What Species Is Responsible for Strand Scission in the Reaction of $[Fe^{II}EDTA]^{2-}$ and H_2O_2 with DNA?

Wendy Knapp Pogozelski,† Timothy J. McNeese,‡ and Thomas D. Tullius*

Contribution from the Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

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Abstract: The reaction of ferrous iron with hydrogen peroxide (the Fenton reaction) has been widely used as a means of inducing strand cleavage in DNA, generally for the purpose of "footprinting" protein-DNA complexes or studying DNA structure. The identity of the DNA-oxidizing species produced in the reaction, however, has been an issue of debate. By comparing γ-radiolysis-induced DNA cleavage patterns in a variety of buffers with those generated by the reaction of $[Fe(EDTA)]^{2-} + H_2O_2$ in the presence of ascorbate, we report that under the conditions used for a typical footprinting experiment, results are consistent with production of the hydroxyl radical as the oxidant responsible for DNA strand scission. We also cite experiments that argue against the participation of a high-valent iron-oxo complex in DNA cutting.

Introduction

Oxygen-derived species, formed in the presence of transition metal ions, are known to damage DNA *in vitro* and *in vivo*.[1] One metal-dependent method of producing a DNA oxidant that has come under much scrutiny is the reaction of Fe^{II} with H_2O_2. One hundred years after Fenton reported the powerful oxidation properties of this combination of reagents,[2] researchers are still debating the identity of the chemical species produced. Haber and Weiss proposed that the reactive species generated in this reaction is the hydroxyl radical (•OH), produced as following:[3]

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \qquad (1)$$

However, this conclusion has been challenged. Although results such as the hydroxylation of aromatic compounds[4] and a demonstrated independence of ionic strength[5] are consistent with predictions that the hydroxyl radical is the oxidizing agent produced,[1] several researchers have claimed otherwise. Walling, for example, proposed that when an iron chelator such as EDTA is used, some hydroxyl radicals may be trapped by the iron complex immediately after formation to create a "caged" reactive species.[6] Based on stoichiometry and low measured rates of reaction with expected scavenging molecules, some researchers have suggested that a high-valent iron-oxo complex, similar to that generated by cytochrome P-450, is produced instead.[7-11] When the oxidation of DNA by the Fenton reagent is considered, some contend that both the hydroxyl radical and an oxidizing iron species are formed.[12,13] Others claim further that while both species are generated, the iron-oxo product is responsible for DNA damage.[14] Lohman and co-workers, in comparing rates of degradation of single-stranded DNA with double-stranded DNA, also proposed the participation of a high-valent iron-oxo species.[15] The issue is further complicated by suggestions that pH,[12] hydrogen peroxide concentration, and the nature of the chelating agent may also play a role.[13,16]

Our laboratory uses a version of the Fenton reaction, Fe^{II}•-EDTA + H_2O_2 + ascorbic acid, to cleave DNA and obtain information about its structure[17-19] and interactions with other molecules.[20,21] Hence, it was of great interest for us to establish what chemical species is responsible for this reaction. Here we show evidence which suggests that with the reaction conditions used for a typical footprinting experiment, the hydroxyl radical is indeed responsible for DNA scission.

Experimental Section

Reagents. Materials were purchased from the following suppliers: ammonium ferrous sulfate, ammonium ferric sulfate dodecahydrate, hydrogen peroxide, thiourea, EDTA, and sodium phosphate, Aldrich; sodium ascorbate, Sigma; sodium acetate, sodium citrate, and sodium propionate, Baker; HEPES (*N*-[2-hydroxylethyl]piperazine-*N*′-[2-

* Author to whom correspondence should be addressed.
† Present address: Naval Research Lab, Code 6615, Washington, D.C. 20375-5345.
‡ Present address: Department of Chemistry, Loyola College, 4501 N. Charles St., Baltimore, MD 21210.
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ethanesulfonic acid]) and MES (2-[*N*-morpholino]ethanesulfonic acid), United States Biochemicals; restriction enzymes, Klenow fragment of DNA polymerase I, and polynucleotide kinase, New England Biolabs; calf alkaline phosphatase, Boehringer Mannheim; acrylamide and bis-acrylamide, IBI; ^{32}P-labeled nucleotides, Amersham.

DNA Substrates. The plasmids pUC18 and pHC624 were amplified and purified by CsCl gradient centrifugation or by chromatography on a Pharmacia FPLC system using Sephacryl S-500 and RPC-5 columns. Plasmid pHC 624 was a gift from Dr. Jacob Lebowitz, University of Alabama, Birmingham.[22] The plasmid MPclone114, containing the bent A-tract sequence $(A_5N_5)_5$, was amplified from a sample prepared by M. A. Price.[23] A DNA fragment of defined sequence and length was obtained from a plasmid by digestion with restriction enzymes. Preparation of 5′-^{32}P-labeled DNA required treatment with calf alkaline phosphatase to remove the 5′-phosphate group. This phosphate was then replaced with radioactive phosphate from [$\gamma-^{32}$P]dATP using the enzyme polynucleotide kinase. A second restriction digest yielded a fragment of the desired size with only one of its strands radiolabeled.

