# Substrate Specificity of *Schizosaccharomyces pombe* Nth Protein for Products of Oxidative DNA Damage

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*Received July 9, 1997; Revised Manuscript Received October 2, 1997*

ABSTRACT: A gene from *Schizosaccharomyces pombe*, which encodes a protein with a strong sequence similarity to the Nth protein of *Escherichia coli*, has recently been identified [Roldán-Arjona, T., Anselmino, C., and Lindahl, T. (1996) *Nucleic Acids Res. 24*, 3307−3312]. The functional analysis of this eukaryotic enzyme indicated that it is a homologue of *E. coli* Nth protein. The gene has been subcloned and the protein (Nth-Spo) purified to apparent homogeneity. We investigated the substrate specificity of this eukaryotic enzyme for modified bases in oxidatively damaged DNA, using the technique of gas chromatography/isotope-dilution mass spectrometry (GC/IDMS). DNA substrates containing up to 17 types of modified bases were prepared by $\gamma$-irradiation or by treatment with $H_2O_2$ in the presence of Fe(III)−EDTA or Cu(II). The results revealed an efficient excision of five pyrimidine-derived lesions, 5-hydroxycytosine, thymine glycol, 5-hydroxy-6-hydrothymine, 5,6-dihydroxycytosine, and 5-hydroxyuracil. None of the other pyrimidine or purine lesions was excised. Excision was measured as a function of enzyme concentration, time, substrate concentration, and temperature. Kinetic constants were determined. Although some DNA base lesions removed by Nth-Spo protein were similar to those previously described for *E. coli* Nth protein, differences between substrate specificities of these two enzymes were noted.

Oxidative DNA damage produced by endogenously and exogenously generated reactive oxygen species has been implicated in mutagenesis, carcinogenesis, and aging (*1*). Among oxygen-derived species, the hydroxyl radical (•OH)[1] is highly reactive, producing a variety of lesions in DNA (*2*, *3*). DNA lesions may lead to biological consequences for organisms, such as mutagenesis and lethality (*4*). Most of these lesions have been shown to be substrates for enzymes engaged in DNA repair in bacteria and mammalian cells (*5*−*7*). Recently, a gene has been identified in *Schizosaccharomyces pombe*, which encodes a protein with a strong sequence similarity to the Nth protein of *Escherichia coli* (Nth-Eco) (*8*). This gene has been subcloned and the protein (Nth-Spo) purified to apparent homogeneity. The properties of this eukaryotic enzyme indicated that it is a homologue of Nth-Eco protein. Nth-Spo is a 40.2 kDa protein of 355 amino acids and possesses both a glycosylase activity on different types of DNA substrates with damage to pyrimidines and an AP lyase activity. Urea and thymine glycol have been shown to be excised by Nth-Spo protein from double-stranded DNA (*8*). Differences in activity toward DNA lesions between this enzyme and its prokaryotic counterpart have also been demonstrated. On the other hand, it is not known whether Nth-Spo protein possesses any activity toward other DNA lesions.

In the present work, we investigated the excision of modified bases from oxidatively damaged DNA by Nth-Spo protein, using the technique of gas chromatography/isotope-dilution mass spectrometry (GC/IDMS). This technique permits precise identification and quantification of a multitude of pyrimidine- and purine-derived lesions in DNA (*9*). It is also well-suited for the determination of substrate specificities of DNA repair enzymes for excision of a variety of lesions from DNA (*10*−*12*).

## EXPERIMENTAL PROCEDURES

*Materials.*[2] Modified DNA bases, their stable isotope-labeled analogues, and other materials for GC/MS were obtained as described (*9*). Irradiated or $H_2O_2$-treated DNA samples were prepared as described (*12*). $H_2O_2$/Cu-treated DNA was prepared in the same manner as $H_2O_2$/Fe−EDTA-treated DNA by replacing Fe(III)−EDTA by Cu(II). The purification of homogenous Nth-Spo protein was as previously described (*8*).

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[1] Abbreviations: •OH, hydroxyl radical; $e_{aq}^-$, hydrated electron; GC/IDMS-SIM, gas chromatography/isotope-dilution mass spectrometry with selected-ion monitoring; Nth-Eco protein, Nth protein of *Escherichia coli*; Nth-Spo, *Schizosaccharomyces pombe* homodogue of *Escherichia coli* Nth protein; 5-OH-Cyt, 5-hydroxycytosine, 5,6-diOH-Cyt, 5,6-dihydroxycytosine; 5-OH-6-HThy, 5-hydroxy-6-hydrothymine; 5-OH-6-HUra, 5-hydroxy-6-hydrouracil; 5-OH-Ura, 5-hydroxyuracil; Thy gly, thymine glycol; Ura gly, uracil glycol; $E_a$, activation energy.

