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Characterization of a Spin-Labeled Abasic Double-Stranded DNA Substrate for Apurinic/Apyrimidinic Endonuclease[¶]

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Abbreviations used:

AP endo, apurinic/aprimidinic endonuclease; ASR, abasic site repair, bp, base pair; ds, double-stranded; ESR, electron spin resonance; HDP, heat degradation product; MOMD, microscopic order/macroscopic disorder, SRLS, slowly relaxing local structure, ss, single-stranded

Introduction

Abasic site repair (ASR) refers to one of several repair pathways that generate and repair an abasic site in double stranded DNA (1-4). The number of abasic sites generated per mammalian cell per day could be as high as 10^6 /cell/day.(5) In eukaryotes, abasic sites can be generated through DNA-damaging agents such as ionizing radiation , reactive oxygen species and chemotherapeutic agents or through a subset of the DNA-repair enzymes known as DNA glycosylases. ASR is important not only in maintaining the integrity of nuclear DNA but also in protecting mitochondrial DNA against reactive oxygen species generated during reduction of O_2 .(6) Abasic sites are unstable and highly mutagenic (7-13). They retard DNA polymerase (13-16) and inhibit both topoisomerase I leading to permanent DNA damage and premature cell death (17), and topoisomerase II, causing DNA double strand breaks.(18)

The ASR system involves an AP endonuclease, a lyase, a DNA polymerase and a DNA ligase (See references (19) and (20)). The major AP endonuclease in humans is called apurinic/apyrimidinic endonuclease (AP endo, also known as HAP1, APE 1 and Apex). Its role is to make a single nick at the 5' position of the abasic site, which generates a 3'-hydroxyl on the upstream strand and a 5'-phosphodeoxyribose (dRP) on the downstream strand. The cleaved abasic site can then be excised by a lyase, probably DNA polymerase β , thus creating a single nucleotide gap that is filled by a DNA polymerase, most likely DNA polymerase β , and re-ligated through the action of a DNA ligase. The cleaved abasic site can also be displaced by one of several DNA polymerases as they add two to six nucleosides at the 3' end of the repairing strand. The displaced downstream strand is

cleaved by flap endonuclease 1, whereupon the nick is sealed by a ligase to regenerate the repaired double-stranded helix.

Even though the co-crystals of AP endo with its substrate or product are available,(21) a dynamic picture of how AP endo interacts with DNA containing an abasic site is needed in order to understand its mode of action. One potential technique for probing such dynamic interactions is electron spin-labeling wherein a stable nitroxide is attached to a specific residue on the biopolymer under study. Site-directed spin labeling (SDSL) ESR(22) has become an increasingly important probe of structure and dynamics in biopolymers because of its sensitivity to motion, probe-probe interactions and local environment. Given all the inferences about possible conformational change that AP endo and particularly its substrate DNA might undergo during binding and cleavage,(20,21) this methodology is optimal for detecting and describing the changes. We have recently reported a high-frequency ESR investigation of a new cytosine spin-label in a single-stranded spin-labeled pentamer, TTC*TT in aqueous solution.(23) In this report we present results on a double stranded oligonucleotide with the spin label in various positions relative to a U or an abasic site residue and examine its flexibility by high field ESR.

For studying AP endo-DNA interactions, it is essential to use double-stranded DNA, as the enzyme fails to cleave an abasic site in single-stranded DNA. We report here the first high-field ESR measurements from double-stranded spin-labeled 23-mers. We also present an initial evaluation of the suitability of spin-labeled DNA for studying interactions with the repair enzyme AP endo. We find that it is possible to enzymatically prepare spin-labeled abasic oligomers as substrates, and that AP endo will bind the substrate when it is labeled 3' to the abasic site, but not when the spin label is 5' to the abasic site.

