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A Zinc Linchpin Motif in the MUTYH Glycosylase Interdomain Connector Is Required for Efficient Repair of DNA Damage

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Supporting Information

ABSTRACT: Mammalian MutY glycosylases have a unique architecture that features an interdomain connector (IDC) that joins the catalytic N-terminal domain and 8-oxoguanine (OG) recognition C-terminal domain. The IDC has been shown to be a hub for interactions with protein partners involved in coordinating downstream repair events and signaling apoptosis. Herein, a previously unidentified zinc ion and its coordination by three Cys residues of the IDC region of eukaryotic MutY organisms were characterized by mutagenesis, ICP-MS, and EXAFS. *In vitro* kinetics and cellular assays on WT and Cys to Ser mutants have revealed an important function for zinc coordination on overall protein stability, iron−sulfur cluster insertion, and ability to mediate DNA damage repair. We propose that this "zinc linchpin" motif serves to structurally organize the IDC and coordinate the damage recognition and base excision functions of the C- and N-terminal domains.

The bacterial Base Excision Repair (BER) glycosylase MutY and its human homologue MUTYH prevent DNA mutations by removing adenine from 8-oxo-7,8-dihydroguanine (OG):A mismatches.[1] OG forms in DNA as a result of oxidative stress and directs polymerases to misincorporate A; failure to remove inappropriately inserted As by MUTYH leads to G:C to T:A transversion mutations.[1] In human cells, the mutagenic consequences of OG are also stalled by the OG glycosylase (hOGG1) that removes OG in base pairs with C and the MTH1 hydrolase that hydrolyzes dOGTP in the nucleotide pool. After damaged base removal, other BER enzymes cleave and resect the abasic site, insert an undamaged nucleotide, and ligate the backbone.[1−3] Defective repair of OG:A mispairs by inherited MUTYH variants has been linked to colorectal cancer, a syndrome termed MUTYH-associated polyposis (MAP).[1,4,5]

The bacterial and mammalian enzymes share significant homology in catalytic and DNA binding domains;[6−8] however, MUTYH contains an additional sequence not present in bacterial MutYs (Figure 1). Sequence alignment of over 50 known and predicted MutY homologues from eukaryotic and prokaryotic organisms revealed the presence of seven well-conserved Cys residues (Figure 1C). Four highly conserved Cys residues coordinate the $[4Fe−4S]^{2+}$ cluster located in the N-terminal domain of MutY enzymes.[2,6] Unexpectedly, three additional conserved Cys residues are present in mammalian MutYs within the interdomain connector (IDC) that links the catalytic N-terminal and the OG-recognition C-terminal domains. Notably, several proteins have been shown to interact with the IDC, including proteins involved in BER (AP endonuclease, APE1)[9,10] and the DNA damage response (Rad9-Hus1-Rad1 (9-1-1)).[11] The Cys residues are contained within a short conserved sequence common for metal binding motifs (Cys-X_6-Cys-X_2-Cys). However, no metal ion was observed coordinated to the Cys residues in the crystal structure of a truncated form of MUTYH (Figure 1).[10] The absence of a metal ion in the MUTYH structure may be due to loss during purification and may explain the absence of electron density near the Cys residues.

Samples of purified murine MutY (Mutyh)[12] were analyzed for trace metals using inductively coupled plasma-mass spectrometry (ICP-MS). Data from several Mutyh preparations contained an average of 2.5 mol of Fe and 0.6 mol of Zn per mol of protein, consistent with coordination of a $[4Fe−4S]^{2+}$ cluster and the presence of a zinc ion (Table 1). A truncated form of Mutyh lacking the C-terminal domain (MutyhΔ333−515) contained an average of 2.8 mol of Fe and 0.4 mol of Zn per mol of protein (Table S2), indicating that the residues coordinating zinc are located within the first 332 residues of Mutyh. Notably, the Zn^{2+} ion in Mutyh was amenable to removal by chelation with 1,10-phenanthroline (Table 1).

