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The use of 2-hydroperoxytetrahydrofuran as a reagent to sequence cytosine and to probe non-Watson–Crick DNA structures

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ABSTRACT

2-Hydroperoxytetrahydrofuran (THF-OOH) can be employed to sequence cytosine (C) and to probe for non-canonical DNA structures involving C. Using ^{32}P-labeled oligomers and a DNA restriction fragment, it is demonstrated that THF-OOH has a strong preference for Cs in single-stranded (s-s) DNA regions, and in bulges, loops and mismatches. The reactivity of C is diminished below pH 6.0, but is not affected by substitution of 5-methylcytosine. To demonstrate the utility of the reagent, it is directly compared to methoxylamine and chloroacetaldehyde, two other reagents commonly used to chemically probe C residues in non-Watson–Crick DNA structures.

INTRODUCTION

The development of chemicals for use as primary sequencing reagents and as probes of secondary and tertiary DNA structure is an area of on going interest. We have previously reported that 2-hydroperoxytetrahydrofuran (THF-OOH; see Fig. 1 for structures) reacts selectively with C in DNA restriction fragments to afford heat labile modifications that can be transformed with alkali into strand breaks (1). We now detail the reactions of THF-OOH and 2-hydroperoxytetrahydropyran (THP-OOH) with single-stranded (s-s) and double-stranded (d-s) DNA, and in d-s DNA containing mismatch, bulge and loop structures (see Fig. 2 for sequences). To demonstrate the value of the reagent as a sequencing tool, the results obtained using THF-OOH are directly compared to those from methoxylamine and chloroacetaldehyde, two other reagents that are commonly used to sequence non-canonical DNA structures containing C residues.

METHODS

Reagents

The syntheses of THF-OOH and THP-OOH have been described (1,2). Basically, 2,3-dihydrofuran (26 g, 0.37 mol) is slowly added to an ice-cold solution of H_2O_2 (30%, 57 g, 0.50 mol) and concentrated H_2SO_4 (0.1 ml). After the addition, the solution is stirred for an additional 45 min with the temperature maintained below 10°C. The reaction mixture is then saturated with solid NH_4Cl and extracted with CH_2Cl_2. The combined CH_2Cl_2 extracts are then extracted with 20% aqueous NaOH and the aqueous layer washed with CH_2Cl_2 and neutralized to slightly above pH 7.2 with HOAc while maintaining the temperature below 10°C. This aqueous solution is saturated with NH_4Cl, extracted with CH_2Cl_2 and the combined extracts washed with cold 10% $NaHCO_3$, dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield 14.7 g (38% yield) of THF-OOH as a clear liquid. The reagents were stored neat at –24°C for 6 months without any significant degradation as measured by ^{1}H-NMR and DNA reactivity. Free methoxylamine (Aldrich) is not stable in air and was prepared from the hydrochloride as needed using diethylamine to adjust the pH as required (3). Chloroacetaldehyde (Aldrich) was also made up immediately prior to use (4).

Preparation of DNA substrates

The 2′-deoxyoligonucleotides were synthesized on an ABI instrument using standard phosphoramidite chemistry and purified by C8 reverse phase HPLC. The restriction fragments were prepared from a clone containing the promotor region for the coat protein of the canine parvovirus (5), using *Nco*I and *Hin*dIII (New England Biolabs) digestion as previously described (6). The restriction fragments and oligomers were 5′ end-labeled using [γ-^{32}P]ATP (Amersham) and T4 kinase (BRL), and then purified on non-denaturing polyacrylamide gels. In one case DNA was end-labeled with ^{35}S.

Reaction conditions

THF-OOH. Unless specified otherwise, the 5′-^{32}P-labeled DNA (with or without a complement strand) and 100 μM sonicated calf thymus DNA were incubated with THF-OOH (or THP-OOH) in 10 mM sodium cacodylate buffer, 1 mM EDTA (pH 7.0) in a final reaction volume of 30 μl. Reaction parameters, including peroxide concentration, incubation time, temperature, salt concentration, pH, etc., were varied as specified in the figure legends.

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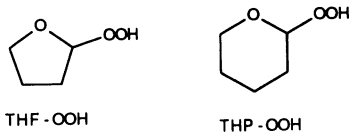

Figure 1. Structures of THF-OOH and THP-OOH.

