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Substrate specificity and sequence dependent activity of the *Saccharomyces cerevisiae* 3-methyladenine DNA glycosylase (Mag)

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Abstract

DNA glycosylases initiate base excision repair by first binding, then excising aberrant DNA bases. *S. cerevisiae* encodes a 3-methyladenine (3MeA) DNA glycosylase, Mag, that recognizes 3MeA and various other DNA lesions including 1,*N*⁶-ethenoadenine (εA), hypoxanthine (Hx) and abasic (AP) sites. In the present study, we explore the relative substrate specificity of Mag for these lesions and in addition, show that Mag also recognizes cisplatin cross-linked adducts, but does not catalyze their excision. Through competition binding and activity studies, we show that in the context of a random DNA sequence Mag binds εA and AP-sites the most tightly, followed by the cross-linked 1,2-d(ApG) cisplatin adduct. While εA binding and excision by Mag was robust in this sequence context, binding and excision of Hx was extremely poor. We further studied the recognition of εA and Hx by Mag, when these lesions are present at different positions within A:T and G:C tracts. Overall, εA was slightly less well excised from each position within the A:T and G:C tracts compared to excision from the random sequence, whereas Hx excision was greatly increased in these sequence contexts (by up to 7-fold) compared to the random sequence. However, given most sequence contexts, Mag had a clear preference for εA relative to Hx, except in the TTXTT (X= εA or Hx) sequence context from which Mag removed both lesions with almost equal efficiency. We discuss how DNA sequence context affects base excision by various 3MeA DNA glycosylases.

Keywords

Base excision repair; 3-methyladenine DNA glycosylase; Substrate specificity; Sequence dependent activity

1. Introduction

DNA is constantly assaulted by various exogenous and endogenous agents resulting in damage, which if left unrepaired, can lead to mutation and cell death [1]. Inappropriate DNA structures can result from replication errors, from reaction with chemicals, reactive oxygen species and radiation, and also from spontaneous hydrolysis resulting in deamination and depurination. Alkylating agents readily induce cell death and mutation [2–4], and a variety of DNA repair

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systems have evolved to repair alkylated DNA bases, among which, Base Excision Repair (BER) is one of the best studied [5–7]. BER is initiated by DNA glycosylases that specifically recognize abnormal DNA bases in a vast excess of normal bases and catalyze their removal via hydrolysis of the *N*-glycosyl bond [6,8]. The resulting abasic site (AP-site) can be cleaved by an AP-endonuclease followed by insertion of the correct nucleotide by DNA polymerase, trimming of the 5' terminus, and sealing of the nick by DNA ligase.

Mechanistically, DNA glycosylases can be divided into monofunctional and bifunctional DNA glycosylases [6]. Monofunctional enzymes can only excise the target base resulting in an AP-site, as described above. In contrast, bifunctional enzymes catalyze both base excision and AP-site cleavage reactions. 3-methyladenine (3MeA) DNA glycosylases exist in both prokaryotes and eukaryotes; they are all monofunctional and can remove various types of alkylated DNA bases. With the exception of Tag from *E. coli* and Mag1 from *Schizosaccharomyces pombe*, most of the 3MeA DNA glycosylases excise an exceptionally wide range of structurally diverse damaged bases that result from alkylation, deamination and in few cases even oxidation [9]. These lesions include 3MeA, 3-methylguanine (3MeG), 7-methylguanine (7MeG), ϵ A, Hx, 3,*N*²-ethenoguanine (ϵ G), and 7,8-dihydro-8-oxoguanine (8-oxoG). Owing to such broad substrate specificity, 3-methyladenine DNA glycosylases help to protect against a wide range of toxic and mutagenic DNA damaging agents [9].

The budding yeast *Saccharomyces cerevisiae*, upon exposure to non-lethal levels of alkylating agents, induces the expression of Mag, the 3MeA DNA glycosylase encoded by the *MAG* gene [10]. Mag shares significant structural and functional homology with the similarly inducible *E. coli* 3MeA DNA glycosylase, namely AlkA (Figure 1A). The *S. pombe* Mag1 protein also shares significant sequence similarity with the *E. coli* AlkA and *S. cerevisiae* Mag DNA glycosylases [11]. Comparisons of Mag and AlkA showed that Mag is more efficient than AlkA in excising ϵ A from duplex DNA [12] and that AlkA is more efficient than Mag in Hx excision [13]. In further comparison, the mammalian counterparts of AlkA and Mag, namely the human AAG and mouse Aag enzymes (also known as MPG and Mpg respectively), are relatively much more efficient at excising both ϵ A and Hx DNA lesions. Here we further characterize the activity of the *S. cerevisiae* Mag enzyme on ϵ A and Hx substrates, and compare this to its ability to act upon a number of other DNA substrates.

The crystal structure of AlkA in complex with a double stranded DNA containing a 1-aza-deoxyribose abasic nucleotide indicated that AlkA is a member of the Helix-hairpin-Helix (HhH) superfamily of DNA glycosylases (Figure 1B) [14]. In order to flip the target nucleotide out of the DNA stack so that the base is inserted into its active site, AlkA induces a 66° bend of the DNA backbone at the site of damage. The AlkA-DNA complex structure also suggests an *S_N1*-type reaction mechanism catalyzed by the essential Asp238 residue to cleave the glycosyl bond (Figure 1B). While the crystal structure lacked the damaged base in its active site pocket, modeling of 3MeA in the active site indicated that the alkylated base would stack against Trp272 through cation- π interaction, and that this probably stabilizes the extrahelical conformation of the 3MeA base. Given the sequence similarity of Mag with AlkA, one can predict that Mag may also apply DNA bending and nucleotide flipping for the recognition and catalysis of the damaged base. Interestingly, another crystal structure of AlkA in complex with the Hx free-base showed that the damaged base, probably representing a reaction product, is not stacked against Trp272, but rather stacked between Trp218 and Tyr239 [15] (Figure 1B); whether this stacking interaction is reserved only for the free base reaction product or exists before cleavage of the glycosyl bond remains to be determined.

