Structural Resolution of Human Insulin with a New Ligand

Farihah Chowdhury

2019-2020 Research Plan

1. RATIONALE

Insulin is a hormone that aids in controlling the level of glucose in the blood (Goodsell, 2001). These hormones work to regulate energy, memory, and mood (Lee et al., 2016). Insulin is a polypeptide hormone with a molecular weight of 5802 and is secreted from the pancreas. Specifically, insulin has a key role in a variety of physiological processes and is crucial to the study of diabetes and obesity (Wilcox, 2005).

Within the body, insulin levels change in response to sugar levels in the blood. When sugar levels are high, insulin is added to the blood. That signal binds to insulin receptors on the liver, muscle, and fat cells, and is then stored in the form of glycogen or fat. However, the human body can often develop an autoimmune disease known as Diabetes Mellitus. This condition is a result of damage to the function of insulin, such as decreased levels of insulin or complete deficiency. These attributes can be potentially life-threatening if not treated, such as changing the pH of blood or clogging cells from glucose build-up (Goodsell, 2001).

The structure of insulin has already been discovered in 1994 (Ciszak & Smith, 1994). But there is a possibility that all of the ligands that can bind onto insulin have not been found. Ligands are smaller molecules that include cofactors and metabolites. These molecules interact with biological molecules and can bond noncovalently and covalently (PDB). Ligand-protein interactions are vital to the process of drug discovery (Laskowski & Swindells, 2011).

RESEARCH QUESTIONS:

Will soaking human insulin in different chemicals reveal a new ligand with X-ray crystallography?

HYPOTHESIS:

It was hypothesized that chemicals such as EDTA will bind to insulin to create a ligand-protein complex.

2. METHODS

2.1. Insulin and Chemical Crystallization Screening

All of the plates for screening will be made using the hanging drop method. 350µl of the buffer being used will be put into each well, and then a 5µl drop of the insulin solution will be put on a cover slide and placed on top of the well. These plates will then be stored in a temperature controlled refrigerator to form crystals. After crystallogenesis, the crystals will be soaked in a variety of chemicals and stored once again.

2.2. Future analysis of crystals

Specific crystals, ones of good and defined size and shape, will be fished and placed into pucks in liquid nitrogen. The crystals will then be placed into the AMX beamlines to create raster files. The purpose of the AMX beamline is to determine the structure of macromolecules using x-ray diffraction (BNL NSLS II). The raster files will then be converted in CCP4i. The

protein structure will then be analyzed on COOT.

3. SAFETY RISKS:

The synchrotron presents a radiation risk when the beamlines are running. However, the facility is well controlled by Brookhaven National Lab, including numerous alarms and safety precautions put into place. All minors working in the synchrotron also wear TLDs which track the amount of radiation exposure, helping to ensure the safety of everyone. Lastly, many training courses were taken to be fully prepared to operate the beamline and work in the labs.

4. BIBLIOGRAPHY:

Ciszak, E., & Smith, G. (1994). Crystallographic Evidence For Dual Coordination Around Zinc In The T3R3 Human Insulin Hexamer. doi:10.2210/pdb1trz/pdb

Flora, G., Mittal, M., & Flora, S. J. (2015). Medical Countermeasures—Chelation Therapy. *Handbook of Arsenic Toxicology*, 589–626. doi: 10.1016/b978-0-12-418688-0.00026-5

Garg, A., Garg, D., & Garg, D. (2016). Biochemical Significance of EDTA in Human Physiology. *IOSR Journal of Dental and Medical Sciences*, *15*(10).

Goodsell, D. (2001). PDB101: Molecule of the Month: Insulin.Retrieved from https://pdb101.rcsb.org/motm/14

Laskowski, R. A., & Swindells, M. B. (2011). LigPlot : Multiple Ligand–Protein Interaction

Diagrams for Drug Discovery. *Journal of Chemical Information and Modeling, 51*(10), 2778-2786. doi:10.1021/ci200227u

Lee, S., Zabolotny, J. M., Huang, H., Lee, H., & Kim, Y. (2016). Insulin in the nervous system and the mind: Functions in metabolism, memory, and mood. *Molecular Metabolism, 5*(8), 589-601. doi:10.1016/j.molmet.2016.06.011

PDB101: Learn: Guide to Understanding PDB Data: Small Molecule Ligands. (n.d.). Retrieved from https://pdb101.rcsb.org/learn/guide-to-understanding-pdb-data/small-molecule-ligands

Wilcox G. (2005). Insulin and insulin resistance. *The Clinical biochemist. Reviews*, *26*(2), 19–39.

