

# Substrate specificities of the Ntg1 and Ntg2 proteins of *Saccharomyces cerevisiae* for oxidized DNA bases are not identical

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## ABSTRACT

Two genes of *Saccharomyces cerevisiae*, *NTG1* and *NTG2*, encode proteins with a significant sequence homology to the endonuclease III of *Escherichia coli*. The Ntg1 and Ntg2 proteins were overexpressed in *E.coli* and purified to apparent homogeneity. The substrate specificity of Ntg1 and Ntg2 proteins for modified bases in oxidatively damaged DNA was investigated using gas chromatography/isotope-dilution mass spectrometry. The substrate used was calf-thymus DNA exposed to  $\gamma$ -radiation in N<sub>2</sub>O-saturated aqueous solution. The results reveal excision by Ntg1 and Ntg2 proteins of six pyrimidine-derived lesions, 5-hydroxy-6-hydrothymine, 5-hydroxy-6-hydroouracil, 5-hydroxy-5-methylhydantoin, 5-hydroxyuracil, 5-hydroxycytosine and thymine glycol, and two purine-derived lesions, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine from  $\gamma$ -irradiated DNA. In contrast, Ntg1 and Ntg2 proteins do not release 8-hydroxyguanine or 8-hydroxyadenine from  $\gamma$ -irradiated DNA. The Ntg1 and Ntg2 proteins also release 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine from damaged poly(dG-dC).poly(dG-dC). Excision was measured as a function of enzyme concentration and time. Furthermore, kinetic parameters were determined for each lesion. The results show that kinetic constants varied among the different lesions for the same enzyme. We also investigated the capacity of the Ntg1 and Ntg2 proteins to cleave 34mer DNA duplexes containing a single 8-OH-Gua residue mispaired with each of the four DNA bases. The results show that the Ntg1 protein preferentially cleaves a DNA duplex containing 8-OH-Gua mispaired with a guanine. Moreover, the Ntg1 protein releases free 8-OH-Gua from 8-OH-Gua/Gua duplex but not from duplexes containing 8-OH-Gua mispaired with adenine, thymine or cytosine. In contrast, the Ntg2 protein does not incise duplexes containing 8-OH-Gua mispaired with

any of the four DNA bases. These results demonstrate that substrate specificities of the Ntg1 and Ntg2 proteins are similar but not identical and clearly different from that of the endonuclease III of *E.coli* and its homologues in *Schizosaccharomyces pombe* or human cells.

## INTRODUCTION

Reactive oxygen species (ROS) generated in cells either as byproducts of aerobic metabolism or as a consequence of exposure to ionizing radiation and chemical oxidizing agents cause damage to DNA, producing a multiplicity of lesions (1–3). Unrepaired oxidative damage to DNA has been suggested to play a role in cancer, aging and several degenerative pathologies in humans (4–7). In the case of cancer, oxidative DNA damage presumably causes mutations which activate oncogenes or inactivate tumor suppressor genes (8). In most organisms, the repair of oxidatively damaged DNA bases is thought to be primarily mediated by the base excision repair (BER) pathway (9). The first step in the course of BER is the excision of the damaged base by a DNA *N*-glycosylase (10–12). In *Escherichia coli*, the principal activities that are involved in the recognition and removal of oxidatively damaged DNA bases are endonuclease III, endonuclease VIII and Fpg protein (13–19). These three enzymes are DNA glycosylases/AP lyases catalyzing both the cleavage of the glycosylic bond to release damaged bases and the incision of the phosphodiester backbone at the resulting apurinic/aprimidinic (AP) site via  $\beta$ - or  $\beta$ - and  $\delta$ -elimination reactions (20–22). Endonucleases III and VIII process a variety of pyrimidine-derived lesions whereas Fpg protein acts primarily at purine modifications (13–19).

In *Saccharomyces cerevisiae*, the *NTG1* and *NTG2* genes encode proteins whose amino acid sequences are closely related (41% identity, 63% similarity) to each other. Furthermore, Ntg1 and Ntg2 proteins are also related to *E.coli* endonuclease III (24% identity, 51% similarity, and 25% identity, 51% similarity, respectively) (23–27). Both proteins possess the highly conserved helix–hairpin–helix (HhH) motif containing a lysine residue at

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position 2 of the second helix, which is probably involved in the catalytic mechanism (24–28). However, the Ntg2 protein, but not the Ntg1 protein, possesses the [-C-X<sub>6</sub>-C-X<sub>2</sub>-C-X<sub>5</sub>-C-] consensus sequence for an iron–sulfur center found in most of the endonuclease III homologues (29–32). On the other hand, Ntg1 protein, but not Ntg2 protein, has a long, positively charged N-terminus that has been hypothesized to be a mitochondrial transit signal (23–27). Yeast Ntg1 and Ntg2 proteins are DNA glycosylases/AP lyases that cleave DNA containing 5,6-dihydrouracil, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (Me-FapyGua), thymine glycol (Thy gly) and AP sites (23–27). The capacity of the Ntg1 protein to incise oligodeoxynucleotides containing 8-hydroxyguanine (8-OH-Gua) mispaired with the four DNA bases has been reported (27). However, the repair of 8-OH-Gua by the Ntg1 protein was not observed in two other studies (23,26). In addition to Ntg1 and Ntg2 proteins, *S.cerevisiae* possesses a third DNA glycosylase/AP lyase coded for by the *OGG1* gene (33). The Ogg1 protein catalyses the excision 8-OH-Gua and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) from  $\gamma$ -irradiated DNA (34).

