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Molecular Basis of Ice-binding Mechanism of Microbial Antifreeze Proteins

微生物由来不凍タンパク質の
氷結晶結合メカニズムの分子基盤

By

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List of abbreviations

ACW	Anchored clathrate water
AFP	Antifreeze protein
AFGP	Antifreeze glycoprotein
Ala	The amino acid Alanine
Asn	The amino acid Asparagine
<i>ColAFP</i>	AFP from <i>Colwellia</i> sp. strain SLW05
<i>CfAFP</i>	AFP from <i>Choristoneura fumiferana</i>
<i>DcIBP</i>	AFP from <i>Daucus carota</i> (carrot)
<i>E. coli</i>	<i>Escherichia coli</i>
<i>EfcIBP</i>	Ice-binding protein from <i>Euplotes focardii</i>
<i>FcIBP</i>	IBP from <i>Fragilariopsis cylindrus</i>
<i>FfIBP</i>	IBP from <i>Flavobacterium frigoris</i> PS1.
FIPA	fluorescence-based ice plane affinity
IBP	ice-binding protein
IBP _v	IBP from a bacterium within the family Flavobacteriaceae (isolate 3519-10) in the Vostok Ice Core
IBS	ice-binding site
I _h	unit structure of an ice crystal at 1 atmosphere
IR	Ice recrystallization
IRI	Ice recrystallization inhibition
IPTG	isopropyl β-D-1-thiogalactopyranoside
jsAFP	AFP from Japanese smelt fish
KOD	DNA polymerase from <i>Thermococcus kodakarensis</i>
<i>LpAFP</i>	Antifreeze protein from <i>Lolium perenne</i>
<i>LeIBP</i>	IBP from <i>Leucosporidium</i> sp. AY30
LB	Lysogeny Broth/Luria-Bertani
<i>MpAFP</i>	Antifreeze protein from <i>Marinomonus premoensis</i>

MD	molecular dynamics
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
Mw	molecular weight
nfeAFP	type III AFP from notched-fin eelpout
RiAFP	AFP from <i>Rhagium inquisitor</i>
RMSD	root-mean-square deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sfAFP	AFP from snow flea
<i>Sf</i> IBP	AFP from <i>Shewanella frigidimarina</i> , a bacterium
5(6)-TAMRA	Carboxytetramethylrhodamine, mixture of isoforms 5 & 6
TH	Thermal hysteresis
Thr	The amino acid Threonine
<i>Tis</i> AFP	AFP from <i>Typhula ishikariensis</i>
T_f	freezing point
T_m	melting point
<i>Tm</i> AFP	AFP from <i>Tenebrio molitor</i>
Tris	tris(hydroxymethyl)aminomethane
Tyr	The amino acid Tyrosine

Chapter 1

General introduction

1.1. Living environment on earth

To date earth is the only planet serving to exist lives; however, its' living environment is not friendly everywhere. Poles are extremely cold in contrast hot springs are extremely hot and tropical regions are suffering barren desserts. In our globe 71% is water of which 96 % is saline water (Gleick, 1993). Extreme conditions for living are identified as high or low temperature, drought or snow covered, high pressure (deep sea) & salinity, and high acidity/basicity, desiccation and sharp change of temperature (Wharton, *et al.*, 2002; D'Elia, *et al.*, 2018). Microorganisms that live in such an adverse environment are termed as extremophiles. For those live-in high temperature, above 60°C called thermophiles, similarly, Barophiles/Piezophiles live under high pressure, halophiles live in high saline water, Alkaliphiles/Acidophiles survive under extreme pH and Psychrophiles thrive under very low temperature (Wang, *et al.*, 2015; Burg, 2003). Adaptations of extremophiles evolved from wide range of biomolecules (Pakchung, *et al.*, 2005), which are called extremozymes. For example, an enzyme proteolysin identified from a Gram-positive anaerobic thermophilic bacterium *Coprophthermobacter proteolyticus*, was recombinantly expressed into *E. coli*, the enzyme showed high thermal stability (up to 80°C) in the presence of organic solvent and detergent as well as activity across a wide range of pH (Toplak, *et al.*, 2013). The relevant knowledge of the survival mechanism of extremophiles helps us to understand how life can flourish under inhospitable conditions.

1.2. Cold adaptation strategies

Start from the ice ages till to date snow cover is the largest component of cryosphere. According to National Snow and Ice Data Center, total area of covering seasonally or permanently is 46 million square kilometers which is approximately 10% of total earth surface. Moreover, most of the places (~85%) on earth are experiencing low temperature ($\leq 5^{\circ}\text{C}$) (Buzzini, *et al.*, 2012; Wang, *et al.*, 2017). In the harsh cold environments its inhabitants have to challenge many limiting factors, for example, reduced enzyme activity, decreased fluidity of cell membrane, low water and high osmotic pressure due to dehydration as a temperature compensation, altered nutrients' availability and waste products together with low temperature (D'Amico, *et al.*, 2006). However, cryophilic

microorganisms colonize successfully in such hostile habitats. These microorganisms construct their own survival strategies for cold-adaptation such as environmental adaptation e.g. habitat selection, transition into viable but non-culturable state, and molecular adaptation that includes elevation of unsaturated lipid synthesis in cell membrane and production of cold-active enzymes and proteins (Maayer, *et al.*, 2014). Formation of microbial community in specific environment for which key drivers are light (for autotrophic growth and avoidance of UV damage), water availability, and physical stability (Cary, *et al.*, 2010), is another strategy for cold adaptation. Example of such community is hypolithic communities found underside of translucent rocks, which promote their physical stability and desiccation (Cowan, 2009).

In addition, with above mentioned difficulties psychrophilic organisms have to face ice-crystal formation inside/outside of cells at sub-zero temperature which is fatal for living cell. Growing ice-crystal creates an osmotic pressure gradient due to increase concentration of solutes of cell surrounding, which induces dehydration of cell water causing critical damage of the cell. To inhibit this lethal ice they produce cryoprotective substances that include production of polyalcohols (glycerol) and polysaccharide substances (sugars) (Marx, *et al.*, 2009). These cryoprotectants inhibit ice formation by lowering the freezing point of water in a colligative manner. On the other hand, these microbes produce antifreeze proteins (AFP) which depress the freezing point in a non-colligative manner to protect cell from freeze damage (Bar-Dolev, *et al.*, 2012).

1.3. Discovery of antifreeze proteins (AFPs)

In 1950s several expeditions were carried out by some scientists to find out the answer of their curious mind question 'how arctic fishes swim in the water at $\sim -1.9^{\circ}\text{C}$ without freezing, however, their body fluid freezing temperature is approximately -0.8°C (Scholander, *et al.*, 1957). In their journey, it was observed that fishes located in shallow-water as well as deep-water. Water surface normally covered by ice for what shallow-water contained lot of ice crystal which might be very harmful for habitants. Relocation of deep-water species in the shallow-water caused death however, shallow-water species survived either layers of water. A conclusion was drawn by this group that shallow water species depressed their freezing point by producing high level of cryoprotectants i.e.

solutes concentration. However, the solute present in their fluid can only depress half of the required amount what did not have agreement with their conclusion.

After a decade, discovery of antifreeze glycoprotein (AFGP) from Antarctic fish blood and subsequent detection of antifreeze protein from various cold adapted organism revealed the mystery of survival mechanism of cold loving or cold tolerant species. DeVries and his co-workers isolated a protein containing carbohydrate named AFGP from the blood serum of Antarctic marine fishes which depressed the freezing point non-colligatively (DeVries, *et al.*, 1969). Antifreeze proteins are also called ice-binding protein.

1.4. Functions of antifreeze proteins (AFPs)

1.4.1. Freeze avoidance

AFPs which are also called AFPs can bind on single ice crystal to depress the freezing point of water below the equilibrium melting point in a non-colligative manner, resulting thermal hysteresis (TH) Fig. 1.1. AFPs adsorb on ice crystal to block their secondary nucleation which results depression of the freezing point. By this way thermal hysteresis proteins can prevent further growth of bound ice crystal. Polar fishes (Duman, *et al.*, 1976), insects (Tomchaney, *et al.*, 1982), microbes (Duman, *et al.*, 1993) those are lethal to ice formation synthesize these class of AFPs. For example, teleost fishes are living in the cold sea water where the temperature is approximately -1.9°C by avoiding freezing (Scholander, *et al.*, 1957; DeVries, *et al.*, 1969). Freeze-avoidant species produce moderate to high TH. Polar fishes producing AFPs & AFGPs in their blood are moderately active. They can depress the freezing point up to 1°C of millimolar level of protein concentration (Scotter, *et al.* 2006). However, insects have evolved mechanism to survive extreme subzero temperatures which can depress freezing point several degrees even at one tenth of concentration of fish derived AFPs (Duman, *et al.* 2004; Scotter, *et al.* 2006).

1.4.2. Ice shaping

One of the primary features of all AFPs to shape single ice crystal and, this is the indication to find the existence of AFP in the samples. It is the first step to discover a new AFP from natural sources. AFPs possess specificity to bind on single ice crystal that faceting ice crystal. Prism and/or pyramidal plane binding moderately active AFPs, identified by ice etching or modern technique in which AFPs are fluorescent tagged, change the crystal morphology to bipyramid whereas hyperactive insects or fish AFPs form lemon shape ice crystal (Scotter, *et al.*, 2006; Sun, *et al.*, 2014). Microbial AFPs modify the ice crystal in a differential manner, for example, hyperactive *Mp*AFP form hexagonal plate shape, on the other hand, *Tis*AFP8 forms round (spherical) shape crystal (Garnham, *et al.*, 2011; Cheng, *et al.*, 2016). In contrast moderately active microbial *Efc*IBP produce Saturn-shaped ice crystal (Kaleda, *et al.*, 2018). A plant AFP, *Lp*AFP binds on basal and primary prism plane regulate the crystal structure as bipyramid (Middleton, *et al.*, 2012). Moderately active AFPs initiate ice shaping during growth of crystal and hyperactive AFPs do on the melting crystal (Bar-Dolev, *et al.*, 2012). Though ice shaping is a precursor to be an AFP, it is not the main role of AFPs which originates due to the adsorption of AFP on the planes of ice crystal.

1.4.3. Ice recrystallization inhibition (IRI)

Ice recrystallization (IR) is a phenomenon that involve in increasing ice crystal size by Oswald ripening method, in which large ice crystal grow up in expense of smaller ones, in the frozen state. In the ice laden region, it is a widely occurring process which may cause detrimental damage for population living in such environment. AFPs produced by a range of cold adapted organisms can significantly inhibit ice recrystallization (Budke, *et al.*, 2014) by adsorbing on and restrain desorption from ice surface (Fig. 1.2). In the freeze tolerant species ice nucleation takes place frequently in their body fluid or surrounding of their cell are necessary to keep these crystals smaller to mitigate the damage. AFPs producing by these species, however, depress the freezing point a few tenths of a degree can protect the organisms from deleterious IR process. And, to date, all identified AFPs showed IRI activity though no clear correlation is established between

TH & IRI activities (Olijve, *et al.*, 2016). It is also not clearly understood what molecular factors determine the strength of IRI activity.

