A Substrate Recognition Role for the [4Fe-4S]$^{2+}$ Cluster of the DNA Repair Glycosylase MutY†

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ABSTRACT: The *Escherichia coli* DNA repair enzyme MutY plays an important role in the recognition and repair of 7,8-dihydro-8-oxo-2′-deoxyguanosine: 2′-deoxyadenosine (OG:A) mismatches in DNA [Michaels et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7022−7025]. MutY prevents mutations due to misincorporation of A opposite OG during DNA replication by removing the adenine base. This enzyme has significant sequence homology with the [4Fe-4S]$^{2+}$ cluster-containing DNA repair enzyme, endonuclease III [Michaels et al. (1990) *Nucleic Acids Res. 18*, 3841−3845]. In the present study, we have investigated the importance of cluster assembly in folding of MutY. MutY was denatured and then refolded in the presence or absence of ferrous and sulfide ions. Denatured MutY can refold in the presence of ferrous and sulfide ions to provide active enzyme. This suggests the cluster can self-assemble and that this process is facile in vitro. Interestingly, CD spectra and T_m measurements of MutY refolded with and without ferrous and sulfide ions are essentially identical, implying that assembly of the cluster is not required for MutY folding. Additionally, T_m measurements indicated that the [4Fe-4S]$^{2+}$ cluster does not contribute significantly to the overall thermal stability of MutY. Refolded forms of MutY which lack the cluster are unable to perform the adenine glycosylase function and bind to DNA. However, these inactive folded forms regain activity by addition of ferrous and sulfide ions. This indicates that the Fe-S cluster may have a superficial location, allowing for its assembly after folding. More importantly, these results provide evidence that the presence of the [4Fe-4S]$^{2+}$ cluster is critical for the specific recognition of substrate DNA necessary for the adenine glycosylase activity of MutY.

DNA repair is a fundamental part of cellular biochemistry which must be performed efficiently in order to maintain an intact genetic code. Continuous cellular DNA damage occurs as a result of alkylating and oxidative agents, exposure to radiation, spontaneous hydrolysis, and errors during DNA replication (*1*). There are several DNA repair pathways in the cell that recognize and repair a variety of DNA modifications. One of these pathways, the base excision repair (BER) pathway, primarily targets damaged bases and catalyzes N-glycosidic bond hydrolysis to effect damaged base removal (*2*, *3*). The *Escherichia coli* MutY protein is unique among the BER class of enzymes in that it has activity toward a normal base (adenine) base-paired with a damaged base (*4*, *5*). MutY specifically recognizes 7,8-dihydro-8-oxo-2′-deoxyguanosine: 2′-deoxyadenosine (OG:A) mismatches in DNA that result from misincorporation of A opposite oxidatively damaged 2′-deoxyguanosine during DNA replication (*6*, *7*). If this damaged and mismatched base-pair is left unrepaired, a G:C to T:A transversion mutation occurs. MutY prevents these mutations by hydrolysis of the N-glycosidic bond to effect removal of the adenine base from the sugar providing an abasic (AP) site (*4*). Subsequent action of AP endonucleases, DNA polymerase, and DNA ligase produces an OG:C base-pair which is a substrate for the OG glycosylase, MutM (also referred to as Fpg protein). MutM action facilitates eventual restoration of the correct G:C base-pair (*5*). In vitro, MutY also exhibits glycosylase activity toward adenine in G:A and C:A mismatches within DNA; however, the importance of these substrates in vivo remains to be established (*8*, *9*).

MutY shares significant sequence homology with another *E. coli* BER enzyme, endonuclease III, though the two enzymes recognize entirely different substrates (*10*). Endonuclease III (Endo III) displays glycosylase activity for DNA containing damaged pyrimidines such as ring-saturated and ring-fragmented thymine derivatives and cytosine photoproducts (*11*−*13*). The homology between MutY, a 350-amino acid protein, and Endo III, a 211-amino acid protein, spans 181 residues of the N-terminal portion of MutY. The two proteins are 66.3% similar and 23.8% identical in this region. An interesting feature of Endo III is that it contains a [4Fe-4S]$^{2+}$ cluster (*14*). MutY and Endo III share a set of four identically spaced cysteine residues with a spacing (Cys-X_6-Cys-X_2-Cys-X_5-Cys) distinct from that in other Fe-S proteins. In the crystal structure of Endo III (Figure 1), these four cysteines coordinate the [4Fe-4S]$^{2+}$ cluster at the surface of the protein near the C-terminus to form a distinct Fe-S binding domain which has been referred to as the [4Fe-4S]$^{2+}$ cluster loop (FCL) domain (*15*, *16*). Conversion of a lysine residue positioned in a solvent-exposed loop of this Fe-S

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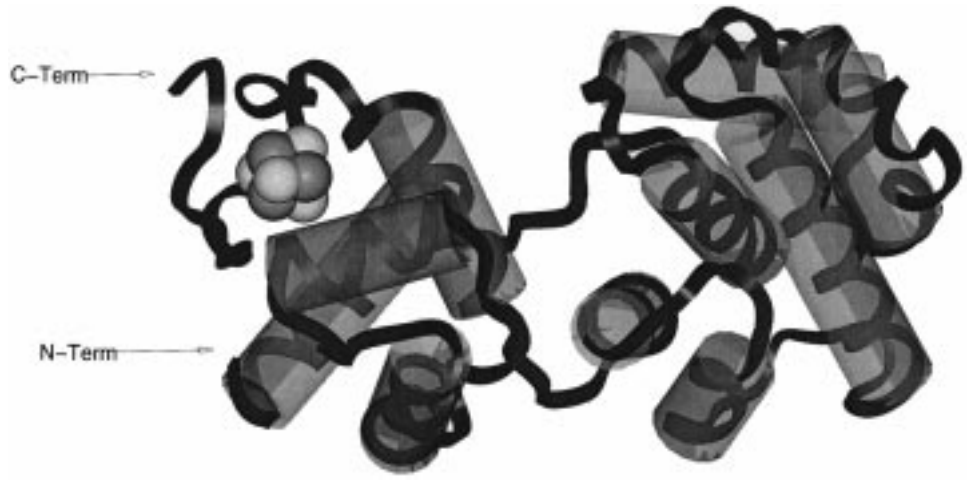


FIGURE 1: Three-dimensional structure of endonuclease III from X-ray crystallographic data (*16*). The iron and sulfide ions of the [4Fe-4S] cluster are depicted with space-filling presentation. Random-coil and turn portions of endonuclease III's polypeptide chain (including the cysteine ligands to the cluster) are depicted as ribbons, while the α-helical portions are highlighted by a barrel representation. The N-terminus and C-terminus have been marked to emphasize that MutY extends from the C-terminus in proximity to the $[4Fe\text{-}4S]^{2+}$. The figure was generated using the Brookhaven PDB file ABK2 and the Biosym/MSI program Insight II (V.95.0) on an SGI workstation.

cluster domain to glutamic acid using site-directed mutagenesis resulted in >100-fold increase in K_m (*16*). This result provides compelling evidence that the FCL domain of Endo III is involved in DNA binding. The conservation of cysteine ligand positioning and identity of amino acids in this region of the two enzymes suggests that MutY also contains a $[4Fe\text{-}4S]^{2+}$ center and that the presence of this cluster may be important for its function. In Endo III, the $[4Fe\text{-}4S]^{2+}$ cluster has a superficial location near the C-terminus of the enzyme (*15*); however, in MutY, the sequence extends considerably beyond the C-terminus of Endo III, providing an additional domain (*10*), and therefore the cluster may not have a superficial location. The proximity of the C-terminal domain to the Fe-S cluster in MutY suggests that this domain may influence the properties of the cluster relative to those in Endo III. The observation that the cluster in Endo III is resistant to oxidation or reduction argues against an electron-transfer role for the metal cluster and has led to the suggestion that its role is primarily structural (*14*).

