Published in final edited form as:

Radiat Res. 2001 May ; 155(5): 687–692.

Electron Paramagnetic Resonance Evidence for a C3' Sugar Radical in Crystalline d(CTCTCGAGAG) X-Irradiated at 4 K

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Abstract

A neutral sugar radical formed by the net loss of hydrogen from C3' has been identified in crystalline DNA X-irradiated at 4 K. Crystals of duplex d(CTCTCGAGAG), known to be of B conformation, were studied using electron paramagnetic resonance (EPR) spectroscopy. The C3' radical was identified by using information from dose saturation, power saturation, thermal annealing, and spectrum simulation. The yield of the C3' radical, $G(C3')$, is $0.03 \pm 0.01 \mu\text{mol/J}$, and its concentration does not appear to saturate up to at least 100 kGy. In the region in which total radical concentration increases linearly with dose, the C3' radical makes up about 4.5% of the total radical population trapped in the oligodeoxynucleotide crystal at 4 K. Based on free base release measured in other oligodeoxynucleotides, we suggest that in d(CTCTCGAGAG) the C3' radical is responsible for about one-third of the strand breakage events.

INTRODUCTION

The EPR spectra of irradiated DNA are dominated by features stemming from radicals derived from the base moieties [see for example refs. (1-4)]. More recent evidence indicates that a significant fraction of radicals must reside on the sugars (see Fig. 1 for structures of the sugar radicals). In one calculation, it was estimated that up to 15% of the radicals initially formed in directly ionized DNA could be sugar radicals (5). In general, the EPR spectra of sugar radicals overlap with the spectra of the DNA base radicals; they are broad and relatively featureless, making positive identification problematic. Furthermore, assuming that each of the five sugar radicals is produced with equal probability means that each sugar radical spectrum would be but 3% of the total DNA spectrum, and thus would be difficult to detect.

That there must be sugar radicals present has been supported by recent work by Razskazovskiy *et al.* demonstrating that there is a sizable amount of base release in X-irradiated oligodeoxynucleotide crystals of DNA, and that this base release must be due to sugar-centered radicals (6). Previous EPR work has described sugar radical species: The presence of C1' and C3'/C4' sugar radicals in hydrated amorphous DNA has been postulated based on obtaining EPR component spectra through spectral subtraction (1). Both C1' and C3' radicals have been implicated in deoxycytidine samples (7), the C3' or C4' radical has been found in high-LET-irradiated lyophilized DNA (8), and the C1' radical has been identified in lyophilized powder DNA (9).

Here we present evidence for the existence of a C3' sugar radical (see Fig. 1) in crystalline oligodeoxynucleotide d(CTCTCGAGAG) produced by the direct-type effect of ionizing

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radiation. Because both the DNA conformation and crystal packing are known to high resolution, advantages accrue in both the identification of radicals and the measurement of their yields. Observed properties that support identification of the C3' radical include dose saturation, power saturation, thermal annealing profile, spectral width, and isotropy of the outer features of the spectra. Estimates for the yield of the C3' radicals in d(CTCTCGAGAG) are provided. We suggest that the C3' radical is a direct precursor to about one-third of the strand breaks resulting from direct ionization of the oligodeoxynucleotide and its solvation shell.

MATERIALS AND METHODS

Crystals of d(CTCTCGAGAG) were grown using oligodeoxynucleotides purchased from Midland Certified Reagent and TIB MolBiol and used without further purification. The crystals were grown from solutions containing the oligodeoxynucleotide, calcium acetate, and 2-methyl-2,4-pentanediol (MPD) following published procedures (10). The DNA is B-form, and the oligodeoxynucleotides are continuously stacked in the crystal: that is, the terminal base pair of one oligodeoxynucleotide abuts the first base pair of the neighboring helical segment, maintaining π -bond overlap. A single crystal of 101 μ g was used in the rotation study, and polycrystalline samples of 48–245 μ g were used in the other experiments.

Most crystals were removed from the mother liquor, carefully dried by application of fine paper wicks, and placed in Charles Supper Company quartz tubes (chosen for the low free radical background produced upon irradiation). Samples were irradiated at 4 K in a Janis Dewar setup (11) with X rays generated by a Varian/Eimac OEG-76H tungsten-target tube operated at 70 keV and 20 mA. The dose rate was 26 kGy/h. Free radical yields were measured in the Q-band Varian E-12 EPR spectrometer by comparing the intensity of the EPR signal at 4 K to that of a ruby standard mounted on the side of the EPR cavity (12).