Experimental

Substrates

The substrates used for the experiments consisted of 23 base pair (bp) double stranded (ds) oligonucleotides with a uracil at position 12 (Figure 2). The ESR label (Figure 1) was positioned on the same strand as the uracil, either 2 bp downstream from the uracil (3'L), or 2 bp upstream from the uracil (5'L). As a control some studies were performed on the same oligonucleotide lacking the spin label. When appropriate, the strand containing the uracil was radioactively labeled at the 5' end by means of T4 polynucleotide kinase (PNK) and [$\gamma^{32}\text{P}$] ATP (3000Ci/mmol, Dupont/NEN, Boston MA) as described in references (24) and (25). The presence of the spin label did not interfere with 5'-end labeling. After end-labeling, PNK was heat inactivated at 70 °C for 5 min, the complementary strand was added and the mixture was slow cooled to promote annealing. The ds oligonucleotide was then phenol extracted to remove kinase and subjected to molecular sieve chromatography using Sephadex G50 to remove unincorporated ATP. To prepare the abasic site, ds oligonucleotide strand was treated with *Escherichia coli* uracil DNA glycosylase (UDG) (Ung, Epicentre Technologies, Madison WI; 1 unit/100 pmol of uracil residues unless specified otherwise) as described previously (24). The reducing agent NaBH₄ (0.1 mM unless otherwise noted) was added during the incubation with UDG to minimize the formation of β -elimination product. UDG treatment was terminated by heating the reaction mixture to 70 °C for 5 min, followed by slow cooling to allow for re-annealing of the DNA strands.

Preparation of spin-labeled DNA

The labeled 23-mer was obtained with a post-polymerization modification of the DNA as described by Budil et al. (23). Briefly, standard phosphoramidite synthesis including a convertible nucleoside (N^4 -triazolo-dU, obtained from Glen Research Corp., Sterling, VA) was used to synthesize the oligomer with N^4 -triazolo-dU at the location to be labeled. Synthesis was accomplished on a commercial DNA synthesizer (Applied Biosystems). The triazolo group on the oligomer was substituted with the protected spin label amine (0.1 M, CH_3CN , 2h, RT > 90% conversion), released from the resin, and purified by reverse-phase HPLC using the same program that has been described previously in detail (23). The major peak from the preparative HPLC was collected and hydrolyzed with 80% aqueous acetic acid to give the spin-labeled oligomer. The final product was purified by reversed-phase HPLC on a C18 column by elution with a mixture of (A) 0.1 M triethyl ammonium acetate buffer, pH 7.1, and (B) CH_3CN , with a gradient from 95% A to 80% A. The most significant byproduct was formed by substitution of unreacted U-triazole by ammonia to generate C at the intended label site.

ESR of spin-labeled DNA

A locally constructed 220 GHz ESR quasi-optical spectrometer was used to obtain high-field ESR spectra of the spin-labeled oligomers. A detailed description of this spectrometer has been given elsewhere (26). Briefly, samples were placed in a thin (15-20 μm) aqueous layer held between two 160-170 μm thick quartz cover slips (ESCO) similar to those described by Barnes and Freed (27). The sample concentrations were approximately 1 mM. The labeled oligonucleotides were dissolved in 50 mM HEPES-NaOH, pH 7.4 containing

0.1 mM EDTA. The volume of sample in the active area of the sample cell was approximately 300 nL, although 6 μ l was typically used to fill the cell. Spectra were obtained using standard field-modulation methods with a modulation amplitude of 1.5 G at 100 kHz.

Slow-motional 220 GHz spectra were analyzed using the nonlinear least-squares program NLSL described by Budil *et al.*(28) All spectra from labeled oligomers were fitted using the Microscopic Order Macroscopic Disorder (MOMD) model introduced by Freed and co-workers (29). This model assumes that the label is influenced by an orienting potential that produces microscopic ordering of the molecule relative to an axis that is fixed with respect to the DNA. The potential is given by equation [1]:

$$U(\theta) = \frac{c_0^2}{2}(3 \cos^2 \theta - 1) \quad [1]$$

where θ is the angle between the z -axis of the diffusion tensor and the local director and c_0^2 is an adjustable parameter expressed in units of kT . A diffusion tilt angle $\beta_d = \pi/2$ was introduced so that the diffusion z -axis coincides with the magnetic x -axis (i.e., the direction of the N—O bond).

