Charge dependence of Fe(II)-catalyzed DNA cleavage

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ABSTRACT

The effect of charge of the Fe(II) reagent used to induce DNA strand cleavage reactions in the presence of a source of reducing equivalents is investigated using two oligonucleotide models. The first consists of the two strands dA₂₀ and dT₂₀, and an equimolar complex between them. The second is a short four-arm branched DNA complex composed of four 16-mer strands. In the former case, cleavage of the 1:1 complex by three reagents with different formal charge, Fe(II)·EDTA²⁻, Fe(II)·EDDA and Fe²⁺, is comparable in rate to that of the individual dT₂₀ and the dA₂₀ strands. While the three reagents show similar cleavage rates for the duplex and single stranded molecules, they give distinctive cutting patterns in the DNA tetramer, consistent with the presence of a site of excess negative charge at the branch point. Scission induced by Fe(II)·EDTA²⁻ shows lower reactivity at the branch site relative to duplex controls, whereas Fe(II)²⁺ shows enhanced reactivity. Formally neutral Fe(II)·EDDA shows weak loss of cutting reactivity at the branch. The position of attack by Fe(II)²⁺ in the branched tetramer is shifted with respect to those of Fe(II)·EDTA²⁻ or Fe(II)·EDDA; a slower migrating species is also detected in the scission of dA₂₀·dT₂₀ duplex by Fe(II) reaction. These results suggest that the Fe(II)²⁺ reaction proceeds by a different mechanism from the other agents. The difference in cutting profiles induced by the neutral and negatively charged chelated complexes is consistent with a local electrostatic repulsion of a negatively charged source of radicals, not a positively charged one.

INTRODUCTION

Footprinting experiments based on enzymatic (1,2) and chemical reagents (3–8) are widely used to define the structures of DNA and RNA and their complexes with ligands. Relatively little is known about the detailed mechanisms underlying strand scission by the latter. A number of chemically reactive probes rely on active radical intermediates released by Fe(II) in the presence of an electron donor, probably via a Fenton reaction (9). This reaction is also thought to play a major role in mediating cell damage by oxidants such as H₂O₂ (10). In the presence of

oxygen or peroxide, oxygen radical species are generated as intermediates in the oxidation of a reduced metal (10), as in

Of the products of the reaction, hydroxyl radical is the most probable source of chemical damage. Since the hydroxyl radical is short-lived, damage produced by the reaction is amplified by a source of electrons, thiols or ascorbate for example, which reoxidize the metal so that it functions catalytically. In selecting Fe(II)·EDTA²⁻ as a probe for DNA, Tullius and Dombrowski (5,6) argue that radical formation is likely to occur in the solvent, because the negatively charged complex should be repelled from DNA. In this view, the hydroxyl radicals diffuse to sites on the DNA from the surrounding solvent with relatively little effect from the DNA itself, a desirable feature in a footprinting reagent. If this is so, one anticipates that the reagent should also cleave single stranded DNA; the report by Lohman's group (11) that single strands are refractory to Fe(II)·EDTA²⁻ induced scission is thus surprising. They propose an alternative mechanism in which a positively charged intermediate, conceivably ferryl radical [Fe(IV)O]⁺, interacts preferentially with duplex DNA in order to explain the lack of reactivity of single strands. In support of this idea, they report that increasing salt concentration reduces the cleavage rate, despite the fact that the electrostatic repulsion between the negative EDTA complex and the duplex should be diminished in high salt, favoring attack by this reagent.

Our purpose here is to test the effect of charge of the Fe(II) compound used to generate radicals on the rate and products of DNA scission reactions. We find that rates of single strand cleavage are not sensitive to the charge of three reagents tested: Fe(II)·EDTA²⁻, Fe(II)·EDDA and Fe(II)²⁺, monitored at two different salt concentrations, or different from those of duplex cleavage. A slower migrating species is also detected in the scission of duplex DNA by Fe(II) reaction, and not in the other cases. This species contains both the A and T strands, since label on either strand is seen at this position (data for the labeled A strand not shown.)

