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DNA FOOTPRINTING WITH THE HYDROXYL RADICAL

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The Fenton reaction of iron(II) EDTA with hydrgen peroxide, performed in the presence of ascorbate ion, has proven to be useful as a probe of structure in DNA systems. Two aspects of this chemistry are discussed: the identity of the active DNA cleaving agent produced by this reagent, and the application of the Fenton reaction to the determination of the structure of the Holliday junction, the four-stranded DNA molecule that is a key intermediate in recombination. The cleavage pattern of the Holliday junction has pseudo-twofold symmetry, putting important constraints on possible structures.

KEY WORDS: Iron(II) EDTA, DNA structure, gamma radiation, Holliday junction, hydroxyl radical.

INTRODUCTION

Over the past several years my group has been developing the Fenton reaction of iron (II) EDTA with hydrogen peroxide for use as a tool for structure determination in DNA systems.[1] This reagent cleaves the backbone of DNA with no preference for any of the four nucleotide bases. The cleavage pattern is easily visualized by electrophoresis of the reaction products on a DNA sequencing gel. While the cleavage pattern of a "normal" DNA molecule is nearly featureless, we found that the cleavage pattern of DNA that is "perturbed"in some way can be analyzed to give structural details at the level of the individual nucleotides.

For example, a protein bound to a specific site in a DNA molecule protects the DNA backbone at the binding site from cleavage by the Fenton reagent.[2] The resulting "footprint" gives a high-resolution view of the contacts of the protein with the DNA backbone. The cleavage pattern of DNA bound to a calcium phosphate precipitate appears sinusoidal, with a period of around 10 nucleotides per repeat.[3] This cleavage periodicity gives directly the helical repeat (the number of base pairs per turn of the double helix) of the DNA molecule. DNA that is stably curved or bent as the consequence of its sequence exhibits a characteristic cleavage pattern that we interpreted as indicating that the minor groove of a bent DNA molecule periodically narrows and widens.[4]

In this contribution I outline recent work on two aspects of DNA cleavage mediated by the Fenton reaction. I first present experimental evidence that bears on the question of the identity of the chemical species that is responsible for the Fenton reaction-induced cleavage of DNA. I then discuss our studies of the structure of the Holiday junction, a four-stranded DNA molecule that is a key intermediate in genetic recombination. The Fenton reaction turns out to provide important information on the symmetry and structure of the Holliday junction.

MATERIALS AND METHODS

λ Repressor-DNA Complex

Formation of the complex of the bacteriophage λ repressor with O_R1 operator DNA, and cleavage of the DNA *via* gamma radiolysis or treatment with the iron(II) EDTA/hydrogen peroxide/ascorbate reagent, was performed as described.[5]

Holliday Junction

The four strands of J1, and their complementary strands, were synthesized by Professor Ned Seeman. J1 was annealed and shown to run as a single band under native conditions in polyacrylamide gel electrophoresis, as described.[6] Cleavage of J1 by the iron(II) EDTA/hydrogen peroxide/ascorbate reagent was performed as described.[6]

RESULTS AND DISCUSSION

How Does the Fenton Reaction Cause DNA Cleavage?

There has been much discussion in the literature over the century since the Fenton reaction was discovered[7] concerning the chemical species produced in this reaction. In our work we have used a particular version of the Fenton reaction, developed by Udenfriend,[8] that involves the reaction of the EDTA complex of iron(II) with hydrogen peroxide in the presence of ascorbate ion (reaction 1):

$$[Fe(EDTA)]^{2-} + H_2O_2 \underset{\text{ascorbate}}{\rightleftharpoons} [Fe(EDTA)]^{1-} + OH^- + \cdot OH \qquad (1)$$

(Ascorbate serves to reduce the iron(III) product back to iron(II), so that micromolar concentrations of reagent are sufficient for DNA cleavage at the levels we require). We chose the EDTA complex of iron because of its negative charge, which should preclude binding of the metal complex to the anionic DNA molecule.[1] We presume that the hydroxyl radical is the actual agent that causes cleavage of the DNA backbone, *via* a process that involves abstraction of a hydrogen atom from a deoxyribose leading subsequently to the breakdown of the sugar and production of a gap in the DNA chain.[9] Another possibility for the identity of the proximate cleavage agent is a ferryl (Fe=O) species.[10] I present below evidence that suggest to us that the cleavage agent is the hydroxyl radical, although more work in this area is clearly needed.

