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Crystal Growth and Engineering

Antifreeze Protein Crystallization

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1. INTRODUCTION

Life in the deep sea, on eternal snow, or in the desert has forced bacteria, fungi, plants, insects, and vertebrates to adapt to a wide range of environmental conditions. Making the distinction between ice and liquid water is a crucial part of effective freezing management, but it is also one of the most difficult recognition tasks in biology. It is the class of proteins called Antifreeze proteins that regulate the transition between the solid and liquid states of water, an essential component of all living organisms.

The freezing point of water can be lowered by antifreeze proteins (AFP) without affecting the melting point, and this property has earned AFP widespread recognition for its capacity to prevent the formation of ice crystals. As a result of adsorption inhibition, they have been proven to prevent the development of ice crystals. The presence of Antifreeze proteins drastically alters crystal shape, size, quantity, and growth rate. They are a vast group of macromolecules with a wide range of structures, but they all share the ability to affect water's thermal hysteresis - the non-equilibrium decrease of its freezing point and to prevent ice from recrystallizing - growth of large ice crystals at the expense of small ones. These proteins are not only essential for life to continue at low temperatures, but also have significant medical and practical applications, such as organ storage for later transplantation.

2. SCIENTIFIC RATIONALE:

Thermodynamics of Crystallization

Crystallization usually takes place by the process of undercooling which is the lowering of temperature below melting point. The process of crystallization, like any other phase transition process, follows the Second Law of Thermodynamics. It is accompanied by the release of heat, ‘Heat of Fusion’, which results in an overall increase in the entropy of the universe.

For Crystallization to be favorable:

$$H_{solid} - H_{liquid} < TS_{Solid} - TS_{liquid} \quad (H \text{ and } S \text{ are Enthalpy and Entropy}) \quad (1)$$

$$G_{solid} < G_{liquid} \quad (G \text{ is the Gibbs Free Energy}) \quad (2)$$

Kinetics of Crystallization

The kinetics of crystallization at a specific temperature, pressure is determined by the supersaturation. Further, kinetics are studied for two main processes occurring during crystallization which are growth and nucleation.

- **Nucleation:** The first step prior to crystal growth, which occurs due to homophase/ heterophase density fluctuations. It is identified by the formation of an ensemble of new phases.

  Nucleation is further classified as primary and secondary nucleation. The governing equations for nucleation are:

  $$\frac{dN}{dt} = k_n(c - c^*)^n \quad (Primary\ Nucleation) \quad (3)$$

  Here, N is Number Density of Nuclei

  k_n is rate Constant

  (c-c*) is supersaturation in terms of Concentration

  n is an empirical constant

  $$\frac{dN}{dt} = k_1 M_T^{\ j}(c - c^*)^b \quad (Secondary\ Nucleation) \quad (4)$$

  Here, k_1 is rate constant

  M_T is suspension density

  j and b are empirical constants

- **Growth:** It is the aggregation of nuclei after nucleation has taken place. Feasibility of growth of new phase (say α) from existing phase (say β) is governed by:

  $$\mu_\alpha > \mu_\beta \quad (for\ \beta\ phase\ to\ grow) \quad (5)$$

As the process of crystallization is controlled by both thermodynamic and kinetic factors, it is highly variable and complex to control. Following Case Studies provide a method to crystallize a special kind of protein, which retards the formation of ice, the Anti-Freeze Protein (AFP).

Antifreeze Proteins

A class of proteins known as antifreeze proteins (AFPs) shields organisms from death by freezing in severe environments. A wide variety of organisms, including plants, bacteria, fungus, and insects, exhibit these. By attaching to and blocking the development of immature ice crystals, these proteins are able to use their unusual architectures to impede freezing. The process of thermal hysteresis, which AFPs can facilitate, protects organisms from damage in subfreezing temperatures. When the freezing point is lowered without correspondingly changing the melting point, we observe a hysteresis gap.

Evolution: Sea level glaciation occurred between 1-2 million years ago in the Northern hemisphere and between 10-30 million years ago in the Antarctic, and this may account for the astonishing diversity of AFPS. There are two reasons why several protein types carry the same function: Even though ice is made up entirely of water molecules, it nevertheless has a variety of binding sites, which means that different AFPS respond in various ways. The five forms of AFPS differ in their main structure of A.A when folded into a working protein, may have commonalities in their 3-D or tertiary structure, and give the same binding with ice.

Name change: Antifreeze proteins (AFPs) have recently been renamed ice structuring proteins (ISPs) to more accurately reflect their role and to disassociate them from ethylene glycol, the active ingredient in vehicle antifreeze.

Properties:

Thermal Hysteresis- It is the result of a decrease in apparent freezing temperature that does not impact the melting point. This leads AFPs to be 200 to 300 times more effective than in perfect solutions at freezing point depressions.

