Crystallization of a Bacterial Single Stranded Annealing DNA Repair Protein

Alburuj Rahman, Charles Bell, Jinwei Hu

Erratum: This article was originally published without crediting Charles Bell and Jinwei Hu as co-authors. The article has been updated. 07/11/14.

ABSTRACT Single-stranded annealing (SSA) proteins bind to single-stranded DNA (ssDNA) and promote the pairing of homologous DNA strands, a process that is involved in the repair of double-stranded breaks. This work focuses on RecT and β protein, from Escherichia coli and bacteriophage lambda, respectively, which are two classic examples of SSA proteins. The human protein Rad52 promotes the same reactions as RecT and β, and is involved in multiple DNA repair pathways. Cells and DNA have mechanisms that repair mutations and failure to do so may lead to cancer. Chemotherapy requires study of repair mechanisms. Therefore, it is important to understand the DNA repair processes of RecT and β. Electron Microscopy studies propose that the proteins form oligomeric rings and helical filaments, but information on the fold of the proteins and the DNA binding sites on the surface of the oligomers is not available. This information can be obtained from high resolution images of a protein's structure. The aim of this study is to determine the mechanism by which SSA proteins bind to ssDNA and promote the annealing of complimentary DNA strands. This will increase our general understanding of how proteins recognize and repair damaged sites of DNA in cells. Our approach will employ the powerful technique of x-ray crystallography to determine the three-dimensional structure of SSA proteins at high resolution. This technique requires that the SSA protein be crystallized. A sample of RecT or β is purified and screened for initial crystals which are then optimized to grow larger crystals. Once large crystals form, x-ray diffraction is used to solve the three-dimensional atomic structure of the protein, preferably in complex with DNA substrates. The first large crystals of RecT that can diffract to 6-7 Angstroms have been formed in the lab, which is a significant step toward understanding the protein repair mechanism, which will ultimately provide a foundation for improved applications in cancer treatment.

INTRODUCTION

Protein atomic structures reveal the fundamental processes of life and can be studied by protein crystallography. Protein crystallography is a powerful technique employed to determine the structure of proteins and protein-DNA complexes by creating crystals and using the crystals to diffract x-rays. The main bottleneck in the process of protein crystallography is to produce structurally and internally well-ordered crystals. Such crystals allow for x-ray diffraction to produce high-resolution images of a protein. Other challenges when investigating a protein's structure include difficulty with expression, purification, or stability [12].

Novel approaches have been made for the improvement of the crystallization process. One study has discovered new methods to effectively study the structures of proteins by creating a hybrid model to predict the outcome of protein crystallization by using experimental and sequence data to "prioritize and direct efforts of structural genomics" (Zucker et al.) [14]. Films have been examined after x-ray diffraction for analysis of the level of bioactivity on the film (Hader et al.) [13]. New techniques, such as evaporation (to determine the phase of a drop at any point in time) and solubility diagrams (to conserve more protein than the amount used in optimization trays), have been administered to produce more accurate and defined crystals and minimize the challenges (Talreja et al.) [12].

Single-stranded annealing (SSA) proteins bind single-stranded DNA (ssDNA) and promote the pairing of homologous DNA strands. This is an important step in the repair of double-stranded breaks by homologous recombination [11]. RecT from Escherichia coli (E. Coli) binds to ssDNA and is involved in recombination by promoting the annealing of complimentary DNA strands [11]. In eukaryotes, Rad52, a 400 residue protein, promotes the same reactions as RecT and Redβ (β protein), and is involved in DNA synthesis and multiple DNA repair pathways [2]. Although Rad52 shares the same functions as RecT and β protein, this study

focuses on the bacterial proteins because they are easier to obtain in the large quantities needed for structural analysis. Also, RecT and β protein are being employed in powerful new biotechnology applications, such as a new method for genetic engineering known as "recombineering" [2].

The human genome consists of sequences of DNA, which are subject to mutations. Cells and DNA have mechanisms to repair mutations; however, failure to do this may lead to cancer. Therefore, it is important to understand how the DNA repair processes of RecT and β protein work so that such mistakes can be prevented.

Both RecT and β protein have been found to promote the same reactions as Rad52 (Hall et al.) [2,7]. Electron Microscopy (EM) studies show that RecT and β protein form oligomeric rings and helical filaments (Passy et al.) [2,10]. Furthermore it has been predicted that the β protein forms oligomeric rings with the ssDNA bound along the outside of the ring, and helical filaments formed on the duplex product of annealing (Passy et al.) [2,10]. β protein has been found to bind weakly to ssDNA, not to all double-stranded DNA (dsDNA), but very tightly to the duplex product of annealing (Karakousis et al.) [2,8]. Another recent study shows that Rad52 and the RecT/β protein family are related to one another (Erler et al.) [2,5]. This all indicates that RecT and β protein are effective alternatives to Rad52 and that some of their functions have been revealed.

