N-terminal Extension of N-Methylpurine DNA Glycosylase Is Required for Turnover in Hypoxanthine Excision Reaction*

Sanjay Adhikari, Aykut Üren, and Rabindra Roy

From the Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, D. C. 20057

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 N-terminal extension on MPG hypoxanthine (Hx) cleavage activity. Our results showed that MPG lacking N-terminal extension excises hypoxanthine with significantly reduced efficiency, one-third of that exhibited by full-length MPG under similar conditions. Steady-state kinetics showed full-length MPG has higher \( V_{\text{max}} \) and lower \( K_{\text{m}} \) than N\( \Delta \)100 MPG. Real time binding experiments by surface plasmon resonance spectroscopy suggested that truncation can substantially increase the equilibrium binding constant of MPG toward Hx, but under single-turnover conditions there is apparently no effect on catalytic chemistry; however, the truncation of the N-terminal tail affected the turnover of the enzyme significantly under multiple turnover conditions. Real time binding experiments by surface plasmon resonance spectroscopy further showed that N\( \Delta \)100 MPG binds approximately six times more tightly toward its product apurinic/apyrimidinic site than the substrate, whereas full-length MPG similarly binds to both the substrate and the product. We thereby conclude that the N-terminal tail in MPG plays a critical role in overcoming the product inhibition, which is achieved by reducing the differences of MPG binding affinity toward Hx and apurinic/apyrimidinic sites and thus is essential for the Hx cleavage reaction of MPG. The results from this study also affirm the need for reinvestigation of full-length MPG for its enzymatic and structural properties, which are currently available mostly for the truncated protein.

Cellular DNA is continuously damaged by various 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 pathway, beginning with cleavage 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 study, we showed that MPG is organized into three distinct domains with a protease-hypersensitive \( \sim \)100-amino acid region at the N terminus (13). We also found that truncated (N\( \Delta \)100C\( \Delta \)18) and full-length enzymes retained similar binding and kinetic properties toward \( \varepsilon \)A (7). Later, several x-ray structures of human truncated MPG in complex with \( \varepsilon \)A or control DNA were published with the notion that the seemingly unstructured (protease-sensitive) N-terminal extension may hinder crystallization of MPG (14–16). But different studies by us and others showed that the N-terminal extension of MPG could be critical for recognition of substrates such as 3-methylguanine, 7-methylguanine, and 1,\( N^2 \)-etheno guanine (1,\( N^2 \)-eG) adducts (9, 17). Notably, there is a report that truncated and full-length forms of human MPG do not show a significant difference in Hx cleavage activity, although the long incubation period, instability of the full-length MPG, and insufficient kinetic details could have contributed to such results (11). Hx is one of the preferred substrates elucidated so far for MPG or related enzymes that are ubiquitously present in all organisms, including humans (18). Moreover, Hx was shown to be significantly mutagenic (19, 20).

In the present study, we have demonstrated through analyzing individual intermediate kinetic steps that the N-terminal tail is crucial for MPG binding to Hx, dissociation from its product AP site, and its overall turnover. However, it does not have an effect on catalysis of Hx-containing DNA. The results from this study also underscore the need for the reinvestigation of full-length MPG for its enzymatic and structural properties, which are currently available in literature primarily for the truncated protein.

MATERIALS AND METHODS

Purification of Recombinant Mouse MPG—Mouse MPG wild type was purified as previously described (7).

Cloning and Purification of N\( \Delta \)100 MPG—An expression construct encoding N\( \Delta \)100 mMPG was prepared by ligating a

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1 To whom correspondence should be addressed: Lombardi Comprehensive Cancer Center, LL level, S-122, 3800 Reservoir Rd., NW, Georgetown University Medical Center, Washington, D. C. 20057. Tel.: 202-687-7390; Fax: 202-687-1068; E-mail: rr228@georgetown.edu.

2 The abbreviations used are: MPG, N-methylpurine DNA glycosylase; AP, apurinic/apyrimidinic; Hx, hypoxanthine; \( \varepsilon \)A, 1,\( N^2 \)-etheno adenine; 1,\( N^2 \)-eG, 1,\( N^2 \)-etheno guanine; STO, single-turnover; PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol.
PCR product containing the MPG coding sequence but lacking the first 100 amino acid residues at the NdeI and EcoRI sites of the pRSCT vector. PCR was carried out using a pair of mouse MPG (N4348) construct as template and the primers (5'-CATATGGACCATTTCTGGCCGGCTA-3' and 5'-GAATTCTTACTTTTGAACAAATTAAAGCCCCCC-3'). The primers allowed the introduction of NdeI and EcoRI sites at the 5' and 3' ends, respectively.