Radiolabeling at the 3′-end first required an enzymatic digest that produced a staggered cut (leaving at least one overhanging nucleotide). The Klenow fragment of DNA polymerase I was then used to catalyze attachment of an [α-^{32}P]-labeled nucleoside triphosphate that was complementary to the overhanging nucleotide. The appropriate dideoxynucleotide was included in the reaction for increased labeling efficiency. As with 5′-end-labeled fragments, a second enzymatic cut yielded a smaller fragment with only one of its strands labeled.

The desired DNA molecule was isolated by electrophoresis on a native polyacrylamide gel (cross-linked with bis-acrylamide in a ratio of 1:31), identified by autoradiography with Kodak XAR-5 X-ray film, excised, and eluted in "Crush and Soak" buffer.[24] The eluate was collected by centrifugation through glass wool. The DNA was precipitated with ethanol, rinsed with 70% ethanol, lyophilized, and brought to a concentration of 1000 Geiger counter counts (10 000 dpm as measured by a scintillation counter) per μL in the desired buffer. Generally, TE Buffer (10 mM Tris (pH 8), 1 mM EDTA) was used.

Reaction of Fe and Fe Complexes with DNA. Reactions of $[Fe(EDTA)]^{2-}$ and H_2O_2 with DNA proceeded as follows: a few picomoles of DNA in 10 mM buffer (generally 10−20000 Geiger counter counts) and 10 mM buffer were combined to yield a total volume of 70 μL. Although TE (pH 8) was the most commonly used buffer, HEPES (pH 7.5), MES (pH 6.1), phosphate (pH 2.1−12.3), acetate (pH 4.7), propionate (pH 4.5), and citrate (pH 5.0) also were used in the buffer experiments. The Fe^{II}·EDTA solution was prepared by mixing equal volumes of 0.2 mM $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ and 0.4 mM EDTA. Ten microliters each of 0.3% H_2O_2, 10 mM sodium ascorbate, and 0.1 mM Fe:0.2 mM EDTA were pre-mixed on the inside wall of an Eppendorf tube containing DNA and buffer and then immediately added to the DNA solution and allowed to react for 2 min. Reactions in phosphate buffer proceeded for 30 s. Reactions were stopped by addition of 100 μL of a stop solution containing 10 mM thiourea, 30 mM EDTA, and 0.6 mM sodium acetate. These conditions were determined to result in no more than one strand break per DNA molecule.[25]

When $[Fe(H_2O)_6]^{2+}$ was used in place of $[Fe(EDTA)]^{2-}$, concentrations and conditions remained the same except that Tris buffer was used instead of Tris/EDTA (TE) buffer.

DNA was precipitated from the reaction mixture first by the addition of 2.5 volumes of ethanol. A second precipitation was performed by dissolution of the DNA in 200 μL of 0.3 M sodium acetate, and then 600 μL of 100% ethanol was added. The precipitate was rinsed with 70% ethanol and dried in a vacuum concentrator. DNA was dissolved in 4 μL of formamide buffer[24] and denatured at 90 °C for 5 min before being loaded onto a denaturing polyacrylamide gel. The acrylamide gel was cross-linked with bis-acrylamide in a ratio of 19:1 in a solution containing 1× TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA) and 8.3 M urea. Electrophoresis was carried out in 1× TBE buffer on a Hoefer Pokerface apparatus preheated to a gel plate temperature of 50 °C. After electrophoresis the gel was transferred to Whatman 3MM chromatography paper and dried. The gel was then exposed either to a Molecular Dynamics PhosphorImager plate or to Kodak XAR-5 X-ray film. The imaging plate was scanned by a Molecular Dynamics Model 400E PhosphorImager (Sunnyvale, CA); the autoradiograph was scanned by a Molecular Dynamics Model 300E scanning densitometer.

γ-radiolysis of DNA. An Eppendorf tube containing a few picomoles of ^{32}P-end-labeled DNA in 100 μL of 10 mM buffer (generally Tris/EDTA) was taped to the wall of a Shepherd ^{137}Cs γ-ray irradiator as close as possible to the source. The sample was irradiated for 5 to 7 min at room temperature. Following radiolysis, the DNA was precipitated as for the iron reactions and loaded onto a denaturing polyacrylamide gel as described above.

