1: (A) Trapping activities of C199A-MutY from the insoluble fraction by denaturation and renaturation steps. A/GO-containing 20-mer DNA was incubated with 1 μL of a 10-, 50-, and 250-fold dilution of the renatured protein sample at 37 °C for 30 min. In lanes 2−4, the sample was dialyzed with buffer containing ammonium sulfide and ferrous ammonium sulfate (+), and in lanes 5−7, the sample was dialyzed without ammonium sulfide and ferrous ammonium sulfate (−). Wild-type MutY (72 fmol) was used as a control in lane 1. The positions of free DNA (F) and the covalent complex (C) are indicated. (B) Binding activities of C199A-MutY from the insoluble fraction by denaturation and renaturation steps. A/GO-containing 20-mer DNA was incubated with MutY at 37 °C for 30 min. Wild-type MutY (4 fmol) were incubated with A/GO-containing DNA in lane 1. Lanes 2−5 used 1 μL of a 25-, 50-, 100-, and 200-fold dilution of the renatured C199A protein sample dialyzed with buffer containing ammonium sulfide and ferrous ammonium sulfate (+). Lanes 6 and 7 used 1 μL of a 25- and 50-fold dilution of C199A dialyzed without ammonium sulfide and ferrous ammonium sulfate (−). The positions of free DNA (F) and the MutY−DNA complex (B) are indicated.

results of Golinelli et al. (*26*). To determine the function of the C199A-MutY protein, we first tried to renature the mutant protein from insoluble cell debris according to the procedures described by Tsai-Wu et al. (*11*). Two renaturation buffers were used: one with ammonium sulfide and ferrous ammonium sulfate and one without. As shown in Figure 1, only the enzyme renatured in the present of iron and sulfur had trapping and binding activities on A/GO-containing DNA. The glycosylase activity of the renatured protein was not easily detected because some other nucleases were present in the extracts (data not shown). The results of Figure 1 indicate that the presence of iron in the refolding process is important to the C199A-MutY activity.

Expression and Purification of the GB1-Tagged MutY Protein. To more fully characterize the properties of the C199A-MutY protein, the stability of the protein needs to be improved for purification. We then tried to express the mutant protein as fusion proteins. We employed the strategy described by Huth et al. (*31*) to express the mutant MutY as a fusion with the binding domain of streptococcal protein G (GB1) at its N-terminus. The 62 amino acid residues of the GB1 domain are MQYKLALNGKTLKGETTTEAVDAA-TAEKVFKQYANDNGVDGEWTYDDATKTFTV-TEPGGPAS. As expected, the solubility of the GB1-C199A-MutY protein was improved substantially. We were able to purify 1.5 mg of the mutant protein from 24 g of PR70/DE3/pGEV-C199A cell paste. In contrast, 125 mg of the GB1-tagged wild-type MutY was purified from the same amount of cell paste. As judged on a 10% SDS−polyacrylamide gel, both GB1-C199A-MutY and GB1-MutY proteins were purified to >98% homogeneity (Figure 2). The GB1-

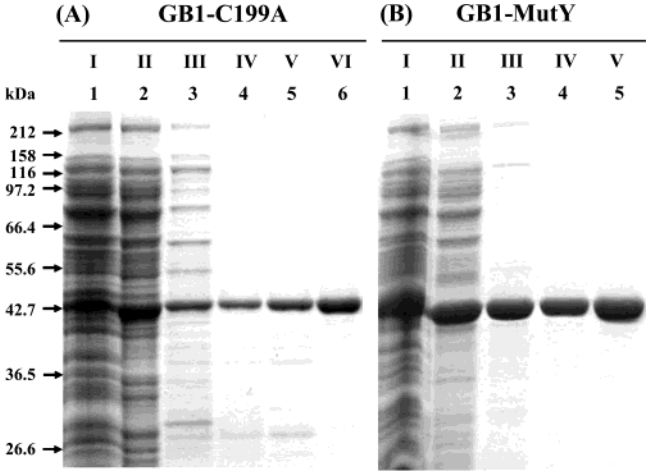

FIGURE 2: SDS−polyacrylamide gel analysis of GB1-C199A-MutY and C199A-MutY. The proteins were separated on a 10% polyacrylamide gel in the presence of SDS and stained with Coomassie Blue. (A) Purification of GB1-C199A-MutY. The positions of protein markers (New England Biolabs, broad range) are indicated with arrows. Lanes 1−6 are fractions I (11 μg, post streptomycin sulfate), II (10 μg, post ammonium sulfate), III (3 μg, post phosphocellulose), IV (1.5 μg, post hydroxylapatite), V (2 μg, post heparin), and VI (2 μg, post MonoS), respectively. (B) Purification of GB1-MutY. Lanes 1−5 are fractions I (11 μg, post streptomycin sulfate), II (8 μg, post ammonium sulfate), III (3.5 μg, post phosphocellulose), IV (2.5 μg, post hydroxylapatite), and V (3.8 μg, post heparin), respectively.