1.4.4. Some special functions of AFPs

At least two multidomain AFPs containing tandem B1g domains isolated from Antarctic bacteria (one is gigantic 1.5 MDa *MpAFP* and the other is *SfIBP*) which play a primary role to adhere the host organism on ice and that is why they are called as ice-binding adhesion protein (Garnham, *et al.*, 2011 & Vance, *et al.*, 2018). Both of them reduced freezing point by several degrees and *SfIBP* showed very high IRI ability even at subnanomolar level.

Another subset of AFP is ice nucleating protein (INP), a membrane associated protein which promote heterogeneous nucleating at subzero temperatures. This class of proteins are reported from plants, insects and bacteria. Their physiological role thought to hamper supercooling and induce freezing to control the location and growth of ice (Duman, 2015). For example, INPs nucleate ice in the extracellular fluid that can protect the cytoplasm of the cell. INPs & AFPs function inversely, however, it is hypothesized that their working principle likely to be the same to induce or inhibit freezing (Graether, *et al.*, 2001). A recent molecular dynamic simulation study revealed and proposed that INP possess anchored clathrate or ice-like waters for promoting ice nucleation (Hudait, *et al.*, 2018).

1.5. Identification of ice planes by fluorescently tagged AFP

All AFPs bind on specific plane of ice which was demonstrated by a technique named ice etching developed by Dr. Charles Knight in 1991 (Knight, *et al.*, 1991). In this technique, hemispherical single ice-crystal was grown in AFP solution. In this process, AFP bound to the growing ice as a microlayer. Then, the hemisphere kept into refrigerator at -20 °C for several hours to sublime so that bound proteins would be appeared as patches on the hemisphere. By relating these patches to Miller-Bravais indices, AFP bound planes could be determined.

Fluorescence-based ice plane affinity (FIPA) is a modified technique (Garnham, *et al.*, 2010; Basu, *et al.*, 2014) in which fluorescently tagged AFP-solution is used. Bound AFP molecules are visualized under specific wavelength of light to determine their bound ice-

planes, illustrated in Fig. 1.3 a, b. FIPA technique is comparatively easy, less time consuming and have identified target ice-planes of many AFPs.

1.6. Structure of AFPs

Protein structure is a key factor for its function. All AFPs adsorb on ice crystal to arrest its growth or to inhibit IR at subzero temperatures which is the basic role. However, AFPs possess a range of structural diversity. This incredible structural diversity of AFPs are predicted to be a result of convergent evolution in different organisms ([Chen, 1997](#)). On the other hand, distinct structural AFPs identified from genetically distant species, such as, DUF3494 domain containing IBPs having discontinuous β -solenoid with a long α -helix parallel to the axis of β -solenoid, isolated from bacteria, fungi, diatoms, algae and yeast which thought to be originated from horizontal gene transfer ([Raymond, et al., 2012](#)). To date, a good number of 3D structure of AFP molecules have been solved which can be divided as α -helix or globular type from fish, β -solenoid or polyproline type II coil from insect, and DUF3494 domain with discontinuous β -solenoid from microbes and β -solenoid type AFP also obtained from bacterium and plant ([Bar-Dolev, et al., 2016](#)).

1.6.1. Fish AFPs

Ice-binding protein from fish was first isolated in 1960s from Antarctic icefishes which was actually glycoprotein ([DeVries, et al., 1969](#)). Antifreeze glycoprotein (AFGP) contains amino acid repeating unit $-(\text{Ala-Ala-Thr})_n-$ bonded with carbohydrate moiety through hydroxyl oxygen of Thr residues (Fig. 1.4-a) AFGP presents in the blood of fishes as a mixture of several isoforms. These isoforms are structurally same but different in size due to variation in their repeating units, which varies from 4 to 50 accordingly molecular mass 2.6 kDa to 33.7 kDa ([Harding, et al., 2003](#)). AFGP showed different rate of migration on electrophoresis gel depending on which Antarctic notothenioid's glycoproteins were classified from AFGP 1 to 8 ([Feeney, 1974](#)).

One of the most studied AFPs is Type I polypeptide. Type I AFP has been reported from flounder or flat fishes winter flounder, *Pseudopleuronectes americanus* ([Duman, et al., 1976](#)); yellowtail flounder, *Limanda ferruginea* ([Scott, et al., 1987](#)); American plaice,

Hippoglossoides platessoides (Gauthier, *et al.*, 2005); & barfin plaice, *Liposetta pinnifasciata* (Mahatabuddin, *et al.*, 2017) as well as from cunner (*Tautoglabrus adspersus*) (Hobbs, *et al.*, 2011), sculpin, *Myoxocephalus octodecimspinosus* (longhorn sculpin) (Deng, *et al.*, 1997) & *Myoxocephalus scorpius* (shorthorn sculpin) (Low, *et al.*, 1998). This AFP type can be short (~ 60 aa) or long (~ 100 or more aa) in their amino acid sequence. Smaller Type I AFPs contain 11aa repeat varying from 2 to 4 whereas long isoforms don't maintain this repeat in their sequence. However, both type I AFPs are alanine rich (> 60%) and highly α -helical (Gauthier, *et al.*, 2005). The first X-ray structure of α -helical type I AFP from winter flounder was reported in 1995 which consist of 37 amino acid residues (Sicheri, *et al.*, 1995) (Fig. 1.4-b). A long isoform of winter flounder was discovered in 2004 (Marshall, *et al.*, 2004) and structure was solved in 2014 (Fig. 1.4-c) revealing its dimer formation with increased activity like insect AFPs (Sun, *et al.*, 2014). A 40-residue type I AFP from barfin plaice (a founder fish) shows that the AFP molecule oligomerized (tetramer 2.7% & octamer 0.7%) at higher concentration that integrate additional ability to bind on ice (Mahatabuddin, *et al.*, 2017).

Globular type II and type III AFP another two classes of AFPs. Type II AFP acquires C-type lectin structure which can be sub-divided into Ca^{2+} -dependent (Liu, *et al.*, 2007) or independent (Gronwald, *et al.*, 1998). Their structure consists of two helices with several β -strands in two β -sheets. The structure stabilizes by five disulfide bonds (Fig. 1.4-d) with intramolecular hydrogen bonds. 3D structure of type III AFP was reported in 1996 (Jia, *et al.*, 1996). It consists of short and imperfect β -strands and one turn of α -helix which makes a compact, angular structure. The unique difference of type III with type II is there is no disulfide bond instead they have many ' β -structure like' interstrand main-chain hydrogen bond that give the protein a rigid, globular fold. These two proteins are shown in (Fig. 1.4-d, e).

1.6.2. Insect AFPs

Insect AFPs commonly form regular β -helical structure without having a hydrophobic core, which compensates by consecutive disulfide bonds through the β -helix core and intramolecular hydrogen bonds (Liou, *et al.*, 2000) to give proper folding of the protein molecule. An insect, snow flea produces a Glycine rich AFP having polyproline type II-

like fold (Mok, *et al.*, 2010) without hydrophobic residues or disulfide bond in the core (Fig. 1.5-a). It has two disulfide bonds at the two edges of flat faces (Fig. 1.5-c). Another insect midge (*Chironomidae*) contained seven rank of tyrosine facing outward interrupting its regularity, flatness as well as antifreeze activity (Basu, *et al.*, 2016) (Fig. 1.5-d). Insect AFPs identified to date commonly possess repeating motif of Threonine either two ranks (Fig. 1.5-a) (Liou, *et al.*, 2000) or four ranks (Fig. 1.5-b) (Hakim, *et al.*, 2013). These repeating motifs of Threonine thought to be responsible to bind the protein to ice planes with the help of anchored clathrate water mechanism (Hudait, *et al.*, 2018).

1.6.3. Plant AFPs

Plants in the cold region of the earth also resist sub-zero temperature. AFPs from plant makes their host freeze-tolerant, which allow the formation of ice in their tissue without damaging by ice recrystallization inhibition (IRI) (Sidebottom, *et al.*, 2000). Two structures of AFP from plant are solved. A perennial grass, *Lolium perenne* (*LpAFP*), synthesized an AFP with β -helical structure having two extensive, flat surfaces 'a' and 'b' (Kuiper, *et al.*, 2001). However, crystal structure of *LpAFP* shows that the flatness of non-IBS 'b' surface disrupted by a bulge protruded from N-terminal (Middleton, *et al.*, 2012). On the other hand, 'a' surface makes a parallel array of hydrophilic residues, Out of 16 residues in this array 5 occupied by Thr, 6 by Ser, 1 by Asn, and rest of the four positions occupied by Val (2), Ala (1), Ile (1) (Fig. 1.6-a). However, these array lacks the regularity of insect AFPs. In addition, the side chain of these residues show conformational variability that revealed by crystal structure. Unlike from insect AFPs the *LpAFP* forms core with 2 arrays sidechains of hydrophobic residues come into the middle of 'a' & 'b' face and other two arrays formed by hydrophilic sidechains come into the core at the two edges of β -roll (Fig. 1.6-a). Another structure for carrot (*Daucus carota*) AFP (*DcIBP*), have recently been solved as a structural homolog of a non-AFP from green bean, polygalacturonase-inhibiting protein (PGIP2) (Fig. 1.6-c), having leucine-rich repeat motif formed a curved solenoid structure (Fig. 1.6-b).

1.6.4. Microbial AFPs

AFPs identified from microbes are bigger in size than AFPs from fishes and insects having molecular weight greater than 20 kDa. Almost all microbial AFPs identified till

the date belong to a family 'Domain of Unknown Region 3494' (DUF3494). This protein fold possesses a common structural feature with right-handed triangular β -helix having a long α -helix parallel to the axis of β -helix. The crystal structure of this protein fold was first reported simultaneously from our lab and another lab for *Tis*AFP6 (Kondo, *et al.*, 2012) and *Le*IBP (Lee, *et al.*, 2012) (Fig. 1.7-a, b). This class of protein also shows high sequence identity (~50%). However, this fold of protein lacks the repetitive motif that of insect AFPs although some of them showed very high activity like insect AFPs. These AFPs vary in their capping structure at the end of the β -helix upon which they are divided into group 1, group 2 and group 3 (Arai, *et al.*, 2019) (Fig. 1.7 a-f). Group 1 and group 2 both have large capping structure where the later one contained a disulfide bond. In contrast, group 3 has short capping structure. An exceptional AFP isolated from a bacterium, *Marinomonas primoryensis* which is a part of a gigantic (1.5 MDa) protein that localized on the cell surface of the bacterium and it is a Ca^{2+} -dependent AFP (Garnham, *et al.*, 2008). This protein helps the bacterium to anchor on ice. Crystal structure of the AFP fold (34 kDa) shows that it has xGTGND repeat which makes this surface flat and they hold 13 Ca^{2+} ions align down this surface (Garnham, *et al.*, 2011) (Fig. 1.7g).