Thermal melting studies

All melting experiments were performed in an HP 8452 Diode Array Spectrophotometer controlled by a computer interface. A NESLAB RTE-110 variable temperature bath was used to control the solution temperature inside the Hellma quartz cuvette. Temperature inside the cuvette was measured by a thermocouple inserted through a tight-fitting septum. The instrument was blanked on 1 ml of HEPES buffer prior to data acquisition. Samples were preheated to 90°C and allowed to cool to room temperature until the baseline had

stabilized. They were then heated to 105°C at $\sim 2\text{ }^{\circ}\text{C min}^{-1}$ while recording absorbance at 260 nm and temperature every 30 s.

UDG titration experiments

Double-stranded oligonucleotides 3'L, 5'L and U (4 mM) were incubated with UDG at UDG/oligo ratios of 1 U/100 pmol U residues, 1 U/75 pmol U residues, 1 U/50 pmol U residues, 1 U/25 pmol U residues and 1 U/10 pmol U residues in HEPES buffer in a 60 μL volume. The reaction was allowed to proceed at 37°C for 30 min, after which UDG was inactivated by heating at 70°C for 5 min. Oligonucleotide in HEPES buffer but without UDG was included as a control. Twenty μL samples were taken from each substrate mix and either left at room temperature or treated with 0.3 M K_2HPO_4 and brought to 70°C for 150 minutes followed by further addition of 1.5 M K_2HPO_4 to 0.5 M (final concentration). The original oligonucleotide was resolved from the resulting β -elimination product by denaturing polyacrylamide gel (15% gel) electrophoresis in the presence of 8 M urea, after which the dried gel were exposed to a phosphorImager screen and scanned with a Storm 840 phosphorImager. The distribution of substrate and product was quantified by using ImageQuant software.

Single turnover AP endo binding experiments:

AP endo was obtained by purification of the enzyme after expression in *E. coli* strain BL21DE3pLys from the pXC53 vector as described earlier (24). To determine whether AP endo could recognize and bind spin labeled ds oligomers containing an abasic site, 4 nM enzyme and 4 nM substrate (final concentration) were mixed in the presence of 4 mM

EDTA for time intervals between 0 and 30 s. After binding, 10 mM Mg^{2+} (final concentration) and trap (heparin 100 mg/ml and HDP 23 nM) were added to allow cleavage of substrate that was already bound to enzyme. HDP was the heat degradation product of an 11mer ds oligonucleotide containing an abasic site at position 6(24). After a 20 s incubation with Mg^{2+} and trap, the reaction was terminated by adding EDTA to a final concentration of 87 mM. Substrate and product were resolved and quantitated as described above.

Results and Discussion

High-field ESR of labeled ds DNA

The high-field ESR spectra from the labeled double-stranded 23-mers (Figure 3) were strikingly different from that of labeled monomer in aqueous solution, which consisted of a single broad line (23). The most prominent feature of the spectra for both labeled double-stranded 23-mers was a well-resolved low-field (“x”) peak, which remained at a relatively constant position at near-physiological temperatures. The “y” and “z” peaks that normally appear in rigid limit spectra were not observed; instead, these peaks were significantly broadened and shifted together into a single, wide feature on the high-field side of the spectrum. These features are qualitatively representative of high-field spectra observed from shorter, single-stranded spin-labeled oligomers in viscous media and are consistent with preferred rotation about the x-axis and significant ordering of this axis relative to the DNA.(23)

The spectra were analyzed using the MOMD model described in the Experimental section. The rotational diffusion constants of the probe, R_{\perp} and R_{\parallel} , and the ordering potential coefficients c_0^2 determined for the 3’L and 5’L species are given in Table 2. The

resulting parameters were quite similar for the two sites, suggesting that there was no positional variation in the local structure and probe ordering near the U site. The local ordering determined from fitting the MOMD model to the 220 GHz spectra was approximately 0.55 for each of the two labeled ds DNA species studies.

