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EPR Detection of an Electron Scavenging Contaminant in Irradiated Deoxyoligonucleotides: One-Electron Reduced Benzoyl

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Abstract

Our lab investigated damage of DNA due to ionizing radiation using electron paramagnetic resonance (EPR). Through studies focused on one-electron-reduction of synthetic oligodeoxynucleotides containing only thymine and adenine, we discovered the significant presence of a contaminant in all samples. The contaminant was observed to have a reduction potential greater than that of thymine. In addition, the contaminant yielded a sharp EPR singlet when it was one-electron reduced that interfered with the distinctive doublet of one-electron reduced thymine. We determined that the contaminant contained a benzoyl group, a chemical used in to protect the amine group of adenine during oligodeoxynucleotide synthesis. Derivatives of benzoyl and 16 different oligomer sequences were prepared in a LiCl glass and studied using EPR after X-irradiating at 4K. This treatment selectively creates one-electron reduced radicals. Synthetic derivatives were used to develop an EPR benchmark of the benzoyl radical. Using this, along with the known spectra of one-electron reduced nucleobases, we performed component analysis of the EPR signal from each sample. This analysis revealed that 2–9% of adenines, in the commercially synthesized oligomers delivered to us, were left contaminated with benzoyl. We concluded that the presence of benzoyl is a potential source of error in a variety of experiments utilizing synthesized oligodeoxynucleotides.

INTRODUCTION

Synthesized oligodeoxynucleotides are utilized in a large number of sectors for both industrial and academic purposes. An application of interest to us is the use of synthetic DNA to study damage produced in DNA by the direct effect of ionizing radiation.^{1–3} Studies by electron paramagnetic resonance (EPR) spectroscopy have shown that the primary intermediates to radiation damage consist of free radicals produced by one-electron oxidation and one-electron reduction.⁴ The work reported here stems from our studies on one-electron reduction of DNA.

Electrons ejected by ionizing radiation inevitably thermalize and add preferentially to the DNA base stack.⁵ The excess electron transfers between bases until trapped by the bases having the largest reduction potential.⁶ The reduction potentials (vs normal hydrogen electrode (NHE)) of the four nucleosides at pH7.0 are 1.7V–thymidine (Thd), 1.6 V–deoxycytidine (Cyd), 1.42 V–deoxyadenosine (Ado), and 1.29 V–deoxyguanosine (Guo).⁷ These potentials lead to electron trapping predominantly at thymine (Thy) and cytosine (Cyt) in duplex DNA.^{8,9}

Currently, we are investigating the competition between Thy and adenine (Ade) for electron trapping. For Thy, this produces the thymine radical anion, $\text{Thy}^{\bullet-}$, while for Ade, the radical anion protonates at N3, giving the neutral $\text{Ade}(\text{N3+H})^{\bullet}$ radical.⁷ These radical structures are shown in Scheme 1. An informative approach to studying these nucleobase radicals involves performing EPR on oligodeoxynucleotides dissolved in 8 M LiCl and cooled to 77 or 4 K.¹⁰ Upon cooling, these solutions form a glass. X-irradiation of this glass results in electrons selectively trapped by nucleobases, leaving electron loss centers to be trapped by the $\text{LiCl:H}_2\text{O}$ matrix as $\text{Cl}_2^{\bullet-}$. The later gives an EPR spectrum that is too broad to interfere with the sharp spectrum of one-electron reduced nucleobases.^{11,12}

In the course of our studies, we encountered a discrepancy between recent results and earlier observations^{9,13} on oligodeoxynucleotides in LiCl glasses at 4 K. This prompted us to focus on sample purity with specific attention to changes that have occurred over the past decade in the methods used during oligodeoxynucleotide synthesis. The use of benzoyl (Bz) to protect the amine of Ade¹⁴ (see benzoylated adenine (Bz-Ade) in Scheme 1) was of particular interest. While published values of reduction potentials at pH7.0 for Bz derivatives are not available, they are expected to be preferential electron traps based on the reduction potential of acetophenone (~ 1.9 V/NHE, pH 7.0).¹⁵ The goal of the work reported here was to identify the impurity in synthetic oligodeoxynucleotides and estimate the level of contamination.