For single crystal rotations, crystals were mounted at the end of an open quartz tube. The crystals were attached using small amounts of rubber cement. The angle of rotation was measured to $\pm 1^\circ$ using a Parker goniometer head. The orientation of the rotation axis relative to the crystallographic axes was not determined.

Annealing was accomplished by raising the sample about 60 cm above the EPR cavity to the heater position. Samples were allowed to warm to the stated temperature for at least 10 min. Temperatures were controlled to $\pm 3\%$ by a Lakeshore Cryotronics temperature controller. The sample was then returned to the EPR detection cavity and allowed to equilibrate for at least 10 min before spectra were recorded.

Computer simulations were performed on a Gateway2000 (Pentium II) computer using software developed in our laboratory (13). Starting values for the hyperfine coupling tensors were derived from the work of Close (5).

RESULTS AND DISCUSSION

This paper focuses on the flanking lines, indicated by the arrows in Fig. 2, of the EPR spectra of d(CTCTGAGAG) X-irradiated at 4 K. Results from dose and power saturation studies, thermal annealing, and spectral simulation provide strong evidence that these flanking lines are due to the C3' sugar radical.

Selected EPR spectra resulting from the rotation of a representative single crystal of d(CTCTCGAGAG) through 180° are shown in Fig. 2. Both the features of the spectra and the degree of anisotropy evident in the central regions of the spectra are consistent with the presence of one-electron oxidized guanine (14) and one-electron reduced cytosine (15). The features of

interest, indicated by the arrows, have a larger spectral width than the more abundant base radicals. Also, these wing features are relatively isotropic.

Care was taken to eliminate the possibility that these features of the spectra could be the result of the presence of MPD (see Materials and Methods above) on the crystal surface or in the disordered hydration layer within the crystal. Irradiation and observation of MPD at 4 K results in a spectrum with broad wings and a spectral extent that is >14.0 mT. Features of the spectra consistent with that of MPD were not detected in the wings of the d(CTCTCGAGAG) spectrum, nor have these features been identified in any of 16 other crystal types for which care was exercised in removing the mother liquor.

Dose Saturation

When irradiated, all DNA samples give an initial linear response in the concentration of trapped radicals. At some point, the radical concentration reaches a level at which further incident radiation begins to destroy as well as produce radicals. At the concentration at which destruction equals production, the dose–response curve is in saturation [see for example ref. (16)]. Different chemical species demonstrate different dose saturation characteristics. For sugar-centered radicals, the cross section for destruction is smaller than that for base-centered radicals (8). This is most likely because the sugar radicals are neutral in charge and the lost proton is displaced a larger distance. It is well known that base-centered electrons and holes are able to transfer between bases (17–22). As a result, the efficiency of accumulation of radicals on the bases is somewhat diminished by the electron/hole mobility between stacked bases. The sugar radicals are more deeply trapped due to irreversible deprotonation events; consequently they are less mobile, and there is a smaller probability of electron return.

Three EPR spectra of a polycrystalline d(CTCTCGAGAG) sample irradiated with doses of 20, 50 and 100 kGy are presented in Fig. 3. In comparing these spectra, there is a shift in the relative intensities of the central peak (generally associated with base radicals) and the flanking lines. The radical, reflected by the wing features of the spectrum, saturates more slowly with dose than the features in the center of the spectrum. This is consistent with the outer features of the spectrum being derived from a sugar radical.

Power Saturation

In general, the power saturation of the sugar radicals differs from that of base radicals, with the sugar radicals saturating at higher microwave powers. This technique has been used previously to distinguish contributions from different free radical components (2,7,9,23). The wing features of the d(CTCTCGAGAG) spectra power-saturate more slowly than the central features (data not shown). This is consistent with the wing lines arising from sugar-centered radicals and the central features arising primarily from base-centered radicals.

Thermal Annealing

The intensity of the outer spectral features was monitored as the sample was warmed. The wing lines diminished in intensity and annealed out entirely between 80 K and 100 K, as seen in Fig. 4. This concurrence of disappearance suggests that both the up-field and down-field wing features stem from the same radical.