[2] 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.

S0006-2960(97)01660-7 CCC: $15.00 
Published on Web 01/13/1998

*Enzymatic Assays*. Aliquots of 100 $\mu$g of DNA samples were dried in a SpeedVac under vacuum. Samples were dissolved in the incubation mixture containing 50 mM phosphate buffer (pH 7.4), 100 mM KCl, 2 mM EDTA, and 2 mM dithiothreitol. Depending on the experiment, various amounts of Nth-Spo protein were added to each mixture. The total volume of the mixture was 110 $\mu$L. Three replicates of each mixture were incubated at 37 °C in a water bath. Incubation time varied depending on the experiment As controls, DNA substrates were incubated with inactivated enzyme or without the enzyme. The inactivation of the enzyme was achieved by heating at 140 °C for 15 min.

For determination of excision as a function of the substrate concentration, 15, 20, 25, 35, 50, and 75 $\mu$g of damaged DNA were mixed with 85, 80, 75, 65, 50, and 25 $\mu$g of undamaged DNA, respectively. Additional samples containing 100 $\mu$g of damaged or undamaged DNA were also used. Three replicates of these samples were incubated with or without 2 $\mu$g of Nth-Spo protein at 37 °C for 30 min. For determination of excision as a function of incubation temperature, three replicates of 100 $\mu$g aliquots of damaged DNA were incubated with or without 2 $\mu$g of Nth-Spo protein at 15, 20, 25, 30, 37, or 45 °C for 30 min.

Following incubation, DNA samples were precipitated with 270 $\mu$L of cold ethanol, kept at −20 °C for 2 h, and centrifuged at 10 000 rpm for 30 min at 4 °C. DNA pellets and supernatant fractions were separated. The recovery of DNA by precipitation with ethanol was near 100%.

*Analysis by Gas Chromatography/Mass Spectrometry*. Aliquots of stable isotope-labeled 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 hydrolysates 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 hydrolysates of pellets were derivatized and then analyzed by GC/IDMS with selected-ion monitoring (SIM) as described (*13*).

## RESULTS

The purpose of this work was to determine the ability of Nth-Spo protein to excise modified bases from oxidatively damaged DNA. Previously, the excision by Nth-Spo protein of urea and thymine glycol from DNA treated with $KMnO_4$ or $OsO_4$ was demonstrated (*8*). An AP lyase activity of this enzyme on UV- and $\gamma$-irradiated DNA was also shown. However, no other modified bases were shown to be the substrates of Nth-Spo protein. In the present work, we used four oxidatively damaged DNA substrates to examine the substrate specificity of Nth-Spo protein. These DNA substrates were prepared by exposure of buffered aqueous solutions of DNA to $\gamma$-irradiation under $N_2O$ or air or to $H_2O_2$ in the presence of Fe(III)−EDTA or Cu(II). They contained a multiplicity of modified bases resulting from reactions of free radicals, i.e., $^{\bullet}OH$, hydrated electron ($e_{aq}^-$), and H atom, with all four DNA bases as was determined by GC/IDMS. These modified bases were 5-hydroxycytosine (5-OH-Cyt), 5,6-dihydroxycytosine (5,6-diOH-Cyt), 5-hydroxyuracil (5-OH-Ura), 5-hydroxy-6-hydrouracil (5-OH-6-HUra), 5,6-dihydroxyuracil (5,6-diOH-Ura), 5,6-dihydrouracil (5,6-diHUra), 5-hydroxyhydantoin, thymine glycol (Thy gly), 5-hydroxy-6-hydrothymine (5-OH-6-HThy), 5,6-dihydrothymine, 5-hydroxy-5-methylhydantoin (5-OH-5-Me-Hyd), 5-(hydroxymethyl)uracil (5-OHMeUra), 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 8-hydroxyguanine, 4,6-diamino-5-formamidopyrimidine, 8-hydroxyadenine, and 2-hydroxyadenine. Except for 5-OHMeUra, the uracil derivatives result from either spontaneous or hydrolysis-induced deamination of the analogous cytosine derivatives (*2*, *11*). Of the modifies bases, 5-OH-6-HUra, 5,6-diHUra, 5-OH-6-HThy, and 5,6-diHThy were identified in DNA $\gamma$-irradiated under $N_2O$ only, because oxygen inhibits their formation (*14*, *15*). The structures of modified bases listed above can be found elsewhere (*9*).

