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Crystallization and preliminary X-ray analysis of human MTH1 with a homogeneous N-terminus

Human MTH1 (hMTH1) is an enzyme that hydrolyses several oxidized purine nucleoside triphosphates to their corresponding nucleoside monophosphates. Crystallographic studies have shown that the accurate mode of interaction between 8-oxoguanine and hMTH1 cannot be understood without determining the positions of the H atoms, as can be observed in neutron and/or ultrahigh-resolution X-ray diffraction studies. The hMTH1 protein prepared in the original expression system from *Escherichia coli* did not appear to be suitable for obtaining high-quality crystals because the hMTH1 protein had heterogeneous N-termini of Met1 and Gly2 that resulted from N-terminal Met excision by methionine aminopeptidase from the *E. coli* host. To obtain homogeneous hMTH1, the Gly at the second position was replaced by Lys. As a result, mutant hMTH1 protein [hMTH1(G2K)] with a homogeneous N-terminus could be prepared and high-quality crystals which diffracted to near 1.1 Å resolution using synchrotron radiation were produced. The new crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 46.36$, $b = 47.58$, $c = 123.89$ Å.

1. Introduction

Oxidative stress is considered to be one of the causes of many common diseases, including cancer. Reactive oxygen species generated under normal cellular metabolic conditions damage DNA, RNA and their precursor nucleotides (Lindahl, 1993). Among the base oxidations by reactive oxygen species, the abundantly generated 8-oxoguanine (8-oxoG) is one of the most serious base lesions (Friedberg, 1995). 8-oxoG can form a base pair with cytosine and adenine, and DNA and RNA polymerases incorporate 8-oxoG into the nascent strand opposite adenine and cytosine in the template with almost equal efficiency, so that A:T to C:G transversion occurs (Kasai, 2002). Usually, 8-oxoG in DNA is repaired by DNA glycosylases such as MutM (*Escherichia coli*) and Ogg1 (human), and adenine mispaired with 8-oxoG is removed by MutY and its homologues (Tsuzuki *et al.*, 2007). Organisms come equipped with elaborate mechanisms to prevent the misincorporation of 8-oxoG in DNA and RNA, as well as DNA-repair systems. *E. coli* MutT and human MutT homologue 1 (hMTH1; GenBank D38594) hydrolyse 8-oxo-dGTP to 8-oxo-dGMP (Maki & Sekiguchi, 1992; Mo *et al.*, 1992). MutT also hydrolyses 8-oxo-dGDP to 8-oxo-dGMP. 8-oxo-dGDP can be phosphorylated to 8-oxo-dGTP by the cellular kinase (Ito *et al.*, 2005). In humans, MTH3 (NUDT18) and NUDT5 hydrolyse 8-oxo-dGDP (Takagi *et al.*, 2012; Ishibashi *et al.*, 2003; Arimori *et al.*, 2011). Thus, the hydrolysis of mutagenic 8-oxo-dGTP and 8-oxo-dGDP by these enzymes leads to the avoidance of replicational errors; that is, spontaneous mutations are prevented. These enzymes can hydrolyse the corresponding ribonucleotides and avoid transcriptional errors; that is, abnormal RNA and protein synthesis (Taddei *et al.*, 1997; Ishibashi *et al.*, 2005). In addition to 8-oxo-dGTP, hMTH1 hydrolyses the oxidized adenine nucleotides 2-oxo-dATP, 2-oxo-ATP and 8-oxo-dATP with similar efficiency (Fujikawa *et al.*, 1999, 2001).

We have reported the crystal structures of MutT in the apo and holo forms and in a complex form with the product 8-oxo-dGMP. These structures have revealed that MutT specifically recognizes 8-oxoguanine nucleotides through a number of hydrogen bonds by a ligand-binding-induced conformational change (Nakamura *et al.*,

2010). These features are supported by the molecular-dynamics simulation method (Higuchi *et al.*, 2011). As previously mentioned, hMTH1 has a broad substrate specificity for several oxidized purine nucleotides (Fujikawa *et al.*, 1999, 2001). As MutT and hMTH1 belong to the Nudix hydrolase superfamily (Bessman *et al.*, 1996), the structural basis of their differing substrate specificity is an interesting subject.

