Formation of cytosine glycol and 5,6-dihydroxycytosine in deoxyribonucleic acid on treatment with osmium tetroxide

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OsO₄ selectively forms thymine glycol lesions in DNA. In the past, OsO₄-treated DNA has been used as a substrate in studies of DNA repair utilizing base-excision repair enzymes such as DNA glycosylases. There is, however, no information available on the chemical identity of other OsO₄-induced base lesions in DNA. A complete knowledge of such DNA lesions may be of importance for repair studies. Using a methodology developed recently for characterization of oxidative base damage in DNA, we provide evidence for the formation of cytosine glycol and 5,6-dihydroxycytosine moieties, in addition to thymine glycol, in DNA on treatment with OsO₄. For this purpose, samples of OsO₄-treated DNA were hydrolysed with formic acid, then trimethylsilylated and analysed by capillary gas chromatography–mass spectrometry. In addition to thymine glycol, 5-hydroxyuracil (isobarbituric acid), 5-hydroxycytosine and 5,6-dihydroxyuracil (isodialuric acid or dialuric acid) were identified in OsO₄-treated DNA. It is suggested that 5-hydroxyuracil was formed by formic acid-induced deamination and dehydration of cytosine glycol, which was the actual oxidation product of the cytosine moiety in DNA. 5-Hydroxycytosine obviously resulted from dehydration of cytosine glycol, and 5,6-dihydroxyuracil from deamination of 5,6-dihydroxycytosine. This scheme was supported by the presence of 5-hydroxyuracil, uracil glycol and 5,6-dihydroxyuracil in OsO₄-treated cytosine. Treatment of OsO₄-treated cytosine with formic acid caused the complete conversion of uracil glycol into 5-hydroxyuracil. The implications of these findings relative to studies of DNA repair are discussed.

INTRODUCTION

Oxidative damage to DNA caused by reactive oxygen species, e.g. hydroxyl radicals, unless repaired by cellular repair processes, may have detrimental effects in living cells, such as mutagenesis, carcinogenesis and cell death (Painter, 1980; Ames, 1983; Friedberg, 1984; Cerutti, 1985). Oxidative damage can also be introduced into DNA by reagents such as OsO₄ and KMnO₄, which react readily with unsaturated organic compounds to give *cis*-glycols among other products (Hofmann, 1912; Criegee, 1936; Schröder, 1980). Investigations have shown that OsO₄ or KMnO₄ react with thymine in oligonucleotides and DNA to form *cis*-thymine glycol (Bayley & Jones, 1959; Burton & Riley, 1966; Beer *et al.*, 1966; Darby *et al.*, 1967; Iida & Hayatsu, 1971; Frenkel *et al.*, 1981). The reactivity of OsO₄ with DNA bases other than thymine has been reported to be minimal or absent (Beer *et al.*, 1966). However, under vigorous conditions, permanganate oxidation of DNA has been reported to lead to some ureido residues of thymine, cytosine and guanine (Bayley & Jones, 1959; Darby *et al.*, 1967). Formation of 5-hydroxy-5-methylbarbituric acid on oxidation of thymine with KMnO₄ has also been observed (Iida & Hayatsu, 1970, 1971). The reactions of Os(VIII) reagents with cytosine in the presence of some ligands have been reported to form oxo-Os(VI) esters of

uracil (Chang *et al.*, 1981). However, no products from the reaction of OsO₄ with cytosine in DNA have yet been reported.

In the past, the production of altered bases in DNA was initiated in order to provide a suitable substrate for studies of DNA repair. OsO₄ has been used commonly as the chemical reagent of choice in studies of thymine glycol release from DNA by DNA glycosylases (Hariharan & Cerutti, 1974; Gates & Linn, 1977; Nes, 1980; Demple & Linn, 1980; Brent, 1983), on the basis of its selective conversion of thymine in DNA into thymine glycol. Typically, reaction mixtures included OsO₄-treated DNA and either cell extracts or partially purified enzymes. Those studies, however, have been conducted without any information on the possible OsO₄-induced products of DNA bases other than thymine. A complete knowledge of base alterations in OsO₄-treated DNA, in addition to thymine glycol, may be of great importance for the studies of DNA repair that use this reagent as well as other similarly acting oxidative agents in the synthesis of DNA substrates.

The present study was undertaken to elucidate other possible OsO₄-induced products in DNA. For this purpose we employed an assay that has been developed for characterization of oxidative DNA base damage (Dizdaroğlu, 1984, 1985). Here we present evidence that cytosine glycol and 5,6-dihydroxycytosine moieties are formed in DNA on treatment with OsO₄.