Several studies on the DNA sequence dependent catalytic activity of 3MeA DNA glycosylases [13,16,17] have shown that these enzymes exhibit remarkable differences in their catalytic activity, depending upon the DNA sequence surrounding the lesion. The mouse Aag 3MeA

DNA glycosylase exhibits differences in Hx removal rates (but not ϵ A removal rates), when the lesion is present at different positions within an A:T tract [17]. Here we set out to understand the influence of local DNA conformation on the ability of the *S. cerevisiae* Mag enzyme to bind and excise ϵ A and Hx lesions, especially when present in tracks of A:T or G:C repeats. We show that DNA sequence context greatly influences Mag's ability to excise Hx, but only modestly affects ϵ A excision. We also show that Mag specifically binds cross-linked 1,2-d (ApG) cisplatin adducts in duplex DNA, but does not catalyze glycosyl bond cleavage at either of the cross-linked bases.

2. Materials and methods

2.1. Chemicals and Enzymes

Polyacrylamide gel electrophoresis (PAGE) purified oligonucleotide substrates were from Integrated DNA technologies. Polynucleotide kinase (PNK) was from New England Biolabs, 32 P- γ ATP was from PerkinElmer and Cisplatin from Sigma. Sephadex G-25 quick spin columns were from Amersham-Pharmacia.

2.2. Mag enzyme preparation

The homogenous preparation of purified recombinant *S. cerevisiae* Mag enzyme was a gift from Dr. Tom Ellenberger (Washington University School of Medicine, MO). As evidenced by SDS-PAGE analysis, the final purified Mag was ~99% pure and was stored in the buffer containing 20 mM Tris-HCl pH 7.8, 100 mM KCl, 10% Glycerol and 5 mM DTT (DL-Dithiothreitol).

2.3. Oligonucleotide substrates and 32 P-labeling

Oligonucleotide substrates (Table 1) were quantified by extinction co-efficient method using UV absorption at 260 nm. The lesion containing strand (G in case of G:T mismatch) was labeled on its 5' end with 32 P- γ ATP using PNK and the labeled strand was annealed with the complementary strand. The unincorporated 32 P- γ ATP was removed using Sephadex G-25 quick spin columns. Platination reaction for 1,2-d(ApG)Pt oligonucleotide (Table 1) was carried out in 5mM Na₃PO₄ pH 7.4 at 37°C for about 20 hours followed by purification on denaturing PAGE, as described before [18]. The platination sites were confirmed by Maxam-Gilbert sequencing [19,20]. The 1,2-d(ApG) cisplatin adduct containing strand was labeled on its 5' end with 32 P- γ ATP as described above and annealed with the complementary strand.

2.4. Gel mobility shift assays

Gel mobility shift assays were performed in 10 μ l reaction mixture containing 1 $\times$ binding buffer (4 mM Tris pH 7.8, 20 mM KCl, 30 mM NaCl, 0.5 mM EDTA, 1 mM β -mercaptoethanol, 10% Glycerol and 10 ng of salmon sperm DNA), Mag at the indicated concentration (100 or 200 nM) and 1 nM 32 P-labeled oligonucleotide. Incubation of the reaction mixture was at 16°C for 15 minutes followed by electrophoresis in 6% polyacrylamide gel using 1 $\times$ TBE buffer at 150 V for 120 min at 4°C. The dried gel was exposed by Molecular Dynamics Phosphorimaging.

2.5. Competition binding experiments

Competition binding assays were performed by titrating increasing amounts of unlabeled competitor DNA into the binding reaction mixture. Reactions were set up in 10 μ l volumes that contained 1 $\times$ binding buffer, 20 nM of 32 P-labeled CA ϵ AGT oligonucleotide, 1 μ M purified Mag and the competitor DNA. Samples were incubated at 16°C for 15 minutes followed by electrophoresis on 6% polyacrylamide gel using 1 $\times$ TBE buffer at 150 V for 120 min at 4°C. The gel was dried and subject to phosphorimaging. The bands corresponding to

bound and free ^{32}P -labeled CA ϵ AGT were quantified using Molecular Dynamics PhosphorImager. The experiment with each competitor was repeated at least three times. In order to determine the IC_{50} (50% inhibitory concentration), competition data was fitted to the sigmoidal dose response curve (equation-1) by non-linear least square analysis method using GraphPad Prism (GraphPad software, Inc.).

$$Y = Y_{\min} + (Y_{\max} - Y_{\min}) / (1 + 10^{\text{LogIC}_{50} - X}) \quad (1)$$

Where X is the logarithm of competitor concentration, $Y_{\max}$ and $Y_{\min}$ are the maximum and minimum values of % bound (Y) and LogIC_{50} is the logarithm of IC_{50} . The binding affinity (K_d) for ϵA competitor was calculated by fitting the competition binding data to the equation-2, using the GraphPad Prism.

$$Y = B_{\max} * [\text{labelled DNA}] / ([\text{labelled DNA}] + X + K_d) + \text{NS} \quad (2)$$