The presence of a $[4Fe\text{-}4S]^{2+}$ cluster in these two DNA repair enzymes was surprising since proteins containing iron-sulfur clusters were originally thought to be primarily involved in electron-transfer reactions (*17*−*19*). For example, ferredoxins (Fd)[1] and high-potential iron-sulfur proteins (HiPIPs) are well-characterized iron-sulfur proteins which participate in electron-transfer reactions. However, many nonredox roles for Fe-S clusters in proteins have been uncovered, and Fe-S clusters are increasingly appreciated as pervasive and versatile cofactors (*20*, *21*). A striking example of this is the Fe-S cluster in the Kreb's-cycle enzyme aconitase that participates in the catalysis of a dehydration/rehydration reaction in the interconversion of citrate and isocitrate (*22*). Interestingly, aconitase has homology to the iron regulatory protein IRP-1, in which the assembly and disassembly of the cluster affects the protein's ability to bind RNA. This is believed to serve as a mechanism for regulating the translation of ferritin and transferrin receptor mRNA.

The synthesis, assembly, and insertion of Fe-S clusters in vivo is not well understood. The potential toxicity of iron and sulfide suggests that incorporation of Fe-S clusters into proteins in vivo may require the coordinated action of specific proteins. In *Azotobactor vinelandii*, the NifS protein is required for full activation of nitrogenase proteins and participates as the source of inorganic sulfide for metal cluster assembly (*23*, *24*). Recently, a protein in *E. coli* has been identified that is similar to NifS with respect to its sequence and functional properties (*25*, *26*). Alternatively, the ability to prepare synthetic iron-sulfur clusters by the process of spontaneous self-assembly (*27*, *28*) and the ability to reconstitute proteins containing [4Fe-4S] clusters from the apoprotein in the presence of iron and sulfide ions (*29*, *30*) suggests that a self-assembly mechanism may be used in vivo. Metal clusters may also be assembled using a combination of enzymatic and nonenzymatic methods.

A related issue is the interplay between protein folding and cluster assembly. Recent NMR work with *Chromatium vinosum* HiPIP has indicated that a stable and partially folded intermediate forms upon re-folding apo-HiPIP in the presence of ferrous ion (*31*). Upon addition of sulfide ions, the [4Fe-4S] cluster forms, and this reconstituted form exhibits the native protein structure by NMR. In addition, apo-HiPIP does not exhibit significant tertiary or secondary structure, and therefore these results indicate the importance of [4Fe-4S] cluster assembly in HiPIP protein folding.

MutY activity can be recovered from denaturing polyacrylamide gels in the presence of ferrous and sulfide ions, providing circumstantial evidence that MutY contains an Fe-S cluster and that the cluster can be reassembled (*9*). An advantage of using a nonenzymatic method for reconstitution of the Fe-S cluster is the potential to identify and characterize

[1] Abbreviations: BER, base-excision repair; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; AP, apurinic/apyrmidinic; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; OG, 8-oxo-7,8-dihydro-2′-deoxyguanosine or 8-hydroxy-2′-deoxyguanosine; HPLC, high-performance liquid chromatography; Gu-HCl, guanidine hydrochloride; CD, circular dichroism; DTT, dithiothreitol; TBE, tris-borate-EDTA buffer; HiPIP, high-potential iron-sulfur protein; Fd, ferredoxin; SSDNA, single-stranded DNA.

intermediate states as was observed in *C. vinosum* HiPIP. In the present study, we have investigated the importance of $[4Fe\text{-}4S]^{2+}$ cluster assembly in MutY folding. Specifically, MutY was denatured and then refolded in the presence or absence of ferrous and sulfide ions. Circular dichroism spectra were used to determine the extent of MutY folding compared to the native enzyme. These data suggest that MutY can fold to a near native and stable conformation without assembly of the $[4Fe\text{-}4S]^{2+}$ cluster. The enzymatic activity and DNA binding properties of forms refolded in the presence or absence of added ferrous and sulfide ions were determined using adenine glycosylase enzyme assays and gel retardation assays, respectively. These results establish the importance of the $[4Fe\text{-}4S]^{2+}$ cluster of MutY for specific recognition of damaged and mismatched DNA.

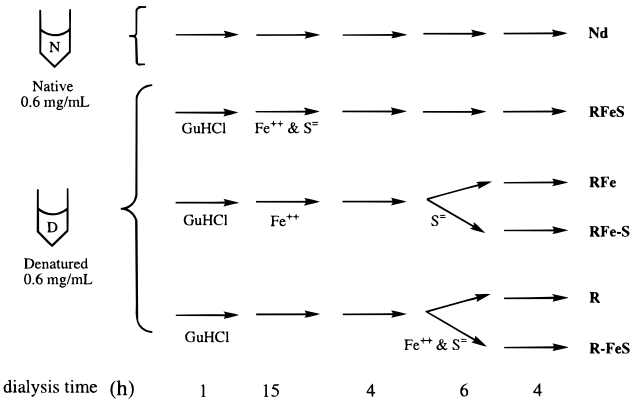
EXPERIMENTAL PROCEDURES

General Methods, Materials, and Instrumentation. All standard chemicals and biochemicals were purchased from Fisher Scientific. Ammonium iron(II) sulfate and ammonium sulfide were purchased from Aldrich. Dialysis cassettes were purchased from Pierce. Reagents for Bradford assays were obtained from Bio-Rad. T4-polynucleotide kinase was purchased from USB. [γ-^{32}P]ATP and X-ray film (Hyperfilm-MP) were obtained from Amersham. Distilled, deionized water (Milli-Q) was used for all aqueous reactions and dilutions. All 2-cyanoethyl phosphoramidites were purchased from Applied Biosystems, Inc. (ABI), except 7,8-dihydro-8-oxo-2′-deoxyguanosine and 2′-deoxyformycin phosphoramidites which were synthesized as reported previously (*32*, *33*). Oligonucleotides were synthesized on an ABI 392B automated DNA synthesizer using the manufacturer's protocols. Oligonucleotides were purified and handled as previously described (*33*). Circular dichroism (CD) spectra were obtained using an AVIV 62A DS circular dichroism spectrometer. UV−visible spectroscopy was performed in a Hewlett-Packard 8452A Diode Array Spectrophotometer. Storage phosphor autoradiography was performed using a Molecular Dynamics STORM 840 Phosphorimager.

MutY Purification and Activity. MutY was purified as described previously (*33*). MutY aliquots were stored in liquid nitrogen after purification, and the concentration of the aliquots was determined using the method of Bradford (*34*). The amount of active MutY was determined by an active site titration method (*35*) and is estimated to be approximately 50% for the enzyme preparations used in these experiments (data not shown). The concentrations listed throughout this paper were not corrected for active enzyme concentration and represent the concentration determined by the Bradford method.

The MutY adenine glycosylase activity assay was performed as described previously (*33*) using the 30-base-pair duplex d(5′-CGATCATGGAGCCAC**X**AGCTCCCGTTA-CAG-3′)•d(3′-GCTAGTACCTCGGTG**Y**TCGAGGGCAAT-GTC-5′), where **Y** represents OG, and **X** represents A. Reactions containing 10 nM 30-mer duplex and 600 nM MutY were incubated 30 min at 37 °C in 20 mM Tris/HCl, pH 7.5, 10 mM EDTA, and 0.1 μg/μL BSA. The reactions were then quenched with NaOH to a final concentration of 0.1 M and by heating at 90 °C. Subsequently, 10 μL of denaturing loading dye (80% formamide, 0.025% xylene cylanol, 0.025% bromophenol blue in TBE) was added to the samples, which were then heat denatured and electrophoresed on a 15% denaturing polyacrylamide gel in 1× TBE buffer. The gel was then exposed to autoradiography film or Phosphorimager screen. The intensity of DNA bands from Phosphorimager analysis was quantitated using Image Quant software (Molecular Dynamics). The procedure for the active site titration determinations was similar except that [MutY] < [DNA] and a number of incubation times ranging from 15 s to 1 h were used (*35*).