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Abbreviations used: thymine glycol, 5,6-dihydroxy-5,6-dihydrothymine; uracil glycol, 5,6-dihydroxy-5,6-dihydrouracil; cytosine glycol, 5,6-dihydroxy-5,6-dihydrocytosine; g.c.–m.s., gas chromatography–mass spectrometry.

MATERIALS AND METHODS

Materials

Calf thymus DNA, 5-hydroxyuracil (isobarbituric acid), cytosine, 5-methylcytosine, thymine, uracil, bis-(trimethylsilyl)trifluoroacetamide and 5,6-dihydrothymine were purchased from Sigma Chemical Co. Acetonitrile was obtained from Pierce Chemical Co. OsO_4 was purchased from Aldrich Chemical Co. Dialysis membranes (Spectrapor; Arthur H. Thomas Co.) had a reported M_r cut-off of approx. 12000. Water purified through a Millipore system was used for all purposes. 5,6-Dihydroxyuracil (isodialuric acid) was synthesized by oxidation of isobarbituric acid with Br_2 (Behrend & Roosen, 1889). *cis*-Thymine glycol and *cis*-uracil glycol were isolated from OsO_4 -treated thymine and uracil respectively by h.p.l.c.

Oxidation of DNA and bases with OsO_4

Numerous variations have been reported in the method for oxidizing DNA with OsO_4 . DNA was denatured to react with OsO_4 by using heat (Beer *et al.*, 1966; Hariharan & Cerutti, 1974; Brent, 1983) or alkali (Gates & Linn, 1977; Nes, 1980). After the reaction of OsO_4 with DNA, unused OsO_4 was removed by either freeze-drying or diethyl ether extraction. The protocol employed in this work reflects a general attempt to duplicate the typical methods used for the synthesis of thymine glycol in DNA in several studies of DNA repair.

An aqueous solution of calf thymus DNA (0.4 mg/ml) was extensively dialysed against water and then treated with OsO_4 (2%) at 60 °C for 1 h. The solution, now coloured black, was cooled on ice and centrifuged to remove a precipitate. The supernatant was extracted three times with CCl_4 to remove excess OsO_4 , dialysed against water and freeze-dried. For control purposes, a portion of the dialysed DNA solution was mixed with OsO_4 , immediately extracted with CCl_4 and dialysed against water.

Thymine, cytosine and uracil were treated with OsO_4 as above, but without dialysis.

Hydrolysis with formic acid

To remove bases from the sugar-phosphate backbone of DNA, 1 mg of each sample was treated with 1 ml of concentrated formic acid (88%) in evacuated and sealed tubes at 150 °C for 30 min. After hydrolysis, samples were dried *in vacuo* in a desiccator before trimethylsilylation. For comparison, samples of OsO_4 -treated cytosine were also treated with formic acid in the same manner.

Trimethylsilylation

Samples were trimethylsilylated in Teflon-capped Hypovials (Pierce Chemical Co.) with 0.2 ml of bis (trimethylsilyl)trifluoroacetamide/acetonitrile (1:1, v/v) mixture by heating for 15 min at 130 °C.

G.c.-m.s.

A Hewlett-Packard model 5880A microprocessor-controlled gas chromatograph interfaced to a Hewlett-Packard model 5970A Mass Selective Detector was used. The injection port and interface were both maintained at 250 °C. Separations were carried out by using a fused-silica capillary column (25 m $\times$ 0.2 mm internal diam.) coated with cross-linked SE-54 (5% phenylmethylsilicone; film thickness 0.11 μm) (Hewlett-Packard). Helium

was used as the carrier gas with a linear velocity of 23.4 cm/s through the column. The split ratio was 10:1. Mass spectra were taken at 70 eV. The temperature of the ion source was approx. 200 °C.

H.p.l.c.

Separations were carried out with a Hewlett-Packard model 1090 microprocessor-controlled liquid chromatograph equipped with a model HP 1040A high-speed spectrophotometric detector and a 10 cm $\times$ 0.21 cm Hypersil ODS microbore reversed-phase column (particle size 5 μm ; Hewlett-Packard). For semi-preparative separations, a Supelcosil LC-8-DB column (25 cm $\times$ 1 cm; particle size 5 μm ; Supelco) was used.

