Deoxyribonucleic Acid–Protein and Deoxyribonucleic Acid Interstrand Cross-Links Induced in Isolated Chromatin by Hydrogen Peroxide and Ferrous Ethylenediaminetetraacetate Chelates†

Stephen A. Lesko,* Jean-Louis Drocourt, and Shu-Uin Yang

ABSTRACT: DNA–protein and DNA interstrand cross-links were induced in isolated chromatin after treatment with H_2O_2 and ferrous ethylenediaminetetraacetate (EDTA). Retention of DNA on membrane filters after heating of chromatin in a dissociating solvent indicated the presence of a stable linkage between DNA and protein. Treatment of protein-free DNA with H_2O_2/Fe^{2+}-EDTA did not result in enhanced filter retention. Incubation of cross-linked chromatin with proteinase K completely eliminated filter retention. Resistance to S_1 nuclease after a denaturation–renaturation cycle was used to detect DNA interstrand cross-links. Heating the treated chromatin at 45 °C for 16 h and $NaBH_4$ reduction enhanced the extent of interstrand cross-linking. The following data are consistent with, but do not totally prove, the hypothesis that cross-links are induced by hydroxyl radicals generated in Fenton-type reactions: (1) cross-linking was inhibited by hydroxyl radical scavengers; (2) the degree of inhibition of DNA interstrand cross-links correlated very closely with the rate constants of the scavengers for reaction with hydroxyl radicals; (3) cross-linking was eliminated or greatly reduced by catalase; (4) the extent of cross-linking was directly related to the concentration of Fe^{2+}-EDTA. Partial inhibition of cross-linking by superoxide dismutase indicates that superoxide-driven Fenton chemistry is involved. The data indicate that DNA cross-linking may play a role in the manifestation of the biological activity of agents or systems that generate reactive hydroxyl radicals.

It is now recognized that the same reduced oxygen species ($O_2^{-}\cdot$, H_2O_2, OH·)[1] involved in radiation damage (Greenstock, 1981) can be formed in essential metabolic processes (Greenstock, 1981; Fridovich, 1978; Finkelstein et al., 1980) or by the action of cellular enzymes on certain xenobiotics (Greenstock, 1981; Fridovich, 1978; Finkelstein et al., 1980; Goodman & Hochstein, 1977). Ionizing radiation and oxygen radical generating systems have similar actions on the genetic apparatus with respect to DNA strand scission (Repine et al., 1981; Bradley & Erickson, 1981; Lesko et al., 1980, Brawn & Fridovich, 1981) and DNA base alterations (Schellenberg, 1979). DNA–protein cross-links are induced in isolated chromatin (Mee & Adelstein, 1979; Olinski et al., 1981) and in mammalian cells (Mee & Adelstein, 1979; Fornace & Little, 1977) by ionizing radiation, but the complementary study has not been done with an oxygen radical generating system. DNA interstrand cross-links have been reported upon treatment of calf thymus DNA in vitro with H_2O_2 alone or in the presence of $FeCl_2$ (Massie et al., 1972).

Ionizing radiation is both mutagenic and carcinogenic (Cerutti, 1974). Therefore, it is important to determine the nature of the lesions induced in the genetic apparatus by activated oxygen and the species responsible for the event. This investigation represents our first step in an extensive study to compare and an extrapolate the effects of ionizing radiation and oxygen radials at several levels of biological organizations, viz., purified chromatin, isolated nuclei, and intact mammalian cells.

The present study was initiated to determine if the OH· (generated from H_2O_2/Fe^{2+}-EDTA) can induce DNA–protein cross-links or DNA interstrand cross-links in isolated chromatin. The evidence for the presence of OH· in this system is considerable (Lown et al., 1978; Walling, 1975; Tomat & Rigo, 1976; Repine et al., 1981). We have used retention on nitrocellulose filters to assay for the presence of DNA–protein cross-links because it is a very sensitive but simple technique (Strniste & Rall, 1976). A systematic study was undertaken to examine salt conditions so that quantitative data and low background values could be obtained. DNA interstrand cross-linking was also assayed with a sensitive but simple technique that involves using single-strand-specific nuclease S_1 to resolve single- from double-stranded DNA after a denaturation–renaturation cycle (Ben-Hur et al., 1979).

Experimental Procedures

Cell Culture. A fibroblastic cell line, BP6T, established from benzo[*a*]pyrene-transformed Syrian hamster embryo cells (Barrett et al., 1979) was used for preparation of chromatin. Cells were grown in 850-cm^2 roller bottles by using IBR modified Dulbecco's reinforced medium supplemented with 0.22 g of $NaHCO_3$/100 mL and 10% fetal bovine serum. So that chromatin could be labeled, the medium contained 1 μCi/mL of [*methyl*-^{3}H]thymidine (100 μCi/μmol) for 24–36 h.