Why does *S.cerevisiae* possess two homologues of *E.coli* endonuclease III? One possibility is that Ntg1 and Ntg2 proteins may have overlapping but non-identical substrate specificities. To investigate such a possibility, we measured the excision by Ntg1 and Ntg2 proteins of modified bases from DNA  $\gamma$ -irradiated in N<sub>2</sub>O-saturated aqueous solution. We utilized the technique of gas chromatography/isotope dilution mass spectrometry (GC/IDMS) for measurement of the excision of lesions and their kinetic parameters. The substrate specificity of Ntg1 and Ntg2 proteins was also investigated using damaged poly(dG-dC).poly(dG-dC) and 34mer oligodeoxynucleotides containing Me-FapyGua or 8-OH-Gua, respectively. The results show that both Ntg1 and Ntg2 proteins release several pyrimidine-derived lesions and formamidopyrimidines. They also show that Ntg1 protein, but not Ntg2 protein, catalyses the cleavage of DNA fragments containing 8-OH-Gua mispaired with a guanine.

## MATERIALS AND METHODS

### Materials

Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Modified DNA bases, their stable isotope-labelled analogues and other materials for GC/IDMS were obtained as described previously (35). Calf thymus DNA and poly(dG-dC).poly(dG-dC) were purchased from Sigma and Boehringer, respectively. Yeast Ogg1 protein was purified as described (28). Restriction endonucleases, DNA polymerases and T4 DNA ligase were from New England Biolabs and Boehringer.

### Preparation of DNA substrate

Calf thymus DNA was dissolved in 10 mM phosphate buffer (pH 7.4, 0.3 mg/ml) and extensively dialysed against the same buffer. Aliquots of the DNA solution were bubbled with N<sub>2</sub>O for 60 min and irradiated with  $\gamma$ -rays in a <sup>60</sup>Co  $\gamma$ -source at a dose of 80 Gy (dose rate of 45 Gy/min). Subsequently, DNA solutions were dialysed against 10 mM phosphate buffer for 18 h at 4°C.

Phosphate buffer was changed three times during the course of dialysis (36).

### PCR cloning of *NTG1*

Plasmid pUC19-NTG1 (23) (a kind gift of Dr Magnar Bjoras, Oslo, Norway) was used as a template in a PCR reaction to amplify the *NTG1* gene. Primer 1: 5'-CCGGAATTCATGCAAA-AGATCAGTAAATAC-3'; primer 2: 5'-CACTCTGCAGTTAG-TCCTCTACTTTAACAGAAA-3'. The amplified DNA fragment containing *NTG1* was incubated with *EcoRI* and *PstI* restriction enzymes and cloned into plasmid pKK-223-3 (Pharmacia) previously digested with *EcoRI* and *PstI* restriction enzymes yielding the pNTG1-1 plasmid.

### PCR cloning of *NTG2*

*Saccharomyces cerevisiae* genomic DNA was used as a template in a PCR reaction to amplify the *NTG2* gene. Primer 1: 5'-ACAG-TAGTCATGAGAGAGGAAAGTAGGTCTAGG-3'; primer 2: 5'-AGCCCAAGCTTCTATTTTTTCTGTGTCTTTTC-3'. The amplified DNA fragment containing *NTG2* was incubated with *BspHI* and *HindIII* restriction enzymes and cloned into plasmid pTrc99A (Pharmacia) previously digested with *NcoI* and *HindIII* restriction enzymes yielding the pNTG2-1 plasmid.

### Purification of the Ntg1 and Ntg2 proteins

*Escherichia coli* strain BH160 (AB1157 but *nth::Kan'*, *fpg::Kan'*) from our laboratory stock, harbouring pNTG1-1 or pNTG2-1, was grown at 37°C in LB broth medium containing ampicillin (100  $\mu$ g/ml). Cell cultures were grown until OD<sub>600</sub> = 0.8 and supplemented with 0.5 mM isopropyl  $\beta$ -D-thiogalactopyranoside and incubated for 3 h at 37°C. The cells (15 and 26 g for Ntg1 or Ntg2 protein preparations, respectively) were resuspended in buffer [25 mM Tris-HCl pH 7.6, 2 mM Na<sub>2</sub>EDTA, 5% (v/v) glycerol, 250 mM NaCl and 1 mM phenylmethylsulfonyl fluoride] supplemented with 0.5 mg/ml lysozyme. The lysate was centrifuged at 130 000 g for 45 min at 2°C. The supernatant was the crude extract fraction. The purification procedure for Ntg1 and Ntg2 proteins includes a QMA anion exchange column (Waters-ACELL) to separate nucleic acids from proteins followed by a Phospho-Ultrogel cation exchange column (IBF-LKB) and an AcA44 gel filtration column (IBF-LKB). For Ntg1 protein, the purification was terminated after an FPLC MonoQ HR5/5 chromatography (Pharmacia). For Ntg2 protein, the purification was terminated by double-stranded DNA cellulose (Sigma) and FPLC MonoS HR5/5 chromatographies. The Ntg1 and Ntg2 proteins were purified to apparent homogeneity and stored at -20°C in a buffer containing 25 mM Tris-HCl (pH 7.6), 2 mM Na<sub>2</sub>EDTA, 100 mM NaCl and 30% glycerol (v/v) at a final concentration of 0.24 and 0.32 mg protein/ml, respectively. Protein concentration was determined by the method of Bradford (37).

