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EXCISED DAMAGED BASE DETERMINES THE TURNOVER OF HUMAN N-METHYLPURINE-DNA GLYCOSYLASE

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Abstract

N-Methylpurine-DNA glycosylase (MPG) initiates base excision repair in DNA by removing a wide variety of alkylated, deaminated, and lipid peroxidation-induced purine adducts. In this study, we tested the role of excised base on MPG's enzymatic activity. After the reaction, MPG produced two products: free damaged base and AP-site containing DNA. Our results showed that MPG excises 1, N⁶-ethenoadenine (εA) from εA-containing oligonucleotide (εA-DNA) at a similar or slightly increased efficiency than it does hypoxanthine (Hx) from Hx-containing oligonucleotide (Hx-DNA) under similar conditions. Real-time binding experiments by surface plasmon resonance (SPR) spectroscopy suggested that both the substrate DNAs have a similar equilibrium binding constant (K_D) towards MPG, but under single-turnover (STO) condition there is apparently no effect on catalytic chemistry; however, the turnover of the enzyme under multiple-turnover (MTO) condition is higher for εA-DNA than it is for Hx-DNA. Real time binding experiments by SPR spectroscopy further showed that the dissociation of MPG from its product, AP-site containing DNA, is faster than the overall turnover of either Hx-or εA-DNA reaction is. We thereby conclude that the excised base plays a critical role in product inhibition and, hence, is essential for MPG's glycosylase activity. Thus, the results provide the first evidence that the excised base rather than AP-site could be rate-limiting for DNA-glycosylase reactions.

1. INTRODUCTION

Cellular DNA is continuously damaged by endogenous or exogenous chemical or physical agents. Multiple DNA repair pathways repair damaged bases and prevent cell death and mutations responsible for genomic instability, cancer and aging [1–3]. In all organisms, repair of DNA-containing small adducts, as well as altered and abnormal bases, occurs primarily via the base excision repair (BER) pathway, beginning with removal of the base by a DNA glycosylase. Mammalian N-Methylpurine-DNA glycosylase (MPG), a monofunctional glycosylase, is known to excise at least 17 structurally diverse modified purine bases, including toxic and mutagenic alkylated, deaminated and etheno adducts from both the major and minor grooves of duplex DNA [4–12].

In our previous studies, we showed that MPG is organized into three distinct domains with a protease hyper sensitive ~100 amino acid region at the amino terminus, and these domains are

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important in overcoming the product inhibition [13,14]. Although it has been shown that MPG is essential, most of its enzymatic and structural properties available in literature are primarily for the truncated protein [15,16]. The major reasons for this include extremely poor expression of full-length hMPG, which made its purification extremely difficult requiring a multi-step process [10]. We have recently overcome this problem and have purified a stable full-length human MPG, using a modified approach [17].

In the present study, we have used this newly purified stable full-length human MPG to investigate the role of modified base on its overall reaction. We demonstrated, through analyzing individual intermediate kinetic steps, that the excised base is crucial for MPG's dissociation from its products after the excision reaction, and consequently for its overall turnover. However, a modified base does not have an effect on either the chemistry (glycosidic bond cleaving) or lesion binding step. Furthermore, the type of excised modified base (ϵ A or Hx), rather than the common AP-site containing DNA (AP-DNA), becomes critical in product inhibition for MPG reaction.

2. MATERIALS AND METHODS

2.1 Purification of Recombinant human MPG

Human full-length MPG wild-type (WT) was purified as previously described [17].

2.2 Preparation of Oligonucleotide Substrates

Hx- and ϵ A-containing 50-mer oligonucleotides with the sequence 5'-TCGAGGATCCTGAGCTCGAGTCGACGXTCGCGAATTCTGCGGATCCAAGC-3' (where X represents Hx or ϵ A) were purchased from Operon Technologies (Alameda, CA) and Gene Link (Hawthorne, NY). The complementary oligonucleotide containing T opposite Hx was synthesized by the Recombinant DNA Laboratory Core Facility at the University of Texas Medical Branch (Galveston, TX). The oligonucleotides were purified on a sequencing gel. The Hx or ϵ A oligonucleotide was labeled at the 5' end, using T4 polynucleotide kinase and 32 P-ATP, and annealed to complementary oligonucleotide to prepare 32 P-end-labeled duplex oligonucleotide as described previously [18].