Isolation of Chromatin and DNA. Chromatin was isolated as described by Pantazis et al. (1979). Nuclei were released by rupturing the cells in a glass–teflon homogenizer in 10 mM KCl–2.5 mM $MgCl_2$–0.1% Triton X-100–10 mM Tris–1 mM DTT, pH 8.1, and then washed 3 times with 105 mM NaCl–25 mM EDTA–10 mM Tris, pH 8. Chromatin was released and swollen by successive washes in 10, 7.5, 5, 2.5, and 1 mM Tris, pH 8, containing 1 mM DTT. The chromatin gel was sheared for 90 s at a setting of 40 with a Virtis 45. The homogenate was centrifuged at 15000*g* to obtain the clarified chromatin supernatant that was extensively dialyzed against 1 mM $NaClO_4$–0.01 mM PMSF. All operations were carried

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[1] Abbreviations: $O_2^{-}\cdot$, superoxide radical anion; OH·, hydroxyl radical; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; Gdn·HCl, guanidine hydrochloride; SLS, sodium lauroyl sarcosinate; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide; *t*-BuOH, *tert*-butyl alcohol.

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out at 4 °C. The sheared, clarified chromatin exhibited the following spectral ratios: $A_{260}/A_{230} = 0.97$; $A_{260}/A_{280} = 1.68$; maximum/minimum = 1.36; $A_{260}/A_{320} = 42$.

DNA was purified from sheared chromatin as described by Saffitz & Caplan (1978) and dialyzed against 1 mM $NaClO_4$. This purified DNA has an $s^0_{20,w}$ of 12.96, which corresponds to a molecular weight of 2×10^6. Its spectral ratios were as follows: $A_{260}/A_{230} = 2.30$; $A_{260}/A_{280} = 1.80$.

The concentration of purified DNA and sheared chromatin in terms of moles of nucleotides was determined by using a molar extinction coefficient of 6.6×10^3 M^{-1} cm^{-1} at 258 nm.

Incubation Conditions. Incubations were carried out at 37 °C in unbuffered 10 mM $NaClO_4$ containing 100 μM chromatin or DNA (in terms of monomeric units), various concentrations of H_2O_2, and various concentrations of $FeSO_4$ or $FeCl_3$ and EDTA at a molar ratio of iron/EDTA of 1.0. Iron salts were freshly prepared just before use. The pH of each reaction mixture was measured immediately before and after incubation and was found to be approximately 4 when the Fe^{2+}-EDTA concentration was 200 μM and H_2O_2/DNA was 10/1. The pH varied from about 3.7–3.8 at 300 μM Fe^{2+}-EDTA to 4.7–4.9 at 50 μM Fe^{2+}-EDTA. To adjust the pH of the reactions, HCl or NaOH was added to the stock EDTA solution.

Filter Assay for DNA–Protein Cross-Linking. [^{3}H]-Chromatin or [^{3}H]DNA samples (125 μL containing about 4 μg of DNA, 10 000–20 000 cpm/μg) were mixed with 3 mL of either 3 M Gdn·HCl (Sigma grade 1)–10 mM Tris–1 mM EDTA–0.25% SLS, pH 7.5 (dissociating conditions), or 20 mM Tris–2 mM EDTA, pH 7.5 (nondissociating conditions). The sample in 3 M Gdn·HCl was heated at 40 °C for 15 min. Three aliquots (1 mL) of each sample were filtered through individual presoaked Millipore filters (type HA) with gentle suction. Each filter was washed with either 30 mL of 20 mM Tris–2 mM EDTA of 3 M Gdn·HCl–0.25% SLS–10 mM Tris–1 mM EDTA at a flow rate of about 3 mL/min. Filters exposed to 3 M Gdn·HCl were washed with 10 mL of 20 mM Tris–2 mM EDTA to remove the residual salt. The filters were oven-dried (70 °C) and placed in scintillation vials with 10 mL of Ready-Solv MP cocktail (Beckman) and counted in a liquid scintillation spectrometer. For determination of total counts per sample, 20 μL of each incubation mixture was spotted on dry filters, dried, and counted.