Where Y is the total binding, $B_{\max}$ is the maximum specific binding response, X is the logarithm of competitor concentration, K_d is the binding affinity and NS is a non-specific binding term. The K_d obtained for ϵA ($K_{d-\epsilon\text{A}}$) was used to calculate the K_d for AP-site and 1,2-d(ApG) competitors using the equation-3.

$$K_d = \text{IC}_{50} / 1 + ([\text{labelled DNA}] / K_{d-\epsilon\text{A}}) \quad (3)$$

Where K_d is the binding affinity of AP-site or 1,2-d(ApG) competitors to Mag, IC_{50} is the 50% inhibitory concentration for AP-site or 1,2-d(ApG) competitors obtained using equation-1 and $K_{d-\epsilon\text{A}}$ is the K_d value obtained for ϵA using equation-2.

2.6. DNA glycosylase assays

DNA glycosylase assays were set up in 10 μl reaction samples containing 1 $\times$ glycosylase assay buffer (20 mM Tris-HCl pH 7.8, 100 mM KCl, 2 mM EDTA, 1 mM EGTA and 5 mM β -mercaptoethanol), 2 nM ^{32}P -labeled oligonucleotide and 580 nM of Mag. Samples were incubated at 37°C for 60 minutes. Reaction was stopped by the addition of 1.2 μl of 1M NaOH and heated at 70°C for 30 minutes. This treatment cleaved the AP-sites created due to removal of the damaged base. 11.2 μl of Formamide dye was added into this mixture and the products were resolved on 20% denaturing Urea-PAGE, using 1 $\times$ TBE buffer at 400 V for 90 minutes at room temperature. The extent of substrate cleavage was quantified and analyzed by phosphorimaging. DNA glycosylase assays in the presence of competitor were carried out in 10 μl reaction samples containing 1 $\times$ glycosylase assay buffer, 2 nM ^{32}P -labeled oligonucleotide, 2000 nM cold competitor and 580 nM of Mag. The reaction was followed as a function of time and the sample at each time point was subjected to hot alkali treatment and the products were resolved on 20% denaturing Urea-PAGE. The final results obtained represent the average of three independent experiments.

2.7. DNA glycosylase assay under single turnover (STO) conditions

To ensure STO conditions, Mag concentration was kept in large excess of substrate concentration. Reactions were set up in 10 μl volumes containing 1 $\times$ glycosylase assay buffer, 2 nM ^{32}P -labeled oligonucleotide and 1.47 μM of Mag and the samples were incubated at 37°C. At each time point the reaction was stopped by the addition of 1.2 μl of 1M NaOH and heated at 70°C for 30 minutes. This was followed by the addition of 11.2 μl of Formamide dye and the products were resolved on 20% denaturing Urea-PAGE. The gels were subject to phosphorimaging and the bands were quantified using Kodak-1D scientific imaging software (Kodak scientific imaging systems). k_{obs} values were calculated by fitting the data from three

independent experiments to the exponential function (equation-4) using GraphPad Prism (GraphPad software, Inc.).

$$Y = Y_{\max} * (1 - e^{-k * t}) \quad (4)$$

Where Y represents the percentage cleaved, $Y_{\max}$ is the maximum percentage of product formed at the last time point of incubation, k is the observed rate of cleavage (k_{obs}) and t is the time. It should be noted that Mag glycosylase activity diminishes with time under our assay conditions and therefore the measured k_{obs} value should not be interpreted as an absolute value.

3. Results

3.1. Recognition of different types of DNA lesions by Mag

Initially we tested the binding of Mag to different base lesions present in DNA duplexes with a random base sequence (CAXGT, G:T mismatch and 1,2-d(ApG)Pt, see Table 1). Duplex DNA labeled on the lesion containing strand was incubated with purified Mag and the resulting complex was visualized by gel-shift analysis. Mag was tested for its ability to bind duplexes containing the following: an ϵ A, a 1,2-d(ApG) cisplatin adduct, a Hx, a G:T mismatch or the AP-site analogue tetrahydrofuran (THF) (Table 1 and Figure 1C); for simplicity we refer to the THF as an AP-site. As evidenced by the shifted bands, Mag showed strong binding to the oligonucleotide duplexes containing an AP-site and significant binding to the duplexes containing an ϵ A lesion (Figure 2A). We had previously shown that the human AAG enzyme binds DNA oligonucleotides containing cisplatin cross-linked DNA base adducts [18], although not as strongly as it binds ϵ A containing DNA. Here we show that Mag also binds duplex DNA containing the 1,2-d(ApG) cisplatin adduct, but, as for the AAG enzyme, Mag's binding to this lesion was weaker than that for ϵ A and AP-site containing DNA (Figure 2A). Surprisingly, Mag exhibited no apparent binding to the duplex containing Hx in the random sequence context, and as expected Mag showed no binding to the undamaged duplex or that containing a G:T mismatch (Figure 2A). In summary, Mag exhibited strong binding to the duplexes containing ϵ A or an AP-site, weak binding to the duplex with 1,2-d(ApG) cisplatin adduct and no apparent binding to the duplexes with Hx, with a G:T mismatch or with no damage. We went on to test whether and how well Mag excises these base lesions in this sequence context, excluding the AP-site (Figure 2B). Mag displayed robust activity for ϵ A excision, and to our surprise (given that Mag did not appear to bind the Hx-containing DNA, Figure 2A), Mag also displayed significant Hx excision, albeit not as robust as that for ϵ A. Interestingly, although Mag could bind 1,2-d(ApG) cisplatin adducts, it did not cleave either of the glycosyl bonds associated with this intrastrand DNA cross-link. Finally, Mag activity on the undamaged duplex and on the duplex containing a G:T mismatch was undetectable (Figure 2B).