2.3 MPG-Mediated Excision Activity Assay

The full-length MPG protein (1–3 nM) was incubated separately with 5' - 32 P-labeled Hx- or ϵ A-containing duplex oligonucleotide (Hx- and ϵ A-DNA) substrates (4 nM) for 10 min at 37°C in an assay buffer (25 mM HEPES-KOH, pH 7.9, 0.5 mM DTT, 10 μ g/ml nuclease free BSA, 150 mM NaCl and 10% glycerol) in a total volume of 20 μ l. The reaction was stopped by inactivating the enzyme at 75°C for 5 min. The products containing the AP-sites were then quantitatively cleaved into smaller fragments by incubating them with a large excess amount (100 ng) of AP endonuclease at 37°C for 10 min after the concentration of Mg^{2+} was adjusted to 5 mM. The reaction mixture was then mixed with 40 μ l of loading buffer containing 1x DNA dye (diluted from blue-orange 6x loading dye; Promega, Madison, WI), 85% formamide, and 0.03 N NaOH and heated at 95°C for 5 min. The samples were resolved by electrophoresis at 60°C using Criterion gel (BioRad, Hercules, CA) containing 20% polyacrylamide and 7 M urea. Radioactivity in the incised oligonucleotides was quantified by exposing the gel to x-ray films and measuring the band intensities using an imager (Chemigenius Bioimaging System, Frederick, MD) with quantification software (Syngene Inc., San Diego, CA) [17].

2.4 DNA Binding Studies Using Surface Plasmon Resonance

A 50-mer duplex oligonucleotide containing an Hx, ϵ A or abasic site (tetrahydrofuran) at the 26th position from the 5' end of one strand was used for measuring enzyme-DNA interactions.

Oligonucleotides were biotinylated and immobilized on streptavidin-coated Biacore chips [14]. Then, we measured the binding parameters of full-length human MPG for Hx- and ϵ A-DNA using (0–30 nM) or for AP-DNA using (0–15 nM) protein in a binding buffer (10 mM HEPES-KOH pH 7.6, 150 mM NaCl and 0.05% surfactant P20, Biacore, Uppsala, Sweden) at 7°C or 37°C. The MPGs at various concentrations were injected, and the surface plasmon resonance (SPR) units were measured with 60 sec injections. Following each injection, the chip was regenerated with 1M NaCl. The binding kinetics for Hx-, ϵ A- or AP-DNA were established with a series of MPG concentrations. The Langmuir isotherms (1:1 binding) at various protein concentrations allowed us to calculate the kinetic binding parameters based on on/off rates and protein concentrations.

2.5 Single-turnover (STO) Kinetic Study

The full-length MPG (54 nM) was incubated individually with 1 nM of 5'-³²P-labeled Hx- or ϵ A-DNA substrate at 37°C in an assay buffer (25 mM HEPES-KOH, pH 7.6, 10 μ g/ml nuclease-free BSA, 150 mM NaCl, 0.5 mM DTT and 10% glycerol) in a total volume of 100 μ l. Aliquots of 5 μ l were taken out at different time points (0–6 min) and heat inactivated at 80°C in a pre-heated micro centrifuge tube. The products containing the AP-sites were quantitatively cleaved into smaller fragments, followed by resolution on denaturing gels. Radioactivity in the incised oligonucleotide was also quantified as described in the activity assay.

2.6 Burst Analysis

The enzyme (6.5 nM) was incubated individually with 5'-³²P-labeled Hx- or ϵ A-DNA (75 nM) at 37°C under conditions similar to those described in the STO kinetic study.