Irradiation of EDTA and $[Fe(EDTA)]^{2-}$. A sample containing 0.1 mM Fe^{II} and 0.2 mM EDTA in a volume of 100 μL, or a sample consisting of 100 μL of 0.4 mM EDTA, was irradiated under conditions identical to those used to cut DNA for 5−7 min in a ^{137}Cs irradiator. The sample containing irradiated EDTA was combined with an equal volume of 0.2 mM Fe^{II} to produce a solution containing 0.1 mM Fe and 0.2 mM EDTA. For each experiment, a 10-μL sample of either irradiated $[Fe(EDTA)]^{2-}$ or $[Fe(EDTA)]^{2-}$ containing irradiated EDTA was combined with 10 μL of 0.3% H_2O_2 and 10 μL of 10 mM ascorbate. DNA cleavage patterns were compared with the patterns resulting from the use of the same concentrations of non-irradiated reagents.

Experiments Varying the EDTA/Fe Ratio. Five 200-μL samples of 20 mM Fe^{II} were mixed with equal volumes of 10, 20, 40, 100, and 200 mM EDTA, respectively. The resulting solutions contained EDTA/Fe ratios of 0.5, 1.0, 2.0, 5.0, and 10.0, respectively. Ten microliters of each Fe·EDTA solution were mixed with 10 μL of 88 mM H_2O_2 and 10 μL of 100 mM sodium ascorbate. DNA cleavage reactions proceeded as detailed above. Reaction products were separated on a denaturing gel as described above.

Results

The reaction of $[Fe^{II}EDTA]^{2-}$ with H_2O_2 is well-known as a technique for studying the structure of DNA and for "footprinting" protein−DNA or drug−DNA complexes.[17−20] Information from these experiments comes from differences in the accessibility of the reactive species to various regions of the DNA molecule. Therefore the identity of this damaging species is important for correct interpretation of the resulting cutting patterns.

The particular version of the reaction used in such experiments varies slightly from the original Fenton reaction and the conditions used in much of the literature in that Fe^{II} is coordinated with the hexadentate polyaminocarboxylic acid ligand ethylenediaminetetraacetic acid ($[EDTA]^{4-}$). This ligand increases the amount of iron in solution by hindering formation of $Fe(OH)_3$ and iron oxide precipitates. In addition it gives the complex an overall 2− charge, so that the iron complex does not interact with polyanionic DNA.[26] A typical reaction mixture also contains buffer to maintain constant pH and ascorbate to regenerate Fe^{II} from Fe^{III}. When ascorbate and EDTA are included, the reaction is known as the Udenfriend version of the Fenton reaction.[27] Reactions are carried out under "single-hit" kinetics; that is, concentrations of reagents and reaction times are selected so that no more than one cleavage event occurs per DNA molecule.[25]

The reactive species in this reaction is believed to oxidize DNA by abstracting hydrogen from the deoxyribose ring,

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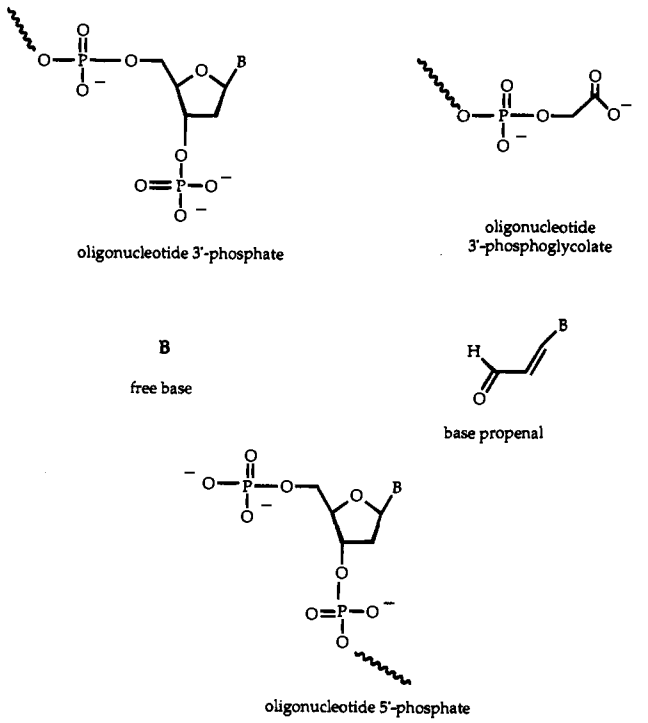

Figure 1. Products of DNA scission induced by the reaction of $[Fe(EDTA)]^{2-}$ + H_2O_2 + ascorbate.

leading to sugar rearrangement and strand scission.[28] The scission creates DNA fragments that terminate in a 5′-phosphate, 3′-phosphate, or 3′-phosphoglycolate group. Thus, when the molecule is radiolabeled at its 3′ end, only one type of product, DNA fragments with 5′-phosphate termini, are detected, while 5′-labeled DNA can show up to two products at each nucleotide position. The ratio of 3′-termini has been shown to depend on reaction conditions.[29,30] Fragments with 3′-phosphate ends always appear in greater yield and have a slightly slower electrophoretic gel mobility than do the 3′-phosphoglycolate products. Nucleic acid bases that are free in solution as well as bases with a propenal moiety attached also have been identified.[31,32] These products are pictured in Figure 1.

