Iron(II)–Ethylenediaminetetraacetic Acid Catalyzed Cleavage of DNA Is Highly Specific for Duplex DNA†

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ABSTRACT: We show that iron(II)–EDTA-catalyzed cleavage of duplex DNA is much more rapid than cleavage of single-stranded DNA that does not form intramolecular base pairs. Comparisons of the extent of cleavage of the fully single-stranded oligonucleotides $d(pT)_{70}$, $d(pA)_{70}$, and $d(pC)_{35}$ and the duplex DNA $d(pT)_{70}$·$d(pA)_{70}$ indicate that the extent of cleavage increases significantly upon formation of the duplex structure. These observations indicate that accessibility of the DNA sugars to the presumed cleaving agent, hydroxyl radical, is not the major determinant for cleavage and that most likely a direct interaction between Fe(II) and the DNA is required. As a result, the interpretation of DNA cleavage experiments performed with this reagent to obtain detailed structural information should be pursued with caution until the mechanism of the cleavage reaction is better understood.

There is great interest in the use of chemical and enzymological reagents that are capable of cleaving the sugar–phosphate backbone of DNA in order to detect complexes between proteins and nucleic acids (Galas & Schmitz, 1978; Tullius & Dombroski, 1986; Brenowitz et al., 1986). These "footprinting" techniques have also been applied to examine the interaction of small ligands, such as dyes and drugs, with duplex DNA (Hertzberg & Dervan, 1984) and RNA (Kean et al., 1985). Most enzymological probes have limited resolution due to their size and resulting inability to access sites that are directly adjacent to the binding site of interest. As a result, much smaller chemical probes have been used recently to overcome this problem and obtain a higher resolution "footprint" of the nucleic acid binding site (Tullius & Dombroski, 1986; Hertzberg & Dervan, 1984; Tullius, 1987; Tullius et al., 1987; Spassky & Sigman, 1985). These chemical agents have also been used to study some structural characteristics of duplex DNA in solution (Tullius & Dombroski, 1985; Burkhoff & Tullius, 1987) including some unusual DNA structures (Churchill et al., 1988; Chen et al., 1988).

Iron(II)–EDTA has been used as a chemical reagent to cleave duplex DNA; the active species are thought to be hydroxyl radicals that are formed through the reduction of hydrogen peroxide, and these can abstract a hydrogen atom from the deoxyribose sugar of the DNA backbone, resulting in strand cleavage (Hertzberg & Dervan, 1984; Tullius & Dombroski, 1985). This reagent seems ideal for these purposes, since it is very small and the overall negative charge of the $[Fe(II)–EDTA]^{2-}$ complex would seem to preclude its direct interaction with the nucleic acid. It has been suggested that in the case of free iron(II)–EDTA the hydroxyl radicals that are formed in solution diffuse into the vicinity of the DNA and effect strand cleavage (Tullius, 1987; Tullius & Dombroski, 1985). Quite high-resolution footprints have been obtained with iron(II)–EDTA for the binding of bacteriophage λ cro and cI proteins to their specific binding sites on the O_R1 operator (Tullius & Dombroski, 1986). We have attempted to use Fe(II)–EDTA for cleavage studies with single-stranded DNA that does not form intramolecular or intermolecular base pairs. However, we have found that the cleavage of single-stranded DNA catalyzed by Fe(II)–EDTA is surprisingly slow when compared to the cleavage of duplex DNA. These results bear on the mechanism by which cleavage may occur, as well as the interpretation of experiments performed with this cleavage reagent.

Materials and Methods

Reagents and Buffers. All chemicals were of reagent grade; all solutions were made with distilled and deionized (Milli-Q) water. The standard buffer was buffer T, which is 10 mM Tris [tris(hydroxymethyl)aminomethane], pH 8.1, and 0.1 mM Na_3EDTA (ethylenediaminetetraacetic acid). $(NH_4)_2Fe(SO_4)_2·6H_2O$ and EDTA (disodium salt, dihydrate) (Gold label) were from Aldrich, L-ascorbate (sodium salt) and thiourea were from Sigma, and hydrogen peroxide was from Baker.