S_1 Nuclease Assay for DNA Interstrand Cross-Linking. A modification of the procedure described by Ben-Hur et al. (1979) was used. After incubation, catalase (20 μg/mL) was added to all reaction mixtures. Two aliquots containing about 4 μg of DNA were removed. One was placed on ice and the other was incubated at 45 °C for 16 h. Five micromoles of $NaBH_4$ in 0.05 M phosphate buffer, pH 7.8, was added, and then the reactions were placed on ice for 30 min. Acetone (136 μmol) was added to scavenge any remaining $NaBH_4$. The samples were then incubated with proteinase K (200 μg/mL) for 1 h at 37 °C in 0.15% $NaDodSO_4$. After digestion of protein, the DNA samples (0.16 mL) were denatured for 15 min in the dark by addition of 0.5 mL of 0.04 N NaOH–1.19 M NaCl. The samples were then brought to pH 4.5 by addition of 0.7 mL of 0.051 N acetic acid and assayed for resistance to S_1 nuclease. After addition of 5 μg/mL denaturated calf thymus DNA, 5 μmol of zinc acetate, and 1 μmol of mercaptoethanol, the samples were divided into two tubes (0.5 mL each). S_1 nuclease (0.05 IU) was added to one tube. both tubes were incubated for 1 h at 37 °C. After addition of carrier BSA, the reaction was stopped with an equal volume of cold 14% trichloracetic acid. The DNA was allowed to precipitate for 15 min in the cold and then the precipitates were trapped on glass fiber filters. The filters were oven-dried (70 °C), and radioactivity was measured in a scintillation spectrometer after addition of 5 mL of betafluor cocktail (National Diagnostics).

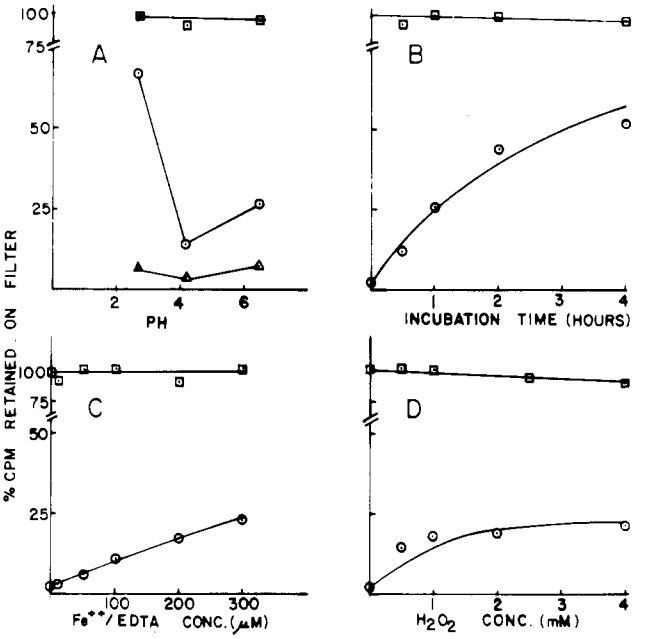

FIGURE 1: DNA–protein cross-linking induced by H_2O_2 and Fe^{2+}-EDTA. Effect of pH, incubation time and H_2O_2 and Fe^{2+}-EDTA concentrations on percentage of [^{3}H]DNA retained on Millipore filters after filtration in dissociating solvent (3 M guanidine hydrochloride–0.25% sodium lauroyl sarcosinate) (○) and in nondissociating solvent (20 mM Tris–2 mM EDTA) (□). Standard incubations were conducted at 37 °C for 1 h at pH ~4 and contained 1 mM H_2O_2, 200 μM Fe^{2+}-EDTA, and 100 μM [^{3}H]chromatin (as DNA nucleotide units). (△) H_2O_2 eliminated from the reaction.

Other Methods. Catalase activity was measured by the procedure of Beers & Sizer (1952). H_2O_2 concentration was determined spectrophotometrically at 240 nm by using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} (Bergmeyer et al., 1970). Total iron and ferrous iron contents of freshly prepared stock solutions used for addition to the incubations were measured colorimetrically with 2,2′-bipyridyl at 522 nm (Snell & Snell, 1949). Superoxide dismutase was completely and irreviersibly inactivated by incubation with H_2O_2 at pH 10 as described by Hodgson & Fridovich (1975).

Results

Filter Retention Assay. The utility of the membrane filter assay for the rapid detection of DNA–protein cross-links in isolated chromatin has been demonstrated by Strniste & Rall (1976). The technique utilizes the differential behavior of protein and DNA on filtration through Millipore filters; free double-stranded DNA passes through the filter whereas protein or DNA–protein complexes are bound to the filter. When chromatin is subjected to dissociating conditions, any DNA that is covalently bound to protein will be retained by the filter. For quantitation it is necessary to show that essentially all the chromatin is retained by the filter under nondissociating conditions. With the low-salt conditions used here, viz., 20 mM Tris–2 mM EDTA (pH 7.5), over 90% of treated or untreated chromatin was consistently retained on the filters (Figure 1). The amount of DNA retained on the filters upon dissociation of cross-linked or untreated chromatin was dependent upon the concentrations of both Gdn·HCl and detergent (Table I). Detergent was required to get low background levels in controls. However, 5 M Gdn·HCl plus SLS

Table I: Effect of Guanidine Hydrochloride and Sodium Lauroyl Sarcosinate on Filter Retention of [^{3}H]Chromatin