Comparison with γ-radiolysis. The first test to identify the reactive species produced in these experiments was to compare the DNA products from the $[Fe(EDTA)]^{2-}$ + H_2O_2 reaction with those of a known hydroxyl-radical-producing system. Water, when subjected to γ-rays, forms hydroxyl radicals in measurable yield.[33,34] Such a system is iron free; it oxidatively damages DNA and its scission products are identical to those of $[Fe(EDTA)]^{2-}$ and H_2O_2-induced cleavage.[31,32] In addition, this system has been shown to produce similar protein−DNA footprints to those generated by $[Fe(EDTA)]^{2-}$ and H_2O_2 in the presence of ascorbate.[35]

We chose to study a DNA fragment that yields a distinctive pattern when treated with Fe•EDTA and H_2O_2. Bent DNA

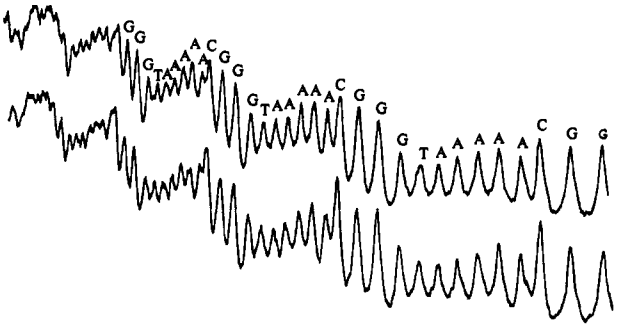

Figure 2. Comparison between bent A-tract DNA cleavage patterns generated by Fenton/Udenfriend chemistry and γ-radiolysis. Shown are densitometer scans of a 5′-^{32}P-end-labeled restriction fragment from MPclone114 (pUC18 with $(A_5N_5)_5$ insert) run on a 25% denaturing gel. Each peak in the pattern represents a population of DNA molecules that were cleaved at a particular nucleotide. Peaks are labeled with the nucleotide at which cleavage occurred. The two scans are aligned horizontally. Top: 2 mM Fe:4 mM EDTA, 88 mM H_2O_2, 2 mM ascorbate final concentrations in 10 mM TE buffer. Bottom: 4 min ^{137}Cs γ-radiation in 100 μL of 10 mM TE buffer.

containing runs of adenines (A-tracts) phased with the helical repeat of the DNA has been found to have a unique sinusoidal cutting pattern in which cleavage decreases smoothly within the A-tract and then increases outside that region. This pattern has been shown to correlate with the variations in width of the minor groove that occur within A-tracts.[18,36] Radiolabeled samples of bent DNA were treated either with γ-rays or with $[Fe(EDTA)]^{2-}$, H_2O_2, and ascorbate, and the reaction products were separated by denaturing gel electrophoresis. As the densitometer scans in Figure 2 show, not only did bands co-migrate, as would be expected for chemically-identical DNA scission products, but the relative amounts of each product at the various nucleotide positions were the same. In fact, the two cutting patterns are virtually superimposable. The oxidant in both reactions appears to recognize the same features of the unusual DNA structure; in this case, the feature appears to be the narrow minor groove in A-tracts.

This experiment strongly supports the role of the hydroxyl radical in the cleavage of DNA by the Fe•EDTA/H_2O_2/ascorbate system. However, two other explanations for these results must be considered: (1) a species other than the hydroxyl radical but common to both systems is oxidizing DNA (i.e., a buffer-mediated radical) or (2) the two systems produce different oxidants, but these show identical selectivity for DNA microstructure.