Scheme 1: Diagrammatic Representation of the Procedure Used in Refolding of Denatured MutY[a]



[a] Each dialysis step is represented by an arrow. In all cases, the dialysis was performed at 4 °C in buffer A with addition of specific components that are indicated above the arrows. The ferrous and sulfide ion concentrations were 0.2 mM. The dialysis times are indicated at the bottom of the figure. The abbreviations distinguish the samples treatment in the dialysis procedures: N, native MutY; Nd, dialyzed native MutY; D, denatured MutY; RFeS, MutY refolded in the presence of Fe^{2+} and S^{2-}; RFe, MutY folded in the presence of Fe^{2+} only; R, MutY refolded in the absence of added Fe^{2+} and S^{2-}; RFe-S, MutY refolded in the presence of Fe^{2+} with subsequent addition of S^{2-}; R-FeS, MutY refolded in the absence of Fe^{2+} and S^{2-} with subsequent addition of Fe^{2+} and S^{2-}.

MutY Denaturation. A denaturation mixture containing 0.6 mg/mL MutY and 6 M Gu-HCl in buffer A (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, and 10% glycerol) was incubated at 4 °C for at least 1 h. In parallel, a MutY sample containing the same components except Gu-HCl was prepared and incubated for an identical period. CD and UV−visible spectra of the denatured and native enzyme samples were obtained to confirm the denaturation procedure was effective. The denatured protein was dialyzed against buffer A-containing 6 M Gu-HCl and 0.1 mM EDTA for 6 h to ensure removal of metal ions prior to refolding procedures.

Refolding. The procedure for refolding of denatured MutY is illustrated in Scheme 1. The denatured enzyme was aliquoted into five samples which were placed in five dialysis cassettes and dialyzed for approximately 12−15 h at 4 °C in buffer A which either lacked or contained ferrous and sulfide ions. One of the five aliquots was dialyzed with buffer A (500 mL) containing 0.2 mM ammonium sulfide and 0.2 mM iron(II) ammonium sulfate. Two of the five aliquots were dialyzed with buffer A alone (500 mL). The remaining two aliquots were dialyzed with buffer A-containing 0.2 mM iron(II) ammonium sulfate. After the overnight dialysis, the five aliquots were dialyzed against fresh buffer A to eliminate excess ions in solution. Subse-

quently, one of the two aliquots that had been dialyzed against buffer A plus Fe(II) was dialyzed in buffer A-containing 0.2 mM ammonium sulfide. Similarly, one of the two aliquots originally dialyzed against buffer A was then dialyzed in buffer containing 0.2 mM Fe(II) and 0.2 mM ammonium sulfide. After these dialysis procedures, all samples were further dialyzed against buffer A for several hours to remove excess ions. In parallel, a native MutY sample was dialyzed against buffer A (designated **Nd**) for the same time period as the other samples to serve as a control for the extensive dialysis treatment. After this procedure, there were six distinct samples which were removed from the dialysis slides and labeled as follows: **Nd**, native protein dialyzed; **RFeS**, refolded in the presence of ferrous and sulfide ions; **RFe**, refolded in the presence of ferrous ions only; **R**, refolded in buffer A without added ions; **RFe-S**, refolded in the presence of ferrous ion, then dialyzed with sulfide ions; **R-FeS**, refolded in buffer A alone and then dialyzed in the presence of ferrous ion and sulfide. The concentration of each sample was determined using the Bradford method to account for loss of MutY due to binding to the dialysis membrane, aggregation, and change in sample volume during the dialysis (*34*). The amount of recovered enzyme after this treatment varies considerably, depending on the buffer conditions used. To perform CD experiments, 1/2× buffer A was used to minimize interference from the buffer. The denaturation/refolding procedure was repeated 13 times using three different MutY preparations. The results obtained were the same for different preparations of MutY. Adenine glycosylase activity was determined on refolded samples from all 13 experiments giving identical results. Quantitative determinations of active enzyme concentrations using the active site titration method were determined on three separate refolding experiments and yielded similar results.

CD Experiments and T_m Measurements. CD spectra on 10 different folding experiments were taken of the refolded samples between 200 and 270 nm in a 1 cm quartz cuvette. The ellipticity of the different samples was obtained in 1/2× buffer A and converted to the appropriate mean residue molar ellipticity values (*36*) using the concentration determined by the Bradford method. The samples were diluted to give approximately the same ellipticitiy value at 222 nm (∼20 mdeg in a 1 cm cell) for the T_m determinations. T_m values were determined by measuring the CD signal at 222 nm from 10 to 70 °C with temperature steps of one or two degrees. An equilibration time of 1 min was used at each temperature step. The data were plotted and fit using the program Ultrafit with the equation, $y = [\min + (\max - \min)]/\{1 + e^{[-k(x-T_m)]}\}$, to determine the temperature at which 50% of the CD signal had disappeared which is defined as the T_m.

Gel Retardation Assay. Gel retardation assays were performed on four different refolding experiments using the same 30 base-pair duplex described previously except that **X** = 2′-deoxyformycin (F). This duplex has been shown to have high affinity for MutY but is not enzymatically processed (*33*). The OG-containing strand of the duplex was 5′-end-labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase following the manufacturer's protocol. Reactions of 2 nM duplex and 25, 75, or 1.25 μM MutY samples were incubated for 20 min at 25 °C in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM Na_2EDTA, 1 mM DTT, 7.5% glycerol, 0.1 mg/mL BSA. MutY samples were diluted to the desired concentrations using dilution buffer (20 mM Tris-HCl, pH 7.5, 10 mM Na_2EDTA, 20% glycerol). Nondenaturing loading dye was added to the samples which were loaded onto 6% nondenaturing polyacrylamide gels (17 cm × 14 cm × 0.3 cm) and then electrophoresed at 150 V for 1.5 h with 0.5× TBE at 4 °C. The dried gels were exposed to Phosphorimager screens (Molecular Dynamics) or X-ray film (Amersham).

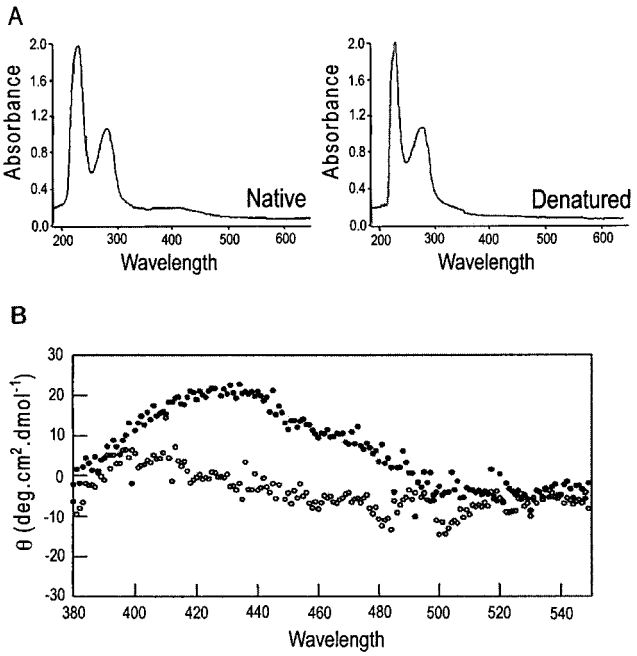


FIGURE 2: (A) UV−visible spectra of native MutY in buffer A (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 2 mM 2-mercaptoethanol, 0.1 mM Na_2EDTA, and 10% glycerol) and denatured MutY in buffer A and 6 M GuHCl. The concentration in both spectra is 0.6 mg/mL. (B) CD spectra in the visible region (250−650 nm) of native MutY in buffer A (●) and denatured MutY in buffer A plus 6 M GuHCl (○). Both spectra were obtained from a 0.6 mg/mL solution of MutY, and the measured ellipticity values were converted to mean residue molar ellipticity.