Our high-field results are consistent with previous estimates of local ordering based on low-frequency ESR of dsDNA labeled with a 5-atom flexible tether (30,31). At lower fields, it is necessary to account for both the global tumbling of the DNA and the local motion of the label using the sophisticated Slowly Relaxing Local Structure (SRLS) model (32). In their analysis of the low-field results, Liang *et al.* (31) used a hydrodynamic model to estimate the global tumbling rate for the DNA and determined the local motion parameters by least-squares fitting. They found two populations of spin probes, including a small relatively disordered fraction, and a predominant, highly ordered fraction with order parameter $S=0.61$. Thus the MOMD model provided an accurate picture of local ordering from high-field spectra even in small or intermediate-sized ds DNA oligomers, without the need to apply a hydrodynamic model to describe the global motions.

AP endo binding experiments

To determine whether AP endo was able to bind abasic site-containing ds oligonucleotide when a spin label was present two nucleotides away from either side of the abasic site, we employed single turnover kinetic methodology (24). Our experiments indicated that single turnover data provide a more accurate reflection of binding as related to potential enzymatic activity than the more traditional electromobility shift assay. (M. Fattal, N. O'Regan and P. Strauss, manuscript in preparation)

Thermal melting studies were first used to confirm the double-strandedness of the 3' and 5' spin labeled oligonucleotides. The melting point for the ds 23-mer was 47 °C, while the presence of the spin label in either location decreased the melting point temperature by 7.8 °C and 6.2 °C respectively. These data indicated that the spin labeled 23-mers were somewhat less stable than the parent without the spin label, although they were double stranded under the room temperature conditions employed in this series of experiments. The observed change in melting point was larger than that previously observed in melting studies of spin-labeled poly dA·dU(33). However, the ratio of spin label to the number of bases was approximately 0.02 in the present study, significantly higher than that used in the previous studies(< 0.01). Given the relatively short length of the AP endo substrate (23-mer) used in our studies, the observed change in melting temperature is not surprising. A more stringent test of the intactness of the ds DNA was the ability of UDG and AP endo to bind to and process the DNA.

After labeling the 5' end of the U-containing strand with [³²P] and forming the ds oligonucleotide, the parent ds oligonucleotide and the two spin labeled oligonucleotides were treated with UDG to generate the abasic site at position 11 (See Methods). To ensure that the presence of the spin-label did not interfere with creation of the abasic site, the two spin-labeled substrates were titrated with UDG. UDG removed uracil from all the three substrates used, even at the lowest concentration of 1 U/100 pmol uracil residues, which is the concentration used in binding experiments (data not shown).

Figure 4 compares the $[ES]_e$ for 3'L, 5'L and the non-spin-labeled ds 23-mer. Equilibrium levels of $[ES]$ for non-labeled oligonucleotide were reached before 5 s, as expected for these concentrations of substrate and enzyme from previously published

studies,(4) while equilibrium levels of [ES] for 3'L were reached by 8-10 s. The K_d for this 23-mer parent ds oligonucleotide with a single abasic site at position 11 was 60 nM, while that for the 3'L ds oligonucleotide with a single abasic site was 28 nM. As binding to the 5'L ds oligonucleotide with a single abasic site was unstable, it was not possible to report a $[ES]_e$ or a K_d for that substrate under these conditions. These results indicate that the enzyme ability to bind to the DNA is directional and that any disturbances made to the substrate in the 5' position relative to the abasic site have a significant effect on the enzyme-substrate interaction.