Previously, we reported the solution structure of hMTH1 and the substrate-binding region using the NMR method and the preliminary X-ray analyses of hMTH1 in complexes with 8-oxo-dGMP and 2-oxo-dATP (Mishima *et al.*, 2004; Nakamura *et al.*, 2006). Recently, crystal structures of hMTH1 with and without 8-oxo-dGMP have been reported (Svensson *et al.*, 2011). These results revealed that hMTH1 binds 8-oxo-dGMP without any conformational change and that Asp119 or Asp120 needs to be protonated for recognition of the 8-oxoguanine base in the complex of hMTH1 with 8-oxo-dGMP. These crystals were grown at pH 4.0 (Nakamura *et al.*, 2006; Svensson *et al.*, 2011). Therefore, the crystallization of hMTH1 complexed with substrate at a neutral pH is required. Also, in order to establish the protonation site of Asp, neutron and ultrahigh-resolution X-ray diffraction studies are essential.

We attempted to obtain crystals under neutral conditions and/or with high quality, but all of the trials were unsuccessful. When the N-terminal sequence analysis of an active-site mutant was carried out (unpublished data), we found that the active-site mutant hMTH1 had heterogeneous N-termini of Met1 and Gly2 that resulted from excision of the N-terminal Met by methionine aminopeptidase (MAP) from the *E. coli* host (Takano *et al.*, 1999). N-terminal sequence analysis of wild-type hMTH1 showed the same result as that for the active-site mutant. One of the reasons why wild-type hMTH1 protein prepared from *E. coli* using the wild-type hMTH1 expression vector (pET8c/hMTH1) was unable to produce high-quality crystals seemed to be the heterogeneous N-termini of Met1 and Gly2. MAP can remove the N-terminal Met residue when the second residue is relatively small and/or uncharged. Therefore, we designed a mutant resistant to MAP, the G2K mutant, as the Lys residue is relatively large and charged. *E. coli* MutT also has Lys as the second residue and the N-terminal region of MutT has an ordered structure (Nakamura *et al.*, 2010).

Here, we report the expression, purification, characterization, crystallization and preliminary X-ray analysis of the G2K mutant of hMTH1 [hMTH1(G2K)] with a homogeneous N-terminus. Crystals of the mutant were obtained under neutral conditions and the quality of the diffraction was very high.

2. Materials and methods

2.1. Mutagenesis, protein expression and purification

The wild-type hMTH1 (156 amino acids; $M_r = 17\,951$; GenBank D38594) expression vector described previously (pET8c/hMTH1; Yakushiji *et al.*, 1997) harbours the full-length hMTH1 sequence between *Bam*HI and *Nco*I on the pET8c vector (Novagen). A plasmid harbouring the hMTH1(G2K) mutant, named pET8c/hMTH1(G2K), was prepared using the QuikChange site-directed mutagenesis kit (Stratagene). PCR amplifications were performed using pET8c/hMTH1 as a template with appropriate primers, 5'-ATACCATGAAAGCCTCCACGCTCTATACCC-3' and 5'-GGG-TATAGAGCCTGGAGGCTTTCATGGTAT-3' (the mutation site from Gly to Lys is indicated in bold), and the PCR product was digested with *Dpn*I to eliminate the template plasmid. The introduction of the mutation into the pET8c/hMTH1(G2K) plasmid was verified by sequencing.

The pET8c/hMTH1(G2K) plasmid was transformed into *E. coli* strain BL21 (DE3) and the cells were grown in Luria-Bertani broth to an OD₆₆₀ of 0.6 at 310 K. Protein expression was induced by the addition of 0.01 mM isopropyl β -D-1-thiogalactopyranoside for 6 h and the cells were harvested by centrifugation. The hMTH1(G2K) protein was purified as described previously for the wild-type protein (Nakamura *et al.*, 2006). A DEAE-Sepharose column, ammonium sulfate precipitation, a Phenyl-Sepharose column and a HiLoad 16/60 Superdex 75 pg column were used. The purified protein was concentrated to 10 mg ml⁻¹ in 20 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 5% glycerol, 1 mM β -mercaptoethanol (Fig. 1).

2.2. Amino-acid sequence analysis and enzyme assays

N-terminal sequence analyses of the purified wild-type and mutant hMTH1s were performed using an Applied Biosystems Precise Sequencer.