RESULTS

UV−Visible and CD Spectra of MutY. The absorbance spectrum of MutY is shown in Figure 2A. Absorption feature centered around 410 nm is attributed to thiolate-to-iron charge-transfer transitions from cysteines ligating the $[4Fe\text{-}4S]^{2+}$ cluster. The relative position of this charge-transfer transition is analogous to that observed for endonuclease III (*14*). Native MutY is EPR silent as expected for a $[4Fe\text{-}4S]^{2+}$ cluster (data not shown). Upon denaturation, the absorbance features (Figure 2) in the visible region around 410 nm disappear. This is consistent with disassembly of the Fe-S cluster upon unfolding of the enzyme.

The circular dichroism spectrum for native MutY in the range 200 to 270 nm is shown in Figure 3A. The ellipticity in this range is distinctive for each protein and depends on its secondary structure. The X-ray structure of Endo III indicates a highly α-helical secondary structure (*16*). The shape of the CD spectrum of MutY is consistent with that expected for a protein structure that is primarily α-helical (*37*); however, the magnitude of the CD signal for native MutY is lower than expected for a purely α-helical protein.

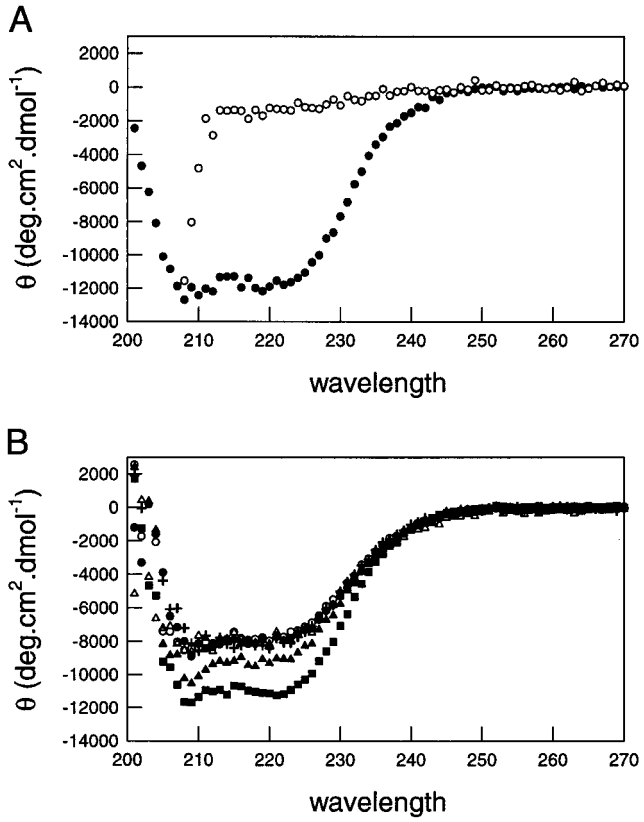


FIGURE 3: (A) CD spectra in the UV region (200−270 nm) of native (●) and denatured (○) MutY. The ellipticity of a 0.6 mg/mL solution was measured and converted to molar ellipticity. The buffer used was 1/2× buffer A. (B) CD spectra in the UV region (200−270 nm) at 25 °C in 1/2× buffer A of the dialyzed native MutY Nd (■) and various refolded forms of MutY, **RFeS** (○), **R-FeS** (+), **RFe-S** (▲), **R** (●), and **RFe** (△). The concentration of the samples used for the CD measurements ranged from 0.1 to 0.05 mg/mL as determined by the Bradford method. The observed ellipticity was converted into units of mean residue molar ellipticity. The data shown are from a single refolding experiment and are representative of the CD measurements obtained on 10 different refolding experiments.

This may be a consequence of the additional C-terminal domain of MutY and overestimation of the protein concentration by the Bradford method (*38*). Upon denaturation of MutY with 6 M Gu-HCl, the characteristic CD signal centered at 222 nm is lost, consistent with loss of secondary structure. The small negative ellipticity values that are observed at lower wavelengths are due to the random-coil form of the protein and high concentrations of Gu-HCl.

The CD spectrum of MutY in the visible region (Figure 2B) is attributed to the [4Fe-4S] cluster. The observation of features in the CD spectrum associated with the Fe-S cluster indicates that protein folding around the cluster produces a chiral enviroment for the Fe-S cluster. A somewhat similar CD signature in the visible region has been observed previously with [4Fe-4S] cluster containing protein *A. vinelandii* ferredoxin I (*38*). MutY's characteristic visible CD features are lost upon denaturation (Figure 2B) which is consistent with disassembly of the cluster upon protein denaturation.

Refolding Experiments. To investigate the ability of MutY to refold in the presence and absence of ferrous and sulfide ions, a series of refolding experiments were performed as outlined in Scheme 1. In typical experiments, MutY was denatured with Gu-HCl and dialyzed extensively against Gu-HCl and EDTA to effectively remove metal ions. The Gu-HCl was then removed by dialysis against buffer A without Gu-HCl to allow for slow refolding of the enzyme. In this subsequent dialysis step, ferrous and sulfide ions were either present or absent to determine whether cluster assembly is required for the folding process. This procedure resulted in the preparation of five distinct refolded and stable forms of MutY. Denatured MutY refolded in the absence of added ferrous or sulfide ions generated a cluster-free form of MutY (**R**). Subsequent dialysis of a sample of **R** with ferrous and sulfide ions, provided a new form of MutY (**R-FeS**) in which the cluster is presumed to have assembled. Denatured MutY samples which were renatured in the presence of ferrous and sulfide ions or ferrous ions alone are referred to as samples **RFeS** and **RFe**, respectively. The **RFe** form of MutY was assumed to contain bound Fe(II) ions, and with subsequent addition of sulfide ions provided the refolded sample designated, **RFe-S**. After the dialysis procedures, the protein concentration for each sample was determined, and the samples were analyzed by CD, adenine glycosylase assays, and DNA binding assays.

The concentration of the recovered samples was consistently low (0.05−0.01 mg/mL) due to precipitation and adhesion to the dialysis membrane. Experiments in the presence of BSA result in more recovered enzyme. BSA not only stabilizes MutY in solution but may also reduce the loss of MutY due to dialysis membrane binding and aggregation. However, BSA interferes with protein concentration and CD measurements and therefore was not used in the present study. For the purpose of obtaining larger amounts of MutY, refolding experiments in the presence of BSA or with shorter dialysis steps may provide better results.

UV−visible spectra of the refolded forms **R** and **RFe** are consistent with the lack of an Fe-S cluster (data not shown). However, with the refolded forms in which assembly of the cluster is expected, **RFeS**, **RFe-S**, **R-FeS**, we have not been able to unambiguously provide UV−visible evidence for the presence of the cluster. For example, concentrated samples of **RFeS** are yellow in color, but the UV−visible spectrum does not have a well-defined feature at 410 nm as in the native enzyme. Instead there is a very broad absorption feature which appears as a shoulder on the protein 280 nm band and extends out to 450 nm. This broad absorption feature we believe is due to FeS bound to the surface of MutY and masks the ability to observe the 410 nm band. A variety of purification methods were attempted to try to remove these Fe impurities; however, these attempts have been unsuccessful. The samples contain Fe, though we cannot establish whether this is due to the surface bound FeS or the Fe-S cluster. EPR spectra of the refolded samples are EPR silent, consistent with the native enzyme, but this does not provide direct evidence for the presence of the Fe-S cluster.