3.2. Mag glycosylase activity in the presence of competitors

In order to further explore Mag's ability to recognize different substrates, we monitored Mag activity in the presence of various competitors. Mag-mediated ϵ A excision activity was followed as a function of time, in the presence of various competitors. Duplex DNA substrate with ϵ A in the random sequence context was incubated with Mag in the presence of 2000 nM cold competitor DNA containing either an ϵ A, an AP-site, a Hx, a 1,2-d(ApG) cisplatin adduct, a G:T mismatch or no damage at all and Mag activity on the ϵ A containing substrate was followed as a function of time (Figure 3). As expected, Mag exhibits maximal activity in the absence of any competitor (Figure 3A and 3D) and the addition of undamaged duplex DNA only slightly inhibited Mag's activity on the ϵ A substrate (Figure 3B and 3D). However, unlabeled competitor duplexes containing ϵ A or an AP-site strongly inhibited Mag activity (Figure 3A and 3D), and that containing the 1,2-d(ApG) cisplatin adduct showed moderate

inhibition of activity (Figure 3B and 3D). Competition by Hx and G:T mismatch containing duplexes were similar to that by undamaged DNA (Figure 3B–3D). Taken together, the relative ability of each lesion to inhibit Mag's base excision activity on an ϵ A substrate, paralleled their ability to bind the lesion containing duplexes in our initial binding experiments. However, it should be noted that the glycosylase activity as measured here, reflects a combination of both lesion binding and glycosyl bond cleavage. In order to specifically address relative lesion binding, which is the affinity of Mag for binding different base lesions, we also performed competition binding studies.

3.3. Competition binding studies

Competition binding studies were performed using gel mobility shift assays. Mag was monitored for its ability to bind 32 P-labeled ϵ A containing duplex DNA (in the context of the random sequence), in the presence of increasing concentrations of cold competitor DNA that was either undamaged, or contained one of the other four base lesions, or contained a G:T mismatch (also in the context of the random sequence). DNA competitor concentration was varied from 12.5 nM to 2000 nM and the 50% inhibitory concentration (IC_{50}) for each competitor was calculated by fitting the competition binding data to equation-1 (Materials and Methods). The K_d value for ϵ A competitor was calculated using equation-2, and those for AP-site and 1,2-d(ApG) competitors calculated using equation-3 (Materials and Methods). The results are summarized in Figure 4. The ϵ A (Figure 4A) and AP-site (Figure 4B) containing DNA duplexes were the best competitors with IC_{50} (and K_d) values of 195 ± 1.4 nM ($K_d = 181.1 \pm 1.7$ nM) and 195.1 ± 1.1 nM ($K_d = 175.8$ nM), respectively (Figure 4C), indicating that Mag actually binds the ϵ A and AP-site containing DNA with roughly equal affinity. This was surprising, given the results from initial binding experiments (Figure 2A). However, the apparent results may be explained by the probable removal of ϵ A by Mag during the gel mobility shift assays. In agreement with the initial binding experiments (Figure 2A) and competition activity studies (Figure 3), the 1,2-d(ApG) cisplatin adduct was a poor competitor (relative to ϵ A and AP-site), but was nevertheless a significant competitor with an IC_{50} of 390 ± 1.1 nM ($K_d = 351.4$ nM) (Figure 4B and 4C). Similarly, the undamaged DNA duplex (Figure 4A), and the duplexes containing Hx (Figure 4A) and G:T mismatch (Figure 4B) were very poor competitors (with IC_{50} 's >2000 nM), and significantly poorer than the 1,2-d(ApG) cisplatin adduct. These results conclusively showed that among the different DNA lesions used in this study, Mag recognizes ϵ A and AP-site containing DNA duplexes with relatively higher affinity, compared to the duplex containing 1,2-d(ApG) cisplatin adduct that is recognized with moderate affinity. In addition, we confirmed again that Mag can recognize cisplatin cross-linked adducts in the duplex DNA.

3.4. Sequence dependent recognition of ϵ A and Hx lesions by Mag

Having shown that among the various substrates tested in this study, Mag is only catalytically active on the duplexes containing ϵ A or Hx, we set out to understand the sequence dependent recognition of these lesions by Mag. Although it has not been demonstrated directly, it seems highly likely that Mag recognizes and cleaves its substrate bases by a nucleotide flipping mechanism. As has been shown for other 3MeA DNA glycosylases, the feasibility of nucleotide flipping can differ according to the architecture and stability of the target base within its base pair and within in its local neighborhood DNA sequence context [16,17,21]. We predicted that the catalytic efficiency of Mag for ϵ A and Hx base lesions may be significantly affected by DNA sequence in the neighborhood of the base lesion. The data already presented shows that Mag binds and removes ϵ A lesions more efficiently than Hx when these lesions are embedded in a random sequence context. Here we assess the ability of Mag to recognize ϵ A and Hx situated at different positions in polynucleotide repeat sequences. ϵ A or Hx lesions were located at the X-position of AAXAA, TTXTT, GGXGG, CCXCC, A₅X and T₅X containing oligonucleotides (see Table 1). In a representative gel shown in Figure 5A, Mag bound robustly

to ϵ A in all six sequence contexts, although binding to the GG ϵ AGG duplex was clearly the weakest (Figure 5A). In contrast, Mag showed weak binding to Hx present in any of the sequence contexts (Figure 5B), as evidenced by the absence of distinctly shifted band on the gel. Moreover, we tested the ability of Mag to excise ϵ A and Hx when present in the aforementioned polynucleotide repeats. For any given any sequence context, Mag clearly excised ϵ A more efficiently than Hx (Supplementary Figure 1). However, it was also clear that sequence context did influence excision for each lesion and the Mag activity was therefore assessed in a more quantitative manner for ϵ A or Hx, when present in each sequence context.