% SLS	Gdn·HCl concn (M)	% [^{3}H]chromatin retained on filter: control chromatin	% [^{3}H]chromatin retained on filter: cross-linked chromatin[a]
0.1	3	2.5	6.1
0.25	3	2.3	6.0
0.5	3	1.8	3.9
0	5	22.7	
0.1	5	0.9	3.3
0.25	5	0.6	1.5
0.5	5	0.4	1.4

[a] Irradiated with near-UV light for 1 h in the presence of H_2O_2; H_2O_2/DNA base = 10/1 [see Hartman et al. (1979)].

at 0.1, 0.25, or 0.5% substantially lowered the binding to Millipore filters of DNA–protein complexes (Table I). In this investigation it was found that 3 M Gdn·HCl–0.25% SLS–10 mM Tris–1 mM EDTA, pH 7.5, gave reasonably low background levels for controls (2.2 ± 0.5 or 1.1 ± 0.6% for two different chromatin preparations) while allowing for filter retention of DNA–protein complexes.

Induction of DNA–Protein Cross-Links by H_2O_2/Fe^{2+}-EDTA. Incubation of [*thymine*-^{3}H]chromatin with EDTA-chelated Fe^{2+} and H_2O_2 resulted in the formation of DNA–protein cross-links as measured by enhanced retention of [^{3}H]DNA on Millipore filters in dissociating conditions. The extent of cross-linking was dependent upon pH, incubation time, and Fe^{2+}-EDTA concentration (Figure 1). There was a dramatic increase in the extent of cross-linking when the pH was lowered to around 2.7 which required the presence of H_2O_2 (Figure 1A). The amount of cross-linking was always higher at pH 6.5 than at pH 4. Cross-linking increased with time (Figure 1B) and was directly proportional to the amount of ferrous ion added (Figure 1C). Raising the concentration of H_2O_2 from 0.51 mM (H_2O_2/DNA base = 5/1) to 4.1 mM (H_2O_2/DNA base = 40/1) had only a small effect (an increase from 15 to 22%) on the amount of DNA cross-linked to protein (Figure 1D). There was some day to day variation in the extent of cross-linking. Under the conditions most commonly used 1 h, 200 μM Fe^{2+}-EDTA, H_2O_2/DNA base = 10/1, pH ~4, the percentage of DNA retained on the filters was 18.4 ± 4.5 and 19.0 ± 3.9 for two different chromatin preparations (total of fifteen determinations; each represents the average of three filtrations). Little, if any, cross-linking occurred when H_2O_2 or Fe^{2+}-EDTA was not present in the reaction mixtures (Table II). Filter retention was almost completely eliminated when cross-linked chromatin was incubated with 200 μg/mL proteinase K in 3 M Gdn·HCl–0.25% SLS, pH 7.5 (Table II). Substituting $FeSO_4$ with $FeCl_3$ substantially lowered the amount of DNA–protein cross-linking (Table II). Purified [^{3}H]DNA did not bind to Millipore filters under conditions that induced filter retention of [^{3}H]chromatin (Table II). Elimination of EDTA from the reaction mixtures resulted in complete retention of [^{3}H]chromatin or [^{3}H]DNA on Millipore filters under dissociating conditions (Table II). Therefore, it was necessary to use chelated iron to conduct the cross-linking experiments. EDTA stimulates the autoxidation of ferrous ion in aqueous solution (Caspary et al., 1981). To determine the valence state of the iron added to the incubation mixtures, we measured the content of total iron and ferrous iron in the freshly prepared EDTA-Fe^{2+} stock solutions. Only 45% of the total iron could be detected with 2,2′-bipyridyl, and 61% of this was in the ferrous form. Chelation of iron with EDTA appears to render more than half of this complex inaccessible for the formation of a colored product with 2,2′-bipyridyl since 100% of the iron could be detected in the ferrous form when freshly prepared in water in the absence of EDTA.

Table II: Effect of Reactants and Proteinase K on Filter Retention of [^{3}H]Chromatin and [^{3}H]DNA[a]

exptl conditions	% cpm retained on filter[c]: [^{3}H]chromatin $FeSO_4$	[^{3}H]chromatin $FeCl_3$	[^{3}H]DNA $FeSO_4$
complete system	18.4	5.5	0.8
minus H_2O_2	1.4	2.8	
minus EDTA	104.0		104.0
minus Fe-EDTA	2.0	4.3	
minus Fe	2.6	2.0	
minus Fe-EDTA, H_2O_2	1.6	1.6	0.3
plus proteinase K[b]	0.8		
plus proteinase K minus H_2O_2/Fe-EDTA	0.2		

[a] Incubation conditions: 37 °C, 1 h, 200 μM Fe-EDTA, pH ~4, and H_2O_2/DNA base = 10. [b] Cross-linked [^{3}H]chromatin was incubated at 37 °C for 2 h with proteinase K (200 μg/mL) in 3 M guanidine hydrochloride–0.25% sodium lauroyl sarcosinate, pH 7.5, before filtration. [c] Values represent an average of at least two experiments; each experiment is an average of three filtrations.

