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Expression, purification and characterization of codon optimized human *N*-methylpurine-DNA glycosylase from *Escherichia coli*

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Abstract

N-methylpurine-DNA glycosylase (MPG), a ubiquitous DNA repair enzyme, initiates excision repair of several *N*-alkylpurine adducts, deaminated and lipid peroxidation-induced purine adducts. MPG from human and mouse has previously been cloned and expressed. However, due to the poor expression level in *Escherichia coli* (*E. coli*) and multi-step purification process of full-length MPG, most successful attempts have been limited by extremely poor yield and stability. Here, we have optimized the codons within the first 5 residues of human MPG (hMPG) to the best used codons for *E. coli* and expressed full-length hMPG in large amounts. This high expression level in conjunction with a strikingly high isoelectric point (9.65) of hMPG, in fact, helped purify the enzyme in a single step. A previously well-characterized monoclonal antibody having an epitope in the N-terminal tail could detect this codon-optimized hMPG protein. Surface plasmon resonance studies showed an equilibrium binding constant (K_D) of 0.25 nM. Steady-state enzyme kinetics showed an apparent K_m of 5.3 nM and k_{cat} of 0.2 min⁻¹ of MPG for the hypoxanthine (Hx) cleavage reaction. Moreover, hMPG had an optimal activity at pH 7.5 and 100 mM KCl. Unlike the previous reports by others, this newly purified full-length hMPG is appreciably stable at high temperature, such as 50°C. Thus, this study indicates that this improved expression and purification system will facilitate large scale production and purification of a stable human MPG protein for further biochemical, biophysical and structure-function analysis.

Introduction

Cellular DNA is continuously exposed to endogenous or exogenous chemical or physical agents that induce DNA lesions. DNA base damage threatens genomic stability and cellular viability. Multiple DNA repair pathways exist in all organisms, from bacteria to humans, to preserve the integrity of the genome [1]. If not repaired, damaged bases could be mutagenic [2] and/or cause cell death by blocking DNA replication [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 cleavage of the base by a DNA glycosylase [1-5]. Monofunctional DNA glycosylases, such as *N*-methylpurine-DNA glycosylase (MPG) and uracil-DNA glycosylase, use an activated water molecule as a nucleophile to generate an apurinic or apyrimidinic (AP) site in DNA. Mammalian MPG is known to excise at least 17 structurally diverse modified bases from DNA [6]. These lesions include 3-alkylpurine, 7-

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alkylguanine, 1,*N*⁶-ethenoadenine (εA), *N*²,3-ethenoguanine, and hypoxanthine (Hx), all of which are purine derivatives [7–12]. Recently, we showed that Mg²⁺ could inhibit MPG's activity by abrogating its substrate binding and decreasing the active enzyme concentration, and the Mg²⁺-mediated inhibition might be considered as a regulator for balanced BER as all the downstream BER enzymes, other than MPG, require Mg²⁺ for their optimum repair activity [13].

Besides the importance of MPG, most of the enzymatic and structural properties, which are currently available in literature, are primarily for the truncated protein. The major reasons include extremely poor expression of full-length hMPG, which made its purification extremely difficult, and so the latter required a multi-step process [10]. Also, we have 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 amino and carboxyl termini, respectively, and wild-type enzyme had similar kinetic and binding properties for εA [7]. Since then, there were several reports on the crystallographic structures of similarly truncated protein in complex with εA or control DNA [14–16]. But our recent studies indicate that the structural information on εA-truncated MPG complex is not sufficient and does not reveal the full scenario for other DNA adducts including Hx [17,18].

Moreover, we have reported that we could easily express and purify ΔExon 1 hMPG in large amounts from *E.coli* [17]. Therefore, in this study we have optimized the codon within the 1st exon sequence of hMPG to the best used codons for *E.coli* without changing the amino acid residues. The codon optimized sequence has improved the expression and purification process significantly, so that we could purify full-length hMPG in a single step. The purified active protein showed similar properties as reported earlier [10] but with improved thermal stability. In the future, this expression and purification system could be used for large scale purification of full-length hMPG that may lead to the crystallographic and spectroscopic studies which require active and stable protein in large amounts.