3.5. Sequence dependent DNA glycosylase activity under single turnover conditions

In order to measure the differences between the sequence dependent catalytic activities of Mag, we performed DNA glycosylase assays under single turnover (STO) conditions. Mag levels were kept in large molar excess of the substrate concentration to ensure STO conditions. The k_{obs} values were calculated by fitting the data to equation-4 (Materials and Methods). Mag excision rates for ϵ A from each sequence context, including the random sequence context for reference, are shown in Figures 6A–6C, and those for Hx excision are shown in Figures 6D–6F. Table 2 summarizes all of these results and indicates the calculated excision rates relative to that in the random sequence context. Mag exhibited the highest preference to remove ϵ A from the CA ϵ AGT random sequence duplex with the $k_{\text{obs}} = 0.44 \pm 0.03 \text{ min}^{-1}$. The removal of ϵ A present in any other polynucleotide repeat sequence was weaker compared to CA ϵ AGT duplex (Figure 6A–6C, Table 2). The maximum reduction in the rate (~ 3 -fold) was observed for A5 ϵ A and GG ϵ AGG duplexes and for the rest of sequences a smaller decrease in the rate was observed (~ 1 – 1.5 -fold, Table 2). In stark contrast, Mag more efficiently removed Hx from all of the polynucleotide repeats, compared to the CAHxGT random sequence duplex, on which it displayed the weakest activity (Figure 6D–6F, Table 2) with $k_{\text{obs}} = 0.037 \pm 0.003 \text{ min}^{-1}$. The largest rate increases were for the removal of Hx from the AAHxAA sequence context (7-fold) followed by the TTHxTT context (6.5-fold), with k_{obs} values equal to 0.26 ± 0.02 and $0.24 \pm 0.02 \text{ min}^{-1}$, respectively. This was followed by T5Hx (~ 4 -fold) and CCHxCC (~ 3 -fold) with k_{obs} values equal to 0.16 ± 0.01 and $0.116 \pm 0.007 \text{ min}^{-1}$, respectively. The smallest, but still significant increases in the reaction rate were for A5Hx and GGHxGG sequence contexts (Table 2).

Given any type of base lesion (ϵ A or Hx), the activity of Mag consistently varied within the repeats. For example, Mag removal of ϵ A or Hx from AAXAA was greater than from A5X (Figure 6A and 6D) and removal of Hx from TTHxTT was greater than from T5Hx (Figure 6E). However, Mag showed a negligible difference in the rate for ϵ A removal between TT ϵ ATT and T5 ϵ A sequences (Figure 6B). The rate of ϵ A or Hx removal by Mag was always significantly greater from the CCXCC duplex, compared to the GGXGG duplex (Figure 6C and 6F). Interestingly, though Mag preferentially removed ϵ A compared to Hx from the majority of sequence contexts, its activity on these two lesions was very similar in the TTXTT sequence context (Table 2).

4. Discussion

The budding yeast *S. cerevisiae* protects against DNA alkylation damage by inducing Mag upon the exposure to alkylating agents [10,22]. Mag shares significant sequence homology with *E. coli* AlkA (Figure 1A), which is known to remove various damaged and normal DNA bases [23]. Previous studies have shown that similar to AlkA, Mag has a wide substrate specificity and can remove a variety of alkylated bases (3MeA, 3MeG, 7MeG, 7-chloroethylguanine and 7-hydroxyethylguanine) including ϵ A, Hx and normal guanine [9]. Interestingly, the overexpression of Mag in yeast increases spontaneous mutation rates by up to 600-fold [24], perhaps due to the non-specific removal of undamaged purines and the

generation of excess AP-sites [25]. Given the importance of Mag in *S. cerevisiae*, we further probed the substrate specificity of Mag enzyme and demonstrated that Mag's efficiency for removing ϵ A and Hx lesions is affected by the DNA sequence context.

Previously, relative to AlkA the activity of Mag was shown to be ~7-fold higher and ~4-fold lower for the removal of ϵ A and Hx lesions, respectively [12,13]. However both enzymes have higher activity for ϵ A compared to Hx, with the latter being the poorer substrate for both enzymes. Although previous studies have characterized the DNA glycosylase activity of Mag to remove ϵ A and Hx lesions, to date no published studies have shed light on the binding affinity of Mag to these lesions. Our binding and competition studies show that Mag binds the ϵ A lesion containing DNA duplex with high affinity, relative to the Hx lesion containing duplex for which Mag showed extremely poor affinity (Figure 2–Figure 4). The specific recognition of ϵ A and Hx lesions by Mag can be best discussed based on the available crystal structures of AlkA and human AAG. ϵ A has an alkene group attached between the N1 and N⁶ positions of adenine that abolishes its ability to form Watson-crick base pair. In contrast, Hx is a deaminated form of adenine and can still form a base pair with either thymine or cytosine. Therefore the only specificity determinant positions for recognition by DNA glycosylases would be the N⁶ of ϵ A and the O⁶ of Hx (Figure 1C). In the crystal structure of AlkA complexed with Hx free base, the specific recognition of Hx is made through a hydrogen bond donated from main chain amide of Leu125 to O⁶ of Hx [15] (Figure 1B). A similar type of specific interaction is seen in the complex structure of human AAG with ϵ A containing DNA [26], where the specificity for ϵ A recognition comes through a hydrogen bond formed between main chain amide of His136 and N⁶ of ϵ A. Looking at these structures, one can propose that Mag may recognize the N⁶ of ϵ A or the O⁶ of Hx through specific hydrogen bonds, which otherwise would not be accepted by N⁶ of normal adenine.