Comparison of Buffers. The first of these alternate possibilities was tested by comparing the effects of various buffers on DNA cutting patterns. Earlier work showed that many common biological buffers quench the cleavage of DNA by the Fe•EDTA/H_2O_2/ascorbate system.[20] In the experiments shown in Figure 3, DNA samples in either 10 mM Tris/EDTA (pH 8.0) or 10 mM phosphate (pH 8.0) buffer were treated with γ-rays or Fe•EDTA, H_2O_2, and ascorbate. Products of the reactions were separated on a denaturing polyacrylamide gel. As expected, both buffers quenched the cutting reaction somewhat. However, Tris buffer is believed to quench radicals by hydrogen abstraction, while phosphate quenches by an electron transfer mechanism. Hydrogen abstraction is several orders of magnitude faster than the electron transfer process.[6] Hence the amount of DNA cutting in phosphate buffer was greater than that seen in Tris/EDTA, and reaction times were scaled accordingly. As Figure 3 shows, cleavage patterns are

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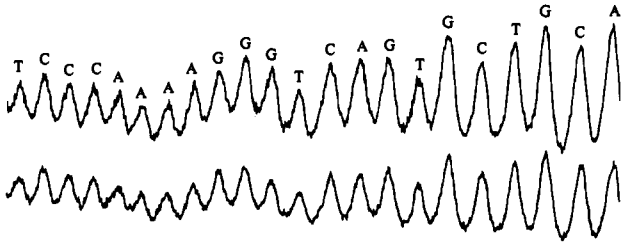

Figure 3. Comparison between DNA cleavage patterns generated by (top) Fenton/Udenfriend chemistry in phosphate buffer and (bottom) γ-radiolysis in TE buffer. Shown are densitometer scans of a 3′-^{32}P-end-labeled restriction fragment from pUC18 run on an 8% denaturing gel. Top: 10 μM Fe:20 μM EDTA, 8.8 mM H_2O_2, 1 mM ascorbate (30 s reaction) in 10 mM phosphate buffer, pH 8.0. Bottom: 5 min ^{137}Cs γ-radiation in 100 μL of 10 mM TE buffer, pH 8.0.

identical, regardless of the buffer or cutting method used. These results support the notion that there is a common agent in all of the reactions. In subsequent experiments, the list of buffers tested was expanded to include sulfonic and carboxylic acids (data not shown). Likewise, cutting reactions in these solutions showed similar quenching trends and identical cleavage profiles.

The observation that identical DNA cleavage patterns are produced by both γ-rays and the Fe·EDTA/H_2O_2/ascorbate system in buffers of different chemical makeup precludes significant participation of a buffer-mediated radical in the oxidation of DNA. Furthermore, the buffers showed identical quenching trends in the Fe·EDTA/H_2O_2/ascorbate and γ-radiolysis systems; this quenching is indicative of a freely diffusible reactive species. Since buffer and water are the only other species common to both sets of experiments, this result gives further support to the idea that the hydroxyl radical is oxidizing DNA in both cutting systems. One should note that quenching experiments should be interpreted with care. Certain scavengers such as alcohols may do more than quench the radicals; they may undergo additional oxidation or reduction reactions with Fe^{II}, Fe^{III}, or H_2O_2.[37] (The observation that ferrous or ferric complexes can undergo reactions in addition to the decomposition of peroxide also suggests that comparisons with γ-radiolysis should be made with care. However, the iron complexes used for footprinting are negatively charged and, as will be demonstrated later, are not expected to react with DNA.)

The other explanation for the identical DNA patterns is the idea that the hydroxyl radical (produced by γ-rays) and a different species (produced by the Fe·EDTA/H_2O_2/ascorbate system) react with DNA in an identical manner. While this hypothesis is difficult to prove or disprove directly, the following experiments lead us to rule out the participation of the proposed[7-15] high-valent iron−oxo species.

Tests for EDTA Degradation. In footprinting reactions, iron is part of a negatively-charged complex, $[Fe(EDTA)]^{2-}$. An iron−oxo species such as $[Fe^{IV}O]^{2+}$ or $[Fe^{V}{=}O]^{3+}$ complexed with EDTA would still be negatively charged (2− or 1−, respectively) and would be repelled by the DNA phosphodiester backbone.[26] One would expect such a complex to react with DNA only if the EDTA were somehow degraded or made to dissociate from the iron. Displacement of the intact ligand is unfavorable, however. The association constant for Fe^{II} with EDTA is very high,[38] $K = 10^{14.26}$ and reactions contain twice as much chelator as metal. An appreciable amount of neutral or positively-charged complex could only be formed if the ligand were somehow degraded. We wished to determine whether or not hydroxyl radical could react with EDTA to produce a neutral or positively-charged species.