To gain further insight into the scission reaction, we have carried out a comparison of the rate of scission of a branched DNA tetramer with that of a set of control duplexes. We find that the charge of the reagent that generates radicals influences the kinetics of cleavage at the base pairs flanking the branch. Whereas Fe(II)·EDTA²⁻ leads to protection of positions 2:8,9 and 4:8,9 (Figure 1) from scission relative to other positions in the duplex controls (12), in the case of Fe(II)·EDDA this protection is reduced. On the other hand, Fe(II)²⁺ shows

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Figure 1. The sequence and structure of the immobile branched junction J1. The upper panels show the junction in schematic, while the lower panel is a representation of what the junction might look like if it were formed from 10.5-fold B-DNA. The junction is composed of four strands of DNA, indicated in the upper panels by Arabic numerals. Each strand participates in forming two double helical arms, indicated by Roman numerals. The structural conclusion of previous work (12) is indicated in this figure, by stacking arm I on arm II and arm III on arm IV to form two helical domains. The antiparallel conformer of J1 shown is the one we believe to be the predominant form in solution (22). Our nomenclature indicates strand number in bold-face, with the specific residue number counting from the 5' end denoting the presumed site of cleavage, leaving a fragment actually one unit shorter. For example, cleavage at site 1:9 indicates that the probe has attacked the sugar of residue 9 in strand 1, leaving a major 5'-labeled chain fragment containing 8 nucleosides, with 5'-phosphate and 3'-phosphomonoester termini.

enhanced scission, but at positions 2:7,8 and 4:7,8. Thus, we believe that the Fe(II)^{2+} reaction may occur on the DNA, and differs in mechanism from the other two. The $\text{Fe(II)} \cdot \text{EDTA}^{2-}$ or $\text{Fe(II)} \cdot \text{EDDA}$ results are consistent with repulsion of a negatively charged species by the local excess of negative phosphate charge we have postulated to exist at the branch site (13). On the other hand the Fe(II)^{2+} reaction behaves as if a positive intermediate is involved.

MATERIALS AND METHODS

Nucleic acids

The dA_{20} and dT_{20} strands were purchased from Pharmacia, and used without further purification. The 16 mer DNA strands used in experiments with branched molecules are synthesized on an ABI 380B automated synthesizer, using standard phosphoramidite

chemistry (14). Strands are purified after removal from the synthetic support and deprotection by preparative HPLC on a Du Pont Zorbax Bio Series oligonucleotide column, using a gradient of NaCl in a solvent system containing 20% acetonitrile and 80% 0.02 M sodium phosphate. Fractions from the major peak are collected, concentrated, desalted and lyophilized.

Phosphorylation and strand purification

20 μg of a specific DNA strand is dissolved in 10 μL of a solution containing 66 mM Tris·HCl, 1 mM spermidine, 10 mM MgCl_2 , 15 mM dithiothreitol (DTT), 0.2 mg/ml nuclease free bovine serum albumin (BSA) from BRL, and mixed with 5 μL of 1.25 mM [$\gamma\text{-}^{32}\text{P}$] ATP (10 mCi/ml) and 2 units of T4 polynucleotide kinase (Boehringer) for 15 minutes at 37°C. This reaction is quenched by adding 1 mM of cold ATP and 1 unit of T4 polynucleotide kinase, and stopped by freezing in dry ice. The mixture is rapidly heated for 5 minutes at 70°C, cooled to room temperature, and loaded on a 20% denaturing polyacrylamide gel. This gel is run at 2000 V (ca. 50 V/cm) for 3 hrs at room temperature, exposed briefly to X-ray film (Kodak XOMat AR), the band corresponding to 16 mers is excised, and soaked overnight at 37°C in 0.5 ml of buffer (0.5 M ammonium acetate, 1 mM EDTA). This material is centrifuged for 5 minutes at room temperature in a microfuge, and the gel re-extracted with 0.1 ml of the same buffer, precipitated twice with ethanol, and lyophilized.

Annealing Reactions

Junctions are formed by annealing a stoichiometric mix of strands at 16 μM concentration in 50 mM Tris·HCl, pH 7.5, with 10 mM MgCl_2 . An Eppendorf tube containing the solution is immersed in boiling water for two minutes, cooled slowly to room temperature, and finally chilled to 4°C. Double stranded controls are formed similarly, adding a stoichiometric concentration of cold strand complementary to the labeled junction strand. In the case of dA_{20} and dT_{20} , a single band corresponding to the 1:1 duplex $\text{dA}_{20} \cdot \text{dT}_{20}$ forms on mixing the two strands when these species are run on a native polyacrylamide gel at 4°C. The duplex migrates more slowly than the dT_{20} strand, but faster than the triple helix, $\text{dA}_{20} \cdot (\text{dT}_{20})_2$, formed in the presence of excess dT_{20} , as detected by autoradiography of labeled dT_{20} and dA_{20} strands and these complexes.

Gel electrophoresis

Native polyacrylamide gel electrophoresis. Native gels (20%) are run at 4°C for 24 hrs at 125 V. The buffer system contains 40 mM Tris, pH 8.0, 20 mM acetic acids, 2 mM EDTA and 50 mM NaCl. The gels are exposed for 60 min without an intensifying screen. **Denaturing gels.** The samples of cleavage reactions are taken up in formamide loading buffer, heated briefly to 90°C, cooled, then run on a denaturing polyacrylamide gel (20%) for 3 hrs at 2000 V (ca 50 V/cm) and 40°C. No dyes are added in these runs. The gel is dried immediately on a vacuum drying apparatus (Hoefler), and exposed at room temperature to film without an intensifier screen.