Both Ntg1 and Ntg2 proteins were purified using the excision of [<sup>3</sup>H]Me-FapyGua as an activity assay. The [<sup>3</sup>H]Me-FapyGua-poly(dG-dC).poly(dG-dC) substrate was prepared as previously described (38). The assay mixture (100  $\mu$ l) contained 25 mM Tris-HCl (pH 7.6), 100 mM KCl, [<sup>3</sup>H]Me-FapyGua-poly(dG-dC).poly(dG-dC) and 5  $\mu$ l of column fractions. The reaction was carried out at 37°C for 15 min. Ethanol-soluble radioactive material was quantified and the chemical nature of this material was monitored by HPLC as described (38). One unit releases

1 pmol of Me-FapyGua in 15 min at 37°C. The Ntg1 and Ntg2 proteins were also purified to apparent homogeneity from *E. coli* BL21 harbouring plasmid SCR1-pGEX-2T or SCR2-pRESETA as previously described (26).

### Enzymatic assays and GC/IDMS

Irradiated and control DNA samples (100 µg) were dried in a SpeedVac under vacuum. DNA samples were dissolved in phosphate buffer (50 mM final concentration, pH 7.4) containing 100 mM KCl, 2 mM Na<sub>2</sub>EDTA and 2 mM dithiothreitol. Depending on the experiment, various amounts of Ntg1 or Ntg2 proteins were added to the mixture. The total volume of the mixture was 110 µl. Three replicates of each mixture were incubated at 37°C in a water bath for periods of time depending on the experiment. As controls, DNA samples were incubated with heat inactivated enzyme (140°C for 15 min) or without enzyme. The kinetic constants were determined as described (36,39). The amount of Ntg1 protein or Ntg2 protein was 2 µg/100 µg of DNA in 110 µl of the incubation mixture, corresponding to a concentration of 395 or 413 nM, respectively. Three replicates of DNA samples were incubated with or without each enzyme at 37°C. Following incubation, DNA samples were precipitated with 270 µl of cold ethanol, kept at -20°C for 2 h, and centrifuged at 10 000 r.p.m. for 30 min at 4°C. Subsequently, DNA pellets and supernatant fractions were separated.

Aliquots of stable isotope-labelled analogues of modified DNA bases were added as internal standards to pellets with known DNA amounts and to supernatant fractions. Pellets were dried under vacuum in a SpeedVac and then hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed tubes at 140°C for 30 min. The hydrolyzates were lyophilized in vials for 18 h. Supernatant fractions were freed from ethanol under vacuum in a SpeedVac and subsequently lyophilized for 18 h without prior hydrolysis. Both lyophilized supernatant fractions and hydrolyzates of DNA pellets were derivatized and analyzed by GC/IDMS (36,39).

### Assay for 8-OH-Gua/N nicking activity

The 34mer oligodeoxynucleotide used in this study [5'-GGCTTCA-TCGTTGTC(8-OHGua)CAGACCTGGTGGATACCG-3'] (40) was a kind gift of Drs A. Guy and J. Cadet, CEA-Grenoble, France. The four complementary sequences were also synthesized (Oligo-Express, France). The 8-OH-Gua containing strand was <sup>32</sup>P-labelled and annealed with each complementary sequence yielding the four possible 8-OH-Gua/N duplexes as described (40). In a standard reaction (10 µl final volume), 100 fmol of <sup>32</sup>P-labelled 8-OH-Gua/N duplex were incubated in reaction buffer (25 mM Tris-HCl pH 7.6, 2 mM Na<sub>2</sub>EDTA, 50 mM NaCl) with Ntg1 or Ntg2 proteins. The reactions were performed at 37°C for 15 min. Reactions were stopped by adding 6 µl of formamide dye and subjected to 7 M urea-20% PAGE (40).

### 8-OH-Gua DNA glycosylase assay

The assay mixture (50 µl) was as described for the nicking assay but contained 50 pmol of unlabelled 8-OH-Gua/N 34mer DNA duplexes and 2 µg Ntg1 or 0.1–1 µg Ogg1 proteins, respectively. The products of the reactions were analysed by HPLC with electrochemical detection (ECD) as described (33).