Although, through many studies, the main functions of these proteins have been revealed, the structures of RecT and β protein had not been solved. There are no crystal structures of RecT and β protein homolog available [2]. There is no information on the fold of the proteins and the suggested DNA binding sites on the oligomers are approximations [2]. A study has produced a low-resolution image of the proteins, however, a high-resolution structure is not yet available [2]. This study aims to determine the mechanism by which SSA proteins bind to ssDNA and promote the annealing of complimentary DNA strands. Protein crystallography and x-ray diffraction will be used to solve the three-dimensional structure of RecT and β protein at atomic resolution.

METHODS

Purification

SSA protein that had been prepared for crystallization through purification was studied. A 231 residue bacterial protein sample of RecT, Lactobacillus planatarum (LPRect231), was cloned into a plasmid for expression of the N-terminal fragment in E. Coli. The protein was purified by Ni2+ -affinity and anion exchange chromatography, concentrated to 20-30 mg/ml, and frozen at -80° Celsius. A similar procedure was carried out for β protein.

β176 and β177, two N-terminal fragments of β protein, were purified. A plasmid was initially transformed into BL21AI competent cells. Cell cultures were then grown in LB medium. The cells were resuspended and sonicated to lyse the cell and extract the protein. Ion exchange chromatography, which separates protein molecules based on their polar charge, and affinity chromatography, which separates the protein based on an engineered tag, was used to purify the protein. Several SDS-Page Gels were run to examine the purity of the protein. The protein was finally concentrated.

Screening

Screening is the process by which versatile premade reservoir agents and solutions are mixed with protein DNA complexes to find preliminary crystals or "hits," which can then be used for optimization of the hit conditions. LPRect231 was initially tested with the Wizard I™ and II™ screening kits from Emerald Biosystems and then with the Salt Rx™ kits from Hampton Research [Fig. A]. The Wizard™ kits contain all different types of agents, and the Salt Rx™ kits contain all different varieties of salt compounds. Although Wizard I™ and II were sparse-matrix screens, our preliminary crystals were found with the salts. The method used for crystallization was hanging drop vapor diffusion, in which the 2 to 4 µl drops of protein, reservoir, and/or DNA becomes supersaturated and more concentrated as water diffuses from the drop to the more concentrated reservoir solution in the well below it. This technique is described in detail by Chayen and Saridakis [4].

Eventually, LPRect231 and β protein complexes were tested with several other screening kits through the Mosquito instrument. After trays were completed when using screens, one-three days were usually needed before being crystal formation was noticeable.

Optimization

The objective of optimizing is to grow larger, high-quality, three-dimensional crystals by finely manipulating the conditions of a hit. A typical setup is

Fig. A. A salt Rx screening kit

shown in Fig. B. Usually, 500 µl to 1 ml of reservoir were prepared for each of the twenty-four wells in a tray by mixing different buffers, stock solutions, and water. The LPRect231 protein was mixed with DNA and dilution buffer Tris pH 8.0 to make a protein DNA complex and to lower the concentration of LPRect231 to 10 mg/ml. β protein was only mixed with Tris pH 8.0 when making its complex as no crystals were grew with it when mixed with DNA.

Initially, four DNA lengths were studied: 32, 36, 40, and 44-mer length nucleotides in the polymer Poly-dT, named DT32, DT36, DT40, and DT44 respectively for this study. The preliminary crystals [Fig. C] grew from Salt Rx set 2 only for DT40, (although DT32 and DT44 were also used) under the conditions: 3.5 M Sodium Formate with Tris pH 8.5. Optimization of the given conditions was carried out using Sodium Formate as the precipitating agent and four buffers pH 8-9 including Tris. Larger crystals formed under the same conditions as the hit from the previous screening. After further optimization, variables such as pH, temperature, protein concentration, DNA length, precipitating agents, cryo protection and drop volume were varied in efforts to improve the quality and size of the crystals.

Fig. B. A typical setup for optimization with various solutions (top), reservoir cocktails (bottom-left) and crystal tray (bottom-right).