1 = 5'-C¹ A T G C⁵ C⁶ C⁷ G C⁹ G C¹¹ C¹² C¹³ G G T A C¹⁸
2 = 3'-G T A C G G G C G C G G G C C A T G
3= 3'-G T A C G G G C C C G G G C C A T G
4 = 3'-G T A C G G G C T C G G G C C A T G
5 = 3'-G T A C G G G C A C G G G C C A T G
6 = 3'-G T A C G G G C _ C G G G C C A T G
7 = 3'-G T A C G G G C G C_4G G G C C A T G
8 = 3'-G T A C G C G C G C G G G C C A T G
9 = 3'-G T A C G G G mC G C G G G mC C A T G

Figure 2. Sequence of deoxynucleotide oligomers 1–9.

Incubations were stopped by cooling the reaction in ice and precipitation of the DNA by addition of NaOAc and cold EtOH. The precipitated DNA was washed with EtOH and dried *in vacuo*.

Methoxylamine. In a total volume of 100 µl, the ^{32}P-labeled and calf thymus DNA (same as above) in 25 µl of 10 mM cacodylate containing 1 mM EDTA and 200 mM NaCl were incubated with 4 M methoxylamine (75 µl) for 10 min at 24°C. The reaction was terminated by adding 3 M NaOAc and EtOH, cooling and centrifugation. The washed DNA pellet was then dried *in vacuo*.

Chloroacetaldehyde. DNA samples (same as above) were dissolved in 100 µl of 10 mM cacodylate, 1 mM EDTA buffer (pH 7.0) containing 200 mM NaCl, and then 2 µl chloroacetaldehyde added. The incubation was maintained at 24°C for 2 h, the reaction terminated by precipitation of the DNA (NaOAc and EtOH), and the DNA pellet thoroughly washed and then dried *in vacuo*. The samples were dissolved in 5 µl of 10 mM Tris, 1 mM EDTA (pH 7.0) buffer and further treated with hydrazine (25 µl) and 20 µl of 5 M NaCl. After precipitation with EtOH and washing, the samples were dried *in vacuo*.

Generation of strand breaks

The THF-OOH treated DNA was heated at 90°C for 15 min in 30 µl of 10 mM Tris, 1 mM EDTA buffer (pH 7.0) to convert THF-OOH induced lesions into abasic sites; however, when the incubations were performed at elevated temperatures this step was unnecessary. The DNA was then precipitated, washed and dried. For the methoxylamine and chloroacetaldehyde reactions, the neutral thermal hydrolysis step was omitted. Precipitated DNA samples were taken up in 1 M piperidine (100 µl) and heated for 30 min at 90°C to generate strand breaks. After removal of piperidine *in vacuo*, the DNA was suspended in loading buffer (80% deionized formamide, 50 mM Tris–borate, pH 8.3, 1 mM EDTA) with (for restriction fragments) or without (for oligomers) marker dyes and denatured by heating at 90°C for 1 min, followed by cooling in ice. The DNA was electrophoresed

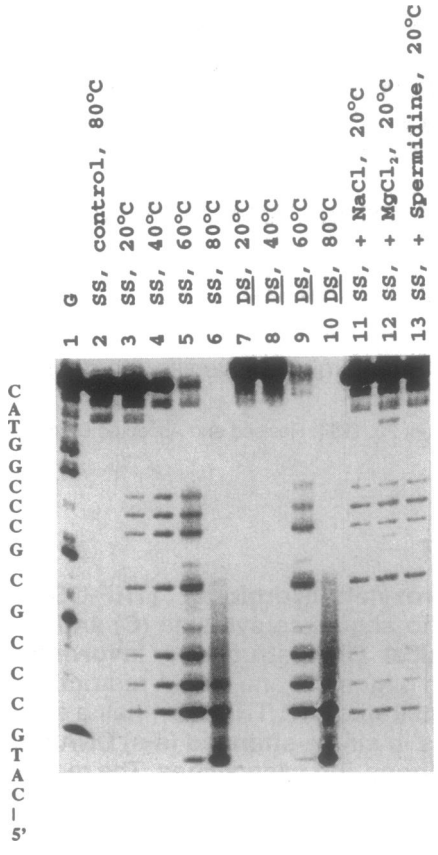

Figure 3. Temperature response for the reaction (1 h incubation) of 1 M THF-OOH with oligomer 1 (s-s) or 1+2 (d-s): lanes 1–13, 10 mM sodium cacodylate, 1 mM EDTA buffer (pH 7.0); lane 11, 200 mM NaCl; lane 12, 20 mM $MgCl_2$; lane 13, 250 µM spermidine.

in a 12% (restriction fragments) or 20% (oligomers) 7.8 M urea polyacrylamide denaturing gel at 75 W (~55°C). Standard Maxam–Gilbert G and G + A reaction lanes were included as sequence markers (7). Control lane DNA received the same treatment except it was not incubated with C modifying reagent. The gel was then exposed to a phosphorimager screen and analyzed using a Molecular Dynamics PhosphorImager.