Hydroxyl radical cleavage reactions

The procedure described by Churchill et al. (12) for $\text{Fe(II)} \cdot \text{EDTA}$ cutting of DNA junctions is used, except that the ratio of labeled to unlabeled strands is kept nearly stoichiometric. $\text{Fe(II)} \cdot \text{EDTA}^{2-}$ cleavage of the duplexes and junctions is carried out at 4°C for 2 min at the following final concentrations

Figure 2. Fe(II)-catalyzed radical cleavage of duplex DNA and unstructured single-stranded DNA. Lane A is 5'-³²P end-labeled d(pT)₂₀ untreated control. Lanes B–G are treated in 10 mM Tris·HCl (pH7.5) plus 50 mM NaCl; lanes H–M in 10 mM Tris·HCl (pH7.5) plus 200 mM NaCl. Lanes B, D, F, H, J and L are duplex d(pA)₂₀·d(pT)₂₀ in which the d(pT)₂₀ has been labeled at its 5' end with ³²P; lanes C, E, G, I, K and M are 5'-³²P end-labeled d(pT)₂₀. Lanes B, C, H and I are treated with 100 μM Fe(II), 200 μM EDTA, 0.15% H₂O₂ and 1 mM ascorbate. Lanes D, E, J and K are treated with 100 μM Fe(II), 400 μM EDDA, 0.15% H₂O₂ and 1 mM ascorbate. Lanes F, G, L and M are treated with 100 μM Fe(II), 0.15% H₂O₂ and 1 mM ascorbate. An autoradiograph of the gel is shown.

of the reagents (100 μM iron (II)/200 μM EDTA/0.15% H₂O₂/1 mM ascorbate); Fe(II)·EDDA cleavage (100 μM iron/400 μM EDDA/0.15% H₂O₂/1 mM ascorbate); Fe(II) cleavage (100 mM iron/0.15% H₂O₂/1 mM ascorbate). These reactions are stopped by addition of thiourea. Dried samples are loaded onto a 20% sequencing gel following ethanol precipitation.

Densitometry

Autoradiographs are scanned on a Hoefer GS300 densitometer, without baseline corrections.

RESULTS

Scission of dA₂₀, dT₂₀ and their 1:1 complex

Before investigating the reactivity of this system, it is necessary to establish that the strands associate under the experimental conditions used. As described in Methods, comparison of the mobility of dT₂₀ with an equimolar annealed mixture of 1:1

dA₂₀/dT₂₀ shows that the latter runs as a single species on native polyacrylamide gels, with lower mobility than the single stranded dT₂₀, while the triple helix, dA₂₀·(dT₂₀)₂ is slower still (data not shown). Under the conditions of the cutting experiments, the mobility of the 1:1 mixture corresponds to that of the dA₂₀·dT₂₀ duplex on a native gel, with no trace of label at the position corresponding to triple helix. Figure 2 shows the results of a cleavage experiment on dA₂₀, dT₂₀ and their 1:1 complex, using three Fe(II) reagents of different charge. Data at two different salt concentrations, 50 mM and 200 mM NaCl, are shown. Jesewska et al (11) report that cleavage of dA₇₀·dT₇₀ by Fe(II)·EDTA²⁻ is retarded by higher salt concentrations, and no such effect is seen in 20 mers, which should be less sensitive to electrostatic effects. Even more striking is the fact that the Fe(II) reagent in the scission of duplexes produces a new species not observed with the other reagents or single stranded DNA: a new band appears at a position migrating more slowly than the 20 mer strands on a denaturing gel (see Figure 2). While

it is likely that this band is the product of a radical recombination process, in which the two strands are cross-linked, we have not characterized it in detail.

Differential effects on scission of a branched tetramer

Footprinting experiments have provided much of the fundamental information available concerning the conformation of DNA junctions, stable branched DNAs formed from oligonucleotide strands. $\text{Fe(II)} \cdot \text{EDTA}^{2-}$ footprinting of J1 reveals a pattern of protection from cutting at the base pairs flanking the branch on two strands of this tetramer, 2 and 4, while the other two strands have profiles very close to their profiles in fully duplex controls (12). This experiment is repeated for comparison in the top four panels of Figure 3, each corresponding to labeling one of the strands. Differences between the duplex and tetramer profiles can be seen at positions 2:8,9 and 4:8,9; the scission at these sites is reduced compared to the same sequence in duplex. This observation has led to the suggestion that the tetramer is organized into two adjacent duplexes (12), strands 2 and 4 forming the cross-

over strands while the other two are relatively unperturbed from their normal duplex environment. While indirect, this inference is consistent with electrophoretic mobility measurements on similar junctions (15,16), a transient electric birefringence measurement (17) and with results of a fluorescence energy transfer experiment on junctions labeled with pairs of donor-acceptor fluorophores on the ends of different arms (18). Our interpretation of this protection has been that positions 8 and 9 in strands 2 and 4 are sterically excluded from radicals released in the solvent (12). Use of the reagents with different charges suggests that a significant electrostatic effect is involved as well.