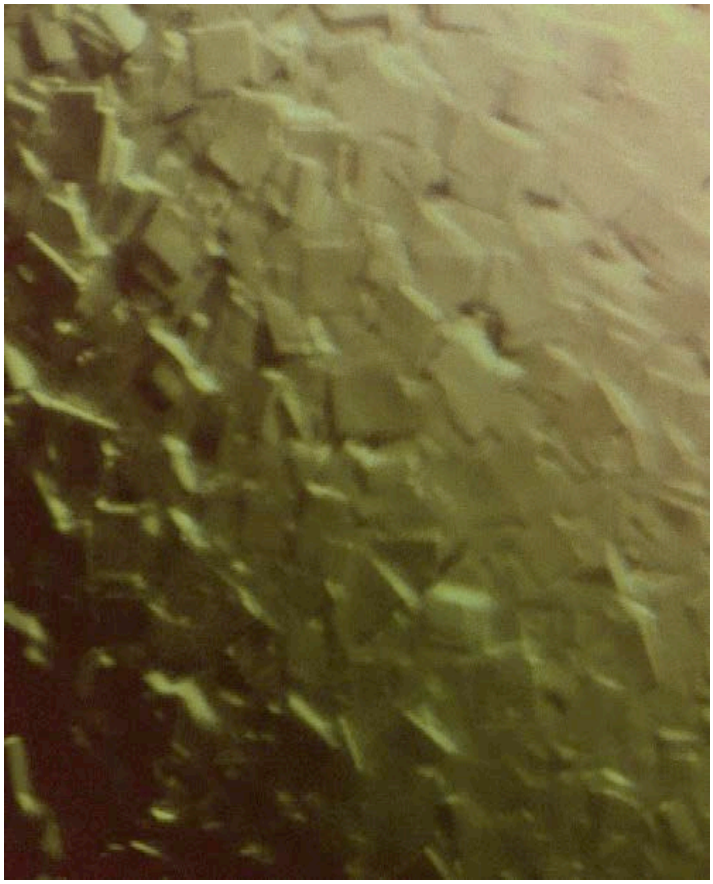

Fig. C: Preliminary crystals grown with Sodium Formate

Optimizations were then completed with a new crystal condition, with precipitating agent Peg 3350, found through a screening kit, Index. Experiments with this were carried out with different DNA lengths such as 38, 40, 42 and 40 5' Phosphate-mer, and various buffers.

Analysis of Trays

Two to three days after completing a tray, the hanging drops were analyzed under a dissecting microscope, in search of crystals. Observations and notes were taken every few days recording the saturation phases of each drop including: the number of clear drops, precipitate build-up, and growth of crystals. After finding new crystals, further optimization of the hit conditions was carried out, honing in closer to the hit's molarity and

Fig. 1: A sheet of small crystals, formed by a Sodium Formate optimization, similar to the crystals formed in the initial salt screen

increasing drop sizes. Film pictures of the best crystals were taken and then several of the crystals were used for x-ray diffraction. By continuing to optimize the crystals, the goal was to improve the internal structure and size of the crystals to get strong diffraction patterns.

RESULTS

LPRect231 was purified and then used with sparse-matrix screens Emerald II™, a disperse compounds kit, producing no crystals. When used with the Salt Rx™ screening kit, crystals formed at the condition of 3.5 M Sodium Formate with buffer Tris pH 8.5 and with the 40-mer (DT40) protein DNA complex [Fig. 1]. The 40-mer DNAs are polydeoxythymines opposed to being composed of a sequence of different nucleotide bases. Only the 40-mer DNA protein complex was used to optimize the hit; other DNA lengths such as 32 and 44-mer were unable to crystallize under those conditions.

Further optimization with Sodium Formate 2.0–4.5M with buffers pH 6.5-9.0 was carried out [Table 1], with crystals only growing in the initial hit condition. This was further refined to 3.2–3.7M of the same precipitating agent with buffers of pH 8.0-9.5 [Table 2]. A sheet of crystals [Fig. 2] and the largest crystals grew from hanging drops of 3.2-3.4M Sodium Formate with buffer Hepes pH 9.0 [Fig. 3]. The crystals grown in this condition were not birefringent, but were three-dimensional and larger than those grown in the initial hit [Fig. 3]. However, the crystals did not provide any diffraction patterns or spots [Fig. 4].

For the next optimization, several factors were varied (Table 3). In another optimization, the main precipitating agent, Sodium Formate, was replaced with Potassium Thiocyanate (5.0M), Sodium Malonate (3.2M), Potassium Hydrogen Phospha te (4.0M), and Sodium Acetate (4.0M) as they share similar properties, however no crystals formed. After several more optimizations, crystal grown from 2.8M Sodium Formate with 0.1M Hepes pH 9.0 and at 30 mg/ml of protein, diffracted up to 22 Angstroms through the use of a capillary [Fig. 5].

LPRect231 Protein Concentration	Precipitating Agent	Buffer	Drop Sizes (protein + reservoir)
10 mg/ml	2.0-4.5M Sodium Formate	0.1M Sodium Carodylate pH 6.5	2 µl + 2 µl
10 mg/ml	2.0-4.5M Sodium Formate	0.1M Imidagodo pH 7.5	2 µl + 2 µl
10 mg/ml	2.0-4.5M Sodium Formate	0.1M Tris pH 8.5	2 µl + 2 µl
10 mg/ml	2.0-4.5M Sodium Formate	0.1M Hepes pH 9.0	2 µl + 2 µl

Table 1: An optimization with Sodium Formate 2.0–4.5M with buffers pH 6.5-9.0

LPRect231 Protein Concentration	Precipitating Agent	Buffer	Drop Sizes (protein + reservoir)
10 mg/ml	3.2-3.7M Sodium Formate	0.1M Hepes pH 8.0	2 µl + 2 µl
10 mg/ml	3.2-3.7M Sodium Formate	0.1M Tris pH 8.5	2 µl + 2 µl
10 mg/ml	3.2-3.7M Sodium Formate	0.1M Hepes pH 9.0	2 µl + 2 µl
10 mg/ml	3.2-3.7M Sodium Formate	0.1M Ches pH 9.5	2 µl + 2 µl

