Vol. 169, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1990 Pages 87-94

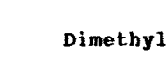
PREFERENTIAL MODIFICATION OF GUANINE BASES IN DNA BY DIMETHYLDIOXIRANE AND ITS APPLICATION TO DNA SEQUENCING

R. Jeremy H. Davies[1], Derek R. Boyd[2], Shiv Kumar[1], Narain D. Sharma[2] and Clarke Stevenson[1]

[1]Division of Biochemistry, School of Biology and Biochemistry, and [2]School of Chemistry, Queen's University, Belfast BT9 7BL, Northern Ireland

Received March 16, 1990

Summary: From gel sequencing experiments with ^{32}P-end-labelled oligodeoxyribonucleotides, it is shown that treatment of DNA with the powerful oxidant dimethyldioxirane, followed by heating in piperidine, causes selective strand scission at the sites of guanine bases. The same specificity for cleavage at guanine was observed with a 45-mer labelled at either the 3'- or 5'-end and with a single and double stranded 34-mer. On account of its speed and operational simplicity, modification with dimethyldioxirane is proposed as a practicable alternative to conventional chemical sequencing procedures for locating guanine bases in DNA.

Dimethyldioxirane  is a new, versatile, and extremely powerful oxidising agent which is capable of inserting an oxygen atom into a wide range of organic functional groups under mild, neutral reaction conditions (1,2). It can, for example, cleanly oxidise amines to nitro compounds, hydroxylamines or amine N-oxides (2) and converts imines into nitrones (3). Product isolation is simplified because both dimethyldioxirane and its reaction by-product acetone are highly volatile. Although the biochemistry of oxidative damage to DNA has been extensively studied (4-7), particularly in relation to the effects of ionising radiation (8), there is currently no information concerning how DNA and its components are modified by dimethyldioxirane. In this communication, we show that treatment of DNA with dimethyldioxirane produces lesions (of unknown structure) predominantly at the sites of guanine bases. Subsequent heating of the modified DNA with piperidine causes selective chain cleavage which can be used to map the position of guanine residues in sequencing experiments.

MATERIALS AND METHODS

Materials: Dimethyldioxirane was prepared by adding potassium monoperoxysulphate (oxone) to a mixture of acetone and aqueous sodium bicarbonate, at room temperature, as described by Murray and Jeyaraman (9).

0006-291X/90 $1.50

It was isolated as a solution in acetone by distillation from the generation vessel into a series of cold traps (9) maintained at dry ice-acetone temperature. The concentration of dimethyldioxirane in this stock solution was determined by iodometric titration (10). In this procedure, the dimethyldioxirane solution is diluted with 60% acetic acid in acetone and then mixed with a saturated aqueous solution of potassium iodide; the iodine liberated is titrated with standardized sodium thiosulphate. In practice, stock solutions of dimethyldioxirane in acetone were found to have concentrations in the range 50-100 mM. They were stored at -70°C and diluted as required with a 1:1 acetone/water mixture at 0°C.

2'-Deoxyribonucleosides and calf thymus DNA were obtained from Sigma; the DNA was sheared and deproteinised before use (11). Synthetic oligodeoxyribonucleotides were assembled following standard procedures with an Applied Biosystems 380 B DNA Synthesiser using β-cyanoethyl-*N*,*N*-diisopropyl-amino-phosphoramidite reagents; after deprotection they were purified by electrophoresis on 20% polyacrylamide gels containing 7 M urea and recovered by the elution by diffusion technique (12). [γ-^{32}P]ATP (110 TBq mmol^{-1}), [α-^{32}P]ddATP (110 TBq mmol^{-1}), T4 polynucleotide kinase (*E.coli* B), and terminal deoxynucleotidyl transferase (calf thymus) were obtained from Amersham International.