## RESULTS

### Purification of Ntg1 and Ntg2 proteins of *S.cerevisiae*

To overproduce the Ntg1 and Ntg2 proteins of *S.cerevisiae*, their coding sequences were PCR-amplified and cloned into expression vectors pKK223-3 or pTrc99A yielding plasmid pNTG1-1 and pNTG2-1, respectively. The Ntg1 and Ntg2 proteins were purified from *E. coli* BH160 (*fpg*<sup>-</sup>, *nth*<sup>-</sup>) harbouring pNTG1-1 and pNTG2-1. The release of Me-FapyGua from [<sup>3</sup>H]Me-FapyGua-poly(dG-dC).poly(dG-dC) was used as an activity assay in the course of the purification procedure. The Ntg1 and Ntg2 proteins used in this study are non-tagged proteins purified to apparent homogeneity. The purity of Ntg1 and Ntg2 proteins was assessed by the observation of a single protein band on an SDS-PAGE with a molecular weight of 43 kDa (data not shown). Moreover, N-terminal sequences of Ntg1 and Ntg2 proteins were determined and were identical to those deduced from the nucleotide sequence of *NTG1* and *NTG2*. The specific activities of Ntg1 and Ntg2 proteins for the excision of Me-FapyGua were 32 and 30 kilounits/mg of protein, respectively. The Ntg1 and Ntg2 proteins were also purified to apparent homogeneity from *E. coli* strain BL21 harbouring plasmid SCR1-pGEX-2T or SCR2-pRESETA as previously described (26).

### Excision of modified bases by Ntg1 and Ntg2 proteins from γ-irradiated DNA

To investigate the ability of Ntg1 and Ntg2 proteins to excise oxidatively damaged DNA bases, we used, as a substrate, calf-thymus DNA exposed to γ-radiation in N<sub>2</sub>O-saturated aqueous solution. Sixteen modified bases in this DNA substrate can be identified and quantified using GC/IDMS (36; Table 1). Of these lesions, Ntg1 and Ntg2 proteins both efficiently excise 5-hydroxy-6-hydrothymine (5-OH-6-HThy), 5-hydroxy-6-hydrouracil (5-OH-6-HUra), 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 5-hydroxyuracil (5-OH-Ura), 5-hydroxycytosine (5-OH-Cyt), Thy gly, 4,6-diamino-5-formamidopyrimidine (FapyAde) and FapyGua. The chemical structure of the lesions excised by Ntg1 and Ntg2 proteins is illustrated in Figure 1. The excision was demonstrated by the appearance of the lesions in the supernatant fraction of γ-irradiated DNA incubated with active Ntg1 or Ntg2 enzymes (36). Furthermore, the amounts of these lesions found in the supernatant fraction were similar to the amounts missing in the pellet fraction of the same DNA sample. Essentially no excision of the lesions illustrated in Figure 1 was observed when γ-irradiated DNA samples were incubated in the presence of heat inactivated Ntg1 or Ntg2 proteins or without enzyme (data not shown). The remaining lesions identified in γ-irradiated DNA, such as 8-hydroxyadenine (8-OH-Ade) and 8-OH-Gua, were not excised at a detectable rate from the γ-irradiated DNA by active Ntg1 or Ntg2 proteins. The same substrate specificity was determined using the Ntg1 (Scr1) protein or the Ntg2 (Scr2) protein purified to homogeneity from *E. coli* strain BL21 harbouring plasmid SCR1pGEX-2T or SCR2-pRESETA (26 and data not shown).

Figure 2 illustrates excision of 5-OH-Ura, 5-OH-Cyt and FapyAde by Ntg1 and Ntg2 proteins as a function of enzyme amounts. The levels of the lesions excised increased with the enzyme amounts approaching a plateau above 3 µg of Ntg1 or Ntg2 proteins (Fig. 2). An amount of 2 µg of Ntg1 or Ntg2 proteins/100 µg of DNA was used for all subsequent experiments.

**Table 1.** Kinetic constants for excision of pyrimidine and purine lesions from DNA exposed to  $\gamma$ -radiation in  $N_2O$  by Ntg1 and Ntg2 proteins

	$k_{cat} \cdot 10^3 \text{ (min}^{-1}\text{)}$		$K_M \text{ (nM)}$		$k_{cat}/K_M \cdot 10^5 \text{ (min}^{-1} \cdot \text{nM}^{-1}\text{)}$	
	Ntg1 (1)	Ntg2 (2)	Ntg1 (1)	Ntg2 (2)	Ntg1 (1)	Ntg2 (2)
(1) 5-OH-6-HThy	37.5±1.5 <sup>a,g</sup>	40.4±1.8 <sup>c-e,g</sup>	250±21 <sup>a,g</sup>	178±21 <sup>a,g</sup>	15.0±0.6 <sup>a,g</sup>	22.7±1.0 <sup>a,g</sup>
(2) 5-OH-6-HUra	63.5±19.7 <sup>b</sup>	43.2±4.2 <sup>c-e,g</sup>	721±263 <sup>e,g</sup>	557±67 <sup>e,g</sup>	8.8±2.7 <sup>b,g</sup>	7.8±0.8 <sup>b,g</sup>
(3) 5-OH-5-MeHyd	24.6±3.7 <sup>e,g</sup>	-	755±132 <sup>e,g</sup>	-	3.3±0.5	-
(4) 5-OH-Ura	50.9±8.0 <sup>h</sup>	23.6±2.0 <sup>f,g</sup>	997±200 <sup>e,g,h</sup>	431±60 <sup>e,g</sup>	5.1±0.8 <sup>e</sup>	5.5±0.5 <sup>e,g</sup>
(5) 5-OH-Cyt	55.9±12.7 <sup>b</sup>	17.6±0.9 <sup>f,g</sup>	1380±364 <sup>e,g,h</sup>	419±26 <sup>e,g</sup>	4.1±0.9 <sup>e</sup>	4.2±0.2 <sup>e,g</sup>
(6) Thy gly	71.6±10.5 <sup>b</sup>	16.2±1.2 <sup>f,g</sup>	3250±560 <sup>f,h</sup>	1000±95 <sup>g</sup>	2.2±0.3 <sup>f,g</sup>	1.6±0.1 <sup>f</sup>
(7) FapyAde	64.3±8.7 <sup>b</sup>	38.8±3.8 <sup>e</sup>	1305±212 <sup>g</sup>	824±110 <sup>g</sup>	4.9±0.7	4.7±0.5 <sup>g</sup>
(8) FapyGua	88.6±4.7 <sup>b</sup>	60.5±1.6	2460±182	2529±87	3.6±0.5	2.4±0.6