So far, studies on the interaction of Mag with AP-site containing DNA have not been reported. In this study we explored this interaction, using a DNA substrate containing the AP-site analogue, THF. Binding and competition studies clearly established that Mag recognizes THF containing DNA with extremely high affinity (Figure 2–Figure 4). The crystal structure of AlkA in complex with DNA containing an oxacarbenium ion (reaction intermediate) mimic, namely 1-aza-deoxyribose (1-aza-dR), showed that the catalytic Asp238 is in direct contact with N1' of 1-aza-dR (Figure 1B) [14]. In turn AlkA was shown to bind 1-aza-dR containing DNA with much higher affinity (K_d =100 pM), compared to THF containing DNA (K_d =45 nM) [14,27]. This implies that Asp238 directly participates in the catalytic reaction by aiding in the development and stabilization of an oxacarbenium ion intermediate. Even though it appears that Mag binds THF containing DNA with relatively low affinity compared to AlkA, given the extensive homology between Mag and AlkA across AlkA's active site region (Figure 1A), it seems likely that Mag's Asp209 also interacts with the oxacarbenium ion/AP-site and uses a catalytic mechanism similar to that of AlkA. Indeed, expression of a Mag-D209N mutant protein fails to complement the alkylation sensitive phenotype of a *MAG* deletion yeast strain, indicating that Asp209 is important for the catalytic activity (data not shown). However, detailed structural and functional studies are needed to confirm the proposed role of this residue.

Cisplatin is commonly used for cancer chemotherapy [28–30]. The toxicity of cisplatin is believed to arise from its ability to damage DNA through the formation of intra/inter-strand platinated cross-linked base adducts [31,32] and the consequent recognition of adducts by various cellular proteins [33,34]. The genome-wide transcriptional response and the sensitivity/toxicity profiles of *S. cerevisiae* cells upon exposure to different DNA damaging and anticancer agents, including Cisplatin have been studied [35–37]. The 1,2-d(ApG) cisplatin intrastrand adduct (Figure 1C) comprises approximately 25% of the cisplatin induced DNA cross-links [33,38] and it has been shown to distort the DNA duplex by ~55° bend towards the major groove [39]. We hypothesized that, similar to human AAG [18], Mag may also recognize the

bent DNA structures induced by cisplatin cross-linked adducts. DNA binding and glycosylase assays showed that Mag binds the 1,2-d(ApG) cisplatin intrastrand DNA adduct containing duplex, but fails to exhibit any DNA glycosylase activity at the lesion (Figure 2). Further, competition studies showed that 1,2-d(ApG)Pt competitor DNA significantly competes for both ϵ A excision (Figure 3) and ϵ A binding (Figure 4) by Mag. The role and the consequence of abortive complex formed between Cisplatin adduct and Mag/AAG is not yet clear. However, it is possible that the bound glycosylases stimulate nucleotide excision (NER) repair pathway, which is thought to be involved in the repair of DNA cross-links. It would be interesting to determine whether other DNA glycosylases can also bind cisplatin DNA intrastrand cross-link adducts.

The crystal structure of a G:T mismatch containing DNA showed that the G:T pair adopts a wobble structure, with thymine projecting into the major groove and the guanine into the minor groove [40]. This induces a slight bend of the DNA helix towards the minor groove, even though the global conformation of the helix is largely unchanged. Previously, AlkA was shown to recognize and remove the normal guanines from the G:T mismatches [23]. In another study, both AlkA and Mag were shown to remove undamaged guanines from the DNA [41]. Therefore, in light of Mag's homology to AlkA, we predicted that Mag would also recognize G:T mismatches. Our studies clearly showed that Mag does not bind to duplex DNA with a G:T mismatch and in turn fails to remove guanine from the mismatch (Figure 2–Figure 4). It is surprising that two such close homologs (AlkA and Mag) should behave differently with respect to the removal of normal guanine from G:T mismatch. Previous biochemical studies showed that AlkA possesses an indiscriminate active site in that it exhibits similar rate enhancements (relative to spontaneous base release) for the excision of a structurally diverse set of damaged and undamaged purines bases [14,23]. This was interpreted to indicate that the efficiency of the AlkA catalyzed reaction is not dictated by specific structural recognition of each base lesion, but rather, primarily by the innate stability of *N*-glycosyl bond of each substrate [23]. In contrast, the human AAG enzyme exhibits very different rate enhancements for the excision of structurally diverse base lesions, suggesting that the catalytic reaction of human AAG is not primarily dictated by the stability of the *N*-glycosyl bond [42,43]. Taken together in the context of Mag, one can infer that Mag has an active site that is not as versatile as that of AlkA and speculate that catalysis by Mag is not primarily driven by the stability of *N*-glycosyl bond.

