Unfolding the structure of *Clostridium perfringens*

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Abstract

Clostridium perfringens is an anaerobic, Gram-positive bacteria typically found in the human intestinal tract. *C. perfringens* contains an enzyme called azoreductase (AzoC) which cleaves azo dyes. Azo dyes are widely used in many parts of consumerism, including the food and textile industries. Certain metabolic azo dye products can cause cancer creating concern among the medical and environmental community. However, it is difficult to predict how the enzyme will react with a particular azo dye given that the actual structure of the enzyme is unknown. Determining the structure of the enzyme would allow for a better understanding of the physiological role of the enzyme, which could aide in medicinal practices and solving environmental problems. This study used crystallography methods to produce protein crystals. AzoC protein was extracted and purified from an *E.coli* expression system. The experimental approach used to optimize the protein crystallization conditions involved minor changes associated with the pH, salt concentration, substrate dyes, and buffers, using a 96 well Additive Screen or 24 Custom Screen plates. The screen plates created were observed daily for crystal growth. Sufficiently formed crystals were collected, frozen and transported to the National Synchrotron Light Source for X-ray diffraction. The results will provide important structure information for AzoC, which will impact the medical and environmental community.

Introduction

•*Clostridium perfringens* is an anaerobic, Gram-positive bacteria that can be found in the human intestinal tract. *C. perfringens* contains an azoreductase enzyme, which cleaves azo dyes. Azo dyes are found in many parts of consumerism, including food, pharmaceuticals, and clothing. These dyes are reduced by the azoreducatse enzymes located within the body and produce aromatic amines, which is a pre-cursor for cancer. Azo dyes are also an environmental concern as they toxify the environment after disposal.
•AzoC is unique in that its primary sequence is dramatically different to other azoreductases as there is less than 12% identity with other known microbial azoreductases. Thereby, generating reliable 3-D images using computational methods are not possible. Currently, there is no 3-D image of AzoC as crystallization of the protein has not be done.
•The purpose of this study is to determine the 3-D structure of AzoC, a heterologous expressed protein. The approach used is the sitting drop crystallization method.

Materials and Methods

•The AzoC gene was inserted into the pET15b plasmid (N-terminal His tag) and expressed in *E. coli* (DE strain).
•The AzoC protein was auto-induced using non-inducing MDAG-135 media (37°C, 0.6 O.D.), followed by auto-inducing media, ZYM 5052 (20°C, overnight).
•Cell lysate was collected using the Bugbuster method (Novagen).
•The AzoC protein was purified via the Ni-NTA column method (Qiagen). Washes contained 20mM, 100mM, 200mM, and 500mM Imidazole. The presence of pure AzoC was confirmed by SDS-PAGE gel.
•The AzoC sample was concentrated using centrifugal filter units (Amicon, 30,000 M.W.) to 5mL and loaded onto the FPLC size exclusion column (Sephadex G-50)
•From the FPLC, AzoC was monitored using the UV absorbance at 280 nm. Fractions (10mL) representing AzoC were collected, combined, and concentrated to 10-20mg/mL.
• The concentrated protein sample was used for protein crystallization (Sitting drop method). A Phoenix robot (Art Robins Instruments) was used to setup 96-well plate for an Additive Screen (Hampton Research company). 24 well plates were used to further optimize positive results (manual loading of samples).Protein crystallization plates were incubated at 10°C and check every day using a 40X microscope.
•Condition #1 (02M Na2H/KH2 phosphate, 20% PEG 3350, and 0.1M BisTris proprane pH 7.5) was used in the Additive Screens.(96 well conditions)

Results

•Figure 1 shows the SDS gel and the corresponding proteins associated with the protein purification process.
•Figure 2 is an example of a 96 well plate used in the crystallization process (blue dots represent protein and dye substrate).
•Table 1 summarizes the conditions and observed crystals. Photos for all crystals taken with a microscope Nikon camera.
•Table 1, Conditions A, B, and C are Additive Screens with corresponding crystal images (Figures A,B, C)
•Table 1, Conditions D (w/o dye) and E (w/ dye) are 24-well plate optimizations with corresponding crystal images (Figures D and E).
•Figure 4 shows a salt crystal X-ray diffraction pattern obtained from the National Synchotron Light Source, which is indicative of a salt crystal. Protein crystal patterns would have more centralized black dots in the center of the image.