In enzyme-activity assays, the reaction mixture consisted of 5 nM enzyme, 10 μ M 8-oxo-dGTP (TriLink BioTechnologies), 80 μ g ml⁻¹ bovine serum albumin, 4 mM MgCl₂, 4 mM NaCl, 8 mM DTT, 10% glycerol and 20 mM Tris-HCl pH 8.0 with minor modifications as described by Sakai *et al.* (2002). The reaction was carried out at 303 K for 5 min and was terminated by adding SDS (1% final concentration). After the hydrolytic reaction, nucleotides were separated by HPLC using a Wakopak Handy ODS column (4.6 $\times$ 250 mm; Wako Pure Chemical Industries Ltd, Osaka, Japan) equilibrated with 0.1 M KH₂PO₄/K₂HPO₄ pH 7.0 containing 10% methanol. Nucleotides were quantified by measuring the area of ultraviolet absorbance at a wavelength of 293 nm (Bialkowski & Kasprzak, 1998).

2.3. Crystallization and data collection

Initial crystallization screening of hMTH1(G2K) and the 8-oxo-dGTP complex [hMTH1(G2K)-8-oxo-dGTP] was carried out by the hanging-drop vapour-diffusion method at 288 K using the Wizard I, Wizard II, Wizard III and Precipitant Synergy kits (Emerald BioSystems). 0.5 μ l protein solution, 0.5 μ l 10 mM 8-oxo-dGTP solution (dissolved in distilled water) and 1 μ l reservoir solution were mixed and the mixture was equilibrated against 0.3 ml reservoir

Figure 1
17% SDS-PAGE of purified hMTH1(G2K). Lane M, molecular-mass markers (labelled in kDa); lane 1, 100 μ g purified hMTH1(G2K). The gel was stained with Coomassie Brilliant Blue.

solution. Single crystals were grown over a month under several neutral pH conditions (pH 6.5–8.0). Based on these conditions, the types of buffers and the concentrations of salts were optimized. Large single crystals were obtained by streak-seeding over 1–2 d using a reservoir solution consisting of 1.0 M sodium citrate, 0.1 M cacodylate–HCl pH 6.5, 0.2 M NaCl (Fig. 2, Table 1) or 1.0 M sodium citrate, 0.1 M Tris–HCl pH 7.0, 0.2 M NaCl.

These crystals were transferred into a cryoprotectant solution composed of the reservoir solution containing 20% glycerol and were flash-cooled in a nitrogen stream at 100 K. Diffraction data were collected at 100 K on beamlines BL44XU and BL41XU at Spring-8, Harima, Japan and on beamline NW12A at the Photon Factory Advanced Ring, Tsukuba, Japan. All data were processed, integrated and scaled with *HKL-2000* (Otwinowski & Minor, 1997).

3. Results and discussion

The expression of hMTH1(G2K) was more effective than that of wild-type hMTH1. The protein was purified to near-homogeneity (Fig. 1). Typically, 10–12 mg hMTH1(G2K) was obtained per litre of culture, which was a higher yield than that of the wild type (1.5–3 mg). To check the N-terminal sequence of the purified hMTH1(G2K), an amino-acid sequence analysis was carried out. The first six N-terminal amino-acid residues were determined to be MKASRL without any detectable alterations. Each amino acid was detected at an amount of 10 to 50 pmol in each cycle. The limit of detection was about 0.1 pmol. This showed that hMTH1(G2K) is homogeneous, having a unique N-terminal residue. The 8-oxo-dGTPase activity assay confirmed that hMTH1(G2K) and wild-type hMTH1 exhibit quite similar activities; their specific activities (one unit of enzyme activity produces 1 pmol of 8-oxo-dGMP per min) were $1.7 (3) \times 10^4$ and $1.8 (4) \times 10^4$ units μg^{-1} , respectively.

We obtained crystals of hMTH1 in a new crystal form under neutral pH conditions using the N-terminal mutant hMTH1(G2K) as a homogeneous crystallization sample. The crystal form of hMTH1(G2K) differed from all of the other previously reported crystal forms of wild-type hMTH1 (Nakamura *et al.*, 2006). Moreover, the crystals of hMTH1(G2K) were highly suitable for high-resolution structure determination in comparison with the wild-type hMTH1