Pathogenesis- The majority of sequenced antifreeze proteins are homologous to pathogenesis-related proteins. As a result of a pathogenic infection, they are released into the apoplast.

Freeze tolerance versus freeze avoidance:

Freeze avoidant: These organisms are capable of preventing their body fluids from freezing entirely. Typically, the AFP function can be defeated at extremely cold temperatures, resulting in fast ice development and mortality.

Freeze tolerant: These organisms are able to endure the freezing of their body fluids. It is believed that some freeze-tolerant animals utilise AFPs as cryoprotectants to counteract the damage of freezing, but not freezing itself.

Mechanism of action

An important part of crystallization is nucleation, the process of forming a stable crystal nucleus, and another is the spread of ice crystals by the growth of the nucleus. When the temperature changes, ice recrystallizes, and nucleation typically happens around an alien molecule. The physical harm they do to cells and tissues is magnified. Inhibition of growth by AFPS is believed to occur via a mechanism of adsorption inhibition. They prevent the thermodynamically preferred development of ice by adhering to non-basal planes of ice. Some AFPS, likely those with a flat, hard surface, interact with ice more easily than others due to the Van-der-Waals force surface complementarity.

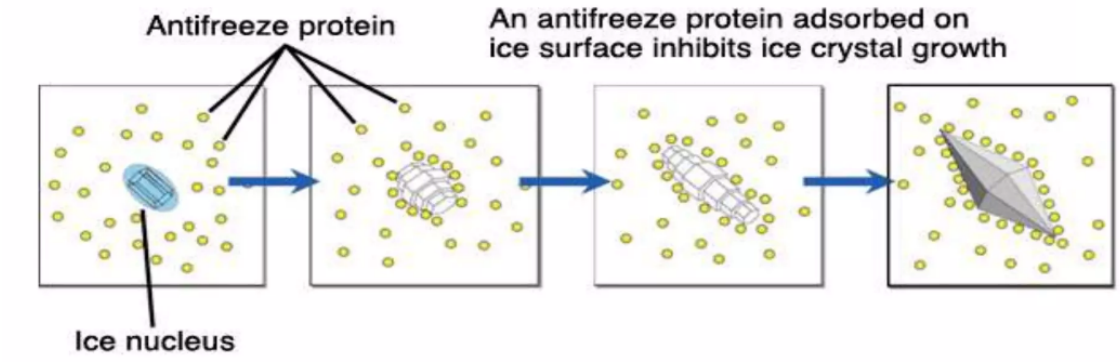

Fig. 1: *Formation and Propagation of a Stable Crystal Nucleus*

3. CASE STUDY I

Crystallisation of Ice-Binding Protein from Antarctic Yeast Leucosporidium sp. AY30

Freezing causes biomolecules to become denatured and ruptures cell membranes, all of which are detrimental effects for living things. Freezing also raises the plasma concentration of ions and other solutes. Some organisms that have adapted to cold environments are able to live in temperatures much below freezing because they produce proteins that can bind to ice crystals and stop their growth. Ice-binding proteins (IBPs), which also include antifreeze proteins (AFPs), have had their properties characterized, and it has been discovered that IBPs have the ability to restrict the growth of ice by binding to certain ice planes and reducing the freezing point. AFPs have been found in bacteria, plants, invertebrates, and fish, and they have been characterized according to their structures and thermal hysteresis (TH) values. The TH value refers to the difference between the freezing point and the melting point of the molecule. Recent research has shown that IBPs may be found in bacteria, diatoms, and fungus, and that these organisms can be grouped together in a unique cluster on a phylogenetic tree. The snow mold Typhula ishikariensis was where the first ice-active fungal protein ($25\ kDa$) was discovered. Another snow mold, Corpinus psychromobidus, yielded a protein with a similar N-terminal sequence to the one discovered in Typhula ishikariensis. Diatoms that live in sea ice, a bacterium that lives in sea ice, and a bacteria that lives in the deep ice core of the Antarctic ice sheet all contain ice-binding proteins that are similar to one another. It has been hypothesized that intracellular barrier proteins (IBPs) reduce the risk of cell injury by limiting the refreezing of extracellular ice. During the course of this case study, we are going to learn about the structure and function of the ice-binding protein that was isolated from Leucosporidium sp. AY30 (LeIBP). We overexpressed and crystallized LeIBP in order to get a deeper comprehension, and we got preliminary X-ray crystallographic data as the first step towards determining the protein's three-dimensional structure.