End-labelled oligonucleotides: The 34-mer 5'-CCCAGTAGATTCGTGACAAAGCCGCATAACC-TCC-3' was labelled with ^{32}P at its 5'-end by the action of T4 polynucleotide kinase and [γ-^{32}P]ATP (13). The 45-mer 5'-GAAGGCTGGCATGGCGCTCTGTGTGGGCTGCACA-GTAGGGGCCAC-3' was also labelled at its 5'-end by this method or at its 3'-end by the action of terminal deoxynucleotidyl transferase and [α-^{32}P]ddATP (14). Double stranded molecules of the end-labelled 34-mer were prepared by annealing it with an unlabelled synthetic oligonucleotide of complementary sequence. An equimolar mixture of the two strands dissolved in 100 mM Tris-HCl buffer, pH 8.0, was heated to 92°C and allowed to cool slowly to room temperature; the duplex was recovered by ethanol precipitation (11).

Treatment of 2'-deoxyribonucleosides with dimethyldioxirane: Aqueous solutions (500 µl, 20 mM) of each of the deoxyribonucleosides 2'-deoxy-adenosine, 2'-deoxyguanosine, 2'-deoxycytidine, 5-methyl-2'-deoxycytidine and thymidine were mixed with an equimolar quantity of dimethyldioxirane (110 µl, 90 mM in acetone) in Eppendorf vials and maintained at 22°C for 1 h; the reaction mixtures were then lyophilised (Savant Speedvac) and reconstituted in water (500 µl). To determine the extent of reaction, the samples were analysed by reversed-phase HPLC on a µ Bondapak C_{18} column (300 x 4 mm) with equipment described elsewhere (15). After sample injection, the column was eluted, at a flow rate of 0.8 ml min^{-1}, with 0.05% aqueous trifluoroacetic acid for 5 min followed by a linear gradient to 0.05% trifluoroacetic acid containing 30% methanol after 15 min; elution profiles were monitored at 254 nm. In each case, the peak corresponding to the unchanged deoxyribonucleoside starting material was collected and then quantified by UV spectrophotometry.

Gel sequencing of oligonucleotides: After base-specific chemical modification and cleavage, ^{32}P-end-labelled oligonucleotides were fractionated on sequencing gels (40 x 18 cm, 0.3 mm thick) containing 16% polyacrylamide and 7 M urea. Electrophoresis was performed, at 45°C and 25 W, on an LKB Macrophor gel electrophoresis system; the gels were subsequently visualised by autoradiography at -70°C (Fuji X-ray film).

Base-specific cleavages at G, T, and C + T were carried out according to Maxam and Gilbert (13); the method of Banaszuk et al. (16) was used to cleave DNA at A + G.

Action of dimethyldioxirane on synthetic oligonucleotides: Reactions between oligonucleotides and dimethyldioxirane were carried out in siliconised 1.5 ml Eppendorf vials. The ^{32}P-end-labelled oligonucleotide (20-40 pmol) was dissolved in water (5 µl) and mixed with 1 µl of aqueous calf thymus DNA (4 mg ml^{-1}). The solution of oligonucleotide plus carrier DNA was maintained at 0°C while freshly diluted dimethyldioxirane (20 µl; 0.1 - 2 mM) was added. After mixing, the reaction vial was held in a water bath at 20°C for 2 min before being placed in another water bath at 85°C for a further 5 min. The chilled contents of the vial were then lyophilised prior to treatment with freshly diluted 1 M piperidine (40 µl) which was carried out by heating in sealed glass capillary tubes at 90°C for 30 min. After chilling in ice, the piperidine solutions were transferred to new Eppendorf vials, dried, and then lyophilised three times from 30 µl of water. The pellets were dissolved in 80% formamide loading solution (11) and loaded onto a denaturing polyacrylamide sequencing gel which was run under the conditions described above.