<sup>a</sup>Statistically different from the value in line 2.

<sup>b</sup>Statistically different from the value in line 3.

<sup>c</sup>Statistically different from the value in line 4.

<sup>d</sup>Statistically different from the value in line 5.

<sup>e</sup>Statistically different from the value in line 6.

<sup>f</sup>Statistically different from the value in line 7.

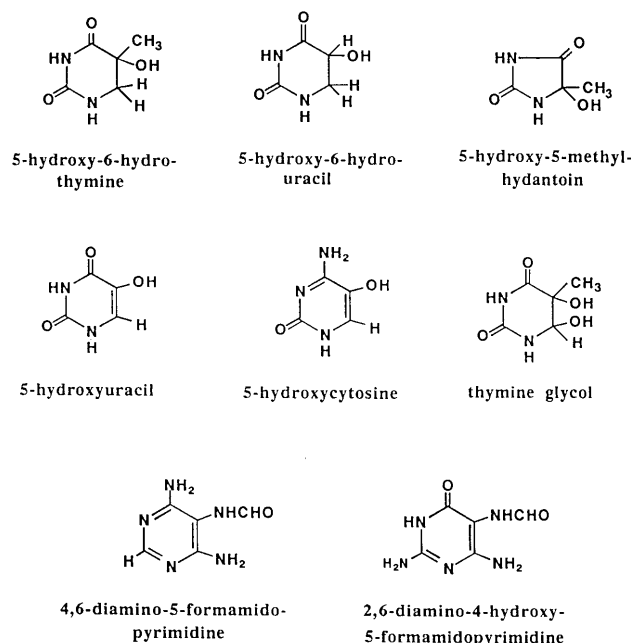
<sup>g</sup>Statistically different from the value in line 8.

<sup>h</sup>Statistically different from the value in column 2.

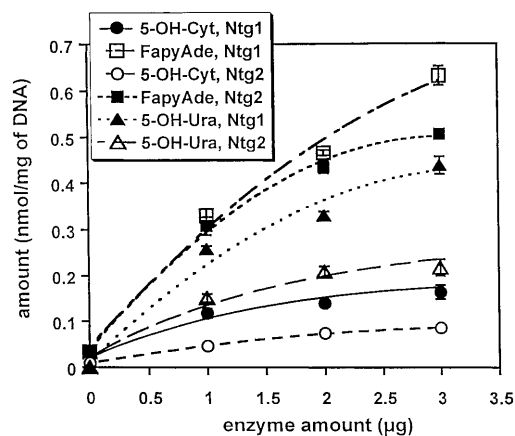
Values represent the mean  $\pm$  standard deviation ( $k_{cat} = V_{max}/[\text{enzyme}]$ , [Ntg1 protein], 395 nM; [Ntg2 protein], 413 nM. Amounts of lesions in  $\gamma$ -irradiated DNA (nmol/mg DNA): 5-OH-Ura, 1.42; 5-OH-6-HThy, 1.13; 5-OH-6HUra, 0.46; Thy gly, 1.31; 5-OH-Cyt, 0.75; 5-OH-5-MeHyd, 0.35; 5,6-diHThy, 0.46; 5,6-diHUra, 0.59; 5-OH-Hyd, 0.21; 5-OH-MeUra, 0.17; 5,6-diOHUra, 0.21; FapyAde, 1.65; FapyGua, 4.47; 2-OH-Ade, 0.05; 8-OH-Ade, 0.74; 8-OH-Gua, 3.27.

Figure 3 illustrates the time dependence of excision of FapyAde by Ntg1 and Ntg2 proteins. No additional excision was observed at times >60 min (Fig. 3). This was also true for any other lesion excised.