Characterization of Refolded MutY Forms Using CD Spectroscopy. Circular dichroism spectra (Figure 3B) in the region of 200−270 nm were taken of approximately equal concentrations (∼0.05 mg/mL) of the various refolded forms and converted to the appropriate units of molar ellipticity [θ]. The shape of the CD signals for the refolded forms is similar to that observed for the dialyzed native enzyme (**Nd**). However, the refolding procedure appears not to completely

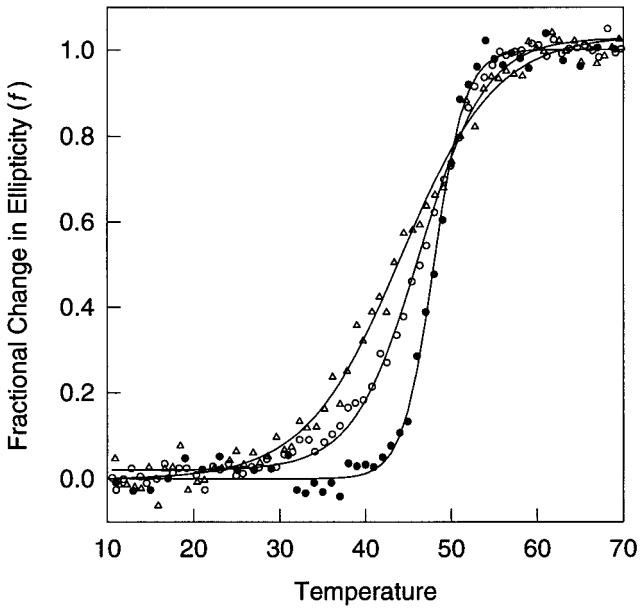


FIGURE 4: Temperature stability of dialyzed native **Nd** (●) and refolded forms **R** (△) and **RFeS** (○) of MutY. The change in ellipticity of the different samples upon heat denaturation was monitored at 222 nm. Samples (~ 0.02 mg/mL) were diluted to give approximately the same initial ellipticity value at 222 nm of ~20 mdeg in a 1 mm cell path length. The ellipticity was converted to a fractional change in mean residue molar ellipticity (*f*) where for the folded form, $f = 1$, and for the denatured form, $f = 0$. The temperature range was 10−70 °C with temperature readings taken every one degree.

reestablish the native structure, since the molar ellipticity values are somewhat less for the refolded samples compared to the **Nd** enzyme. However, the CD spectra for all five refolded forms, **RFeS**, **RFe**, **R**, **RFe-S**, and **R-FeS**, are nearly identical to each other within the errors of the experiment. These results show that the enzyme is able to refold to a significant degree and that this refolding does not depend on the presence of added ferrous and sulfide ions. This suggests that assembly of the cluster is not required to significantly refold MutY. The concentration of the **RFeS**, **RFe-S**, and **R-FeS** refolded forms was too low to observe the CD feature in the visible region.

To investigate the involvement of the $[4Fe\text{-}4S]^{2+}$ cluster in the stability of the enzyme, a series of experiments using CD to monitor the temperature stability of the various refolded forms were undertaken. In these melting experiments, the ellipticity of the samples at 222 nm was monitored as a function of temperature. Representative T_m plots for the **Nd**, **RFeS**, and **R** samples are shown in Figure 4. Melting temperature (T_m) values for each sample were determined as the temperature at which 50% of the total change in the ellipticity is observed. The T_m values for all of the samples are reported in Table 1. The **R** and **RFe** forms exhibit T_m values which are essentially identical within experimental error to those measured for the dialyzed native enzyme and the refolded forms in which the Fe-S cluster was reconstituted. These data shows that the $[4Fe\text{-}4S]^{2+}$ cluster does not provide *overall* stability to the native MutY enzyme. The similarity in T_m values between the MutY samples containing or lacking the Fe-S cluster suggests a similarity in the global structure of these forms. However, the T_m plots for the **R** and **RFeS** samples in Figure 4 indicate more conformational heterogeneity compared to the **Nd** enzyme. This may be a consequence of the presence of partially folded enzyme in the refolded samples.

Table 1: Melting Temperatures (T_m) of Refolded Forms of MutY[a]

MutY form	$T_m \pm 2$ (°C)
Nd	46
RFeS	43
RFe	42
R	42
RFe-S	43
R-FeS	42

[a] T_m values were determined by monitoring the change in the CD signal at 222 nm, as described in the Experimental Procedures. Each T_m value is an average of four separate determinations on four different refolding experiments. Representative temperature plots for **Nd**, **RFeS**, and **R** forms are illustrated in Figure 4.

Adenine Glycosylase Activity of Refolded Samples. The adenine glycosylase activity of the refolded forms of MutY was assayed using a 30-base-pair oligonucleotide harboring a central OG:A base-pair which had been ^{32}P end-labeled on the 5′-end of the A-containing strand. The substrate duplex (10 nM) was incubated with the various refolded and native forms of MutY (~600 nM) and then quenched after 30 min with NaOH. The base treatment also induces strand scission at the abasic site to produce a 14-nucleotide fragment that can be separated from the full-length oligonucleotide using denaturing polyacrylamide gel electrophoresis. The autoradiogram of a representative experiment is shown in Figure 5. Under these assay conditions with the native enzyme, all of the substrate is converted to product. High concentrations of the refolded samples were used to maximize the ability to detect low levels of adenine glycosylase activity. In addition, concentrations for the enzymatic assays were within the same order of magnitude as those used in the CD experiments to allow for direct comparisons between folding and enzyme activity. In these experiments, MutY enzyme refolded in the presence of ferrous and sulfide ions (**RFeS**) is enzymatically active. However, MutY samples which were refolded without ferrous and sulfide ions (**R**) or with ferrous ions alone (**RFe**) exhibit no detectable adenine glycosylase activity. MutY samples which were refolded in the absence of ferrous and sulfide ions and subsequently dialyzed against solutions of ferrous and sulfide ions were enzymatically active (**R-FeS**). These results suggest that an intact Fe-S cluster is required for the adenine glycosylase activity and that the cluster self-assembles under these conditions, producing enzymatically active MutY. Interestingly, MutY samples refolded in the presence of ferrous ion alone were inactive, but became active upon the addition of sulfide ions (**RFe-S**). This implies that the **RFe** form is a relatively stable intermediate containing Fe(II), such that addition of sulfide ions provides an enzymatically active sample. The ability of the refolded inactive forms **RFe** and **R** to generate active MutY suggests a superficial location for the cluster, such that protein unfolding is not necessary for cluster formation. Additionally, this result implies that the structural properties of the **RFe** and **R** forms is similar to the native enzyme such that reconstitution of the cluster affords active MutY enzyme.