The PCR products were then subcloned in pCR2.1-TOPO cloning vector digested with NdeI and EcoRI and subcloned into expression vector pRSCT at NdeI/EcoRI sites, allowing us to express a nonfusion NΔ100 mMPG protein. The identity of the construct was confirmed by DNA sequencing. NΔ100 mMPG was overexpressed in E. coli BL21(DE3) cells and purified to near electrophoretic homogeneity. The purification was carried out as follows. The conditions for cell growth and induction of protein expression were as described previously (9).

For purification, the cells were harvested from 1 liter of culture in 50 ml of Buffer A (20 mM PIPES, pH 6.1, 10% glycerol, 50 mM NaCl, 0.1% Tween 20, 1 mM DTT). The cells were then lysed as described previously (7). The lysate was clarified by centrifugation at 15,000 rpm for 30 min, and the supernatant was applied onto tandemly attached ion exchange columns consisting of Q Sepharose (5 ml) and SP Sepharose (1 ml), which were pre-equilibrated with Buffer A. The columns were washed with 10 column volumes of Buffer A. The Q column was then detached, and the SP column was eluted with a gradient of 0-100% of Buffer B (Buffer A plus 600 mM NaCl) in Buffer A. The peak fractions containing NΔ100 mMPG, tested by SDS-PAGE, were pooled and diluted three times with Buffer C (20 mM HEPES, pH 7.5, 1 M NaCl, 0.8 M ammonium sulfate, 1 mM DTT) before loading onto a hydrophobic phenyl Sepharose column that was pre-equilibrated with Buffer C. After washing with Buffer C, the protein was eluted using a linear gradient of Buffer D (20 mM HEPES, pH 7.5, 5% glycerol, 1 mM DTT). The peak fractions were pooled, dialyzed against Buffer E (Tris-HCl, pH 7.5, 10% glycerol, 50 mM NaCl, and 1 mM DTT), and stored at −80 °C in aliquots for future use.

Oligonucleotide Substrates Preparation—Hx and a containing 50-mer oligonucleotide with the sequence 5'-TCGGAGGTCTCTGAGGCTGCGAGGTXCGGAATCTCTCCGAATC-3' (where X represents Hx) 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 laboratory Core Facility at the University of Texas Medical Branch opposite Hx was synthesized by the Recombinant DNA Laboratory Core Facility at the University of Texas Medical Branch. The complementary oligonucleotide containing T opposite Hx was synthesized by the Recombinant DNA Laboratory Core Facility at the University of Texas Medical Branch.

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MPG-mediated Excision Activity Assay—The MPG proteins, mouse full-length (15–30 nm) and NΔ100 (15–30 nm) were individually incubated with 5'-32P-labeled Hx-containing duplex oligonucleotide substrates (4–8 nm) for 10 min at 37 °C in an assay buffer (25 mM HEPES-KOH, pH 7.9, 0.5 mM DTT, 10 μg/ml nuclease-free bovine serum albumin, 150 mM NaCl and 10% glycerol) in a total volume of 20 μl. The reaction was stopped by inactivating the enzyme at 75 °C for 5 min. The products containing the AP sites were analyzed as described previously (21).

Steady-state Kinetic Study—The full-length or truncated enzyme (8.5 nm) was incubated with 5'-32P-labeled Hx-containing duplex oligonucleotide (0–60 nm) substrates for 5 min at 37 °C under assay conditions similar to those described above. The reaction products were also analyzed and quantified as described for the activity assay.

DNA Binding Studies Using Surface Plasmon Resonance—A 50-mer duplex oligonucleotide containing an Hx 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 (21). Then we measured the binding parameters of truncated (0–300 nm) or full-length MPG (0–25 nm) for Hx and abasic site using a binding buffer (10 mM HEPES-KOH pH 7.6, 150 mM NaCl, and 0.5% surfactant) at 25 °C. The MPGs at various concentrations were injected, and the surface plasmon resonance units were measured, with 60-s injections. Following each injection, the chip was regenerated with 1 M NaCl. The binding kinetics for oligonucleotides containing Hx or AP sites 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.

