Dehydration, deamination and enzymatic repair of cytosine glycols from oxidized poly(dG-dC) and poly(dI-dC)

Sébastien Tremblay¹ and J. Richard Wagner¹,²,*

¹Department of Nuclear Medicine and Radiobiology, Faculty of Medicine and Health Sciences, Université de Sherbrooke and ²Research Center in Aging, Geriatrics University Institute of Sherbrooke, Sherbrooke, Québec, Canada J1H 4C4.

Received June 13, 2007; Revised October 23, 2007; Accepted October 25, 2007

ABSTRACT

Cytosine glycols (5,6-dihydroxy-5,6-dihydrocytosine) are initial products of cytosine oxidation. Because these products are not stable, virtually all biological studies have focused on the stable oxidation products of cytosine, including 5-hydroxycytosine, uracil glycols and 5-hydroxyuracil. Previously, we reported that the lifetime of cytosine glycols was greatly enhanced in double-stranded DNA, thus implicating these products in DNA repair and mutagenesis. In the present work, cytosine and uracil glycols were generated in double-stranded alternating co-polymers by oxidation with KMnO₄. The half-life of cytosine glycols in poly(dG-dC) was 6.5 h giving a ratio of dehydration to deamination of 5:1. At high substrate concentrations, the excision of cytosine glycols from poly(dG-dC) by purified endonuclease III was comparable to that of uracil glycols, whereas the excision of these substrates was 5-fold greater than that of 5-hydroxycytosine. Kinetic studies revealed that the V_max was several fold higher for the excision of cytosine glycols compared to 5-hydroxycytosine. In contrast to cytosine glycols, uracil glycols did not undergo detectable dehydration to 5-hydroxyuracil. Replacing poly(dG-dC) for poly(dI-dC) gave similar results with respect to the lifetime and excision of cytosine glycols. This work demonstrates the formation of cytosine glycols in DNA and their removal by base excision repair.

INTRODUCTION

Reactive oxygen species are constantly generated by endogenous processes, such as aerobic respiration, phagocytosis and by exposure to ionizing radiation (1). The reaction of H₂O₂ with DNA-bound metal ions, i.e. Fe²⁺, appears to be a major source of endogenous oxidative DNA damage (2). In cellular DNA, the formation of oxidative DNA damage is counterbalanced by repair, involving an array of DNA repair proteins, which maintain a low steady state level of potentially mutagenic damage (3). Oxidation of cytosine involves saturation of the 5,6-double bond of cytosine, rendering the exocyclic amino group susceptible to deamination, i.e. conversion of the amino to a carbonyl group (4). Because these groups dictate base pairing in duplex DNA, both thermally and oxidatively induced deamination are efficient mechanisms of GC → AT transition mutations. The most common mutation in the genome of aerobic organisms is GC → AT transitions based on the analysis of mutations within the lacI gene in bacteria, lacI transgenes in rodents and the HPRT gene in rodents and humans (5–7). The same bias toward GC → AT transitions is observed with oxidants, such as H₂O₂ and ionizing radiation (8,9). Recently, Loeb and co-workers (10) reported that GC → AT transitions represented 81% of all spontaneous mutagenic events within mitochondria DNA using a sensitive assay for mutagenesis known as random mutation capture. Again the oxidation of cytosine is likely a major contributor to GC → AT transitions. In contrast, GC → AT transitions do not arise from the oxidation of G because this damage either blocks replication or leads to transversions (GC → TA; e.g. 8-oxo-7,8-dihydroguanine).

The majority of studies on cytosine oxidation has focused on three modifications: uracil glycols, 5-hydroxycytosine and 5-hydroxyuracil (11). These modifications are believed to arise from intermediate cytosine glycols, which undergo deamination to uracil glycols, dehydration to 5-hydroxycytosine, or both deamination and dehydration to 5-hydroxyuracil (4,12). These modifications are substrates for numerous DNA repair proteins, including Nth homologues (Endo III, hNTH1), Nei-like homologues (Endo VIII, yNtg1/
yNtg2, hNeil1/hNeil2), uracil N-glycosylases (Ung and Smug1) and Nfo-like endonucleases with nucleotide incision activity (Apn1, Ape1) (13–16). The mutagenic potential of the above cytosine products has also been studied. The specific incorporation of 5-hydroxycytosine into M13 led to a relatively low frequency of GC→AT transition mutations in host Escherichia coli [0.05%; (17)]; however, 5-hydroxycytosine may be mutagenic in certain sequence context (18,19). In contrast, the incorporation of uracil glycols and 5-hydroxyuraicil into the DNA of E. coli led to a relatively high mutation frequency [>80%; (16,19)]. This may be explained by the initial deamination of oxidized cytosine intermediates (e.g. the deamination of cytosine glycols to uracil glycols). Thus, DNA polymerases predominantly insert A opposite to uracil glycols and 5-hydroxyuraicil, leading to GC→AT transitions after a round of replication. Finally, it is noteworthy that deficiencies in base excision repair associated with the repair of cytosine lesions tend to increase spontaneous and oxidant-induced mutations. For example, E. coli that are deficient in both Endo III and Endo VIII are hypersensitive to ionizing radiation (17). The mutagenic repair associated with the repair of cytosine lesions tend to increase spontaneous and oxidant-induced mutations. For example, E. coli that are deficient in both Endo III and Endo VIII are hypersensitive to ionizing radiation (17). The mutagenic

