Reaction of Single-Stranded DNA with Hydroxyl Radical Generated by Iron(II)–Ethylenediaminetetraacetic Acid

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ABSTRACT: This study demonstrates that the reaction of Fe(II)–EDTA and hydrogen peroxide with the single-stranded nucleic acids $d(pT)_{70}$ and a 29-base sequence containing a mixture of bases results in substantial damage which is not directly detected by gel electrophoresis. Cleavage of the DNA sugar backbone is enhanced significantly after the samples are incubated at 90 °C in the presence of piperidine. The latter reaction is used in traditional Maxam–Gilbert DNA sequencing to detect base damage, and the current results are consistent with reaction of the hydroxyl radical with the bases in single-stranded DNA (although reaction with sugar may also produce adducts that are uncleaved but labile to cleavage by piperidine). We propose that hydroxyl radicals may react preferentially with the nucleic acid bases in ssDNA and that reaction of the sugars in dsDNA is dominant because the bases are sequestered within the double helix. These results have implications both for the study of single-stranded DNA binding protein binding sites and for the interpretation of experiments using the hydroxyl radical to probe DNA structure or to footprint double-stranded DNA binding protein binding sites.

There has been widespread use of chemical reagents and enzymes which can react with the ribose–phosphate backbone of nucleic acids to provide a "footprint" of the binding site of double-stranded DNA (dsDNA) binding proteins (Tullius et al., 1987). One technique utilizes hydroxyl radicals produced by the reaction of Fe(II)–EDTA and hydrogen peroxide to cleave one of the strands (Hertzberg & Dervan, 1984; Tullius & Dombroski, 1985). The chemistry involved is thought to be an attack on the ribose moiety by the hydroxyl radical, with subsequent displacement of the 3′-phosphoryl leading to strand cleavage. When a protein is bound to dsDNA, the ribose rings at the protein/dsDNA interface are far less accessible to hydroxyl radicals than those in unbound regions, and the rate of cleavage at the interface is therefore significantly reduced. The low molecular weight of the hydroxyl radical provides a map or footprint of the protein binding site with base-pair resolution.

One recent study of the applicability of the hydroxyl radical as a probe for single-stranded DNA (ssDNA)–protein complexes presented evidence demonstrating that the extent of strand cleavage was higher in double- vs single-stranded DNA (Jezewska et al., 1989, 1990). The authors concluded this to be due to some required interaction between the Fe(II)–EDTA complex and duplex DNA. This explanation is inconsistent with the production of hydroxyl radicals from Fe(II)–EDTA and peroxide in the absence (Floyd, 1982) or presence of dsDNA (Floyd, 1981) and the high reactivity of hydroxyl radicals toward the ribose moiety of nucleotides (von Sonntag, 1987). Furthermore, another recent study showed that the reaction of Fe(II)–EDTA and hydrogen peroxide with single-stranded and double-stranded samples of both RNA and DNA does cause strand cleavage in both types of nucleic acid and that the reactivity of the hydroxyl radical for nucleic acids is unaffected by secondary structure (Celander & Cech, 1990).

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The results presented in this paper show that the hydroxyl radical does indeed react with ssDNA, but we propose that the sites of reaction are preferentially the bases in ssDNA. Only at high concentrations of reagents is any direct strand cleavage observed. We show that the Fe(II)–EDTA/hydrogen peroxide system can be combined with piperidine-mediated strand cleavage to footprint ssDNA. These results also have implications for the interpretation of hydroxyl radical "footprinting" experiments involving dsDNA.

Materials and Methods

Reagents. All chemicals were of reagent grade and used without further purification except for piperidine, which was freshly distilled. The $(NH_4)_2Fe(SO_4)_2$ (Aldrich) was stored under vacuum, and the Fe(II)–EDTA complex was prepared within 30 min of each experiment. H_2O_2 was diluted freshly before each experiment from a 30% solution. Sodium ascorbate was diluted freshly from a 1.0 M stock stored at −80 °C.

