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

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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 H_2O_2 with DNA-bound metal ions, i.e. Fe^{2+}, 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 H_2O_2 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/

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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-hydroxyuracil 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-hydroxyuracil, 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 and H_2O_2 and display a high frequency of spontaneous mutations (20). In addition, when the activities of both Smug1 and Ung proteins are compromised in mammalian cells, the frequency of spontaneous GC→AT transition mutations rises to as much as 10-fold higher than that in wild type cells (21).

Previously, we reported that the lifetime of cytosine glycols was greatly enhanced in double-stranded calf thymus DNA compared to the free nucleoside (half-life is 28 h for DNA compared to 50 min for the nucleoside; (12)). This suggests that cytosine glycols are substrates for base excision repair and if they are not repaired, they may contribute to mutagenesis during DNA replication. Moreover, the inability to repair cytosine glycols gives time for these products to undergo deamination and transform into products with an extremely high mutagenic potential (i.e. uracil glycols). For the above reasons, it is important to examine cytosine glycols in DNA and determine the specificity of DNA repair proteins toward this damage. In the present work, we have oxidized alternating heteroduplexes [poly(dG-dC) or poly(dI-dC)] with $KMnO_4$ such that the main product in polymers is either cytosine glycols or 5-hydroxycytosine. We have developed several methods to measure cytosine glycols in DNA and in the supernatant of DNA–enzyme mixtures. Using these methods, we confirm the presence of cytosine glycols in oxidized polymers, examine their decomposition (half-life and extent of deamination) and determine the kinetics of their excision by Endo III as a model system of base excision repair.

MATERIALS AND METHODS

Chemicals

Water was prepared by double distillation in glass followed by passage through a water purification system (resistivity is $18.3\ M\Omega/cm$; EASY pure, Barnstead). Chemicals were of the highest available purity.

Sodium chloride (NaCl), potassium permanganate ($KMnO_4$), sodium metabisulfite ($Na_2O_5S_2$), sodium hydroxide (NaOH) and formic acid (CH_2O_2) 88% were purchased from Fluka; *N,O-bis*(trimethylsilyl)trifluoroacetamide (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.

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 $0.25\ mm \times 30\ m$ 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 $^{15}N_2^{13}C$-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 $0.6 \times 25\ cm$ C18 ODS-AQ column (YMC) 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

(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 nucleosides 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 KMnO$_4$

The standard procedure for the oxidation of poly(dG-dC) and poly(dI-dC) involved the addition of KMnO$_4$ (final concentration is 1–5 mM) to a solution of polymer (0.5 μg/μl; 1 absorbance unit = 50 μg/ml) containing sodium phosphate buffer (25 mM; pH 3–10) and NaCl (0–3 M). The reaction was allowed to proceed at room temperature for specific times (0–4 h). It was terminated by the addition of 2 mM EDTA (final concentration) and Na$_2$O$_5$S$_2$ (two equivalents) followed by the addition of NaCl (0.5 M) and then isopropanol (50% v/v) to precipitate the polymer. For precipitation, the samples were kept at −20°C for 30 min prior to centrifugation at 13 200 g at 4°C for 40 min. The pellets were subsequently dissolved in 25 μl of water and dialyzed overnight against 4 l of water at 4°C. Dialysis was carried out using a microdialyzer apparatus (Spectra/Por 16 wells, Spectrum) equipped with 12 000–14 000 MWCO regenerated cellulose membranes (Spectra/Por). The membranes were prepared by boiling them in water containing 1 mM EDTA and 2% bicarbonate for 10 min followed by extensive washing with water and 1 mM EDTA.

Excision of cytosine products by Endo III

Poly(dG-dC) and poly(dI-dC) (0.5 μg/μl) were oxidized by exposure to 1 mM KMnO$_4$ for 2 h in a solution of 2 M NaCl. The reaction was stopped and DNA was precipitated as described above. The pellets were subsequently dissolved in TE buffer (10 mM Tris–HCl, 100 mM NaCl and 1 mM EDTA) and dialyzed overnight against 4 l of water at 4°C (as above). The polymers were divided into two fractions: one fraction was stored at −20°C to preserve the amount of cytosine glycols, denoted as freshly oxidized polymer; and the other fraction was incubated at 37°C in 10 mM Tris–Cl (pH 7.0), 100 mM NaCl and 1 mM EDTA for 4 days to completely transform cytosine glycols into 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 KMnO$_4$ (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 (K_m and V_{max}) were determined by Hanes plots of the data [concentration of substrate/velocity (y-axis) vs concentration of substrate (x-axis)] according to Equation (1).