Methodology

Initially cloning, overexpression and purification of protein was done. After that crystallization trials were performed using the sitting-drop vapor diffusion method to set up 96-well IntelliPlates at 295 K. Crystal growth was scaled up to the hanging drop vapor-diffusion method in 24-well VDX plates at 295 K. Crystals appeared within 2 d and grew to full size within two weeks. For data collection, crystals were first cryoprotected using Paratone-N and then flash-cooled at 100 K in a liquid-nitrogen stream. Data sets were collected to a resolution of 1.5 Å from a native crystal on beamline 4A and to a resolution of 2.0 Å from a selenomethionine substituted crystal on beamline 6C at the Pohang Light Source at 100 K. A total of 360 images were collected with an oscillation angle of 1°. The following multiple wavelength anomalous diffraction (MAD) data sets were collected: peak (0.97935 Å), inflection (0.97958 Å) and high-energy remote (0.97168 Å) from the selenium absorption edge. The data sets were indexed, processed and scaled using the HKL-2000 software package.

Discussion

It was possible to clone the gene from Leucosporidium sp. that codes for the LeIBP protein. In order to conduct structural research, the protein was first overexpressed in E. coli and then purified. It was determined that LeIBP has a molecular weight of 24.9 kDa and contains a total of 261 amino acids. The tetragonal space group P43212 was the one that the native crystal belonged to. In addition, the crystal with the selenomethionine substitution was a member of the space group P43212.The VM value of the native crystal was 2.55 Å 3 Da^{-1}, which indicated that the crystal most likely included two molecules per asymmetric unit and had a solvent content of 51.81%. Furthermore, the result indicated that the crystal was found in its natural state. We carried out a procedure known as single-wavelength anomalous diffraction (SAD) phasing, however we only used the peak data. The positioning of the Se-atoms, the preliminary computations of the phase, and the phase improvement brought about by the density change. A total of four selenium sites in the asymmetric unit were determined.

Potential and Future Aspects

This study can open wide perspectives for detailed study of ice binding proteins. The ice binding proteins are remarkable ice crystal regulators for frozen foods. LeIBP decreases the oxidative stress during vitrification.

4. CASE STUDY II:

Crystallization of Snow Flea Antifreeze Protein and Structure

The focus of this case study is to discuss the crystallization of Antifreeze Protein found in a Snow Flea (sfAFP). The sfAFP is one of the two protein isoforms obtained from snow flea homogenates. We make use of 3 processes viz. Chemical Synthesis, Crystallization and X-Ray Crystallography to study the crystal structure of sfAFP in detail.

Crystallization of L-sfAFP: The sfAFP is prepared using chemical synthesis and this is further used for its extensive crystallization. After performing crystallization trials for over 6 months, with varying concentrations of protein, $(NH_4)_2SO_4$ and varying temperature, it is observed that it was very difficult to obtain crystals. It is impossible (with present technical advancements) to crystallize large derivatives of L-sfAFP.

Crystallization of Racemic Mixture: A racemic mixture of L-sfAFP and D-sfAFP is prepared using chemical synthesis. In case of crystallization of Racemic Solutions, it is observed that we can successfully get crystals in 50% of the total cases.

Crystallization of Quasi Racemic Mixture: Quasi Racemate crystals are obtained by co-crystallizing of D-sfAFP and L enantiomer of sfAFP containing one atom of Selenium, L-Se-sfAFP. It is comparatively easy to obtain Quasi Racemate crystals at a wide range of conditions. Further its crystals have nearly the same structure as that of true Racemic Crystals.

These two features of Quasi Racemate crystals mainly lead to their use to study the crystal structure of Racemic Crystals also.

General Process for Protein Crystallization

Our aim is to crystallize the sfAFP and from the crystals obtained, our goal is to study its structure.

The following processes take place while crystallization takes place:

1) **Chemical Synthesis of L-sfAFP and Analogues:** The polypeptide chain of sfAFP is prepared by Native Chemical Ligation of 4 peptides, Thz 1-12-thioester, Thz 13-27-thioester, Thz 28-42-thioester and Cys 43-81. For preparation of L-Se-sfAFP, pseudo-Se-GIn is incorporated in Thz 1-12-thioester peptide.
2) **Dissolution:** Second step is the dissolution of sfAFP in about $10 - 40$ mg/mL of distilled water.
3) **Vapor Diffusion:** Crystals are grown in hanging drops at around $23^{\circ}C$ by the method of Vapor Diffusion, with an equimolar concentration of protein solution and reservoir solution.

After these steps are performed, we can directly obtain the grown crystals of L-sfAFP.

Racemic Mixture of sfAFP is obtained at two different concentrations (equimolar concentrations of L-sfAFP and D-sfAFP), 38 ml/mL and 19 mg/mL. Final step is to screen the racemic mixture using Sparse Matrix Room Temperature screens. Using different sets of conditions, crystals are obtained after 1 day and 10 days.

