Mobility measurements by EPR of the anthracis repressor protein

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Abstract

Metal-binding regulatory proteins belong to an important class of proteins that help bacteria maintain metal homeostasis. In 2003, a protein belonging to this family was discovered in *Bacillus anthracis*, the bacteria responsible for anthrax.[1] I invested the metal-binding behavior of this protein, the anthracis repressor (AntR). In particular, I used continuous wave electron paramagnetic resonance (CW EPR) to measure the local mobility changes caused by the addition of Zn(II) and the target DNA of AntR. Although definite conclusions cannot be made at this point about the addition of DNA to AntR, the addition of Zn(II) reduced the intermolecular mobility of AntR at three different sites.

Introduction

The Anthracis repressor, or AntR, is a 142 residue protein that was recently identified and cloned from *Bacillus anthracis*.[2] It belongs to the Diphtheria toxin Repressor (DtxR) family, a family of metal-binding repressors found in bacteria. Many members of the DtxR family have been well-characterized and their crystal structures have been solved, but AntR has not been characterized and its structure has not been solved. AntR does, however, share an 89% similarity with one of the members of the DtxR family: MntR, a protein that has been characterized, and whose crystal structure is known. Like AntR, MntR is a 142 residue protein similar to DtxR, except that AntR and

MntR have slightly different metal-binding behavior, and they are smaller than DtxR because they do not contain an SH3-like domain near their C-terminal.[3] Also, in MntR, dimerization occurs in both the holo and apo forms, whereas in other DtxR homologues, metal binding affects dimerization.[4] Because of these similarities, MntR will be used as basis for studying the structural changes due to metal-binding of AntR.

I am examining the effects of metal binding on the intermolecular mobility of dimerized AntR at three sites through the use of electron paramagnetic resonance, or EPR, performed on spin labeled mutants. The mutant residues to be studied are 37, 104, and 124.

Experimental

K. Sen developed the experimental procedure described below.

Materials and methods: Expression, purification, and spin labeling

Wild type AntR was received as an insert in the pet11b plasmid. Mutagenesis was performed on the wild type AntR, producing single cysteine mutants at residues 37, 104, and 124. The plasmids with the mutated sequences were transformed into BL21-CodonPlus(DE3)-RIL competent *E. coli* cells and grown in TB media using a self-induction protocol. The cells were lysed with a microfluidizer in the presence of the protease inhibitor PMSF. The solutions were centrifuged at 20,000 RPM for 30 minutes to remove the insoluble cell walls from the soluble protein. Following the centrifugation, the supernatant was filtered and loaded on an anion exchange column at pH 8.5 in a 50 mM tris buffer, and a NaCl concentration ranging from an initial value of 0 to a final value of 2 M. The protein eluted from the column and collected, as confirmed by a SDS PAGE gel of the fractions. The fractions containing AntR mutants were mixed with an

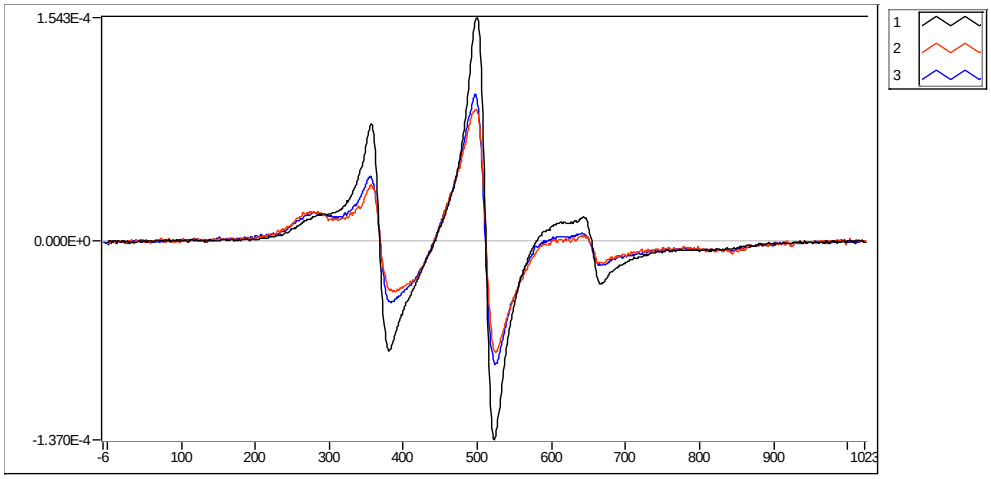
equal amount of 8 M urea, and incubated at 37ºC for 10 minutes to ensure complete unfolding. EDTA was added to a final concentration of 20 mM remove trace metals, and DTT was added to a final concentration of 10 mM to reduce cystine. This solution was dialyzed using an 8,000 molecular weight cutoff membrane for three times in 10 mM Hepes with 500 mM NaCl at pH 7.1 ± 0.1. The protein solution was loaded onto a desalting column in 10 mM Hepes with 500 mM NaCl, pH 7.1 ± 0.1, and the resulting fractions were pooled, divided into three, and spin labeled with three different spin labels. The three spin labels are maleimide spin label (MSL), methanethiosulfonate spin label (MTSSL), and iodoacetamide spin label (IASL). The solutions incubated for 30 minutes at 37ºC, and then they were dialyzed four times in the presence of Chelex, against 10 mM Hepes with 500 mM NaCl at pH 7.1 ± 0.1. The solutions were concentrated with an Amicon concentrator to concentrations around 100 mM.