Oligodeoxyribonucleotides. Oligonucleotides were synthesized on an Applied Biosystems 380 B automated synthesizer, with phosphoramidite chemistry, and were purified by HPLC on a Nucleogen DEAE 60-7 column (Rainin) as previously described (Lohman & Bujalowski, 1988). The oligonucleotides were labeled at their 5′ ends by reaction with T4 polynucleotide kinase and [γ-^{32}P]ATP (Maniatis et al., 1982). The $d(pT)_{70}$·$d(pA)_{70}$ duplex was formed by mixing [in 10 mM Tris-HCl (pH 7.4), 50 mM KCl] 5′-^{32}P end-labeled $d(pT)_{70}$ [3.2×10^{-6} M (molecules)] with a slight excess of unlabeled $d(pA)_{70}$ so that all of the $d(pT)_{70}$ was part of the duplex.

Iron(II)–EDTA-Catalyzed Cleavage of DNA. The procedure for Fe(II)–EDTA-catalyzed cleavage of the DNA was that of Tullius et al. (1987). We used three different concentrations of cleavage reagents [w $(NH_4)_2Fe(SO_4)_2·6H_2O$–x EDTA–y H_2O_2–z sodium ascorbate] in our studies. The final concentrations of the reagents in the three cleavage reactions

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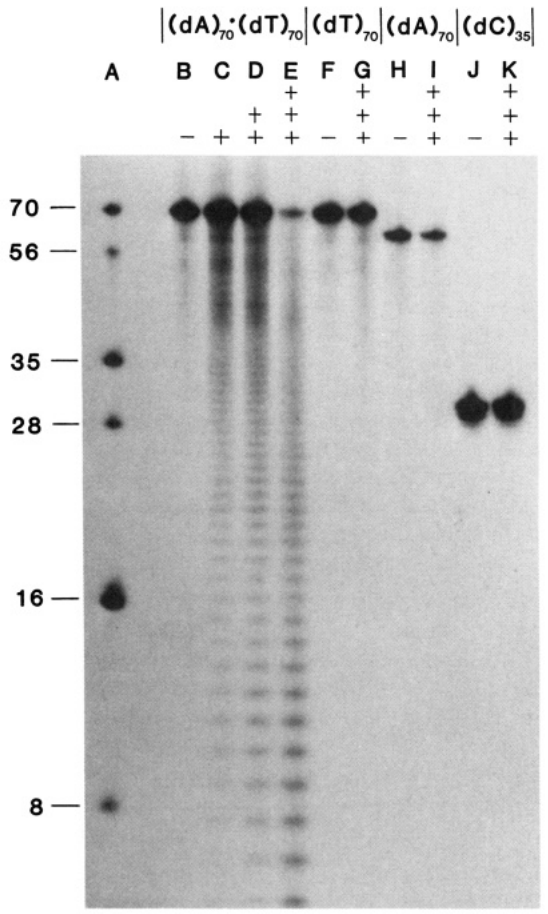

FIGURE 1: Fe(II)–EDTA-catalyzed cleavage of duplex DNA is much more rapid than cleavage of unstructured single-stranded DNA. (Lane A) Oligothymidylate molecular weight standards; (lanes B–E) duplex $d(pA)_{70}$·$d(pT)_{70}$ in which the $d(pT)_{70}$ has been labeled at its 5′ end with ^{32}P; (lanes F and G) 5′-^{32}P end-labeled $d(pT)_{70}$; (lanes H and I) 5′-^{32}P end-labeled $d(pA)_{70}$; (lanes J and K) 5′-^{32}P end-labeled $d(pC)_{35}$. Lanes marked with (−) (lanes B, F, H, and J) were untreated. Lane C (+) was treated with 20 μM Fe(II), 40 μM EDTA, 0.06% H_2O_2, and 1 mM ascorbate. Lane D (++) was treated with 50 μM Fe(II), 100 μM EDTA, 0.15% H_2O_2, and 1 mM ascorbate. Lanes marked with (+++) (lanes E, G, I, and K) were treated with 200 μM Fe(II), 400 μM EDTA, 0.5% H_2O_2, and 1 mM ascorbate.

