Characterization of an 8-Oxoguanine DNA Glycosylase from Methanococcus jannaschii

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A thermostable 8-oxoguanine (oxoG) DNA glycosylase from Methanococcus jannaschii has been expressed in Escherichia coli, purified, and characterized. The enzyme, which has been named mjOgg, belongs to the same diverse DNA glycosylase superfamily as the 8-oxoguanine DNA glycosylases from yeast (yOgg1) and human (hOgg1) but is substantially different in sequence. In addition, unlike its eukaryotic counterparts, which have a strong preference for oxoG-C base pairs, mjOgg has little specificity for the base opposite oxoG. mjOgg has both DNA glycosylase and DNA lyase (β-elimination) activity, and the combined glycosylase/lyase activity occurs at a rate comparable with the glycosylase activity alone. Mutation of Lys-129, analogous to Lys-241 of MutM from E. coli, abolishes glycosylase activity.

8-Oxoguanine (oxoG) is a prevalent lesion caused by oxidizing agents or ionizing radiation and is highly mutagenic (1, 2). DNA glycosylases with specificity for oxoG have been characterized from bacterial and eukaryotic species (3–11). Interestingly, the enzymes do not seem to belong to the same family, rather appearing to be an example of convergent evolution toward a similar function. The oxoG DNA glycosylases identified from yeast and mammals (5–11) belong to a superfamily of sequence-related DNA glycosylases whose members have a wide range of specificities. In contrast, the prokaryotic enzymes, as typified by E. coli MutM (Fpg) (3, 4), appear to comprise their own distinct sequence family, requiring zinc for 8-oxoguanine-DNA glycosylase activity (12).

As part of a study of DNA glycosylase sequences, we recently screened several new DNA glycosylase superfamily homologs for base excision activity. In the course of that screen, we found that Methanococcus jannaschii open reading frame MJ0704 has oxoG DNA glycosylase activity. The specificity of this enzyme for oxoG (and for a second guanine oxidation product, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG)) was established using gas chromatography-mass spectrometry to analyze bases released by the enzyme following treatment of oxidized DNA. Because this enzyme appears to be the first archaeal oxoG DNA glycosylase to be identified, it is important to investigate its properties in detail.

EXPERIMENTAL PROCEDURES

Protein Purification—The mjOgg gene was cloned as described previously (2). The gene was expressed in E. coli as a fusion to maltose-binding protein using the vector pMal-c2 (New England Biolabs). The fusion polypeptide contains a Factor Xa cleavage site that is expected to produce mjOgg with two extra residues (Ile and Ser) at the N-terminal end. The fusion protein was expressed in the mutM– strain NR12389 (provided by Dr. Roel M. Schaaper). The cells were grown in rich broth to mid-log phase and induced with 0.3 mM isopropyl-1-thio-
β-D-galactopyranoside for 3 h. The cell pellet was collected, resuspended in Buffer A (0.5 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol) with the addition of protease inhibitors (Pefabloc SC, leupeptin, and pepstatin), and frozen at −20 °C overnight. The cells were lysed by sonication. The fusion protein was purified by affinity chromatography of the crude bacterial lysate on an amylose column, eluted with 10 mM maltose in Buffer A, and then cleaved using Factor Xa protease (≈12 µg/mg of protein for 42 h at room temperature). The digestion mixture was heated to 65 °C for 50 min, which effectively precipitates any uncleaved fusion protein. Following centrifugation to remove precipitated protein, mjOgg was diluted 5-fold to reduce the salt concentration and was applied to a heparin column. mjOgg was then eluted with Buffer A (0.5 mM NaCl) in an essentially homogeneous form. mjOgg was stored at −20 °C in Buffer A plus 10% glycerol at a concentration of 40 µg/ml.

Substrate Preparation—The two complementary oligonucleotides synthesized were d(5′-GTGACGATCTGCTTCTGCGCCTTCTGCGAGCC-3′) and d(3′-CAGTGCTAGCTCGAGCGAAGACGCCTCGGT-5′), where X stands for A, C, G, or T and Q stands for oxoG. The deprotection of oligomers containing oxoG was supplemented with 0.25 mM β-mercaptoethanol. The oxoG-containing oligonucleotide was 5′-end-labeled using polynucleotide kinase and [γ-32P]ATP and purified with Amersham Pharmacia Biotech G-25 microspin columns. The duplexes were annealed in buffer containing 20 mM Tris, pH 7.4, 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 3% glycerol by heating to 75 °C for 5 min and then cooling to room temperature.