Oligodeoxyribonucleotides. $d(pT)_{70}$ and the mixed-sequence oligonucleotide were synthesized by the phosphoramidite method on a Biosearch 8800 automated synthesizer. The detritylated synthesis products were first purified by HPLC on a Nucleogen DEAE-60 anion-exchange column. The oligonucleotides were labeled with [γ-^{32}P]ATP at the free 5′-hydroxyl ends by T4 polynucleotide kinase (Maniatis et al., 1982) and then purified on a Sephadex G-10 spun column equilibrated in TE buffer (10 mM Tris, pH 7.9, 1.0 mM EDTA). They were further purified by extraction with equal volumes of 1:1 phenol:(24:1 chloroform:isoamyl alcohol) and then 24:1 chloroform:isoamyl alcohol followed by precipitation with three volumes of 1:1 ethanol:acetone at −80 °C. The precipitate was pelleted by centrifugation for 15 min at 14000 rpm in a microcentrifuge, dissolved in 5 μL of a formamide–dye mixture, and then electrophoresed on a 10% polyacrylamide gel. The appropriate bands were identified and excised from the gel, and the labeled ssDNA was eluted into TE

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buffer. The volume of eluant was reduced to 100 μL on a Speed-Vac concentrator and then precipitated as described above. The pellets were washed with 100 μL of 4:1:1 water:ethanol:acetone, dried, and diluted in TE buffer.

ssDNA Cleavage Reactions. Three different cleavage reactions were performed. Reaction volumes were all 10 μL, ssDNA concentrations were 2.0 μM, and the reaction temperatures were 23 °C. The ssDNA stock solutions were spiked with labeled ssDNA in such a way that the final ssDNA concentrations would vary by a maximum of 2%.

The protocol for cutting with dimethyl sulfate (DMS) was adapted from Maniatis et al. (1982); 1 μL of DMS was added to initiate the reaction, and 50 μL of DMS stop was added to quench the reaction after 3 min.

The procedure for reaction with Fe(II)–EDTA was slightly modified from that of Tullius et al. (1987). DNA was diluted into reaction buffer containing 10 mM Tris, pH 7.9, 0.10 mM EDTA, and 2.0 mM $MgCl_2$; 1.0 μL each of 0.4/0.8 mM $(NH_4)_2Fe(SO_4)_2$/EDTA, 25 mM sodium ascorbate, and 1.2% H_2O_2 was spotted onto the wall of a plastic tube containing the DNA, mixed together, and then immediately added to the solution of DNA. The final reagent concentrations were 0.04 mM Fe(II)–EDTA, 0.12% hydrogen peroxide, and 2.5 mM sodium ascorbate. The reactions were quenched after 2 min with 5 μL of 0.2 M thiourea.

The $KMnO_4$ reaction buffer was the same as for the Fe(II)–EDTA reactions. Final permanganate concentrations were 0.067 mM, and the reactions were quenched after 2 min with 10 μL of allyl alcohol.

The reacted ssDNA was purified by extraction and precipitated as described above. The pellets were resuspended in 50 μL of either 1.0 M piperidine or water and either heated to 90 °C for 30 min or kept at room temperature. The samples were then dried on a Speed-Vac, and the residue was washed twice with 10 μL of water. The samples were resuspended in 5 μL of formamide–dye mixture, heated to 90 °C for 30 s, and electrophoresed on an 8% polyacrylamide–7 M urea denaturing gel for 2 h at 2000 V.

Results and Discussion

Figure 1 shows the autoradiograph for the reactions of Fe(II)–EDTA with $d(pT)_{70}$. Control lanes 1–3 demonstrate that heating in the absence or presence of piperidine results in little cleavage of the oligonucleotide. As shown in lane 4, treatment with Fe(II)–EDTA, peroxide, and ascorbate alone also leads to very little cleavage of ssDNA. Subsequent heating to 90 °C increases cleavage significantly (lane 5), but heating in the presence of piperidine brings about extensive cleavage of the ssDNA (lane 6). Analogous results for the reaction with the thymine base specific reagent permanganate (Rubin & Schmid, 1980) are depicted in lanes 7–9.

In Figure 2 the results of similar reactions with a single-stranded mixed-base oligonucleotide of known sequence are shown. Again the controls, lanes 1–3, show little reaction when the unreacted oligonucleotide is heated to 90 °C in the absence or presence of piperidine. Lane 4 shows the Maxam–Gilbert reaction using dimethyl sulfate to cleave preferentially at guanosine bases. The sequence is given along the left side of the autoradiograph. Lanes 5–7 again demonstrate that cleavage of ssDNA reacted with Fe(II)–EDTA is enhanced substantially by heating in the presence of piperidine, but in this case heating alone also leads to increased strand cleavage. These results are consistent with the known chemistry of these reactants and substrates. Piperidine reacts with nucleic acids at residues containing modified bases by inducing β-elimination at the 1′-carbon, and the intermediate undergoes further reaction leading to cleavage of the 3′-phosphoryl bond (Maxam & Gilbert, 1980).