Kinetic parameters for the excision of lesions, from  $\gamma$ -irradiated DNA, by Ntg1 and Ntg2 proteins were measured by varying the substrate concentration (36,39). The amounts of the lesions found in supernatants were used to determine the kinetic constants. Initial velocities were estimated on the basis of the time dependency of excision. The concentration ranges of the excised lesions were: 5-OH-6-HThy, 0.11–1.02  $\mu\text{M}$ ; 5-OH-6-HUra, 0.092–0.42  $\mu\text{M}$ ; 5-OH-5-MeHyd, 0.11–0.32  $\mu\text{M}$ ; 5-OH-Ura, 0.17–1.29  $\mu\text{M}$ ; 5-OH-Cyt, 0.11–0.68  $\mu\text{M}$ ; Thy gly, 0.32–1.19  $\mu\text{M}$ ; FapyAde, 0.21–1.50  $\mu\text{M}$ ; and FapyGua, 0.97–4.07  $\mu\text{M}$ . The DNA glycosylase activity of Ntg1 and Ntg2 proteins on the excised lesions followed Michaelis–Menten kinetics. The kinetic constants and their standard deviations ( $n = 6$ ) were determined using Lineweaver–Burk plots (41) and a linear least-square analysis of the data. The kinetic parameters as well as the specificity constants,  $k_{cat}/K_M$ , are given in Table 1. The excision of 5-OH-5-MeHyd by Ntg2 protein is clearly observed but due to the low level of product released, it did not allow the calculation of kinetic parameters (Table 1). The results show that 5-OH-6-HThy and 5-OH-6-HUra are excised with the highest specificity constants by both Ntg1 and Ntg2 proteins (Table 1). Although Ntg1 and Ntg2 proteins excise the same lesions, we observed significant differences between these enzymes in terms of  $k_{cat}$  and  $K_M$  values for the excision of several lesions (Table 1). For example,  $k_{cat}$  values for the excision of 5-OH-Cyt and Thy gly by the Ntg1 protein are 3- and 5-fold higher than that for the same lesions by the Ntg2 protein (Table 1). It should be emphasized that

**Figure 1.** Structure of the substrates of the Ntg1 and Ntg2 proteins of *S.cerevisiae* identified by GC/IDMS.

these kinetic parameters represent values for enzymes purified from a bacterial overexpression system and may or may not reflect the kinetic parameters of the native enzymes expressed in yeast.



**Figure 2.** Excision of 5-OH-Ura, 5-OH-Cyt and FapyAde by Ntg1 and Ntg2 proteins as a function of enzyme amount. Calf thymus DNA  $\gamma$ -irradiated under  $N_2O$  (100  $\mu$ g) was used as a substrate for Ntg1 and Ntg2 proteins. The incubation time was 30 min. The amounts given on the y-axis represent those found in the supernatant fractions after GC/IDMS analysis. One nmol of a lesion corresponds to 32 lesions/ $10^5$  DNA bases.

### Excision of Me-FapyGua by the Ntg1 and Ntg2 proteins

The excision of Me-FapyGua by Ntg1 and Ntg2 proteins was measured using [ $^3H$ ]Me-FapyGua-poly(dG-dC).poly(dG-dC), as a substrate. The results show that both Ntg1 and Ntg2 proteins release Me-FapyGua as a free base (38 and data not shown). The kinetic constants for excision of Me-FapyGua by Ntg1 and Ntg2 proteins are very similar (Table 2). This study allowed us to compare the kinetic constants for the excision of products from substrates containing a single lesion [Me-FapyGua-poly(dG-dC).poly(dG-dC)] or a multiplicity of lesions ( $\gamma$ -irradiated DNA). Comparison of the values given for the excision by the Ntg1 protein of FapyGua (Table 1) and Me-FapyGua (Table 2) indicates similar  $k_{cat}$  values, 0.23 and 0.089  $min^{-1}$ , but very different  $K_M$  values, 10 and 2460 nM, respectively. This was also true for the Ntg2 protein. The large difference in term of  $K_M$  values probably reflects competitive inhibition between the different lesions in  $\gamma$ -irradiated DNA.

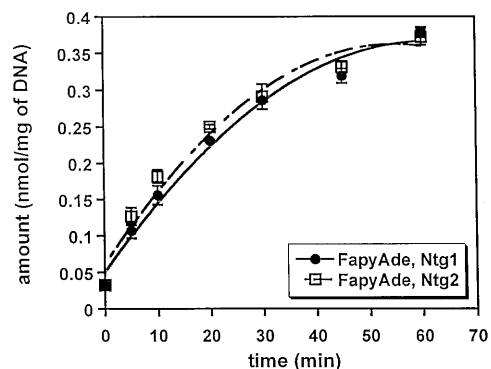
**Table 2.** Kinetic constants for excision of Me-FapyGua by Ntg1 and Ntg2 proteins of *S.cerevisiae*

Protein	$k_{cat} \times 10^3$ ( $min^{-1}$ )	$K_M$ (nM)	$k_{cat}/K_M \times 10^5$ ( $min^{-1}.nM^{-1}$ )
Ntg1	230	10	2300
Ntg2	350	21	1766

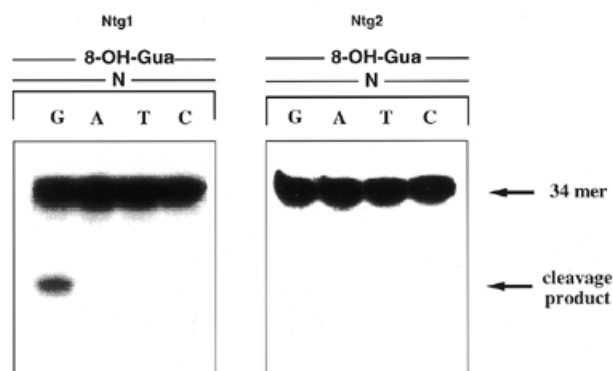
Lineweaver-Burk plot was used for the determination of kinetic constants of Ntg1 and Ntg2 proteins for the excision of Me-FapyGua. The substrate concentration [S] was given as the concentration of Me-FapyGua base.