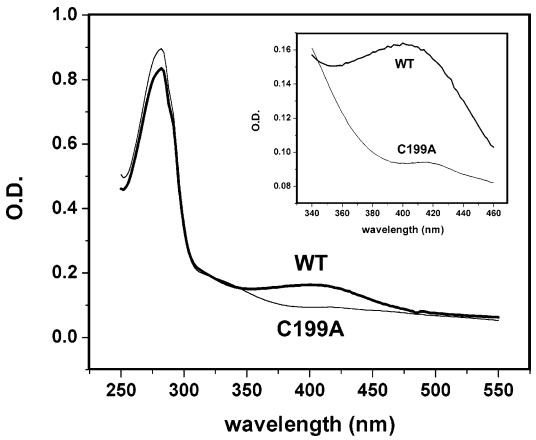

FIGURE 3: UV−visible absorption spectra of GB1-MutY (thick line) and GB1-C199A-MutY (thin line). The inset is the enlarged visible absorption spectra.

C199A-MutY protein required one additional column step (MonoS) to be purified to homogeneity. There was a substantial portion of the GB1-C199A-MutY protein which did not bind to the phosphocellulose column.

The Iron−Sulfur Cluster of the GB1-C199A-MutY Protein. The color of the purified GB1-C199A-MutY protein was slightly pink as compared to the brown color of the wild-type protein. The absorption spectrum of GB1-C199A-MutY was different from that of the wild-type enzyme (Figure 3). The visible absorption of GB1-C199A-MutY peaked at 415 nm while that of the wild-type enzyme peaked at 400 nm (a red shift at about 15 nm). In addition, the visible absorption of GB1-C199A-MutY was much weaker than that of the wild-type enzyme (see the inset in Figure 3). Iron analyses of the purified GB1-C199A-MutY and GB1-MutY by chemical methods yielded 2.7 and 3.8 Fe per protein molecule, respectively. Because the 62-residue GB1 domain

contains no Cys and His, iron in this domain is extremely unlikely. Therefore, the GB1-C199A-MutY protein contains a 3-Fe iron−sulfur cluster.

DNA Binding Activity of the GB1-C199A-MutY Protein. With the purified GB1-C199A-MutY protein, we compared the binding affinities of the mutant protein to that of the wild-type protein. Using gel retardation assays, the apparent dissociation constant (K_d) values of the GB1-C199A-MutY protein with A/G- and A/GO-containing 20-mer DNA were measured to be 26.2 and 0.173 nM, respectively (Table 1). The K_d values of the GB1-MutY protein with A/G- and A/GO-containing 20-mer DNA were 21.9 and 0.119 nM, respectively. Thus, the K_d values of the GB1-C199A-MutY protein with DNA are slightly higher than those of the wild-type enzyme. Compared to untagged MutY with K_d values of 5.3 and 0.066 nM (*18*), the GB1-tagged wild-type MutY has 4- and 2-fold higher K_d values with A/G- and A/GO-containing 20-mer DNA, respectively.

Catalytic Activities of the GB1-C199A-MutY Protein. To test whether mutant MutY with a modified iron−sulfur cluster is catalytically active, we measured its adenine glycosylase activity on A/G and A/GO mismatches. As shown in Figure 4, at lower protein concentrations (enzyme to DNA ratios less than 1), the GB1-C199A-MutY protein had slightly lower glycosylase activities toward both A/G and A/GO mismatches than the wild-type enzyme. However, at concentrations higher than 3 and 0.2 nM, both enzymes had similar activities with A/G and A/GO mismatches, respectively.

Because of the slow turnover rate of MutY (*39*, *40*), the steady-state kinetics of the MutY reaction, as measured at 37 °C for 30 min, may not reflect the true reactivity. Thus, we used single-turnover glycosylase kinetics to compare the activities of GB1-MutY and GB1-C199A-MutY. A minimal kinetic mechanism of MutY has been proposed (*40*): MutY first binds to DNA substrate with binding constant k_1, removes the adenine base from the substrate to form a product containing an apurinic/apyrimidinic (AP) site with rate constant k_2, and then dissociates from the product with dissociation constant k_3. Time−course studies with enzyme concentrations higher than the DNA concentration and above the K_d were used to determine the extent of glycosylase activity on both A/G and A/GO substrates. Because the cleavage rates on A/GO-containing DNA with GB1-MutY and GB1-C199A-MutY are too fast for manual measurement, the reactions were carried out at 4 °C. Because some fractions of DNA molecules of the mismatch-containing 20-mer oligonucleotides become single stranded, only 30−40% of DNA are cleaved by MutY (for example, see Figure 4). To avoid DNA being single stranded, the 44-mer DNA substrates were used in these single-turnover kinetic experiments in the presence of bovine serum albumin. As shown in Figure 5 and Table 1, the rates of cleavage of both A/G- and A/GO-containing DNA for GB1-C199A-MutY (squares) and GB1-MutY (circles) are comparable. Thus, GB1-C199A-MutY has normal glycosylase activities on A/G- and A/GO-containing DNA, as compared with GB1-MutY. The results of Figure 5 do not contradict with those of Figure 4 because the time−course analyses were performed with proteins at higher concentrations (enzyme to DNA ratios higher than 14) and at lower temperatures than those in Figure 4.