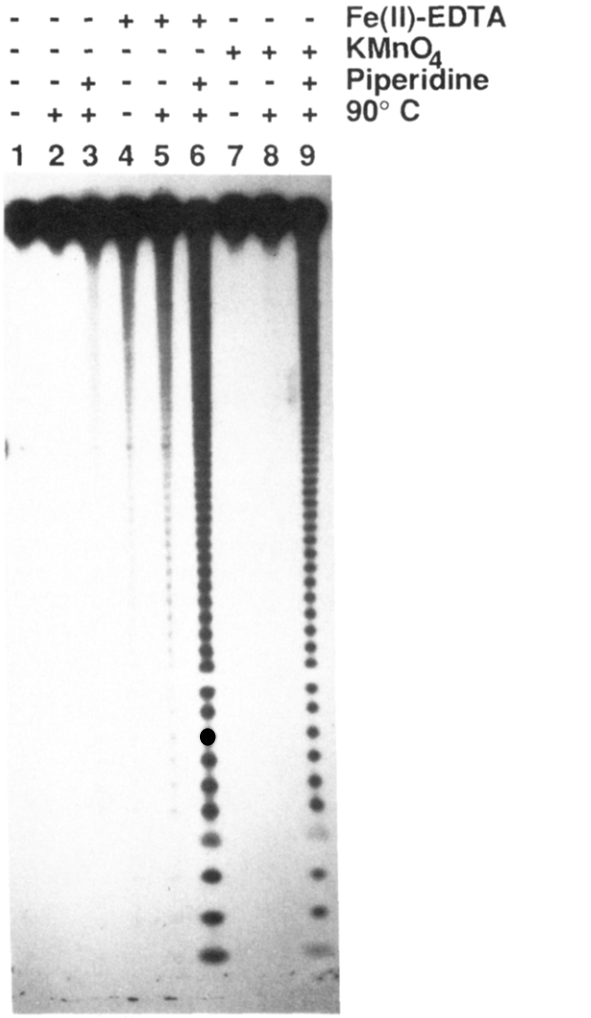

FIGURE 1: Cleavage of $d(pT)_{70}$ with Fe(II)–EDTA and potassium permanganate. The (+) and (−) signs above the autoradiograph indicate which reagents and conditions were used to prepare the samples in each lane. Fe(II)–EDTA indicates the reaction of hydroxyl radicals with $d(pT)_{70}$. $KMnO_4$ indicates the reaction of permanganate with $d(pT)_{70}$. Piperidine indicates the addition of piperidine prior to heating to 90 °C. 90 °C indicates heating for 30 min at 90 °C. The experimental details are presented under Materials and Methods.

Hydroxyl radicals can react with the ribose moiety of nucleic acids by abstracting a hydrogen atom (von Sonntag, 1987). Some of the resultant radical intermediates further rearrange to bring about cleavage of the phosphodiester linkage at the 3′-position. This reaction is the basis for the dsDNA footprinting technique of Tullius and others. However, hydroxyl radicals can also form adducts with the bases of DNA, leading to oxidation and ring opening. For example, ESR studies have shown that the hydroxyl radical adds to the 5-carbon of thymidine with no detectable reaction with the ribose group [Hildenbrand et al., 1989; but see also Kuwabara et al. (1989)]. The pyrimidine 5–6 double bond appears to be particularly reactive with hydroxyl radical (Kuwabara et al., 1989; Hiraoka et al., 1990). It seems generally true that the reactivity of the hydroxyl radical toward the ribose moiety of nucleosides, nucleotides, and single-stranded oligonucleotides and nucleic acids is considerably less than that toward the purine and pyrimidine rings (von Sonntag, 1987).

From these results we propose that the somewhat lower extent of strand cleavage observed for ssDNA [$d(pT)_{70}$] vs dsDNA [$d(pT)_{70}$·$dA(pA)_{69}$] by Jezewska et al. (1989, 1990)