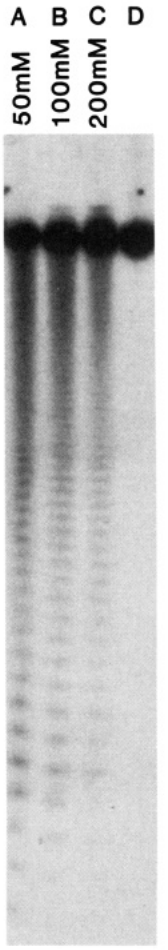

FIGURE 2: Fe(II)–EDTA-catalyzed cleavage of duplex DNA decreases with increasing KCl concentration. Samples of duplex DNA [d-$(pA)_{70}$·$d(pT)_{70}$] in which the $d(pT)_{70}$ was labeled at its 5′ end with ^{32}P were treated with 200 μM Fe(II), 400 μM EDTA, 0.5% H_2O_2, and 1 mM ascorbate in 10 mM Tris-HCl (pH 7) plus the indicated KCl concentration. Electrophoresis was through 15% polyacrylamide in the presence of 7 M urea. (Lane A) 50 mM KCl + Fe(II)–EDTA; (lane B) 100 mM KCl + Fe(II)–EDTA; (lane C) 200 mM KCl + Fe(II)–EDTA; (lane D) no Fe(II)–EDTA.

were (*w*:*x*:*y*:*z*) 20 μM:40 μM:0.06%:1 mM, 50 μM:100 μM:0.15%:1 mM, and 200 μM:400 μM:0.5%:1 mM. The oligodeoxyribonucleotides were prepared for the cleavage reaction by diluting the radioactively labeled DNA sample to a volume of 70 μL in 10 mM Tris-HCl (pH 7.0) at ~22 °C. The cleavage reactions were initiated as described by Tullius et al. (1987) by adding 10 μL each of the premixed Fe(II)–EDTA stock, the H_2O_2 stock, and the ascorbate stock. The approximate final concentrations of the oligonucleotides, in a final volume of 100 μL, were 2.0×10^{-8} M $d(pT)_{70}$, 2×10^{-8} M $d(pA)_{70}$, 1.5×10^{-8} M $d(pC)_{35}$, and 4.6×10^{-8} M d-$(pT)_{70}$·$d(pA)_{70}$ duplex. The reactions were incubated for 90 s at ~22 °C and quenched by the addition of 0.1 M thiourea (10 μL), 3 M sodium acetate (25 μL), and absolute ethanol (750 μL) (Tullius et al., 1987). The DNA was precipitated at −70 °C, the pellet was washed with 70% ethanol and dried in a Speed-Vac concentrator, and the pellet was dissolved in a formamide–dye mixture (Maniatis et al., 1982). Electrophoresis was performed at 400 V for 4–5 h through a denaturing polyacrylamide gel [15% acrylamide, 0.73% *N*,*N*′-methylenebis(acrylamide), 7 M urea]. Gels were dried onto filter paper and viewed by autoradiography using preflashed XAR-5 film (Kodak).

Results and Discussion

Iron(II)–EDTA-Catalyzed Cleavage of DNA Is Much More Rapid for Duplex Than for Single-Stranded DNA. We were interested in using iron(II)–EDTA to attempt to obtain a high-resolution footprint of proteins that bind to single-stranded DNA, e.g., the *Escherichia coli* SSB protein, in which the ss DNA wraps around the SSB tetramer (Lohman et al., 1988). However, during the course of these studies, we observed that the cleavage of ss DNA catalyzed by this reagent is quite slow compared to cleavage of duplex DNA. This is shown quite dramatically in Figure 1, where we directly compare the ability of iron(II)–EDTA to catalyze the cleavage of three single-stranded DNA oligonucleotides, $d(pT)_{70}$, d-$(pA)_{70}$, and $d(pC)_{35}$, with its ability to cleave the duplex formed between $d(pT)_{70}$ and $d(pA)_{70}$. Lanes C–E show the results of treatment of the DNA duplex with three different concentrations of cleavage reagents, each possessing increasing cutting ability. The conditions used were as follows: (lane C) 20 μM Fe(II), 40 μM EDTA, 0.06% H_2O_2, and 1 mM ascorbate; (lane D) 50 μM Fe(II), 100 μM EDTA, 0.15% H_2O_2, and 1 mM ascorbate; (lane E) 200 μM Fe(II), 400 μM EDTA, 0.5% H_2O_2, and 1 mM ascorbate. The concentrations of the cleavage reagents used in the experiments in Figure 1 are all higher than those typically used in previous studies of