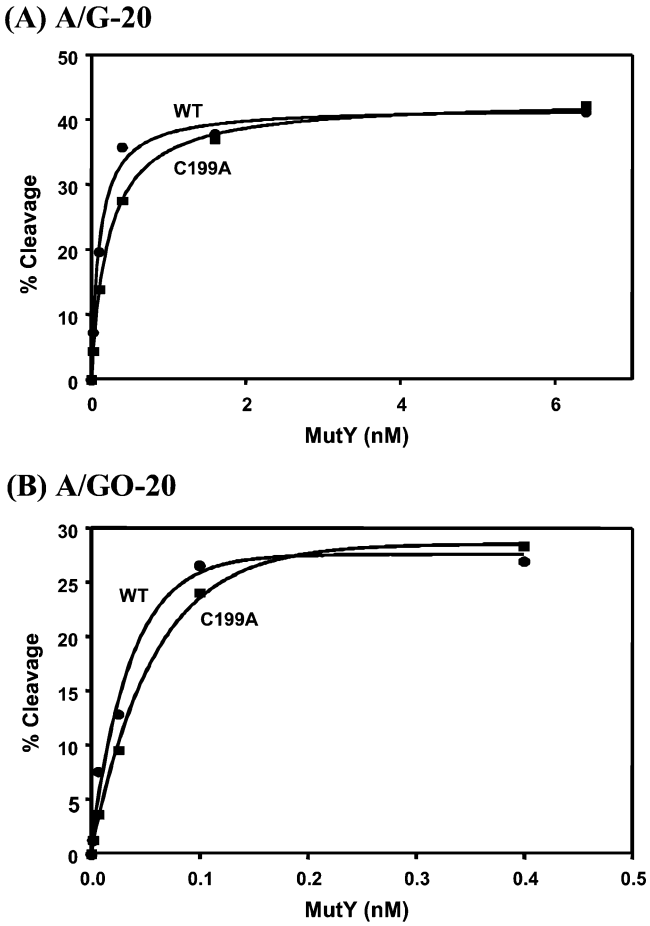

FIGURE 4: Glycosylase activities of wild-type (WT, circles) and C199A mutant (squares) GEV-MutY. A/G- (A) and A/GO- (B) containing 20-mer DNA (1.8 fmol) was incubated with different protein concentrations in a 10 μL reaction at 37 °C for 30 min. After the reaction, the products were dried, resuspended in formamide dye, heated at 90 °C for 2 min, and analyzed on a 14% denaturing sequencing gel. Data were from PhosphorImager quantitative analyses of gel images over three experiments. Percentages of DNA cleaved were plotted versus protein concentrations.

In the trapping assay in the presence of sodium borohydride, GB1-C199A-MutY protein produced about 2-fold less of the covalent protein−DNA complex on A/G-containing DNA than GB1-MutY at all protein concentrations tested (Figure 6A). With A/GO mismatch, when GB1-C199A-MutY to DNA ratios were less than 1, the extent of protein−DNA covalent complex formation was less than that of GB1-MutY (Figure 6B). However, the trapping activities of both enzymes were nearly identical with A/GO-containing DNA when enzyme to DNA ratios were higher than 1 (Figure 6B).

In Vivo Complementation Activity of C199A-MutY and GB1-C199A-MutY. The *E. coli mutY* mutant is a mutator because it fails to correct replication errors. It has been shown that *mutY* mutants have higher mutation frequencies than wild-type cells (*41*) as measured by rifampicin resistance and Lac^+ reversion (*8*, *9*). Rifampicin-resistant cells contain mutations in the gene of RNA polymerase. In the absence of functional MutY, a high level of mutations in the rifampicin binding site of RNA polymerase renders the cell resistant to rifampicin. As shown in Table 2, PR70 (*mutY*) had a 52-fold higher mutation frequency than wild-type PR8 cells. Expression of GB1-MutY or GB1-C199A-MutY from plasmids in PR70/DE3 (*mutY*) cells significantly reduced the