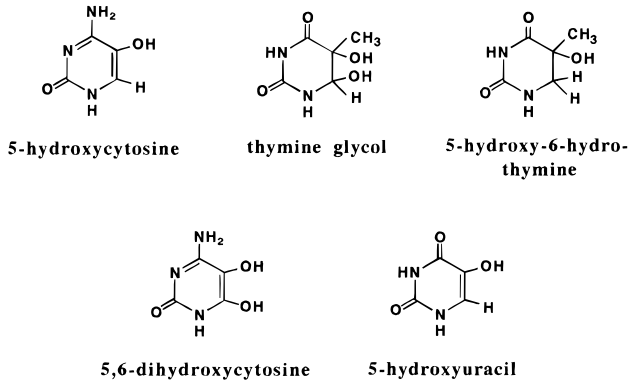


FIGURE 1: Structures of the substrates of Nth-Spo protein.

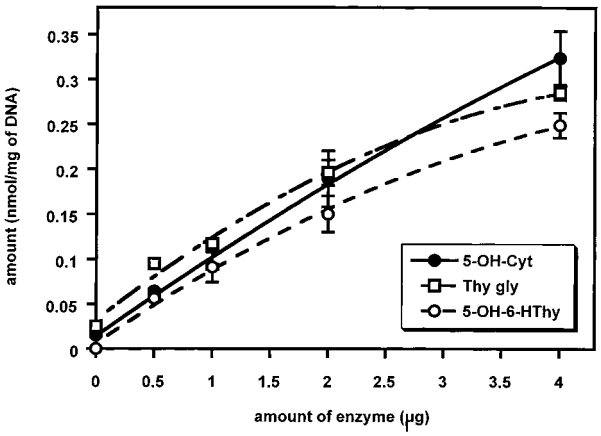


FIGURE 2: Excision of 5-OH-Cyt, Thy gly, and 5-OH-6-HThy by Nth-Spo protein as a function of the enzyme amount. DNA $\gamma$-irradiated under $N_2O$ was used. Incubation time was 30 min. The amounts given on the *y*-axis represent those found in the supernatant fractions. One nanomole of a lesion corresponds to ≈32 lesions/$10^5$ DNA bases.

Nth-Spo protein efficiently excised 5-OH-Cyt, Thy gly, 5-OH-6-HThy, 5-OH-Ura, and 5,6-diOH-Cyt (see structures in Figure 1). The amounts of these modified bases in DNA pellets incubated with active enzyme were significantly reduced when compared with those in DNA pellets incubated with inactivated enzyme or without enzyme. Their excision was confirmed by their appearance in supernatants fractions of DNA substrates incubated with active enzyme. The amounts found in the supernatant fractions of DNA samples incubated with active enzyme were similar to those removed from the pellets of the same DNA (data not shown).

Table 1: Concentration Range of the Lesions Used for Determination of Kinetic Constants and Amounts of the Modified Bases in DNA Substrates

| DNA substrate | 5-OH-Cyt | Thy gly | 5-OH-6-HThy | 5,6-diOH-Cyt | 5-OH-Ura |
|---|---|---|---|---|---|
| | | (A) Concentration Range of Lesion ($\mu$M) | | | |
| irradiation/$N_2O$ | 0.23−1.10 | 0.33−1.32 | 0.23−1.51 | 0.02−0.10 | 0.04−0.21 |
| irradiation/air | 0.34−1.22 | 0.63−2.29 | | 0.07−0.28 | 0.12−0.59 |
| $H_2O_2$/Fe−EDTA | 0.42−1.98 | 0.84−3.92 | | 0.09−0.78 | 0.22−0.92 |
| $H_2O_2$/Cu | 0.49−1.91 | 1.24−3.89 | | 0.09−0.57 | 0.15−0.55 |
| | | (B) Amounts of the Modified Bases in DNA Substrates (lesions/$10^5$ DNA bases[a]) | | | |
| irradiation/$N_2O$ | 39.1 ± 0.6 | 46.7 ± 5.8 | 53.1 ± 1.9 | 3.8 ± 0.6 | 7.4 ± 1.3 |
| irradiation/air | 42.9 ± 0.3 | 80.6 ± 1.0 | | 9.6 ± 0.6 | 20.8 ± 0.3 |
| $H_2O_2$/Fe−EDTA | 70.1 ± 7.0 | 138 ± 6 | | 27.5 ± 1.6 | 32.6 ± 3.8 |
| $H_2O_2$/Cu | 67.2 ± 8.0 | 137 ± 2 | | 20.2 ± 0.6 | 19.2 ± 2.2 |

[a] Values represent the mean ± standard deviation ($n = 3$).