MATERIALS AND METHODS

DNA oligomers were purchased from Integrated DNA Technologies (IDT) and SynGen, either desalted or high-performance liquid chromatography (HPLC) purified, and were used without further purification. Of these oligomers, five of the sequences were palindromic: $(\text{TTTTAAAA})_2$, $(\text{T})_8(\text{A})_8$, $(\text{AATT})_4$, $(\text{AT})_8$, and $(\text{TA})_8$. The duplex form is designated, for example, by $(\text{AATT})_4:(\text{AATT})_4$ and $(\text{AT})_8:(\text{AT})_8$. Six of the sequences were nonpalindromic with duplex structures varying from $(\text{A}(\text{T})_7\text{A})_2:(\text{T}(\text{A})_7\text{T})_2$ to $(\text{ATTA})_4:(\text{TAAT})_4$, and two duplexes were formed by homologous single strands, $\text{A}_{16}:\text{T}_{16}$ and $\text{A}_{10}:\text{T}_{10}$. In all cases, the strand termini were phosphate free. *N*-Benzoyl-D-threonine (Bz-Thr in Scheme 1) was purchased from Sigma Aldrich and used as delivered. Instead of purified Bz, which is highly toxic, the oligomer $(\text{AT})_8:(\text{AT})_8$ with the Bz protection left in place was purchased from SynGen. The structure of Bz-Ade is provided in Scheme 1. Bz-Thr and Bz-Ade were employed as authentic benzoylated compounds.

The oligomers were dissolved in 8 M LiCl solutions to a monomer concentration of 27.7 mM. Under standard atmosphere without degassing, a volume of 3 μL of solution was placed in high purity quartz tubes with an outer diameter of 0.7 mm obtained from Charles Supper Company. The samples were inserted into a Janis liquid helium cryostat and cooled to 4 K in approximately 10 min. At temperatures below $\sim 200\text{K}$, this solution becomes a transparent glass.

Samples were X-irradiated at 4 K using a Varian/Eimac OEG-76H tungsten target tube operated at 70 kV, 20 mA, and filtered by 40 μm aluminum foil at a dose rate of 0.75 kGy/

min. After a dose of 3 kGy at 4K, EPR spectra were recorded at 4K using Q-band (35 GHz) microwave frequencies. Hydrogen atoms, created by the radiation and trapped in the LiCl matrix, were used as an internal *g*-standard with an assumed isotropic *g* value of 2.0022.^{16,17}

Benchmarks for one-electron reduced Thy, Ade, and two Bz derivatives were calculated by simulating experimental EPR spectra recorded under conditions where the desired radical was isolated. Simulations were performed using PowderSim software developed in house.¹⁸ A detailed description of this approach has been published for one-electron reduced Thy.¹⁹ Using a weighted sum of the three benchmarks, a best fit was determined for the EPR spectrum of a series of one-electron reduced DNA oligomers. This component analysis, also performed by our PowderSim software, gave the relative intensities of each of the benchmarks and, thereby, the concentration of each radical species.

RESULTS

The EPR spectrum of the one-electron reduced duplex (AATT)₄:(AATT)₄ shown in Figure 1a adopts the form of a broadened doublet. An attempt to simulate this spectrum with just two components, Thy^{•−}, a sharp doublet, and Ade(N3+H)[•], a broad singlet (Figure 1b and 1c, respectively), gave poor results because the spectral extent of Ade(N3+H)[•] exceeds that of the reduced oligomer (8.4 mT vs 7.0 mT, respectively). The relative spectral extents of one-electron reduced (AATT)₄:(AATT)₄ and one-electron reduced Ade is provided in Figure 2a. This indicates that another radical had to be formed by one-electron reduction of a contaminant, i.e., not from a DNA constituent.

The contaminant of interest must possess a reduction potential greater than that of Thy (1.7 V vsNHE²⁰ at pH 7.0) and give a singlet spectrum narrower than that of Ade(N3+H)[•]. Since the LiCl solution alone gives no such signal,¹¹ the synthetic DNA must carry the contaminant. Because Bz was used to protect the amine group of Ade during synthesis, it was a likely candidate. To test this, we purchased benzoylated-(AT)⁸ from SynGen, which was an oligodeoxynucleotide of sequence (AT)⁸ synthesized without the deprotection protocol. That sample gave the singlet spectrum with a spectral extent of 6.5 mT and an isotropic *g* value of 2.00237(5), the simulation of which is shown in Figure 1d. Confirmation that this singlet can be attributed to one-electron reduced Bz was obtained performing the same experiment on Bz-Thr. A singlet of 5.4 mT spectral extent and *g* value of 2.00237(8) was observed. This is strong evidence that the contaminant in DNA is Bz-Ade. In the AT-mers, the singlet spectrum may be due to either Bz-Ade^{•−} or Bz-Ade(O+H)[•], the later being more likely because the pH of 8 M LiCl is ~6 and the proton affinity of Bz-Ade^{•−} is predictably high.