1.7. Ice-binding site (IBS) of AFPs

AFP molecules possess diversity in their structure for a convergent function of ice binding to inhibit the growth of bound crystal until a certain degree of cooling, which is related to the strength of antifreeze activity of the molecule. IBS consists of amino acid residues which interact with ice to anchor the protein molecule on it. It is surprising that only selective residues on specific face or surface of the entire protein molecule can interact with ice. Like the structure of AFPs, IBSs are also diverse in their sequence, conformation and structure. However, some common features of IBS are identified, for example, hydrophobicity by acquisition of hydrophobic amino acid residues, flatness, in addition they are relatively extensive. Type I AFPs form its IBS on alanine-rich face having a flanking row of Thr & Ala in opposite sides (Graham, *et al.*, 2008). IBS of type II AFP is the flat & hydrophobic face which include calcium ion for calcium dependent species. The calcium ion binds with sidechain of the IBS residues to provide their specific conformation and rigidity to bind on ice (Arai, *et al.*, 2019). A type III AFP from notched

fin eel pout constructs a compound IBS, two adjacent sites inclined to each other at 150° that recognize two different ice surfaces (Garnham, *et al.*, 2010). β -helical AFP from insects compile their IBS forming ladder of TxT (T for amino acid Threonine & x for any amino acid) with a definite distance between two adjacent Thr which is the best matching with the water distance on ice surface leading to bind the AFP on ice surface strongly (Graether, *et al.*, 2000). Subsequent study of the structure by X-ray crystallography finds that these conserved Thr (TxT) expose their sidechains in identical orientations formulating the most regular as well as flat IBS (Liou, *et al.*, 2000). Microbial AFPs work out the most complicated IBS. Some of that can bind with ice strongly like insect (Hanada, *et al.*, 2014) and other interact with ice similar to fish AFP (Lee, 2012).

1.8. Hyperactive vs. Moderately active

With the discovery of many new AFPs it was observed that they show some similarity in their activity, for example, fish derived AFPs commonly produce lower TH (~1 °C) with bipyramid ice shape. On the other hand, insect AFPs depress the freezing point several fold higher than fish AFPs even at one-tenth of concentration with lemon shape ice-crystal (Scotter, *et al.*, 2006). Bipyramidal ice crystal formed in the fish AFPs solutions grows from the two tips of the bipyramid when the solution cool below freezing temperature. In contrast, lemon shape crystal in the presence of insect AFPs grow in a hexagonal dendritic pattern (Hakim, *et al.*, 2013). Scotter *et al.* classified AFPs considering TH into hyperactive and moderately active. Hyperactive AFPs can produce TH 2-5 °C which is 10-100 times higher than moderately active AFPs when compared at same concentration. Ice plane to which AFPs bind determine by etching or FIPA (Basu, *et al.*, 2013) technique revealed that hyperactive AFPs always target on basal plane (Bar-Dolev, *et al.*, 2016) including prism and pyramidal plane whereas moderately active AFPs bind on prism and/or pyramidal plane (Garnham, *et al.*, 2010) without basal plane (Loewen, *et al.*, 1999). Microbial AFPs show wide range of activity including hyperactivity and moderate activity. Some of the microbial AFPs can reduce freezing point similar to fish AFPs, however, they can adsorb on basal plane e.g. *Tis*AFP6 (Kondo, *et al.*, 2012), *Le*IBP (Lee, *et al.*, 2012), *Fc*IBP (Bayer-Giraldi, *et al.*, 2018) which was described as hallmark for

hyperactivity. As these microbial AFPs cannot stop basal plane growth below the T_m probably they have weak affinity toward basal plane.

1.9. Structure function correlation

To understand the functional mechanism of any protein properly its structural information is very important. X-ray crystallography provides a wide number of AFP structures which show their structural diversity, however, it is surprising that all of them are binding on ice plane to depress the freezing point. From the crystal structure of *MpAFP* it is observed that ice-like lattice water orders on IBS by its hydrophobic effect which are anchored on the protein surface via hydrogen bonds either with backbone or sidechain ([Garnham, et al., 2011](#)). The distances among these anchored waters have perfect position match with ice waters on basal and prism planes that might be technique to bind the protein on ice planes. The authors termed this AFP:ice interactions as ‘anchored clathrate’ mechanism of AFP function. 3D structure of insect AFP from *Rhagium inquisitor* (*RiAFP*), *Rhagium mordax* (*RmAFP*) reveals two layers of ice-like waters (tetrahedral or pentagonal pattern) on their wide flat IBS that can match with ice plane waters to bind the protein on it ([Hakim, et al., 2013](#) & [Ye, et al., 2020](#)). Similarly, polypentagonal hydration waters network observed on the surface of an activity improved variant of type III AFP ([Mahatabuddin, et al., 2018](#)). Molecular dynamic simulation of insect AFPs divulge three distinct anchored clathrate motifs of ice-binding ([Hudait, et al., 2018](#)). It is not clearly understood yet that all AFPs recognize ice planes through unique mechanism or not. That is why more crystal structure may contribute to discover the true mechanism of AFP action.

1.10. Solubility of IBPs that relates its function

Solubility of protein is important for its biological function. Fish type I, II, III AFPs can shape single ice-crystal to a hexagonal disc shape to bipyramid depending on their concentration ([Mahatabuddin, et al., 2016](#)). Type I AFP, BpAFP from barfin plaice showed concentration dependent oligomerization which enable this AFP to extend its target ice plane from pyramidal to entire ice planes including basal plane ([Mahatabuddin,](#)

et al., 2017). It is also supersoluble (650 mg/mL) in cold deionized water and transition from moderate activity to hyperactivity with increasing concentration, however, it is highly alanine-rich (75%) protein. A recent research has been reported by Tsuda, *et al.* in which Type II, III and AFGP shows solubility 200 mg/mL, 100 mg/ml and 1800 mg/ml respectively (Tsuda, *et al.*, 2020) at 4 °C. Surprisingly, at room temperature these proteins show lower solubility which might be appeared due to their thermal instability. All these protein shows very high TH at higher concentration (Tsuda, *et al.*, 2020).

1.11. Ligand of AFPs

The toughest recognition problem in biology considered antifreeze proteins binding to its ligand, which is a nascent ice nucleus. In ice, water molecules are three-dimensionally arranged to form its crystal lattice. The hexagonal ice crystal lattice is denoted by I_h and characterized by three equivalent a -axes (a_1, a_2, a_3) normal to the c -axis. The I_h crystal creates under atmospheric pressure (1 atm) with its six-fold symmetry. Planes of ice crystal are defined by Miller-Bravais indices (Hobbs, 1974). The planes can be categorized into basal, prism (primary and secondary) and pyramidal plane. Basal plane is perpendicular to c -axis. Primary and secondary prism planes are parallel to c -axis. On the other hand, pyramidal plane makes acute angle with c -axis. All ice planes are represented in fig. 1.9. AFPs can bind on these ice planes and change the crystal morphology as hexagonal bipyramid (Mahatabuddin, *et al.*, 2017), hexagonal plate (Cheng, *et al.*, 2011), lemon (Scotter, *et al.*, 2006) or spherical (Cheng, *et al.*, 2016).

1.12. Aims and objectives

Since 1960s AFPs are discovering and adding a wide number to its variety from different species of cold acclimated environments. In the recent time, a large number of crystal structure of AFP molecules are solved. It shows their structural diversity as well as their differences in binding strength on ice surface or activity. And their IBS amino acid sequences and structures are also diverse. Regardless of their diversity in IBS or overall structure, all AFPs bind on ice surface and depress the freezing point. This convergent function instigates to assume a common mechanism for all AFPs.

For insect AFP, regular array of Threonine either in two rank or four rank organize the

water molecules on its surface that are well fitted on waters of ice planes (basal as well as prism planes) (Liou, *et al.*, 2000; Hakim, *et al.*, 2013) (Fig. 1.5-a, b) which are called ‘ice-like waters’. In 2011, Garnham, *et al.* observed ‘ice-like waters’ on the IBS of an ice adhesin bacterium *Marinomonus primoriensis*, MpAFP and they proposed anchored clathrate water (ACW) mechanism for ice-binding of MpAFP as well as for other AFPs (Garnham, *et al.*, 2011). Polypentagonal ice-like waters-network is observed for fish type III and hyperactive MAXI (Sun, *et al.*, 2014; Mahatabuddin, *et al.*, 2018).

Microbial AFPs are evolved with a common scaffold as triangular irregular β -helix. Despite of their structural similarity, they have poorly conserved IBS without any repetitive motive as found in insects. However, they are showing wide range of activity as hyperactive to moderately active and follow a complex binding mechanism. This variation of activity showed by AFPs from different species were reported from isoforms of a same species, TisAFP6 as moderately active whereas TisAFP8 as hyperactive. Hanada, *et al.* and Cheng, *et al.* individually proposed the existence of compound ice-binding site of hyperactive ColAFP and TisAFP8. However, it is not clarified that how compound IBS organize water structure on its surface to offer the molecule to bind on different ice planes.

To reveal this unsolved mechanism, I worked on other isoform from the same species, *Typhula ishikariensis*, TisAFP7, which shows high sequence identity with both moderately active isoform TisAFP6 (87%) and hyperactive isoform TisAFP8 (90%). I performed functional and structural analysis to find out the molecular basis of their activity.

Figure 1.1. Illustration of thermal hysteresis (TH). In the upper panel 1, 2, 3 indicate the continuous growth of ice crystal in absence of AFP. In the lower panel red dots are indicating the AFP molecule in the solution.