The results obtained are supported by previous data in which a second abasic site is positioned on the opposite strand and either 5' or 3' relative to the first. If the second abasic site is located to the 3' side of the abasic site no significant reduction of binding and nicking activity is seen; however, if the second abasic site is positioned 5' to the first, enzymatic activity is greatly decreased, especially if the second abasic site is located 3 bp away from the first. (34) Surprisingly, the cocrystal of enzyme and substrate formed in the presence of Mn^{2+} indicates direct interaction of the enzyme at the phosphate between residues located -1 and -2 *and* between residues +1 and +2 to the abasic site.(21) We therefore expected that the spin label in both positions might have interfered with enzymatic binding and subsequent cleavage. The observed activity of AP endo with 3'L thus suggests that the active enzyme substrate complex is considerably more flexible than is indicated by the crystal structure. Further support for this notion comes from the apparent inability to crystallize the enzyme in its native conformation at pH 7.4 in the presence of Mg^{2+} .

Conclusion

We have presented the first observation of spin-labeled ds DNA by high-field ESR. The high-field ESR line shape was analyzed for spin-labels at two locations on a double-stranded 23-mer. Application of the microscopic order, macroscopic disorder (MOMD) model to high-field spectra gives a local order parameter of approximately 0.55, in excellent agreement with recent low-field studies using the more complex slowly relaxing local structure (SRLS) model. The perturbation of the dsDNA introduced by the spin label was evaluated using both thermal melting studies and enzymatic assays of the activity of UDG and AP endo. A ds 23-mer oligonucleotide containing a central U residue in one strand with the spin label displaced in either direction by two base pairs can be phosphorylated at its 5' end with polynucleotide kinase and undergo processing by UDG to give an abasic substrate for AP endo. However, AP endo will only bind and cleave when the label is 3' to the abasic site.

One of the long-term objectives of this work is to study interactions between AP endo and its substrate using spin labels on the oligonucleotide substrate itself. Aside from a study of EcoRI and its spin-labeled substrate by Keyes *et al.*(35), there has been relatively little work reported on DNA-protein interactions using spin-labeled DNA. They utilized labels with a flexible 5-atom tether and monitored the local order parameter S of probes placed 6, 9, and 11 base pairs from the EcoRI binding site. Only a slight increase in ordering was observed for the most proximal position, which was attributed to bending of the DNA induced by protein binding rather than direct label-protein interactions. The results of the present study suggest that double label experiments with spin labels on both

the protein and the ds DNA substrate should allow one to monitor structural changes induced by ligand binding with a high degree of precision.

Table 1

Magnetic Parameters of Cytosine Spin Label*

| | |
|--------------------|----------------|
| $g_x^{\#}$ | 2.00879 |
| $g_y^{\#}$ | 2.00586 |
| $g_z^{\#}$ | 2.00210 |
| A_x/Gauss | 5.5 ± 0.3 |
| A_y/Gauss | 5.8 ± 0.3 |
| A_z/Gauss | 37.0 ± 0.2 |

*Determined by least-squares analysis of rigid limit 220 GHz spectrum of cytosine spin label

[#]Estimated uncertainty in g-values is 5×10^{-5}

Table 2

Dynamic parameters for spin label motion determined by least-squares fitting to the spectra shown in Figure 3 using the MOMD model described in the text.

| | 5'L | 3'L |
|---------------------|----------------------------------|----------------------------------|
| $R_{ }$ | $5.1 \times 10^8 \text{ s}^{-1}$ | $5.6 \times 10^8 \text{ s}^{-1}$ |
| R_{\perp} | $1.3 \times 10^8 \text{ s}^{-1}$ | $1.1 \times 10^8 \text{ s}^{-1}$ |
| c_0^2 | 2.3 kT | 2.6 kT |
| $\langle S \rangle$ | 0.53 | 0.55 |

Figure Captions

Figure 1

Structure of cytosine-analog spin label.

Figure 2.

Sequences of spin-labeled double-stranded DNA substrates used in this study. (a) unlabeled oligonucleotide U, with a uracil placed at the location where the abasic site will be generated. (b) oligonucleotide 3'L, spin-labeled 2 base pairs 3' to the abasic site; (c) oligonucleotide 5'L, spin-labeled 2 base pairs 5' to the abasic site.

