The ensemble reactions of hydroxyl radical exhibit no specificity for primary or secondary structure of DNA

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ABSTRACT

Hydroxyl radical reacts at numerous sites within nucleic acids to form a wide range of derivatives yet the conformational specificity of only one of these processes, direct strand fragmentation, has received much attention to date. Since the deleterious effects of this radical are not likely limited to strand fragmentation in vivo, this report examined the conformational specificity expressed in a more general manner. For this, modification of DNA was induced by the hydroxyl radical generating system of H_2O_2 and Fe-EDTA. The ensemble rate of oxidation (nucleobase + deoxyribose backbone) was determined from the overall consumption of a series of oligonucleotides that were designed to model random coils and double helices containing complementary and noncomplementary base pairing. The resulting pseudo-first order rate constants derived from this model system were relatively unaffected by nucleotide sequence or secondary structure and varied from only 0.022 to 0.048 s^{-1} . Consequently, the indiscriminant nature of hydroxyl radical appears to persist beyond strand fragmentation to include nucleobase oxidation as well.

INTRODUCTION

Hydroxyl radical oxidation of nucleic acids is the subject of two very active and quite distinct research areas. As a diagnostic agent, this radical is widely applied to footprinting of nucleic acids and their complexes. Alternatively as a physiological and endogenous oxidant, this radical is central to many toxicological and radiological investigations. A comprehensive description of hydroxyl radical interaction with DNA (and RNA) will ultimately draw from both perspectives.

Radical-based footprinting relies on the direct strand scission of polynucleotides that results from oxidative attack of the sugar backbone. In most every example, the array of nucleic acid fragments formed under these conditions is separated by gel electrophoresis, visualized by autoradiography and, when necessary, quantified by densitometry (1). These protocols have been used to characterize the folding (2–7), binding (8,9) and cross-linking (10,11) of nucleic acids ranging in size from small oligonucleotide models to high molecular weight polymers. The success of these studies has depended in part on the insensitivity of spontaneous fragmentation to nucleotide sequence (primary)

and duplex (secondary) structure (5,12,13). However, the general nature of hydroxyl radical modification of nucleic acids should not be casually extrapolated from monitoring just this single type of product. Strand fragmentation due to sugar oxidation is only one of many reactions between hydroxyl radical and DNA (or RNA).

Radiological investigations have in fact identified numerous pathways for hydroxyl radical oxidation of DNA. In general, the nucleobases react preferentially over the sugar moiety by 4- to 6-fold and each purine and pyrimidine forms a characteristic series of modified derivatives (14–17). Many of the products of these pathways, for example 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 8-oxoguanine and 5,6-dihydroxycytosine, have become diagnostic for the intermediacy of this radical (18,19). In addition, another derivative, thymine (and thymidine) glycol, has been used to assess oxidative damage sustained by DNA in vivo (20). The conformational specificity of these dominant nucleobase reactions has yet to receive adequate attention (21) despite its potential physiological importance. The determinants controlling hydroxyl radical reaction of the sugar backbone cannot be equated to those controlling the modification of nucleobases without further analysis.

In this report, a kinetic study has been undertaken to determine the sequence- and structure-dependence of DNA oxidation effected by the ensemble reactions of hydroxyl radical (sugar and base oxidation). The overall progress of reaction was monitored by consumption of the parent oligonucleotides used to model a range of DNA structures. Chromatographic rather than electrophoretic methods provided the most convenient method for resolving and quantifying these starting materials. The resulting rate constants were used to represent the collective properties of all detectable oxidative processes operating at their natural efficiency. Such analysis complements the more typical focus on individual products of nucleic acid modification and consequently additional insight into the radical degradation of DNA is described here.

EXPERIMENTAL PROCEDURES

Materials

All commercial chemicals (reagent grade) were used directly; water was distilled, deionized and filtered (Nanopure, Sybron/Barnstead). Solutions used for modification of DNA were prepared freshly each day. Oligonucleotides were synthesized

via standard solid-phase cyanoethyl phosphoramidite chemistry on DuPont or Biosearch equipment and purified by reverse phase chromatography. The homogeneity of each isolated strand (14 and 15 bases in length, see Table 1) was analyzed and, when necessary, further purified by the anion exchange methods described below. DNA concentrations were determined per mol of oligonucleotide by using ϵ_{260} values estimated from the sum of the deoxynucleotides and their effects on near neighbors (22). Duplex structures (3–6 μ M) were annealed by slowly cooling (>3 hr) a heat denatured mixture of DNA strands in 10 mM potassium phosphate pH 7 and 100 mM NaCl (23).

Methods

Hydroxyl Radical Oxidation of DNA. Radical generation and subsequent modification of DNA were based on the methods of Tullius et al. (1). The appropriately prepared oligonucleotides (3–6 μ M) were combined with 100 mM NaCl, 10 mM potassium phosphate pH 7, 250 μ M ascorbate and 25 μ M FeEDTA; oxidation was initiated by the addition of 2.5 mM H_2O_2 . Aliquots (100 μ L) were removed during the initial 45 s (commonly at 0, 15, 30 and 45 s) of the reaction and quenched with mannitol (670 mM). These samples were then individually dialyzed against 1 mM EDTA pH 8 (twice) and water (once), dried in a Speed-Vac concentrator and finally resuspended in 11.5 mM NaOH for direct chromatographic analysis. The full alkaline lability of products was identified by treating the dialyzed samples with 0.2 M piperidine at 90°C for 30 min prior to chromatographic analysis.