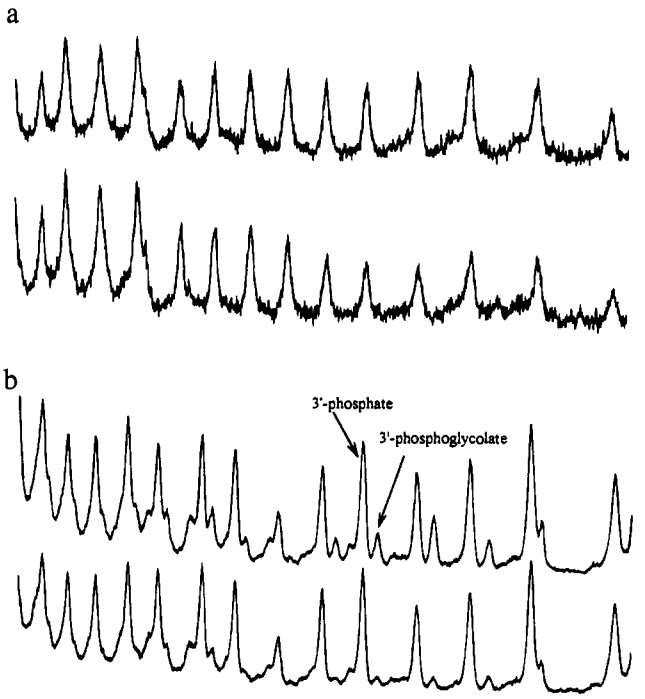

Figure 4. (a) Comparison between DNA cleavage patterns generated by (top) Fenton/Udenfriend chemistry in which EDTA was treated with γ-rays and (bottom) Fenton/Udenfriend chemistry in which EDTA was untreated. Shown are densitometer scans of a 5′-^{32}P-end-labeled restriction fragment from pHC624 separated on a 25% denaturing gel. Top: 10 μM Fe^{II} chelated with 20 μM EDTA that had been subjected to 5 min of ^{137}Cs γ-irradiation; 8.8 mM H_2O_2, 1 mM ascorbate final concentrations in 10 mM TE buffer. Bottom: 10 μM Fe:20 μM EDTA, 8.8 mM H_2O_2, 1 mM ascorbate final concentrations in 10 mM TE buffer. (b) Comparison between DNA cleavage patterns using chelated and unchelated Fe^{II}. Shown are PhosphorImager scans of a 5′-^{32}P-end-labeled oligonucleotide annealed to its unlabeled complement and run on a 25% denaturing gel. Top: 0.1 mM $[Fe(H_2O)_6]^{2+}$, 88 mM H_2O_2, 10 mM ascorbate final concentrations in 10 mM Tris buffer. Bottom: 0.1 mM Fe:0.2 mM EDTA, 88 mM H_2O_2, 10 mM ascorbate final concentrations in 10 mM TE buffer. The larger peak in each pair represents a DNA strand with a 3′-phosphate end; the smaller peak has a 3′-phosphoglycolate end.

To test for degradation of $[Fe(EDTA)]^{2-}$ by the hydroxyl radical, EDTA and Fe·EDTA solutions were subjected to γ-ray-induced hydroxyl radicals, using the same conditions that result in single-hit cleavage of DNA. In other words, the EDTA and Fe·EDTA solutions were subjected to the same flux of radicals by γ-radiolysis as the DNA presumably receives when the source of cleavage is the reaction of $[Fe(EDTA)]^{2-} + H_2O_2$ in the presence of ascorbate. The treated EDTA or Fe·EDTA was then used to oxidize DNA by the Fe·EDTA + H_2O_2 + ascorbate method. Both sets of reaction products were separated on a denaturing gel. As is shown in Figure 4a, the γ-ray-treated EDTA and $[Fe(EDTA)]^{2-}$ produced the same DNA-cutting pattern and were as effective in cleavage as the untreated reagents. The ratios of 3′-termini were also identical. By contrast, as Figure 4b shows, unchelated iron(II) produces a different pattern than that generated by $[Fe(EDTA)]^{2-}$. Although trends in the intensities of the 3′-phosphate peaks are the same, reaction with unchelated iron results in a higher proportion of the 3′-phosphoglycolate product. The patterns compared in Figure 4a do not show this characteristic. This assay suggests that the hydroxyl radical does not degrade EDTA to an extent that results in appreciable amounts of free iron or positively-charged species.

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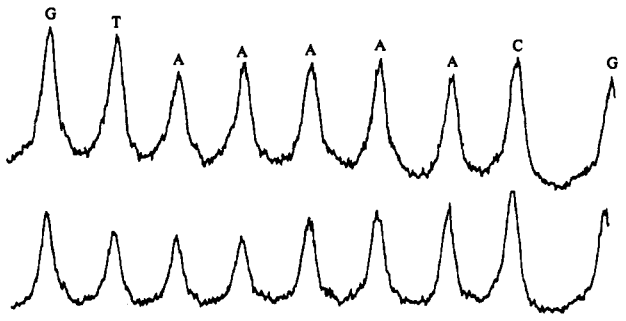

Figure 5. Comparison between DNA cleavage patterns generated by Fenton/Udenfriend chemistry using different EDTA/Fe^{II} ratios. Shown are densitometer scans of a 5′-^{32}P-end-labeled restriction fragment from MPclone114 (pUC 18 with an $(A_5N_5)_5$ insert) run on a 25% denaturing gel. Top: EDTA/Fe ratio = 1:2 (1 mM EDTA:2 mM Fe), 88 mM H_2O_2, 10 mM ascorbate final concentrations in 10 mM TE buffer. Bottom: EDTA/Fe ratio = 1:10 (20 mM EDTA:2 mM Fe, 88 mM H_2O_2, 10 mM ascorbate final concentrations in 10 mM TE buffer).