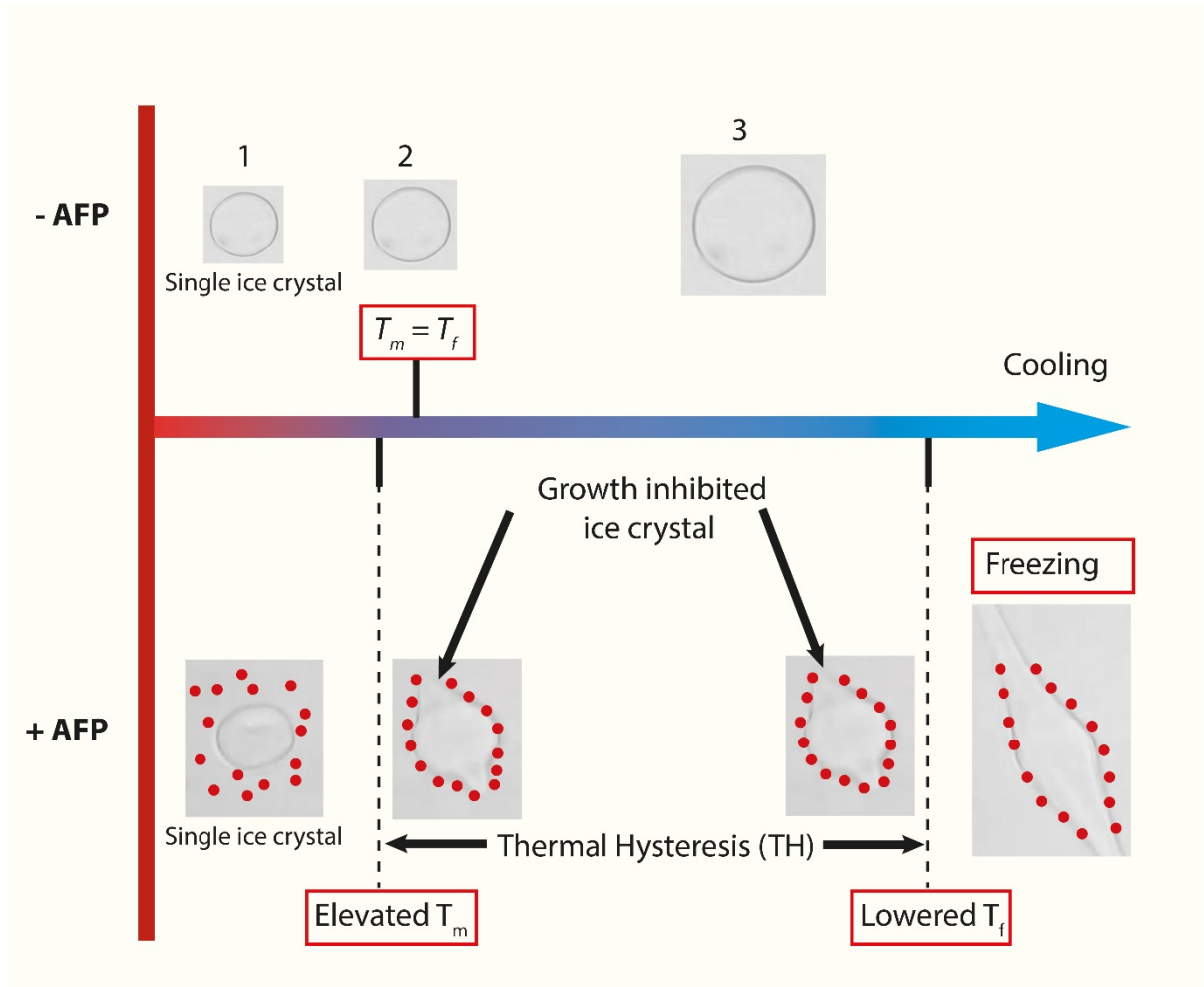


Figure 1.2. Ice recrystallization in the presence and absence of AFP in the solution. Lower panel represents zero-minute ice crystal and upper panel for 60 minutes. All images are snapshot from recorded video of experiment. 1, 2, 3 are three representative ice crystal which are becoming bigger via ice recrystallization.

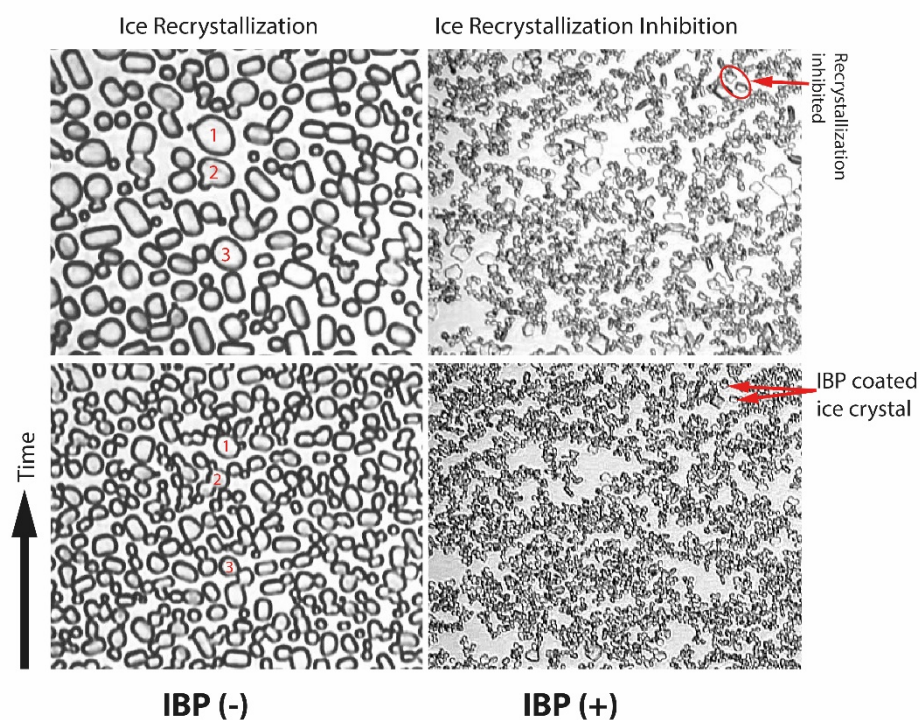
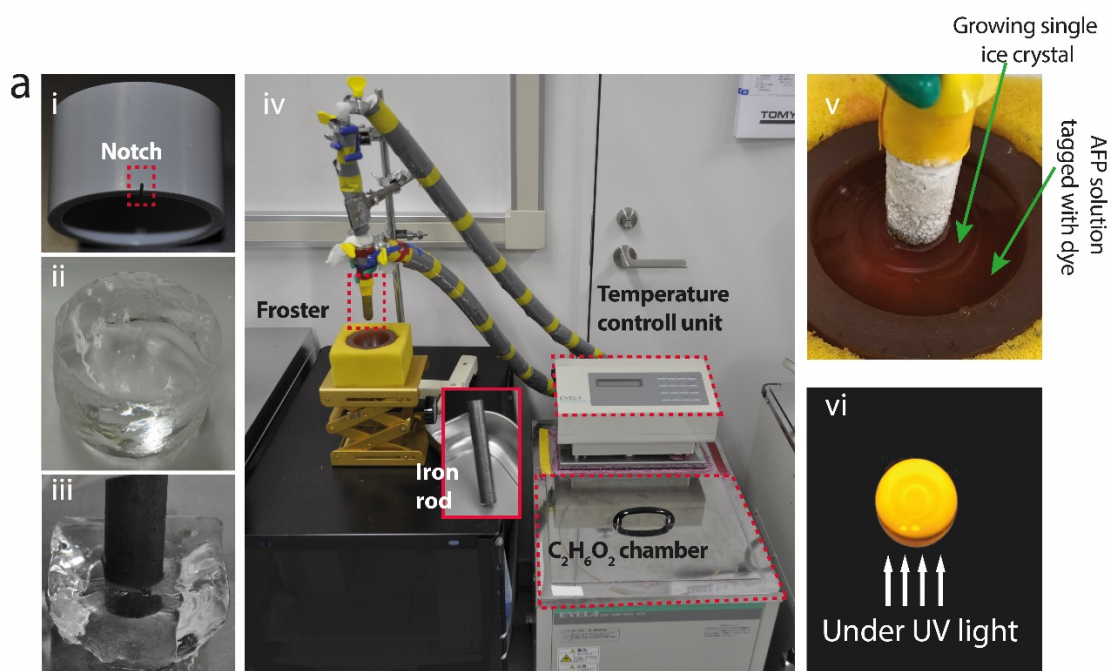


Figure 1.3. (a) Experimental set up for fluorescence-based ice plane affinity (FIPA) analysis. i) Polyvinyl chloride (PVC) pipe which is used to prepare single ice crystal. On the edge of the pipe there is a notch through which cold water enter into it. ii) A single ice crystal prepared using the PVC. iii) Creating a hole to mount the crystal with froster through this hole. iv) The instrument use to grow the single ice crystal in fluorescence-tagged AFP solution which includes ethylene glycol chamber and temperature control, froster and insulated AFP solution container. v) Crystal growing in the fluorescence labelled AFP solution. vi) A single crystal hemisphere grown into AFP solution visualizing bound AFP under UV light.

(b) Identification of pyramidal plane binding. i) A typical pyramidal plane in a single ice crystal. ii) A star mark created on the basal plane of single ice crystal which representing prism planes of the crystal. iii) A crystal mounted via basal plane perpendicular to the froster. iv) The crystal mounted through primary prism plane of the crystal. v) Illustration of typical pyramidal plane binding when mounting as iii. vi) Illustration of typical pyramidal plane binding when mounting as iv.



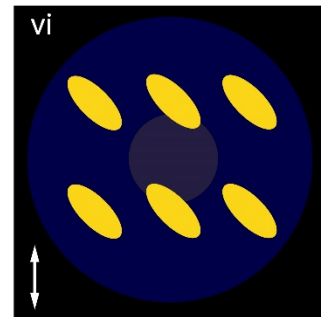
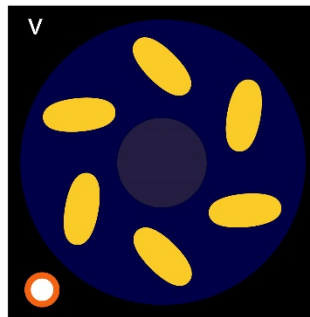
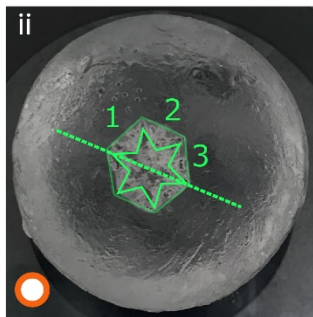
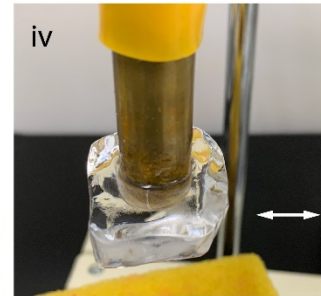
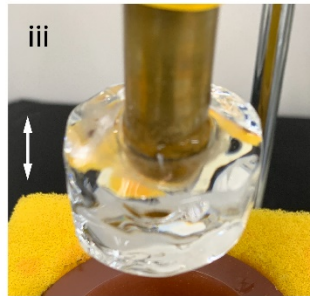
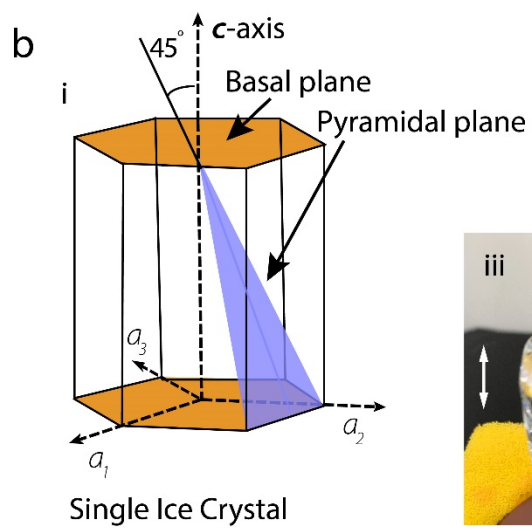


Figure 1.4. 3D structure of antifreeze proteins from fish. (a) Antifreeze glycoprotein (AFGP) isolated from Antarctic notothenioid. (b) Crystal structure of Type I AFP from winter flounder. (c) Crystal structure of hyperactive type I AFP (MAXI). (d) Type II AFP from long-snout poacher (*Brachyopsis rostratus*). Five disulfide bonds are denoted by numbers 1, 2, 3, 4, 5 in red color. (e) Type III AFP from eel pout (*Macrozoarces americanus*).