$$\frac{[\text{lesion}]}{v} = \frac{[\text{lesion}]}{V_{max}} + \frac{K_m}{V_{max}} \qquad \mathbf{1}$$

where [lesion] is the concentration of either cytosine glycol or 5-hydroxycytosine, V_{max} is the maximum enzymatic reaction velocity, v is the initial reaction velocity and K_m is the Michaelis–Menten constant.
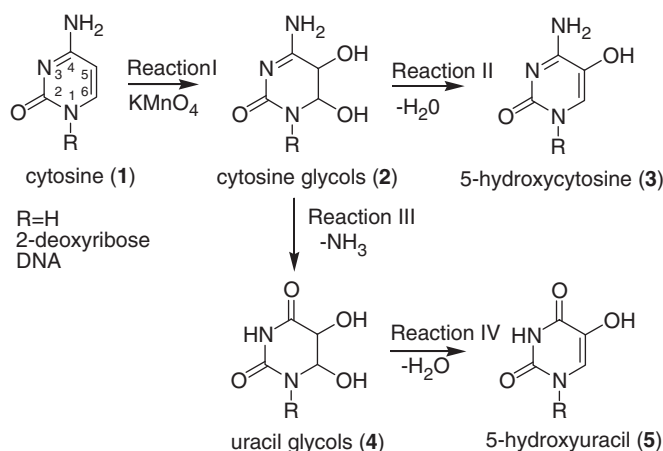
Figure 1. Formation and decomposition of cytosine glycols. Cytosine (**1**) was oxidized to cytosine glycols (**2**) by $KMnO_4$ (Reaction I; Figure 1). Cytosine glycols (**2**) decomposed by either dehydration to 5-hydroxycytosine (**3**; Reaction II) or deamination to uracil glycols (**4**; Reaction III). During acid hydrolysis of DNA, cytosine glycols (**2**) and uracil glycols (**4**) are converted to 5-hydroxycytosine (**3**) and 5-hydroxyuracil (**5**), respectively (Reactions II and IV; Figure 1). Thus, the amount of 5-hydroxycytosine obtained by acid hydrolysis corresponds to the sum of cytosine glycols (**2**) and 5-hydroxycytosine (**3**), whereas the amount of 5-hydroxyuracil (**5**) corresponds to the sum of uracil glycols (**4**) and 5-hydroxyuracil (**5**).

RESULTS

Oxidation of poly(dG-dC) and poly(dI-dC) by $KMnO_4$

The oxidation of alternating heteroduplexes containing cytosine was achieved using $KMnO_4$ (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 $KMnO_4$ 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 $KMnO_4$ gives a uniform distribution of damage. Although $KMnO_4$ 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-hydroxycytosine (Reaction II) or deamination to uracil glycols (Reaction III) when DNA is hydrolyzed to monomers (Figure 1). Thus, we developed a number of methods to indirectly measure cytosine glycols from oxidized polymers. The first step in these methods involved the release of cytosine glycols from the polymers. This was achieved by either treatment with hot formic acid which hydrolyses the polymer to its component nucleobases or mild enzymatic digestion with P1 nuclease and alkaline phosphatase which hydrolyzes the polymers into its component 2-deoxyribose nucleosides. In addition, cytosine glycols and other cytosine oxidation products, 5-hydroxycytosine and uracil glycols, were excised from polymers by treatment with purified Endo III. The modifications were subsequently detected by either GC/MS (for nucleobases) or HPLC/EC (for nucleobases and nucleosides; Figure 2). In the case of formic acid hydrolysis, cytosine glycols are quantitatively converted to 5-hydroxycytosine without any detectable deamination and thus, the measured 5-hydroxycytosine represents the sum of cytosine glycols and 5-hydroxycytosine. Likewise, uracil glycols are quantitatively converted to 5-hydroxyuracil under these conditions.