Single-Turnover (STO) Kinetic Study—The full-length (20–40 nm) and truncated enzymes (450–900 nm) were individually incubated with 2 nm of 5'-32P-labeled Hx-containing duplex oligonucleotide substrates at 37 °C in an assay buffer (25 mM HEPES-KOH, pH 7.9, 10 μg/ml nuclease-free bovine serum albumin, 0.5 mM DTT, 150 mM NaCl, and 10% glycerol) in a total volume of 100 μl. Aliquots of 5 μl were taken out at different time points (0–17 min) and heat-inactivated at 80 °C in a preheated 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.

Burst Analysis—The enzymes (10 nm) were individually incubated with 5'-32P-labeled Hx-containing duplex oligonucleotide (84–112 nm) at 37 °C under conditions similar to those described in the STO kinetic study.

RESULTS

Purification of NΔ100 MPG and MPG-mediated Excision Activity Assay—The wild type and NΔ100 MPGs were purified using the methods described under “Materials and Methods.” Both the proteins were 85–88% pure electrophoretically (Fig. 1). Then the activity of the purified full-length and truncated MPG was measured using Hx as a substrate. The full-length MPG had 2–3-fold more activity than the truncated protein at different enzyme:substrate (2–4:1) molar ratios, indicating that the overall decreasing effect on product formation during the Hx reaction can most likely be attributed to the deletion of the
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N-terminal extension. Thus, it appears that the N-terminal extension plays a role in the excision activity of MPG toward Hx (Fig. 2).

**Steady-state Kinetic Study**—To understand the mechanism of reduced activity of truncated MPG, we then compared the steady-state kinetic parameters for both the full-length and truncated MPGs. We measured the full-length as in a previous study (21) but included NΔ100 MPG here and found that NΔ100 MPG has significantly higher $K_m$ and lower $V_{max}$ compared with its full-length counterpart (Table 1).

**Mechanism Analysis to Understand the Role of N-terminal Tail in Hx-MPG Reaction**—To have further insight into this mechanism, we used both surface plasmon resonance spectroscopy and pre-steady-state kinetics. In Fig. 3 we described the basic reaction steps of MPG, which are slightly modified from our published scheme (21). Previously, we hypothesized that the cleaved base will be diffused spontaneously, but that may not be the case. The cleaved base may still remain bound with the enzyme by a specific hydrogen bond (details under “Discussion”). Therefore, the dissociation of both products from MPG may contribute to the overall product dissociation rate. Using surface plasmon resonance, we measured the binding of MPG toward substrate (Hx) and product (abasic site). Pre-steady-state kinetic analysis provides the opportunity to identify the intermediate reaction step(s) that might be affected by the N-terminal tail. We took advantage of the slow reaction rates of MPG and measured the effect of the N-terminal tail on the glycosidic bond cleavage (catalysis) step by STO kinetics and the product dissociation step by multiple turnover reaction conditions.

**Hx Binding Studies Using Surface Plasmon Resonance**—In search of a mechanism of modulation of MPG activity by N-terminal extension, we examined the MPG-Hx binding using a Biacore-T100 (Biacore, Uppsala, Sweden). Our results showed that the equilibrium binding constant ($K_d$) is 0.15 nM for the full-length protein, whereas it is 25 nM for the truncated one (Fig. 4). Apparently, the major effect of N-terminal extension on $K_d$ is primarily due to the high rate of microscopic association ($k_{on}$, $4.03 \pm 0.4 \times 10^8 \text{M}^{-1} \text{s}^{-1}$) for full-length MPG, which is close to the diffusion-controlled limit ($10^9 \text{M}^{-1} \text{s}^{-1}$) (22). Thus, the N-terminal extension plays a significant role in Hx recognition and binding.

**STO Kinetics**—Prompted by the observation that the N-terminal tail could play a critical role in product formation in Hx-MPG reactions, we tested whether the N-terminal tail affects any of the catalytic intermediate steps other than the binding step. We conducted STO kinetics with full-length MPG proteins to measure the $k_{chem}$ (21, 23). The reaction was performed at substrate and enzyme concentrations of 2 and 20–40 nM for full-length enzyme or 450–900 nM for truncated enzyme, respectively. The data were analyzed using the first order rate equation,

$$[P]_t = A_0[1 - \exp(-k_{obs}t)]$$  \hspace{1cm} (Eq. 1)

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 of 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 $k_{chem}$. Two different enzyme substrate ratios for both of the proteins provide similar values of $k_{chem}$, ensuring the enzymatic reactions following STO conditions. However, the full-length and NΔ100 MPG have similar $k_{chem}$ (0.3 ± 0.03) (Fig. 5), indicating the minimal effect of N-terminal extension on the chemistry step of MPG-Hx reaction.