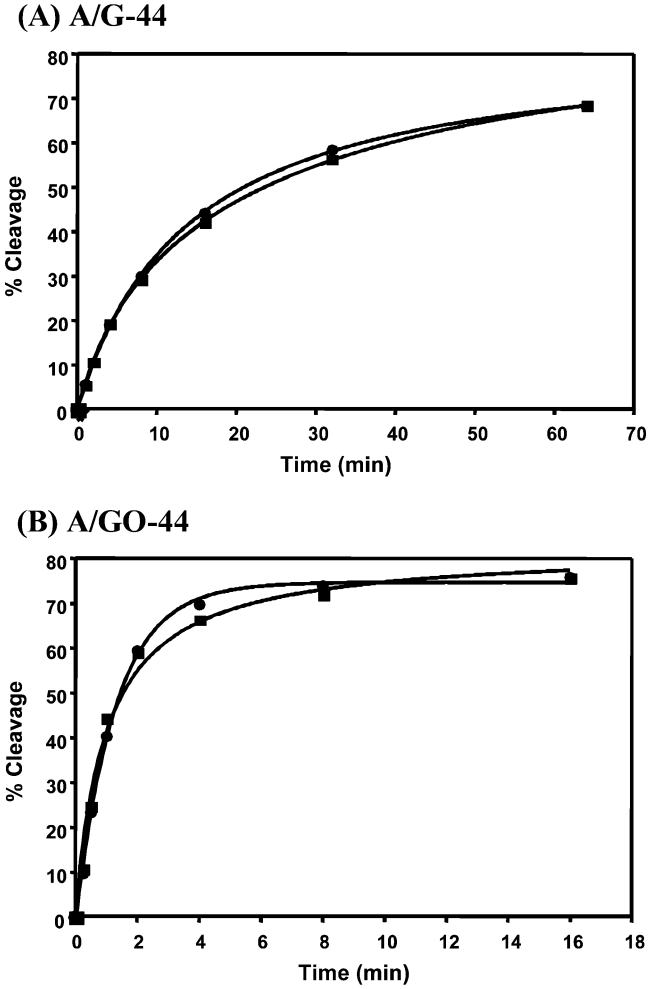

FIGURE 5: Time−course studies of glycosylase activities of wild-type (WT, circles) and C199A mutant (squares) GEV-MutY. (A) A/G-containing 44-mer DNA (1.8 fmol) was incubated with 720 fmol (72 nM) of proteins at 22 °C, and (B) A/GO-containing 44-mer DNA (1.8 fmol) was incubated with 14.4 fmol (1.44 nM) of proteins at 4 °C. After the reaction, the products were treated with 0.1 N NaOH as described in Materials and Methods and analyzed on a 14% denaturing sequencing gel. Data were from PhosphorImager quantitative analyses of gel images over three experiments. Percentages of DNA cleaved were plotted as a function of time.

Table 1: Apparent Dissociation Constants (K_d) and Rate Constants (K_2) of MutY Mutants for Mismatch-Containing DNA

	A/G		A/GO	
enzyme	K_d[a] (nM)	k_2[b] (min^{-1})	K_d[a] (nM)	k_2[c] (min^{-1})
GB1-WT	21.9 ± 6.1	0.082 ± 0.020	0.119 ± 0.018	0.88 ± 0.12
GB1-C199A	26.2 ± 5.6	0.076 ± 0.018	0.173 ± 0.049	0.72 ± 0.13

[a] K_d values were determined from three experiments using nine protein concentrations with 20-mer oligonucleotides. [b] k_2 rate constants were determined from three single-turnover experiments as in Figure 5A using 72 nM MutY and 0.18 nM 44-mer A/G-containing DNA at room temperature. [c] k_2 rate constants were determined from three single-turnover experiments as in Figure 5B using 1.44 nM MutY and 0.18 nM 44-mer A/GO-containing DNA at 4 °C. The errors reported are the standard deviations of the averages.

mutation frequencies (Table 2, lines 4 and 5). Thus, GB1-C199A-MutY like wild-type GB1-MutY is able to complement the chromosomal *mutY* mutation. The CC104/*mutY-mutM* cells with double mutations at *mutY* and *mutM* genes have a very high mutation frequency (*7*) (Table 2, line 7).