Table III: Effect of Hydroxyl Radical Scavengers, Catalase, and Superoxide Dismutase on DNA–Protein Cross-Linking Induced by H_2O_2/Fe^{2+}-EDTA[a]

addition	concn	% inhibition of filter retention: pH 2.7[b]	pH 4	pH 6.5[b]
Me_2SO	0.25 M	68.5	56.8	93.3
Me_2SO	0.56 M		73.2	
Me_2SO	1.12 M		81.4	
t-BuOH	0.25 M	53.5	51.5	92.9
thiourea	0.25 M		66.3	94.1
mannitol	0.25 M	67.4	53.5	71.5
urea	0.25 M	0		
catalase	1 μg/mL			98
inactivated catalase[c]	1 μg/mL			0
superoxide dismutase	1 μg/mL			50
inactivated superoxide[d] dismutase	1 μg/mL			0
BSA	1 μg/mL			0

[a] Incubation conditions: 37 °C, 1 h, 200 μM Fe^{2+}-EDTA, and H_2O_2/DNA base = 10. [b] pH was adjusted by adding HCl or NaOH to stock EDTA solutions. [c] Heated at 100 °C for 15 min. [d] Treated with H_2O_2, pH 10, for 1 h at 23 °C (Hodgson & Fridovich, 1975).

Effect of Specific Enzymes and OH· Scavengers on DNA–Protein Cross-Links. In order to investigate the mechanism of DNA–protein cross-linking by H_2O_2/Fe^{2+}-EDTA, we examined the effects of catalase, superoxide dismutase, and OH· scavengers. Table III shows that effective scavengers of OH· such as Me_2SO, *t*-BuOH, thiourea, and mannitol inhibited filter retention of [^{3}H]chromatin. In contrast, urea (a weak scavenger of OH·) did not decrease filter retention. To examine the effects of catalase and superoxide dismutase, it was necessary to raise the pH of the reactions to 6.5. Table III shows that catalase (1 μg/mL) almost completely inhibited DNA–protein cross-linking at pH 6.5. Superoxide dismutase inhibited filter retention approximately 50%. Superoxide dismutase was found to be free of catalase activity. Heat-inactivated catalase, H_2O_2-inactivated superoxide dismutase, and BSA had no effect on filter retention, thereby eliminating nonspecific effects.

DNA Interstrand Cross-Linking. Incubation of [^{3}H]-chromatin with EDTA-Fe^{2+} and H_2O_2 resulted in interstrand

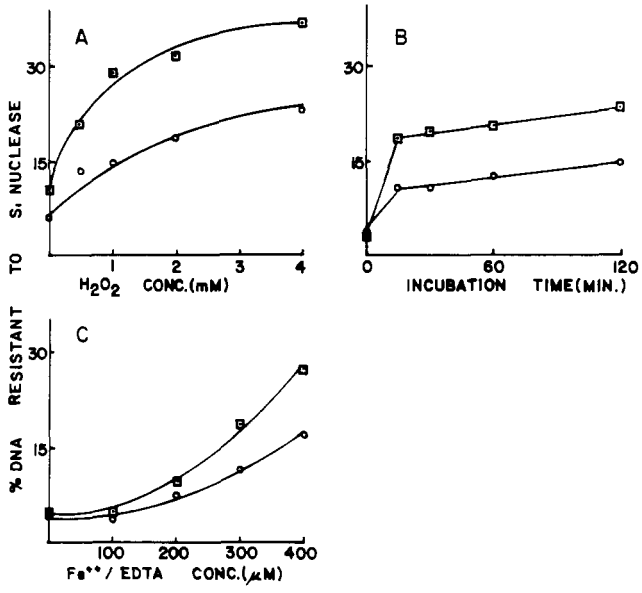

FIGURE 2: DNA interstrand cross-linking induced by H_2O_2 and Fe^{2+}-EDTA. Effect of incubation time and H_2O_2 and Fe^{2+}-EDTA concentrations on percent of [^{3}H]DNA resistant to S_1 nuclease after a denaturation–renaturation cycle. Incubation conditions were identical with those in Figure 1 except that pH was ~6.5. (○) No subsequent heating; (□) heated at 45 °C for 16 h before assay.

