Site Selective Formation of Thymine Glycol-Containing Oligodeoxynucleotides by Oxidation with Osmium Tetroxide and Bipyridine-Tethered Oligonucleotide

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Received February 7, 2000

Thymine glycol (5,6-dihydroxy-5,6-dihydrothymidine) is a major oxidation product induced by ionizing radiation and endogenous oxidation of DNA.[1−4] It is reported that thymine glycol is not strongly mutagenic[5,6] but efficiently blocks DNA replication either one residue before or at the site of damage.[7−9] NMR studies showed that thymine glycol induces significant and localized structural change of duplex DNA, with the base being largely extrahelical.[10] Thymine glycol-containing oligodeoxynucleotides (ODNs) are usually prepared by oxidation of single strand ODNs with osmium tetroxide or potassium permanganate, followed by HPLC separation from other oxidation products.[11] Due to the limitation of this oxidation method for only ODNs containing a single thymine, a different approach employing enzymatic incorporation of thymine glycol into 3′-end of ODN with terminal deoxynucleotidyl transferase has been investigated.[12−14] To develop a conceptually advanced and more versatile method for the synthesis of thymine glycol-containing ODNs with high overall efficiency and wide sequence applicability, we have investigated site-selective thymine (T) oxidation of ODNs with osmium tetroxide in the presence of bipyridine-tethered complementary ODN. By the use of bipyridine-tethered ODN, we demonstrated a highly selective modification of a targeted single thymine into thymine glycol in a 35-mer ODN containing 14 thymine residues.[15] A combination of this T-selective oxidation and subsequent hot piperidine treatment constitutes a practically useful method for cutting DNA at any desired T residue of single-stranded DNA.

Most transition metal-mediated oxidation of DNA proceeds either by hydrogen abstraction from sugar backbone, leading to direct strand cleavage,[16] or by one-electron transfer from G to produce radical cations not only at a proximal G but also at distal Gs due to the accompanying hole migration.[17] These oxidation methods are not suitable for the synthesis of ODNs containing a single oxidized nucleobase at desired sites. Oxidation of ODN with osmium tetroxide is known to proceed selectively at T residues in a single strand region.[9,18,19] Coordination of osmium to a bidentate ligand, 2,2′-bipyridine (Bpy), can accelerate the oxidation by 10^4-fold to produce a stable osmate complex.[19] These results suggest that T in the vicinity of osmium−bipyridine complex would be oxidized more easily than those being apart from the complex (Figure 1). The target 35-mer d(T_1CG T_4AT_6 GT_8G T_{10}CT_{12} GGC CAC CT_{20}G T_{22}CT_{24} CT_{26}G T_{28}GT_{30}AT_{32}G CT_{35}) (**ODN1**) contains a sequence (C_{11}−C_{25}) complementary to 15-mer d(GAG ACA GGT GGC CAG) (**ODN2**) to form a partial duplex possessing single-stranded overhangs of 10-bases long at both 3′- and 5′-ends (Figure 2). Bipyridine-tethered oligonucleotide (**BpyODN2**) was synthesized by a coupling of modified **ODN2** possessing an aminohexyl linker at the 5′-end with (6-(2-pyridyl)-2-pyridyl)methyl (2,5-dioxopyrrolidinyloxy)formate. Since the thymine glycol site is piperidine-labile,[19] we examined the T oxidation of **ODN1** in the presence of **BpyODN2** that is followed by PAGE analysis after hot piperidine treatment.