Thermal stability

The denaturation of oligomers 1+2–8 and 7 was monitored as a function of temperature in the same 10 mM cacodylate buffer with 200 mM NaCl and 1 mM EDTA using UV absorbance at 260 nm. T_m values were calculated by plotting $d(A_{260})/dT$ versus T (Table 1).

RESULTS

THF-OOH reacts with DNA to afford modified Cs that can be selectively converted into apyrimidinic sites by heating at 90°C for 15 min (1). The generation of strand breaks with uniform specificity at all Cs in s-s oligomeric DNA is demonstrated in Figure 3 (lanes 3–6). A similar result is observed when a d-s restriction fragment is incubated for prolonged periods at 37°C (1), or when the DNA is first denatured by heating to >60°C (Figs 4 and 5, lanes 8–11 and 17–21). The kinetic preference of

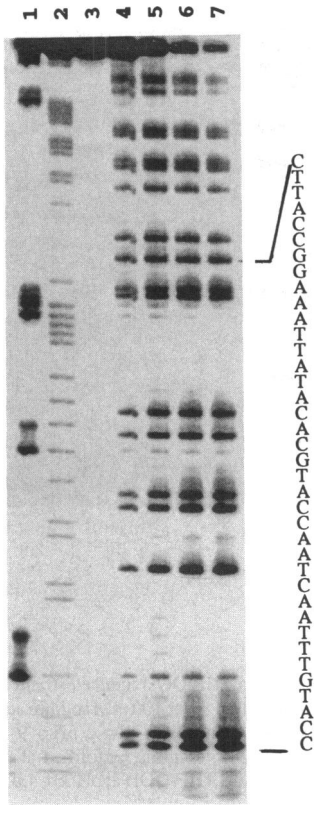

Figure 4. The effect of incubation time on the reaction of 1 M THF-OOH with 5′-^{32}P-labeled 85 bp fragment in 10 mM sodium cacodylate buffer, 1 mM EDTA (pH 7.0) at 80°C: lane 1, G; lane 2, G + A; lane 3, control (80°C for 30 min); lanes 4–7, reaction time of 5, 10, 20 and 30 min, respectively. The first C shown in the sequence is C^{233} (5).

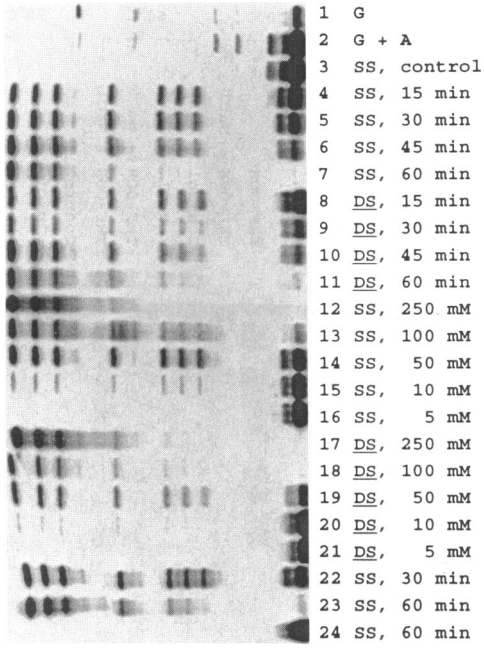

Figure 5. Time course and concentration effect for the reaction of THF-OOH with oligomer 1 (s-s) or 1+2 (d-s) in 10 mM sodium cacodylate buffer, 1 mM EDTA (pH 7.0) at 80°C: lanes 22 and 23, the neutral thermal hydrolysis was omitted but the DNA was treated with piperidine; lane 24, the neutral thermal hydrolysis and the piperidine treatments were omitted.

THF-OOH for s-s versus d-s DNA is evident (Fig. 3, lanes 3 versus 7). The same specificity for C in s-s DNA is also observed for THP-OOH (data not shown). Experiments to determine whether methanolic or aqueous stock solutions of THF-OOH could be stored for extended periods of time without compromising its reactivity were performed. The results (data not shown) show that solutions of 30% (v/v) THF-OOH in methanol or deionized water can be stored at 5°C for >4 weeks without any change in the activity of THF-OOH.