RESULTS AND DISCUSSION

In preliminary experiments the relative reactivity of the 2'-deoxyribonucleoside components of DNA towards dimethyldioxirane was assessed. Each deoxyribonucleoside was treated with an equimolar quantity of dimethyldioxirane at 22°C for 1 h following which the amount of unchanged deoxyribonucleoside was estimated by HPLC analysis. Extensive destruction of the starting material (80%) occurred with deoxyguanosine (dG) and thymidine (dT). The HPLC profiles of the reaction products (Fig. 1 (a) and (b)) indicate that dG and dT are converted mainly into non-UV absorbing species which lack a conjugated π-electron chromophore. Deoxycytidine (dC) and 5-methyldeoxycytidine (m^5dC) were degraded to the extent of 30% and 20% respectively and the HPLC profiles (Fig. 1 (c) and (d)) show that some UV-absorbing products are formed. There was very little (< 5%) destruction of deoxyadenosine (dA). After reaction, its HPLC profile (not shown) revealed no new peaks. However, the UV spectrum of the recovered dA indicated that limited conversion to the N(1)-oxide had occurred, this substance co-eluting with dA on HPLC. Based on these results, the susceptibility of the deoxyribonucleosides towards oxidation by dimethyldioxirane is in the order dG ~ dT > dC ~ m^5dC > dA. This trend was confirmed when the experiments were repeated with 2 mol of dimethyldioxirane per mol of deoxyribonucleoside. There was now total destruction of dG and dT and a proportionate increase in degradation of the others. The nature of the oxidation products derived from the individual deoxyribonucleosides has not yet been determined but is under investigation.

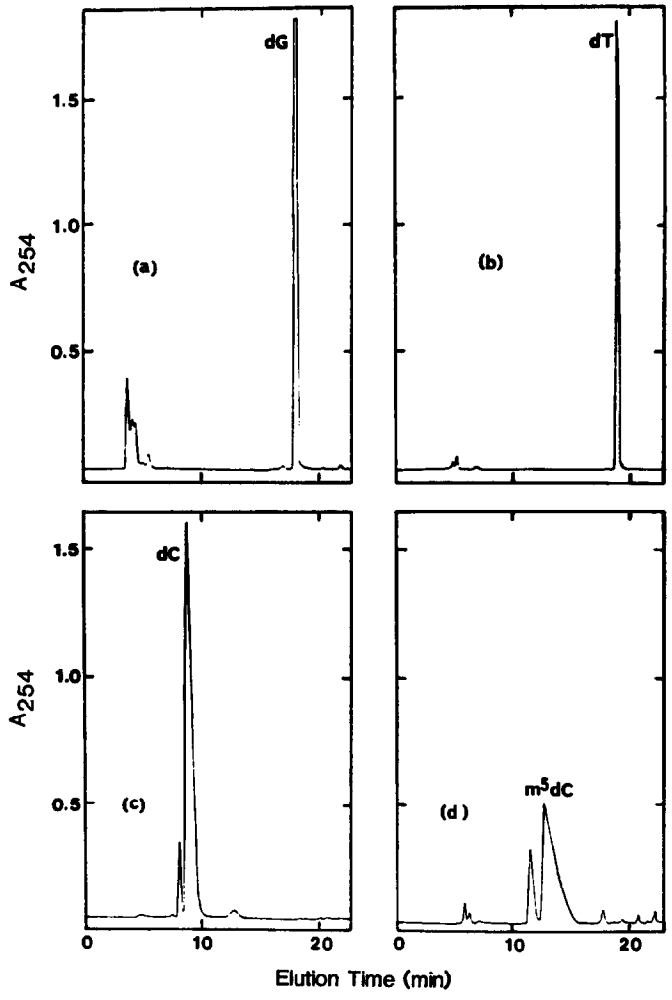


Fig. 1. HPLC elution profiles (at 254 nm) of 2'-deoxyribonucleosides treated with an equimolar quantity of dimethyldioxirane: (a) deoxyguanosine (dG), (b) thymidine (dT), (c) deoxycytidine (dC), (d) 5-methyldeoxycytidine (m^5dC). Details of the HPLC system are given under Methods.

The modification of nucleotide residues in DNA by dimethyldioxirane was explored by gel sequencing experiments with ^{32}P-end-labelled synthetic oligonucleotides containing 34 or 45 bases. An approximately 10-fold excess of carrier DNA was mixed with the labelled oligonucleotide before treating it with dimethyldioxirane. An amount of dimethyldioxirane, in the range 0.3 to 3 mol per mol of total nucleotide phosphate, was added to each sample and allowed to react for 2 min at 20°C before heating to 85°C to decompose residual dimethyldioxirane. The oligonucleotides were then analysed for the presence of frank chain breaks or base labile cleavage sites on DNA sequencing gels with reference to fragments produced by conventional chemical sequencing reactions.