**Acid hydrolysis and GC/MS**

All operations were carried out in silicon capped glass vials (300 μl), which were sealed under an atmosphere of nitrogen between steps. Acidic hydrolysis was achieved by heating polymers for 40 min at 145°C in 100 μl of 88% formic acid. Samples were then dried under vacuum using a speed-vac apparatus (Savant). The corresponding trimethylsilyl derivatives of DNA bases were obtained by derivatization at 120°C for 25 min using a 1:3 mixture (total volume = 50 μl) of anhydrous acetonitrile and BSTFA containing 1% TMCS. The analysis of modified bases was carried out by GC/MS (Model QP5050A, Shimadzu) equipped with a 0.25 mm × 30 m XTI-5 column (Restek) with helium as carrier gas at a flow rate of 2 ml/min. The initial temperature of the column was set at 125°C for 2 min and it was increased at a rate of 5.2°C/min for 30 min and then held at 280°C for an additional 12 min. The temperature of the injector and detector were 250°C and 280°C, respectively. Ionization was carried out by collision with 70 eV electrons. Authentic standards of 5-hydroxycytosine and 5-hydroxyuracil were prepared by an established method (22). Stable isotopes (+ 3 amu) of cis and trans uracil glycols, 5-hydroxycytosine, 5-hydroxyuracil were prepared from 15N2-labeled urea (Cambridge Isotopes), as previously described (23,24).

**Acid hydrolysis and HPLC/EC**

The same acid hydrolysis protocol was used for HPLC/EC and GC/MS analysis (see above). HPLC analysis of modified nucleobases was performed using a dual pump HPLC (Model 616, Waters) with a solvent controller (Model 600S, Waters), attached to an automated injector (Model 717 plus, Waters), PDA detector (Model 996, Waters) and an electrochemical detector (Coulochem II Model 5200, ESA Associates) equipped with an electrochemical cell (Model 5011, ESA). Data were acquired using an AD converter (SAT/IN, Waters). HPLC and data acquisition was controlled by Millenium software (Version 3.2, Waters). For the separation of modified nucleobases, we used a 0.6 × 25 cm C18 ODS-AQ column (YM) at a flow rate of 1.2 ml/min with a mixture of sodium phosphate (25 mM) and sodium acetate (2.5 mM) at pH 5.5 as the mobile phase. 5-Hydroxycytosine and 5-hydroxyuracil were detected at the first electrode.

**MATERIALS AND METHODS**

**Chemicals**

Water was prepared by double distillation in glass followed by passage through a water purification system (resistivity is 18.3 MΩ·cm; EASY pure, Barnstead). Chemicals were of the highest available purity. Sodium chloride (NaCl), potassium permanganate (KMnO4), sodium metabisulfite (Na2O5S2), sodium hydroxide (NaOH) and formic acid (CH2O2) 88% were purchased from Fluka; N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were purchased from Supelco, EDTA was purchased from Sigma; phosphoric acid 88%, cytosine, guanine were obtained from Aldrich; and the alternating heteroduplexes [poly(dG-dC) and poly(dI-dC)] from Amersham Pharmacia Biotech. Purified endonuclease III DNA N-glycosylase (Endo III) and formamidopyrimidine DNA N-glycosylase (Fpg) were kindly provided by Serge Boiteux, Fontenay aux Roses, France.
(75 mV vs Pd reference) and second electrode (350 mV) of the electrochemical detector, respectively. The yield of damage was calculated from a ratio of 5-hydroxycytosine (or 5-hydroxyuracil) obtained by electrochemical detection, to nonmodified cytosine, obtained by UV detection at 260 nm, on the same chromatographic run.