crosslinking of DNA as measured by resistance to S_1 nuclease after a denaturation–renaturation cycle. Heating the chromatin at 45 °C for 16 h after H_2O_2/Fe^{2+}-EDTA treatment enhanced the extent of cross-linking (Figure 2). Interstrand cross-linking was also enhanced by treatment of the reacted and/or reacted–heated chromatin with $NaBH_4$ (data not shown). Cross-linking was dependent upon H_2O_2 concentration (Figure 2A) and was directly proportional to the amount of ferrous ion added when the concentration exceeded 100 μM Fe^{2+}-EDTA (Figure 2C). Cross-linking occurs quite rapidly (within 15 min); further incubation from 15 min to 2 h resulted in only a small increase (Figure 2B). There was day to day variation in the extent of cross-linking. Under the conditions most commonly used, 1 h, 200 μM Fe^{2+}-EDTA, H_2O_2/DNA base = 10, and pH ~4, the percentage of DNA resistant to S_1 nuclease was 16.6 ± 1.8%. The value of untreated controls was 4.0 ± 0.6%. As shown in Table IV, Me_2SO, thiourea, *t*-BuOH, and urea inhibited DNA interstrand cross-linking in close relationship to their effectiveness of OH· scavengers. The degree of inhibition was dependent upon the concentration of scavenger. However, at 250 mM these scavengers were less effective in inhibiting DNA interstrand cross-linking compared to DNA–protein cross-linking under identical reaction conditions. Catalase or superoxide dismutase at 1 μg/mL inhibits DNA interstrand cross-linking, and like the OH· scavengers, their effectiveness is diminished and more variable when compared to the DNA–protein cross-linking reaction (data not shown). This may occur because interstrand cross-linking must take place within the DNA helix where the potential for inhibiting the reaction is restricted or limited. The OH· would not be expected to diffuse very far from its site of formation.

Discussion

This investigation has shown that incubation of isolated chromatin with H_2O_2/Fe^{2+}-EDTA results in the induction of DNA–protein cross-links measured by an enhanced retention of [^{3}H]DNA on Millipore filters under dissociating conditions. This technique has been used previously to demonstrate DNA–protein cross-linking in isolated chromatin by UV light (Strniste & Rall, 1976) and γ irradiation (Mee & Adelstein, 1979). We have used 3 M Gdn·HCl–10 mM Tris–1 mM EDTA–0.25% SLS, pH 7.5, as the dissociating solvent because it resulted in consistently lower background values (1–2%) for filter retention of untreated controls compared to those of 3M NaCl–0.5% SLS used in the studies cited above.

Table IV: Effect of Hydroxyl Radical Scavengers on DNA Interstrand Cross-Linking Induced by H_2O_2/Fe^{2+}-EDTA[a]

addition	concn (M)	k_2 (M^{-1} s^{-1})[b]	% inhibition of resistance to nuclease S_1
Me_2SO	0.25	5.8×10^9	32.9
Me_2SO	0.50		85.7
t-BuOH	0.25	5.2×10^8	6.4
t-BuOH	0.50		31.7
thiourea	0.25	4.7×10^9	40.2
thiourea	0.50		63.8

[a] Incubation conditions: 37 °C, 1 h, 200 μM Fe^{2+}-EDTA, pH ~4, and H_2O_2/DNA = 10. [b] k_2 is the second-order rate constant for each reaction with OH· (Dorfman & Adams, 1973).

The evidence that filter retention of [^{3}H]DNA results from a covalent linkage to protein is as follows: (1) incubation of cross-linked chromatin with proteinase K in dissociating solvent eliminates filter binding; (2) conditions that enhance filter retentions of [^{3}H]chromatin do not enhance filter retentions of naked [^{3}H]DNA purified from chromatin; (3) cross-linked chromatin is stable to 3 M Gdn·HCl–0.25% SLS at 40 °C while untreated control chromatin is almost completely dissociated.

H_2O_2/Fe^{2+}-EDTA treatment also induces DNA interstrand cross-links in isolated chromatin as measured by a increase in the fraction of double-stranded DNA resistant to S_1 nuclease following a denaturation–renaturation cycle. This technique has been used previously to measure DNA interstrand cross-linking in *Escherichia coli* treated with psoralen plus 360-nm light (Ben-Hur et al., 1979). In our study, cross-linking was enhanced by further incubation of the treated chromatin at 45 °C for 16 h. This may result from the hydrolysis of modified DNA bases to give to apurinic and apyrimidinic sites that can then initiate interstrand cross-linking. Burnotte & Verly (1972) have reported on the induction of DNA interstrand cross-linking after heating methylated DNA at 50 °C for 4 h. There is evidence that saturation of the thymine double bond (e.g., 5,6-dihydroxydihydrothymine-type products) substantially decreases the stability of the N-glycosidic bond (Dunlap & Cerutti, 1975). Such saturated products are formed when thymine is reacted with H_2O_2/Fe^{2+} and the reactions occur quickly (Schellenberg, 1979), which is analogous to the kinetics of our cross-linking system. Alternately, the enhancement of cross-linking seen upon heating may be due to malondialdehyde, which could result from hydrolysis of DNA base–CH=CHCH type products. Base–propenals are released from treatment of DNA with bleomycin (Giloni et al., 1981). Since cross-linking is also increased by teatment with $NaBH_4$ before exposure to high pH for denaturation, stabilization of a Schiff's base by $NaBH_4$ reduction could be involved (Mirzabekov et al., 1978). Cross-linking by either of the mechanisms postulated above could occur via a Schiff's base since free aldehyde functions would be available in both cases.