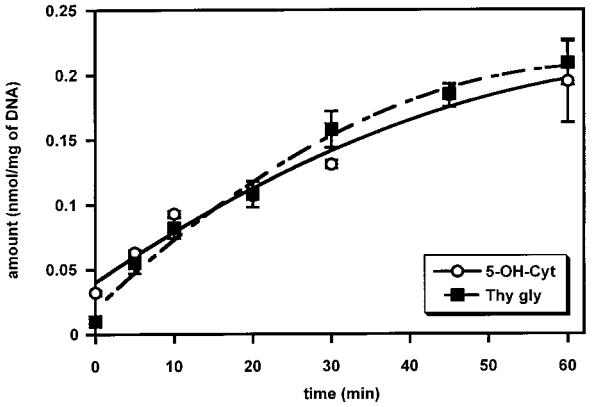


FIGURE 3: Excision of 5-OH-Cyt and Thy gly by Nth-Spo protein as a function of incubation time. DNA $\gamma$-irradiated under $N_2O$ was used. Enzyme amount was 2 $\mu$g/100 $\mu$g of DNA. The amounts given on the *y*-axis represent those found in the supernatant fractions. One nanomole of a lesion corresponds to ≈32 lesions/$10^5$ DNA bases.

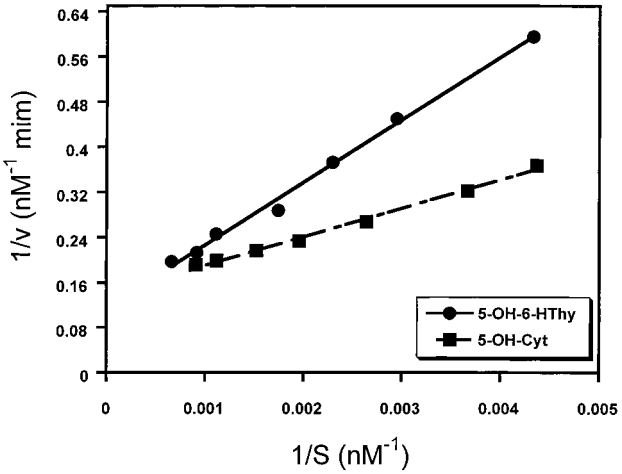


FIGURE 4: Lineweaver−Burk plots for excision of 5-OH-Cyt and 5-OH-6-HThy by Nth-Spo protein from DNA $\gamma$-irradiated under $N_2O$. The amounts of these modified bases found in the supernatant fractions were used to determine the initial velocity.

Excision of modified bases by Nth-Spo protein increased with the amount of enzyme and the incubation time. Figure 2 illustrates excision of 5-OH-Cyt, Thy gly, and 5-OH-6-HThy as a function of the amount of Nth-Spo protein in the reaction mixture. The level of removed lesion increased approaching a plateau at enzyme amounts above 4 $\mu$g. Similar profiles were obtained when excision was analyzed as a function of the incubation time. Figure 3 illustrates the example of 5-OH-Cyt and Thy gly, with no additional excision following incubations longer than 60 min.

Excision was determined as a function of substrate concentration. Measurements were performed following a 30 min incubation of DNA samples with active enzyme. The amounts of excised products in supernatant fractions, which were in good agreement with those removed from DNA pellets, were used for measurement of kinetic constants. Lineweaver−Burk plots representing the reciprocal of initial velocity versus the reciprocal of substrate concentration were utilized to determine the kinetic constants (*16*). Initial velocities were estimated using the plots of time dependency of excision for each excised lesion. Figure 4 illustrates two representative Lineweaver−Burk plots. Table 1 shows the concentration range of excised lesions in DNA samples used for these measurements and the amounts of these lesions in DNA substrates. The kinetic constants and standard deviations ($n = 7$) were obtained by a linear least-squares analysis of the data and are given in Tables 2−4. In the case of $H_2O_2$/Cu-treated DNA, the kinetic constants for excision of 5-OH-Ura could not be reliably determined because of low levels of excision at low substrate concentrations.

Excision was determined as a function of temperature in the range of 15−45 °C. Data were analyzed according to the Arrhenius equation (*16*). Linear relationships were obtained when the logarithm of the first-order rate constants was plotted against the reciprocal of the absolute temperature. Two examples of Arrhenius plots are illustrated in Figure 5. Activation energies ($E_a$) and standard deviations ($n = 6$) were obtained by linear least-squares analysis of the data. The results are given in Table 5. Activation energies of 5,6-diOH-Cyt and 5-OH-Ura in the case of DNA irradiated under $N_2O$ and $E_a$ of 5-OH-Ura in the case of $H_2O_2$/Cu-treated DNA could not be reliably determined because of low levels of excision at low temperatures.