Table 2: An optimization of 3.2–3.7M of Sodium Formate with buffers of pH 8.0-9.5

After screening LPRect231 with 36, 40, and 44-mer DNA with the Axygen Custom VI and Index screens, two new hits formed. The crystal condition from the Axygen kit was 0.48M Magnesium Sulfate with buffer Na-K-Phosphate pH 6.5, which formed crystals for 36 and 40-mer. The condition of the crystal from the Index kit was 2% Peg 3350 with 15% Tascimate pH 7.0 and buffer 0.1M Hepes pH 7.0, which formed needle-like crystals for 36-mer. After optimizing the hit with Peg 3350, the needle structures were reproduced for 1.0-3.5% Peg 3350 for pH 7.0-8.5 [Fig. 6]. However, no crystals were produced after optimizing the Magnesium Sulfate hit,

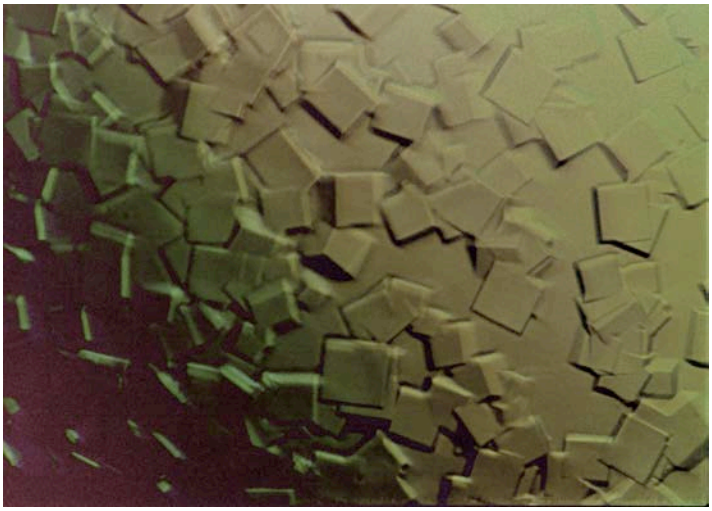

Fig. 2. A sheet of large crystals grown from 3.3M Sodium Formate with buffer 0.1M Herpes pH 9.0

Fig. 3. The largest, three-dimensional crystals grown from 3.2M Sodium Formate with buffer 0.1M Herpes pH9.0

only precipitate and some spherical structure formed on the hanging drops.

Additives, which are supplemental chemicals added to reservoirs, were used on the Peg 3350 condition once the largest needles were obtained. A new condition with Peg 3350 was found that had solid, rectangular crystals. Experiments with this were carried out with different DNA lengths such as 38, 40, and 42-mer, and buffers. Eventually, large, three-dimensional, and birefringent crystals [Fig. 7] were grown with LPRecT231

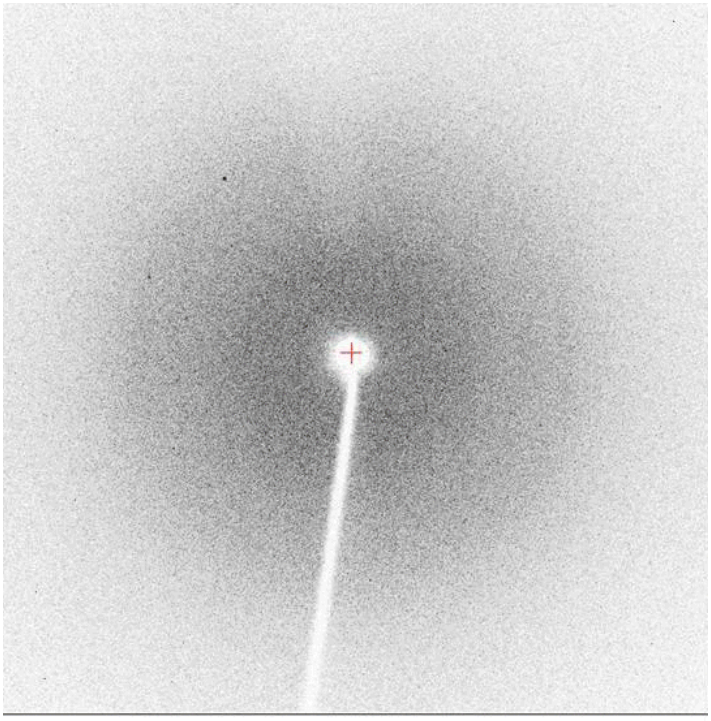

Fig. 4. The diffraction image produced with the largest three-dimensional crystal (3.2M Sodium Formate with Hepes pH 9.0). The crystal failed to diffract, resulting in no diffraction spots or patterns.