DNA sequence has a significant effect on the efficiency of DNA replication, on the susceptibility of DNA to chemical and physical damage, and on the rate of DNA repair [17, 44–47]. Several studies have shown that the sequence adjacent to the lesion base has significant effects on the thermodynamic stability and global conformation of the duplex, and that the efficiency of lesion removal by human AAG and mouse Aag is significantly affected by sequences adjacent to the lesion [16,17,21,48]. However, to date no studies on the sequence dependent activity of Mag have been reported. Therefore, we set out to understand the ability of Mag to remove ϵ A and Hx lesions present in different positions within polynucleotide repeats. The activity assays were performed under single turnover conditions, i.e., with a vast excess of enzyme versus substrate. Similar to mouse Aag [17], Mag exhibits large differences in the sequence dependent excision of Hx, but only modest differences in the sequence dependent excision of ϵ A. Mag removed Hx from the AAHxAA and TTHxTT duplexes at a ~7-fold greater rate than from the CAHxGT random sequence duplex. Interestingly, Mag was better able to remove both ϵ A and Hx from the middle of polyA and polyT runs (AAXAA and TTXTT), than from the ends of such runs (A5X or T5X). This presumably results from the significant structural deviation of the polyA:T tracts compared to that of normal B-form DNA. For polyA:T tract DNA, the width of the minor groove progressively decreases in the 5' to 3' direction. Thus, in the A5X and T5X duplexes, the base lesions are present in the region of narrowed minor groove, and this could pose a structural hindrance for Mag to efficiently flip

the lesions into its active site to perform further catalysis. For the AAXAA and TTXTT duplexes, the minor groove width at the target base should be wider relative to that for the A5X and T5X duplexes, and thus the target base should be relatively more amenable to Mag mediated flipping in the AAXAA and TTXTT sequence contexts than in the A5X and T5X sequence contexts. Supporting this hypothesis, the mouse Aag removed Hx from AAHxAA more efficiently than from the A4Hx sequences [17]. Interestingly, while Aag removed Hx from T5Hx more efficiently than from A4Hx, it removed ϵ A at similar rates from each sequence context. In contrast, Mag consistently showed higher activity to remove ϵ A or Hx from T5X, compared to A5X sequences (Table 2).

The sequence dependent studies on human AAG showed that there is a significant correlation between the thermodynamic stability of the DNA duplex (conferred by the base pairs flanking an ϵ A or a Hx lesion), and the efficiency of base excision [16,21]. The results from one study of AAG on Hx lesions, showed that lower duplex stability correlated with an increased Hx excision [16]. Likewise, Mag excises Hx more efficiently from the thermally less stable AAHxAA and TTHxTT duplexes, compared to that from the more stable GGHxGG and CCHxCC duplexes (Table 2). Another study showed that AAG is 3–5 fold more efficient at removing ϵ A from the relatively more stable GGeAGG and CC ϵ ACC duplexes, compared to the relatively less stable AA ϵ AAA and TT ϵ ATT duplexes [21]. However, this pattern was not observed for Mag mediated ϵ A excision; unlike AAG, Mag showed similar excision of ϵ A from AA ϵ AAA, TT ϵ ATT and CC ϵ ACC duplexes, but more efficient excision from the GGeAGG duplex (Table 2). This implies that the efficiency of ϵ A excision by Mag depends on factors other than, or in addition to, the thermodynamic stability of the DNA duplex. It is clear that the neighborhood of a damaged DNA base has a significant effect on the catalytic activity of DNA repair enzymes. This effect, along with the fact that DNA sequences affect the susceptibility of DNA to DNA damaging agents, contributes to the fact that there are mutational hot spots and cold spots in the genome of all organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Sequence alignment of *S. cerevisiae* Mag with *E. coli* AlkA, generated using ClustalW program [49], available at www.ebi.ac.uk. The amino acid residues absolutely conserved between two proteins are highlighted in the green boxes. The catalytic residue, Asp238 of AlkA and the corresponding residue Asp209 of Mag are indicated by an arrow. The secondary structure of AlkA aligned with its sequence is shown at the bottom. The α -helices are indicated as rectangles, β -sheets as arrows and loops as lines. The highly conserved Helix-hairpin-Helix motif of AlkA is highlighted in magenta. (B) The active site of AlkA bound to 1-azadeoxyribose (1-aza-dR) containing DNA (PDB ID 1DIZ) [14]. The amino acid residues are shown green and the 1-aza-dR containing DNA as yellow. The interactions between Asp238,

1-aza-dR and amino acids are shown as dotted lines. (C) The chemical structures of different DNA lesions used in this study.

Figure 2.

Recognition of different DNA lesions by Mag. (A) Gel mobility shift assays for the binding of Mag to oligonucleotide duplexes containing different DNA lesions. Reaction samples containing 1nM ^{32}P -labelled oligonucleotide were incubated in the absence or in the presence of 100 and 200 nM of Mag protein. (B) DNA glycosylase activity of Mag tested for the removal of different base lesions. Reaction samples containing 2 nM of ^{32}P -labelled oligonucleotide duplex were incubated with 580 nM Mag at 37°C and the samples were taken out at indicated time points.

Figure 3.