The reaction of THF-OOH with s-s DNA oligomers at 80°C shows clear time (Fig. 5, lanes 4–7) and dose (Fig. 5, lanes 12–16) responses. The co-addition of inorganic or organic cations does not alter the cleavage pattern with s-s DNA substrates (Fig. 3, lanes 11–13). Similarly, increasing the temperature of the reaction increases the rate of DNA cleavage (Fig. 3, lanes 3–6), although the temperature effect is not observed with d-s DNA (1+2) until 60°C is approached (Fig. 3, lane 9). In the same buffer that the DNA cleavage reactions are run, the T_m of 1+2 is 62°C (Table 1). As expected, the stabilization of duplex DNA by cations does shift the temperature at which cleavage is observed (Fig. 6, lane 9 versus 10). However, salt concentration does not affect the reaction of THF-OOH with DNA once the DNA has denatured (Fig. 6, lane 13 versus 14). Obviously the results for incubations containing s-s and d-s DNA are the same once the d-s DNA has denatured (Fig. 3, lane 5 versus lane 9). At the higher temperatures the neutral thermal hydrolysis step that is required at room temperature becomes redundant (Fig. 5, lanes 22 and 23), but the treatment with hot alkali to generate the strand breaks is still necessary (Fig. 5, lane 24).

Table 1. Melting temperatures for DNA oligomers[a]

DNA	Description	T_m (°C)
1+2	normal	71.5
1+2	normal	62.0 (no NaCl)
1+3	C–C mismatch	59.8
1+4	C–T mismatch	60.5
1+5	C–A mismatch	63.2
1+6	single C bulge	56.3
1+7	C_3 loop	57.0
1+8	C–C mismatch	60.5
7	single-strand	60.8

[a]Studies performed in 10 mM sodium cacodylate buffer (pH 7.0) + 200 mM NaCl, except as noted.

In one set of experiments the DNA target was 5′-labeled with ^{35}S, and the incubations with THF-OOH were performed as described for the ^{32}P-labeled material. However, the THF-OOH excised the ^{35}S-labeled from the end of the DNA leaving unlabeled material.

Experiments to determine how pH changes affected the cleavage reaction indicate that rate of reaction is pH dependent below, but not above, pH 6 (Fig. 7). As expected, some

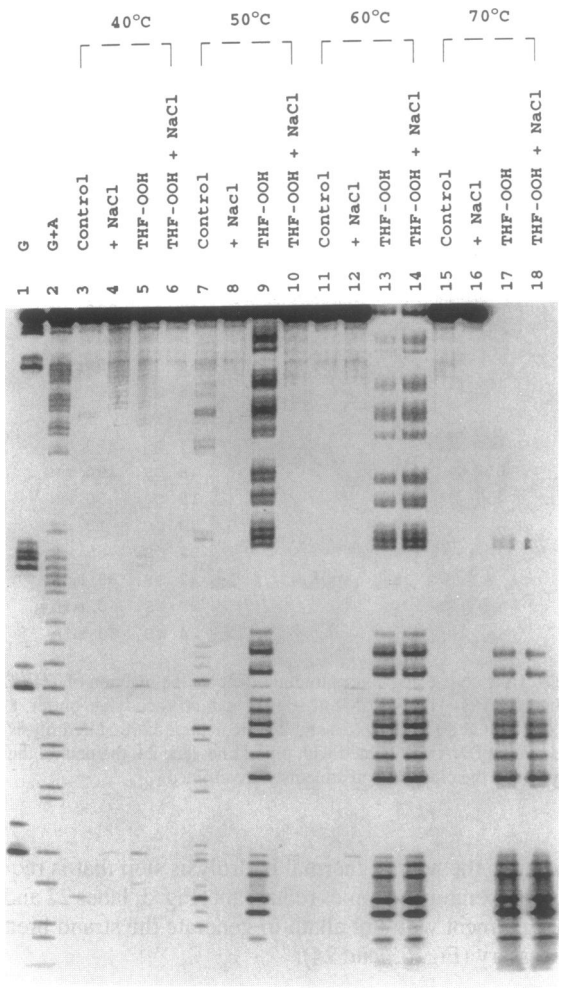

Figure 6. The effect of 200 mM NaCl on the temperature-dependent cleavage of 5′-^{32}P-labeled 85 bp restriction fragment by 1 M THF-OOH in 10 mM sodium cacodylate, 1 mM EDTA (pH 7.0) with 1 h incubation: lanes 4, 6, 8, 10, 12, 14, 16 and 18, NaCl concentration is 200 mM. The same sequence is shown in Figure 4.