## DISCUSSION

The results provide evidence that Nth-Spo protein possesses an activity for a number of pyrimidine-derived lesions in DNA. This work confirms the excision of Thy gly, which was previously reported (*8*). Urea, which was also reported to be excised, was not measured. The present work is the first to report on the excision of other pyrimidine-derived lesions from oxidatively damaged DNA by this enzyme. Four different DNA substrates were used for these studies. Each

Table 2: Catalytic Constants for Excision of Pyrimidine Lesions from DNA by Nth-Spo Protein

| | $k_{cat} \times 10^3$ (min$^{-1}$)[a] | | | | |
|---|---|---|---|---|---|
| DNA substrate | 5-OH-Cyt | Thy gly | 5-OH-6-HThy | 5,6-diOH-Cyt | 5-OH-Ura |
| irradiation/$N_2O$ | $16.0 \pm 0.5^{b-f}$ | $38.0 \pm 6.6^{c,e-g}$ | $19.0 \pm 1.4^{e,f}$ | $4.0 \pm 0.5^{b,c,f,h}$ | $6.0 \pm 0.47^{b}$ |
| irradiation/air | $18.0 \pm 1.3^{b-f}$ | $46.0 \pm 5.1^{c,e,f}$ | | $7.2 \pm 1.2^{c}$ | $5.5 \pm 0.3^{b}$ |
| $H_2O_2$/Fe−EDTA | $50.0 \pm 6.4^{e,f}$ | $52.0 \pm 5.9^{c,e,f}$ | | $7.6 \pm 0.7^{c}$ | $9.9 \pm 1.1$ |
| $H_2O_2$/Cu | $51.5 \pm 12.5^{d,e}$ | $240 \pm 65^{e}$ | | $14 \pm 2.2$ | |

[a] Values represent the mean ± standard deviation ($n = 7$). $k_{cat} = V_{max}$/[enzyme]. [b] Statistically different ($P < 0.05$) from the value in line 3. [c] Statistically different ($P < 0.05$) from the value in line 4. [d] Statistically different ($P < 0.05$) from the value in column 3. [e] Statistically different ($P < 0.05$) from the value in column 5. [f] Statistically different ($P < 0.05$) from the value in column 6. [g] Statistically different ($P < 0.05$) from the value in column 4. [h] Statistically different ($P < 0.05$) from the value in line 2.

Table 3: Michaelis Constants for Excision of Pyrimidine Lesions from DNA by Nth-Spo Protein

| | $K_M$ (nM)[a] | | | | |
|---|---|---|---|---|---|
| DNA substrate | 5-OH-Cyt | Thy gly | 5-OH-6-HThy | 5,6-diOH-Cyt | 5-OH-Ura |
| irradiation/$N_2O$ | $360 \pm 16^{b-h}$ | $1741 \pm 332^{b-d,f,g}$ | $963 \pm 76^{f,g}$ | $111 \pm 18^{b-d,g}$ | $173 \pm 15^{b,c}$ |
| irradiation/air | $725 \pm 65^{c,f-h}$ | $3083 \pm 355^{f,g}$ | | $380 \pm 65$ | $403 \pm 20^{c}$ |
| $H_2O_2$/Fe−EDTA | $4394 \pm 578^{d,f,g}$ | $4774 \pm 561^{f,g}$ | | $467 \pm 46$ | $679 \pm 85$ |
| $H_2O_2$/Cu | $1059 \pm 292^{f,h}$ | $6423 \pm 1808^{f}$ | | $500 \pm 85$ | |

[a] Values represent the mean ± standard deviation ($n = 7$). [b] Statistically different ($P < 0.05$) from the value in line 2. [c] Statistically different ($P < 0.05$) from the value in line 3. [d] Statistically different ($P < 0.05$) from the value in line 4. [e] Statistically different ($P < 0.05$) from the value in column 4. [f] Statistically different ($P < 0.05$) from the value in column 5. [g] Statistically different ($P < 0.05$) from the value in column 6. [h] Statistically different ($P < 0.05$) from the value in column 3.