Iron/H_2O_2 systems are extrememly complex, allowing for a number of reactions to occur as shown (Walling, 1975):

$$\text{EDTA-Fe}^{2+} + H_2O_2 \rightarrow \text{EDTA-Fe}^{3+} + \text{OH}\cdot + \text{OH}^- \quad (1)$$

$$\text{EDTA-Fe}^{2+} + O_2 \rightarrow \text{EDTA-Fe}^{3+} + O_2^{-}\cdot \quad (2)$$

$$\text{EDTA-Fe}^{3+} + H_2O_2 \rightarrow \text{EDTA-Fe}^{2+} + H^+ + HO_2\cdot \quad (3)$$

$$OH\cdot + H_2O_2 \rightarrow H_2O + HO_2\cdot \quad (4)$$

$$EDTA\text{-}Fe^{3+} + HO_2\cdot \rightarrow EDTA\text{-}Fe^{2+} + O_2 + H^+ \quad (5)$$

$$EDTA\text{-}Fe^{2+} + OH\cdot \rightarrow EDTA\text{-}Fe^{3+} + OH^- \quad (6)$$

Our data are consistent with the hypothesis that DNA–protein and DNA interstrand cross-linking is mediated by OH·; however, due to the complexity of the system, the hypothesis has not been totally proven. The evidence for implicating the OH· is as follows: (1) cross-linking is inhibited by OH· scavengers; (2) the degree of inhibition of interstrand cross-linking correlates closely with the rate constants of the scavengers for reaction with OH·; (3) cross-linking is greatly reduced by catalase; (4) extent of cross-linking is directly related to the amount of Fe^{2+}-EDTA added to the incubations. There are several reasons why $O_2^{-}\cdot$ has not been considered as the active inducer of the cross-linking observed. (1) A number of reports indicate that $O_2^{-}\cdot$ is relatively unreactive with organic substances of biological importance (Fee, 1980); (2) elimination of H_2O_2 from the reaction results in little, if any, enhancement of filter retention above the control value (Table II); (3) the cross-linking reactions are only inhibited about 50% or less by superoxide dismutase. It is postulated that the inhibition observed with superoxide dismutase is due to the requirement of $O_2^{-}\cdot$ to serve as the reducing agent for recycling the metal ion (see reaction 5) after oxidation in reaction 1.

There was a dramatic increase in DNA–protein cross-linking when the pH was lowered to about 2.7. In their study on UV-light-induced DNA–protein cross-linking, Strniste & Rall (1976) attributed such an increase to the chromatin being in a more condensed form such that DNA–protein interactions become more intimate and thus in an advantageous position for cross-linking to occur. In the present experiments, some of the increased amount of cross-linking may also be attributed to more optimum conditions for the Fenton reaction (see reaction 1) at lower pH (Fee, 1980).

In our conditions, there was 100% retention of naked DNA and chromatin on Millipore filters in dissociating conditions when EDTA was eliminated from the reactions. This most likely results from the condensation of DNA molecules into a more compact form or the formation of aggregates. Mandelkern et al. (1981) have reported bundle formation in DNA solutions exposed to 40–120 M $FeCl_3$. Polyvalent cations and trivalent $Co(NH)^{3+}$ have been reported to cause condensation of DNA (Wilson & Bloomfield, 1979; Widom & Baldwin, 1980).

The main objective of this investigation was to determine if OH· can induce DNA–protein and interstrand cross-links in isolated chromatin. The data presented strongly suggest that OH· generated from H_2O_2 in a Fenton-type reaction does indeed induce such lesions. In contrast, it has been recently reported that exogenous H_2O_2 does not induce DNA–protein crosslinks in Chinese hamster V-79 cells (Bradley & Erickson, 1981). However, it did induce DNA strand scission in these cells. DNA–protein cross-links are induced in isolated chromatin (Mee & Adelstein, 1979, Olinski et al., 1981) and in mammalian cells (Mee & Aldestein, 1979; Fornace & Little, 1977) by ionizing radiation and the OH· has been implicated. OH· formed from H_2O_2 in metal-catalyzed reactions have been reported to induce other types of DNA damage in vitro such as DNA strand scission (Repine et al., 1981; Lesko et al., 1980; Brawn & Fridovich, 1981) as well as DNA base and sugar alterations (Schellenberg, 1979; Floyd, 1981). Similar types of damage to the genetic apparatus are induced by ionizing radiation in vitro using purified DNA (Repine et al., 1981; Armel et al., 1977; Remsen & Roti Roti, 1977) and in mammalian cells (Bradley & Erickson, 1981; Millar et al., 1981; Roots & Okada, 1972); the formation of such lesions was inhibited by hydroxyl radical scavengers. Although H_2O_2 and ionizing radiation both produce hydroxyl radicals, only X-ray has been reported to be mutagenic in Chinese hamster V-79 cells. Mutation at the hypoxanthine–guanine phosphoribosyltransferase locus was not detectable when V-79 cells were treated with exogenous H_2O_2 (Bradley & Erickson, 1981). The implications of the above findings are that it still remains a very challenging problem to assign a specific biological effect to a particular reduced oxygen species or to a particular lesion in the genetic apparatus. Careful studies are needed to compare and extrapolate the effects of ionizing radiation and oxygen radicals from purified chromatin, through isolated nuclei to intact mammalian cells with biochemical and biological equivalent doses. Such studies are presently being pursued in this laboratory.