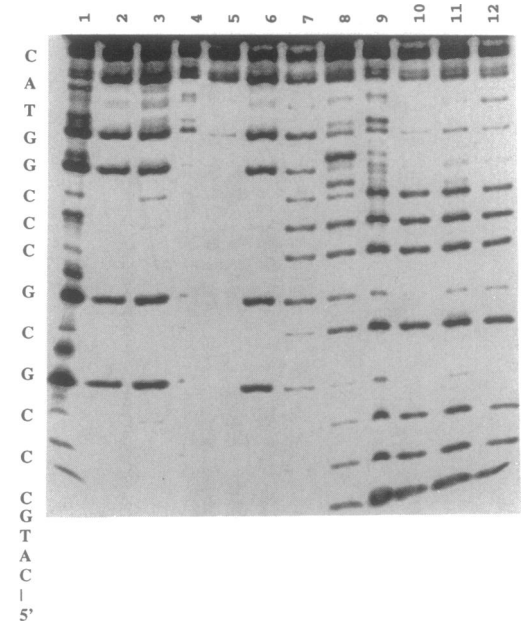

Figure 7. The effect of pH on THF-OOH induced strand breaks in oligomer 1 (s-s) in 100 mM glycine/HCl (pH 3), or 100 mM sodium acetate/acetic acid (pH 4 and 5), or 100 mM potassium phosphate/borax (pH 6–9) at 24°C for 4 h: lane 1, G; lane 2, G + A; lanes 3–5, controls performed at pH 3.0, 6.0 and 9.0, respectively; lanes 6–12, 50 mM THF-OOH at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0, respectively.

depurination is also observed in the incubations performed in the more acidic buffers.

The reaction of THF-OOH with oligomers 1+3–5 demonstrate that this reagent is sensitive to the presence of non-Watson–Crick base pairing motifs induced by the creation of mismatches at C. Several independent experiments consistently show the following order of reactivity of THF-OOH with the three mismatched substrates: C–C (1+3) >> C–T (1+4) > C–A (1+5) (Fig. 8, lanes 7–9). The creation of a bulge at the same C by deleting the Watson–Crick G in the complementary strand (1+6) causes a hyperreactivity which exceeds that observed for any of the mismatches (Fig. 8, lane 11). Using a duplex containing a C_3-loop structure (1+7) does not alter the reactivity of any of the Cs in strand 1 (Fig. 8, lane 6). However, the three unpaired Cs in strand 7 (Cs 10–12) of the 1+7 duplex are hyperreactive to THF-OOH (Fig. 8, lane 16). The C (C-9) that is 5′ to the unpaired C_3 stretch also shows significant reactivity, although clearly less than the Cs designed to be in the loop. As can be seen in Figure 8 (lane 15), oligomer-7 as a s-s target shows a reactivity with THF-OOH at 24°C that indicates secondary structure. In this case it is C-12 that is the most reactive base in the C_4 run. However, at higher temperature (above the T_m) all Cs show equivalent reactivity. The presence of secondary structure is also confirmed by the melting experiment which shows non-cooperative melting for 7 with a calculated T_m of ~61°C (Table 1). This TM value is only ~10°C lower than that of normal duplex 1+2 which suggests that 7 forms a duplex structure(s) with extensive base pairing.

In order to assess the practical utility of THF-OOH as a probe of DNA structure, the reactions of methoxylamine and chloroacetaldehyde were performed using the same oligomeric DNA targets as already described for THF-OOH. As expected (3), methoxylamine also prefers s-s DNA and can detect mismatches and bulges (Fig. 9). However, the bands derived from the base-paired Cs are readily observed making the unequivocal assignment of non-Watson–Crick pairing motifs somewhat subjective. In addition, the secondary structure seen in 7 using methoxylamine is not as clearly resolved, specifically the relative intensities of the bands in the C_4 run (Fig. 9, lanes 13 and 14).

The reactions of chloroacetaldehyde involve initial treatment of the DNA target with chloroacetaldehyde followed by reaction with hydrazine and generation of strand breaks with hot piperidine (4). The results from these reactions are compared to those for hydrazine alone and hyperreactivity indicates non-canonical DNA structure. As previously reported, the results demonstrate (Fig. 10) that chloroacetaldehyde also prefers s-s DNA (4,8,9). However, analysis of the data requires substraction of the hydrazine 'control' lane and it is not trivial to distinguish the mismatched Cs from those involved in normal H-bonding arrangements.

Finally, substitution of 5-methylcytosine for C in the oligomers 9 and 1+9, did not qualitatively or quantitatively affect the cleavage pattern induced by THF-OOH (data not shown).