The cleavage agent is diffusible

A number of experiments from the group of P.B. Dervan[11] graphically demonstrate that the DNA cleavage agent produced by iron(II) EDTA diffuses over several Ångstroms. Dervan and his coworkers have attached a derivative of EDTA to a large variety of molecules that bind to DNA. Addition of iron(II) and a reducing agent transforms these DNA binding molecules into potent DNA cleavers. When the molecule binds to a specific site on DNA, cleavage occurs not at a single nucleotide, but over a range of a half-dozen or so nucleotides on each DNA strand. The cleavage pattern is approximately Gaussian in shape, peaking at a particular nucleotide and

falling off to either side. The participation of a small diffusible species in the cleavage event is clear from such a pattern.

Other DNA cleavage reagents, in particular bis(*o*-phenanthroline)copper(I) and derivatives,[12] that also break the DNA backbone oxidatively, cleave much more site-specifically. Instead of a Gaussian-like distribution, one or two nucleotide positions are cleaved at each binding site. It therefore is probable that the two classes of reagent, iron EDTA and copper phenanthroline, involve different types of cleavage agent. A metal-oxo species is likely for the copper reagents, while with the iron EDTA-based molecules the hydroxyl radical is a plausible participant.

In our work with the Udenfriend reaction system (reaction 1), we have noted that radical scavengers strongly inhibit the cleavage reaction.[13] For example, the cleavage rate is noticeably greater for DNA samples dissolved in 10mM compared to 50 mM Tris · Cl buffer. This effect, also known in radiation biology, likely results from the ability of the hydroxyl radical to react with the Tris molecule. Indeed, DNA cleavage reactions performed in phosphate buffer proceed at a far higher rate.[13] Phosphate does not scavenge the hydroxyl radical. Glycerol is a particularly efficient scavenger, and so footprinting experiments must be performed on protein-DNA complexes dissolved in buffers that are as free of glycerol as practicable.[13]

Other evidence that the Udenfriend system cleaves DNA *via* a diffusible agent comes from experiments demonstrating that aquaferrous ion cleaves DNA much faster than does iron(II) EDTA (G. Shafer and T.D. Tullius, unpublished). The aquaferrous cation likely binds to the phosphates of the DNA backbone, thus generating the cleavage agent very near the DNA backbone, while iron(II) EDTA produces a cleavage agent that must diffuse some distance through solution (and thus be exposed to other scavenger molecules) before reaching the DNA molecule. Cleavage by aquaferrous ion is much less sensitive to radical scavengers (G. Shafer and T.D. Tullius, unpublished), additional evidence that this cation is bound near to the DNA backbone.

We think that the DNA cleavage agent in the Udenfriend system is not cationic (as a ferryl species that dissociates from EDTA would be), because addition of excess unlabeled DNA has very little effect on the rate of cleavage of a small amount of radioactively-labeled DNA by iron(II) EDTA (J. Hayes and T.D. Tullius, unpublished). If a cationic species that binds to DNA *were* the active cleavage agent, the excess polyanionic DNA should efficiently sequester the cleaving species and thus lower the effective rate of cleavage of the labeled DNA.

Gamma rays and iron(II) EDTA give the same cleavage patterns for DNA and DNA-protein complexes

One way to test whether cleavage of DNA by the Udenfriend system involves the hydroxyl radical is to use an independent means of producing this radical, and then compare the properties of the two DNA cleavage reactions. A convenient way to generate the hydroxyl radical is by radiolysis of water with gamma radiation (reaction 2):

$$H_2O \xrightarrow{h\nu} \cdot H + \cdot OH \qquad (2)$$

Haseltine and coworkers showed that gamma ray-induced DNA cleavage leaves phosphate and phosphoglycolate ends on the resulting gap in the DNA strand.[14,15]

DNA cleavage products with these end groups have characteristic mobilities on a DNA sequencing electrophoresis gel. These workers also noted that the ferrous ion-induced DNA cleavage reaction gave products with gel mobilities similar to those of the gamma ray reaction products.[14] We observe that the iron(II) EDTA/hydrogen peroxide/ascorbate system also leads to cleavage products with mobilities characteristic of phosphate and phosphoglycolate end groups. In addition, we have found that free nucleic bases are produced in the cleavage reaction (G. Shafer and T.D. Tullius, unpublished), another characteristic of the gamma ray-induced DNA cleavage reaction.