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
Beamline	BL44XU, SPring-8
Wavelength (Å)	0.9
Beam size (μm)	50 × 50
Detector	DIP-6040
Crystal-to-detector distance (mm)	210
Oscillation range (°)	0.5
Images	360
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 46.36, b = 47.58, c = 123.89$
Resolution (Å)	50.00–1.22 (1.24–1.22)
Unique reflections	81536 (3965)
Multiplicity	6.4 (5.6)
Completeness (%)	99.5 (98.2)
$\langle I/\sigma(I) \rangle$	57.4 (8.39)
$R_{\text{merge}}^\dagger$ (%)	5.7 (37.4)
Current refinement	
Resolution range (Å)	43.44–1.222 (1.254–1.222)
Used reflections	81421 (5807)
No. of atoms	Protein, 2574; nucleotide, 164; water, 464
Completeness (%)	99.3 (96.4)
$R_{\text{work}}^\ddagger/R_{\text{free}}^\S$ (%)	16.2 (15.0)/18.7 (19.6)
R.m.s.d. in bond lengths (Å)	0.013
R.m.s.d. in angles (°)	1.77

$^\dagger R_{\text{merge}} = 100 \times \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean value of $I_i(hkl)$. $^\ddagger R_{\text{work}} = 100 \times \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$. $^\S R_{\text{free}}$ was calculated from the test set (5% of the total data).

crystals, which diffracted to a highest resolution of ~ 1.8 Å (Nakamura *et al.*, 2006; Svensson *et al.*, 2011). The best data set was obtained from the crystal at pH 6.5 and was collected to a resolution of 1.22 Å on beamline BL44XU at SPring-8. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 46.36, b = 47.58, c = 123.89$ Å. There are two complexes in the asymmetric unit, with a volume per unit molecular weight of the protein of $1.9 \text{ Å}^3 \text{ Da}^{-1}$ and a calculated solvent content of 36% (Matthews, 1968). The data-collection statistics for the crystal at pH 6.5 are given in Table 1.

Molecular-replacement searches were carried out with *MOLREP* (Vagin & Teplyakov, 2010) using our refined structure of the complex of wild-type hMTH1 with 8-oxo-dGMP (unpublished data) as a search model, and one clear solution was obtained with an initial R factor of 45.8%. The $2F_o - F_c$ map after an initial cycle of refinement ($R = 30.7\%$ and $R_{\text{free}} = 33.3\%$) using *CNS* (Brünger *et al.*, 1998) at

Figure 2

X-ray diffraction images of an hMTH1(G2K) crystal grown at pH 6.5. (a) Diffraction image. The red concentric circles indicate the resolution rings. (b) Close-up of high-resolution shells. (c) Crystals of hMTH1(G2K) in complex with 8-oxo-dGTP.

1.8 Å resolution showed ambiguous density for 8-oxo-dGTP and the mutated N-terminal region from Met1. This feature is the same as was found for the isomorphous crystal grown at pH 7.0. In this crystal form, two molecules in the asymmetric unit interact with each other through their mutated N-terminal regions, and this interaction seems to contribute to the crystal packing related to high resolution. The current refinement statistics for the crystal at pH 6.5 are given in Table 1.

The recognition scheme of 8-oxo-dGTP at pH 6.5 and pH 7.0 was almost identical to that observed in the crystal of the complex of wild-type hMTH1 and 8-oxo-dGTP obtained at pH 4.0 (unpublished data). This means that Asp119 or Asp120 needs to be protonated for recognition of the 8-oxoguanine base at neutral pH. Asp119 and Asp120 are located near negatively charged residues and are surrounded by many hydrophobic residues. In such cases, it is well known that high pK_a values are observed for Asp residues (Forsyth *et al.*, 2002). The determination of the protonation site of Asp requires neutron and ultrahigh-resolution X-ray diffraction studies. Preparation of large crystals for neutron diffraction is in progress. To obtain high-quality crystals, crystallization in space is also being attempted.

The conformations of the β - and γ -phosphates of 8-oxo-dGTP in the crystals of hMTH1(G2K) at neutral pH conditions were different from those in the wild-type crystal at pH 4.0. The β - and γ -phosphates at neutral pH were located nearer to the Nudix motif. We investigated whether or not 8-oxo-dGTP was hydrolysed to 8-oxo-dGMP in the crystal at pH 6.5 when the crystal was soaked in 50 mM Mn^{2+} solution at pH 7.4. A crystallographic analysis of a crystal soaked in 50 mM Mn^{2+} solution for 2 d showed that 8-oxo-dGTP hydrolysis occurred in the crystal, although hydrolysis did not occur in the wild-type crystal at pH 4.0.

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