3. RESULTS

3.1 MPG-Mediated Excision Activity Assay

The activity of the purified full-length MPG was measured using Hx- and ϵ A-DNA as substrates. The full-length MPG had moderately higher activity for ϵ A-DNA than for Hx-DNA depending on the molar ratio of enzyme and substrate concentrations (Fig. 1 A and B). The results suggest that the efficiency of product formation by MPG may be moderately affected by the modified base. Detailed analysis of the intermediate steps was then conducted to gain further insight.

3.2 Mechanism Analysis to Understand the Role of Modified Base in MPG Reaction

In Fig. 1C, we propose a series of MPG reaction steps, which are slightly modified from our previously published scheme [17]. We had hypothesized that the removed base would be diffused spontaneously, but in reality that may not be the case. The excised base may still remain bound with the enzyme by a specific hydrogen bond (details in the “Discussion” section). Therefore, the dissociation of products, excised base and AP-DNA, from MPG may contribute to the overall product dissociation rate. To further elucidate this mechanism, we used both SPR spectroscopy and pre-steady-state kinetics. Using SPR, we measured the DNA binding of MPG towards substrates (Hx- and ϵ A-DNA) and the product (AP-DNA). Pre-steady-state kinetic analysis provides the opportunity to identify the intermediate reaction step (s) that might be affected by the modified base. We took advantage of MPG's slow reaction rates and measured the effect of the modified base on the glycosidic bond cleavage (chemistry) step by STO kinetics and the product dissociation step by multiple-turnover (MTO) reaction conditions. Note that the calculated k_{pd} by burst analysis must be the slower of the two rate constants (k_{pd1} and k_{pd2}) for two products, such as AP-DNA and the excised base (Fig. 1C).

3.3 Hx-/εA-DNA Binding Studies Using Surface Plasmon Resonance

In search of a mechanism for MPG activity, we examined the MPG-Hx-/εA-DNA binding at 7°C using a Biacore-T100 (Biacore, Uppsala, Sweden). Our results showed that the equilibrium binding constant (K_D) is 1.57 nM and 2.25 nM for Hx- and εA-DNA, respectively (Fig 2 A, B, and C). Apparently, there is no major effect of the modified bases on the substrate binding of the full-length MPG.

3.4 STO Kinetics

Prompted by the observation that the εA- and Hx-DNA is different in product formation in MPG reactions, we tested whether the modified base containing oligonucleotides are different in any of the catalytic intermediate steps other than the binding step. We conducted STO kinetics with full-length MPG protein to measure the k_{chem} [14,18]. The reaction was performed at substrate (εA-/Hx-DNA) and enzyme concentrations of 1 and 54 nM, respectively. Data were analyzed using the first-order rate equation (1): $[P]_t = A_0 \{1 - \exp(-k_{obs} t)\}$, where A_0 represents the amplitude of the exponential phase, and k_{obs} is the observed rate constant associated with the reaction process. Under the STO conditions ($[E] \gg [S]$), all the substrate molecules should remain bound by enzymes. The binding step should not affect the rate of product formation, and hence, under these conditions k_{obs} can be considered as k_{chem} . However, the full-length MPG has a similar k_{chem} or both εA- ($1.3 \pm 0.2 \text{ min}^{-1}$) and Hx- ($1.4 \pm 2 \text{ min}^{-1}$) containing oligonucleotides (Fig. 3A and B), indicating minimal effect of the modified base on the chemistry step of the MPG-mediated reaction.