As evidenced in Fig. 2 (lanes 10 and 15), exposure of DNA to dimethyldioxirane under the conditions employed did not cause appreciable scission of the deoxyribose-phosphate backbone. However, heating the treated DNA with piperidine caused selective cleavage at the sites of guanine bases, the fragments produced having the same mobility as those generated by the standard guanine-specific Maxam-Gilbert procedure. Similar results were obtained for both the single and double stranded 34-mer (Fig. 2, lanes 7 to 9

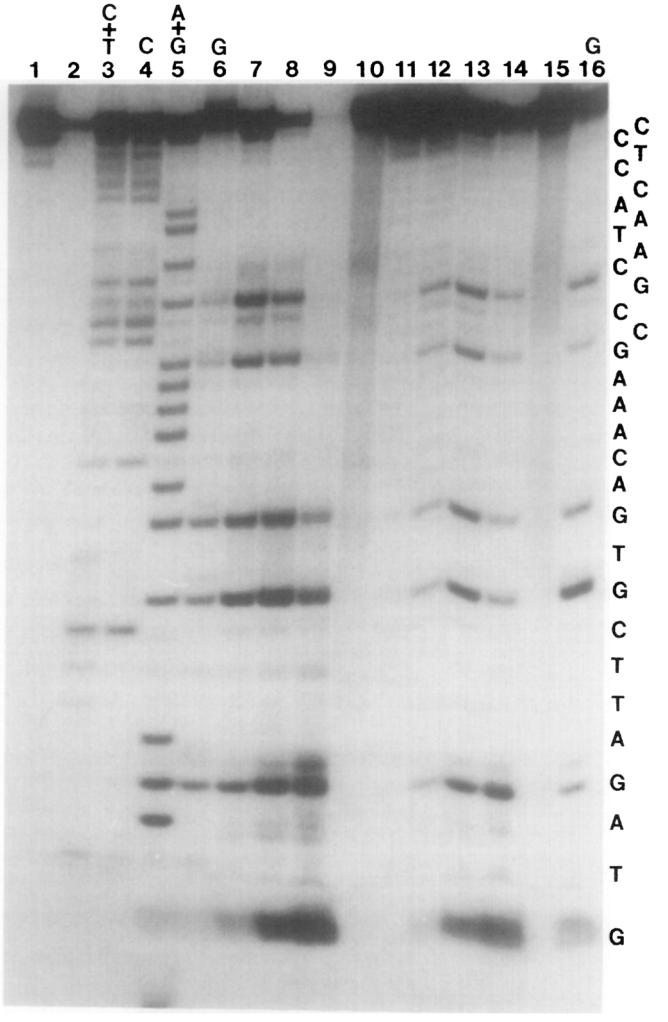


Fig. 2. Autoradiograph of [5'-^{32}P] end-labelled single and double stranded 34-mer treated with dimethyldioxirane and piperidine. Lanes 1 and 2: uncut 34-mer before and after heating with piperidine; lanes 3 to 6 and 16: products of Maxam-Gilbert sequencing reactions (13) as indicated; lanes 7 to 9: single stranded 34-mer (20 pmol) plus carrier DNA (4 µg) reacted with 7, 14 and 22 nmol of dimethyldioxirane respectively then heated with piperidine; lanes 11 to 14: double stranded 34-mer (20 pmol) plus carrier DNA (4 µg) reacted with 7, 14, 22 and 36 nmol of dimethyldioxirane respectively then heated with piperidine; lanes 10 and 15: same as lanes 9 and 14 but without piperidine treatment.