Figure 3

220 GHz ESR spectra of double-stranded DNA 23-mers (a) 3'L and (b) 5'L. 220 GHz ESR spectra of a double-stranded DNA 23-mer labeled 2 bases in the 5' direction from the center (lower strand in Figure 2b) obtained as a function of temperature. Solid lines show least-squares fits of the ESR line shape using the MOMD model as described in the text.

Figure 4.

Binding of AP endo to ESR-labeled (3'L or 5'L) and non-spin-labeled oligonucleotide (none). Enzyme was incubated under single turnover conditions with AP endo for 0, 5, 6, 8, 10, 15, 20, 30, 40 seconds, after which Mg^{2+} /trap were added. The reaction was allowed to proceed for 20 seconds and stopped with EDTA.

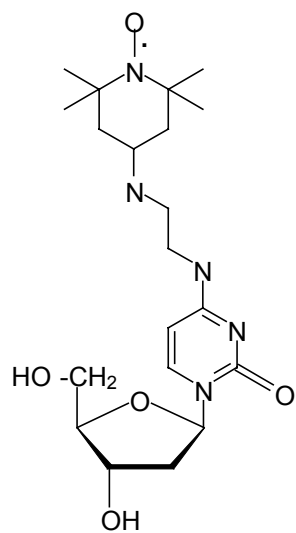


Figure 1
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a.

5' - CTCTCCATACAUACATATCCTCT - 3'
3' - GAGAGGTATGTATGTATAGGAGA - 5'

b.

5' - CTCTCCATACAUAC^LATATCCTCT - 3'
3' - GAGAGGTATGTATG TATAGGAGA - 5'

c.

5' - CTCTCCATAC^LAUACATATCCTCT - 3'
3' - GAGAGGTATG TATGTATAGGAGA - 5'

Figure 2
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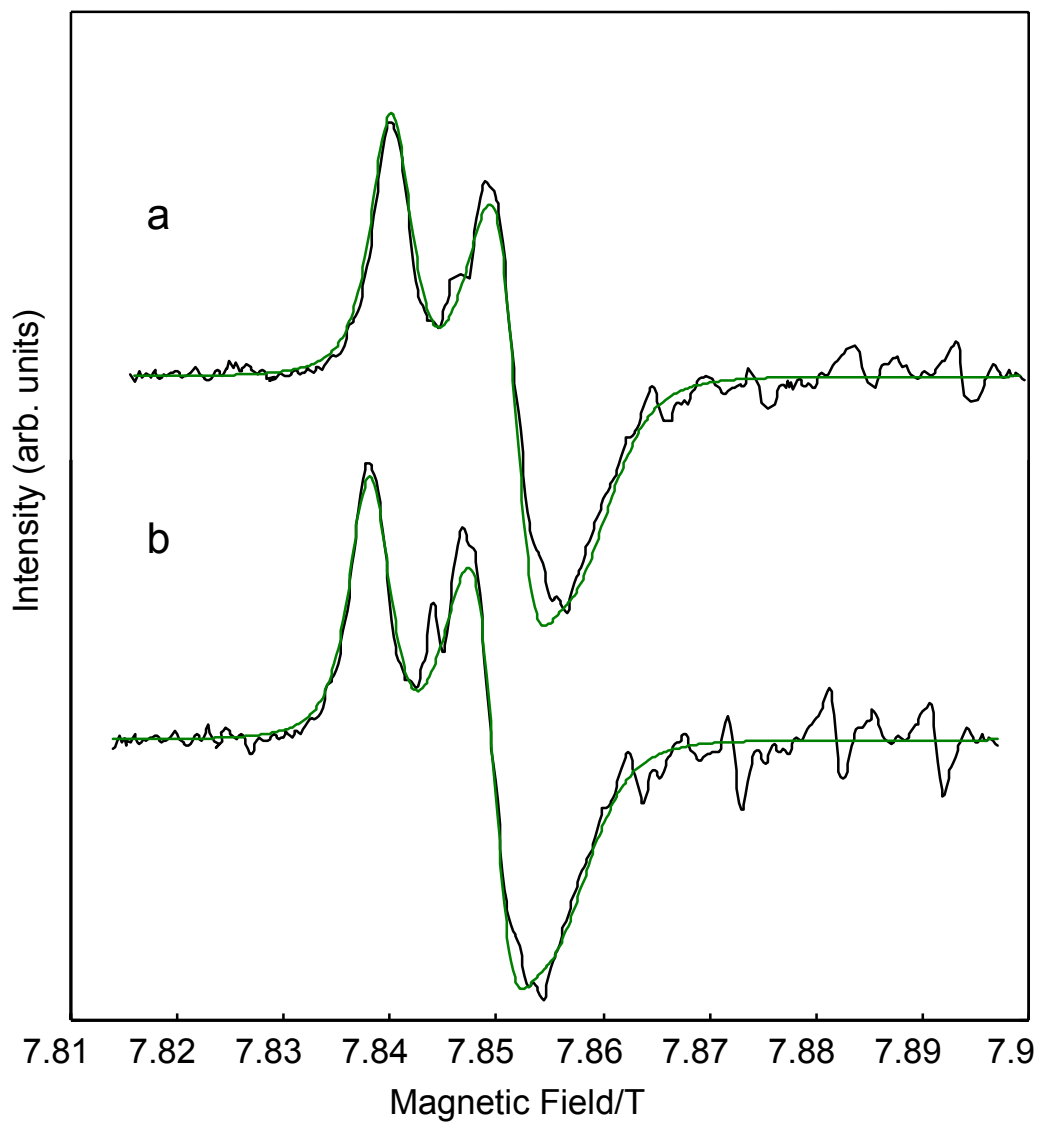