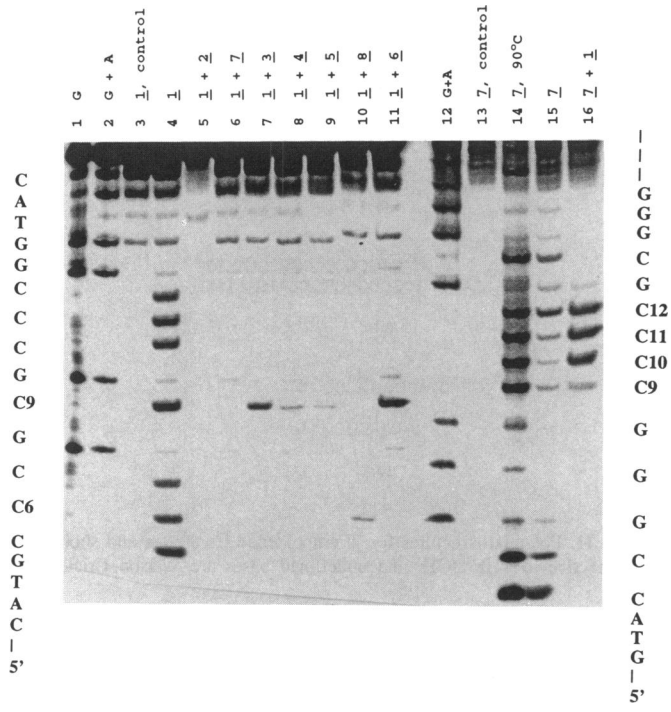

Figure 8. Reaction (2 h incubation) of 1 M THF-OOH in 10 mM sodium cacodylate buffer, 1 mM EDTA (pH 7.0) containing 200 mM NaCl at 24°C (unless specified otherwise) with oligomers 1, 7 and 1+2–7: lanes 1–11, 1 is 5′-^{32}P-end-labeled; lanes 12–16, 7 is 5′-^{32}P-end-labeled.

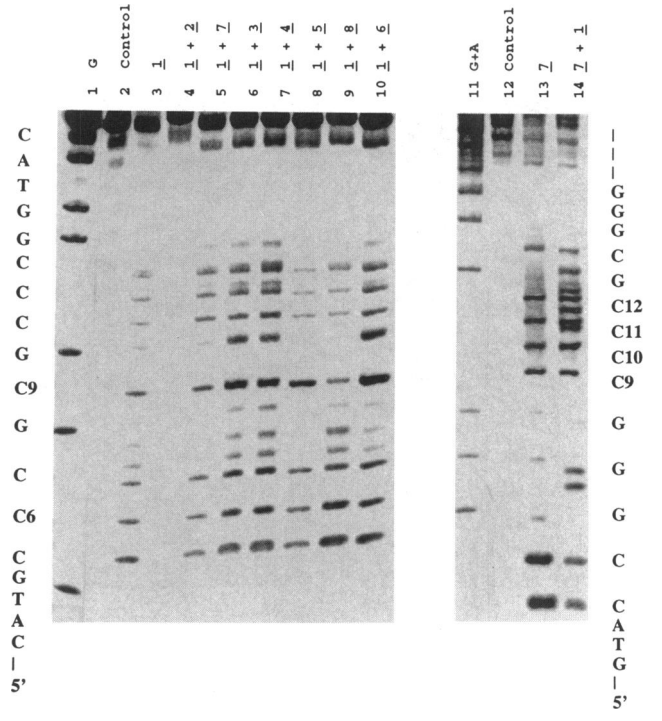

Figure 9. Reaction (10 min incubation) of 3 M methoxylamine in 10 mM sodium cacodylate buffer, 1 mM EDTA (pH 7.0) containing 200 mM NaCl at 24°C (unless specified otherwise) with oligomers 1, 7 and 1+2–7: lanes 1–10, 1 is 5′-^{32}P-end-labeled; lanes 11–14, 7 is 5′-^{32}P-end-labeled.