RESULTS AND DISCUSSION

DNA samples were hydrolysed with formic acid and subsequently analysed by capillary g.c.-m.s. by using a method described previously (Dizdaroglu, 1985). Fig. 1 shows a total-ion chromatogram obtained from a trimethylsilylated formic acid hydrolysate of OsO_4 -treated DNA. Peaks 1-4, 13 and 14 represent the trimethylsilyl derivatives of phosphoric acid, 2-deoxyribose, thymine, cytosine, adenine and guanine respectively. Peak 5 corresponds to the trimethylsilyl derivative of 5-methylcytosine, for which authentic material was available. Calf thymus DNA is known to have a 5-methylcytosine content of about 1.3% (Adams *et al.*, 1981). Peak 7 also represents cytosine but with an additional trimethylsilyl group attached to its amino group, as revealed both by comparison with authentic material and by its mass spectrum. Peaks marked with 'x' were also present in control samples. Peak 10, corresponding to the trimethylsilyl derivative of *cis*-thymine glycol, was verified by the use of authentic material and by comparison of its mass spectrum with previously published data (Dizdaroglu, 1984). *trans*-Thymine glycol was also observed (peak 12 in Fig. 1); however, its presence was due to the partial isomerization of *cis*-thymine glycol by acidic treatment (Iida & Hayatsu, 1970). The isomerization was confirmed in the present work by g.c.-m.s. analysis of formic acid-treated *cis*-thymine glycol. Peak 8 appeared to correspond to a sugar derivative; however, its origin could not be defined.

Peaks 6, 9 and 11 in Fig. 1 represent the trimethylsilyl derivatives of 5-hydroxyuracil (isobarbituric acid), 5-hydroxycytosine and 5,6-dihydroxyuracil (isodialuric acid or dialuric acid) respectively. Of these three, authentic materials were available for isobarbituric acid and isodialuric acid (for the mass spectra of their trimethylsilyl derivatives see Dizdaroglu, 1985). The trimethylsilyl derivative of dialuric acid is known to be identical with that of isodialuric acid as a consequence of enolization (Schuchmann & von Sonntag, 1983). 5-Hydroxycytosine was identified on the basis of the known mass spectrum and gas-chromatographic behaviour of its trimethylsilyl derivative (Dizdaroglu, 1984). The two uracil derivatives discussed above are believed to be derived from some OsO_4 -induced products of the cytosine moiety of DNA by acid treatment. For example, it is well known that deamination and dehydration readily occur with the cytosine derivatives saturated at the C-5-C-6 double bond (Green & Cohen, 1957; Evans *et al.*, 1975; Taguchi *et al.*, 1977; Teoule & Cadet, 1978). It is therefore suggested that 5-hydroxyuracil was formed

Fig. 1. Total-ion chromatogram obtained from a trimethylsilylated formic acid hydrolysate of OsO₄-treated calf thymus DNA

The column, a fused-silica capillary (25 m × 0.2 mm internal diam.) coated with cross-linked SE-54, was programmed after 3 min at 100 °C from 100 to 250 °C at 7 °C/min. For other details see the Materials and methods section.

Scheme 1. Acid-induced formation of 5-hydroxycytosine and 5-hydroxyuracil from cytosine glycol

by formic acid-induced deamination and dehydration of cytosine glycol, which was the actual oxidation product of the cytosine moiety of DNA. 5-Hydroxycytosine was most probably the result of the dehydration of the same compound (Scheme 1).

The presence of 5,6-dihydroxyuracil (peak 11 in Fig. 1) can be explained analogously to the formation of 5-hydroxy-5-methylbarbituric acid from thymine on permanganate oxidation (Iida & Hayatsu, 1970, 1971) (Scheme 2). The actual oxidation products of cytosine, i.e. structures (I) and (II), were not observed in DNA. As the calf thymus DNA used in this work did not contain uracil, the uracil derivatives discussed above could not have been formed from uracil in DNA.

In order to support the suggestion that 5-hydroxyuracil and 5,6-dihydroxyuracil were derived from the cytosine moiety of DNA, cytosine was also treated with OsO₄, and analysed by g.c.-m.s. and h.p.l.c. before and after formic acid treatment. Fig. 2 shows a total-ion chromatogram obtained from a sample of OsO₄-treated cytosine after trimethylsilylation. Peaks 1 and 3 represent cytosine, and correspond to peaks 4 and 7 in Fig. 1. Peak 2 represents

the trimethylsilyl derivative of 5-hydroxyuracil (as peak 6 in Fig. 1). A mass spectrum taken from peak 4 is illustrated in Fig. 3. A molecular ion (M^{+}) and a characteristic $M - CH_3$ ion were observed at m/z 434 and 419 respectively. This mass spectrum was assigned to the trimethylsilyl derivative of 5,6-dihydroxy-5,6-dihydro-uracil (uracil glycol), which shows a fragmentation pattern similar to that of the trimethylsilyl derivative of thymine glycol (for a comparison of the mass spectra see Dizdaroğlu, 1984). Authentic uracil glycol was also available, obtained from OsO₄ oxidation of uracil. Since cytosine used here did not contain uracil, the presence of uracil glycol in OsO₄-treated cytosine can only be explained by deamination of cytosine glycol, which was the actual oxidation product of cytosine. However, it is not known whether the deamination occurred spontaneously in aqueous solution or was induced by trimethylsilylation.