Quasi Racemate Crystals are obtained by following the same procedure we followed for Racemic Mixture. For most of the sets of conditions, we get the crystals after 1 day.

Crystal Structure

The crystal structure of the sfAFP crystallized using above methods is examined using X Ray Diffraction. A sample of crystals of L-sfAFP, Racemic Mixture and Quasi Racemate are taken. These are then transferred to cryoprotectant solution (mixture of reservoir solution and $20\%\ v/v$ glycerol) and analyzed at $100K$ using three different wavelengths. This procedure of determining X-Ray structure of sfAFP is commonly called 'Multiple Wavelength Anomalous Dispersion' (MAD)

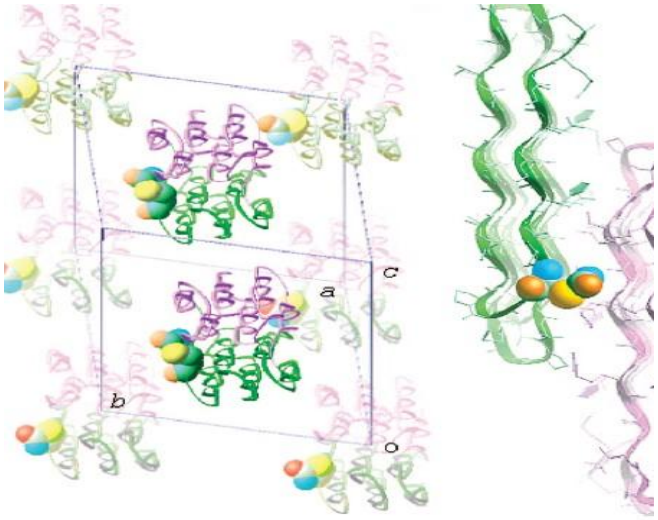

Fig. 2: *Schematic of Quasi Racemate Crystals*

The results of X-Ray Crystallography are tabulated below: (a, b, c, α, β and γ are cell parameters)

Cell Parameter	**L-sfAFP**	**Racemic Mixture**	**Quasi Racemic**
a (in Å)	16.70	28.60	28.64
b (in Å)	74.28	32.40	32.36
c (in Å)	17.69	59.85	59.70
α	–	88.69°	88.62°
β	102.2°	89.19°	89.31°
γ	–	73.41°	72.84°

Potential and Future Aspects

The Antifreeze Protein found in snow flea is of great practical importance. Researchers from Queen's University have found out that sfAFP can be used to increase the shelf life of human organs. It can lower the freezing point by about $6^{\circ}C$. Further, it breaks at higher temperatures, which means that it will easily break in the human system. Apart from the medical field, it finds applications in the food industry also as it can resist ice crystallization in frozen foods. Although the main problem is that with current scientific progress, it is not feasible and economic for us to obtain sfAFP from a Snow Flea.

5. SUMMARY & CONCLUSION:

Antifreeze proteins (AFP) can lower water's freezing point without impacting its melting point, preventing ice crystal formation. They prevent ice crystals by inhibiting adsorption. Antifreeze proteins change crystal structure, size, quantity, and growth rate. They are a big set of macromolecules with diverse structures, but they all affect water's thermal hysteresis, the non-equilibrium reduction of its freezing point, and prevent ice from recrystallizing, the formation of large ice crystals at the expense of small ones. Undercooling below the melting point causes crystallization. Growth and nucleation kinetics are also examined. The 2 Case Studies show how to crystallize an antifreeze protein (AFP). The first case study was about the structure and crystallization of yeast ice binding protein and second was about crystallization of snow flea antifreeze protein. Antifreeze proteins (AFPs) protect organisms from freezing death. These proteins prevent freezing by binding to and inhibiting immature ice crystal formation. AFPs enhance thermal hysteresis, which protects organisms under sub freezing conditions. Multiple protein types perform the same job for two reasons: Even though ice is composed of water molecules, it has several binding sites, therefore different

AFPS react differently. Extreme cold temperatures can disable AFP, causing quick ice formation and death. When the temperature changes, ice recrystallizes around a foreign molecule. AFPS inhibits growth through adsorption.

6. FUTURE ASPECT:

Natural selection has resulted in creatures that can survive in frigid environments. The emergence of AFPs exemplifies a fundamental step toward realizing the gravity of the self-defense situation. Our knowledge of AFP's applications has grown rapidly over the past decade, confirming the protein's diverse medical and industrial potential. Recent advances in structural and physicochemical biology and genetic techniques have made it possible for us to get an unprecedented understanding of the mechanisms by which AFP protects membranes and binds to ice. Numerous studies have demonstrated the numerous ways in which AFPs can be put to good use. These helpful macromolecules have enormous potential, but their widespread application has been hampered by the lengthy purification process required to extract AFPs.

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