Spectrum Simulation

The wing features are relatively isotropic and have a spectral width of 6.9–7.3 mT. This width is consistent with an unpaired electron localized on a carbon and coupled to at least two β hydrogens. While two radicals located on the deoxyribose moiety can be used to fit these features, they cannot be fitted adequately by base radicals that have been identified in irradiated

DNA and DNA components. A computer simulation of the C3' sugar radical was made using the hyperfine interaction of three β hydrogens (two at C2', one at C4') with couplings calculated by Close (5), a slightly anisotropic g tensor, and 0.68 mT line width. A representative simulated spectrum compared to an experimental spectrum of d(CTCTCGAGAG) irradiated with 26 kGy is shown in Fig. 5.

Similar simulations were carried out for C1', C2', C4' and C5'-centered radicals (structures shown in Fig. 1) (5). The extents of the spectra of the C1', C4' and C5'-centered radicals were much too narrow to account for the wing features. These radicals, however, may be present in the sample and may be contributing to the spectral intensity in the center of the spectra.

While the extent of the spectrum of the C2' radical was somewhat comparable to that of the experimental spectrum, its outer features were significantly broader, and the best-fit simulation required an anisotropy in the g tensor that is physically unrealistic. The C2' powder spectrum has broader features due to the H2' hyperfine interaction. H2' is alpha to the main locus of unpaired electron density, and its hyperfine interaction is therefore very anisotropic. If the C2' radicals are randomly oriented, this large hyperfine anisotropy results in broad lines and poor resolution. A powder simulation therefore gives a poor fit to the wing lines of the d(CTCTCGAGAG) spectra. If the sites of C2' radical formation did not vary in conformation or orientation, sharp well-resolved lines would be observed for any given orientation of the crystal. But under these circumstances, the anisotropy should be apparent when the crystal is rotated about almost any axis. The one exception is the axis normal to the plane containing the intermediate principal axis and a vector lying in the plane of the minimum and maximum principal axes that has the magnitude of the intermediate principal value. But, for this exception to manifest itself, it is also necessary that all of the C2' radicals be magnetically equivalent at all of the crystal orientations where spectra were recorded. This is extremely unlikely, because there are 10 possible sites for C2' radical formation in one duplex of d(CTCTCGAGAG), which are magnetically inequivalent, and there are 2 magnetically distinct duplexes related to the C2 space group of the crystal. Given that two planes of data, acquired by rotation of two different single crystals of d(CTCTCGAGAG), did not exhibit anisotropic behavior (for an example, see Fig. 2), we conclude that the wing lines of interest are not due to the C2' radical. Based on simulations of the spectra and the observed lack of anisotropy, the C3' radical is the only good candidate for explaining the wing line features of the EPR spectrum.

Yield

The concentration of C3' radicals was estimated by scaling the outermost features of the simulated spectrum to the wing line features of the experimental spectra. The intensity of the simulated spectrum was then compared to that of the ruby standard. The concentrations, in free radicals/g, were then plotted as a function of X-ray dose. The resultant dose-response curve is shown in Fig. 6. From the slope of the least-squares fit line, the yield of sugar radicals is estimated at $G(\text{C3}') = 0.03 \pm 0.01 \mu\text{mol/J}$, or about 4.5% the yield of total radicals for crystals of d(CTCTCGAGAG) (12). The yields of base release in closely related oligodeoxynucleotide crystals due to direct-type effects, and thus strand breaks, have been measured to be $\sim 0.1 \mu\text{mol/J}$ [(6) and unpublished data]. The ratio $G(\text{C3}')/G(\text{free base})$ is $0.03/0.1$, suggesting that about one-third of the strand breaks arising from direct-type damage to d(CTCTCGAGAG) stem from the C3' radical. In addition, we note that the dose response appears to be linear to 100 kGy. This is consistent with the yield of base release fragments of d(CGCG) and d(CGCGCG), which also appears to be linear to ≥ 100 kGy. These latter results are described in an upcoming publication.