### Excision of 8-OH-Gua from oligodeoxynucleotides containing 8-OH-Gua mispaired with guanine by the Ntg1 protein

The repair of 8-OH-Gua by the Ntg1 and Ntg2 proteins was investigated using, as substrates, 34mer oligodeoxynucleotides containing a single 8-OH-Gua placed opposite each of the four



**Figure 3.** Excision of FapyAde by Ntg1 and Ntg2 proteins as a function of incubation time at 37°C. Calf thymus DNA  $\gamma$ -irradiated under  $N_2O$  (100  $\mu$ g) was used as a substrate for Ntg1 and Ntg2 proteins. Enzyme amount was 2  $\mu$ g per assay. The amounts given on the y-axis represent those found in the supernatant fractions after GC/IDMS analysis. One nmol of a lesion corresponds to 32 lesions/ $10^5$  DNA bases.



**Figure 4.** Cleavage of 34mer oligodeoxynucleotides containing 8-OH-Gua mispaired with each of the four DNA bases by the Ntg1 and Ntg2 proteins. The 8-OH-Gua containing strand was labeled with  $^{32}P$  at the 5'-end and annealed with one of the complementary sequences carrying one of the four DNA bases opposite to 8-OH-Gua yielding 8-OH-Gua/N duplexes. These DNA duplexes (100 fmol) were incubated at 37°C for 15 min with 20 ng of Ntg1 or Ntg2 proteins. The products of the reaction were separated on denaturing 20% PAGE containing 7 M urea.

DNA bases. Figure 4 shows that the Ntg1 protein cleaves the 8-OH-Gua/Gua duplex whereas 8-OH-Gua/Thy, 8-OH-Gua/Cyt and 8-OH-Gua/Ade duplexes are not incised. Quantitative analysis reveals that the Ntg1 protein cleaves 8-OH-Gua/Gua duplex at least 10-fold more efficiently than the three other duplexes. Our results also show that the Ntg2 protein does not incise any of the four 8-OH-Gua/N duplexes (Fig. 4). It should be noted that the rate of incision of 8-OH-Gua/Gua substrate by the Ntg1 protein is 50-fold slower than that of 8-OH-Gua/Cyt by the Ogg1 protein (28 and data not shown). The marked, if not exclusive, preference of the Ntg1 protein for 8-OH-Gua/Gua substrate was observed for three sequence contexts, [Thy (8-OH-Gua) Ade], [Ade (8-OH-Gua) Ade] and [Cyt (8-OH-Gua) Cyt] (Fig. 4 and data not shown). The same results were obtained using Ntg1 and Ntg2 proteins purified from *E.coli* strain BL21

harbouring plasmid SCR1-pGEX-2T or SCR2-pRESETA (26) and a 37mer oligodeoxynucleotide containing a single 8-OH-Gua in the [Cyt, 8-OH-Gua, Ade] context (data not shown).

The capacity of the Ntg1 protein to release 8-OH-Gua as a free base was also investigated using, as substrates, the same four 8-OH-Gua/N 34mer DNA duplexes. The release of 8-OH-Gua was determined using HPLC with electrochemical detection (28,33). Table 3 shows that the Ntg1 protein releases 8-OH-Gua from 8-OH-Gua/Gua duplex but not from the three other DNA duplexes. For comparison, we have measured the excision of 8-OH-Gua from the same substrates, by the Ogg1 protein. The results confirm that Ogg1 preferentially releases 8-OH-Gua from the 8-OH-Gua/Cyt DNA duplex (28; Table 3). Furthermore, they show that the rate of excision of 8-OH-Gua from 8-OH-Gua/Gua duplex by the Ntg1 protein is 65-fold slower than that of 8-OH-Gua from 8-OH-Gua/Cyt duplex by the Ogg1 protein (Table 3).

**Table 3.** Excision of 8-OH-Gua from 34mer oligodeoxynucleotides containing 8-OH-Gua mispaired with each of the four DNA bases by Ntg1 and Ogg1 proteins

Protein	8-OH-Gua DNA glycosylase activity (kilounits/mg protein)			
	8-OH-Gua/Gua	8-OH-Gua/Ade	8-OH-Gua/Thy	8-OH-Gua/Cyt
Ntg1	0.96	<0.1	<0.1	<0.1
Ogg1	1.9	<0.2	25.5	62.0

The 34mer oligodeoxynucleotides used in this study are identical to those used in the nicking assays (Fig. 4). Each 8-OH-Gua/N duplex (100 pmol) was incubated in the presence of 2 µg of Ntg1 or 0.1–1 µg of Ogg1 for 30 min at 37°C. The products of the reactions were separated by HPLC and analyzed by ECD as previously described (33). One unit releases 1 pmol of 8-OH-Gua in 15 min at 37°C.