The adenine glycosylase assay was also used to perform an active site titration on a representative set of samples to compare native enzyme activity to that recovered from MutY

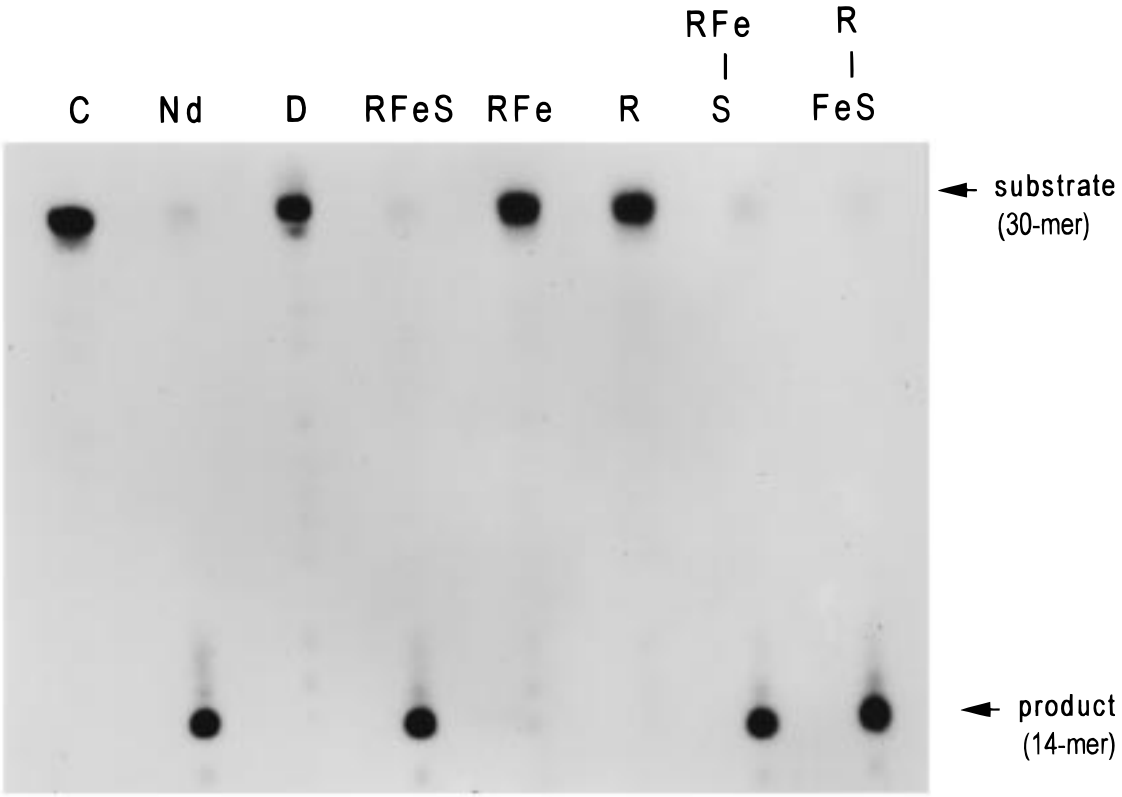


FIGURE 5: Autoradiogram of adenine glycosylase assay of native MutY and various refolded forms of MutY with a 30-base-pair duplex containing a central OG:A mismatch. Prior to annealing of the duplex, the A-containing strand was ^{32}P end-labeled on the 5′ end. All samples contained 10 nM of the substrate duplex and 600 nM of the relevant MutY form. The reaction was allowed to proceed for 30 min and was quenched with NaOH. The base treatment also serves to cleave the DNA at the abasic site produced by MutY's enzymatic action to produce a 14-nucleotide ^{32}P end-labeled fragment. The amount of substrate remaining at this time is represented by a full-length ^{32}P end-labeled 30-mer oligonucleotide, while the amount of product produced is represented by the ^{32}P end-labeled 14-base oligonucleotide. Lane C, control lane with no added MutY; lane Nd, dialyzed native MutY; lane D, denatured MutY; lane RFeS, MutY refolded in the presence of Fe^{2+} and S^{2-}; lane RFe, MutY refolded in the presence of Fe^{2+} only; lane R, MutY refolded in the absence of added Fe^{2+} and S^{2-}; lane RFe-S, MutY refolded in the presence of Fe^{2+} with subsequent addition of S^{2-}; lane R-FeS, MutY refolded in the absence of Fe^{2+} and S^{2-} with subsequent addition of Fe^{2+} and S^{2-}.

protein refolded in the presence of ferrous and sulfide ions (data not shown). The activity of the refolded forms compared to the dialyzed native enzyme was 30, 26, and 20% for the **RFe-S**, **RFeS**, and **R-FeS** forms, respectively. This amount of recovered activity is not surprising, since we have found that MutY loses activity within days at 4 °C and with excessive handling.

DNA Binding Properties of Refolded Forms. In order to determine if the lack of glycosylase activity for MutY enzyme refolded in the absence of ferrous and sulfide ions (**R**) or presence of ferrous ions alone (**RFe**) is due to inefficient DNA binding, experiments were performed to evaluate DNA binding to a MutY substrate analogue DNA duplex. DNA duplexes containing a 2′-deoxyformycin-A (F):OG base-pair have been shown to effectively mimic the corresponding A:OG duplex in terms of specific recognition by MutY (*33*). The advantage of using the F analogue instead of A in duplexes to determine DNA binding is that complications arising from enzymatic turnover are removed. In these experiments, a nondenaturing gel retardation method was used to monitor specific complex formation between the different MutY samples and the OG:F-containing duplex. The enzyme concentrations used in this experiment are above the K_d for MutY with an OG:F duplex (~1 nM), but below the K_d for nonspecific DNA binding (~300 nM) of MutY (*33*). These conditions were chosen to optimize observation of only specific binding to the substrate analogue duplex. These binding assays were performed on six different refolding experiments and gave reproducible results. A representative phosphor image for a set of refolded samples is shown in Figure 6A. In these gel retardation experiments, native MutY (**N**) forms a large amount of specific complex (>90%) as evidenced by a retarded band in the nondenaturing gel. With the refolded MutY samples, a band with a retarded mobility representative of the specific MutY−OG:F duplex DNA complex is only observed in the samples which have enzyme activity. This indicates that only the **RFeS**, **R-FeS**, and **RFe-S** are able to bind to the OG:F duplex under these conditions.

Some differences in binding affinity of the various active refolded forms are evident in this experiment. However, the % MutY−DNA complex in each case cannot be compared directly to percent active enzyme concentration since the DNA and MutY concentrations used in the binding experiments are in the same range as the K_d. However, under these conditions, the observed amount of MutY−DNA complex is extremely sensitive to small concentration differences in active enzyme in the sample. The native enzyme which had been subjected to the extensive dialysis procedure (**Nd**) forms slightly less of the specific complex at the lower MutY concentration (25 nM) than native MutY (**N**) which had not been dialyzed. This may indicate some loss of enzyme