The second set of panels in Figure 3 corresponds to a similar experiment to that in the top, but using $\text{Fe(II)} \cdot \text{EDDA}$ rather than EDTA as the iron reagent. The resulting pattern shows diminished protection from scission at the same positions (2:8,9 and 4:8,9) as in the top panel. Finally, the last set of panels in Figure 3 corresponds to use of Fe(II) as the reagent. Instead of protection of sites near the branch, enhanced reactivity is observed. The enhancement now occurs at 2:7,8 and 4:7,8 rather than the positions 2:8,9 and 4:8,9 noted above.

Figure 3. Densitometric traces of the patterns of scission of J1 and control duplexes by different reagents. Each column of this figure corresponds to labeling the numbered strand of J1 as shown in Figure 1, and contains three panels. Panel A shows profiles of scission induced by $\text{Fe(II)} \cdot \text{EDTA}$; panel B those for $\text{Fe(II)} \cdot \text{EDDA}$; panel C those for Fe(II) alone. Each panel contains two scans, one corresponding to the strand labeled in the tetramer (designated J), and the other with the strand labeled in linear duplex formed by pairing it with its fully complementary strand (designated ds). The branch point lies between positions 8 and 9 on each strand. Filled circles are used to indicate sites of *protection* in the junction relative to duplexes, and black bands sites of *enhancement* in the junction.

DISCUSSION

These observations provide new information about the DNA scission reaction induced by radicals generated by oxidation of Fe(II) in reagents of different charge. With all three reagents tested, Fe(II)·EDTA²⁻, Fe(II)·EDDA and Fe(II)²⁺, the rate of scission of duplex dA₂₀·dT₂₀ is close to that of the unpaired single strands in both 50 mM and 200 mM NaCl, an observation at variance with the result of a similar experiment on dA₇₀·dT₇₀ (11). Since the lifetime of OH radicals in water is short, it is not unreasonable that the local electrostatic environment of the latter duplex might exhibit polyelectrolyte effects not present in the shorter chains we use here. Neither the charge of the Fe(II) reagent nor the salt concentration exerts a strong effect on the rates of scission of single strands or duplexes in our case.

By contrast, in the branched tetramer J1 the charge of the iron reagent plays a major role. The protection from scission of the bases flanking the putative cross-over strands in J1 observed with Fe(II)·EDTA²⁻ (12) and Fe(II)·EDDA suggests a simple model in which two factors determine the degree of inhibition observed. The first is the proximity of two phosphates at the branch point (12), which presents a local excess of negative charge in this vicinity (13). The second factor corresponds to a steric effect, exerted perhaps by crowding of groups in the two cross-over strands at the branch. Thus we assume that the extent of protection observed with neutral Fe(II)·EDDA can be attributed to the second factor, while the increased effect seen in the case of the Fe(II)·EDTA²⁻ reflects both electrostatic repulsion and steric exclusion. According to this model, the major protection from Fe(II)·EDTA²⁻ induced cutting at the branch then represents electrostatic repulsion of the radical initiating species from the DNA.

The different behavior of Fe(II) suggests that another mechanism is involved in this case, since the sites differentially affected shift in the branched tetramer, and a higher molecular weight species than intact strand appears in the scission of dA₂₀·dT₂₀ duplex. It is interesting that both the positively charged (OP)₂Cu(I) reagent (7,8,19) and Dervan's reagent, MPE·Fe(II), in which the Fe(II) group is tethered via EDTA to a positively charged methidium ring system (3,4), show enhanced cutting activity near the branch point in J1 and other junctions (13,20,21). However, the positions that show strongest differential effects between duplex and junction in scission due to these two reagents differ from each other as well as from those seen with Fe(II)²⁺ as one might expect.

None of the observations we have made implicates a positively charged intermediate in the mechanism of cleavage by Fe(II)·EDTA²⁻ in short DNA chains at least. Instead the response of the branched tetramer to the Fe(II)·EDTA²⁻ reagent is consistent with repulsion of a negatively charged source of short lived hydroxyl radicals from the local environment of a negatively charged target.

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