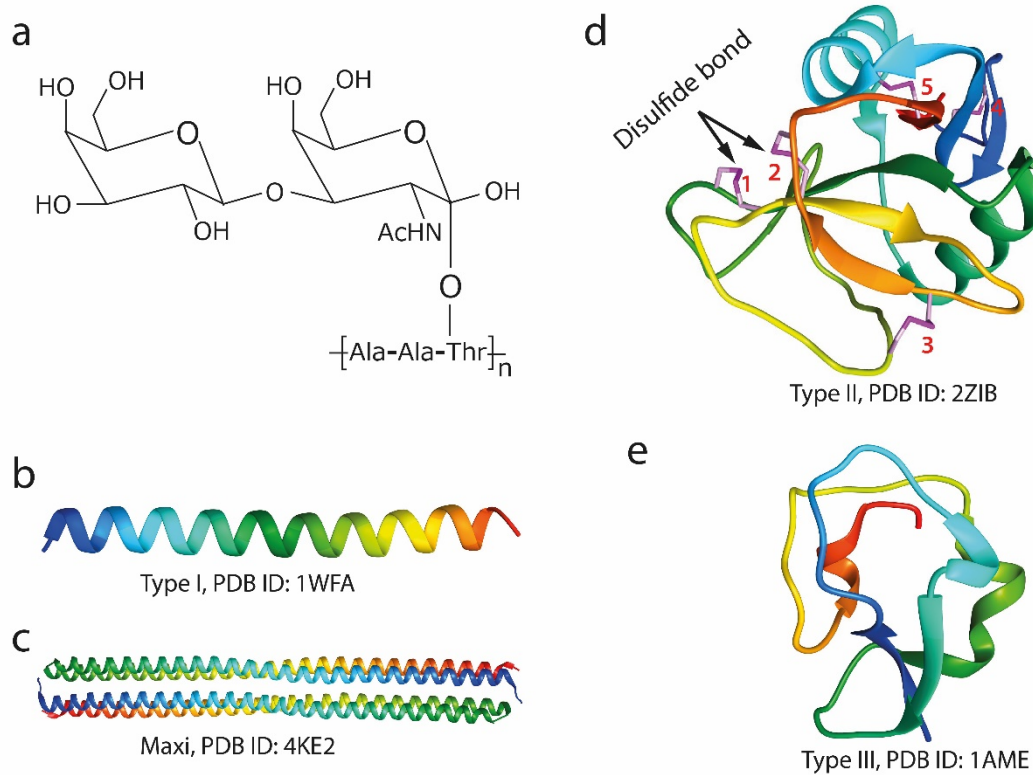


Figure 1.5. Crystal structure of insect AFPs. (a) structure of AFP from an insect *Tenebrio molitor* showing the regular array of Threonine residues on its ice-binding site (IBS). Inside core contained 8 consecutive disulfide bonds yellow color. (b) AFP from *Rhagium inquisitor*. Four rank of Threonine is shown on its IBS. (c) The AFP from snow flea. (d) Model of midge AFP, a fly collected from Lake Ontario, Canada. Seven Tyrosine residue aligned on its IBS.

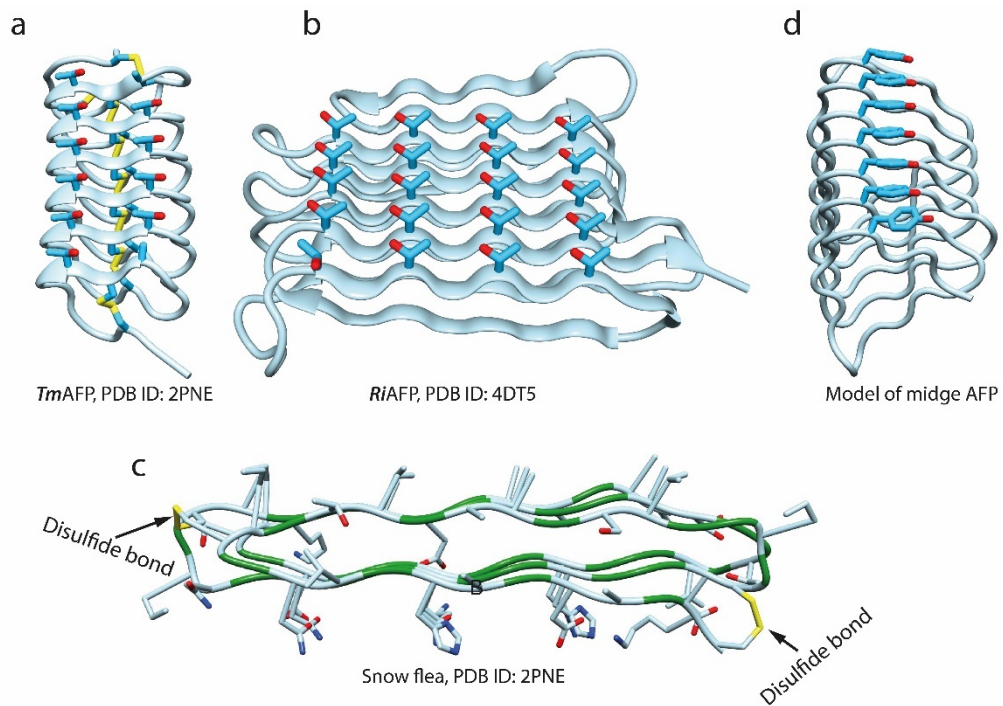


Figure 1.6. X-ray structure of AFPs from plants. (a) AFP from a ryegrass *Lolium perenne*. (b) A carrot AFP with long curve solenoid structure. (c) A plant polygalacturanase inhibitor protein (PGIP) from bean. It is a non-AFP protein with identical structure and 42.7% identity with carrot IBP.

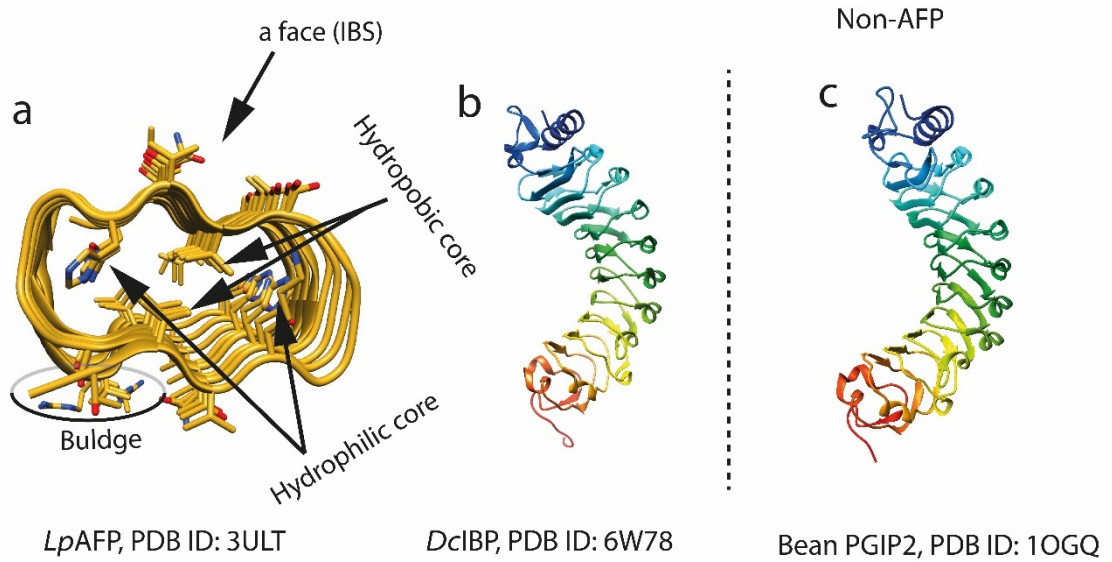


Figure 1.7. Microbial AFPs. Microbial AFPs form a same fold with different length of capping structure which colored in blue in each molecule. Group 1: *Tis*AFP6 (PDB ID: 3V3N) and *Le*IBP (PDB ID: 3UYU); Group 2: *Col*AFP (PDB ID: 3WP9) and *Ff*IBP (PDB ID: 4NU2); Group 3: *Anp*AFP (PDB ID: 7BWY) and *Efc*IBP (PDB ID: 6EIO). Exceptional microbial AFP from *Marinomonus premoensis* *Mp*AFP (PDB ID: 3P4G)

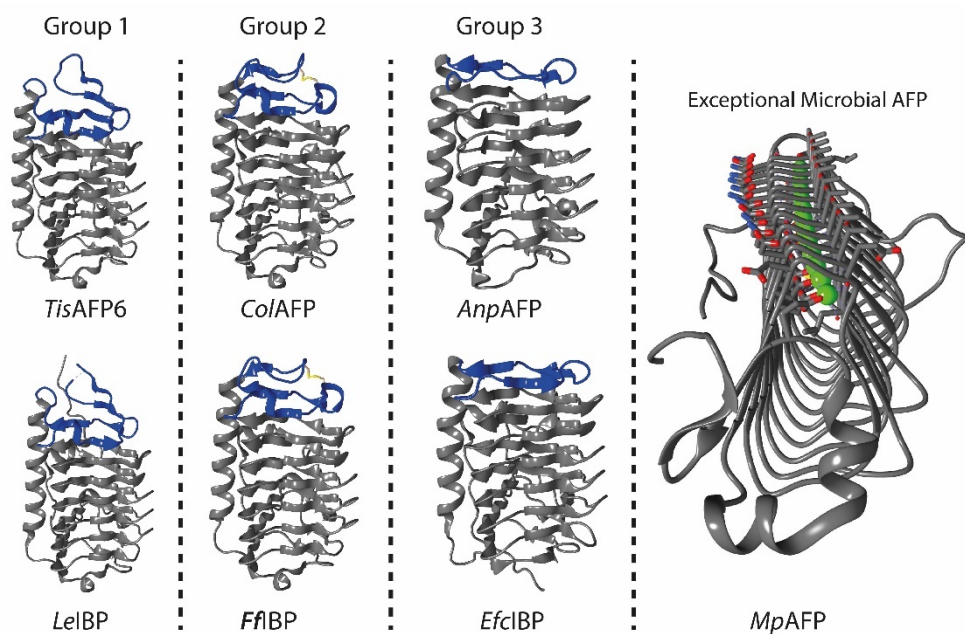


Figure 1.8. Ice-binding sites (IBS) of fish and insects AFPs. (a) Type II AFP (jsAFP) IBS is denoted by the red circle. (b) Compound IBS from fish Type III (nfeAFP) Two adjacent IBS, prism plane binding site colored purple and pyramidal plane binding site colored pink. (c) & (d) Distances between perfectly positioned Threonine arrayed on the IBS of insect AFPs from *Christonura funiferana* CfAFP and *Tenebio molitor* TmAFP.