One of the most convincing pieces of evidence for the involvement of the same cleaving species in gamma ray- and iron(II) EDTA-induced DNA cleavage reactions comes from footprinting experiments on the bacteriophage λ repressor. We found that the cleavage patterns of the naked DNA molecule containing the repressor binding site, as well as the repressor-DNA complex, were virtually identical using either iron(II) EDTA/hydrogen peroxide/ascorbate or gamma irradiation to induce DNA cleavage.[5] The footprints of the repressor-DNA complex produced by both methods are shown in Figure 1. The overall patterns of protected and exposed nucleotides, as well as the fine details of the pattern (the relative intensities of the bands in the patterns), are remarkably similar. While both systems generate a variety of reactive species (ferryl complexes in Fenton chemistry, the hydrogen atom and the hydrated electron in gamma radiolysis), the only common species is the hydroxyl

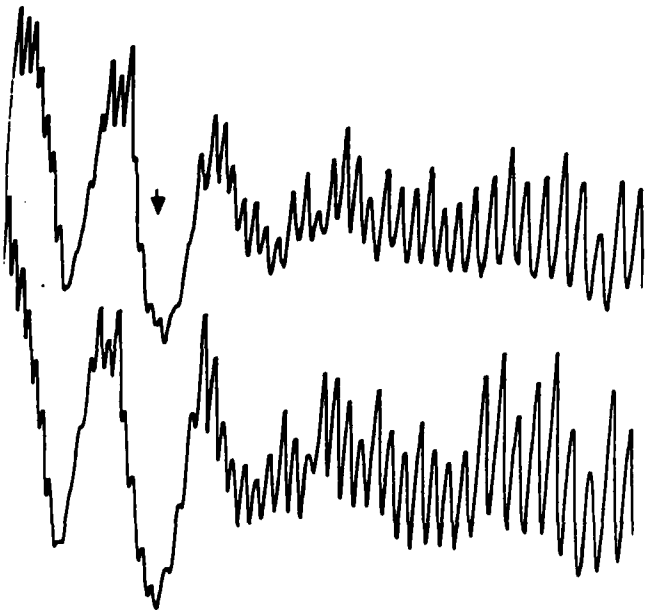

FIGURE 1. Comparison of gamma ray and iron(II) EDTA footprints of the bacteriophage λ repressor, bound to the O_R1 operator sequence. Densitometer scans are shown of lanes from a DNA sequencing electrophoresis gel on which was separated the products of cleavage of the λ repressor/DNA complex. Each peak represents cleavage at a particular nucleotide in the DNA sequence. The area of a band is proportional to the cleavage rate. Upper trace, cleavage induced by gamma irradiation. Lower trace, cleavage induced by iron(II) EDTA/hydrogen peroxide/ascorbate.

radical. Thus we think that these footprinting patterns offer suggestive evidence for the participation of the hydroxyl radical in iron(II) EDTA-induced cleavage of DNA.

Using the Fenton Reaction to Determine the Structure of the Holliday Junction

I now describe our application of the Fenton reaction to a very interesting problem in DNA structural chemistry. In DNA recombination processes, segments of DNA similar in sequence exchange positions; the topological complications of such processes are still not completely clarified. One of the key intermediates in recombination is a four-stranded DNA molecule called the Holliday junction[16] (Figure 2). While natural Holliday junctions have been observed, their inherent instability (because of the built-in sequence symmetry of the four strands) makes them difficult subjects for detailed structural study.

A few years ago Seeman[17] designed a four-stranded DNA molecule, called J1 (Figure 3), whose lower sequence symmetry gives rise to a stable, immobile four-way DNA junction. Several questions of structure and dynamics now become possible to address because of the stable nature of J1. For example, do two particular strands form the crossover region and two remain essentially helical[18] (Figure 2), or are all four strands structurally equivalent (Figure 3)? If the structure has twofold symmetry, are the two arms of the structure parallel (as in Figure 2), or are they antiparallel? What governs the process of branch migration, during which the crossover position moves from one end of the four-way junction to the other? Our cleavage experiments[6]

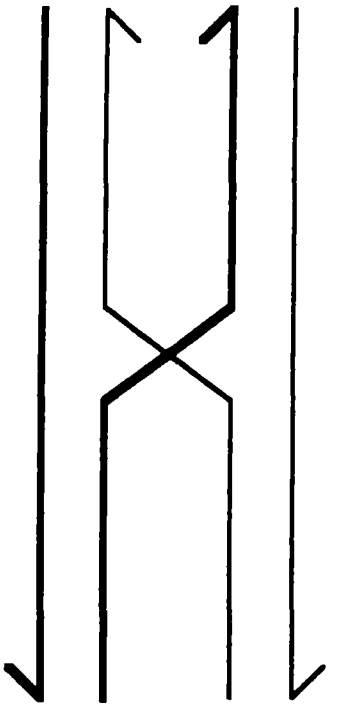

FIGURE 2. Schematic diagram of the Holliday junction, a four-stranded DNA molecule that is a key intermediate in recombination.