## DISCUSSION

Previous studies have demonstrated the presence of DNA glycosylases/AP lyases involved in the repair of oxidatively damaged DNA bases in *S.cerevisiae* (42,43). These activities were thought to be the homologues of the Fpg protein or the endonuclease III of *E.coli*. Recently, yeast genes coding for these DNA glycosylases have been cloned in different laboratories. The *OGG1* gene codes for a functional homologue of the Fpg protein which excises FapyGua and 8-OH-Gua from  $\gamma$ -irradiated DNA (28,33,34). The *NTG1* and *NTG2* genes of *S.cerevisiae* encode proteins whose amino acid sequences are closely related to each other and to the endonuclease III of *E.coli* (24–27). In this study, we have investigated the substrate specificity of the Ntg1 and Ntg2 proteins using damaged DNA substrates. The results show that both Ntg1 and Ntg2 proteins excise six pyrimidine-derived lesions, 5-OH-6-HThy, 5-OH-6-HUra, 5-OH-MeHyd, 5-OH-Ura, 5-OH-Cyt and Thy gly, and two purine-derived lesions, FapyAde and FapyGua, but not 8-OH-Ade and 8-OH-Gua from  $\gamma$ -irradiated DNA. However, a comparison of the kinetics of excision of each lesion by Ntg1 and Ntg2 proteins reveals significant differences between excision rates. This study also confirms the excision of Me-FapyGua by both Ntg1 and Ntg2 proteins (23,26). In addition, Ntg1 and Ntg2 proteins have been shown to cleave 37mer oligodeoxynucleotide containing a single 5,6-dihydrouracil lesion (26). Previous results obtained with partially purified yeast redoxendonuclease (42) or purified Ntg1 and Ntg2 proteins (P.W.D. and M.D., unpublished results) showed that these

enzymes are capable of cleaving UV-irradiated DNA at guanine sites. We recently reported that UV-irradiation of DNA causes formation of FapyGua and FapyAde (44). For this reason, the results of the present work showing the excision of FapyGua and FapyAde by Ntg1 and Ntg2 proteins are in agreement with those previous observations (42).

In this study, we also show that Ntg1 protein, but not the Ntg2 protein, can act on oligodeoxynucleotide duplexes containing a single 8-OH-Gua mispaired with a guanine. The Ntg1 protein not only cleaves 8-OH-Gua/Gua duplex but also releases 8-OH-Gua as a free base from the same substrate. The repair of 8-OH-Gua by the Ntg1 protein is characterised by its strong specificity in favour of 8-OH-Gua mispaired with a guanine and its modest efficiency. Indeed, Ogg1 releases 8-OH-Gua 65-fold more rapidly than Ntg1. These results are in agreement with the present study where we did not observe excision of 8-OH-Gua, paired with a cytosine, from  $\gamma$ -irradiated calf thymus DNA by Ntg1 or Ntg2 proteins. On the other hand, these results are to some extent contradictory with recently published studies (23,27). Eide *et al.* (23) did not observe cleavage of 8-OH-Gua containing DNA duplexes by the Ntg1 protein. These authors used a crude extract fraction as a source of Ntg1 which may not contain enough activity to observe cleavage of 8-OH-Gua/N duplexes. In contrast, Bruner *et al.* (27) showed that purified Ntg1 protein cleaves, with identical efficiency, the four 8-OH-Gua/N duplexes. We do not have an explanation for this contradictory result. However, the procedures used to purify the Ntg1 protein were different. We purified the Ntg1 protein under native conditions whereas Bruner *et al.* (27) used a protein which was denatured and renatured. It should be noted that the preferential action of Ogg1/Ntg1 at 8-OH-Gua/Gua was previously observed by the same research group (45). The specific recognition of 8-OH-Gua mispaired with a guanine was initially described as a property of a Me-FapyGua DNA glycosylase activity purified from wild-type yeast (43). Several lines of evidence suggest that the DNA glycosylase activity purified by de Oliveira *et al.* (43) is the Ntg1 protein. These activities bind a MonoQ column whereas Ogg1 or Ntg2 proteins do not. They are primarily Me-FapyGua DNA glycosylases but they also cleave 8-OH-Gua/Gua DNA duplex with a low efficiency. What is the biological signification of the repair of 8-OH-Gua mispaired with a guanine by the Ntg1 protein? We do not know! The repair of 8-OH-Ade from the 8-OH-Ade/Cyt duplex by the Ogg1 protein raises the same question (40). In fact, these unexpected substrate specificities may reflect molecular mechanisms used by these enzymes to recognize and/or excise damaged bases in DNA, and do not necessarily reveal biological functions. To conclude, our results show that Ntg1 and Ntg2 proteins exhibit original, very similar but non-identical substrate specificities. Unlike *E.coli* endonuclease III and its *Schizosaccharomyces pombe* (46) and human homologues (M.D., unpublished results), Ntg1 and Ntg2 proteins repair purine-derived lesions such as FapyAde, FapyGua and Me-Fapy-Gua.

The substrate specificities of Ntg1 and Ntg2 proteins clearly suggest that one of the biological roles of these enzymes is to release lethal lesions such as Thy gly and formamidopyrimidines from oxidatively damaged DNA (25). The repair of 8-OH-Gua by Ntg1 but not Ntg2 protein indicates that these proteins can recognize different products in damaged DNA. This observation may suggest overlapping but non-identical biological roles for Ntg1 and Ntg2 proteins. The elucidation of the respective biological

roles of Ntg1 and Ntg2 proteins will await characterisation of *ntg1* and *ntg2* mutants, identification of other substrates and analysis of their subcellular locations.

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