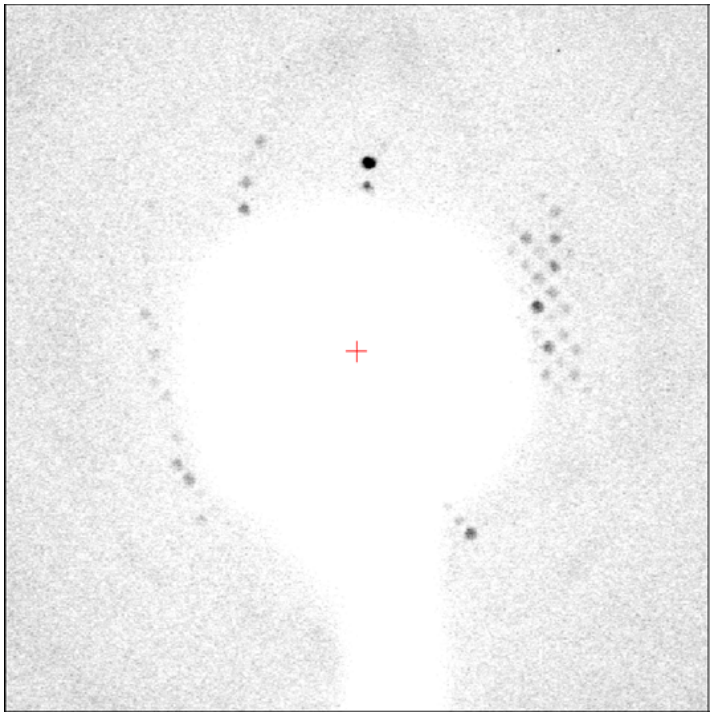

Fig. 5. The diffraction image produced, with the use of a capillary, of a crystal grown from 2.8M Sodium Formate with 0.1M Hepes pH 9.0 and at 30 mg/ml of protein

and DT38 at the condition: 0.5% Peg 3350, 16% Tascimate, 200 mM ZnSO4, and Hepes pH 7.5. Also, similarly large crystals were grown with LPRecT231 10 mg/ml with DT40 5 Prime Phosphate at the condition: 0.4% Peg 3350, 16% Tascimate, 230 mM ZnSO4, and Hepes pH 7.5. They diffracted up to 6-7 Angstroms with the use of a capillary (room-temperature setting). [Fig. 8-9].

Crystal experiments with β protein without x-ray diffraction. β crystals were initially grown from a hit with the condition: 0.064M Sodium Malonate with a buffer of pH 7-7.5. The condition was optimized until crystal needles grew with β in the condition: 0.192M Sodium Malonate with 15% Glycerol and 0.1M Hepes pH 7.5. A new hit crystal condition for β was discovered: 0.2M Ammonium Acetate with 15% 2-Propanol and 0.1M Tris pH 8.5. This condition was further optimized by: varying buffer pH, varying 2-Propanol concentration, adding multiple alcohol precipitating agents (i.e. Ethylene Glycol. Hexane Diol, MPD,

LPRect231 Protein Concentration	Precipitating Agent	Buffer	Drop Sizes (protein + reservoir)
10 mg/ml	2.95-3.20M Sodium Formate	0.1M Hepes pH 9.0	2 µl + 2 µl
10 mg/ml	3.40-3.50M Sodium Formate	0.1M Tris pH 8.5	2 µl + 2 µl
10 mg/ml	3.20M Sodium Formate	0.1M Hepes pH 9.0	varied from 1+1 µl to 3+1 µl to 1.5+0.5 µl
15 mg/ml	2.7-3.2M Sodium Formate	0.1M Hepes pH 9.0	2 µl + 2 µl

Table 3: A Sodium Formate optimization testing several variables.

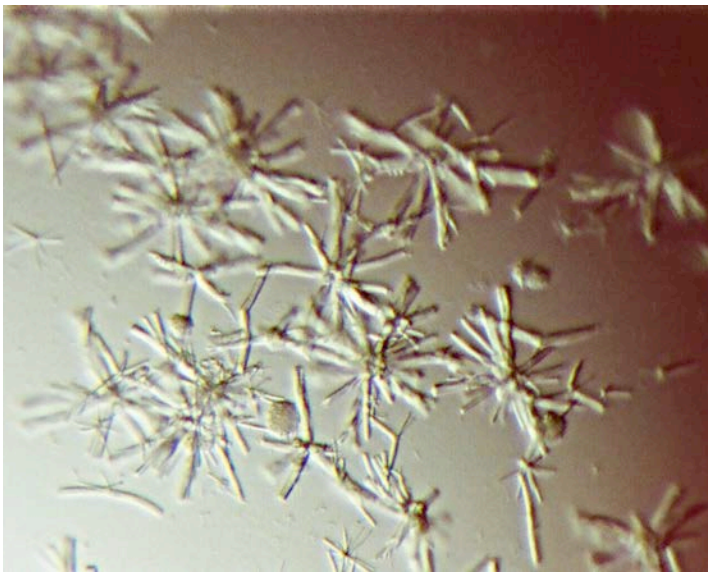

Fig. 6. Crystal needles formed for the Peg 3350 optimization from 2% Peg 3350 (50% stock) with Hepes pH 7.5 (1M)