3.5 Burst Analysis

Next, we attempted to measure the rate of product release (k_{pd}) and active enzyme concentration available for the reaction [14,18]. The extremely slow turnover rate of MPG during excision of Hx provided the opportunity to perform burst analysis under the reaction conditions of $[S] \gg [E]$, where the substrate and enzyme concentrations were 75 nM and 6.5 nM, respectively. The data were fit to the equation (2): $[P]_t = A_0 \{1 - \exp(-k_{obs} t)\} + k_{ss} t$. Plot of product concentration (P_t) versus time (t) can be analyzed using equation (2), as before [14,18], to determine the kinetic parameters, A_0 (amplitude of the burst) and k_{ss} (slope of the linear phase; turnover). We previously found a k_{pd} value ($0.016 \pm 0.001 \text{ min}^{-1}$) for mMPG which was low and apparently rate-limiting in the MPG-mediated multi-step reaction process [18]. Here, with human full-length MPG, we found a much higher k_{pd} value, but notably the latter was nearly twice as high for εA ($0.00435 + 0.00057 \text{ sec}^{-1}$) as it was for Hx ($0.00235 + 0.00022 \text{ sec}^{-1}$) (Fig. 4A and B). Therefore, it is evident that the type of modified base can regulate the product dissociation rate or MPG's turnover in a significant fashion.

3.6 AP-DNA Binding Studies Using Surface Plasmon Resonance

Further, we asked what the rate of dissociation of MPG from AP-site is and whether it is faster or slower than the overall product dissociation. We directly measured the binding kinetics of full-length MPG to AP-site at 37°C using a Biacore-T100. Our results showed an apparent K_D of 0.71 nM (Fig. 5 A and B). Apparently, at 37°C MPG has a dissociation rate of 0.011 sec^{-1} . Thus, the modified bases appear to play a critical role in product inhibition as they likely dissociate more slowly than the AP-DNA and emphasize their rate-limiting function in MPG's turnover.

4. DISCUSSION

Most, if not all, DNA glycosylases show some degree of product inhibition, because their dissociation from the product, AP-site, is rate-limiting for enzymatic turnover [19–22]. This property is most pronounced in the mismatch-specific uracil glycosylase (MUG) family

proteins. The eukaryotic thymine-DNA-glycosylase bind AP-sites with exceptional strength and are thus fully product inhibited [23,24], whereas the bacterial MUG protein turns over, albeit at a very low rate [25]. The bifunctional, DNA glycosylases, such as NTH1 and OGG1, also remain tightly bound to their AP lyase product [26,27,28].

For MPG, it is also thought that after the base is removed, the AP-site is the limiting factor for product dissociation. Product inhibition by excised free base, the second reaction product, to reduce enzymatic activity of MPG or any DNA glycosylase has been tried without much success in the past [8]. In the current study as the reaction proceeds towards the pseudo-single-turnover condition, the difference in product formation is diminished (Fig. 1). Interestingly, the K_D s for at least two different substrates (Hx- and ϵ A-DNA) differ very little (Fig. 2). Also there is no difference in the reaction rate under single-turnover condition for Hx- and ϵ A-DNA, but the difference is apparent under multiple-turnover condition (Fig. 3 and Fig. 4).

Being a broad substrate enzyme, MPG must be flexible for DNA binding in order to recognize DNA lesions of varied structures. In fact, for similar reasons T. Ellenberger and his colleagues proposed that a “nonspecific catalytic mechanism” must be met for an enzyme to succeed as a generalist as one of the major criteria, which comes “at the expense of catalytic power” [29]. That minimal difference in binding does not have any effect on overall catalysis. In our previous study, we showed that although the K_D was apparently ~160-fold less for N Δ 100 mouse MPG (mMPG) compared to full-length protein, the overall product formation was only ~3-fold different. One possible explanation was that the binding of full-length mMPG to Hx was extremely fast, as evident from the K_D value of 0.15 nM compared to its chemistry and turnover step [14]. The same logic possibly holds true for this study. That study and a paper by Saparbaev et al also indicated the importance of N-terminal tail in the turnover [14,30]. Moreover, in a recent study Dr. L.D. Samson and colleagues showed the requirement of N-terminal for a variety of new substrates of MPG [31]. As N-terminal tail seems to be important for MPG’s reaction, it is highly recommended to do the detailed characterization of full-length, rather than the truncated MPG. To date, most studies reported using the truncated form of MPG. To our knowledge, this is the first detailed pre-steady state kinetic characterization of stable human full-length MPG.