**Enzymatic hydrolysis and HPLC/EC**

Polymers were hydrolyzed to a mixture of nucleobases by enzymatic digestion. Ten micrograms of oxidized polymers were incubated at 50°C for 30 min with 5 units of P1 nuclease (Roche) in 40 μl of 10 mM sodium acetate (pH 4.8). Following digestion with P1 nuclease, the pH of the solution was adjusted to pH 7 by the addition of 5 μl of 1.2 M ammonium acetate and 5 units of alkaline phosphatase (Roche) was added to hydrolyze the phosphate group. Protein was removed from the sample by the addition of 50 μl of chloroform. The sample was analysed by HPLC using a 0.6 × 25 cm C18 ODS-AQ column (YMC) at a flow rate of 1.2 ml/min and 25 mM sodium phosphate (pH 5.5) plus 2.5 mM sodium acetate as the mobile phase. 5-Hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine were quantified using the electrochemical detector with a window of oxidation between 50 and 350 mV (Model 5011, ESA). As described above for nucleobases, the yield of damage was normalized to the amount of nonmodified 2'-deoxycytidine obtained by UV detection at 260 nm.

**Oxidation of polymers by KMnO4**

The standard procedure for the oxidation of poly(dG-dC) and poly(dI-dC) involved the addition of KMnO4 (final concentration is 1–5 mM) to a solution of polymer and poly(dI-dC) involved the addition of KMnO4 (0.1–2 mM). The amount of total damage was calculated from a ratio of 5-hydroxycytosine nucleoside. As before, the excision of cytosine glycol and 5-hydroxycytosine, denoted as heat-treated polymer. Enzymatic excision of cytosine products from DNA was examined in a mixture of oxidized polymer (50 μg) and Endo III (1 μg) in 50 μl of 100 mM sodium phosphate (pH 7.4). Before starting the reaction, the mixture was dialyzed 45 min against 100 mM sodium phosphate at 4°C to remove glycerol. At this point, equal amounts of four stable isotopes (+3 amu) were added as internal standards, which included 5-hydroxycytosine, 5-hydroxyuracil, and the cis and trans isomers of uracil glycols. The enzymatic reaction was terminated by the addition of 10 volumes of cold acetone, followed by storage at −20°C and centrifugation at 13 200 g for 30 min to precipitate polymers and protein. The supernatant was removed and dried under vacuum. Modified nucleobases were trimethylsilylated and subjected to GC/MS analysis, as described above.

**Kinetics of excision by Endo III**

To vary the concentration of substrate, the oxidation of poly(dG-dC) was carried out at different concentrations of KMnO4 (0.1–2 mM). The amount of total damage (cytosine glycol and 5-hydroxycytosine) was estimated in each sample by enzymatic digestion and HPLC/EC analysis of 5-hydroxycytosine nucleoside. As before, the excision of cytosine glycol and 5-hydroxycytosine from oxidized poly(dG-dC) was determined by comparing fresh polymer that contains cytosine glycol with heat-treated polymer that contains 5-hydroxycytosine. In this case, however, the release of cytosine glycol and 5-hydroxycytosine was monitored by HPLC/EC, which requires relatively small amounts of substrate compared to GC/MS analysis. The velocity of excision (pmol/min/ng protein) at a given substrate concentration was calculated from the average release of cytosine glycol and 5-hydroxycytosine after incubation with Endo III for 15, 30, 45 min at 37°C). Immediately before analysis, the samples were filtered through a 3000 MW cutoff filter to remove Endo III and the filtrate was incubated for 1 h at 37°C to convert cytosine glycols to 5-hydroxycytosine. Kinetic parameters (Km and Vmax) were determined by Hanes plots of the data [concentration of substrate/velocity (y-axis) vs concentration of substrate (x-axis)] according to Equation (1).

\[
\frac{[\text{lesion}]}{v} = \frac{[\text{lesion}]}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}}
\]

where [lesion] is the concentration of either cytosine glycol or 5-hydroxycytosine, Vmax is the maximum enzymatic reaction velocity, v is the initial reaction velocity and Km is the Michaelis–Menten constant.
RESULTS
Oxidation of poly(dG-dC) and poly(dI-dC) by KMnO₄

The oxidation of alternating heteroduplexes containing cytosine was achieved using KMnO₄ (Reaction I, Figure 1). Under these conditions, <1% of the total cytosine was oxidized giving yields of damage in the range of 1–10 modifications per 1000 nonmodified cytosine. The formation of cytosine and uracil glycols in polymers was linear as a function of reaction time (0–4 h) and KMnO₄ concentration (0.1–2 mM). The yield of products increased with ionic strength of the reaction mixture (0–3 M NaCl). This effect may be attributed to electrostatic repulsion between the negative charges of DNA and the attacking permanganate anions (25). It should also be noted that the oxidation of polymers by KMnO₄ gives a uniform distribution of damage. Although KMnO₄ is known to react 10–20-fold more efficiently with single stranded compared to double stranded regions (25), the fact that the formation of damage was linear as a function of time of exposure indicates that the percentage of single-stranded regions in polymers was negligible. Furthermore, pre-incubation of polymers with S1 nuclease to remove single-stranded or looped sequences did not affect the yield of cytosine and uracil glycols, indicating that the oxidation of cytosine takes place in double-stranded regions of the polymers.