2-Ethoxyethanol, Ammonium Formate, Ammonium Citrate, Ammonium Sulfate, Tri-Sodium Citrate, Potassium Phosphate, and Glycerol), and varying drop ratio. Glycerol was the main cryo protectant for LPRect231, but it caused smaller sheets of crystals to form when mixed with a β condition. Crystals were grown in up to 10% Glycerol. 2-Propanol allowed for larger crystals to form, but only when 5% of 2-Propanol was used were crystals able to form. The largest crystals formed when β was used with the following conditions: (1) 0.08M Sodium Malonate pH 8 with Hepes pH 7.0 and 4% Glycerol and (2) 0.05-0.75M Ammonium Phosphate pH 7 with 0.05M NaCl and Hepes pH 7.5. Crystals were also formed with Hepes pH 7.5 alone.

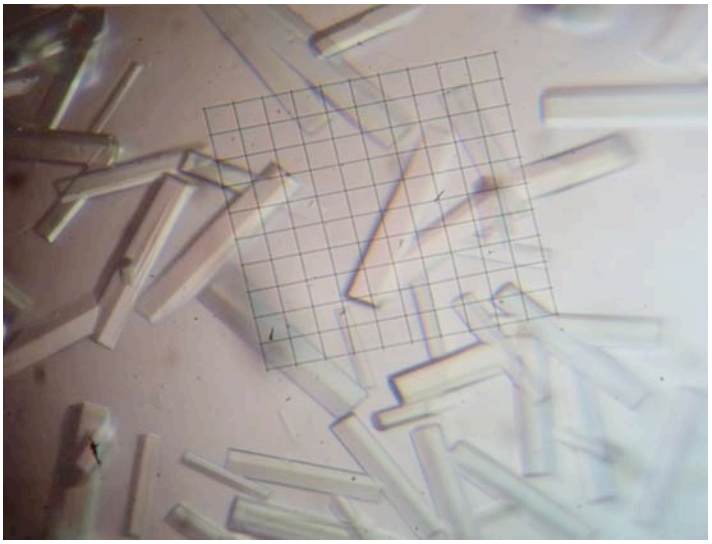

Fig. 7. LPRect231 crystals grown with Peg 3350, Tascimate, Zinc Acetate, and Hepes pH 7.5