Figure 3
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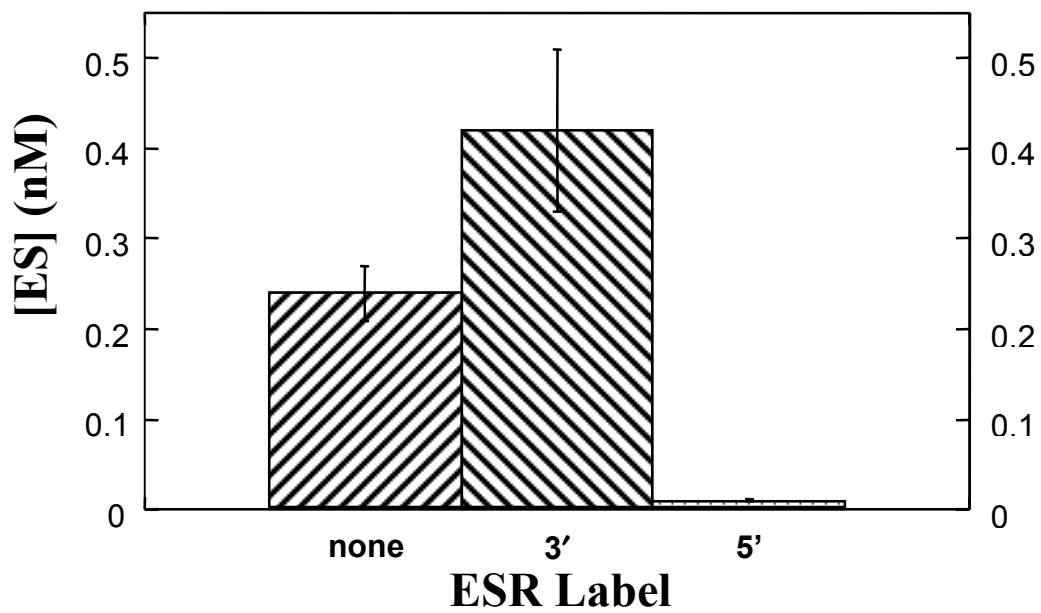


Figure 4
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