In another experiment designed to test the importance of chelator, the ratio of Fe^{II} to EDTA was varied. The results, shown in Figure 5, indicate that DNA cutting mediated by a 1:1 complex of Fe·EDTA is very similar to that seen with a 1:10 ratio of Fe^{II} to EDTA. Cutting patterns were nearly identical and little additional quenching at the higher concentration of EDTA was observed. These observations suggest that EDTA degradation by hydroxyl radical is not a significant contribution to the chemistry at work in this system.

Discussion

The experiments described here, as well as those of other researchers, suggest that identical selectivity of the hydroxyl radical and an iron−oxo species for DNA is highly unlikely. The two oxidants differ not only in charge, as mentioned above, but in size as well. It is unlikely that they would recognize in the same way the sequence and structural variations along the DNA polymer or within an individual nucleotide. A positively-charged metal complex, for example, would be expected to interact preferentially with the phosphate groups along the DNA backbone, while a neutral oxidant would not. High-valent iron species are still relatively uncharacterized thermodynamically and electrochemically. However, Koppenol and Liebman calculated standard reduction potentials for both the ferryl ion (FeO^{2+}) and the hydroxyl radical and concluded that the latter was the stronger oxidant.[39]

Having compared Fenton/Udenfriend-generated DNA products with those produced by γ-radiolysis, the obvious experiment would be to compare the Fenton/Udenfriend DNA products with those produced by a known source of a diffusible high-valent iron−oxo complex. Unfortunately, a demonstrated means for producing this type of species in aqueous solution has not been reported. Although the drug iron(II)·bleomycin is presumed to cleave DNA *via* the intermediacy of an iron−oxo species, this system cuts DNA by a somewhat different mechanism than does the hydroxyl radical.[29,40,41] Bleomycin binds to DNA in the minor groove[42] and is far more regiospecific as demonstrated by its selective abstraction of the 4′-deoxyribose hydrogen.[40,41] In contrast, a freely diffusible species with access to the phosphate groups and both grooves of the DNA molecule might be expected to react with any of the deoxyribose hydrogens. In fact, deuterium isotope effect experiments have shown that, in contrast to bleomycin, the Fe·EDTA/H_2O_2/ascorbate system results in abstraction of at least the 5′- and 4′-hydrogens; a different distribution of products is observed as well.[30]

In an effort to examine the reactivity of high-valent iron species, Bielski and co-workers compared the reactions of Fe(IV) and Fe(V) complexes with various amino acids to those of the hydroxyl radical with the same amino acids. The iron complexes showed more selectivity in their reactions.[43,44] However, although these experiments showed differences between the reactions of the hydroxyl radical and high-valent iron, these experiments did not include hydrogen peroxide and were carried out at high pH.

Another factor to consider is that footprinting experiments are carried out in an excess of ascorbate, a reagent expected to reduce high-valent iron to lower oxidation states. Ascorbate may prevent oxidation of iron to the ferryl [Fe(IV)] or perferryl [(Fe(V)] states. The importance of ascorbate in our system has been demonstrated.[20] When no ascorbate is present, levels of DNA cutting are vastly reduced (data not shown).

Could the "caged" radical, (Fe^{III}−EDTA−·OH), proposed by Walling,[5,6] be responsible for DNA strand scission? Again, the presence of ascorbate, the importance of Fe^{II}, and the charge on EDTA make this unlikely. Such an adduct may be formed transiently. This complex is thought to have a distinctive blue-violet color. We sometimes observe such a color at very high iron concentrations, but it disappears as reagents are mixed prior to reaction with DNA.

Other researchers have reported evidence for hydroxyl radical formation. Walling and co-workers observed that the Fenton reaction was independent of ionic strength[5] and Kallenbach found that the rate and products of DNA cleavage by Fe^{II}EDTA plus H_2O_2 were independent of salt concentration.[45] Both of these results support a neutral reactive species.