Materials and Methods

Construction of expression plasmids for full-length codon-optimized human MPG

Polymerase chain reaction was carried out to generate full-length hMPG using ΔExon1 hMPG expression vector, reported previously [17] as template and a set of primers which include forward primer (5'-CATATGGTGACCCCGGCGTTGCAGATGAAG-3') and the reverse primer (5'-GAATTCTCAGGCCTGTGTGTCCTGCT-3'). The forward primer contains an NdeI site at the 5' end and harbors sequences for Exon 1a with the codons (underlined) best used for translation in *E.coli*. The 3' of the PCR product ended with MPG's internal stop codon and also included an EcoRI site after the stop codon. Thus, a full-length hMPG cDNA with the codons optimized at the N-terminus was amplified. The PCR products were then subcloned in TA cloning vector, digested with NdeI and EcoRI and subcloned into expression vector pRSETB at NdeI/EcoRI sites, allowing us to express a nonfusion hMPG protein. The identity of the construct was confirmed by DNA sequencing.

Purification of full-length human MPG

E. coli BL21(DE3) carrying the construct with the hMPG full-length optimized sequence was grown in magnificent broth (MacConnell Research, CA) at 37°C until the absorbance at 600 nm reached 0.6. The culture was cooled to 25°C and, after the addition of IPTG to 1 mM, was grown at 25°C for 6 h, prior to chilling to 0°C. All subsequent procedures were carried out at 4°C. After the bacteria were harvested by centrifugation, they were resuspended in buffer A (40 mM Tris-HCl, pH 8.5, 120 mM NaCl, and 0.1% Tween-20, 1 mM DTT, 10% glycerol, 1X protease inhibitor [Complete EDTA-free protease inhibitor cocktail tablet, Roche Diagnostics,

IN]) and then sonicated (10×45 s) on ice at full power using a Braun-Sonic U. After centrifugation of the cell lysate (2×30 min at $15,000 \times g$), the supernatant was applied to ion exchange columns, SP Sepharose (1 ml) (Amersham Pharmacia Biotech, Piscataway, NJ). The columns were pre-equilibrated with buffer A. After washing with buffer A, the protein bound with SP Sepharose was eluted with a linear gradient of NaCl (120 to 550 mM) in buffer A. The electrophoretically pure peak fractions were pooled and kept at -80°C in aliquots.

Western blot analysis

Purified MPG (100 ng) was separated by SDS-PAGE (15% polyacrylamide). For Western Blot analysis, proteins were transferred to a nitrocellulose membrane, and the MPG bands were visualized with a previously characterized purified anti-human MPG monoclonal antibody (520-3A; 17) using the enhanced chemi-luminescence protocol (Amersham Life Sciences, Piscataway, NJ).

N-terminal amino acid sequencing

For direct sequencing, SDS-PAGE was performed as above and gels electro-blotted onto PVDF membrane. Membranes were stained for approximately 1 min in a freshly made and filtered 0.1% solution of Ponceau S, and destained in water until protein bands were visible. Protein bands were excised from the membrane and subjected to direct amino acid sequence analysis (Protein Core facility, UTMB, TX; 18).

Preparation of substrates

Hx containing 50-mer oligonucleotides with the sequence 5'-TCGAGGATCCTGAGCTCGAGTCGACG~~X~~TCGCGAATTCTGCGGATCCAAGC-3' (where X represents Hx) was purchased from 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 polyacrylamide sequencing gel. The Hx oligonucleotide was labeled at the 5' end using T4 polynucleotide kinase and $\gamma^{32}\text{P}$ -ATP and annealed to complementary oligonucleotide to prepare ^{32}P -end-labeled duplex oligonucleotide as described previously [13].