Table 4: Specificity Constants for Excision of Pyrimidine Lesions from DNA by Nth-Spo Protein

| | $k_{cat}/K_M \times 10^5$ (min$^{-1}$ × nM$^{-1}$)[a] | | | | |
|---|---|---|---|---|---|
| DNA substrate | 5-OH-Cyt | Thy gly | 5-OH-6-HThy | 5,6-diOH-Cyt | 5-OH-Ura |
| irradiation/$N_2O$ | $4.39 \pm 0.13^{b-g}$ | $2.18 \pm 0.38^{c,g}$ | $1.99 \pm 0.20^{f,g}$ | $3.26 \pm 0.46^{b,c}$ | $3.37 \pm 0.27^{b,c}$ |
| irradiation/air | $2.54 \pm 0.21^{c,d,g}$ | $1.50 \pm 0.1^{h}$ | | $1.88 \pm 0.31$ | $1.40 \pm 0.06$ |
| $H_2O_2$/Fe−EDTA | $1.13 \pm 0.11^{f,h}$ | $1.09 \pm 0.11^{f,h}$ | | $1.63 \pm 0.15^{h}$ | $1.46 \pm 0.17$ |
| $H_2O_2$/Cu | $4.86 \pm 1.18$ | $3.75 \pm 1.02$ | | $2.80 \pm 0.46$ | |

[a] Values represent the mean ± standard deviation ($n = 7$). [b] Statistically different ($P < 0.05$) from the value in line 2. [c] Statistically different ($P < 0.05$) from the value in line 3. [d] Statistically different ($P < 0.05$) from the value in column 3. [e] Statistically different ($P < 0.05$) from the value in column 4. [f] Statistically different ($P < 0.05$) from the value in column 5. [g] Statistically different ($P < 0.05$) from the value in column 6. [h] Statistically different ($P < 0.05$) from the value in line 4.

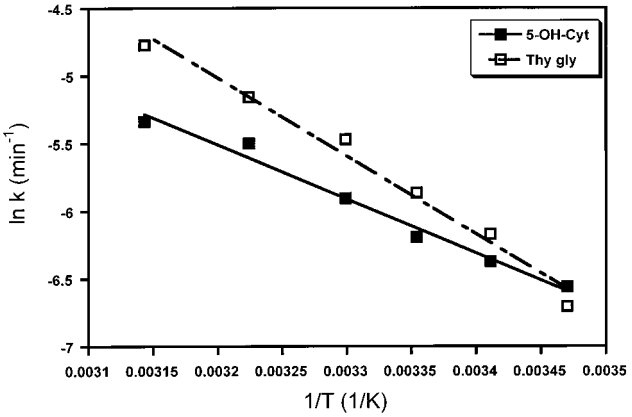


FIGURE 5: Temperature dependence of excision of 5-OH-Cyt and Thy gly by Nth-Spo protein from DNA $\gamma$-irradiated under $N_2O$. The amounts of these modified bases found in the supernatant fractions were used to determine the rate constants.

DNA substrate contained a variety of pyrimidine- and purine-derived lesions, which were formed in DNA by reactions of •OH, and also by reactions of $e_{aq}^-$ and H atom in the case of DNA irradiated under $N_2O$ (*2*, *3*). Thus, this work differs from studies of substrate specificities of repair enzymes that use oligonucleotides having a single lesion embedded at a defined position (*17*−*19*). Five of the 17 identified lesions, 5-OH-Cyt, Thy gly, 5-OH-6-HThy, 5,6-diOH-Cyt, and 5-OH-Ura, were excised by Nth-Spo protein.

The results of the present work indicate that the substrate specificity of Nth-Spo protein differs from that of its *E. coli* counterpart (Nth-Eco protein). Numerous pyrimidine-derived lesions have been identified as substrates of Nth-Eco protein (*11*, *20*−*23*). Four of these lesions, 5,6-diHThy, 5-OH-5-MeHyd, 5,6-diHUra, and 5-OH-Hyd (decarboxylated form of alloxan), were not significantly excised by Nth-Spo protein. On the other hand, 5,6-diOH-Cyt, which is not a substrate of Nth-Eco protein (*11*), was found to be a substrate of Nth-Spo protein. The lesions that both enzymes have as common substrates are 5-OH-Cyt, Thy gly, 5-OH-6-HThy, 5-OH-Ura, and urea (not measured in this work). Table 6 shows the substrates of both enzymes for comparison. It should be pointed out that only DNA $\gamma$-irradiated under $N_2O$ was used in the case of Nth-Eco protein as opposed to four DNA substrates employed in this work. Furthermore, excision kinetics of Nth-Eco have not been determined (*11*, *23*). Thus, a comparison of excision rates will have to await the measurement under experimental conditions similar to those employed in this work. In the same context, these enzymes have been shown to possess different efficiencies in removing Thy gly and urea from $KMnO_4$- and $OsO_4$-treated DNA substrates when the time course of excision