Results

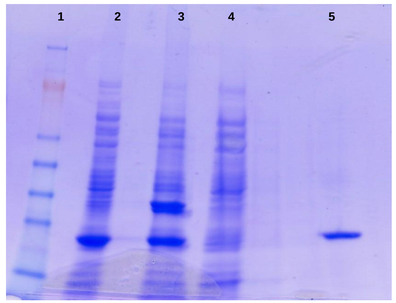


Figure 1: SDS-PAGE Gel in Phosphate Buffer. Lanes 1, MW marker; 2, total lysate; 3, supernatant; 4, flow through; and 5, elusion (AzoC)

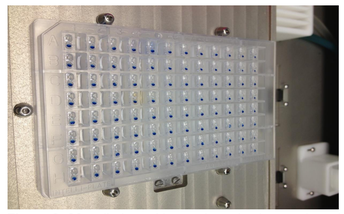


Figure 2: 96-well plate with protein and substrate (azo dye)

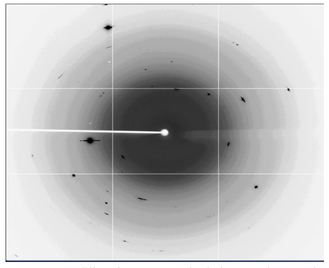


Figure 4: X-ray diffraction pattern depicting a salt crystal.

Table 1

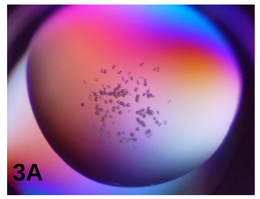
Condition	Crystal Structure
A.) 0.02M Condition #1 with an additive of 0.1M Manganese (II) chloride tetrahydrate	Radial/star shaped (Figure 3A)
B.) 0.02M Condition #1 with an additive of 0.1M Zinc chloride	Circular shaped (Figure 3B)
C.) 0.02M Condition #1 with an additive of 0.1M spermine tetrahydrochloride	3 needle shaped ~7 star shaped (Figure 3C)
D.) 0.02M Condition #1 without substrate	Numerous, needle shaped (Figure 3D)
E.) 0.02M Condition #1 with substrate	Numerous, needle shaped (Figure 3E)

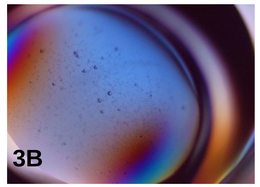
Conclusions

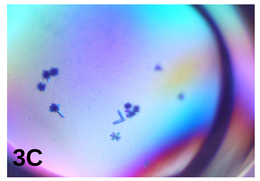
•Most wells from the Additive Screen and Optimization Screen produced precipitate.
•Salt conditions generated salt crystals after 1-3 days, rather than protein crystals.
• Addition of the dye substrate did not result in protein crystals, only salt crystals..
• Each salt condition produced a different salt crystal formation.

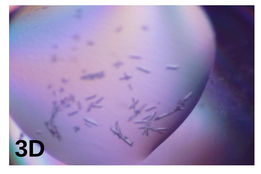
Discussion

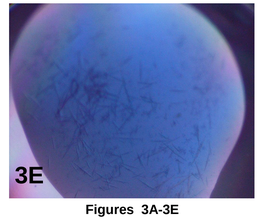
Protein crystal formation for AzoC from *C. perfringens* has proven to be a challenging task. The unique sequence homology and function of AzoC may support this. Based on the results, we determined that the amount and concentration of salts present in the solutions can greatly affect the amount and type of crystals produced. Additives can also influence the shape of the crystal formed. Based on these observations, future efforts will keep the salt concentration as low as possible in the initial solution. In addition, different types of 96 well screens are needed to determine the optimal condition(s) need to produce protein crystals.











Figures 3A-3E

Acknowledgments

I would like to thank Dr. Swaminathan, Dr. Eswaramoorthy, Dr. Kumaran, and Dr. John for all of their support and encouragement with this project. I would also like to thank Brookhaven National Laboratory for sponsoring this internship.

References

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