Oxidation of a partial duplex of 5′-^{32}P-end-labeled **ODN1** and **ODN2** with osmium tetroxide and subsequent hot piperidine treatment did not produce any detectable cleavage bands (Figure 3a, lane 3), implying that oxidation of T residues in **ODN1**−**ODN2** with ligand-free osmium tetroxide is in fact negligible. In sharp contrast, incubation of **ODN1**−**BpyODN2** with osmium tetroxide for only 10 min produces a band of less mobility than **ODN1** (lane 5, bands shown by X), a cross-linked band between two

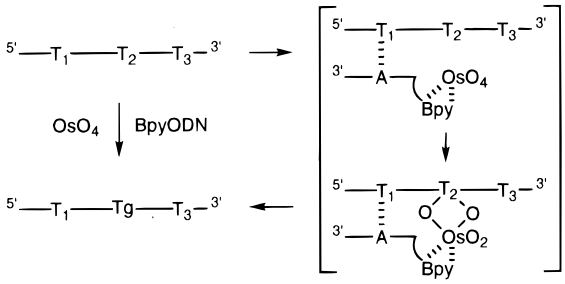

Figure 1. Schematic illustration of thymine (T) modification to thymine glycol (Tg) by osmium tetroxide in the presence of bipyridine-tethered oligonucleotide (BpyODN).

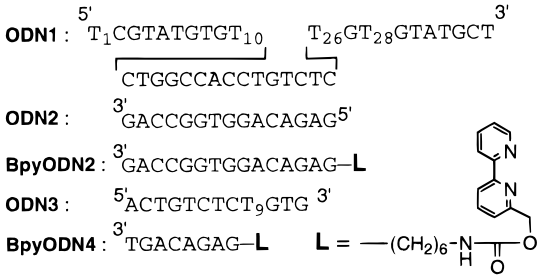

Figure 2. Sequences for the target and bipyridine-tethered oligonucleotides.

(1) Newcomb, T. G.; Loeb, L. A. In *DNA Damage and Repair: DNA Repair in Prokaryotes and Lower Eukaryotes.* Human Press Inc., Totowa, Vol. I., pp 65−84, 1998.
(2) Friedberg, E. C.; Walker, G. C.; Siede, W. *DNA Repair and Mutagenesis*; ASM Press: Washington, 1995.
(3) Téoule, R.; Bonicel, A.; Bert, C.; Cadet, J.; Polverelli, M. *Radiat. Res.* **1974**, *57*, 46−58.
(4) Cathcart, R.; Schwiers, E.; Saul, R. L.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 5633−5637.
(5) Basu, A. K.; Loechler, E. L.; Leadon, S. A.; Essigmann, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7677−7681.
(6) Breimer, L. H.; Lindahl, T. *Biochemistry* **1985**, *24*, 4108−4022.
(7) McNulty, J. M.; Jerkovic, B.; Bolton, P. H.; Basum A. K. *Chem. Res. Toxicol.* **1998**, *11*, 666−673.
(8) Breimer, L. H. *Mol. Carcinog.* **1990**, *3*, 188−197.
(9) Ide, H.; Kow, Y. W.; Wallace, S. S. *Nuc. Acids Res.* **1985**, *13*, 8035−8052.
(10) Kung, H. C.; Bolton, P. H. *J. Biol. Chem.* **1997**, *272*, 9227−9236.
(11) Kao, J. Y.; Goljer, I.; Phan, T. A.; Bolton, P. H. **1993**, *J. Biol. Chem.* **1993**, *268*, 17787−17793.
(12) Hatahet, Z.; Purmal, A. A.; Wallace, S. S. *Nucleic Acids Res.* **1993**, *21*, 1563−1568.
(13) Ide, H.; Wallace, S. S. *Nucleic Acids Res.* **1988**, *16*, 11339−11354.
(14) Ide, H.; Melamede, R. J.; Wallace, S. S. *Biochemistry* **1987**, *26*, 964−969.
(15) For early studies of sequence-specific oxidation of thymine with osmium tetroxide, see: Ford, H.; Chang, C.-H.; Behrman, E. J. *J. Am. Chem. Soc.* **1981**, *103*, 7773−7779.
(16) Pogozelski, W. K.; Tullius, T. D. *Chem. Rev.* **1998**, *98*, 1089−1107.
(17) For a recent review for the oxidation of nucleobase, see: Burrows, C. J.; Muller, J. G. *Chem. Rev.* **1998**, *98*, 1109−1151.
(18) Beer, M.; Stern, S.; Carmalt, D.; Mohlhenrich, K. H. *Biochemistry* **1966**, *5*, 2283−2288.
(19) Palecek, E. *Methods Enzymol.* **1992**, *212*, 139−155.