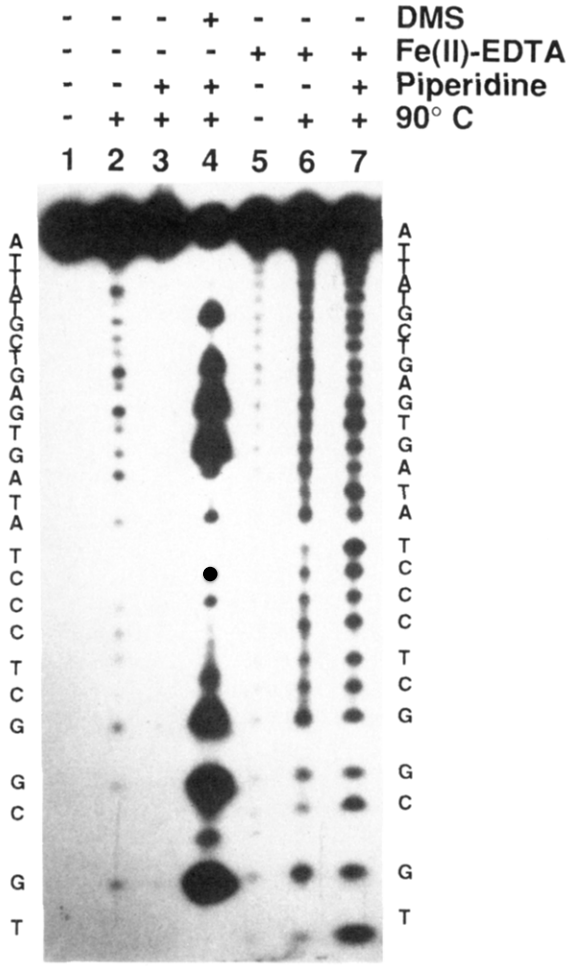

FIGURE 2: Cleavage of a mixed-base oligonucleotide with Fe(II)–EDTA and dimethyl sulfate. The (+) and (−) signs above the autoradiograph indicate which reagents and conditions were used to prepare the samples in each lane. DMS indicates the reaction of dimethyl sulfate with the oligonucleotide. Fe(II)–EDTA indicates the reaction of hydroxyl radicals with the oligonucleotide. Piperidine indicates the addition of piperidine prior to heating to 90 °C. 90 °C indicates heating for 30 min at 90 °C. The sequence of the oligonucleotide is given along both sides of the autoradiograph. The experimental details are presented under Materials and Methods.

is due to a scavenging of the hydroxyl radical in ssDNA by the more reactive thymine base. Although it is difficult to know the transient effective concentration of the hydroxyl radical in this type of experiment, the catalytic Fe(II)–EDTA is the reagent of lowest concentration. In the study of Celander and Cech (1990) the Fe concentration is 0.1–1 mM, compared with 0.02–0.2 mM in the study of Jezewska et al. (1989) and 0.04 mM in the current study. The concentrations of DNA were 20–50 nM in these earlier studies, but the concentration is 2.0 μM in the current work. If the nucleotide bases are inherently more reactive than the sugars, the difference in reactivity may nevertheless become less pronounced as the ratio of radical to DNA is increased.

Fe(II)–EDTA can be used to directly footprint *double-stranded* DNA because the base pairs are sequestered within the double-helix structure. Reactivity of the hydroxyl radical toward the inherently more labile aromatic rings is attenuated to a significant degree, and the ribose group becomes the preferred substrate. For single-stranded DNA (or for double-stranded DNA containing single-stranded regions), one must consider the reactivity of the hydroxyl radical with the base substituents.

These results indicate that Fe(II)–EDTA and some other Maxam–Gilbert reagents can be used to footprint *single-stranded* DNA. Such experiments should be especially useful for the study of proteins that bind to ssDNA, such as the SSB protein of *Escherichia coli* (Prigodich and Martin, unpublished results). Base-specific reagents such as permanganate may also be useful in the study of dsDNA binding proteins, as probes for helix unwinding in regions distant from protein binding sites. The difference in reactivity of the hydroxyl radical toward base and sugar groups is very relevant to the interpretation of footprinting studies of dsDNA with Fe(II)–EDTA or of studies using this approach to determine DNA or RNA secondary structure. Regions of duplex DNA unwound by protein binding adjacent to those regions may appear to be protected by direct contact with the protein when in fact the ribose rings are protected from reaction with hydroxyl radical by the availability of the far more reactive nucleic acid bases. Finally, in the interpretation of hydroxyl radical footprinting studies, one must consider that aromatic or other reactive amino acids may similarly compete for reaction with the hydroxyl radical, thereby casting a larger footprint on the DNA than less reactive amino acids.

Registry No. d(pT), 25086-81-1; Fe(II)–EDTA, 15651-72-6; DMS, 77-78-1; $KMnO_4$, 7722-64-7; H_2O_2, 7722-84-1; hydroxyl radical, 3352-57-6; piperidine, 110-89-4.

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