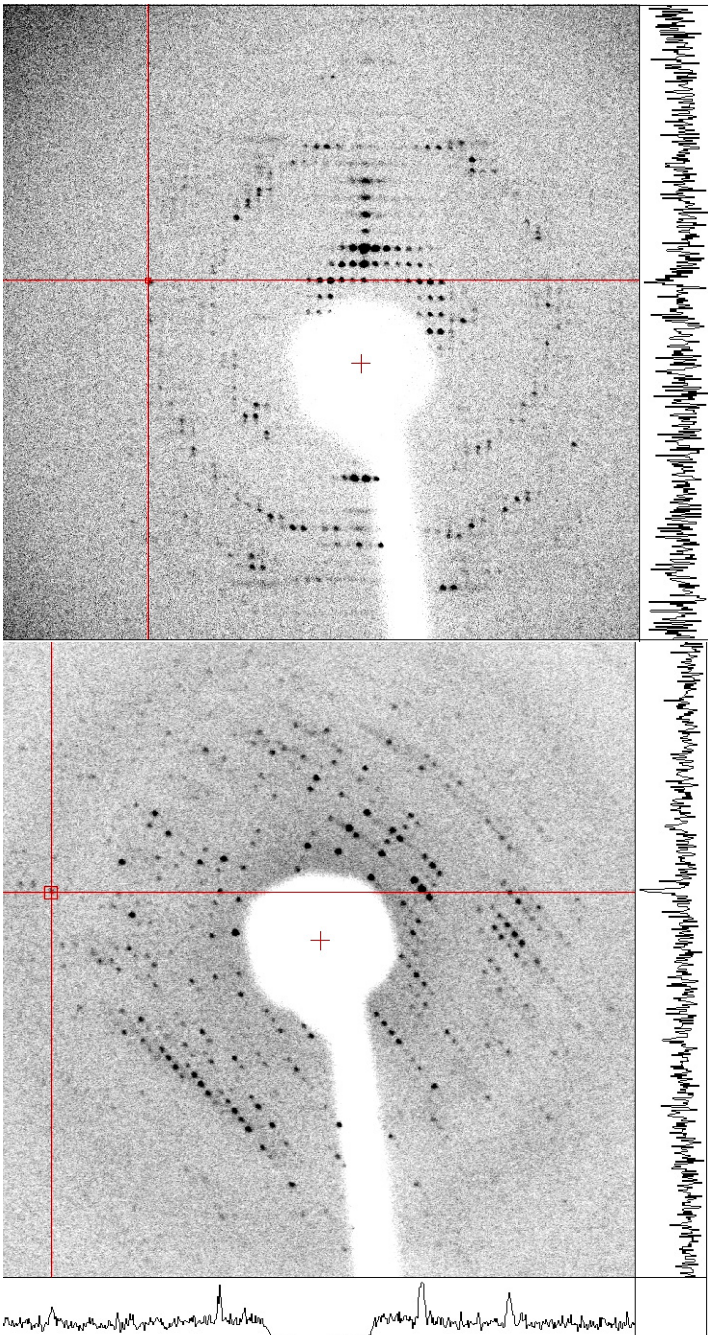

Fig. 8-9: The diffraction spots/pattern of a LPRect231 20 mg/ml 38-mer crystal using a capillary (top). The crystal was grown in the condition: 0.5% Peg 3350 with 16% Tascimate, 200 mM ZnSO4, and 100 mM Hepes pH 7.5. The diffraction/spots of LPRect231 38-mer without the use of a capillary (bottom). The crystal conditions are the same except 3.75% Peg 3350 is used and the crystal is grown in 20% Glycerol.

DISCUSSION

The optimum pH for LPRect231 crystal formation with Sodium Formate was found to be 8.5-9.0. Through optimizations, it became clear that including DNA in the protein complex is necessary for crystal structures to form with Sodium Formate. Also, crystals only form with certain DNA lengths (40-mer, not 32 or 44-mer).

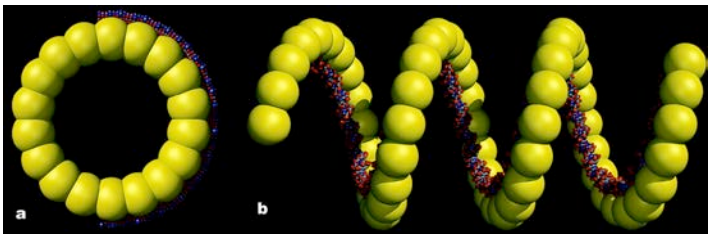

Fig. 10. Oligomeric rings and helical filaments formed by β protein (reference 10)

This specificity supports shows that the notion that DNA is required because it is part of the crystal, rather than it having a non-specific poly-anion effect. This suggests that we have in fact crystallized the protein-DNA complex and not simply the protein alone.

The mechanism by which SSA proteins bind to ssDNA and promote the annealing of complimentary DNA has been proposed through EM studies and graphs and a low-resolution model has been created. It has been proposed that once the rings have formed over the crystal, helical filaments wrap around it [Fig. 10]. Although through other studies, the basic structure of the protein has been proposed, this study is not yet complete and the protein structure has not yet been discovered.

When viewed with a light microscope, the crystals that formed with Sodium Formate were three-dimensional and appeared to be solid and ideal for diffraction. Nevertheless, they did not produce any diffraction spots until a capillary was used. With the use of a capillary, the crystal was diffracted up to 22 Angstroms. However, the crystals that formed with Peg 3350 and ZnSO4 diffracted to 7 Angstroms which is significantly high resolution diffraction. Crystals need to be well-ordered internally in order to form diffraction spots.

Through experimentation, it can be concluded that Sodium Formate can produce large crystals with LPRect231, however the crystals are unable to diffract without the use of a capillary. The largest crystals formed when using 3.2M of Sodium Formate with LPRect231 15 mg/ml opposed to 10 mg/ml or 20 mg/ml. Initially, only the buffer Tris pH 8.5 with Sodium Formate allowed crystals to grow, but after many optimizations, using Hepes pH 7.0 produced the best-quality crystals.

Regarding the optimization with Magnesium Sulfate, no crystals formed with salt precipitating agents similar to Sodium Formate, indicating that other salts could not replace Formate. Also, the initial hit was likely a salt solid rather than a crystal. The crystals grown by the Peg 3350 optimization produced needle-like crystals initially. The crystals began to fragment, indicating that growing larger and well-ordered crystals with similar salts may not be possible through optimization. However, once the new condition, with Peg 3350, found through additives was optimized, very large and internally-stable crystals formed.

The Peg 3350 condition with Zinc Sulfate and LPRect231 has formed the largest, well-structured, and birefringent crystals. Zinc Sulfate, although not the main precipitating agent, was a necessary component in growing the best crystals. Getting well-structured crystals also strictly depended on the Zinc Sulfate concentration. We have found that crystals are able to grow with other Zinc stock solutions, especially Zinc Acetate, in addition to Zinc Sulfate, but we have not yet found any crystals that will grow with Zinc Chloride although multiple experiments were carried out with it.

The β protein did not grow any crystals with DNA, however it was able to grow large, three-dimensional, and very birefringent (when compared to LPRect231) crystals without DNA [Fig. 11]. The recent β crystals have shown no sign of diffraction, however they are still being experimented on to grow them larger and no capillary experiments have been carried out.