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Orientation of Nucleosomes in the Thirty-Nanometer Chromatin Fiber†

Hiroko Yabuki,‡ Nanibhushan Dattagupta, and Donald M. Crothers*

ABSTRACT: We have measured the linear dichroism of Mg^{2+}-stabilized and dimethylsuberimidate cross-linked 30-nm chromatin fibers, using electric fields to produce orientation. The limiting dichroism of Mg^{2+}-stabilized fibers at low monovalent ion concentration is −0.09, with indistinguishable results for avian erythrocyte and calf thymus chromatin. Correction of the apparent dichroism signal for an absorbance change that becomes significant at high electric field yields the dichroism of −0.09 and reveals that the fibers reach saturation of their orientation by about 15 kV/cm. Calf thymus chromatin fibers cross-linked at 100 mM NaCl concentration have a dichroism of +0.05, with a dependence of orientation on field similar to that observed for Mg^{2+}-stabilized fibers. Comparative sedimentation studies of Mg^{2+}-stabilized fibers and fibers in 100 mM cross-linking buffer revealed a 25% sedimentation coefficient increase accompanying the dichroism increase from −0.09 to +0.05. We interpret the results to mean that the nucleosomal disk diameters form an angle of about 30° to the chromatin fiber axis in Mg^{2+}-stabilized fibers at low monovalent ion concentration. When 100 mM NaCl is added, the fiber becomes more compact, and the disk diameter angles increase by 8°, to about 38°. Cross-linking itself may also contribute slightly to fiber compaction. The results are consistent with a large longitudinal compressibility of the 30-nm fiber, a feature required for the bendability necessary when the fiber is further coiled to form structures such as chromosomes. Our results indicate that compression is accommodated by small changes in the angular orientation of the nucleosomal disks.

The detailed structure of the 30-nm chromatin fiber, which is readily visualized in the electron microscope (Ris & Kubai, 1970), remains unknown. The organization of nucleosomal disks in chromatin fibers has been studied by electron microscopy (Finch & Klug, 1976; Rattner & Hamkalo, 1978; Thoma et al., 1979), neutron scattering (Suau et al., 1979), X-ray diffraction (Sperling & Klug, 1977), light scattering (Campbell et al., 1978), electric dichroism (Rill & Van Holde, 1974; Houssier et al., 1977; McGhee et al., 1980; Lee et al., 1981; Lee & Crothers, 1982), and flow dichroism (Tjerneld & Norden, 1982). It has been proposed that in the 10-nm fiber observed at low salt concentration, the nucleosomal disks are arranged with their diameters nearly parallel to the fiber axis. However, this conclusion is not supported by the flow dichroism studies of Tjerneld & Norden (1982). The 30-nm fiber results from further coiling or folding of the 10-nm fiber, which is induced by higher salt concentration or addition of multivalent cations, and occurs over a range of salt concentrations (Thoma et al., 1970; Butler & Thomas, 1980; Thomas & Butler, 1980; Bates et al., 1981).

The linear dichroism of oriented 30-nm chromatin fibers is expected to be a sensitive function of the orientation of nucleosomal disks relative to the fiber axis. Recent experiments by McGhee et al. (1980) and in our laboratory (Lee et al., 1981; Lee & Crothers, 1982) have used electric field orientation to obtain the dichroism of the fiber. The results of those studies left some unresolved discrepancies, which we seek to clear up in this paper. Specifically, McGhee et al. (1980), working with unfixed erythrocyte chromatin at low ionic strength in the presence of Mg^{2+}, found a limiting dichroism of about −0.2 and did not observe saturation of the orientation at increasing electric field. Our experiments (Lee

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06511. *Received April 5, 1982.* Supported by Grant GM 21966 from the National Institutes of Health.
‡ Present address: Institute of Endocrinology, Gunma University, Maebashi, Japan.

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