Glycosylase and Lyase Assays—Unless otherwise noted, the substrates were incubated with mjOgg at 37 or 50 °C in 20 mM Tris, pH 8.5, 80 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. The final concentrations were 8 nM substrate and 0.8 nM mjOgg (10:1 ratio). To measure glycosylase activity (production of abasic DNA), aliquots were treated with 1 M piperidine and incubated at 98 °C for 30 min to cleave the DNA strand at the abasic site. To measure lyase activity (production of backbone-cleaved DNA), aliquots were removed and the reaction was stopped by extraction with phenol/chloroform. All mixtures were ethanol-precipitated, air-dried, resuspended in 80% formamide loading buffer, and electrophoresed on a denaturing gel. The gels were quantitated using a PhosphorImager. The fraction of cleaved DNA was calculated from the intensity of the substrate and product bands. A correction was made for the amount of the substrate that comigrates with the product band in the absence of enzyme (typically ~5% for lyase and ~10% for glycosylase assays). This high background precluded the use of substantially higher substrate:enzyme ratios. Initial reaction rates were obtained by fitting to a modified Michaelis-Menten equation that included an exponential decay term to model the loss of enzyme activity. The pH Profile—The effect of pH was examined using 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 3% glycerol and one of the following: (a) 30 mM MES, pH 6.4; (b) 20 mM Hepes, pH 7.15; (c) 20 mM Tris, pH 7.3; (d)
The enzyme was added to the substrate in a total volume of 50 μM.

Thermal denaturation was performed using a Peltier device and the K129S mutant using an Aviv CD60 spectropolarimeter.

The protein was overexpressed and purified as described for the wild-type protein.

The protein was preincubated with 0, 100, and 500 μg/ml bovine serum albumin for 5 and 30 min in a total volume of 95.5 μl. The substrate was added at the start of the reaction to a concentration of 8 nM.

Circular Dichroism

The raw data were transformed into fraction unfolded and are presented as a function of temperature. The curve was fit to the data assuming a two-state unfolding model.

The K129S mutant was constructed using site-directed mutagenesis by combining polymerase chain reaction fragments that overlap in sequence. Primers 1 and 2 produce the 5'-3' fragment of the final polymerase chain reaction product was inserted into the pMal-c2 vector, and its sequence was verified.

Mutagenesis—The K129S mutant was constructed using site-directed mutagenesis by combining polymerase chain reaction fragments that overlap in sequence. Primers 1 and 2 produce the 5'-3' fragment of the final polymerase chain reaction product was inserted into the pMal-c2 vector, and its sequence was verified.

The protein was overexpressed and purified as described for the wild-type protein.

Circular Dichroism—Circular dichroism data were obtained for mjOgg and the K129S mutant using an Aviv CD60 spectropolarimeter. Thermal denaturation was performed using a Peltier device temperature controller at a rate of 2 °C/min at 222 nm.

RESULTS

mjOgg was readily purified as a fusion protein, proteolytically cleaved, and purified to homogeneity by preferential heat denaturation and heparin chromatography. As expected for a protein from a thermophilic organism, mjOgg is thermally stable, denaturing at 93 °C (Fig. 1).

However, the enzyme loses activity under normal assay conditions. This apparent loss of activity is not due to depletion of substrate because the extent of the reaction is similar even when the DNA concentration is increased 10-fold. It also does not appear to be due to product inhibition because incubation of the enzyme under assay conditions in the absence of substrate led to the complete loss of activity within 1 h (data not shown). To obtain rate constants from these data, the time courses were fit with the assumption that the loss in activity follows an exponential decay once it is diluted into the assay mix. Testing under a variety of conditions (see “Experimental Procedures”) indicated that the protein is more stable in the presence of bovine serum albumin and DNA and less stable in high levels of salt (200 mM NaCl). The protein as purified and stored is quite stable, however. Incubation of the enzyme stock for 3 h at 50 °C decreased the activity by only one-third (data not shown). The enzyme activity is optimal near pH 8.5 (Fig. 2).

The enzyme is 6-fold more active at 50 °C than at 37 °C (Fig. 3). It seems probable that the enzyme is active at significantly higher temperatures, but this was not investigated because of concern for the denaturation of substrate. At both 37 and 50 °C, the rate of the combined glycosylase/lyase activity is very similar to that of the glycosylase activity alone, indicating that the lyase reaction is fast once the base has been excised (Fig. 3).