The initial amount of cytosine glycols in oxidized poly(dG-dC) and poly(dI-dC) was determined by acid hydrolysis and HPLC/EC analysis (Figure 2a). In these analyses, we assume that neither 5-hydroxycytosine nor 5-hydroxyuracil exist in freshly oxidized polymers because both of these products have a relatively low oxidation potential and if formed, they are likely immediately oxidized during treatment with $KMnO_4$ (26,27). Thus, the amount of cytosine glycols in freshly oxidized polymers is equal to the amount of measured 5-hydroxycytosine. In contrast, the amount of cytosine glycols decreased in heat-treated polymers with an increase in uracil glycols (Figure 2a). Although cytosine glycols and 5-hydroxycytosine are indistinguishable in these analyses, we assume that the difference between the total of cytosine glycol and 5-hydroxycytosine (measured as 5-hydroxycytosine) before and after heating equals the amount of cytosine glycols that has converted to uracil glycol. After incubating freshly oxidized polymer at 37°C for several half-lives, we assume that the polymer no longer contains cytosine glycol and that the amount of 5-hydroxycytosine as measured 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 nucleoside 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-hydroxycytosine during digestion (i.e. 5-hydroxycytosine does not inhibit digestion). 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
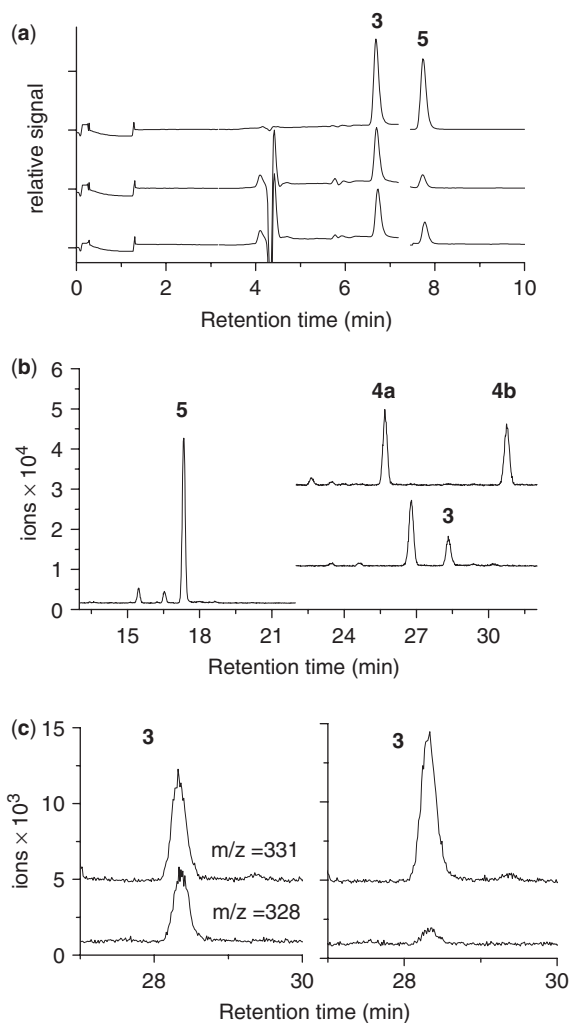
Figure 2. Analyses of cytosine oxidation products. (**a**) HPLC/EC analysis of 5-hydroxycytosine (**3**) and 5-hydroxyuracil (**5**). Top chromatogram–standard compounds; middle chromatogram–freshly oxidized poly(dG-dC) subjected to acid hydrolysis; bottom chromatogram–heat treated oxidized poly(dG-dC) subjected to acid hydrolysis. Products **3** and **5** were detected by electrochemical detection with the oxidation potential at 75 mV and 350 mV, respectively. (**b**) GC/MS analysis of 5-hydroxycytosine (**3**), 5-hydroxyuracil (**5**) and uracil glycols (**4a** and **4b**). The samples were prepared from polymers by either acid hydrolysis or incubation with Endo III followed by trimethylsilylation of the resulting nucleobases. The most abundant ion in the mass spectrum was chosen for selective ion monitoring (molecular ion −15 amu, unless indicated): 5-hydroxycytosine (**3**, m/z 328); 5-hydroxyuracil (**6**, m/z 329); *trans* uracil glycol (**4a**, m/z 245, ion fragment) and *cis* uracil glycol (**4b**, m/z 245, ion fragment). The peak at 27 min was an impurity. c) Quantitation of 5-hydroxycytosine (**3**), released from oxidized poly(dG-dC) by Endo III, was achieved by GC/MS analysis with selective ion monitoring. The amount of 5-hydroxycytosine was determined from the ratio of natural product released by Endo III to the corresponding isotopically labeled 5-hydroxycytosine (+3 amu), which was added before the addition of enzyme. The chromatogram depicts the excision of the 5-hydroxycytosine (**3**) from freshly oxidized poly(dG-dC) (left) and heat-treated polymer (right).

of cytosine oxidation in polymers by $KMnO_4$. 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 $KMnO_4$ 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-hydroxyhydantoin, or alloxan. 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 $KMnO_4$.