After the base is excised from DNA, MPG generates two products: AP-site and the free damaged base. AP-site is a common product of MPG’s excision reaction towards ϵ A- and Hx-DNA. The product dissociation rate, however, is different, ~2-fold, for these two lesions (Fig. 4). Although this difference amounts to less than an order of magnitude, it is possible that such differences could be amplified *in vivo*, given that MPG activity could be affected by various repair modulatory proteins in the cell. This difference can only be explained if the modified base dissociates from the enzyme at a slower rate compared to the AP-site. In fact, we observed much faster dissociation of AP-site at 37°C in SPR experiments than the overall product dissociation of MPG under MTO condition. Furthermore, there is a notable difference in the dissociation rates (k_{pd}) for the modified bases; ϵ A dissociates faster than Hx (Fig 5). Thus, it is tempting to speculate that dissociation rates of MPG from its reaction products would be in the order of AP-DNA > ϵ A > Hx. This, to our knowledge, is the first evidence to show the importance of excised base for any DNA glycosylase in its enzymatic turnover.

Note that, previously, in an elegant study on the role of product inhibition in glycosylase function, S. David and her colleagues demonstrated that the product dissociation rate of MutY (Adenine DNA-glycosylase) from the cleaved DNA product was different for G:A and OG:A mismatch substrates. The authors noted that “the slower dissociation rate of MutY from an OG:(AP site) compared to the corresponding G:(AP-site) is suggestive of specific contacts between MutY and OG that persist after the adenine has been removed.” This result showed

that the orphan base on DNA was responsible for product inhibition [32]. Our study indicates the role of the removed base in product inhibition.

The contribution of excised base in the overall product dissociation can also be explained from the existing crystal structures of MPG. From the tertiary structures of MPG in complexes with ϵ A-containing DNA, it was suggested that the enzyme selects the substrate bases through a combination of hydrogen bonds and the steric constraints of the active site [29]. Several other principles, such as shape and electrostatic properties of the binding pocket which accepts the flipped-out nucleotide, also dictate MPG in selecting its substrates. As revealed from crystal structures, the backbone amide of His-136 donates a hydrogen bond to the etheno N-6 nitrogen of ϵ A [29]. This hydrogen bond can discriminate against adenine, which has a 6-amino group that cannot accept a hydrogen bond [29]. Thus, this hydrogen bond may also be important in discrimination and excision of Hx and ϵ A, and perhaps in the dissociation of the excised bases and overall turnover. Particularly, the dissociation of excised Hx would be slower compared to that of ϵ A, if we assume that the free bases are transiently remaining in the catalytic pocket through the same hydrogen bonding and consider that the oxygen atom at the 6-oxo group of Hx in the Hx-MPG complex forms a stronger hydrogen bond than the nitrogen atom in ϵ A-MPG complex [29]. Similar studies will be required to extend to other DNA glycosylases to examine the participation of excised damaged bases in enzyme turnover and also to test whether this phenomenon is general for reaction mechanisms of DNA glycosylases.

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Abbreviations

BER, base excision repair; AP, Apurinic / apyrimidinic; MPG, N-Methylpurine DNA glycosylase; hNTH1, Human endonucleases III; APE, Apurinic / apyrimidinic endonuclease; Hx, Hypoxanthine; εA, ethenoadenine.