and 11 to 14) though approximately twice the concentration of dimethyldioxirane was required to effect the same extent of cleavage with the duplex. At the highest concentrations used (lanes 9 and 14) there was a bias towards shorter fragments indicating that multiple cleavage sites were being introduced into the oligonucleotide chain. Experiments were also conducted with a single stranded 45-mer labelled with ^{32}P at either its 5'- or 3'-end. On treatment with dimethyldioxirane and piperidine, the 5'-end labelled material (gel not shown) behaved identically to the 34-mer (Fig. 2). Results for the 3'-end labelled molecule are shown in Fig. 3 and likewise illustrate a strong preference for cleavage at the sites of guanine bases. The even

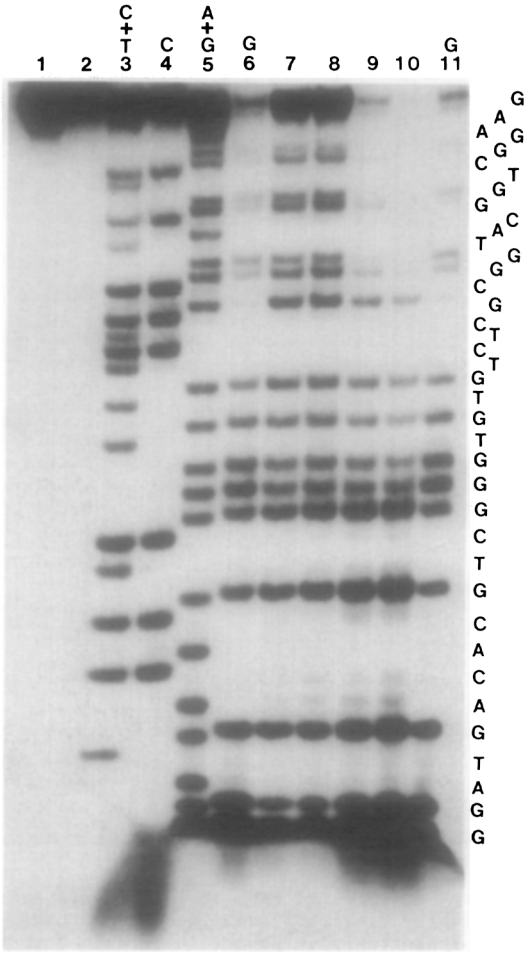


Fig. 3. Autoradiograph of single stranded [3'-^{32}P] end-labelled 45-mer treated with dimethyldioxirane and piperidine. Lanes 1 and 2: uncut 45-mer before and after heating with piperidine; lanes 3 to 6 and 11: products of Maxam-Gilbert sequencing reactions as indicated; lanes 7 to 10, 45-mer (20 pmol) plus carrier DNA (4 µg) reacted with 4, 7, 14 and 22 nmol of dimethyldioxirane respectively then heated with piperidine.

density of the bands in the lanes on the gel suggests that base sequence context has little influence on the reactivity of individual guanines. By appropriate adjustment of the dimethyldioxirane concentration it should be feasible to optimise the cleavage conditions for longer fragments of DNA than those studied here. In view of the reactivity of the individual deoxyribonucleosides, it is probable that other nucleobases in DNA, especially thymine, are modified to some degree by dimethyldioxirane. If so, it would appear that, in contrast to guanine, the resultant lesions do not give rise to chain scission on heating with piperidine.

The experimental protocol described above clearly affords a simple and rapid method for mapping the positions of guanine bases in terminally labelled DNA fragments of known or unknown sequence. As such, it provides a practicable alternative to the conventional guanine-specific chemical sequencing procedures which utilise modification of the DNA by dimethyl sulphate (17). In comparison with these methods, the use of dimethyldioxirane offers several advantages. It is less time-consuming, requires fewer reagents and entails fewer operational steps. There is no necessity for ethanol precipitation so that quantitative recovery of the sample is achieved without the complications of added salt or generation of radioactive supernatant waste solutions. Furthermore, the addition of a quenching agent is avoided because excess dimethyldioxirane can be decomposed to volatile acetone and oxygen, and thus be totally removed, simply by gentle heating. It is noteworthy that dimethyldioxirane in acetone solution is a readily prepared and inexpensive reagent (9) which is stable for many months when stored at $-70^{\circ}C$; at $25^{\circ}C$, it has a half-life of ~48 h.