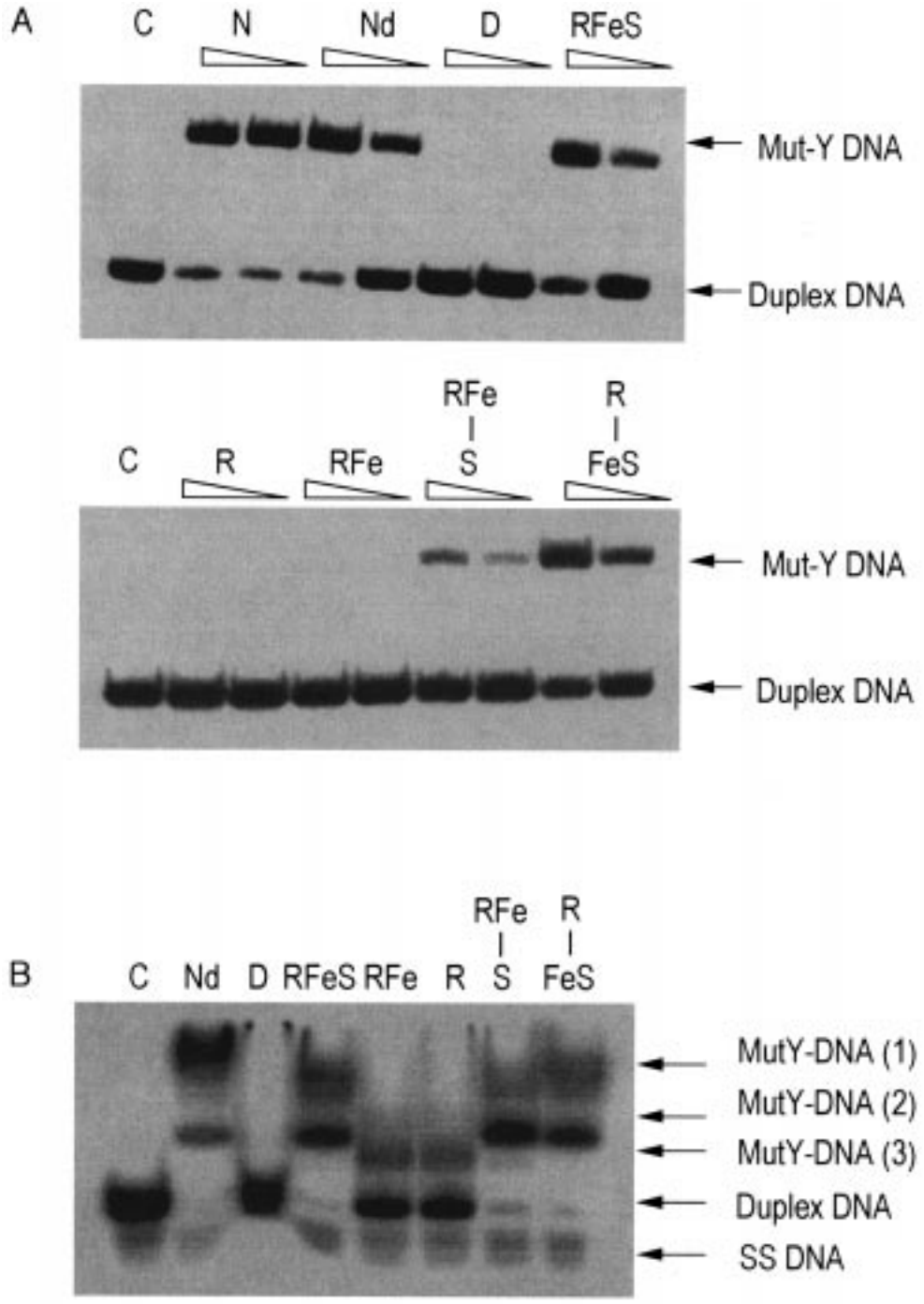


FIGURE 6: (A) Storage phosphor autoradiogram of nondenaturing polyacrylamide gel retardation assay used to detect *specific binding* of MutY with a substrate analogue duplex. The 30-base-pair substrate analogue duplex contains a centrally located OG:F base-pair and the OG-containing strand was ^{32}P end-labeled on the 5′ end. The DNA duplex concentration in all samples was 2 nM, while the MutY concentrations are 75 and 25 nM, respectively. Lane C, control lane with no added MutY; lane N, native MutY enzyme; lane Nd, dialyzed native MutY; lane D, denatured MutY; lane RFeS, MutY refolded in the presence of Fe^{2+} and S^{2-}; lane RFe, MutY folded in the presence of Fe^{2+} only; lane R, MutY refolded in the absence of added Fe^{2+} and S^{2-}; lane RFe-S, MutY refolded in the presence of Fe^{2+} with subsequent addition of S^{2-}; lane R-FeS, MutY refolded in the absence of Fe^{2+} and S^{2-} with subsequent addition of Fe^{2+} and S^{2-}. The low-mobility bands in the nondenaturing gel (top of gel) are representative of the specific MutY−DNA complex, while the high-mobility bands (bottom of the gel) are due to the free DNA duplex. (B) Storage phosphor autoradiogram of nondenaturing gel retardation assay using high enzyme concentrations, such that [MutY] $\gg K_d$ for binding to the substrate analogue DNA. In this experiment, the large enzyme concentration allows for detection of weak binding of the refolded forms of MutY with a substrate analogue duplex. The DNA duplex concentration was 2 nM, while the MutY concentration was 1.25 μM. Lane C, control lane with no added MutY; lane Nd, dialyzed native MutY; lane D, denatured MutY; lane RFeS, MutY refolded in the presence of Fe^{2+} and S^{2-}; Lane RFe, MutY folded in the presence of Fe^{2+} only; lane R, MutY refolded in the absence of added Fe^{2+} and S^{2-}; lane RFe-S, MutY refolded in the presence of Fe^{2+} with subsequent addition of S^{2-}; Lane R-FeS, MutY refolded in the absence of Fe^{2+} and S^{2-} with subsequent addition of Fe^{2+} and S^{2-}. MutY−DNA (1) represents the nonspecific complex of MutY with DNA containing more than one molecule of MutY per DNA duplex. MutY−DNA (2) represents the specific complex formed between MutY and the OG:F duplex with the expected mobility for the specific complex observed in Figure 6A. MutY−DNA (3) signifies a complex between MutY-and OG:F duplex with unusual mobility, distinct from MutY-DNA (2).

activity during the dialysis procedure. The refolded forms, **RFeS**, **RFe-S**, and **R-FeS**, also form less specific complex in the nondenaturing gel than the dialyzed native (**Nd**) sample. This is consistent with the loss of enzyme activity compared to the Nd sample determined by the active site titration. No specific binding is observed for the refolded forms, **R** and **RFe**, which also exhibited no glycosylase activity. Together these results support the conclusion that the absence of glycosylase activity of the refolded forms **R** and **RFe** is due to the inability to bind to substrate DNA. Indeed, these results suggest that the presence of the Fe-S cluster is required for efficient *specific* DNA binding.

Similar experiments (Figure 6B) were also performed to detect weak binding by using a concentration of MutY (μM) which is considerably higher than those in Figure 6A. At this high concentration of dialyzed native MutY (**Nd**, Figure 6B), the predominant bands observed in the gel have very low mobility and are retarded significantly more than the bands observed for the specific MutY−DNA complex evident at lower enzyme concentrations (Figure 6A). These highly retarded bands are presumably due to complexes containing more than one MutY enzyme per 30-base-pair duplex and are representative of nonspecific binding of MutY *in addition to* specific binding. Notably, with the **Nd** sample, a band with a mobility corresponding to specific MutY−OG:F containing duplex complex is observed in addition to the highly retarded bands. The refolded samples, **RFeS**, **R-FeS**, and **RFe-S**, behave similarly to the dialyzed native (**Nd**) enzyme at this enzyme concentration. However, the refolded forms **R** and **RFe** do not appear to bind appreciably to the 30-mer DNA duplex even at these high concentrations and clearly interact with the DNA in a manner significantly different from the rest of the refolded samples. A retarded band representing approximately 30% of the DNA in the sample is observed with the **R** and **RFe** forms, indicating a small amount of weak binding to DNA. Based on the concentrations of enzyme used in this experiment, the magnitude of this binding is significantly less than native MutY binding to an OG:F duplex. Interestingly, this retarded band has a significantly different mobility than the band representing the specific complex for the **Nd**, **RFeS**, **R-FeS**, and **RFe-S** forms. These observations along with those from the low enzyme concentration experiments (Figure 6A) indicate that only the refolded forms, **RFeS**, **R-FeS**, and **RFe-S**, bind tightly to the OG:F duplex in a manner analogous to the native enzyme. These results provide additional support for the conclusion that the [4Fe-4S] cluster in MutY plays a critical role in *specific* DNA binding.

DISCUSSION

MutY and Endo III represent a distinct class of Fe-S proteins involved in the repair of oxidative damage to DNA. In order to provide insight into the role of the $[4Fe\text{-}4S]^{2+}$ center in MutY, we have investigated the self-assembly of the $[4Fe\text{-}4S]^{2+}$ cluster and the refolding of denatured MutY. This has provided the opportunity to determine the functional properties of MutY with and without the presence of the $[4Fe\text{-}4S]^{2+}$ cluster. Our results establish the importance of the $[4Fe\text{-}4S]^{2+}$ cluster in MutY's recognition and repair of DNA. Thus, this work provides an important starting point for delineating the role of the $[4Fe\text{-}4S]^{2+}$ cluster in MutY. Additionally, these investigations provide insight into possible routes for Fe-S cluster assembly in Fe-S proteins in vivo and possible functions for Fe-S cofactors in biology.