the cutting of duplex DNA, which are 10 μM Fe(II), 20 μM EDTA, 0.03% H_2O_2, and 1 mM ascorbate (Tullius & Dombroski, 1986; Tullius et al., 1987). The higher concentrations used here increase the rate of the cleavage reaction (Tullius et al., 1987) and were used in an attempt to obtain better cleavage of single-stranded DNA. All three conditions used in the experiments with duplex DNA shown in Figure 1 show definite cutting of the $d(pT)_{70}$·$d(pA)_{70}$ duplex after treatment for 90 s, and the reactions in lanes C–E, which contained increasing reagent concentrations, show a progressive increase in cleavage of the duplex DNA. However, there was no significant cleavage of any of the single-stranded DNA under the same conditions used to cleave the duplex DNA. Lanes G, I, and K show the results after treatment with the highest concentrations of reagents, and these lanes should be compared with lane E for the duplex DNA. We have not yet made a quantitative comparison of cutting rates among the different ss DNA molecules to determine if these rates differ.

On the basis of the proposed mechanism of action of this reagent (Tullius, 1987), the observation that the iron(II)–EDTA-catalyzed cleavage of duplex DNA occurs much more rapidly than that with ss DNA is surprising. It is clear that the relative solvent accessibility of the DNA backbone is not the main determinant for rapid cutting by this reagent, since the backbone of each of the ss DNA molecules is more accessible to solvent than that of the duplex DNA. Even $d(pA)_{70}$, in which the nucleotides can partially stack, is much less susceptible to cleavage than is duplex DNA. Although we do not have a detailed explanation for these observations at this time, these results indicate that one must be cautious in interpreting the iron(II)–EDTA-catalyzed cleavage patterns of nucleic acids, since they reflect more than just solvent accessibility of the nucleic acid. One possibility is that, in addition to the requirement for solvent accessibility, cleavage is enhanced at positions in the backbone that are strained. On the other hand, it is possible that the iron(II)–EDTA complex does not play as passive a role in the cleavage reaction as has been previously considered. It has been suggested that this reagent does not directly interact with the nucleic acid due to its overall net negative charge and that hydroxyl radicals are generated in solution and diffuse to the site of cleavage on the DNA (Tullius, 1987; Tullius et al., 1987). However, it may be possible that some type of exchange reaction between the EDTA and the DNA can occur to deliver the Fe^{2+}, or some other form of Fe, directly to the DNA, thus producing hydroxyl radicals directly at the site of DNA cleavage. Since duplex DNA binds metal ions with higher affinity than ss DNA (Krakauer, 1974; Record, 1975; Record et al., 1978), this may contribute to the more rapid cutting of duplex DNA over ss DNA. In addition, if hydroxyl radicals were generated at a significant distance from the DNA, it seems most likely that they would react with the buffer components (e.g., 10 mM Tris) before ever reaching the DNA.

We have also observed that the extent of iron(II)–EDTA-catalyzed cleavage is diminished as the concentration of KCl in the reaction mixture is increased from 50 to 200 mM as shown in Figure 2. This suggests that at least one step, before the rate-limiting one, involves an interaction between two species of opposite charge (Record et al., 1976; Lohman, 1986), presumably some form of Fe and DNA. Since the hydroxyl radical is uncharged, this effect does not support the view that hydroxyl radicals are formed at a distance from the DNA and diffuse to the site of cleavage. The decrease in cleavage with increasing KCl concentration suggests that the Fe may be interacting with the DNA, at least transiently. The selectivity of the iron(II)–EDTA reagent for duplex DNA resembles that of the 1,10-phenanthroline–cuprous complex, which is thought to directly interact with duplex DNA, thereby generating the hydroxyl radical at the site of cleavage (Sigman et al., 1979; Marshall et al., 1981). In fact, recent work on the oxidation of saturated hydrocarbons catalyzed by Fe(II) and/or Fe(III) suggests that an iron oxenoid (Fe^{V}=O) may be responsible for the cleavage reaction (Barton et al., 1989); this may also be the active species in the DNA cleavage reactions catalyzed by Fe(II)–EDTA (D. H. R. Barton, personal communication).