Other methods that have been reported to lead to predominant cleavage of DNA at guanine residues include its treatment with diethylpyrocarbonate (18), photolysis in the presence of methylene blue (19), methylamine (20) or the dimethyldiazaperipyrenium dication (21), and excitation with high intensity pulsed laser radiation at 248 nm (22). To date, these approaches have not found routine application in the sequence determination of DNA but diethylpyrocarbonate, in common with dimethylsulphate, is widely used as a chemical probe or 'footprinting' reagent for investigating the recognition of DNA by proteins and other molecules. Depending upon its reactivity towards proteins, dimethyldioxirane may also prove to be useful in this respect. As a potential chemical probe of DNA secondary structure and of the local environment of guanine bases in DNA-protein or DNA-ligand complexes, dimethyldioxirane has the desirable properties of being a highly reactive, uncharged small molecule which is readily soluble in aqueous media at neutral pH

Acknowledgments: This work was supported by SERC grant GR/E 48060. We thank Dr R.M. McGuckin for assistance with the preparation of dimethyldioxirane.

REFERENCES

1. Adam, W., Curci, R., and Edwards, J.O. (1989) Acc. Chem. Res. 22, 205-211.
2. Murray, R.W. (1989) Chem. Rev. 89, 1187-1201.
3. Boyd, D.R., Coulter, P.B., McGuckin, R.M., Sharma, N.D., Jennings, W.B., and Wilson, V.E. (1990) J. Chem. Soc. Perkin Trans. I, in press.
4. Ames, B.N., Saul, R.L., Schwiers, E., Adelman, R., and Cathcart, R. (1985) In Molecular Biology of Aging: Gene Stability and Gene Expression (R.S. Sohal, L.S. Birnbaum and R.G. Cutler, Eds.), pp. 137-144. Raven, New York.
5. Sies, H. (1986) Angew. Chem. Int. Ed. Engl. 25, 1058-1071.
6. Storz, G., Christman, M.F., Sies, H., and Ames, B.N. (1987) Proc. Natl. Acad. Sci. USA 84, 8917-8921.
7. Teebor, G.W., Boorstein, R.J., and Cadet, J. (1988) Int. J. Radiat. Biol. 54, 131-150.
8. von Sonntag, C. (1987) The Chemical Basis of Radiation Biology, Taylor & Francis, London.
9. Murray, R.W., and Jeyaraman, R. (1985) J. Org. Chem. 50, 2847-2853.
10. Adam, W., Chan, Y-Y., Cremer, D., Gauss, J., Scheutzow, D., and Schindler, M. (1987) J. Org. Chem. 52, 2800-2803.
11. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory.
12. Smith, H.O. (1980) Methods Enzymol. 65, 371-380.
13. Maxam, A.M., and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.
14. Yousaf, S.I., Carroll, A.R., and Clarke, B.E. (1984) Gene 27, 309-313.
15. Kumar, S., and Davies, R.J.H. (1987) Photochem. Photobiol. 45, 571-579.
16. Banaszuk, A.M., Deugau, K.V., Sherwood, J., Michalak, M., and Glick, B.R. (1983) Anal. Biochem. 128, 281-286.
17. Ambrose, B.J.B., and Pless, R.C. (1987) Methods Enzymol. 152, 522-538.
18. Krayev, A.S. (1981) FEBS Lett. 130, 19-22.
19. Friedmann, T., and Brown, D.M. (1978) Nucleic Acids Res. 5, 615-622.
20. Saito, I., Sugiyama, H., Matsuura, T., Ueda, K., and Komano, T. (1984) Nucleic Acids Res. 12, 2879-2885.
21. Slama-Schwok, A., Jazwinski, J., Bere, A., Montenay-Garestier, T., Rougee, M., Hélène, C., and Lehn, J.-M. (1989) Biochemistry 28, 3227-3234.
22. Blau, W., Croke, D.T., Kelly, J.M., McConnell, D.J., OhUigin, C., and Van der Putten, W.J.M. (1987) J. Chem. Soc. Chem. Commun. 751-752.