Peak 5 in Fig. 2 represents the trimethylsilyl derivative of 5,6-dihydroxyuracil, also observed in OsO₄-treated DNA, as explained above (peak 11 in Fig. 1). Although the g.c.-m.s. analysis of OsO₄-treated cytosine after

Scheme 2.

Fig. 2. Total-ion chromatogram obtained from a sample of OsO_4 -treated cytosine after trimethylsilylation

Column details were as given in Fig. 1 legend.

formic acid treatment clearly showed the absence of uracil glycol (Fig. 4), 5-hydroxyuracil and 5,6-dihydroxyuracil were present (peaks 2 and 4 in Fig. 4 respectively). The increased amount of 5-hydroxyuracil (compare peaks 2 in Figs. 2 and 4) indicates its formation from uracil glycol by dehydration. In confirmation, formic acid treatment completely converted authentic uracil glycol into 5-hydroxyuracil.

OsO_4 -treated cytosine was also analysed by reversed-phase h.p.l.c., as illustrated in Fig. 5. Cytosine gave a broad and tailing peak under these conditions (peak 5). Compounds represented by peaks 1–4 were collected by the use of a semi-preparative column and subsequently analysed by g.c.–m.s. Peaks 2 and 3 were found to correspond to uracil glycol and 5,6-dihydroxyuracil respectively. Fraction 2 also contained trace amounts of 5-hydroxyuracil. Fractions 1 and 4 contained no uracil or cytosine derivatives.

It is noteworthy that 5,6-dihydroxythymine, which had been tentatively identified in OsO_4 -treated DNA in

Fig. 3. Mass spectrum taken from peak 4 in Fig. 2

Fig. 4. Total-ion chromatogram obtained from a sample of OsO_4 -treated cytosine after formic acid treatment and trimethylsilylation

Column details were as given in Fig. 1 legend.

previous work (Demple & Linn, 1980), was not observed in the present paper. This compound is easily detectable by g.c.-m.s. as demonstrated previously (Dizdaroglu, 1984, 1985), and authentic material was also available. The absence of 5,6-dihydrothymine from OsO_4 -treated DNA confirms the assumption by Demple & Linn (1980) that this compound was most probably not formed in DNA by OsO_4 treatment.

The yields of conversion of thymine and cytosine into the products identified on OsO_4 treatment of DNA under the conditions described in the Materials and methods section were determined by g.c. with a flame ionization detector. Uracil was used as an internal standard and added to the samples before formic acid hydrolysis. The relative molar response factors versus uracil of thymine, thymine glycol, cytosine, 5-hydroxyuracil and 5,6-

dihydroxyuracil were determined to be 0.95, 0.75, 1.1, 0.9 and 0.8 respectively. The relative molar response factor of 5,6-dihydroxycytosine versus uracil was assumed to be 1.0. The results showed that approx. 73% of thymine and 20% of cytosine were converted into products. The ratio of cytosine glycol (in terms of the sum of 5-hydroxyuracil and 5-hydroxycytosine) to 5,6-dihydroxycytosine (in terms of 5,6-dihydroxyuracil) was approx. 4:1.

CONCLUSIONS

The data presented here clearly suggest that, in addition to thymine glycol, cytosine glycol and 5,6-dihydroxycytosine are formed in DNA on OsO_4 treatment, and their subsequent deamination and dehydration give rise to formation of uracil glycol, 5-hydroxycytosine, 5-hydroxyuracil and 5,6-dihydroxyuracil. These results may be of importance to DNA repair studies that use OsO_4 -treated DNA as a substrate for base-excision repair enzymes such as glycosylases.

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Fig. 5. H.p.l.c. profile obtained from a sample of OsO_4 -treated cytosine

The column was microbore Hypersil ODS (10 cm $\times$ 0.21 cm; particle size 5 μm). The flow rate was 0.1 ml/min. The eluent was prepared from water (solvent A) and acetonitrile (solvent B); isocratic elution was with solvent A for 5 min, then gradient elution at 0.5% solvent B/min, at ambient temperature.

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