**Burst Analysis**—Next, we tried to measure the rate of product release

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**FIGURE 1. Purification of MPGS.** SDS-PAGE of purified full-length and NΔ100 MPGs after Coomassie staining. The details of the purification are described under “Materials and Methods.”

**FIGURE 2. MPG-mediated excision of Hx.** Left panel, 15 and 30 nM concentrations of full-length and NΔ100 MPGs were reacted with a 50-mer $^{32}$P-labeled Hx-containing oligonucleotide (8 nM) at 37 °C for 10 min. The details of the reaction conditions are described under “Materials and Methods.” Right panel, data obtained in A were plotted. The data represent the mean values with standard error derived from three independent experiments.
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Effect of N-terminal tail on steady-state kinetics of MPG-Hx reactions

The reaction conditions are described under “Materials and Methods.”

| Protein   | \( k_{on} \) (nM) | \( V_{max} \) (nmol/min/nmol of MPG) | Turnover |
|-----------|-------------------|--------------------------------------|----------|
| MPG       | 35 ± 1.5          | 6 ± 0.2                              | 6.6 ± 0.2 |
| NΔ100 MPG | 84 ± 9            | 1 ± 0.1                              | 1.1 ± 0.1 |

N-terminal Extension

\[
E + S_{1} \rightarrow ES_{1} \rightarrow E-P_{1} + P_{2} + P_{3}
\]

FIGURE 3. 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; \( S_{1} \), the substrate with the modified base (eA or Hx); \( P_{1} \), the excised modified base; \( P_{2} \), the oligonucleotide substrate with AP site after the base is excised. The rate constants are \( k_{on} \), the binding constant between substrate and enzyme; \( k_{cat} \), the catalytic constant at the chemistry step; and \( k_{pd} \), the product dissociation constant. The right arrow (\( \rightarrow \)) denotes regulatory action.

DISCUSSION

N-terminal extension is present in all MPGs from higher eukaryotes. This suggests an evolutionarily conserved critical function of the N-terminal tail for MPG activity. However, neither the structure of MPG including the N-terminal tail nor the role of this extension in enzymatic activity is fully elucidated yet.

In the present study, we have demonstrated that the N-terminal tail is a regulatory domain and is essential for MPG turnover. We found in a previous study that the N-terminal tail also had a modulating effect on the function of a bifunctional DNA glycosylase, such as human endonuclease III, but it inhibited the turnover of this enzyme and in turn its activity (24). In fact, the truncation of the N-terminal tail stimulated human endonucleases III activity at the product dissociation step (24), whereas the present study shows that the truncation of the N-terminal tail inhibited MPG activity. Thus, the N-terminal tail apparently regulates the activity of DNA glycosylases, such as a monofunctional enzyme MPG and a bi-functional NTH1, but in a diverse manner.
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![FIGURE 5. Effect of N-terminal tail on MPG-Hx reaction under single-turnover conditions.](image)

The reaction was performed using different substrate and enzyme concentrations as described under "Materials and Methods." A, effect of N-terminal tail on pre-steady-state kinetic parameters under single-turnover conditions. B, data derived from A were analyzed using the first order rate equation: \[
\frac{d[P]}{dt} = k_{off}[E] - k_{on}[S] + k_{cat}[E][S] - k_{diss}[P]
\] as described under "Results." \(k_{chem}\) catalytic constant at the chemistry step.

![FIGURE 6. Effect of N-terminal tail on MPG-Hx reaction under multiple-turnover conditions.](image)

The reaction was performed using different substrate and enzyme concentrations as described under "Materials and Methods."

We had shown before by systematic deletion analysis of MPG from N and C termini that a minimally sized polypeptide (NΔ100CΔ18) lacking 100 and 18 amino acid residues from the N and C termini, respectively, and wild type enzyme had similar kinetic and binding properties for the N and C termini, respectively, and wild type enzyme had that the mode of action of MPG toward a neutral substrate, such as Hx. From this study, it is apparent the role of the N-terminal tail for MPG activity toward a methylated substrate were positively charged, and the pro- teins were partially purified, so it was of interest to evaluate the C-terminal halves of human and mouse MPG, we found that the N-terminal half is critical for the recognition of 3-methylguanine and 7-methylguanine (9). However, both of the methylated substrates were positively charged, and the proteins were partially purified, so it was of interest to evaluate the role of the N-terminal tail for MPG activity toward a neutral substrate, such as Hx. From this study, it is apparent that the mode of action of MPG toward Hx and εA are strikingly different.