Analysis of cytosine and uracil glycols in DNA

Direct analysis of cytosine glycols in DNA was not possible because they undergo dehydration to 5-hydroxyuracil (Reaction II) during alkaline hydrolysis and HPLC/EC analysis. Although thymine glycols are indistinguishable in these analyses, we assume that the difference between the total amount of 5-hydroxyuracil nucleoside and 5-hydroxycytosine obtained by acid hydrolysis equals 5-hydroxycytosine in the polymer.

An alternative method to measure cytosine glycols in DNA involves enzymatic hydrolysis with P1 nuclease and alkaline phosphatase, followed by the detection of 5-hydroxycytosine and 5-hydroxyuracil nucleosides by HPLC/EC (not shown). Using this method, it was found that the amount of 5-hydroxycytosine and 5-hydroxyuracil nucleosides was the same as that of 5-hydroxycytosine obtained by acid hydrolysis and HPLC/EC analysis. Although thymine glycols are known to inhibit cleavage of the phosphodiester bond by P1 nuclease (28,29), this does not likely affect the digestion of DNA containing cytosine glycols because they undergo dehydration to 5-hydroxyuracil nucleoside. Interestingly, there was no detectable formation of 5-hydroxyuracil nucleoside in freshly oxidized polymers by enzymatic digestion and HPLC/EC analysis. This result indicates that 5-hydroxyuracil is not a product of DNA oxidation by KMnO₄.
of cytosine oxidation in polymers by KMnO₄. In other words, the entirety of 5-hydroxyuracil observed in oxidized polymers by the method of acid hydrolysis and GC/MS analysis may be attributed to the formation of uracil glycols (uracil glycols are quantitatively converted to 5-hydroxyuracil during acid hydrolysis but not during enzymatic digestion at neutral pH; Reaction IV, Figure 1). Furthermore, we did not detect 5-hydroxyuracil nucleoside in heat-treated polymers, indicating that uracil glycols do not undergo dehydration to 5-hydroxyuracil in double-stranded DNA under neutral conditions.

On the basis of acid hydrolysis and GC/MS analysis, the major products observed from the oxidation of poly(dG-dC) and poly(dI-dC) by KMnO₄ were 5-hydroxycytosine and 5-hydroxyuracil (estimated to be 10-fold greater than other known oxidation products of cytosine and guanine). There was no indication for the formation of other products, including 5,6-dihydroxyuracil (dialuric and isodialuric acid), 5-hydroxyhydratoxantin, or alloxyxan. Thus, we conclude that cytosine glycols (measured as 5-hydroxycytosine) and uracil glycols (measured as 5-hydroxyuracil) are the main oxidation products of poly(dG-dC) and poly(dI-dC) by KMnO₄.

**Decomposition of cytosine glycols in oxidized polymers**

The thermal decomposition of cytosine glycols was examined in oxidized polymers by incubation of the polymers at 37°C (Figure 3). The loss of cytosine glycols was accompanied with a corresponding gain of uracil glycols, consistent with the deamination of cytosine glycols to uracil glycols in oxidized polymers (Reaction III, Figure 1). The rates of decomposition and growth were the same (k of 0.10 h⁻¹ or half-life of 6.5 h; Figure 3a). In addition, the size of cytosine glycol loss and uracil glycol gain was comparable, with values of 540 and 350 damages per 1000 nonmodified cytosine, respectively. Taking the average of both values ([540 + 350]/2), the percentage of dehydration and deamination of cytosine glycols in poly(dG-dC) was estimated to be 86% ([3270 – 445]/3270) and 14% (100% – 86%), respectively. The decomposition of cytosine glycols was also examined in oxidized poly(dI-dC) (not shown). In contrast to cytosine glycols in poly(dG-dC), the corresponding lifetime in poly(dI-dC) was 2-fold shorter and the percentage of dehydration was 85%. The decomposition of cytosine glycols in poly(dG-dC) was studied at different pH and salt concentrations (Figure 3b). Interestingly, the rate of decomposition of cytosine glycols was 2-fold greater in acid (pH 5.5) compared to neutral solutions (pH 7 and 8) and markedly increased 3–4-fold in going from 0.15 M to 2.0 M NaCl.