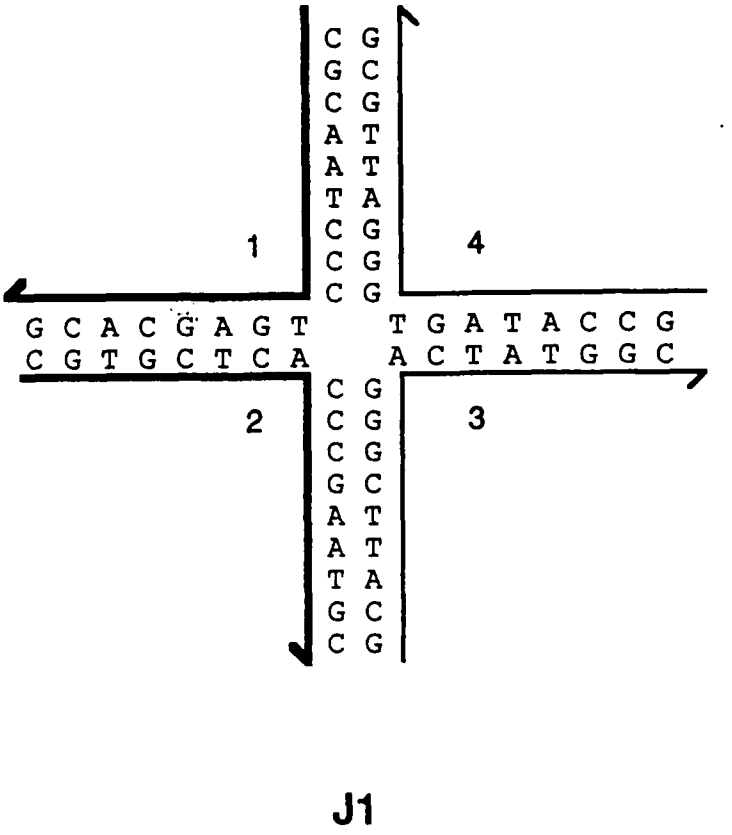

FIGURE 3. J1, a stable model for the Holliday junction.

using the Fenton reaction give a clear answer to the question of the symmetry of the Holliday junction model J1.

Shown in Figure 4 are the cleavage patterns of the four strands of J1. For each strand, the upper pattern is for the strand complexed with an exactly complementary strand in a normal duplex, as a control. The lower cleavage pattern for each strand was produced with that strand in the four-way junction structure (Figure 3). Since each strand of J1 is 16 nucleotides long, positions 8 and 9 flank the four-way junction region. It is evident from Figure 4 that iron(II) EDTA-induced cleavage gives information on each nucleotide of J1. While the cleavage patterns of the strands in the duplex controls are rather uniform, clear differences in cleavage patterns between strands are evident in the junction samples.

Although a detailed analysis of the intensity of each peak in the cleavage pattern might be useful, the overall symmetry of the set of four cleavage patterns gives important information on its own. Strands 2 and 4 are cleaved at very low rates at positions 8 and 9, located at the junction, while for strands 1 and 3 these positions are cleaved at nearly normal rates (compare duplex and junction patterns for each strand). The cleavage patterns of strands 2 and 4 are therefore related by symmetry, as are the patterns of strands 1 and 3, but all four strands do not have the *same*