The first 70 amino acid residues of hMPG were also indis- pensable for 1,N2-εG excision reaction (17), because the N-terminally truncated protein was less active toward 1,N2-εG. However, unlike Hx the 1,N2-εG binding was not affected by truncation; rather, a possible change in the catalytic pocket or lack of amino acids specifically involved in the catalysis of that substrate was suggested to be the cause (17). However, it is important to note that 1,N2-εG has yet to be detected in genomic DNA of biological samples (17). In the present study, we have shown by detailed mechanistic analysis of all three major steps in the MPG-Hx reaction that product formation is affected by the N-terminal tail.

Nonetheless, being a broad substrate enzyme, MPG must be flexible for DNA binding to recognize DNA lesions of varied structures. In fact, for similar reasons O’Brien and Ellenberger (18) 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.” In the future, it would be interesting to study the details of the binding and catalytic mechanisms of full-length MPG toward substrates other than Hx of different structures, including N-3 and N-7 of deoxyguan- nine adducts generated by nitrogen and sulfur mustards (26). Obviously, the structures of these MPG-cleavable adducts containing large modifications, especially at N-7 of deoxyguanine, are very different from those of Hx and εA.

Another interesting point is that the equilibrium binding constant \(K_{ss}\) between MPG and Hx is entirely different for the full-length and truncated versions. The \(K_{ss}\) is apparently ~160-fold less for NΔ100 MPG compared with full-length protein, but overall product formation is only ~3-fold different. One possible explanation could be that compared with its chemistry and turnover step, the binding of MPG to Hx is extremely fast, as is evident from the \(k_{off}\) value of 4.03 ± 0.4 \(\times\) 10^9 M⁻¹ s⁻¹, which is close to the diffusion-controlled limit (10^9 M⁻¹ s⁻¹). Thus, the chemistry or turnover should provide the overall rate determining step. The effect of the N-terminal tail on turnover is significant, because there is no apparent turnover of NΔ100 MPG caused by product inhibition, whereas the latter is less significant for full-length MPG. Interestingly, the \(k_{ss}\) (from surface plasmon resonance studies) of two different forms of MPG for AP site DNA are similar. However, the turnover \(k_{ss}\) of the truncated MPG is extremely slow compared with the wild type protein (Fig. 6). This could be explained by the apparent relative affinity of both the proteins toward Hx and AP site DNA (Figs. 4 and 7). Notably, \(k_{ss}\) is composed of \(k_{pd}\) values for both free base and the AP site containing DNA. Moreover, the \(k_{ss}\) consists of \(k_{pd}\) and the effective rate constant \(k'\) for the process, MPG + Hx-DNA → MPG-AP site DNA (details discussed in Refs. 21, 23). Generally, for obtaining the definite value for product...
dissociation from burst kinetics, it is assumed that \( k_{\text{on}} \) is much slower than the \( k' \), and thus the contribution of the latter is ignored, and \( k_{\text{off}} \) becomes identical to \( k_{\text{off}} \). But huge product inhibition in the case of N-terminally truncated MPG obscures that assumption, and therefore, an accurate \( k'_{\text{on}} \) value for the truncated protein could not be assigned. Therefore, the overall effect on product formation is evidently arising from the alternations in the turnover step, and the overall effect on product formation is undoubtedly important.

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MPG lacking N-terminal tail must collide with the substrates multiple times before engaging in a productive binding leading to catalysis. This indicates that without the N terminus, MPG possibly changes its conformation, or the N terminus in the full-length enzyme helps guide productive interaction with the modified base. Surprisingly, the differences in \( k_{\text{on}} \) values for the subsequent product, an abasic site, are only \(~10\)-fold for these two proteins. However, the apparent lack of an effect for the N terminus for AP site binding further underscores the importance of the N-terminal tail for substrate binding.

In the future, it would be interesting to study in detail the role of the N-terminal tail in substrate binding and perhaps in scanning and base flipping. Furthermore, the re-investigation of biochemistry, kinetics, and structural analysis of full-length MPG (so far elucidated for truncated protein) with different substrates is undoubtedly important.

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