X-ray absorption spectroscopy (XAS) was utilized to probe the zinc coordination sphere within Mutyh. The Zn K-edge EXAFS (extended X-ray absorption fine structure) region of the spectrum of MutyhΔ333−515 was best fit with a model having a mononuclear zinc site coordinated by either three sulfur ligands and two oxygen/nitrogen ligands (bond distances ∼2.32 and 2.08 Å, respectively) or by four sulfurs (bond distance ∼2.31 Å) (Figures S2 and Table S1). The presence of sulfur ligands demonstrated by the EXAFS data is consistent with the three conserved Cys ligands in the IDC. Trace metal analysis showed that C300S Mutyh contained zinc levels similar to WT Mutyh, whereas C307S and C310S had significant reductions in zinc content, demonstrating that both Cys307

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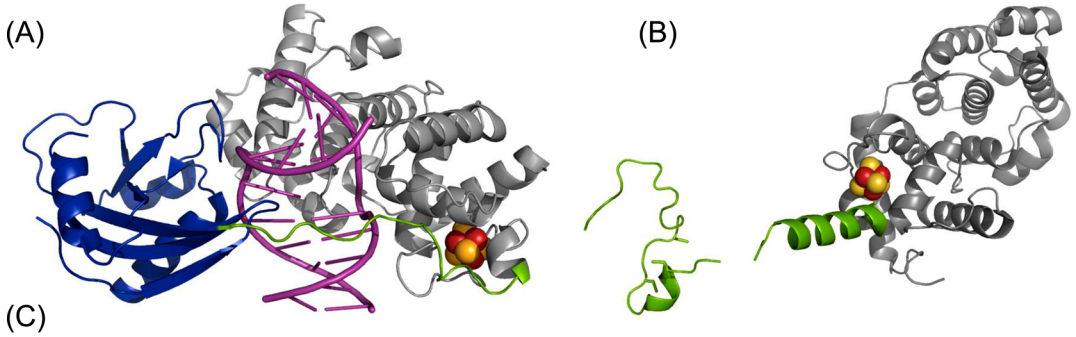

Figure 1. Crystal structures of MutY from *B. stearothermophilus* MutY (PDB: 3FSQ) (A) and the N-terminal fragment of MUTYH (PDB: 3N5N) (B). Color coding is as follows: N-terminal domain, gray; C-terminal domain, blue; IDC, green; DNA, purple. (C) Sequence alignment of the interdomain connector (IDC) region of MutY homologues from eukaryotic and prokaryotic organisms. Conserved Cys ligands coordinating [4Fe–4S]$^{2+}$ and Zn^{2+} are highlighted in yellow and turquoise, respectively.

Table 1. Metal Ion Content of WT and Mutant Mutyh Enzymes by ICP-MS[a]

metal	Mutyh	C300S	C307S	C310S	Zn(−)
Fe	2.5 ± 0.3	2.6 ± 0.2	0.29 ± 0.01	0.84 ± 0.19	3.7 ± 1
Zn	0.6 ± 0.1	0.6 ± 0.02	0.08 ± 0.002	0.09 ± 0.02	0.09 ± 0.02

[a]Values are expressed as molar ratio of metal:protein.

and Cys310 are involved in coordinating the zinc ion (Table 1). Although the EXAFS could be fit with either 4 Cys or 3Cys + 2N/O ligands, structural and sequence analysis does not obviously identify a fourth conserved Cys or His within a reasonable distance from the three Cys residues. Three potential oxygen ligands from conserved residues Asp296, Glu298, and Glu299 are within coordination distance, yet mutagenesis to Ala in Mutyh did not alter zinc binding (Table S2). Consequently, we are unable to definitively assign one of these residues or Cys300 as ligands. Based on the strong evidence for at least three S ligands by EXAFS, the most likely Zn^{2+} ligands are the three Cys residues and two N/O from either the amide backbone or solvent interactions.