Figure 1. MPG mediated excision of Hx

(A) One and three nanomolar concentrations of full-length human MPG were reacted with 50-mer ³²P-labeled Hx- and εA-containing oligonucleotides (4 nM) at 37°C for 10 min. The details of the reaction conditions are described in “Materials and Methods.” (B) Data obtained in Panel A were plotted. Data represent mean values with standard error derived from three independent experiments. (C) Proposed model for reaction mechanism of MPG. The steps and the notations used to indicate the intermediate steps of MPG reaction are as follows: E, the enzyme MPG, S₁, the substrate DNA with the modified base (εA or Hx); P₁, and P₂, the excised modified base and the AP-DNA after the base is excised. The K_d is the equilibrium binding constant between substrate and enzyme, and the rate constants are: k_{chem}, the catalytic constant at the chemistry step, and k_{pd1} and k_{pd2}, the product dissociation constants. The (→) denotes regulatory action.

C

Substrate	K_{on} ($\text{mol}^{-1}\text{sec}^{-1}$)	K_{off} (sec^{-1})	K_D (nM)
Hx-DNA	$(34.1 \pm 0.1) \times 10^5$	$(5.36 \pm 0.01) \times 10^{-3}$	1.57 ± 0.01
ϵ A-DNA	$(19.9 \pm 0.3) \times 10^5$	$(4.46 \pm 0.05) \times 10^{-3}$	2.25 ± 0.01

Figure 2. Langmuir Isotherm of MPG binding to 50-mer biotinylated oligonucleotides containing Hx (A) and ϵ A (B) using Biacore-T100

The experimental results and the fitted curves are represented in red and black lines, respectively. Binding kinetic parameters were obtained using various concentrations of full-length MPG (0, 0.93, 1.875, 3.75, 7.5, 15, 30 nM). (C) Data represent mean values with standard error derived from three independent experiments.

B

Substrate type	k_{chem} (min^{-1})
Hx-DNA	1.4 ± 0.2
ϵ A-DNA	1.3 ± 0.2

Figure 3. Effect of modified base on MPG reaction under single-turnover conditions

The reaction was performed using different substrate and enzyme concentrations as described in “Materials and Methods.” (A) Effect of modified base on pre-steady-state kinetic parameters under single-turnover conditions. (B) Data derived from Panel A were analyzed using the first-order rate equation: $[P]_t = A_0 \{ 1 - \exp(-k_{\text{obs}}t) \}$ as described in “Results.” k_{chem} , catalytic constant at the chemistry step.

B

Substrate Type	Hx-DNA	εA-DNA
A_0 (nM)	4.749+0.248	5.101+0.452
k_{obs} (min^{-1})	1.061+0.145	1.533+0.328
k_{ss} ($\text{min}^{-1}\text{nM}^{-1}$)	0.672+0.034	1.332+0.071
K_{ss}/A_0 (sec^{-1})	0.00235+0.00022	0.00435+0.00057

Figure 4. Effect of N-terminal tail on MPG-Hx reaction under multiple-turnover conditions
 (A) The reaction was performed using substrate and enzyme concentrations of 75 and 6.5 nM, respectively, as described under "Materials and Methods." (B) Data derived from A were analyzed using the rate equation $[P]_t = A_0 [1 - \exp(-k_{obs}t)] + k_{ss}t$ (also described under "Results"). A_0 , active MPG concentration; k_{obs} ($\approx k_{chem}$), burst constant; k_{ss}/A_0 ($\approx k_{pd}$), microscopic product dissociation rate constant/turnover number. The reaction was performed using different substrate and enzyme concentrations as described in "Materials and Methods."

B

K_{on} (mol ⁻¹ sec ⁻¹)	K_{off} (sec ⁻¹)	K_D (nM)
$(158 \pm 2) \times 10^5$	$(11.23 \pm 0.12) \times 10^{-3}$	0.71 ± 0.02

Figure 5. Langmuir Isotherm of (A) MPG binding to 50-mer biotinylated oligonucleotides containing AP-site using Biacore-T100

The experimental results and the fitted curves are represented in red and black lines, respectively. Binding kinetic parameters were obtained using various concentrations of full-length MPG (0, 0.23, 0.47, 0.93, 1.875, 3.75, 7.5, 15nM) (B) Data represent mean values with standard error derived from three independent experiments.