DISCUSSION

The mechanistic details of the C specific formation of labile sites generated by THF-OOH have not yet been completely elucidated. It is expected that a modification at N3-C is involved, because it is known that a heat labile adduct is formed, and that both heat and alkali treatment are required to convert the initial modification into a strand break (1). N3-C modifications are generally heat labile (10). It is assumed that THF-OOH also modifies other nucelobases but that these lesions are not heat or alkali labile. Attack of a THF-OOH derived intermediate at N3-C is consistent with the observed pH dependence and the higher reactivity of s-s over duplex DNA since N3-C is sterically inaccessible and electronically less active in Watson–Crick DNA. As noted above, the reactions of THP-OOH with DNA are indistinguishable from those of THF-OOH. This indicates that the chemistry responsible for the C cleavages is related to the α-peroxyether functionality. We have already reported that simple alkyl peroxides, e.g., cumene and *t*-butyl hydroperoxide, do not yield labile C adducts (1). ESR studies on the decomposition of THF-OOH under reaction conditions suggest that a carbon-centered radical is responsible for the C specificity (1), and both THF-OOH and THP-OOH decompose to form homologous radical species (1,11). The structural features of the peroxide that are required for the C cleavage reaction and the nature of the C modifications are areas of ongoing work. Regardless of the mechanistic details, the data presented show that THF-OOH is an excellent reagent for primary sequencing and for the detection of non-canonical DNA base pairing at C.

Primary sequencing

THF-OOH preferentially reacts with s-s DNA but it can conveniently be used to sequence d-s fragments because its reactivity and C specificity persist even at the high temperatures required to denature large restriction fragments. In fact, at elevated temperatures the incubation times are reduced to 5–10 min, and the requirement for a neutral hydrolysis step, to produce apyrmidinic sites, is also eliminated. The DNA is simply precipitated and treated with hot piperidine to produce strand breaks.

Sequencing non-canonical structures

The preference of THF-OOH for Cs in s-s DNA can be exploited to detect structures in which a C–G base pair is absent or significantly destabilized, i.e., in mismatches, bulges or hairpin structures. It is of note that THF-OOH quantitatively distinguishes between the three mismatches (1+3–5) and that chloroacetaldehyde also shows the same preference: bulge > C–C > C–T > C–A. This reactivity inversely parallels the stabilities of the duplexes (Table 1) and suggests that significant strand separation is required for C modification. This is a reasonable interpretation since C–A and C–T can form two H-bonds in a wobble base pair without significant disruption of the helix (12). However, it is not possible to form more than a single H-bond between two non-protonated Cs and still maintain a B-DNA structure. Methoxylamine also strongly adducts Cs lacking a complementary base (1+6), but differences between the

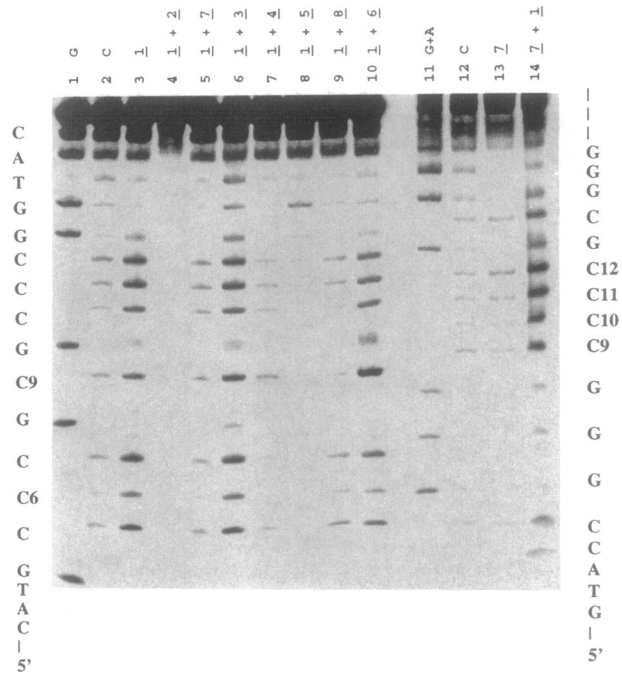

Figure 10. Reaction (2 h incubation) of (~0.16 M) chloroacetaldehyde in 10 mM sodium cacodylate buffer, 1 mM EDTA (pH 7.0) containing 200 mM NaCl at 24°C (unless specified otherwise) with oligomers 1, 7 and 1+2–7: lanes 1–10, 1 is 5′-^{32}P-end-labeled; lanes 12–14, 7 is 5′-^{32}P-end-labeled; lanes 2 and 12, Maxam–Gilbert C lane (7).