Notably, both C307S and C310S exhibited low levels of Fe. The crystal structure of the MUTYH N-terminal fragment (Figure 1B) reveals that the α-helix at the beginning of the IDC extension is stabilized and oriented by coordination of Cys292 to the [4Fe–4S]$^{2+}$ cluster. In addition, Cys292 and other residues surrounding the iron–sulfur cluster are involved in an extensive hydrogen-bond network with the first five residues of the IDC α-helix. The absence of a coordinated Zn^{2+} within this region may influence insertion of the [4Fe–4S]$^{2+}$ cluster cofactor into Mutyh. In addition, the absence of Zn^{2+} may destabilize interactions between the IDC and the cluster coordination domain resulting in lability of the iron–sulfur cluster. Interestingly, mutations of several residues involved in the hydrogen-bond network between the IDC and the [4Fe–4S]$^{2+}$ cluster have been associated with MAP.

The adenine glycosylase activity of C300S, C307S, C310S, and WT Mutyh was monitored using a 5′-[^{32}P]-endlabeled OG:A-containing duplex. Under multiple-turnover conditions ([E] < [S]), Mutyh exhibits burst kinetics due to rate-limiting product release.[12,13] The amplitude of the burst phase corresponds directly to the concentration of active enzyme.[12] Active site titrations revealed significantly reduced active fractions for C307S and C310S Mutyh compared to WT and C300S Mutyh (Table 2). The reduced active fraction of C307S and C310S Mutyh correlates with the reduced levels of zinc and iron associated with these proteins (Table 1). Similarly, samples of Mutyh where the Zn^{2+} had been removed but retained the [4Fe–4S]$^{2+}$ cluster exhibited low levels of active enzyme indicating that loss of Zn^{2+} is sufficient to inactivate the enzyme.

Table 2. Effects on Ability to Prevent Mutations and Adenine Glycosylase Activity by Ser Replacements of the Zn(II) Cys Ligands

enzyme[a]	mutation frequency (f, × 10^{-8})[b]	active fraction (%)[c]	k_2 (min^{-1})[c]
WT	1.9 (1.3–2.1)	28 ± 2	1.5 ± 0.2
C300S/C318S	2.6 (1.3–3.3)	16 ± 4	1.4 ± 0.1
C307S/C325S	22 (15–36)	1.2 ± 0.1	1.3 ± 0.1
C310S/C328S	23 (15–29)	2.1 ± 0.3	1.3 ± 0.1

[a]C300, C307 and C310 in murine Mutyh correspond to C318, C325, and C328 in human MUTYH. [b]Mutation frequencies (95% confidence levels) are measured with the human protein. [c]Active fraction and k_2 were determined with the murine enzyme.

Single-turnover ([E] > [S]) experiments were performed on the Cys-to-Ser mutated enzymes to determine the intrinsic rate of glycosidic bond cleavage (k_2);[12] the measured rate constants were similar to that for WT Mutyh. Taken together, the relative activities of the various Mutyh enzyme forms indicate that the absence of Zn^{2+} alters the fraction of the enzyme capable of properly engaging the damaged substrate and mediating base excision. Of note, many laboratories (including ours) have reported difficulty in overexpressing high concentrations of active MUTYH,[14−16] perhaps due to loss of the Zn^{2+} ion during purification.