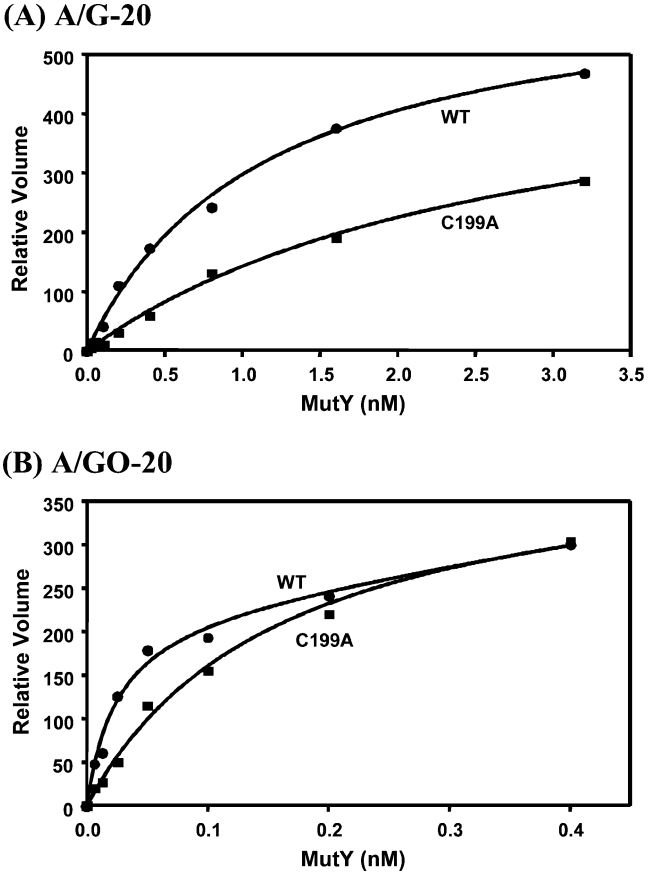

FIGURE 6: Trapping activities of wild-type (WT, circles) and C199A mutant (squares) GEV-MutY. A/G- (A) and A/GO- (B) containing 20-mer DNA (1.8 fmol) was incubated with various amounts of proteins in the presence of $NaBH_4$ at 37 °C for 30 min. The samples were heated at 90 °C for 2 min and separated on a 12% polyacrylamide gel in the presence of SDS. Data were from PhosphorImager quantitative analyses of gel images over three experiments. Volumes of covalent complexes were plotted versus protein concentrations. The percentages of covalent complexes could not be calculated because the free DNA and free [α-^{32}P]dCTP were not separated far enough in the gel.

Surprisingly, when the same plasmids (pGEV-MutY and pGEV-C199A) were transformed into the CC104/*mutYmutM*/DE3 cells, the wild-type GB1-MutY could, but GB1-C199A-MutY could not, complement the mutator phenotype (Table 2, lines 9 and 10). Another surprising finding is that the proteins were not well expressed in this strain. Both GB1-C199A-MutY and GB1-MutY proteins were not visible by Coomassie Blue stain (not shown) and could only be detected by Western blotting (Figure 7, lanes 2 and 3). Because CC104/*mutYmutM* is *lac*I$^-$, T7 RNA polymerase is constitutively expressed from the λDE3 lysogen, and the gene under the control of the T7 promoter is expected to be constitutively expressed. Further investigation revealed that the λDE lysogen was not properly established in the CC104/*mutYmutM* strain. The tester T7 phage with a defective T7 RNA polymerase could only form very tiny plaques on the cells. Repeated isolation of a better lysogen was unsuccessful, and the reason is not clear. Because the expression of GB1-fusion proteins was controlled by the T7 promoter, improper λDE lysogen resulted in poor expression of the proteins. By comparison to a known amount of purified MutY, GB1-C199A-MutY and GB1-MutY were expressed as approximately 1000 and 25000 molecules per CC104/*mutYmutM*

Table 2: Mutation Frequencies of *E. coli MutY* and *MutYmutM* Mutants Expressing Mutant MutY Proteins

line	strain[a]	mutation frequency[b] (Rif^R colonies/ 10^8 cells)	increase (fold)[c]
1	PR8 (WT)	0.65 ± 0.2	1
2	PR70 (*mutY*)	34 ± 5	52
3	PR70/pGEV1 (vector)	18 ± 3	28
4	PR70/pGEV-MutY (WT)	1.5 ± 0.5	2
5	PR70/pGEV-C199A (mutant)	1.0 ± 0.4	2
6	CC104 (WT)	2 ± 0.8	1
7	CC104/*mutYmutM*	1385 ± 696	693
8	CC104/*mutYmutM*/pGEV1 (vector)	279 ± 97	140
9	CC104/*mutYmutM*/pGEV-MutY (WT)	4 ± 0.7	2
10	CC104/*mutYmutM*/pGEV-C199A (mutant)	204 ± 75	102
11	CC104/*mutYmutM*/pET11a (vector)	555 ± 263	278
12	CC104/*mutYmutM*/pMYW1 (WT)	2 ± 0.9	1
13	CC104/*mutYmutM*/pET-C199A (mutant)	559 ± 138	280
14	CC104/*mutYmutM*/pKK223-3 (vector)	452 ± 57	226
15	CC104/*mutYmutM*/pJTW10-12 (WT)	6 ± 2	3
16	CC104/*mutYmutM*/pKK-C199A (mutant)	2 ± 0.6	1