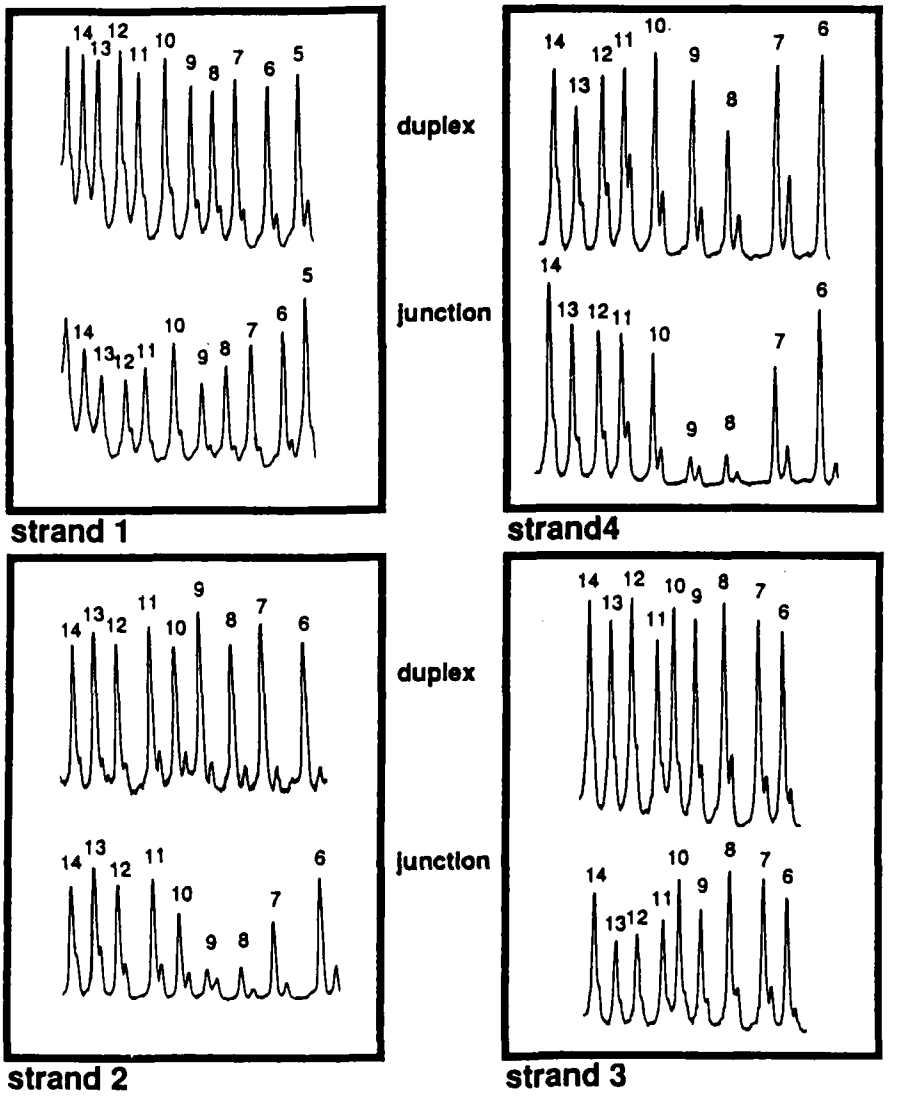

FIGURE 4. Cleavage patterns of J1 induced by iron(II) EDTA/hydrogen peroxide/ascorbate. Each of the four strands of J1 was cleaved while base-paired with its exact complementary strand (duplex) as a control, or while incorporated into the four-way junction (junction). Positions 8 and 9 flank the junction region.

pattern. This twofold symmetry immediately rules out structures like that shown in Figure 3, while it is consistent with structures of the type depicted in Figure 2. We therefore assign strands 2 and 4 of J1 as crossing over in some way, and strands 1 and 3 as adopting a rather normal helical configuration.

An unexpected corollary of these observations is that J1 exists primarily in the structure as discussed above; that is, strands 1 and 3 do not adopt crossover positions, and strands 2 and 4 are not helical. In other experiments, we[19] and others[20,21] have found that the sequence surrounding the junction determines which strands cross

over, and which are helical. A key observation is that that the symmetry of the cleavage pattern directly gives the symmetry of the molecule.

CONCLUSIONS

I have discussed experimental evidence that the Fenton reaction of iron(II) EDTA with hydrogen peroxide cleaves DNA through the agency of a diffusible species, which likely is the hydroxyl radical. This cleavage chemistry can provide useful information on the structures of DNA and DNA-protein complexes. Our work on a model of the four-stranded Holliday junction demonstrates that this structure has inherent pseudo-twofold symmetry, and that particular strands adopt particular configurations (helical or crossed over). Simply observing the symmetry properties of the cleavage pattern of the Holliday junction gives important insights into the structural properties of this intriguing molecule.

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