Table 5: Activation Energies for Excision of Pyrimidine Lesions from DNA by Nth-Spo Protein

| | $E_a$ (kJ·mol$^{-1}$)[a] | | | | |
|---|---|---|---|---|---|
| DNA substrate | 5-OH-Cyt | Thy gly | 5-OH-6-HThy | 5,6-diOH-Cyt | 5-OH-Ura |
| irradiation/$N_2O$ | $33.2 \pm 2.2$[b–e] | $47.9 \pm 3.2$[b,c,e] | $29.9 \pm 1.5$ | | |
| irradiation/air | $22.4 \pm 1.3$[f] | $16.4 \pm 2.5$[f,g] | | $45.1 \pm 6.6$[h] | $24.5 \pm 3.4$ |
| $H_2O_2$/Fe−EDTA | $21.9 \pm 3.4$[f] | $20.2 \pm 0.7$[f,g] | | $35.8 \pm 3.9$ | |
| $H_2O_2$/Cu | $29.1 \pm 3.9$[d] | $49.3 \pm 2.0$ | | $39.9 \pm 5.3$ | |

[a] Values represent the mean ± standard deviation ($n = 6$). [b] Statistically different ($P < 0.05$) from the value in line 2. [c] Statistically different ($P < 0.05$) from the value in line 3. [d] Statistically different ($P < 0.05$) from the value in column 3. [e] Statistically different ($P < 0.05$) from the value in column 4. [f] Statistically different ($P < 0.05$) from the value in column 5. [g] Statistically different ($P < 0.05$) from the value in line 4. [h] Statistically different ($P < 0.05$) from the value in column 6.

Table 6: Substrates of Prokaryotic and Eukaryotic Nth Proteins

| lesion | Nth-Eco[a] | Nth-Spo[b] |
|---|---|---|
| 5,6-diHThy | + | − |
| 5,6-diHUra | + | − |
| 5-OH-5-MeHyd | + | − |
| alloxan | + | − |
| 5-OH-6-HThy | + | + |
| 5-OH-6-HUra | + | − |
| Thy gly | + | + |
| 5,6-diOH-Cyt | − | + |
| 5-OH-Cyt | + | + |
| Ura gly (5-OH-Ura) | + | + |

[a] DNA $\gamma$-irradiated under $N_2O$ was used only. [b] Four DNA substrates were used.

was measured (*8*). They also differ in their efficiencies in incising UV- or $\gamma$-irradiated DNA substrates. It is likely that there is not a unique explanation for the differences in substrate specificities and excision efficiencies between Nth-Spo and Nth-Eco. The two proteins have a high sequence similarity, but the [4Fe-4S] cluster loop motif in Nth-Spo protein shows seven residues instead of five between the last two Cys residues (*8*). Furthermore, the eukaryotic protein is larger (40.2 kDa with 355 amino acids) than the prokaryotic one (23.4 kDa with 211 amino acids), showing regions of nonconserved sequence at the N- and C-termini. It is tempting to speculate that the structure of Nth-Spo protein retains many characteristics of the prokaryotic enzyme, however, it is endowed with the ability to cope with the higher-order chromatin structure of an eukaryotic genome. In this regard, it will be of interest to address directly this question by comparing the repair capacities of Nth-Spo and Nth-Eco on damaged minichromosomes. On the other hand, the differences between substrate specificities of these enzymes also raise the possibility that lesions not removed by Nth-Spo but recognized by Nth-Eco may be substrates of a second Nth-like protein. In this respect, it is interesting to note that a recent report has shown the presence of two similar Nth-like activities in *Saccharomyces cerevisiae* (*24*).