[a] *E. coli* strains PR8 and CC104 contain the wild-type *mutY* gene, PR70 has a mutated *mutY* gene, and CC104/*mutYmutM* is a double mutant of *mutY* and *mutM*. All strains contain λDE3 lysogen, but the lysogen in CC104 derivatives is not properly established. [b] The in vivo activity of MutY is measured by the frequency of rifampicin-resistant colonies by an average of at least three separate experiments. The errors reported are the standard deviations of the averages. [c] Fold increase compared with the respective wild-type (WT) strains.

cell, respectively. The expression of the MutY protein from its own promoter in the chromosome was about 1400 molecules per CC104 cell (Figure 7, lane 9). Thus, the expression level of GB1-C199A-MutY is 25-fold lower than that of GB1-MutY but is only slightly lower than the wild-type CC104 cell. The incapability of GB1-C199A-MutY to function in vivo may be due to its low expression inside the cell or other factors.

To investigate the effect of the GB1 tag on MutY function in vivo, untagged MutY and C199A-MutY were expressed in the CC104/*mutYmutM* strain from either the T7 promoter or *tac* promoter. When plasmids pMYW1 and pET-C199A were transformed into the CC104/*mutYmutM*/DE3 cells, the wild-type MutY could, but C199A-MutY could not, complement the mutator phenotype (Table 2, lines 12 and 13). However, both wild-type MutY and C199A-MutY could complement the mutator phenotype of CC104/*mutYmutM*/DE3 cells when they are controlled by *tac* promoter (Table 2, lines 15 and 16). Western analyses revealed that proteins are expressed about 1000-fold better from the *tac* promoter than from the T7 promoter (Figure 7, compare lanes 4 and 5 to lanes 6 and 7). This differential expression is due to the improper λDE lysogen in the CC104/*mutYmutM* strain. Our results in Table 2, lines 14−16, are consistent with the report of Golinelli et al. (*26*) that untagged C199A-MutY can complement CC104/*mutYmutM* in vivo from a *tac* promoter.

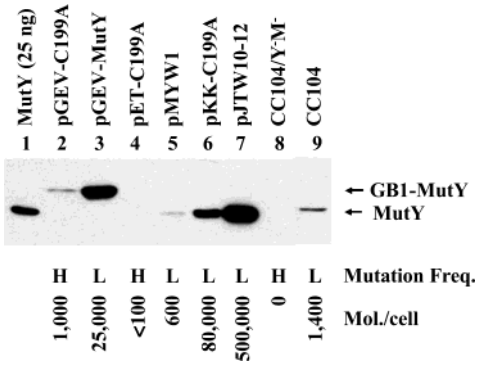

FIGURE 7: Expression levels of MutY and GB1-MutY in the CC104/*mutYmutM*/DE3 strain. The *E. coli* cells were grown overnight in LB media with appropriate antibiotics. Cell paste from 1 mL of the culture was resuspended in cracking dye (60 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% β-mercaptoethanol, and 0.01% bromophenol blue) (0.1 mL per OD unit) and boiled for 10 min. Cell lysates (lanes 2−9) and purified MutY were fractionated by 10% SDS−polyacrylamide gel electrophoresis, and MutY protein was detected by Western blotting with polyclonal antibodies against purified MutY. Lanes: 1, 25 ng of purified MutY; 2−7, extracts of CC104/*mutYmutM*/DE3 containing plasmid pGEV-C199A, pGEV-MutY, pET-C199A, pMYW1, pKK-C199A, or pJTW10-12, respectively; 8 and 9, extracts of CC104/*mutYmutM* and wild-type CC104, respectively. Lanes 6 and 7 (1 μL) contain 10% of the total protein of those loaded onto lanes 2−5, 8, and 9 (10 μL). The positions of MutY and GB1-MutY are indicated. By comparison to the amount of purified MutY in lane 1, the molecules of MutY and GB1-MutY proteins per cell were estimated. The data of high (H) and low (L) mutation frequencies of the cells were taken from Table 2. In a separate gel, 10 μL of the cell lysates of each culture was loaded, stained with Coomassie Blue, and shown to contain an equal amount of proteins (data not shown).

In all cases, C199A-MutY was expressed at least 6-fold less than the wild-type MutY (Figure 7, compare lanes 2, 4, and 6 to lanes 3, 5, and 7). Thus, the C199A mutation may cause the protein to be unstable in vivo. Addition of the GB1 domain at the N-termini of MutY and C199A-MutY increased the expression or stability by more than 20-fold as compared to that of untagged proteins (Figure 7, compare lane 2 to lane 4 and compare lane 3 to lane 5). Interestingly, the GB1-C199A-MutY (Table 2, line 10) could not, but wild-type MutY (Table 2, line 12) could, complement CC104/*mutYmutM*/DE3; even GB1-C199A-MutY was expressed at a higher amount than wild-type MutY (Figure 7, compare lanes 2 and 5). Therefore, other factors such as solubility and activity besides expression level may attribute to the in vivo activity of C199A-MutY.