10.1021/ja0004478 CCC: $19.00
Published on Web 06/17/2000

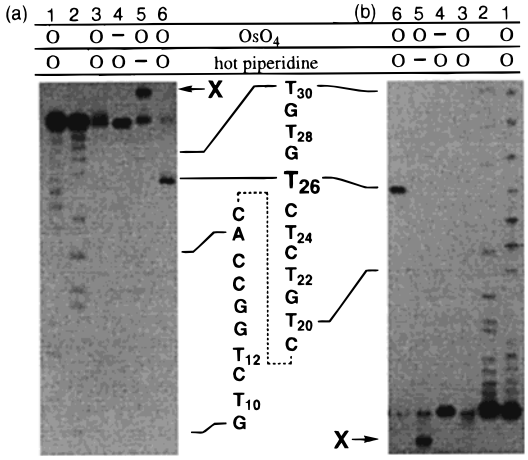

Figure 3. Autoradiograms of a denaturing 15% polyacrylamide/7 M urea gel used to analyze the oxidation of **ODN1** by OsO_4 in the presence of **BpyODN2**. **ODN 1** was labeled at either the (a) 5′- or (b) 3′-end with ^{32}P and annealed with **ODN2** or **BpyODN2** (10 μM) in Tris-HCl buffer (10 mM, pH 7.6) containing NaCl (100 mM) for 12 h at 0 °C. OsO_4 (100 mM) was added, and the resulting mixture was incubated at 25 °C for 10 min. Recovered DNA by ethanol precipitation was analyzed by electrophoresis with or without piperidine treatment (10% v/v, 90 °C, 2 h). Lane 1, T reaction of **ODN1** with OsO_4 (1 mM) and pyridine (2% v/v); lane 2, Maxam−Gilbert G+A sequencing reactions; lane 3, after anealing with **ODN2**; lanes 4−6, after anealing with **BpyODN2**; ODNs in lanes 3, 5, and 6 were treated with OsO_4; all ODNs except in lane 5 were heated with piperidine. A partial base sequence of **ODN1** is shown in the middle. The cross-linked band is marked with **X**. For clarity, the autoradiogram for 3′-end-labeled **ODN1** is shown upside down.

oligomers through cyclic osmate formation. Upon heating the mixture with piperidine, both cross-linked band and the band comigrated with **ODN1** completely disappeared with a concomitant formation of a distinct cleavage band at T_{26} (lane 6).[20] Other T residues in double-stranded and the 5′-side overhang regions were not oxidized at all. Furthermore, the cleavage at T_{28} that is only two bases away from T_{26} was also negligible.

To confirm the cleavage at T in the 3′-side overhang region more definitely, we examined the same oxidation of 3′-^{32}P-end-labeled **ODN1** (Figure 3b). We detected the cleavage band at only T_{26} but not at T_{28}, T_{30}, T_{32}, or T_{35} (lane 6). These results showed that among 14 thymine residues of **ODN1** T_{26} is the sole site to be oxidized by osmium tetroxide in the presence of **BpyODN2**. In contrast, we were able to detect the cleavage band at T_{27} for the ODN containing 5′-$T_{26}T_{27}T_{28}$-3′ sequence instead of 5′-$T_{26}G_{27}T_{28}$-3′ in **ODN1** (Supporting Information). The selectivity for the oxidation of T_{26} and T_{27} was 93:7. Highly selective oxidation of T_{26} observed for **BpyODN2** is primarily due to the proximity effect of an osmium−bipyridine complex to T_{26}.[21,22]