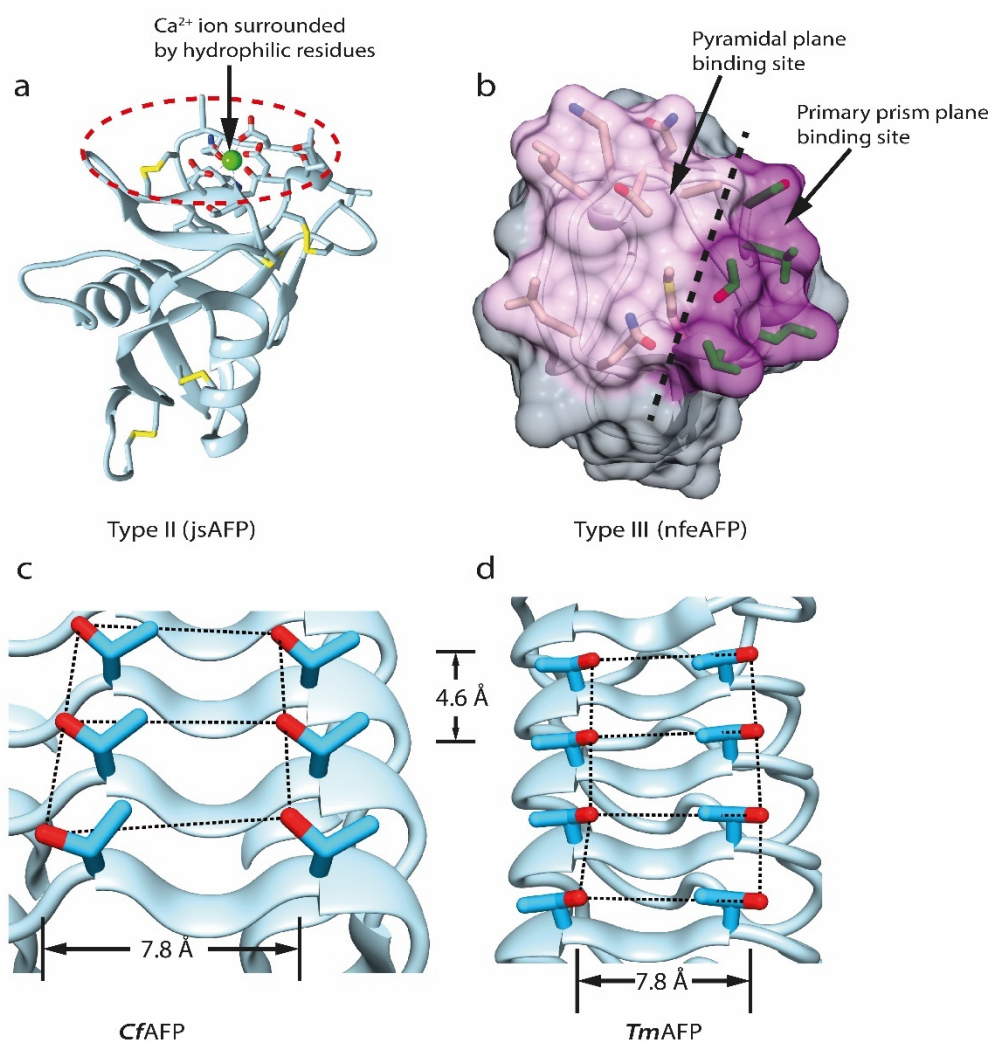
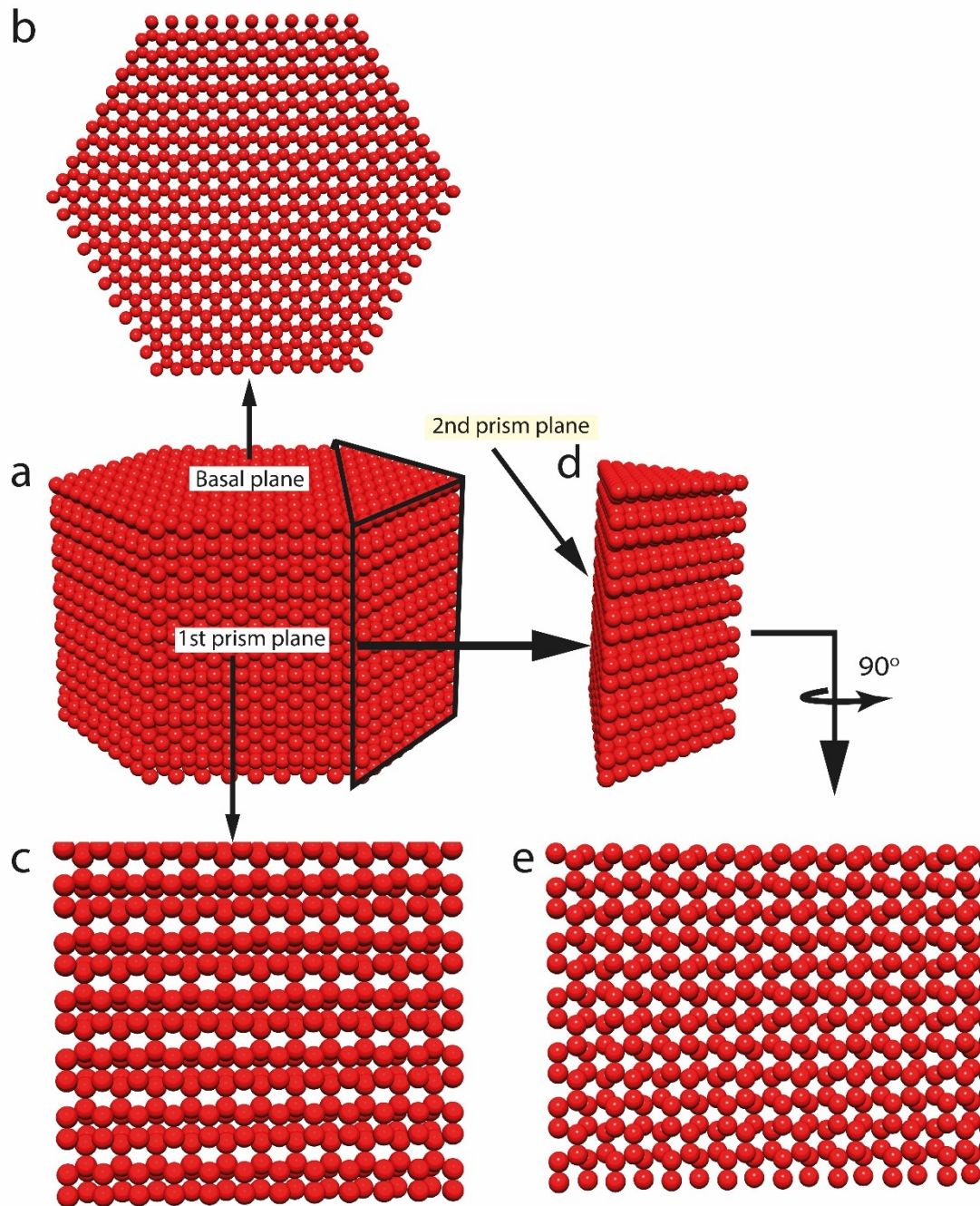


Figure 1.9. Illustration of hexagonal ice crystal (I_h) and its planes. (a) Model of a hexagonal ice crystal. (b) Basal plane. (c) Primary or 1st or 1° prism plane. (d) & (e) Secondary or 2nd or 2° prism plane.



Chapter 2

Characterization of microbial antifreeze protein with intermediate activity suggests that a bound-water network is essential for hyperactivity

Preface

In this chapter the author describes the contents of a published paper in the Scientific Reports: ‘Characterization of microbial antifreeze protein with intermediate activity suggests that a bound-water network is essential for hyperactivity’ by N. M.-Mofiz Uddin Khan, Tatsuya Arai, Sakae Tsuda and Hidemasa Kondo. The contribution of the authors in the manuscript is as Conceptualization, N.M.-M.U.K. and H.K.; Methodology, N.M.-M.U.K., T.A., S.T., and H.K.; Software, N.M.-M.U.K., S.T., and H.K.; Writing – original draft preparation and editing, N.M.-M.U.K. and H.K.; Figure preparation, N.M.-M.U.K. and H.K.

Abstract

Antifreeze proteins (AFPs) inhibit ice growth by adsorbing onto specific ice planes. Microbial AFPs show diverse antifreeze activity and ice plane specificity, while sharing a common molecular scaffold. To probe the molecular mechanisms responsible for AFP activity, we here characterized the antifreeze activity and crystal structure of *Tis*AFP7 from the snow mold fungus *Typhula ishikariensis*. *Tis*AFP7 exhibited intermediate activity, with the ability to bind the basal plane, compared with a hyperactive isoform *Tis*AFP8 and a moderately active isoform *Tis*AFP6. Analysis of the *Tis*AFP7 crystal structure revealed a bound-water network arranged in a zigzag pattern on the surface of the protein’s ice-binding site (IBS). While the three AFP isoforms shared the water network pattern, the network on *Tis*AFP7 IBS was not extensive, which was likely related to its intermediate activity. Analysis of the *Tis*AFP7 crystal structure also revealed the presence of additional water molecules that form a ring-like network surrounding the hydrophobic side chain of a crucial IBS phenylalanine, which might be responsible for the increased adsorption of AFP molecule onto the basal plane. Based on these observations, we propose that the extended water network and hydrophobic hydration at IBS together determine the *Tis*AFP activity.

2.1. Introduction

Organisms inhabiting cold regions have developed diverse strategies to prevent freeze damage. Antifreeze proteins (AFP) are part of one such strategy. These proteins are structurally diverse and produced by various organisms, including fish [Duman, *et al.*, 1976](#)), insects ([Tomchaney, *et al.*, 1982](#)), plants ([Urrutia, *et al.*, 1992](#)), and microbes ([Duman, *et al.*, 1993](#)).