Rate and Product Analysis. Parent oligonucleotides and their derivatives were separated by anion exchange chromatography [Mono Q (Pharmacia)] using a gradient of 0–825 mM NaCl in 11.5 mM NaOH over 50 min (1 mL/min). Elution profiles were monitored at 254 nm and the remaining starting material was quantified by integrating the detector signal. First order rate constants were then calculated by linear regression of a semi-logarithm plot of remaining starting material (%) vs time. The integrated area at zero time was set at 100% and determined from samples that had the quenching agent, mannitol, added prior to the oxidants. Uncertainties were derived from the standard error arising from multiple sets (>2) of data used in each study.

RESULTS & DISCUSSION

The hydroxyl radical has come under increasing scrutiny as the ultimate oxidant of many deleterious reactions in vivo (15,16,24). This species readily modifies the vital target, DNA, and produces numerous deoxynucleotide derivatives through a series of parallel pathways. The conformational selectivity of this ensemble process

is now described below as part of our continuing effort to evaluate the structural origins of hyperreactive and hypermutable sites in DNA (23,25,26). An oligonucleotide model system was chosen in this case to minimize sample heterogeneity and yet allow for the examination of a defined set of secondary conformations.

In general, the specificity of a DNA modifying agent is traditionally described in terms of its profile of nucleic acid products. Characterization of such profiles can provide information on both sequence- and site-dependence of modification. This approach is however less applicable for the specific emphasis of our current study. DNA reactivity is described here by the loss of starting material rather than the formation of product. The resulting rate of oligonucleotide consumption focuses attention on the overall consequences of hydroxyl radical exposure along an entire sequence. Subtle or global effects on sequence reactivity would not necessarily have been apparent from routine data on direct DNA strand scission alone. For example, the frequency of strand fragmentation within a local region of DNA might be enhanced by 100% and still remain unnoticed by product analysis if 10 consecutive fragments were only increased by 10% each. In addition, the multiple measurements necessary to account for all products would introduce significantly more uncertainty than the single measurement necessary for detecting unreacted starting material.

Chromatographic Detection

For oligonucleotide-based studies, anion exchange chromatography is particularly efficient for separating and quantifying reaction mixtures. The column used in this report (Mono Q [Pharmacia]) differentiated oligonucleotides by charge primarily, but hydrophobic interactions also affected column retention. Consequently, denaturing conditions (pH 12) allowed for separation of complementary DNA sequences and their

Figure 1. Anion exchange separation of oligonucleotides 4•1 and their oxidized products resulting from exposure to hydroxyl radical (a) and subsequent derivatization by piperidine (b). Analysis of the unmodified sequences are illustrated in the insert c. Reaction conditions and chromatographic procedures are described in the Methods Section.

Table 1. DNA Sequences Subjected to Hydroxyl Radical Oxidation

duplex structure	#	Oligonucleotides	T_m^a (°C)
fully complementary	1	5'd(CACGGGTGCGCATG)	60–62
	2	3'd(GTGCCACGCGTAC)	
Double Watson–Crick mismatch	1	5'd(CACGGGT ^{TC} GCGCATG)	48–50
	3	3'd(GTGCCCT ^{TT} GCGTAC)	
fully complementary	5	5'd(CACGGGAACGCGATG)	58–60
	3	3'd(GTGCCCTTGCCTAC)	
Single Base Insert	1	5'd(CACGGGT–GCGCATG)	53–55
	4	3'd(GTGCCCA ₇ CGCGTAC)	

^aMelting temperatures were determined previously (25).

products formed by strand scission and base oxidation (23,25–27). The degradation rate of each duplex strand could therefore be monitored simultaneously; differential labeling of the individual strands was not necessary for this study. Also, direct observation of the elution profiles (A_{254}) minimized the manipulations necessary for analysis and allowed for easy automation of this process.

The chromatograms resulting from the discontinuous assay of strand oxidation (Figure 1) were quite reminiscent to those generated after photochemical oxidation of DNA (25). The major products (80% of total) eluted in the expected manner for oligonucleotides that contained an oxidized base (25,26). The retention times of these were again only slightly shorter than their respective unmodified sequences. In conjunction with single strand studies, the material eluting at 38 min derived from 4 ($t_r = 39$ min) whereas the material eluting ($t_r = 40$ min) between the parent sequences derived from 1 ($t_r = 41$ min). The DNA fragments formed from direct strand scission were evident in low abundance and eluted throughout the chromatograms as observed in this example with retention times of 19–37 min (20% of total). These fragments corresponded to the species commonly distinguished by gel electrophoresis. The exact assignment of each fragment was not warranted in this case since no individual or set of these products dominated the profile. When necessary, however, fragments have been identified by co-elution with synthetic standards and quantified by their absorbance at 254 nm (23).