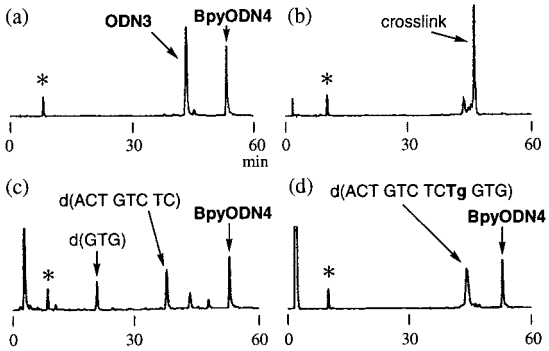

Figure 4. HPLC profiles for the oxidation of an equimolar mixture of **ODN3** and **BpyODN4** (50 μM each) by OsO_4 (100 mM) in Tris-HCl (10 mM) and NaCl (100 mM). (a) A mixture of **ODN3**, **BpyODN4**, and dA (added as an internal standard, marked with *); (b) incubation with OsO_4 for 10 min; further treatment of the mixture with (c) hot piperidine (10% v/v) for 2 h at 90 °C followed by alkaline phosphatase and (d) sodium sulfite (50% saturated solution) for 3 h at 50 °C.

HPLC analysis of the oxidized mixture also confirmed the highly selective oxidation. Upon addition of osmium tetroxide to duplex **ODN3**−**BpyODN4**, a new cross-linked peak appeared with a disappearance of starting oligomers (Figure 4).[23] Cross-linking of two oligomers through cyclic osmate formation was confirmed by observing two molecular ions at 6714.06 (calcd for [M − H − O], 6714.63) and 6697.97 (calcd for [M − H − O_2], 6698.63) by MALDI-TOF-MS. Treatment of this mixture with hot piperidine followed by alkaline phosphatase produced both d(ACT GTC TC) and d(GTG), indicating a selective oxidation at T_9 with a reappearance of **BpyODN4**. Chemical yields for the formation of d(ACT GTC TC) and d(GTG) for average four experiments were 64 and 76%, respectively, whereas **BpyODN4** was recovered in 70% yield. Treatment of the same mixture with sodium sulfite gave rise to the formation of thymine glycol (Tg)-containing ODN d(ACT GTC TC**Tg** GTG) as evidenced by MALDI-TOF-MS (found 3660.60; calcd for [M − H], 3660.41).

The present method for site-specific conversion of thymine to thymine glycol by osmium oxidation and subsequent sulfite reduction would be widely applicable to the synthesis of various thymine glycol-containing ODNs. The site-specific T cleavage by osmium oxidation and hot piperidine treatment constitute a practically useful method for cutting DNA at any desired T sites of single strands, thus providing a useful tool for DNA manipulation.

Supporting Information Available: Synthetic procedures for BpyODN2 and PAGE analysis for T oxidation of an oligomer containing continued T sequence (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) These observations showed that the band comigrated with **ODN1** in lane 5 of Figure 3a and 3b corresponds to oxidized **ODN1** containing thymine glycol at the T_{26} position which was produced by hydrolysis of the osmate complex of the cross-linked product.

(21) The T_{26}/T_{27} selectivity was slightly improved by using a tether with shorter length with concomitant decrease of oxidation efficiency. See Supporting Information.

(22) For proximity effects on the site selectivity of guanine modification, see: (a) Nakatani, K.; Shirai, J.; Sando, S.; Saito, I. *J. Am. Chem. Soc*. **1997**, *119*, 7626−7635. (b) Grant, K. B.; Dervan, P. B. *Biochemistry* **1996**, *35*, 12313−12319.

(23) The oxidation of **ODN3** with osmium tetroxide in the presence of ODN that lacks bipyridine ligand and contains only a primary amino group at the 5′-end was not observed at all under identical conditions.