The decomposition of cytosine glycols in poly(dG-dC) and poly(dI-dC) was examined in strong alkali (phosphate buffer; EDTA; pH 12). For these studies, it was necessary to add EDTA to the reaction in order to protect 5-hydroxycytosine against secondary oxidation which is a problem at high pH (26). When oxidized polymers were treated at pH 12, the amount of cytosine glycols in polymers dropped by ~20% of initial values. In comparison, uracil glycols in polymers completely disappeared after 2 h of treatment at high pH. The behavior of uracil glycols was nearly identical to that reported for thymine glycols in oxidized plasmid DNA (30). The inability to completely destroy cytosine glycols in polymers may be
attributed to the efficient dehydration of cytosine glycols to 5-hydroxycytosine at high pH (12).

Excision of cytosine glycols by Endo III

The excision of cytosine glycols from oxidized polymer by Endo III was studied by comparing the profile of excision products from freshly oxidized polymers, which contained cytosine glycols, with that from heat-treated polymers, which contained 5-hydroxycytosine. For this purpose, each oxidized polymer was divided into two aliquots. The first aliquot was kept at 4°C to preserve cytosine glycols within the polymer, whereas the other aliquot was incubated at 37°C to transform initial cytosine glycols to 5-hydroxycytosine and uracil glycols. The amount of damage in each sample was determined by acid hydrolysis and HPLC/EC analysis. From these analyses, the amount of cytosine glycol (assuming no 5-hydroxycytosine) in freshly oxidized poly(dG-dC) was 2.2, whereas the amount of 5-hydroxycytosine (assuming no cytosine glycols) in the correspondingly heated polymer was 1.7 lesions per 10⁵ nonmodified cytosine (Table 1). This corresponds to a ratio of dehydration to deamination of 78%:22% respectively, in agreement with our decomposition studies.

The release of cytosine oxidation products from oxidized polymers by Endo III was estimated by GC/MS analysis using isotopic dilution to correct for losses of product during sample preparation (Figure 2b and c; Table 1). The results revealed the release of 4.8-fold more cytosine glycols (measured as 5-hydroxycytosine) from freshly oxidized poly(dG-dC) compared to the release of 5-hydroxycytosine from heated polymer (Table 1). Similar results were observed for poly(dI-dC) with a 3.2-fold difference in the efficiency of excision for cytosine glycols (Table 1). The smaller effect observed for poly(dI-dC) polymer may be explained in part by the transformation of cytosine glycols to 5-hydroxycytosine in freshly oxidized poly(dI-dC) before or during reaction with Endo III due to the shorter lifetime of cytosine glycols in poly(dI-dC) (3 h) compared to poly(dG-dC) (6.5 h).

The percent excision of cytosine glycols was comparable to that of uracil glycols using Endo III and freshly oxidized poly(dG-dC) (17.8% compared to 23.3%; Table 1). This suggests that cytosine and uracil glycols are comparable substrates for Endo III. In GC/MS analysis, three oxidation products of uracil were observed in the supernatant of Endo III-polymer reactions. The major product was cis uracil glycol (51%), followed by 5-hydroxyuracil (36%) and trans uracil glycol (13%), where the percentage corresponds to the average yield of each product divided by the total yield of deamination products (Table 1). The predominant release of cis uracil glycol is consistent with the formation of cis products by KMnO₄ oxidation. For example, the yield of cis glycol is several fold greater than that of the corresponding trans glycol from KMnO₄ oxidation of thymine derivatives (31–33). The presence of trans uracil glycol and 5-hydroxyuracil in Endo III/DNA polymer mixtures may be attributed to the transformation of cis uracil glycols during the preparation of samples for GC/MS analysis; for example, a similar profile of the three products was obtained by trimethylsililation and GC/MS analysis of purified cis uracil glycol.

Excision of cytosine glycols by Endo III (kinetic studies)

It was difficult to study the kinetics of excision for Endo III/polymer by acid hydrolysis and GC/MS because of the relatively large amount of polymer required for accurate determination of the products (50 µg of polymer
Table 1. Excision of cytosine and uracil oxidation products for oxidized poly(dG-dC) and poly(dI-dC) by Endo III

| Substrate       | Damage cytosine (2 or 3) pmol/50µg | Percent excision (2 or 3) | Damage uracil (4) pmol/50µg | Percent excision (4) |
|-----------------|-----------------------------------|---------------------------|-----------------------------|----------------------|
| Poly-(dG-dC) fresh | 180 ± 13                           | 17.8 ± 1.1                | 58.9 ± 10.4                 | 23.3 ± 1.7           |
| Poly-(dG-dC) heated | 141 ± 13                           | 3.8 ± 0.3                 | 84.0 ± 4.8                  | 24.5 ± 1.1           |
| Ratio           | 0.74                               |                           | 0.65                        | 0.74                 |
| Poly-(dI-dC) fresh | 433 ± 9                            | 14.0 ± 0.8                | 127.5 ± 3.7                 | 27.1 ± 1.2           |
| Poly-(dI-dC) heated | 378 ± 21                           | 4.4 ± 0.4                 | 128.3 ± 5.9                 | 36.4 ± 1.2           |
| Ratio           | 0.3                                |                           | 0.54                        | 0.74                 |