MPG-mediated excision activity assay

The hMPG protein (15-30 nM) was incubated with 1 nM oligonucleotides in the presence of buffer that differed in pH (5.5-9.6) and ionic strength (15-200 mM KCl). The standard reaction mixtures contained 1 mM DTT, 10 $\mu\text{g/ml}$ nuclease-free BSA, and 10% glycerol with different pH or ionic strength in a total volume of 20 μl . Incubation was at 37°C for 10 min, if not otherwise stated. The reaction was stopped by inactivating the enzyme at 75°C for 5 min, and the products were analyzed as described previously [13]. For the thermal stability study, 300 nM proteins were kept at 50°C , and at different time points (0-30 min) aliquots were transferred to separate assay tubes. Mixtures containing 30 nM hMPG and 1 nM Hx-oligonucleotide substrate were subjected for the activity assay described previously [13].

Steady-state kinetic studies

The full-length hMPG (5 nM) was incubated with 5'- ^{32}P -labeled Hx-containing duplex oligonucleotide (0-20 nM) substrates for 3 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

To further characterize the newly purified full-length hMPG, we examined its binding with Hx-oligo using a Biacore-T100 (Biacore, Uppsala, Sweden). A 50-mer duplex oligonucleotide containing an Hx 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 (13). Then, we measured the binding parameters of full-length hMPG (0-7.5 nM) using a binding buffer (10 mM HEPES-KOH pH 7.6, 150 mM KCl and 0.5 % surfactant) at 5°C. The MPGs at various concentrations were injected, and the surface plasmon resonance units were measured with 60 sec injection. Following each injection, the chip was regenerated with 1M NaCl. The binding kinetics for oligonucleotides containing Hx was 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.

Results

Purification of full-length human MPG

We replaced the original codons (2nd, 4th and 5th) of hMPG with codons best used in *E.coli* (Fig. 1) and found an improved expression of hMPG protein. The full-length hMPG protein was then purified to near homogeneity as shown by SDS-PAGE (Fig. 2A). From 4 L of *E.coli* culture we have purified 6.4 mg protein with an apparent purity of ~95% revealed by coomassie blue staining. The recovery and purification fold were 80% and ~125, respectively (Table 1). The high isoelectric point (pI) of MPG (pI=9.65) allowed us to run an SP-sepharose column under a reasonably high pH of 8.5 where most of the *E.coli* proteins do not bind effectively to the beads.

Western blot analysis and N-terminal amino acid sequencing

We previously characterized a purified moAb 520-3A that recognizes specifically the sequence corresponding to the residues 156 to 246 in the hMPG coding sequence (17). In western blot analysis, this antibody (520-3A) has cross-reacted with purified full-length hMPG (Fig. 2B). Further, the N-terminal sequencing results were similar to what could expect from the coding sequence [10], and thus, confirmed the identity of this protein.

MPG activity assay

The activity of the purified hMPG was measured in the presence of oligonucleotides in buffers adjusted to different pHs (5.5-9.6) (Fig. 3A) and different ionic strength (15-200 mM KCl) (Fig. 3B). Like in earlier reports by others, the hMPG protein showed an optimum activity at pH 7.5 and an ionic strength of 100 mM KCl (10). Most importantly, the protein showed very high thermal stability. At 50°C even 30 minutes of incubation inactivated the protein by only 30%. Thus, contrary to the previous report [10], this newly purified full-length hMPG is stable (Fig. 3C).

Steady-state kinetic study

In a previous study we measured the steady-state kinetic parameters for full-length mouse MPG. Likewise, we measured this for hMPG. The steady-state enzyme kinetics showed an apparent K_m of 5.3 nM and k_{cat} of 0.2 min⁻¹ of hMPG for Hx cleavage reaction.

Hx binding studies using surface plasmon resonance

To further characterize full-length hMPG, we examined the MPG-Hx binding using a Biacore-T100 (Biacore, Uppsala, Sweden) (Fig. 4A). Our results showed an equilibrium binding

constant (K_D) of 0.25 nM (Fig. 4B) for this full-length human protein, similar to that of full-length mouse MPG (18).