DISCUSSION

This paper describes the first biochemical characterization of a mutant protein containing an iron−sulfur cluster ligated by three cysteines. This is possible with purified protein by expressing the mutant MutY as a fusion protein containing the GB1 domain. Although GB1-tagged C199A has improved solubility, it is still expressed far less than the wild-type protein. Without the GB1 domain, the untagged C199A-MutY is low expressed and very insoluble, but a small fraction of that can be renatured to active enzyme containing an iron−sulfur cluster. Because the side chain of Ala is not sufficient to coordinate the iron, the iron−sulfur cluster ligated with three cysteines may dissociate very easily from the apoprotein that is destabilized and folded improperly.

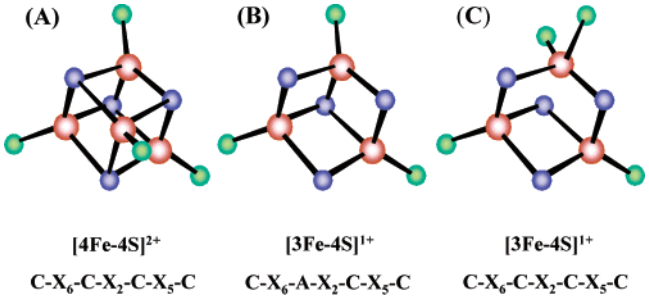

FIGURE 8: Proposed iron−sulfur cluster in C199A-MutY. (A) The normal cubane $[4Fe\text{-}4S]^{2+}$ cluster is ligated by four cysteines in wild-type MutY. (B) A modified cuboidal $[3Fe\text{-}4S]^{1+}$ is ligated by three cysteines in C199A-MutY. (C) An oxidized cuboidal $[3Fe\text{-}4S]^{1+}$ is ligated by four cysteines. Irons are in orange, inorganic sulfides are in blue, and cysteinate sulfurs are in green.

The GB1 domain may promote a stable and folded MutY protein by filling the space of the missing cysteine. Because the GB1 domain does not contain a cysteine residue, it may function as a cap to prevent the iron−sulfur cluster from dissociation from the protein. According to the X-ray structure, the N-terminus of MutY is located close to the iron−sulfur cluster (*22*); thus, the tagged GB1 domain at the N-terminus may fold over to the iron−sulfur cluster. Therefore, with some modification, MutY does not need a completely coordinated [4Fe-4S] cluster for overexpression.

The C199 to A mutation apparently causes a major modification of the cluster coordination, as we observed a red shift of about 15 nm of the visible spectrum. Iron analysis indicates that the GB1-C199A-MutY protein contains a 3-Fe iron−sulfur cluster. We propose that the protein has an $[3Fe\text{-}4S]^{1+}$ cluster as in Figure 8B. Further EPR analysis is required to confirm this. In this cuboidal cluster, each Fe is ligated by three inorganic sulfides and one cysteinate sulfur. Three sulfides bridge two Fe atoms (μ_2-S type), and one sulfide bridges three Fe atoms (μ_3-S type). This cluster is probably different from an oxidized $[3Fe\text{-}4S]^{1+}$ which is ligated by four cysteines (Figure 8C) (*42*). Alternatively, one of the other three cysteines of MutY, C148, C260, or C314, may be used as a ligand to bind the iron−sulfur cluster in the GB1-C199A-MutY protein. This ligand swapping has been reported in *Azotobacter vinelandii* ferredoxin in which a free cysteine, C24, is swapped to the cluster when ligand C20 is mutated to Ala (*43*). The X-ray crystal structure of the N-terminal domain of MutY reveals that the region of Ile191−Cys199 forms a surface-exposed loop, referred to as the iron−sulfur cluster loop (FCL) motif, which is anchored by C192 and C199 (*22*). Of the four conserved cysteines, C199 of MutY is the most exposed to the outer solvent (*22*). It is interesting to note that H199 can ligate an iron but 15−30% of the C199H-MutY protein contains a [3Fe-4S] cluster (*27*). The overall structure of the N-terminal domain of C199H is very similar to that of the wild-type fragment and shows no evidence of ligand swapping (*27*). However, we cannot rule out the possibility of ligand swapping in the GB1-C199A-MutY protein.