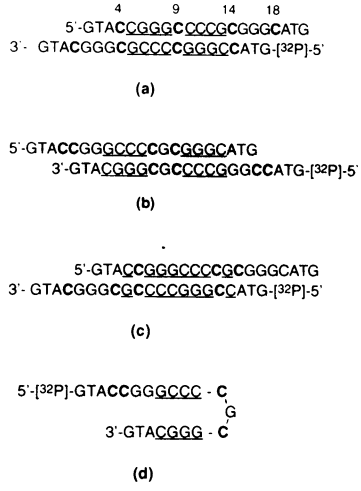

Figure 11. Potential structures for 7: emboldened Cs are s-s and should be hyperreactive to THF-OOH, and underlined bases are Watson–Crick base paired.

intensities of the C bands in the three mismatch targets is less clear. There is also a difference in how the three reagents react with duplex 1+8, where a C–C mismatch has been introduced at the center of a $(G)_3$–$(C)_3$ structure. Chloroacetaldehyde gives bands at the Cs flanking the designed mismatch, while methoxylamine reacts extensively with all three Cs. With THF-OOH, only the band from the C designed to be in a mismatch is intensified.

Another example of the facility of THF-OOH to discern s-s and d-s regions is evident in the case of 5′-[^{32}P]-7 , which has a cleavage pattern atypical of a s-s oligomer. The gel data are consistent with either an intramolecular hairpin (Fig. 11d) and/or only one of the duplexes (Fig. 11b). This interpretation is based on the observation that: (i) the 3′-C in the C4 run is the most reactive of the four, indicating that it spends more time without a complementary base than the other Cs; and (ii) all Cs outside of the C4 region behave as normal s-s bases, as would be predicted due to mismatches and end effects.

The different cleavage patterns for the three reagents may result from the formation of different DNA structures as a consequence of the high concentrations of the reagents and/or the ionic strength of the reaction. It is also possible that the three chemical probes have different reaction selectivities for certain DNA equilibrium structures based on steric and electronic factors. This explanation is reasonable since the major groove reaction site for methoxylamine adduction does not need to be in a completely denatured state to become more accessible, i.e., more reactive. As mentioned above, adduction at N3-C requires complete, albeit local, denaturation of the helix.

It has been reported that in unique circumstances the environment of C in RNA can affect its pKa, as measured by reactivity with dimethyl sulfate (13). Since we have shown that the formation of heat labile sites is pH dependent, THF-OOH should also be a useful probe for the phenomenon of sequence and structural dependent pKa changes.

Practical issues

In comparison to the other reagents that are used to sequence and/or probe non-Watson–Crick structures involving C, THF-OOH has unique advantages. It is relatively stable and can be stored for >6 months without loss of activity. It is also stable at –20°C when dissolved, in the absence of reducing agents, in MeOH (for >6 months) or H_2O (>1 month). In contrast, chloroacetaldehyde is subject to nucleophilic attack by most solvents that are compatible with DNA, and solutions must be made up and standardized prior to its use. Methoxylamine, is relatively stable, but must be stored as the salt to prevent air oxidation, and it is recommended that solutions be prepared immediately before use (3).

The reaction of THF-OOH with C can be analyzed in a straightforward manner that does not require assessment of differential reactivity as does chloroacetaldehyde (4). In terms of its reactivity, we assume that chloroacetaldehyde and THF-OOH modify the same site on C, but THF-OOH does not react at other nucleobases to yield heat labile adducts. Chloroacetaldehyde shows significant cross-reactivity, especially with adenine (4). Methoxylamine, and the related probe hydroxylamine, react with the sp^2 6-position of C that is normally a sterically hindered major groove site in B-DNA. Accordingly, methoxylamine has been used to detect mismatches and B–Z junctions (3,14,15). The pH optimum for its reaction with C is 5.1 (6.1 for hydroxylamine). In contrast, we have demonstrated that THF-OOH has optimum reactivity at neutral pH. Another limitation of methoxylamine (and hydroxylamine) is that they do not efficiently react with 5-methylcytosine (3), whereas THF-OOH does not distinguish between the methylated and unmethylated base. Currently, permanganate at weakly acidic pH (4.3) is used to sequence 5-methylcytosine (16).

One potential limitation of THF-OOH was noticed when the DNA was end-labeled with ^{35}S. The ^{35}S was released from the

DNA, apparently by an oxidative mechanism, leaving unlabeled oligomer. All attempts to resolve this problem were unsuccessful.

Finally, since THF-OOH is a neutral molecule and amphoteric in its solubility properties, it should be able to efficiently penetrate cells. This raises the possibility of using it as an *in vivo* footprinting reagent to map potential H-DNA and Z-DNA structures.

Safety considerations

THF-OOH is a peroxide that can violently explode if heated to its boiling point as a neat liquid (17). However, we have carried out large scale reductions of THF-OOH with Fe(II) or dithiothreitol without any evidence of an exothermic reaction. Both chloroacetaldehyde and methoxylamine must also be used in a hood and treated with caution due to their chemical and biological activities.

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