The reconstitution of Fe-S proteins by treating the apoprotein with ferrous ion, sulfide ions, and β-mercaptoethanol under aerobic conditions is well-documented (*21*, *29*, *39*). In this procedure, some ferrous ion is oxidized to ferric ion to yield the $[4Fe\text{-}4S]^{2+}$ oxidation state of the metal cluster. The present work shows that MutY can be reconstituted by dialysis of denatured MutY against buffer containing ferrous ions, sulfide ions, and β-mercaptoethanol but lacking denaturant to allow for slow refolding of the enzyme and Fe-S cluster assembly. The presence of the Fe-S cluster in the refolded forms **RFeS**, **RFe-S**, and **R-FeS** has not been unambiguously established due to interference from adventitious FeS bound to refolded MutY. However, the forms of the enzyme in which both ferrous and sulfide ions have been added behave in a manner that is similar to the native enzyme, which does contain an Fe-S cluster. In addition, the refolded forms **R** and **RFe** clearly lack the Fe-S cluster as expected from the refolding procedure, and these forms do not behave like the native enzyme. Thus, there seems to be strong circumstantial evidence for the presence of an Fe-S cluster in the refolded MutY samples. Unfortunately, we have been unable to identify conditions in which we can oxidize or reduce the $[4Fe\text{-}4S]^{2+}$ cluster in native MutY which would be expected to provide a useful EPR signature for the cluster distinctive from adventitously bound ferrous or ferric ion.

The similarity of CD signatures and T_m values of the native enzyme versus the enzyme refolded with ferrous and sulfide ions (**RFeS**) indicates that there are no global structural differences between the native and refolded proteins. Furthermore, the reconstituted forms, **RFeS**, **RFe-S**, and **R-FeS**, are enzymatically active, indicating that the refolding procedure provides the active enzyme conformation. A significant amount of active MutY enzyme is produced using this procedure which suggests that self-assembly of the Fe-S cluster in MutY is facile. The ability to provide active MutY upon addition of ferrous and sulfide ions subsequent to enzyme refolding is consistent with a superficial location of the cluster in MutY. The ability to regain enzyme activity by first refolding in the presence of ferrous ion and then subsequent addition of sulfide ions suggests that the refolded enzyme may bind ferrous ions with reasonable affinity, possibly in a manner similar to that observed with *C. vinosum* HiPIP (*31*). However, the treatment of the enzyme with only ferrous ions is not sufficient for enzymatic activity and specific high affinity DNA binding and requires subsequent addition of sulfide ions to provide active MutY. This procedure presumably reconstitutes the Fe-S cluster.

We have also demonstrated that MutY can form a refolded protein structure in the absence of the $[4Fe\text{-}4S]^{2+}$ cluster. This suggests that the Fe-S cluster does not participate appreciably in MutY folding. The CD signatures of MutY refolded in the presence or absence of ferrous and sulfide ions are nearly identical; this indicates that formation of the $[4Fe\text{-}4S]^{2+}$ cluster does not cause a major structural change in the enzyme. Since MutY is likely highly α-helical like Endo III, the CD signature at 220 nm is a reliable indicator of its refolding. Additionally, CD experiments as a function of temperature were used to measure the stability (T_m) of native MutY and its refolded forms. These results indicate

that there is not a significant difference in the stability of MutY in the presence or absence of the Fe-S cluster. Such results are in striking contrast to *C. vinosum* HiPIP in which the [4Fe-4S] cluster appears to be playing a major role in the enzyme's folding process. The results with MutY are also significantly different from those obtained with the [2Fe-2S] ferredoxin, adrenodoxin-A (Adx) (*40*). Thermodynamic measurements established that apo-Adx is considerably less stable than holo-Adx with a $\Delta\Delta H$(denaturation) = 250 kJ/mol and $\Delta T_m = \sim 15$ °C between the two forms. The CD signatures between holo- and apo-Adx were also significantly different, representative of a change in the structure of the protein upon assembly of the [2Fe-2S] cluster. Thus, it appears that MutY is an example of an Fe-S protein in which the metal cluster is not involved in initiating folding of the enzyme or in stabilizing its overall structure.

The Fe-S cluster does play a major role in the adenine glycosylase activity since only the refolded MutY samples in which both ferrous and sulfide ions have been provided are active adenine glycosylases and are able to bind to a substrate analogue DNA duplex. Previous work with Endo III had implicated the region around the $[4Fe\text{-}4S]^{2+}$ cluster (FCL domain) as important for DNA binding by removal of a critical lysine residue. However, though the lysine mutant enzyme had impaired DNA binding affinity, it retained enzyme activity with substrate DNA. The present work illustrates that in the case of MutY, the presence of the $[4Fe\text{-}4S]^{2+}$ cluster is critical for efficient recognition of specific DNA since no specific DNA binding is observed in its absence. The rest of the enzyme appears to be folded, and therefore the $[4Fe\text{-}4S]^{2+}$ cluster may provide the scaffolding for proper positioning of the amino acids in the FCL domain for efficient substrate DNA recognition. If this is the case, the $[4Fe\text{-}4S]^{2+}$ cluster may represent a semimodular DNA binding motif similar to the well-known zinc-finger motif in DNA binding transcription factors and proteins (*41*−*43*). It is also intriguing to consider other properties of the $[4Fe\text{-}4S]^{2+}$ cluster, such as electrostatics and the ability to hydrogen-bond, which may be exploited to enhance specific DNA recognition.

Endo III and MutY belong to a superfamily of base-excision repair enzymes that have a similar three-dimensional fold (*16*, *44*). The hallmark of this class of BER enzymes is the presence of helix-hairpin-helix (HhH) and Gly/Pro-rich-Asp (G/PD) motifs. The HhH motif is found in a variety of DNA binding proteins and likely represents a nonsequence-specific DNA recognition motif (*46*). In Endo III and other members of the superfamily, the HhH-G/PD motif is part of the interdomain cleft where many residues have been found that are essential for catalytic activity. Interestingly, the three-dimensional structure of AlkA, a member of the superfamily, exhibits a similar overall fold as Endo III (*47*). Surprisingly, AlkA lacks the [4Fe-4S] cluster binding domain. The results reported herein show a *critical* role for the [4Fe-4S] in MutY in DNA recognition. The presence or absence of the FCL domain may be related to the substrate specificity of members of this BER superfamily. There may be an important relationship between the HhH-G/PD motif and other DNA binding motifs (like the FCL domain) to fine-tune the active site and DNA binding surface for recognition of a specific type of damaged base or base-pair. Within this superfamily, MutY is specific for primarily one substrate, OG:A base-pairs, while AlkA has broad substrate specificity for a variety of alkylated bases. Accordingly, the basic framework of this superfamily may be modulated in a variety of ways, depending on the specific task of the BER enzyme. Thus, the presence of the [4Fe-4S] may be used to achieve specific substrate recognition by MutY. Further elucidation of the relationship between the various structural motifs in MutY in specific DNA recognition will be aided by X-ray analysis of MutY and a MutY−DNA complex.

In summary, we have shown that the presence of the $[4Fe\text{-}4S]^{2+}$ cluster in MutY is critical for the adenine glycosylase activity of MutY and for specific binding to a substrate analogue duplex. Furthermore, these results illustrate that initiation of folding of MutY is independent of cluster assembly. In addition, the $[4Fe\text{-}4S]^{2+}$ cluster does not appear to play an important role in stabilizing the overall structure of the enzyme. The cluster instead appears to play a more subtle role in providing a critical binding domain for specific recognition of substrate DNA. The ability of the cluster to readily self-assemble even after the protein has folded *may* be a property which is used in vivo to regulate the enzymatic properties of MutY. The possibility that Fe-S proteins participating in oxidatively damaged DNA repair may respond to the oxidizing conditions of the cell by assembly or disassembly of an Fe-S cluster should be considered.

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