EPR methods: Continuous wave (CW) EPR was performed on the samples, in apo form, at room temperature. The room temperature CW EPR was performed on a Bruker EMX spectrometer with a TE_{102} cavity in 50 μL capillaries. Zn(II) was added in a 1:1 ratio to the protein, so that the holo form of the protein could be examined. Target DNA sequences were added to the holo forms of mutants 104 and 37, and CW EPR was performed on these samples as well.

Results and Discussion

The experiments performed to this point suggest that stoichiometric amounts of Zn^{2+} cause mobility changes at sites 37, 104, and 124. The experiments also support the assertion that AntR's structure is similar to that of MntR. These local changes in mobility may provide insight into the structural changes of AntR in response to metal-

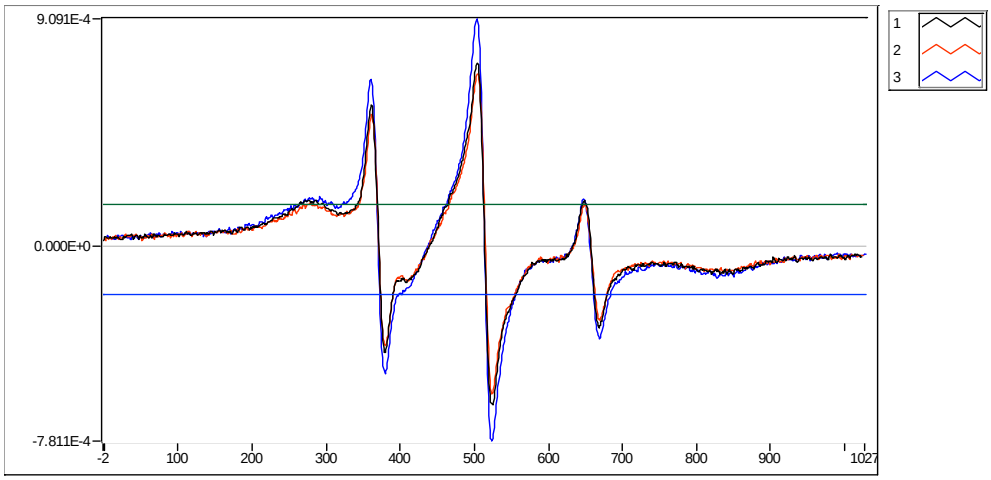
binding. Specifically, these changes will allow us to create global models for the changes that occur as a result of metal binding.



CW spectra for the 37th residue with the spin label MSL. The black line is the apo form, the red line is the holo form from the addition of Zn^{2+}, and the blue line is the holo form plus complimentary DNA.

These experimental set-ups contain certain limitations, however, which restrict our conclusions to preliminary assertions. Data from both the holo form of AntR, and the form containing complimentary DNA may be misleading for a number of reasons. Of all the ions examined in the literature, including Mn^{2+} and Fe^{2+}, AntR binds most strongly to Zn^{2+}.[5] Zinc-bound AntR, however, only weakly activates repressor activity. Therefore, one must consider the possibility that Zn^{2+} may not be physiologically relevant, and AntR may make conformational changes in the presence of Mn^{2+} or Fe^{2+} that do not occur in the presence of the more strongly bound Zn^{2+}. Although this possibility would be difficult to confirm, it still must be addressed before stronger assertions can be made. Secondly, high concentrations of Zn^{2+} appear to cause the protein to precipitate, which may lead to signal broadening if the poorly organized protein is closely packed. Without ultracentrifugation data on the holo form of AntR, definite conclusions cannot be made.

Tests performed on the DNA-bound form of AntR are also inconclusive without global measurements that indicate that the DNA actually binds to the zinc-bound, spin-labeled AntR. In fact, our initial results suggest that DNA binding may not even occur in the presence of zinc, based on the CW EPR spectra for residues 37 and 104.



CW spectra for the 104th residue with the spin label MSL. The black line is the holo and DNA form, the red line is the holo form, and the blue line is the apo form.

This problem can be addressed most directly with a binding assay, followed by circular dichroism to determine if the zinc-bound form of AntR undergoes structural changes with DNA binding.

Conclusions

Three mutants of AntR were expressed, purified, spin-labeled, and studied in the apo, holo, and DNA-bound forms using CW EPR. These preliminary studies suggest that metal binding causes local structural changes, while the addition of complimentary DNA may not affect intermolecular mobility. Further experiments are still necessary to address these issues.

References Cited:

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[5] Sen, Kadir Ilker. *ibid.*