Table 2. Kinetic parameters for the excision of cytosine glycols and 5-hydroxycytosine from oxidized poly(dG-dC) by Endo III

| Substrate                  | $K_m$ (µM) | $V_{max}$ (pmol/min/µg) | $V_{max}/K_m$ |
|----------------------------|------------|-------------------------|---------------|
| 5-hydroxycytosine glycol   | 0.19       | 0.00024 (0.000015)     | 0.0013 (0.00031) |
| cytosine glycol            | 0.45       | 0.00116 (0.000062)     | 0.0026 (0.00025) |

Kinetic parameters were derived from graphs in Figure 4 using the Hanes equation (see Methods and Materials) with the following statistical profile ($n = 10$; $r^2 > 0.98$; $P < 0.0001$). Numbers in parentheses indicate SE calculated from linear regression.

No excision of cytosine glycols by Fpg enzyme

The possibility that Fpg enzyme excises cytosine glycols from oxidized poly(dG-dC) was examined by analysis of enzyme-DNA supernatants as a function of time of incubation, as carried out for Endo III. From these analyses, no release of cytosine glycols was observed from freshly oxidized polymer even at 10-fold higher concentration of enzyme compared to that used for Endo III (not shown). In comparison, Fpg enzyme efficiently hydrolyzed 8-oxo-7,8-dihydroguanine from poly(dG-dC) when exposed to H$_2$O$_2$ and Fe$^{2+}$ in order to produce this damage at comparable levels to that of cytosine glycols in KMnO$_4$-oxidized polymer. Thus, we conclude that cytosine glycols within oxidized polymer are also not substrates for Fpg. In addition, there was no detectable excision of 5-hydroxycytosine from oxidized polymer when the polymer was heated before the addition of enzyme to convert cytosine glycols to 5-hydroxycytosine; thus, 5-hydroxycytosine in oxidized polymer are also not substrates for Fpg. The lack of excision of 5-hydroxycytosine by Fpg is consistent with an early report using gamma-irradiated calf-thymus DNA and GC/MS analysis (34); however, two later studies reported the excision of 5-hydroxycytosine from synthetic oligonucleotides (35,36). The reason for this discrepancy is not clear. One possibility is that oligonucleotides containing 5-hydroxycytosine undergo secondary oxidation under certain conditions to transform into potential substrates for excision by Fpg [i.e. isodialuric acid; (31)].

Figure 4. Plots of reaction velocity ($v$) vs substrate concentration. The substrate was either cytosine glycols (solid circles) or 5-hydroxycytosine (open circles) within freshly oxidized poly(dG-dC) or freshly oxidized and then heated polymer, respectively. The red line represents the best fit of data to an exponential function.
DISCUSSION

The oxidation of DNA bases by KMnO$_4$ follows the order: thymine $>$ cytosine $>$ guanine $>$ adenine (32). The difference in the rate of oxidation between thymine and cytosine varies from 10- to 30-fold for monomers and 30- to 45-fold for single-stranded oligonucleotides and plasmid DNA (25,32,33,37–39). In contrast to pyrimidines, purines are much less reactive. The rate of reaction of KMnO$_4$ with guanine nucleoside is at least 5-fold less than that with cytosine nucleoside under neutral conditions (38). The least reactive DNA base, i.e. adenine, resists oxidation by KMnO$_4$ even under harsh conditions (39). Although the oxidation of inosine by KMnO$_4$ has not been reported, the reactivity of this base is likely comparable to that of adenine in view of the similarities of their structure and oxidation potential. Therefore, cytosine residues in both poly(dG-dC) and poly(dI-dC) are the principle targets (>80%) of oxidation by KMnO$_4$. A number of DNA base oxidation products was reported from KMnO$_4$ oxidation of denatured plasmid DNA using acid hydrolysis and GC/MS analysis (40). Although most of the damage occurred at thymine, the authors reported some damage at cytosine, including 5-hydroxycytosine, 5,6-dihydroxycytosine (dialuric or isodialuric acid) and 5-hydroxyhydantoin (40). In contrast, we only observed the formation of cytosine glycols (measured as 5-hydroxycytosine) and uracil glycols (measured as 5-hydroxycytosine) by acid hydrolysis and GC/MS analysis (40). Although most of the damage occurred at thymine, the authors reported some damage at cytosine, including 5-hydroxycytosine, 5,6-dihydroxycytosine (dialuric or isodialuric acid) and 5-hydroxyhydantoin (40).