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
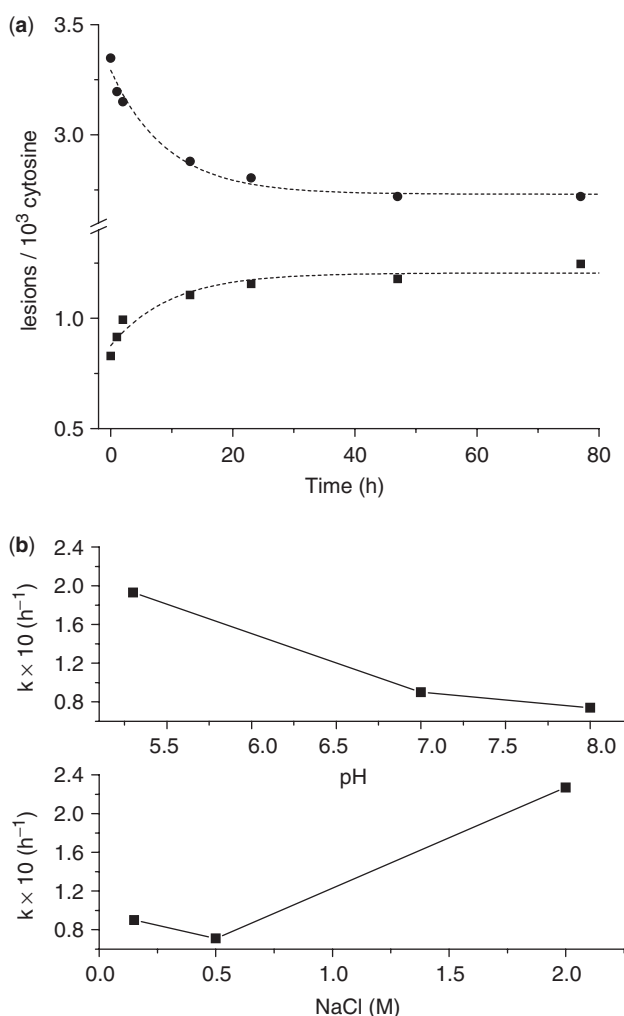
Figure 3. Thermal decomposition of cytosine glycols in oxidized poly(dG-dC). Decomposition was carried out in phosphate buffer (25 mM, pH 7.0) containing 0.15 NaCl and 1 mM EDTA. Analysis of cytosine glycols (solid circles) and uracil glycols (solid squares) was carried out by acid hydrolysis and HPLC/EC. The dashed line represents the best fit of data to an exponential function [$y = y_0 + be$ ($-t/k$)], where y_0 and y are the yield of product at time zero and at specific times of incubation (t), respectively, k is the rate of decomposition or growth and b is a constant. From these analyses, the rate of decomposition of cytosine glycols was $-0.11 \, h^{-1}$ whereas the growth of uracil glycols was $0.10 \, h^{-1}$ ($n = 7$; $r^2 \geqslant 0.94$). Repeated experiments gave similar rates of decomposition and growth. (**b**) Top panel: decomposition of cytosine glycols as a function of pH (5–8) in phosphate buffer (25 mM) at a fixed concentration of NaCl (0.15 M); bottom panel: decomposition of cytosine glycols as a function of salt concentration (0.15–2 M) in phosphate buffer at pH 7.0 (25 mM, pH 7.0). Rates were estimated from the best fit of data to the above exponential function ($n = 7$; $r^2 \geqslant 0.97$).

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 10^3 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 in 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 KMnO$_4$ oxidation. For example, the yield of *cis* glycol is several fold greater than that of the corresponding *trans* glycol from KMnO$_4$ 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 trimethylsilyation 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/polymers 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

	Damage cytosine[a] (**2** or **3**)	Percent excision[b] (**2** or **3**)	Damage uracil[a] (**4a**)	Percent excision[b] (**4a**,**4b**,**5**)	*trans*-Uracil glycol (**4a**)	*cis*-Uracil glycol (**4b**)	5-Hydroxy uracil (**5**)
Poly-(dG-dC) fresh	180 ± 13	17.8 ± 1.1	58.9 ± 10.4	23.3 ± 1.7	4.1 ± 0.3	11.5 ± 1.0	7.6 ± 0.3
Poly-(dG-dC) heated[c]	141 ± 13	3.8 ± 0.3	84.0 ± 4.8	24.5 ± 1.1	1.6 ± 0.6	13.3 ± 0.2	9.6 ± 0.2
Ratio		4.75		0.95			
Poly-(dI-dC) fresh	433 ± 9	14.0 ± 0.8	127.5 ± 3.7	27.1 ± 1.2	4.2 ± 0.1	13.5 ± 0.7	9.3 ± 0.4
Poly-(dI-dC) heated[c]	378 ± 21	4.4 ± 0.4	128.3 ± 5.9	36.4 ± 1.2	4.1 ± 0.2	18.2 ± 0.3	14.0 ± 0.7
Ratio		3.18		0.74			