Surprisingly, the binding and catalytic activities of GB1-C199A-MutY are not significantly different from those of the wild-type enzyme. The apparent K_d values of GB1-C199A-MutY with both A/G and A/8-oxoG mismatches are slightly higher than that of the wild-type MutY protein. The DNA glycosylase activities of GB1-C199A-MutY and the wild-type enzyme are comparable. Our results suggest that the overall structure of GB1-C199A-MutY is not significantly modified although it contains a [3Fe-4S] cluster. The FCL containing the conserved cysteines and positively charged amino acids has been shown to be important for DNA recognition (*28*). Therefore, the FCL may be flexible enough to accommodate A199 when the GB1 domain is at a position to prevent the loss of the iron−sulfur cluster. The major difference between the GB1-C199A-MutY and wild-type proteins is their trapping activities (formation of Schiff base intermediates). The GB1-C199A-MutY mutant has a weaker trapping activity than the wild-type enzyme with A/G mismatches at all protein concentrations and with A/8-oxoG mismatches when enzyme to DNA ratios are less than 1. This may be due to the lower affinity of GB1-C199A-MutY to DNA substrates than that of GB1-MutY (Table 1) or due to the instability of GB1-C199A-MutY at lower concentrations. We and others have shown that K142 is the primary residue to form the Schiff base with DNA (*29*, *44*, *45*). Thus, K142 in GB1-C199A-MutY may be more distant to the active site than that in the wild-type enzyme.

David and co-workers have investigated the effects of iron−sulfur cluster on MutY activity. They reported that C199S and C199H have catalytic activities similar to those of the wild-type enzyme (*26*) and that MutY with an oxidized [3Fe-4S] cluster has kinetic properties similar to those of the wild-type protein (*27*). They were unable to characterize the C199A-MutY mutant because it could not be purified. By tagging with GB1 domain at the N-terminus of C199A-MutY, we were able to characterize this unusual protein. Although the addition of the GB1 domain reduces slightly the MutY affinity to DNA, the catalytic activity of the fusion protein remains essentially the same. Our results are consistent with theirs in that MutY with a [3Fe-4S] cluster is active and the apoprotein has no activity (*25*). The C199A mutant we characterized here is different from the reported C199S and C199H MutY mutants (*27*) because Ser and His can serve as ligands to coordinate the iron−sulfur cluster. Because Ala is not sufficient to coordinate the iron, using Ala to replace the second cysteine in MutY is novel in iron−sulfur proteins.

The C199A mutation causes protein instability in vivo because C199A-MutY and GB1-C199A-MutY were expressed at least 6-fold less than the wild-type MutY. Addition of the GB1 domain at the N-termini of MutY and C199A-MutY increased the expression or stability by more than 20-fold as compared to that of untagged proteins. When GB1-C199A-MutY and untagged C199A-MutY are highly expressed, they can complement *mutY* mutants (Table 2 and Figure 7). However, GB1-C199A-MutY and untagged C199A-MutY cannot complement *mutY* mutants in vivo when both proteins are poorly expressed. This new finding about the in vivo activity of C199A-MutY has not been acknowledged by Golinelli et al. (*26*). Because the apoprotein form of MutY has no activity (*25*) and the absence of iron in the refolding process cannot restore C199A-MutY activity (Figure 1), the in vivo activity observed in the highly expressed C199A-MutY is probably derived from some folded protein molecules which contain a 3-Fe cluster. By quantitative Western blotting analysis, we estimate that there are about 1400 molecules of MutY per wild-type cell. However, 600 copies of MutY per cell are sufficient to

defense against the mutagenic effect of GO lesions. When GB1-C199A-MutY is expressed at about 1000 copies of MutY per CC104/*mutYmutM* cell, it cannot complement *mutY* mutation. Therefore, other factors such as solubility and activity besides expression level may attribute to the in vivo activity of C199A-MutY. In view of the low solubility and instability of C199A-MutY, the C199A mutation in the chromosomal *mutY* gene will probably lead to a mutator phenotype. It turns out that the improper λDE3 lysogen of CC104/*mutYmutM* will offer a more sensitive assay for in vivo complementation because proteins from the T7 promoter are expressed less in this cell than that from the *mutY* own promoter in a wild-type cell.

Atypical coordination of iron−sulfur clusters has been found in natural MutY/endo III enzymes. His and Asp can be found at the second position in these proteins. The side chains of His and Asp are long enough to accommodate the iron. Two endo III proteins in the *Chlamydia* genera (Genbank Accession Numbers AAC68292 and D72029) contain Ile at the second position. These two proteins may have an iron−sulfur cluster like that in GB1-C99A-MutY although aliphatic Ile has a longer side chain than Ala. However, in a closer inspection, the spacer between the third and fourth cysteines is only two residues in *Chlamydia* endo III proteins, and the corresponding spacer is five residues in all of the MutY family. There are two His residues between the first and third cysteines in *Chlamydia* endo III proteins, so it is likely that the iron−sulfur cluster in those proteins may be ligated by three cysteines and one histidine. Nevertheless, using a small residue such as Ala to replace the second cysteine is novel in iron−sulfur proteins.

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