The results reported here suggest that the extent of cleavage catalyzed by the seemingly simple chemical reagent iron(II)–EDTA at any site along a stretch of DNA reflects more than accessibility of that site to hydroxyl radicals, since the rate of cleavage of duplex DNA is much greater than that for single-stranded nucleic acids. As a result, the detailed mechanism of cleavage catalyzed by iron(II)–EDTA must be better understood if it is to be used as a tool for the study of nucleic acid structure, especially those structures containing a mixture of single-stranded and duplex DNA. In that context, iron(II)–EDTA may be a useful probe for the discrimination of duplex from ss regions of nucleic acid and, hence, could be an important tool for mapping RNA secondary structure if the cleavage chemistry is the same as it is for DNA.

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Articles

UV-Induced Pyrimidine Hydrates in DNA Are Repaired by Bacterial and Mammalian DNA Glycosylase Activities†

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ABSTRACT: *Escherichia coli* endonuclease III and mammalian repair enzymes cleave UV-irradiated DNA at AP sites formed by the removal of cytosine photoproducts by the DNA glycosylase activity of these enzymes. Poly(dG-[^{3}H]dC) was UV irradiated and incubated with purified endonuclease III. ^{3}H-Containing material was released in a fashion consistent with Michaelis-Menten kinetics. This ^{3}H material was determined to be cytosine by chromatography in two independent systems and microderivatization. ^{3}H-Containing material was not released from nonirradiated copolymer. When poly(dA-[^{3}H]dU) was UV irradiated, endonuclease III released ^{3}H-containing material that coeluted with uracil hydrate (6-hydroxy-5,6-dihydrouracil). Similar results are obtained by using extracts of HeLa cells. There results indicate that the modified cytosine residue recognized by endonuclease III and the mammalian enzyme is cytosine hydrate (6-hydroxy-5,6-dihydrocytosine). Once released from DNA through DNA-glycosylase action, the compound eliminates water, reverting to cytosine. This is consistent with the known instability of cytosine hydrate. The repairability of cytosine hydrate in DNA suggests that it is stable in DNA and potentially genotoxic.

Endonuclease III of *Escherichia coli* incises DNA damaged by ionizing and UV radiation and oxidizing agents such as osmium tetraoxide (Radman, 1976; Gates & Linn, 1977; Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984; Weiss & Duker, 1986, 1987; Doetsch et al., 1987). Analogous enzyme activities have been identified in yeast and mammalian cells and tissues [Breimer, 1983; Doetsch et al., 1986; Higgins et al., 1987; Lee et al., 1987; Gossett et al., 1988; reviewed in Wallace (1988)]. These enzymes contain two activities that act sequentially: a DNA glycosylase activity, which releases the modified DNA base, and an AP endonuclease activity, which cleaves the DNA backbone at abasic sites (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984). These enzymes release modified thymine residues from DNA exposed to ionizing radiation or osmium tetraoxide (Breimer, 1983; Doetsch et al., 1986; Higgins et al., 1987). The common structural feature of the base modifications recognized by these enzymes is loss of aromaticity of the thymine ring. Subsequently, it has been proposed that enzymatic release is mediated via a transiminization reaction between the ring open form of the modified thymine residue and an enzyme amino group (Kow & Wallace, 1987; Bailly & Verly, 1987).

In contrast to incision at thymine residues after exposure of DNA to ionizing radiation, endonuclease III and the analogous mammalian enzyme activity incise UV-irradiated DNA at cytosine residues, releasing cytosine-derived material (Doetsch et al., 1986; Weiss & Duker, 1986, 1987). The chemical nature of this repairable modified cytosine residue has not been characterized to date. In the experiments described in the succeeding sections, we used UV-irradiated poly(dG-[^{3}H]dC) to demonstrate that the modified cytosine residue is cytosine photohydrate.

Experimental Procedures

Materials

Enzyme. Endonuclease III was purified from the cloned *E. coli nth* gene (Asahara et al., 1989). The enzyme was stored in a 1 mg/mL solution (100 mM potassium phosphate, pH 6.6, 50% glycerol). Enzyme was diluted for enzyme assays with the following buffer: 50 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 0.5 mg/mL molecular grade BSA (from BRL), and 10% glycerol.

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