In order to include the spectrum of Bz-Ade(O+H)[•] as a benchmark, the spectrum was simulated as described under Materials and Methods. The *g*, hyperfine, and line width tensors used to simulate one-electron reduced Bz-Thr and Bz-Ade are provided in Table 1. The tensors in Table 1 can be used to generate these spectra. It should be understood, however, that simulation of singlets of this type does not constrain the tensor elements to a unique solution. While the tensor elements are physically realistic for the radical structure, they represent only one parameter set out of a number of degenerate sets that would give the same spectrum. In Figure 1d, the simulated spectrum of Bz-Ade(O+H)[•] is shown; it reproduces the EPR spectrum of the protected (AT)₈ sample to within the noise of the trace.

Figure 2a overlays the two one-electron reduced benchmarks with the experimental EPR spectrum of one-electron reduced (AATT)₄:(AATT)₄, illustrating the difference in spectral features between one-electron reduced Ade and one-electron reduced Bz-Ade; all three have been placed in *g*-register using trapped hydrogen atoms as a marker. By adding the third

component, Bz-Ade(O+H)[•], we were able to obtain a good fit to the spectrum of (AATT)₄: (AATT)₄. The fit shown in Figure 2b gave an *R* value²¹ of 0.03 and was obtained using 65% Thy^{•-}, 0% Ade(N3+H)[•], and 35% Bz-Ade(O+H)[•]. Also shown in Figure 2b is the residual signal obtained by subtracting the T and A benchmarks from the experimental spectrum. The residual closely conforms to the spectrum of one-electron reduced Bz. Figure 3 shows the result for (AATT)₄: (AATT)₄ along with component ratios that gave comparable fits to the EPR spectrum of 15 other AT-oligomers. For the 16 samples studied, the best fits required from 20% to 60% of the Bz-Ade(O+H)[•] spectral component.

In only two cases, A₁₆:T₁₆ and A₁₀:T₁₀, was electron trapping by Ade observed. For the two oligomers that were HPLC purified, (AT)₈: (AT)₈ and (TA)₈: (TA)₈, the contribution of Bz-Ade(O+H)[•] component was about 1.5–2 times greater than when they were purified via standard desalting.

In order to estimate the level of Bz contamination, known molar ratios of Bz-Thr to 2'-deoxythymidine (dThd) were employed using the same protocol as above. The result (Figure 4) shows that competition for one-electron capture favors dThd over Bz-Thr by 60:40. When dThd is contained within a T-oligomer, T₁₆ in this case, it decreases the probability of electron capture by any single Thy. This can be seen from the results for Bz-Thr mixed with T₁₆ in a 1:1 ratio (which is based on the concentration of monomer Thy). In this case, capture by Thy is less than Bz-Thr by 40:60. Also as shown in Figure 4, trapping by Thy increases approximately in proportion to the relative concentration of T₁₆ as it is increased by 10x and 100x. These observations indicate that Bz-Thr is about 1.5 times better at trapping electrons in the LiCl matrix than any given Thy within a single stranded 16mer.

It is important to note that at the 3 kGy dose used for these samples, the total radical concentration varied from 0.12 to 0.39 mM. Therefore, about one in eight oligomers were one-electron reduced, and the probability of two one-electron reductions of the same oligomer is very small. We know from previous work²² that 3 kGy is within the linear region of the dose response curve, indicating single-track radiation chemistry.

DISCUSSION

Our results can be used to estimate the level of Bz contamination by assuming that the reduction potentials of Bz-Thr and Bz-Ade are identical. Across the 16 samples studied, the probability of trapping by Bz-Ade varied from 1.5 to 0.5 times the probability of trapping by Thy. Considering these extremes, for (AT)₈: (AT)₈ (HPLC purified) there were 16 Ade per duplex and, thereby, for every three duplexes containing Bz-Ade-(O+H)[•] there were two containing Thy^{•-}. Therefore, one out of every 11 (16/1.5) Ade's was benzoylated. At the other extreme, (AATT)₄, one finds that for every other duplex trapping an electron at Thy, one duplex trapped an electron at Bz-Ade. Therefore, one in 32 (16/0.5) Ade was benzoylated.

The A₁₀:T₁₀ duplex had the lowest level of Bz. Under the reasonable assumption that in order to form the Ade(N3+H)[•] radical on a given strand that strand must *not* contain Bz-Ade, and based on the observed ratio of 70% Ade(N3+H)[•]/Bz-Ade-(O+H)[•] = 3.5, the estimate is that one out of 3.5×10 = 35 Ade is benzoylated. The same calculation for A₁₆:T₁₆ gives one Bz-Ade per 53%/38% × 16 = 48 Ade. Therefore, the impurity due to remaining Bz protection, for this series of oligomers, ranged between 2% to 9%. To the degree that Bz-Ade has a higher reduction potential than Bz-Thr, these percentages would be lower.