Addendum:

Title: Potential Pitfalls in the Protein Structure Determination via Protein Crystallography

1. RATIONALE

Protein crystallography is a process that revolutionized the field of biochemistry. The first protein crystals were created in the 1800s, for purposes of purification (McPherson & Gavira, 2014). Then, in the early 1900s, X-ray crystallography was first used to determine the atomic structures of salt crystals. As the decades went on, X-ray crystallography was used to find the structures of many proteins as well as other molecules, such as DNA (Curry, 2015). These structures have been periodically added to the Protein Data Bank (PDB), and the number of released structures has increased exponentially in the last twenty years as various technologies have developed (RSCB, 2020).

Structural protein data can then be used for many practical purposes, such as drug discovery. A high resolution protein structure can be used in complex with ligands to analyze the mechanisms of drug activity (Zheng et al., 2014). Algorithms and code can be used to predict the possible complexes that can form between proteins and ligands, but X-ray crystallography is one of the best ways to validate this data (March-Villa, 2017).

The influx of protein structures added to the PDB is correlated to the decreased level of difficulty to crystallize a protein. Due to this vast volume of structures, there are bound to be issues with the process. Honest errors are made at every point, from almost publishing a structure that is completely wrong to forgetting to check the R factor in model refinement (Wlodawer, 2013). The aim of this project is to address the potential pitfalls

associated with macromolecular crystallogenesis and structural resolution. Pitfalls can occur at any step along the meticulous process, including during crystallization, during data collection, and during data and model refinement.

RESEARCH AIM:

The aim of this project was to address the potential pitfalls associated with macromolecular crystallogenesis and structural resolution.

HYPOTHESIS:

Pitfalls can occur at any step along the meticulous process, including during crystallization, during data collection, and during data and model refinement.

2. METHODOLOGY

Macromolecular crystallography uses the chemistry of supersaturation states of proteins to evaluate protein structure. The protein starts in the mother liquor as an undersaturated solution. Then, the particle concentration moves towards equilibrium as the solution becomes metastable. After, the solution becomes more saturated as the crystal begins to form in the nucleation zone. Finally, the crystals fully form in the precipitation zone when the solution becomes supersaturated (SINE2020, 2016). To make sense of the crystals, the diffraction patterns from the beamline are converted into a 3D protein structure (McPherson & Gavira, 2014). Throughout the crystallization and analysis of bovine thyroglobulin and human insulin structures, the pitfalls in each step were recorded

and discussed.

2.1. Methods of Crystallization

There are multiple methods of crystallization, such as the hanging drop, sitting drop, micro-batch, and microdialysis methods (McPherson & Gavira, 2014). In this project, the hanging drop vapor diffusion method was used for different proteins along with the use of nucleation inducing reagents, such as gelatin. The protein solution was placed on a coverslip over a reservoir of the mother liquor. This method uses vapor diffusion, which allows the protein, buffer, and precipitate to equilibrate with the reservoir liquids to reach the necessary state of supersaturation (Rhodes, 2006).

Every protein has its own unique set of conditions that promote crystallogenesis. In order to ascertain the perfect environment for crystal growth, a multitude of conditions were tested in a systematic way. However, since they are commercially sold, the conditions are difficult to replicate without buying the vial straight from the source, and it is tedious to test every unique solution individually.

2.2. Data Collection and Analysis

After crystals formed, they were fished and placed into a puck immersed in liquid nitrogen. In this research, the Highly Automated Macromolecular Crystallography (AMX) beamline was used to obtain diffraction data from harvested crystals (NSLS II BNL). Once diffraction patterns are obtained, these patterns can be analyzed with a multitude of programs found in CCP4i. The 2D distances between the patterns are converted into pieces

of data that come together to form a 3D protein structure with electron density.