Kinetics of excision of five lesions were measured using four different DNA substrates. It should be emphasized that kinetic constants reported in this work should be considered "apparent" rather than "ideal". In the substrate systems used here, a single enzyme acts on several alternative substrate lesions, and all of them being present simultaneously, each of them will act as competitive inhibitor with respect to the others. As a result, kinetic constants will be different from those observed in the absence of the other substrates, when oligonucleotides having a single lesion embedded at a defined position are used. Excision rates significantly varied among the lesions and DNA substrates. In the case of DNA irradiated under $N_2O$, the concentration ranges of 5-OH-Cyt, Thy gly, and 5-OH-6-HThy were similar. However, $k_{cat}$ and $K_M$ values differed significantly, with Thy gly having the highest values. The specificity constant for 5-OH-Cyt was 2-fold greater than that for Thy gly or 5-OH-6-HThy, indicating that 5-OH-Cyt was the preferred substrate for the enzyme. This was also the case for DNA irradiated under air. In the case of $H_2O_2$/Fe−EDTA- and $H_2O_2$/Cu-treated DNA substrates, the specificity constants of 5-OH-Cyt and Thy gly were similar, indicating a similar preference of the enzyme for excision of these lesions. Since 5-OH-6-HThy was found only in DNA irradiated under $N_2O$, a comparison of its kinetic constants was not possible with those of 5-OH-Cyt and 5-OH-6-HThy in the case of other DNA substrates. The concentrations of 5,6-diOH-Cyt and 5-OH-Ura in DNA substrates were lower than those of the other lesions. Low excision rates were observed for these compounds.

Significant differences were noted between the kinetic constants for the same lesion in different DNA substrates. For 5-OH-Cyt, the excision rates were higher for $H_2O_2$/Fe−EDTA- and $H_2O_2$/Cu-treated DNA substrates than those for irradiated DNA substrates, whereas the excision rate of Thy gly for $H_2O_2$/Cu-treated DNA was highest. $K_M$ values of all lesions significantly differed among DNA substrates. The specificity constants were highest for DNA irradiated under $N_2O$ and for $H_2O_2$/Cu-treated DNA, indicating the preferential excision of the lesions by Nth-Spo protein from these DNA substrates. Interestingly, the $k_{cat}/K_M$ values of both 5-OH-Cyt and Thy gly for excision from $H_2O_2$/Cu-treated DNA were 4-fold higher than those for excision from $H_2O_2$/Fe−EDTA-treated DNA, although the concentration ranges of these compounds in both substrates were similar. Differences between irradiated DNA substrates were also noted. The specificity constants of the lesions for their excision from DNA irradiated under $N_2O$ were significantly greater than those from DNA irradiated under air, indicating the preference of the enzyme for excision from the former DNA substrate. Taken together, these results indicate a strong dependence of excision of pyrimidine lesions by Nth-Spo protein on the nature of DNA substrate.

The measurement of excision as a function of temperature revealed a significant dependence of this reaction on temperature. Excision of both 5-OH-Cyt and Thy gly from DNA irradiated under $N_2O$ and from $H_2O_2$/Cu-treated DNA required more $E_a$ than from the other DNA substrates. Significant differences between activation energies of the lesions within a DNA substrate were also noted. For example, in the case of DNA irradiated under $N_2O$, $E_a$ for

Thy gly excision was significantly higher than that of 5-OH-Cyt and 5-OH-6-HThy. 5,6-diOH-Cyt had the highest $E_a$ for excision from DNA irradiated under air and from $H_2O_2$/Fe−EDTA-treated DNA. The results indicate a significant dependence of activation energies for excision by Nth-Spo protein on the nature of the DNA substrate and a significant difference between activation energies for each DNA substrate tested.

The finding that kinetics of excision of pyrimidine lesions by Nth-Spo protein may vary depending on the nature of DNA substrate is in good agreement with the results of a recent study on kinetics of excision of purine lesions by *E. coli* Fpg protein (*12*), where DNA substrates similar to those used in this work were employed. One possible explanation for the observed differences in excision kinetics is that free radicals generated under different conditions yield a different, sequence-dependent distribution of lesions. The most obvious impact of the different distributions of lesions is through mass action effects on the rates of enzymatic reactions. A more subtle effect of the surrounding sequence may still modulate the formation of lesions or their enzymatic recognition. Another possibility is that the different treatments generate unidentified lesions that may interfere with the removal of the lesions under study. In any case, the fact that the kinetic constants depend on the nature of the DNA substrate does not imply that we cannot reach any conclusions about the specificity of the enzyme. In fact, the relative order of $k_{cat}$ and $K_M$ values for each lesion is the same independent of the nature of the DNA substrate (Thy gly > 5-OH-Cyt > 5-OH-Ura > 5,6-diOH-Cyt).

In conclusion, the Nth protein from *Schizosaccharomyces pombe* excises a number of pyrimidine-derived lesions from oxidatively damaged DNA. The substrate specificity of this enzyme is somewhat different from that of its *E. coli* counterpart, although the two enzymes have some common substrates. Kinetics of excision by Nth-Spo protein depend on the type of excised lesion and on the nature of the DNA substrate. The present work confirms the previously described excision of Thy gly and extends the substrate specificity of Nth-Spo protein to other thymine- and cytosine-derived lesions in DNA.

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BI971660S