Efforts to grow new and better crystals with LPRect231 and β will continue. The next step would be to try testing the protein DNA complexes with other screening kits to get new hits to further optimize and also try using more DNA lengths. More capillary exper-

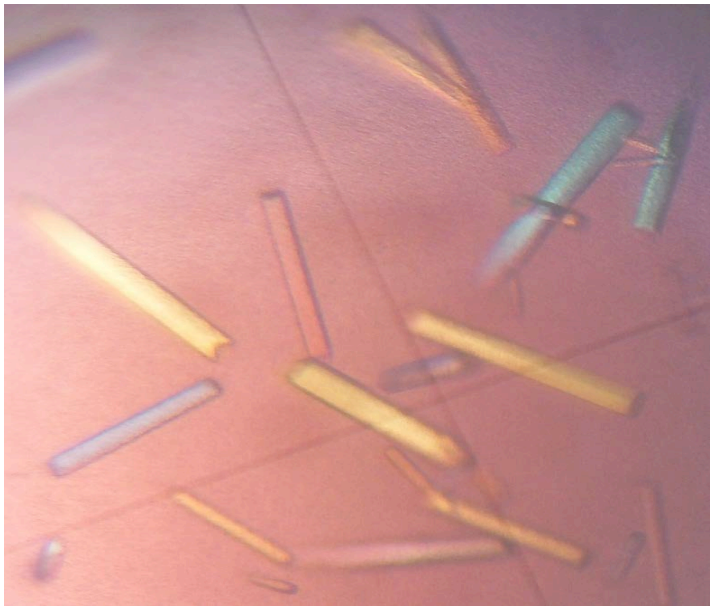

Fig. 11. β177 protein crystals grown with Tris pH 7.5 and 2-Propanol

iments could be carried out to achieve higher quality diffraction and optimizations with β177 could be carried out.

In conclusion, we have made considerable progress in formation of crystals from RecT and β protein. Finding these proteins' structure will help in understanding the DNA repair mechanisms, which is crucial for destroying cancerous cells.

REFERENCES

1. Bell, Charles. Practical Macromolecular Crystallography. Retrieved April 1, 2010. Department of Molecular Biochemistry, the Ohio State University. Lecture Notes for MOLBIOCHEM 840.
2. Bell, Charles. Protein Crystallization Project Description. Retrieved April 8, 2010. Reading.
3. Bell, Charles. Separation and Characterization by Electrophoresis. The Ohio State University. Retrieved April 8, 2010. Lecture Notes from MOLBIOCHEM 761.
4. Chayen, Naomi E., Emmanuel Saridakis. Protein Crystallization: from Purified Protein to Diffraction-quality Crystal. Perspective 2008;5: 147-52.
5. Erler, A., Wegmann, S., Elie-Caille, C., Bradshaw, C.R., Maresca, M., Seidel, R., Habermann, B., Muller, D.J., and Stewart, A.F. Conformational adaptability of Redβ during DNA annealing and implications for its structural relationship with Rad52. J. Mol. Biol. 2009; 391:586-598.
6. Haders, D.J., Kazanecki, C.C., Denhardt, D.T., Riman, R.E. Crystallography engineered, hydrothermally crystallized hydroxyapatite films: an in vitro study of bioactivity. J. Mater. Sci. 2010: 1-12.
7. Hall, S.D., Kane, M.F., and Kolodner, R.D. Identification and characterization of the Escherichia coli RecT protein, a protein encoded by the recE region that promotes renaturation of homologous single-stranded DNA. J. Bacteriol. 1993;175:277-287.
8. Karakousis, G., Ye, N., Li, Z., Chiu, S.K., Reddy, G., and Radding, C.M. The β protein of phage binds preferentially to an intermediate in DNA renaturation. J. Mol. Biol. 1998; 276: 721-731.
9. Kmiec, E., and Holloman, W.K. B protein of bacteriophage l promotes renaturation of DNA. J. Biol. Chem. 1981;256: 12636–12639.
10. Passy, S.I., Yu, X., Li, Z, Radding, C.M., and Egelman, E.H. (1999) Rings and filaments of β protein from bacteriophage suggest a superfamily of recombinant proteins. Proc. Natl. Acad. Sci. USA 96, 4279-4284.
11. Shiraishi K, Hanada K, Iwakura Y, Ikeda H. Roles of RecJ, RecO, and RecR in RecET-mediated illegitimate recombination in Escherichia coli. J. Bacteriol. 2002;184:4715-21.
12. Talreja, S., Perry, S.L., Guha, S., et al. Determination of the Phase Diagram for Soluble and Membrane Proteins. J. Phys. Chem. B 2010:, 4432–4441.
13. Thresher, R.J., Makhov, A.M., Hall, S.D., Kolodner, R., and Griffith, J.D. (1995) Electron microscopic visualization of RecT protein and its complexes with DNA. J. Mol. Biol. 254, 364-371.
14. Zucker, F.H., Stewart, C., Rosa, Jaclyn., et al. Prediction of protein crystallization outcome using a hybrid method. J. Struct. Biol. 2010: 1-10.