The presence of cytosine glycols in poly(dG-dC) and poly(dI-dC) is supported by the transformation of cytosine products (cytosine glycols to uracil glycols) as a function time and the marked difference in the excision of products by Endo III between freshly and heated polymers. In oxidized poly(dG-dC), cytosine glycols (measured as 5-hydroxycytosine) decreased with a half-life of 6.5 h whereas uracil glycols (measured as 5-hydroxycytosine) increased with similar kinetics (Figure 3). Although direct analysis of cytosine glycols is not possible, the only explanation for the concomitant loss of measured 5-hydroxycytosine and gain of measured 5-hydroxycytosine is the deamination of cytosine glycols to uracil glycols. The amount of measured 5-hydroxycytosine reaches a plateau in oxidized polymers after incubation at 37°C, indicating that 5-hydroxycytosine does not undergo deamination to 5-hydroxycytosine. In addition, the effects of pH and salt concentration on the decomposition of cytosine glycols in oxidized polymers were very similar to those observed for cytosine glycoside in aqueous solution (12). The presence of cytosine glycols in oxidized polymers was also supported by the difference in the excision of cytosine oxidation products by Endo III between freshly oxidized and heat-treated polymer (Table 1). The main excision product in freshly oxidized polymers was cytosine glycols whereas the main product in heat-treated samples was 5-hydroxycytosine. Thus, the difference in the excision of products from freshly oxidized and heated polymer by Endo III excision arises from the transformation of polymer containing a good substrate, i.e. cytosine glycols, to one containing a relatively poor substrate, i.e. 5-hydroxycytosine.

Our analysis indicated that uracil glycols but not 5-hydroxycytosine was produced in oxidized poly(dG-dC) and poly(dI-dC) and that 5-hydroxycytosine did not form even after extensive incubation at 37°C. Thus, we conclude that uracil glycols do not undergo dehydrogenation to 5-hydroxycytosine in polymers (Reaction IV; Figure 1). In comparison, pyrimidine photohydrates (6-hydroxy-5, 6-dihydroxycytosine and 6-hydroxy-5,6-dihydroxycytosine) appear to undergo dehydrogenation to uracil within photo-irradiated polymers, e.g. poly(dA-dU) and poly(dG-dC), although the activation energy for the dehydration of uracil photohydrate is much higher than that for cytosine photohydrates (41,42). The lack of dehydration of uracil glycols to 5-hydroxycytosine in polymers suggests that there may be alternative pathways to explain the formation of 5-hydroxycytosine from the free radical oxidation of DNA; for example, the formation of 5-hydroxycytosine by the elimination of H$_2$O$_2$ from intermediate hydroperoxides (4,43).

The present work indicates that the excision of cytosine glycols is comparable to that of uracil glycols and that both of these substrates are more efficiently excised in comparison to 5-hydroxycytosine (Tables 1 and 2). The difference in excision between glycols and 5-hydroxycytosine is consistent with previous studies of pyrimidine glycols. For example, Wallace and co-workers (16,35) reported a 2.3-fold difference in the relative efficiency ($V_{max}/K_m$) for uracil glycols compared to thymine glycols, while the excision of thymine glycols was 7-fold greater than that of 5-hydroxycytosine. The same difference between thymine glycols and 5-hydroxycytosine was also reported by Cadet and co-workers (36). In comparison, the same trend albeit with a smaller difference in excision (1–2 fold) was reported for uracil glycols and either 5-hydroxycytosine or 5-hydroxycytosine (44). The kinetics for the excision of cytosine glycols compared to 5-hydroxycytosine are largely determined by the difference in the $V_{max}$ of excision, which is consistent with the greater susceptibility of cytosine glycols toward acid or base catalyzed N-glycosidic bond cleavage.

Although the relative rates of excision of several products have been compiled for Endo III and various DNA substrates, they have failed to distinguish between the excision of cytosine glycols and 5-hydroxycytosine (23,45). In the present study, the excision of cytosine glycols and 5-hydroxycytosine was determined by comparison of the rates of excision from freshly oxidized and heated polymers. These analyses permit the separation and comparison of the excision of cytosine glycols, uracil glycols and 5-hydroxycytosine (Table 1). The relatively high efficiency of excision for cytosine glycols suggests that Endo III and homologous enzymes in yeast and mammalian cells are active in the repair of cytosine glycols. The removal of cytosine glycols is critical because they undergo deamination to uracil glycols, which probably...
have a higher mutagenic potential and efficiently generate GC→AT transitions.

ACKNOWLEDGEMENTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada. Funding to pay the Open Access publication charges for this article was provided by the Fonds de la Recherche en Santé du Québec.

Conflict of interest statement. None declared.

REFERENCES

1. Valko,M., Leibfritz,D., Moncol,L., Cronin,M.T.D., Mazur,M. and Telser,J. (2007) Free radicals and antioxidants in normal physiological functions and human diseases. Int. J. Biochem. Cell B., 39, 44–53.
2. Henle,E.S. and Linn,S. (1997) Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. J. Biol. Chem., 272, 19095–19098.
3. Bjelland,S. and Seeberg,E. (2003) Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. Mutat. Res.-Fund. Mol. M., 531, 37–80.
4. Wagner,J.R., Decrooz,C., Berger,M. and Cadet,J. (1999) Hydroxyl radical-induced decomposition of 2'-deoxycytidine in aerated aqueous solutions. J. Am. Chem. Soc., 121, 4101–4110.
5. Schaaper,R.M. and Dunn,R.L. (1991) Spontaneous mutation in the Escherichia coli lacI gene. Genetics, 129, 317–326.
6. Zhang,S.L., Glickman,B.W. and de Boer,J.G. (2001) Spontaneous mutation of the lacI gene in Escherichia coli. Proc. Natl Acad. Sci. USA, 98, 4302–4307.
7. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
8. Tkeshelashvili,L.K., McBride,T., Spence,K. and Loeb,L.A. (1991) Mutation spectrum of copper-induced DNA damage. J. Biol. Chem., 266, 6401–6406.
9. Wang,D., Kreutzer,D.A. and Essigmann,J.M. (1998) Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. Mutat. Res.-Fund. Mol. M., 400, 99–115.
10. Vermulst,M., Bielas,J.H., Kujoth,G.C., Ladiges,W.C. and Boer,J.G. (2001) Mutagenicity of the lacI gene in bacteria: absence of species, strain, and insertion-site influence. Environ. Mol. Mutagen., 37, 141–146.
11. Albertini,R.J. (2001) Spontaneous mutation of the lacI gene in bacteria: absence of species, strain, and insertion-site influence. Environ. Mol. Mutagen., 37, 141–146.
12. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
13. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
14. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
15. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
16. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
17. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
18. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
19. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
20. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
21. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
22. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
23. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
24. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
25. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
26. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
27. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
28. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
29. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
30. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
31. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
32. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
33. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
34. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
35. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
36. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
37. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
38. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
39. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
40. Albertini,R.J. (2001) HPRT mutations in humans: biomarkers for mechanistic studies. Mutat. Res.-Rev. Mutat., 489, 1–16.
38. Nawamura, T., Negishi, K. and Hayatsu, H. (1994) 8-Hydroxyguanine is not produced by permanganate oxidation of DNA. *Arch. Biochem. Biophys.*, 311, 523–524.

39. Jones, A.S., Ross, W.G., Takemura, S., Thompson, W.T. and Walker, T.R. (1964) The nucleotide sequence in deoxyribonucleic acids part VI: the preparation and reactions of permanganate-oxidised deoxyribonucleic acid. *J. Chem. Soc.*, 373–378.

40. Akman, S.A., Doroshow, J.H. and Dizdaroglu, M. (1990) Base modifications in plasmid DNA caused by potassium permanganate. *Arch. Biochem. Biophys.*, 282, 202–205.

41. Boorstein, R.J., Hilbert, T.P., Cadet, J., Cunningham, R.P. and Teebor, G.W. (1989) UV-induced pyrimidine hydrates in DNA are repaired by bacterial and mammalian DNA glycosylase activities. *Biochemistry*, 28, 6164–6170.

42. Boorstein, R.J., Hilbert, T.P., Cunningham, R.P. and Teebor, G.W. (1990) Formation and stability of repairable pyrimidine photohydrates in DNA. *Biochemistry*, 29, 10455–10460.

43. Wagner, J.R., van Lier, J.E., Berger, M. and Cadet, J. (1994) Thymidine hydroperoxides - structural assignment, conformational features, and thermal decomposition in water. *J. Am. Chem. Soc.*, 116, 2235–2242.

44. Wang, D. and Essigmann, J.M. (1997) Kinetics of oxidized cytosine repair by endonuclease III of Escherichia coli. *Biochemistry*, 36, 8628–8633.

45. Dizdaroglu, M., Bauche, C., Rodriguez, H. and Laval, J. (2000) *Biochemistry*, 39, 5586–5592.