Rate of Deprotonation

It is known from earlier studies on diethyl phosphates (24) that the $\text{CH}_3\text{C}^*\text{HOP}(\text{O})_2\text{OC}_2\text{H}_5^-$ radical is produced by oxidation, not reduction. We propose, therefore, that the C3' radical is formed by irreversible deprotonation of a one-electron oxidized precursor localized on the phosphate. Oxidation of the phosphate (native charge state -1) results in a neutral free radical. If the C3' radical is to be formed by this heterolytic process (as opposed to homolytic C–H bond cleavage), it follows that the rate of deprotonation at C3' (k_{pt}) must be fast relative to the rate of hole transfer (k_{ht}) to a guanine in the base stack. This condition appears to be readily met. Using the atomic positions determined by X-ray crystallography (10), the distance in d(CTCTCGAGAG) from a 3' or 5' phosphorus to guanine carbon atoms (C2, C4, C5, C6 or C8) is between 4.1 and 11.0 Å. The distance dependence for the rate of hole transfer can be described by the equation (25,26)

$$k_{\text{ht}} = k_0 \exp[-\beta(\Delta r)],$$

where Δr is the distance of closest approach of the donor/acceptor pair and β is the decay factor. The work of Meggers *et al.* has measured a β of $\sim 1.0 \text{ \AA}^{-1}$ for electron transfer from a guanine base to an oxidized sugar (19) and a k_{ET} of 10^8 s^{-1} . This suggests that only a very modest rate for deprotonation from C3', $k_{\text{pt}} \geq 10^8 \text{ s}^{-1}$, is sufficient for radical formation on C3' to compete with hole transfer.

CONCLUSIONS

We have identified a C3' sugar radical in the oligodeoxynucleotide crystal of d(CTCTCGAGAG). Production of the C3' radical appears to be linear with radiation dose to $\geq 100 \text{ kGy}$, and the yield of the C3' radical is $0.03 \pm 0.01 \text{ } \mu\text{mol/J}$. Production of the C3' radical accounts for about 4.5% of the total yield of trapped radicals. It is suggested that the C3' radical may be the precursor for about one-third of the direct-type strand breaks in d(CTCTCGAGAG).

Acknowledgements

The authors thank Kermit R. Mercer for his invaluable technical assistance. This study was supported by PHS Grant 2-R01-CA32546, awarded by the National Cancer Institute. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

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FIG. 1.
Chemical structures of the sugar radicals discussed in this paper.

FIG. 2.

EPR spectra of a single crystal of d(CTCTCGAGAG) irradiated at 4 k with a dose of 22 kGy and rotated about an undetermined axis. Spectra recorded at 4 K are shown at rotation angles of 0°, 45°, 90° and 135°. Scan width is 40 mT. The vertical line indicates the position of $g = 2.0023$. The vertical arrows indicate the spectral features identified as belonging to the C3' sugar radical. Note the comparative lack of anisotropy in the indicated features compared to the features in the central region of the spectra.

FIG. 3. EPR spectra of polycrystalline d(CTCTCGAGAG) irradiated at 4 K with 20, 50 and 100 kGy and recorded at 4 K. Scan width is 20 mT. The vertical arrows indicate the spectral features identified as belonging to the C3' sugar radical.

FIG. 4. EPR spectra of d(CTCTCGAGAG) irradiated at 4 K, annealed and then returned to 4 K for recording. Annealing temperatures are 80, 100, 120, 140, 160, 180, 220, 240 and 260 K. Scan width is 20 mT. The vertical arrows indicate the spectral features identified as belonging to the C3' sugar radical.

FIG. 5.

Q-band EPR spectrum of polycrystalline d(CTCTCGAGAG) after 26 kGy irradiation at 4 K compared to a simulated C3' spectrum generated using two nearly isotropic β -hydrogen couplings [(3.70, 3.71, 3.92 mT), (1.76, 1.86, 1.73 mT)] and one isotropic β -hydrogen coupling (0.9 mT) derived from calculations by Close (5), a slightly anisotropic g tensor (2.0033, 2.0020, 2.0022), and 0.68 mT isotropic line width. The total scan width is 20 mT.

FIG. 6.

Dose-response curve of calculated C3' concentrations as a function of X-ray dose. The concentrations of C3' were determined by comparing the experimental spectra with the simulated spectrum of C3' in B-DNA. The data have been fitted to a least-squares line, and the yield was determined from the slope of this line. A total of eight crystalline and polycrystalline samples were used to determine the yield.