[a]Initial damage in pmol for $50 \, \mu g$ of polymer was estimated by acid hydrolysis and HPLC/EC analysis (Figure 2). $180 \, pmol/50 \, \mu g = 2.2$ lesions per 10^3 nonmodified cytosine assuming an average molecular weight of 308.6 for poly(dG-dC).
[b]Percent excision of initial damage.
[c]Polymer was pre-incubated at $37°C$ to transform cytosine glycols (**2**) to 5-hydroxycytosine (**3**).
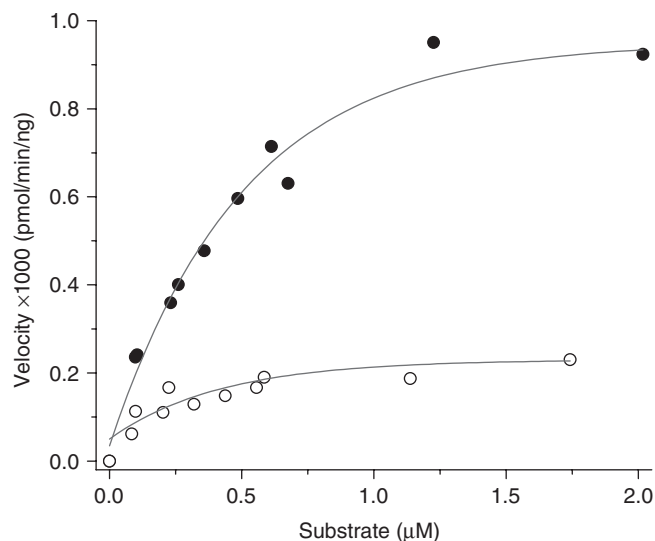

Figure 4. Plots of reaction velocity (*v*) vs substrate concentration. The substrate was either cytosine glycols (solid circles) or 5-hydroxcytosine (open circles) within freshly oxidized poly(dG-dC) or freshly oxidized and then heated polymer, respectively. The red line represents the best fit of data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

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/ng)	V_{max}/K_m
5-hydroxycytosine	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_2O_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 poly(dG-dC) are 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)].

per timepoint). Thus, we developed a more sensitive method to monitor the excision of cytosine glycols and 5-hydroxycytosine from Endo III/polymer mixtures using HPLC/EC (see Materials and Methods). In this method, the excision of cytosine glycols and 5-hydroxycytosine was measured by removing the substrate and enzyme, converting cytosine glycols to 5-hydroxycytosine in the supernatant, followed by the analysis of 5-hydroxycytosine by HPLC/EC. The rate of excision (velocity) for each substrate depended on the concentration of damage of oxidized polymer according to Michaelis–Menten kinetics (Figure 4). The V_{max} was 4.8-fold higher for cytosine glycols compared to 5-hydroxycytosine, whereas the K_m was 2.4-fold higher for cytosine glycols (Table 2). Thus, the ratio (V_{max}/K_m) was ~2-fold higher for cytosine glycols than 5-hydroxycytosine. The excision of cytosine glycols compared to that of 5-hydroxycytosine was higher than 2-fold in previous experiments (Table 1) because the substrate concentration was close to enzyme saturation.

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-dihydroxyuracil (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-hydroxyuracil) by acid hydrolysis and GC/MS. The discrepancies between the two studies may be attributed to differences in experimental conditions; in particular, the oxidation of plasmid DNA in the previous study was carried out with single-stranded DNA, which is more susceptible to oxidation than double-stranded DNA.

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-hydroxyuracil) 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-hydroxyuracil 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-hydroxyuracil. 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 glycol nucleoside 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 glycol but not 5-hydroxyuracil was produced in oxidized poly(dG-dC) and poly(dI-dC) and that 5-hydroxyuracil did not form even after extensive incubation at 37°C. Thus, we conclude that uracil glycols do not undergo dehydration to 5-hydroxyuracil in polymers (Reaction IV; Figure 1). In comparison, pyrimidine photohydrates (6-hydroxy-5, 6-dihydrocytosine and 6-hydroxy-5,6-dihydrouracil) appear to undergo dehydration 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-hydroxyuracil in polymers suggests that there may be alternative pathways to explain the formation of 5-hydroxyuracil from the free radical oxidation of DNA; for example, the formation of 5-hydroxyuracil by the elimination of H_2O_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-hydroxyuracil (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.

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