Discussion

We have shown before by systematic deletion analysis of MPG from N- and C-termini that a minimally sized polypeptide (N Δ 100C Δ 18) lacking 100 and 18 amino acid residues from the amino and carboxyl termini, respectively, and wild-type enzyme had similar kinetic and binding properties for ϵ A [7]. Since then, there were several reports on the crystallographic structures of similarly truncated protein in complex with ϵ A or control DNA [14–16]. But we recently showed that the structural information on ϵ A-truncated MPG complex is not sufficient and does not reveal the full scenario for other DNA adducts including Hx [19]. The N-terminally truncated MPG showed less activity compared to the full-length protein towards Hx substrate. Also, with the help of a highly characterized monoclonal antibody with an epitope in the N-terminal tail, we recently showed that the latter appears to have an important role in substrate discrimination, however, with a differential effect on different substrates [17].

In another previous study with a recombinant chimeric protein containing N- and 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]. Others also showed that the first 70 amino acid residues of hMPG were indispensable for 1,N²- ϵ G excision reaction [20].

Thus, these results overall 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. Importantly, the apparent reason of using the truncated protein was largely due to the instability of the human protein, and the latter resulted from extremely poor expression and thus an inefficient purification process. However, while purifying Δ Exon1 hMPG, we realized that one could improve the expression by optimizing the Exon 1 codons for *E. coli*. Indeed, the codon optimization resulted in ample protein expression. Moreover, the advantageous high pI value (9.65) of hMPG allowed us to select a strong cation-exchange (SP-Sepharose) column and optimum buffer systems, so that most of the *E. coli* protein did not bind to the column, whereas a single linear gradient could purify hMPG in apparent homogeneity in SDS-PAGE. The protein is also highly stable, and thus it can be used in further structural studies, such as X-ray crystallography or biophysical analysis.

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Abbreviations

BER

base excision repair

MPG

N-Methylpurine DNA-glycosylase

Hx

εA

Hypoxanthine

1,N⁶ethenoadenine

WT Sequence:

ATG²GTCACC⁴CCC⁵GCTTTGCAGATGAAG
Val Pro Ala

Codon-optimized Sequence:

ATG²GTGACC⁴CCG⁵GCGTTGCAGATGAAG
Val Pro Ala

Figure 1. Nucleotide sequences of WT and codon-optimized first 5 amino acid residues of hMPG

Figure 2.

(A) Purification of full-length hMPG. The full-length hMPG protein was purified to near homogeneity revealed by coomassie blue staining. The details of the purification are described in “Materials and Methods.” **(B) Western Blot analysis of MPG by monoclonal antibody.** The details of the hybridization conditions are described in “Materials and Methods.”

Figure 3. Properties of the purified hMPG

Dependence of MPG activity on pH (A) and KCl concentration (B) and its thermal stability (C). The relative activity is defined as the ratio of the hypoxanthine cleavage activity at optimum pH or of KCl concentrations to the similar activity at different pH and various concentrations of KCl, whereas the remaining activity is defined as the ratio of hypoxanthine cleavage activity without incubation at 50°C to the similar activity observed following incubation at a given time. Data represent mean values with standard error derived from three independent experiments.

B

k_{on} ($M^{-1}sec^{-1}$)	k_{off} (sec^{-1})	K_D (nM)
$(4.67 \pm 0.09) \times 10^7$	0.0117 ± 0.0002	0.25 ± 0.01

Figure 4. Langmuir Isotherm of full-length hMPG binding to 50-mer biotinylated oligonucleotides containing Hx using Biacore T100

(A) Binding kinetic parameters were obtained using various concentrations of full-length of hMPG (0, 0.47, 0.94, 1.88, 3.75, 7.5 nM). The line for the 0 nM hMPG has merged with the X-axis as blank was subtracted from each run (B) Data represent mean values with standard error derived from triplicate experiments.

Table 1
Purification of full-length hMPG after over expression from *E.coli*.

Purification Step	Total Protein (mg)	Total activity (IU [*])	Specific Activity (IU/mg)	Yield (%)	Purification fold
Crude Extract	1000	80,000	80	100	1
SP-Sepharose	6.4	64,000	10,000	80	125

* One International Unit (IU) is defined as the activity, which cleaves 50% of 0.5 pmole of double stranded DNA oligo substrate containing a hypoxanthine lesion in 10 min at 37°C.