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The effect of Zn^{2+} coordination on the ability of MUTYH to prevent DNA mutations was evaluated using a rifampicin resistance assay.[14] Low MUTYH-mediated repair allows for accumulation of mutations in an RNA polymerase, making rifampicin a less effective block to transcription. The mutation frequency (f) is related to the number of rifampicin resistant colonies. Cells expressing WT or C318S MUTYH exhibited extremely low mutation frequencies while those expressing C325S and C328S MUTYH exhibited dramatically increased mutation frequencies (12-fold) (Tables 2 and S3). These results show that C325S and C328S MUTYH lack the ability to prevent spontaneous mutations in vivo. The inability to prevent mutations correlates with reduced Zn^{2+} levels in the corresponding mutated Mutyh enzymes. Indeed, an even higher increase (19-fold) in mutation frequency is observed in cells expressing the triple mutant C318S/C325S/C328S MUTYH which is unlikely to retain any Zn^{2+} (Table S3). The rifampicin resistance assays, coupled with the metal analysis and adenine glycosylase assays, show that coordination of zinc within the IDC is required for MUTYH-dependent OG:A repair and prevention of DNA mutations.

Zinc sites in proteins are generally classified as structural or catalytic sites, largely based on the Zn^{2+} coordination number and ligand type.[17] Structural Zn^{2+} sites are typically coordinated by two or more Cys ligands, as we have identified in Mutyh. The mechanism for catalysis of glycosidic bond hydrolysis by MutY enzymes does not require Zn^{2+};[18,19] moreover, the Zn^{2+}-coordinating Cys residues in MUTYH are relatively far from the adenine excision pocket. These features suggest that Zn^{2+} coordination provides stability and a defined structure of the IDC region of mammalian MutY enzymes. Structural Zn^{2+} sites often impact catalysis by positioning key residues or protein regions for catalysis and/or substrate binding.[17] Coupling of OG recognition with adenine excision is of particular importance with MUTYH due its charge of finding and removing adenine from within rare OG:A mismatches while ignoring the large excess of T:A base pairs.[1,20] The reduced fraction of catalytically competent Mutyh in the absence of Zn^{2+} suggests that improper folding of the IDC hampers effective OG:A mismatch engagement by the N- and C-terminal domains. Notably, a chimeric protein containing the N- and C-terminal domains of *S. pombe* MutY and the IDC linker of Ec MutY exhibited reduced glycosylase activity and OG:A mismatch affinity; however, affinity for undamaged DNA *increased*.[10] These results suggest an active role of the IDC in mammalian MutY in promoting catalysis by controlling the orientation of the two domains to optimize repair.

Zinc binding sites have also been identified at protein−protein interfaces[17,21] and as structural scaffolds for mediating protein−protein interactions.[17,22] Two residues positioned near the conserved Cys residues in the IDC (V315 and E316 in MUTYH) have been shown to be critical for proper interactions with the 9-1-1 complex.[10] In addition, mutations in the IDC region have deleterious effects on cellular OG:A repair.[23] Of note, many MAP variants are localized to the IDC region[1] and these variations may impact Zn^{2+} ion coordination. The proximity of the Zn-containing IDC and the $[4Fe{-}4S]^{2+}$ cluster suggest that the presence of Zn may alter the stability or redox properties of the $[4Fe{-}4S]^{2+}$cluster.[24] It is also intriguing to consider that the Zn^{2+} site may be altered under conditions of oxidative stress and used in signaling processes.[22,25] Indeed, a reactive cysteine coordinated to Zn^{2+} in PKCα provides for a proposed mechanism for activation of the kinase by reactive oxygen species (ROS) in a process involving release of the Zn^{2+} ion.[26] Given the role of MUTYH in the oxidative stress response, the effect of ROS on the zinc site may reveal a new mechanism for regulating DNA repair.

Herein, we establish the importance of a previously unrecognized Zn^{2+} site in MUTYH and, from XAS, establish its first coordination sphere of ligands. We propose that zinc coordination within the IDC serves a critical function in organizing the N- and C-terminal domains to coordinate OG:A mismatch recognition, adenine extrusion, and excision. Additionally, we suggest that the presence of Zn^{2+} in MUTYH may provide a mechanism to regulate its repair activity and coordination with other cellular processes.

■ ASSOCIATED CONTENT

Supporting Information

Detailed materials and methods, EXAFS data and relevant fits, full ICP metal analysis data, and rifampicin resistance assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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