Hydroxyl radical is competent to oxidize all nucleobases (guanine > adenine > cytosine > thymine [19]) and the principle derivatives formed by this radical (>90% [19]) have previously been shown to induce scission of DNA strands after reaction with piperidine and heat (21,25,28,29). As expected then, much of the material associated with nucleobase oxidation in this study was also found sensitive to piperidine treatment (Figure 1). Anion exchange analysis subsequent to this treatment revealed a decrease in the oligonucleotide products containing modified bases and a corresponding increase (ca 300% by integration) in the strand fragments from DNA scission. However, the rate measurements based on oligonucleotide

consumption were not effected by piperidine. This suggests that the oxidized products labile to piperidine did not co-migrate with the intact parent strands and, since these products predominate, all major pathways of modification were likely monitored by our chromatographic procedure.

Although abasic sites in DNA are produced by some radical processes (30), these species do not appear to form in the presence of hydroxyl radical (31). If abasic sites had formed under our conditions, these would have been detected together with the products of direct strand scission. The alkaline conditions (pH 12, ambient temperature) used to prepare samples for the anion exchange analysis were sufficient to hydrolyze most abasic derivatives and produce DNA strand scission (32).

Oxidation Rate vs Deoxynucleotide Sequence and Composition

Kinetic analysis of the radical reaction was based on the integration of elution profiles equivalent to those illustrated in Figure 1. The loss of parent oligonucleotides followed an exponential decay (Figure 2) and rate constants were calculated from these first order processes (Table 2). From this analysis, hydroxyl radical reacted with striking uniformity. Consumption of the random coils individually formed by 1–5 all proceeded with rate constants that varied from the average [$(10^3 \times k) = 38 \text{ s}^{-1}$] by no more than 30%. Nucleotide sequence therefore did not appear to influence the overall reactivity between hydroxyl radical and oligonucleotides. Nucleotide composition may play a limited role in the net reactivity of a sequence since the most reactive oligonucleotide (3) contained the greatest number of thymines and fewest number of adenines and the least reactive oligonucleotide (5) contained the opposite proportion.

Oxidation Rate vs DNA Conformation

The indiscriminant nature of hydroxyl radical is further revealed by comparing the collection of rate constants used to describe the reactivity of DNA structures ranging from single strand coils to fully complementary duplexes (Table 2). Only slight variation was evident in the overall rate of oligonucleotide consumption in each case. Although strand hybridization generally appeared to suppress the oxidation of DNA, the effect was not great. Accordingly, no unusual effects were induced by the irregular duplex conformations containing either mismatched (1•3) or extrahelical bases (1•4).

Figure 2. Consumption of oligonucleotide 1 from the duplex 1•3 in the presence of hydroxyl radical as determined by discontinuous assay ($^{\circ}$). The theoretical loss of starting material (—) was calculated from the reported rate constant (Table 2).

Table 2. Conformational Dependence of Oligonucleotide Oxidation by Hydroxyl Radical^a

sequence	modification of parent DNA, $10^3 \times k \text{ (s}^{-1}\text{)}$		
	single strand	complementary duplex	irregular duplex
1	37 ± 7	29 ± 3	31 ± 2 (in complex with 3) 28 ± 4 (in complex with 4)
2	35 ± 4	35 ± 5	b
3	48 ± 8	33 ± 4	35 ± 3 (in complex with 1)
4	42 ± 8	b	31 ± 5 (in complex with 1)
5	27 ± 5	22 ± 3	b

^aReaction conditions and kinetic measurements are described in the Methods Section. Exponential decay of the parent DNA was determined for incubations containing an initial concentration of ca $5 \mu\text{M}$ per oligonucleotide strand. First order rate constants were calculated from data collected between 0–45 s as illustrated in Figure 2. ^bNot determined.

Nucleotide stacking within an organized structure of DNA may suppress the attack of hydroxyl radical at the nucleobase but such an effect was hardly detectable in this study. Indeed, all sites of this attack are thought to remain relatively accessible in a typical Watson–Crick double helix (15,33). No current evidence then supports a recent proposal suggesting that nucleobase oxidation dominates the reaction of single strand DNA and sugar oxidation dominates the reaction of duplex DNA (21). This scenario could only have generated the data reported here in the event that deoxyribose modification was activated by helix formation and nucleobase modification was concomitantly inhibited by helix formation.

The described chromatographic and kinetic approach serves to characterize the specificity of hydroxyl radical in a manner distinct from past investigations. Rate constants measured in this manner represent a composite of all detectable derivatization and reflect the full consequences of structural equilibria and dynamics. A similar set of features likely potentiate the hyperreactivity of DNA *in vivo*.

Electrophoretic analysis has consistently demonstrated that direct strand scission of nucleic acids by hydroxyl radical is insensitive to nucleic acid secondary structure (12,13). Our studies support this conclusion and go on to suggest that the lack of selectivity extends beyond the oxidation of the sugar backbone to encompass the ensemble reactions of hydroxyl radical with DNA. The homogeneity of oligonucleotide consumption indicates that this radical had equal access to nucleotides held in either random coils or a variety of regular helical structures.

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