This is consistent with a proposed mechanism for bifunctional glycosylases like mjOgg (14, 15). In this proposal, a lysine common to this subgroup of enzymes (Lys-129 in mjOgg) displaces the base by nucleophilic attack and, in a concerted reaction, forms a Schiff base with the ring-opened sugar that promotes β-elimination and cleavage of the backbone. To verify the importance of Lys-129, it was mutated to serine, and the mutant protein was assayed for DNA glycosylase activity toward both oxoG-C and oxoG-A base pairs. No activity was detected on either strand. The loss of activity is probably not due to disruption of the structure because the thermal denaturation properties of the mutant are nearly the same as those of the wild type (data not shown).

mjOgg was assayed for glycosylase/lyase activity against all four oxoG-N substrates. Unlike other oxoG DNA glycosylases, mjOgg has little specificity for the base opposite oxoG (Fig. 4).

Although there is a slight preference for pyrimidines over purines, the strong preference for oxoG-C base pairs that characterizes yeast and hOgg1 was not observed (7, 12).

DISCUSSION

**M. jannaschii** is a hyperthermophilic methanogen and a member of the euryarchaeotal kingdom of the Archaea. It was originally isolated from a deep sea vent and grows optimally at 85 °C and at pressures up to 200 atm. The entire genome sequence of *M. jannaschii* has been determined, making it the first archael organism to be sequenced in its entirety (16).

mjOgg was originally identified in a screen of several novel DNA glycosylase homologs whose specificity was not readily predicted from considerations of sequence similarity. Fig. 5 illustrates the point that mjOgg is very distantly related to the other known oxoG-specific enzymes that belong to the same glycosylase superfamily. Indeed, the degree of sequence similarity between mjOgg and its eukaryotic counterparts is not dramatically greater than between mjOgg and other members of the superfamily that have very different specificity. As a result, conventional sequence clustering methods fail to group mjOgg with eukaryotic homologs of similar specificity. However, principal component analysis of the sequence family does cluster mjOgg properly with its eukaryotic counterparts.

mjOgg differs from the eukaryotic oxoG DNA glycosylases not only within the common catalytic domain but also in lacking an extended segment N-terminal to this domain (Fig. 5).
The existence of this N-terminal sequence in the eukaryotic enzymes and its absence in mjOgg may be related to differences in specificity. In particular, the N-terminal region of the eukaryotic enzymes could be responsible for their specificity for C opposite the excised oxoG. There is precedence for this suggestion; we have recently shown that MutY, an adenine DNA glycosylase belonging to the same superfamily as mjOgg, uses a C-terminal domain unique to its subfamily to confer opposite-base specificity for oxoguanine over guanine (13).

We have established that mjOgg is an authentic DNA lyase as well as a DNA glycosylase. The lyase reaction probably proceeds through $\beta$-elimination, as has been shown for related enzymes, because the product of the lyase reaction has slightly slower electrophoretic mobility than the product of the piperidine-cleaved glycosylase product. This is the expected result because $\beta$-elimination leaves a 3'-phosphoribosyl moiety rather than the 3'-phosphate left by piperidine cleavage.

Lys-129 is analogous to lysines in other homologs that have been shown to be necessary for lyase activity because they form a Schiff base to the sugar (12). According to the prevailing model for the mechanism of bifunctional DNA glycosylases (14, 15), this lysine has also been proposed to be the nucleophile that cleaves the glycosylic bond. Consistent with this model, mutation to serine resulted in the complete loss of glycosylase activity. However, this experiment, and similar ones in the literature, should be interpreted with caution. The amino acid found at this position is correlated quite strongly with the substrate specificity of the enzyme; homologs that exise oxidized bases (e.g. mjOgg and endonuclease III) tend to have a lysine at this position, those that excise alkylated purines tend to have a tryptophan, and those that excise adenine mispairs tend to have a hydroxyl-containing amino acid. Thus, loss of glycosylase activity could be due to the loss of substrate recog-
nition rather than the loss of an active site nucleophile.

mjOgg adds important information to our understanding of the endoIII/MutY/AlkA superfamily because it is the first oxoG-specific glycosylase in this family to be characterized from a noneukaryotic organism. Although clearly belonging to the same family, mjOgg is remote in sequence from its eukaryotic homologs. This divergence in sequence helps to identify the residues that are most characteristic of oxoG DNA glycosylases and can provide insights into the way these enzymes recognize substrate and how they evolve. mjOgg also differs from the eukaryotic oxoG DNA glycosylases in its relative indifference to the base opposite oxoG. Further characterization of other distantly related members of the family will shed further light on the sequence features responsible for substrate specificity.

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