AFPs have specific affinity for a single ice crystal, to arrest its growth in the direction normal to the bound surface. They noncolligatively depress the non-equilibrium freezing point (T_f) of a solution below the melting point (T_m) ([DeLuca, *et al.*, 1998](#) & [Bar-Dolev *et al.*, 2012](#)) via Gibbs–Thomson effect ([Yeh, *et al.*, 1996](#)). The difference between T_m and non-equilibrium T_f is called thermal hysteresis (TH), which is a prime indicator of the AFP antifreeze activity ([Devies, *et al.*, 2014](#); [Bar-Dolev *et al.*, 2016](#)). Based on the TH value, AFPs are classified into two main groups, namely, moderately active and hyperactive AFPs. Hyperactive AFPs achieve up to 2–5 °C TH, which is 10–100 times that of the moderately active AFPs at identical protein concentrations ([Scotter, *et al.*, 2006](#)). Hyperactivity can be ascribed to the binding affinity to the basal plane of ice as well as prism and pyramidal planes, leading to rapid ice growth (burst) below T_f , and expansion perpendicular to the ice c -axis with a dendritic pattern with a 6-fold symmetry ([Bar-Dolev, *et al.*, 2016](#); [Hakim, *et al.*, 2013](#)). By contrast, typical moderately active AFPs bind to prism and/or pyramidal planes to form a bipyramidal ice between T_m and the non-equilibrium T_f (defined as the TH gap). Below the non-equilibrium T_f , the ice crystal bursts at the two unbound bipyramid tips, which lie along the c -axis, forming a needle-shaped ice ([Davies, *et al.*, 1990](#); [Loewen, *et al.*, 1999](#); [Mahatabuddin, *et al.*, 2017](#)). The specific affinity toward sets of water molecules constituting discrete ice surfaces is attributable to the ice-binding site (IBS), localized on the flat surface of an AFP molecule ([Devies, *et al.*, 2014](#); [Bar-Dolev *et al.*, 2016](#)). IBS in various AFPs show vast amino acid sequence and structure diversity, which implies that each AFP evolved from a different ancestor molecule to adapt to the cold environment by acquiring ice-binding ability ([Bar-Dolev, *et al.*, 2016](#)). Therefore, understanding the detailed molecular mechanism that defines ice-binding specificity is crucial for the elucidation of adaptation to cold environment associated with AFP evolution.

Most microbial AFPs, produced by such microorganisms as fungi (Duman, *et al.*, 1993; Hoshino, *et al.* 2003), diatoms (Bayer-Giraldi, *et al.*, 2011), and bacteria (Raymond, *et al.*, 2007), belong to a widespread family formerly designated as “domain of unknown function” (DUF) 3494 (Vance, *et al.*, 2019) in the Pfam database (El-Gebali, *et al.*, 2019), and currently categorized as “ice-binding–like family”. Characterization of DUF3494 AFPs (or ice-binding proteins; IBPs) revealed their highly diversified antifreeze activities. For example, AFPs from *Colwellia* sp. (ColAFP) (Hanada, *et al.*, 2014), *Flavobacterium frigidis* PS1 (FfIBP) (Do, *et al.*, 2014), *Typhula ishikariensis* isoform 8 (TisAFP8) (Cheng, *et al.*, 2016), and *Shewanella frigidimarina* (SfIBP_1) (Vance, *et al.*, 2018)²³ are hyperactive, whereas those from *Leucosporidium* sp. (LeIBP) (Lee, *et al.*, 2010), *T. ishikariensis* isoform 6 (TisAFP6) (Kondo, *et al.*, 2012), *Antarctomyces psychrotrophicus* (AnpIBP1a) (Arai, *et al.*, 2016), and *Fragilariopsis cylindrus* (FcIBP) (Bayer-Giraldi, *et al.*, 2011) show moderate activity. X-ray crystallographic studies revealed that all these AFPs adopt a similar β -helical structure. The putative IBS of this protein family is located on the flat surface of one β -helix (Hanada, *et al.*, 2014; Cheng, *et al.*, 2016; Kondo, *et al.*, 2012). The level of IBS sequence identity shared by various AFPs is similar to that shared by other regions of these proteins; however, no conserved sequence motifs in IBS have been identified. Based on phylogenetic analysis, microbial AFP genes are presumably propagated via horizontal gene transfer (Arai, *et al.*, 2016; Raymond, *et al.*, 2012). Less is known, however, about the key determinants of hyperactivity and moderate activity of this AFP family.

Three-dimensional structural analyses of hyperactive AFPs from arthropods and bacteria have revealed unique features of the IBS and bound water structure. For instance, insect AFPs form a β -helical structure with repetitive sequence motifs. Conserved residues in these motifs (Thr–X–Thr, where X is any amino acid) are organized into two parallel arrays along the β -helical axis, constructing the IBS on one flat face of the β -helix (Liou, *et al.*, 2000; Graether, *et al.*, 2000). On the array, Thr residues are aligned at constant intervals, matching the distance between the water molecules on the ice plane (Liou, *et al.*, 2000). Further, the hydroxy groups in the side chain of IBS Thr residues anchor the bound water molecules at constant intervals, which match the basal and prism plane of the ice surface (Graether, *et al.*, 2000). According to a molecular dynamics simulation

study, regularly ordered water clathrate gives rise to the highest affinity for the ice basal plane (Chakraborty, *et al.*, 2000). Crystal structure analysis of *MpAFP_RIV* from the Antarctic bacterium *Marinomonas primoryensis* revealed an array of ordered water molecules aligned along the IBS residues. The bound water molecules are anchored to a repetitive motif of IBS residues (Thr–Gly–Asn/Asp) to form ice-like structure, which led to the proposal of the “anchored clathrate water” mechanism for AFP–ice interaction (Garnham, *et al.*, 2011).

IBS of microbial AFPs with the DUF3494 fold lack the repetitive amino acid sequence and consensus motif (Hanada, *et al.*, 2014; Vance, *et al.*, 2018; Kondo, *et al.*, 2012), which implies that a different architecture drives their hyperactivity. Based on studies of *ColAFP* and *TisAFP8*, Hanada *et al.* (2014) and Cheng *et al.* (2016), respectively, proposed that the b-face of IBS be subdivided into the β -sheet and adjacent loop regions, termed a compound IBS, which stick to the discrete ice plane. Based on the crystal structure analysis and ice-docking modeling combined with a mutation study, IBS loop residues were proposed as crucial for the affinity to the basal plane, conferring hyperactivity (Hanada, *et al.*, 2014; Cheng, *et al.*, 2016). Nevertheless, it is still not clear how the compound IBS recognizes the specific ice plane.

The snow mold fungus *T. ishikariensis* secretes AFP (*TisAFP*) at 0 °C (Hoshino, *et al.*, 2003). Culture filtrates contain seven AFP isoforms (*TisAFP2–8*), 223 amino acid residues each. We have previously shown that *TisAFP8* is hyperactive (Cheng, *et al.*, 2016) whereas *TisAFP6* is only moderately active (Kondo, *et al.*, 2012), despite high sequence identity (83%) shared with *TisAFP8*. Another isoform, *TisAFP7*, shares an even higher identity with *TisAFP8* (91%) and *TisAFP6* (87%). We reasoned that *TisAFP7* might be a suitable target molecule for gaining insight into the relationship between AFP antifreeze activity and IBS structure. In the current study, we characterized *TisAFP7* using TH measurements, ice crystal morphology observation, and visualization of AFP-bound ice planes by fluorescence-based ice plane affinity (FIPA) analysis. We also analyzed the crystal structures of *TisAFP7* and its defective mutants. The analysis revealed a distinct bound-water network on IBS, which presumably defines the binding affinity of microbial AFPs for the basal plane. The findings broaden the understanding of cold-adaptation mechanism of psychrophilic and cold-tolerant microorganisms, and will inform the design of artificial compounds with antifreeze properties.

2.2. Materials and methods

2.2.1. Preparation of *TisAFP7* and its mutants

The *TisAFP7* (Genbank accession number BAD02892) gene was codon-optimized for *Escherichia coli* and synthesized by GENEWIZ (South Plainfield, NJ, USA). It was then inserted into the expression vector pET38b (Novagen, Madison, WI, USA). The vector was used to transform *E. coli* BL21 (DE3) (Novagen), and the transformants were plated onto Luria-Bertani (LB) agar containing 30.0 µg/ml kanamycin, and incubated at 37 °C overnight. Site-directed mutagenesis of the *TisAFP7* gene was performed using the KOD plus mutagenesis kit (Toyobo, Osaka, Japan) with primers listed in Supplementary Table S1, and the mutations were confirmed by sequencing. Protein samples of wild-type *TisAFP7* and its mutants were prepared as described by Cheng *et al.* (2016), with a slight modification. Specifically, a bacterial colony was inoculated into 50 ml of LB medium containing 30.0 µg/ml kanamycin, and pre-cultured overnight at 37 °C, with shaking at 130–140 rpm. The culture was then transferred into 1.0 L of fresh LB medium supplemented with 30.0 µg/ml kanamycin, and cultured at 30 °C until the culture OD₆₀₀ reached 0.4–0.8 units. Expression of *TisAFP7* was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were further cultured at 15 °C for 24 h, harvested by centrifuging, and disrupted using high-pressure homogenizer EmulsiFlex-C3 (Avestin Europe, Mannheim, Germany). Cell debris was removed by centrifuging, and the supernatant was dialyzed against 10 mM glycine-HCl (pH 3.0). The dialysate was filtered and loaded onto a cation exchange column Macro-Prep High S (Bio-Rad, Hercules, CA, USA) equilibrated with the same buffer. The adsorbed sample was eluted using a linear gradient of 0–300 mM NaCl. *TisAFP7*-containing fractions were identified by checking antifreeze activity and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and were then dialyzed against 25 mM ammonium bicarbonate (ABC) buffer (pH 7.9). The sample was loaded onto an anion exchange column Macro-Prep High Q (Bio-Rad) equilibrated with same buffer. *TisAFP7* was collected in the flow-through fraction. The purity of the sample was confirmed by SDS-PAGE and analytical size-exclusion chromatography (SEC) using Sephadex G-75 (Cytiva, Marlborough, MA, USA). Purified *TisAFP* preparation was concentrated by

using an ultrafiltration device Amicon Ultra (MilliporeSigma, Burlington, MA, USA). Protein concentration was determined based on the optical absorbance at 280 nm. Wild-type *TisAFP7* eluted as a major fraction after cation-exchange chromatography, and migrated as a single band on SDS-PAGE gel and a single peak after SEC (Supplementary Fig. S2).