Mag glycosylase activity in the presence of competitors. The DNA glycosylase activity of Mag on CAεAGT oligonucleotide duplex was measured in the presence of different competitors. 2 nM 32 P-labeled CAεAGT duplex was incubated with 580 nM Mag and 2000 nM cold competitor at 37°C. The reaction was followed as a function of time. DNA glycosylase assays were carried out, (A) in the absence of any competitor, in the presence of εA or AP-site containing duplex; (B) in the presence of undamaged DNA, 1,2-d(ApG) cisplatin adduct and Hx containing duplex; and (C) in the presence of G:T mismatch containing duplex. (D) Graphical representation of the DNA glycosylase activity of Mag in the absence of any

competitor (◆); and in the presence of cold competitors, undamaged DNA (■), εA (●), Hx (▲), AP-site (○), 1,2-d(ApG) cisplatin adduct (△) and G:T mismatch (□).

Figure 4.

Competition binding studies of Mag. 20 nM ³²P-labeled CAεAGT oligonucleotide duplex was incubated with 1 μM Mag in the presence of various cold oligonucleotide competitors. Increasing concentrations of cold competitors were used to compete the binding of ³²P-labeled CAεAGT duplex by Mag. The resulting products were resolved using gel mobility shift assays. Graphs show the results from competition binding studies fitted to equation-1 (Materials and Methods) to determine the IC₅₀ for each cold competitor used in the reaction with different concentration of competitors; (A) undamaged DNA (■), εA (●) and Hx (▲); and (B) AP-site (○), 1,2-d(ApG) cisplatin adduct (Δ) and G:T mismatch (□) on X-axis, plotted against the

percentage of Mag-bound ^{32}P -labeled CAεAGT duplex on the Y-axis. (C) Summary of calculated IC_{50} and K_d values with standard deviation (SD) for the cold competitors used in the experiment. The results are an average of at least three independent experiments.

A **B** **Figure 5.**

Sequence dependent recognition of εA and Hx lesions by Mag. Gel mobility shift assay results showing the binding of Mag to ³²P-labeled (A) A₅εA, T₅εA, CCεACC, GGεAGG, AAεAAA and TTεATT duplex oligonucleotides; and (B) A₅Hx, T₅Hx, CCHxCC, GGHxGG, AAHxAA and TTHxTT oligonucleotide duplexes. Binding reactions were carried out in the presence of 1 nM ³²P-labelled oligonucleotide and 0, 100 and 200 nM of Mag protein.

Figure 6.

Sequence dependent DNA glycosylase activity of Mag under single turnover conditions. Mag activity was measured for the removal of ϵ A from the oligonucleotide duplexes: (A) CA ϵ AGT (■), A5 ϵ A (▲) and AA ϵ AAA (▼); (B) CA ϵ AGT (■), T5 ϵ A (▲) and TT ϵ ATT (▼) and (C) CA ϵ AGT (■), GG ϵ AGG (▲) and CC ϵ ACC (▼). Mag activity measured for the removal of Hx from the oligonucleotide duplexes: (D) CAHxGT (□), A5Hx (Δ) and AAHxAA (▽); (E) CAHxGT (□), T5Hx (Δ) and TTHxTT (▽) and (F) CAHxGT (□), GGHxGG (Δ) and CCHxCC (▽). The assays were carried out at 37°C in the presence of 2 nM 32 P-labeled oligonucleotide and 1.47 μ M of Mag.

Table 1

List of oligonucleotides used in this study. The position X indicates the lesion εA, Hx, an AP-site analogue tetrahydrofuron (THF) or A (undamaged). The G involved in G:T base pair in the G:T mismatch oligonucleotide and the bases involved in the cisplatin mediated intrastrand cross-link in 1,2-d(ApG)Pt oligonucleotide are highlighted.

Name	Oligonucleotide (5' – 3')
CAXGT	GCA ATC TAG C <u>CAXGT</u> CGA TGT ATG C
G:T mismatch	CCT CTC CTT <u>G</u> AT CTT CTC CTC TCC
1,2-d(ApG)Pt	CCT CTC CTT <u>AG</u> T CTT CTC CTC TCC
AAXAA	GCA ATC TAG C <u>AAXAA</u> CGA TGT ATG C
TTXTT	GCA ATC TAG C <u>TTXTT</u> CGA TGT ATG C
GGXGG	GCA ATC TAG C <u>GGXGG</u> CGA TGT ATG C
CCXCC	GCA ATC TAG C <u>CCXCC</u> CGA TGT ATG C
A5X	GCA ATC TAG C <u>AAAAAX</u> CGA TGT ATG C
T5X	GCA ATC TAG C <u>TTTTTX</u> CGA TGT ATG C

Table 2

Observed rate constants (k_{obs}) with standard deviation (SD) for the Mag mediated reactions under single turnover conditions; k_{obs} was calculated as described in the Materials and Methods. Values in the parenthesis indicate the number of fold increase (+) or decrease (–) in the observed rate constants as compared to that of CAXGT oligonucleotide substrate.

Substrate Oligonucleotide	$k_{\text{obs}} \pm \text{SD min}^{-1}$	
	X = Ethenoadenine (εA)	X = Hypoxanthine (Hx)
CAXGT	0.44 ± 0.03	0.037 ± 0.003
A5X	0.156 ± 0.007 (–2.8)	0.053 ± 0.004 (+1.4)
AAXAA	0.33 ± 0.04 (–1.3)	0.26 ± 0.02 (+7.0)
T5X	0.28 ± 0.03 (–1.6)	0.16 ± 0.01 (+4.3)
TTXTT	0.24 ± 0.01 (–1.8)	0.24 ± 0.02 (+6.5)
GGXGG	0.15 ± 0.01 (–2.9)	0.068 ± 0.006 (+1.8)
CCXCC	0.34 ± 0.02 (–1.3)	0.116 ± 0.007 (+3.1)