By extrapolating the dilution results shown in Figure 4 to the results in Figure 3, EPR can be used to detect Bz-Ade in double stranded AT-oligodeoxynucleotides at a level 1 order of

magnitude below those observed for this series of oligomers, establishing the threshold for detection at about 0.1%.

It is quite interesting that electron addition to Ade was observed at all in duplex DNA and that this required duplex DNA containing one strand consisting only of Ade. The generally held expectation has been that, given the disparity in reduction potentials, 1.7 V for Thy and 1.42 V for Ade²⁰ at pH 7.0, electron capture by Thy should always predominate. The results reported here indicate that the rate of electron trapping by Ade is faster than the rate of electron transfer between strands. The nature of electron transfer in TA-duplex DNA will be the subject of a future work.

CONCLUSION

In a series of commercially synthesized AT-oligodeoxynucleotides, 2%-9% of the Ade's retained the Bz protection group. Samples that were purified via desalting had lower levels of impurity than samples that were HPLC purified. The observed level of impurity has been a confounding factor in our studies of electron transfer in DNA. We believe that there are other applications employing synthesized DNA that will be similarly affected by these levels of impurity. EPR spectroscopy sensitivity is such that it can be used to measure an impurity of Bz-Ade as low as 0.1%.

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Figure 1.

Spectrum of a one-electron reduced oligomer (a) and the benchmarks used to analyze it. The simulations of one-electron reduced Thy (b) and Ade (c) were developed using EPR spectra and tensors from the literature.^{19,23} The simulated spectrum of Bz-Ade (d) was obtained by fitting Bz-protected (AT)₈; the simulated and experimental spectra are effectively identical.

Figure 2.

(a) Spectrum of a one-electron reduced $(AATT)_4:(AATT)_4$ overlaid with the simulated benchmarks of one-electron reduced Ade and one-electron reduced Bz. The spectral extent of $Ade(N3+H)^{\bullet}$ was treated as a distinctive characteristic for component analysis. (b) The same spectrum provided in panel a overlaid with the simulation using one-electron reduced Thy, Ade, and Bz benchmarks. The fit using Bz had an R value of 0.03. In addition, a plot of the residual is shown; it was created by subtracting the T and A benchmarks from the experimental spectrum and it gives a trace in agreement with that of Bz-protected $(AT)_8$.

Figure 3.

Bar graph showing the distribution of electron trapping between Thy, Ade, and Bz-Ade in 16 different duplexes given identical treatment.

Figure 4.

Results from the component analyses for various ratios of Bz-Thr to each Thy base within T₁₆ oligomer (dThd in T₁₆ rows) and to Thy nucleoside (dThd row).

Scheme 1.

Six Structures Relevant to This Study: Benzoylated Threonine (Bz-Thr), Benzoylated Adenine (Bz-Ade), Thymine Radical Anion (Thy^{•-}), N3-Protonated Adenine Radical Anion (Ade(N3+H)[•]) and the Bz-Ade Radical Anion before (Bz-Ade^{•-}) and after Protonation at the Keto Oxygen Bz-Ade((O+H)[•])

Table 1EPR-Spectrum Simulation Parameters for One- Electron Reduced Bz Derivatives^a

	values	$\cos \theta_x$	$\cos \theta_y$	$\cos \theta_z$
g_{xx}	2.0022	0.7753	0.0000	-0.6316
g_{yy}	2.0021	0.0000	1.0000	0.0000
g_{zz}	2.0027	0.6316	0.0000	0.7753
A_{xx}	10	1.0000	0.0000	0.0000
A_{yy}	30	0.0000	1.0000	0.0000
A_{zz}	20	0.0000	0.0000	1.0000
Bz-Thr				
LW_{xx}	41.3	0.9967	0.0000	0.0816
LW_{yy}	27.8	0.0000	1.0000	0.0000
LW_{zz}	25.8	-0.0816	0.0000	0.9967
Bz-Ade				
LW_{xx}	29.3	0.8050	0.5594	0.1975
LW_{yy}	26.5	-0.4264	0.7771	-0.4630
LW_{zz}	65.2	-0.4125	0.2885	0.8641

^aPrinciple values and direction cosines of tensors for g , hyperfine coupling (A), and line width (LW) were diagonalized using the axis system for A as a frame of reference. A and LW are in MHz.