In another set of experiments, Häring *et al.* showed that the abasic sites generated by γ-rays and by an iron(II) complex were recognized similarly by repair endonucleases, while the abasic sites arising from low pH did not show the same recognition patterns. These results support the idea that the same oxidative species is causing DNA damage in both the γ-radiolysis and iron systems.[46]

Work from the laboratory of Dervan provides further evidence for a freely diffusible DNA-oxidizing species in related systems. In these experiments, methidiumpropyl-EDTA was attached to the DNA-binding drug distamycin or to a complementary strand of DNA. When iron(II) and a reducing agent were added, cleavage occurred not at a single nucleotide in the binding site but over several positions, including some on the opposite strand. The cutting frequencies could be plotted as a Gaussian curve, with a maximum amount of cleavage at one nucleotide and frequencies falling off equally on either side.[47−49] Similar results were obtained by Chu and Orgel,[50] who covalently attached EDTA to the 5′-terminus of a 16-base oligonucleotide. When iron(II) and dithiothreitol were added and the oligomer hybrid-

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ized to a 37-base complementary strand, cleavage occurred at up to four nucleotides on either side of the 16-mer's modified 5′-terminus. Such results are strongly indicative of a DNA-cleaving species that is not metal-bound, but freely diffusible.

By contrast, a system in which the DNA oxidant is thought to be metal bound does not show this diffusion pattern. When *o*-phenanthroline was covalently attached to the DNA-binding protein *trp* repressor[51,52] and copper(I) was added, the copper-bound oxidant cleaved DNA at no more than four sites in the region of binding. Cleavage at surrounding nucleotides was not observed.

While the techniques of ESR spectroscopy and spin-trapping have been used to attempt to identify the oxidizing species produced in the Fenton reaction or its variations, little agreement has been achieved. Gilbert and co-workers reported direct evidence for the formation of hydroxyl radical by the reaction of $[Fe(EDTA)]^{2-}$ with H_2O_2,[37,53] while Yamazaki and Piette concluded that both a ferryl species and hydroxyl radical were being produced.[13] Such discrepancies might be resolved, however, if one considers that some of the signals that differ from those attributed to hydroxyl radical actually arise from secondary oxidation by Fe^{III}. Indeed, these arguments were used by Gilbert[37,53] and Walling[6] to explain many of the kinetic observations that first led to the proposal of a high-valent iron–oxo oxidant. For example, Shiga found that the reaction of the Fe·EDTA/H_2O_2 system with ethanol produced the β-radical $\cdot CH_2CH_2OH$, while the Ti^{III}/H_2O_2 system (thought to produce $\cdot OH$) resulted in the α-radical.[54] Gilbert argued that the α-radical was produced in both systems, but that in the iron-containing system, it was rapidly oxidized by Fe^{III}. Burkitt proposed secondary reactions associated with the common spin trap 5,5-dimethyl-1-pyridine *N*-oxide (DMPO) and showed that the ESR spin adduct was more readily oxidized by iron in the presence of EDTA.[55] One should note that ascorbate was not included in these ESR experiments because it silenced the signals.

Many researchers who propose an alternative oxidant to the hydroxyl radical have done so based on the observation that expected hydroxyl radical scavengers sometimes fail to quench the Fenton reaction. However, another explanation has been offered for these findings. Czapski and co-workers have proposed that such results may be indicative of a site-specific or site-directed mechanism, in which radicals are generated at the site of attack and do not diffuse enough for reaction with scavengers.[56] This interpretation is supported by our observation that when the DNA cleavage reaction is mediated by unchelated iron ($[Fe(H_2O)_6]^{2+}$), known hydroxyl radical quenchers such as Tris or glycerol are much less effective in preventing DNA cleavage than when the cleavage reaction is mediated by $[Fe(EDTA)]^{2-}$. Presumably, unchelated iron associates with the DNA phosphates and generates radicals in closer proximity to the DNA molecule, resulting in reduced quenching. The arguments supporting an iron–oxo oxidant, when based on unexpected rates of reaction with scavengers, sometimes include the illogical assumption that this species and the hydroxyl radical have a similar reaction with DNA, but different reactions with scavengers.

Although a number of researchers have proposed that a species other than the hydroxyl radical is responsible for DNA cleavage, many of the experiments were performed in the absence of DNA. Furthermore, few have mimicked the conditions used in footprinting reactions; in particular, most studies have not employed EDTA as a chelator for iron or used ascorbate to regenerate iron(II). It is not our intention to argue against formation of any non-hydroxyl radical species by the Fenton reagent. However, the experiments shown here are consistent with the proposal that under the conditions used in footprinting reactions, the species responsible for DNA cleavage is the hydroxyl radical.

Summary

We have shown that the DNA-cleavage patterns generated by a known source of hydroxyl radicals, the γ-radiolysis of water, are identical to those generated by the reaction of $[Fe(EDTA)]^{2-}$ with H_2O_2 in the presence of ascorbate. The observed patterns are independent of the type of buffer used. Because the two DNA-cleaving systems contain no other components in common, our results provide evidence that the species responsible for DNA damage in both systems is the hydroxyl radical rather than a high-valent iron–oxo species.

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