2.3. Model Refinement

After the raw data was analyzed through CCP4i, the outputted 3D model was fitted to the electron density in COOT. The model was completed using refinement, model building, and validation processes.

To refine the data, multiple techniques were used, such as Auto-zone, Sphere Refinement, Single-Atom Drag, and Ramachandran Refinement, known as Real Space Refinement collectively. Through these processes, the coordinates of the model were adjusted to match the electron density structure factors as much as possible. The bonds, planes, angles, torsions, and non-bonded contacts had to be minimized (Diamond, 1971). To complete the model, some portions needed to be built or removed (Lohkamp, 2011).

3. SAFETY RISKS:

The synchrotron presents a radiation risk when the beamlines are running. However, the facility is well controlled by Brookhaven National Lab, including numerous alarms and safety precautions put into place. All minors working in the synchrotron also wear TLDs which track the amount of radiation exposure, helping to ensure the safety of everyone. Lastly, many training courses were taken to be fully prepared to operate the beamline and work in the labs.

4. BIBLIOGRAPHY

1. **Aldrich: Analytical, Biology, Chemistry & Materials Science products and services. (n.d.). Retrieved from https://www.sigmaaldrich.com/**

2. **Brünger, A.T.(1997). Free R Value: Cross-validation in crystallography, Methods in Enzym. 277, 366-396**
3. **Diamond, R. (1971). Acta Cryst. A 27, 436-452.**
4. **French G.S. and Wilson K.S. (1978). Acta. Cryst., A34, 517.**
5. **Hazes B., unpublished results**
6. **Lohkamp, B. (2011). Model-Building with Coot: An Introduction, low resolution tools.**
7. **March-Vila, E., Pinzi, L., Sturm, N., Tinivella, A., Engkvist, O., Chen, H., & Rastelli, G. (2017). On the integration of in silico drug design methods for drug repurposing. Frontiers in pharmacology, 8, 298.**
8. **McPherson, A., & Gavira, J. A. (2014). Introduction to protein crystallization. Acta Crystallographica Section F: Structural Biology Communications, 70(1), 2-20.**
9. **RCSB Protein Data Bank. (2020). PDB Statistics: Overall Growth of Released Structures Per Year. Retrieved from https://www.rcsb.org/stats/growth/overall**
10. **Rhodes, G. (2006) Crystallography Made Crystal Clear, Third Edition: A Guide for Users of Macromolecular Models, 3rd Ed., Academic Press**
11. **Russo Krauss, I., Merlino, A., Vergara, A., & Sica, F. (2013). An overview of biological macromolecule crystallization. International journal of molecular sciences, 14(6), 11643-11691.**
12. **SINE2020. (2016). France, Germany and Sweden: Phase diagram crystal growth. Retrieved from https://sine2020.eu/about/the-road-to-the-ess/phase-diagram.html**

13. **Stephen Curry (2015) Structural Biology: A Century-long Journey into an Unseen World,Interdisciplinary Science Reviews, 40:3, 308-328, DOI: 10.1179/0308018815Z.000000000120**

14. **Vagin, AA, Steiner, RS, Lebedev, AA, Potterton, L, McNicholas, S, Long, F and Murshudov, GN. (2004). REFMAC5 dictionary: organisation of prior chemical knowledge and guidelines for its use. Acta Cryst. D60: 2284-2295**

15. **Vagin,A.,Teplyakov,A.(1997). MOLREP: an automated program for molecular replacement., J. Appl. Cryst. 30, 1022-1025**

16. **Wlodawer, A., Minor, W., Dauter, Z., & Jaskolski, M. (2013). Protein crystallography for aspiring crystallographers or how to avoid pitfalls and traps in macromolecular structure determination. The FEBS journal, 280(22), 5705-5736.**

17. **Zheng, H., Hou, J., Zimmerman, M. D., Wlodawer, A., & Minor, W. (2014). The future of crystallography in drug discovery. Expert opinion on drug discovery, 9(2), 125–137. doi:10.1517/17460441.2014.872623**