2.2.2. Circular Dichroism (CD) spectroscopy

To examine protein folding and thermal stability of the prepared samples, circular dichroism (CD) spectra were measured for *TisAFP7*, T20Y, A214Y, T20Y/A214Y mutants, *TisAFP6* and *TisAFP8*. CD spectra were measured on a J-725 CD spectrophotometer (JASCO, Tokyo, Japan) equipped with a temperature-controlled sample holder. *TisAFP* samples were prepared at 0.25 mg/ml in 10mM sodium phosphate buffer (pH7.4) and measured at 4 °C in a quartz cuvette of 1mm optical path length. As shown in Supplementary Fig. S3a, all the samples except for T20Y/A214Y exhibits CD spectra with similar profiles, which are typical for the β -sheet structure. CD spectra of T20Y/A214Y is not shown in Supplementary Fig. S3a due to a technical reason for a storage device of the raw data while it showed a similar profile to other samples. Subsequently, the thermal stability of each *TisAFP* sample was observed by monitoring the ellipticity (θ) at 220 nm with heating the samples at 0.2 °C interval from 20 °C to 60 °C at a rate of 1 °C/min. During the temperature scanning, CD spectra were recorded at 4, 20, 30, 40, 50, 60 °C to record the spectral change upon the thermal denaturing process. Typical spectral change is shown in Supplementary Fig. S3b for *TisAFP7*, which was fully denatured at 60 °C. Fraction of unfolded protein was estimated as $(\theta_T - \theta_{20^\circ\text{C}})/(\theta_{60^\circ\text{C}} - \theta_{20^\circ\text{C}})$ and smoothened by averaged over adjacent values within 0.4 °C, then plotted against temperature (T) with 1.0 °C intervals in Supplementary Fig. S3c and d. Denaturation temperature (T_m) was estimated as a midpoint temperature at which 50% of the sample is unfolded. For *TisAFP6*, the heating process was applied up to 70 °C, and $\theta_{70^\circ\text{C}}$ was adopted instead of $\theta_{60^\circ\text{C}}$ as *TisAFP6* exhibited slightly higher thermostability than other isoforms. After the temperature scanning, the sample was cooled back to 4 °C. For all *TisAFP* samples, the denaturated protein was precipitated in the cuvette even after the cooling back process. Also, CD spectrum observed at 4 °C after

heating process revealed the most of samples remain denaturated, as shown in Supplementary Fig. S3b. These observations exhibited that the thermal denaturation of *TisAFP* is the irreversible process whereas the reversible process was reported for fish AFPs (Mahatabuddin, *et al.*, 2017; García-Arribas, *et al.*, 2017; Gauthier, *et al.*, 2005).

2.2.3. Photomicroscopic determination of antifreeze activity

The antifreeze activity of protein samples was evaluated by observing ice crystal growth in AFP solutions by using Leica DMLB100 photomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with Linkam THMS600 sample temperature controller (Linkam Scientific Instruments Ltd., Tadworth, Surrey, UK). A color video 3CCD camera (Sony, Tokyo, Japan) was used to capture the images of ice crystals and the temperature of the solution displayed on the screen.

TH of protein solutions was determined as described by Takamichi *et al.* (2007). Briefly, approximately 0.75 μ l of AFP sample was placed in a center of a glass capillary tube (10–15 mm long), which was then sealed at both ends with mineral oil. The capillary was placed in a copper holder on the cooling stage. To ensure the thermal conductivity, the clearance gap between the capillary and the stage was filled with 20 μ l of ethylene glycol. The sample solution was frozen by cooling to approximately -30°C , subsequently warmed up to obtain a single particle of ice crystal. The melting point was determined as the temperature at which the ice started to melt. The sample was then incubated for 3 min at 0.05°C below the T_m . Finally, the ice crystal was cooled at a rate of $0.1^{\circ}\text{C}/\text{min}$ until rapid growth of the ice crystal was observed. The temperature at which the rapid growth started was recorded as the freezing point, T_f , of the solution. TH measurement was repeated at least three times for each sample at the specified protein concentrations.

2.2.4. FIPA analysis

To determine the *TisAFP7*-bound ice plane, FIPA analysis was performed, as described by Garnham *et al.* (2010) and Basu *et al.* (2014). Briefly, a single ice crystal, approximately 40 mm in diameter and 30–40-mm high, was prepared in a suitably sized polyvinyl chloride pipe by gradually growing from a single seed ice. The single

crystallinity of the ice crystal was checked by using in-house assembled crossed polarizer. The orientation of the crystallographic *a*-axis was confirmed by the direction of a star shape poked on the ice surface after lyophilization for 10 min. The macroscopic ice crystal was then mounted on a brass cold finger cooled at -1°C , with the basal or primary prism plane perpendicular to the cold finger. The mounted ice crystal was immersed in chilled milli-Q water and trimmed to a hemispherical shape with 10-mm diameter. Wild-type *TisAFP7* was labeled with an orange-fluorescent dye [5(6)-TAMRA-X, SE] (Thermo Fisher Scientific, Waltham, MA, USA). The three mutants, *TisAFP7* T20Y, A214Y and T20Y/A214Y, were labeled with NHS-Fluorescein (Thermo Fisher Scientific). AFP samples were incubated with the fluorescent dyes in 0.1 M NaHCO_3 at 25°C for 3 h, and then dialyzed against water to remove free dye. The mounted ice crystal was grown in 35–40 ml of a solution of fluorescently-labeled AFP (0.007 mg/ml), which was slowly incorporated into the specific ice plane in the hemisphere. The AFP solution was stirred every 20 min. The cooling temperature of the cold finger was gradually lowered to between -7°C and -8°C over 3–4 h. The ice hemisphere was detached from the cold finger after achieving a 45-mm diameter, and kept refrigerated at -30°C for 20–30 min prior to observation. The fluorescence of the ice hemisphere was observed under UV light and photographed.

2.2.5. Crystal structure analysis of *TisAFP7* and T20Y mutant.

TisAFP7 and T20Y mutant were crystallized by hanging-drop vapor diffusion method (McPherson, *et al.*, 2014). The initial crystallization conditions were determined by using screening kits, Crystal Screen, Crystal Screen II, and Index (Hampton Research, Aliso Viejo, CA, USA), and Wizard (Rigaku Reagents, Bainbridge Island, WA, USA). For the crystallization trial, the protein solution was concentrated to 30.0 mg/ml for wild-type *TisAFP7* and 28.0 mg/ml for the T20Y mutant in milli-Q water. Then, 1 μl of the protein solution was mixed with an equal volume of a mother liquor of the crystallization solution and incubated at 20°C . A thin plate-like crystal was grown under various conditions, as specified in the kits; all reactions contained polyethylene glycol and polyethylene glycol monomethyl ether 5000. The wild-type *TisAFP7* crystal used for the data collection was grown in 0.2 M ammonium sulfate, 0.1 M 2-(*N*-morpholino)ethanesulfonic acid

monohydrate (pH 6.5), and 30% (w/v) polyethylene glycol monomethyl ether 5000. For *TisAFP7* T20Y, the diffraction data were collected using a crystal grown in 0.2 M MgCl₂, 0.1 M Tris-HCl (pH 8.5), and 30% (w/v) polyethylene glycol 4000. For diffraction data collection, the crystals were picked up and flash-cooled in liquid nitrogen. The diffraction data for both proteins were collected at the beamline BL-1A in Photon Factory, KEK, Tsukuba, Japan (Liebschner, *et al.*, 2016), using 1.1000 Å radiation. The diffraction data were processed using XDS (Kabsch, *et al.*, 2010) and CCP4 (Winn, *et al.*, 2011) software suites version 7.0.055 (<http://ccp4.ac.uk>). The crystal structure of *TisAFP7* was determined by the molecular replacement method using PHENIX (Adams, *et al.*, 2010) and the coordinates of *TisAFP8* (Protein Data Bank 5B5H) as a search model. The initial model was examined and corrected by using Coot (Emsley, *et al.*, 2010), and further refined by using PHENIX (Adams, *et al.*, 2010) and REFMAC5 (Murshudov, *et al.*, 2011). The crystal structure of T20Y mutant was determined by molecular replacement by using the refined structure of *TisAFP7* as a search model and refined by the same procedure as that used for wild-type *TisAFP7*. To survey the bound water molecules of *TisAFP*s, which were crystallized in different space groups, the water molecules were rearranged in equivalent positions around the IBS by applying crystallographic symmetry operation of each space group by using Coot (Emsley, *et al.*, 2010).

2.2.6. Docking with ice plane

To assess the structural similarity of IBS waters and ice waters, the water molecules bound at the IBS loop of *TisAFP7* and a set of water molecules comprising the basal plane of ice were superposed. Eleven water molecules at the IBS loop were sampled for superposition with the basal plane of ice crystal. The optimum orientation of the ice plane was determined by using program LSQKAB version 7.0.055 (Kabsch, *et al.*, 1978) in CCP4 suite, which calculates the presumable minimum distances between bound water molecules and the corresponding ice-plane water molecules. The same calculation was performed for *TisAFP8* and *TisAFP6*. In these analyses, the same numbers of bound water molecules and in positions equivalent to those on *TisAFP7* were sampled.

2.2.7. Molecular Dynamics Analysis

To compare the hydration network on the molecular surface of *TisAFP7*, *TisAFP8*, and *TisAFP6*, we calculated the residence time for water molecules by molecular dynamics (MD) simulations using the program GROMACS 2020 version 2020.1-Ubuntu-2020.1-1 (<http://gromacs.org>) (Abraham, *et al.*, 2015). The crystal structure of each *TisAFP* isoform was solvated in a simulation box and neutralized by adding counter ions (Na⁺ or Cl⁻). After energy minimization and short position-restrained MD simulation, production MD simulation in 5 ns was performed. Berendsen temperature and pressure coupling were applied, along with the OPLS all-atom force field (Jorgensen, *et al.*, 1996) and TIP4P water model (Jorgensen, *et al.*, 1983). The trajectory of the MD simulation was applied to the hbond tool in GROMACS to calculate the residence time of water molecules that are forming hydrogen bonds with the protein surface. The surface of *TisAFP* was divided into a whole molecule, IBS, and non-IBS to compare the water residence time of each region. The water residence time was estimated as a “forward lifetime” of hydrogen bonds, based on the model by references (Luzar, *et al.*, 1996) and (Luzar, *et al.*, 2000).

2.3. Results and discussion

2.3.1. *TisAFP7* shows intermediate TH activity that falls between that of *TisAFP6* and *TisAFP8*

Antifreeze activity of *TisAFP7* was first evaluated by TH measurements, at a series of protein concentrations (0.022–0.23 mM; Fig. 2.1a). TH value increased with an increasing protein concentration with a maximum of 0.95 °C at 0.23 mM protein. We have previously characterized TH activity of the hyperactive isoform *TisAFP8* (Cheng, *et al.*, 2016) and the moderately active isoform *TisAFP6* (Kondo, *et al.*, 2012). *TisAFP8* showed the highest TH (approximately 2 °C) at 0.11 mM, and *TisAFP6* exhibited the lowest TH (approximately 0.6 °C) at 0.35 mM. Comparing the TH values of these AFPs at the same protein concentration, e.g., 0.11 mM, *TisAFP7* TH was approximately 35% that of *TisAFP8*, and 1.15-fold that of *TisAFP6*. Based on the TH values, the antifreeze activity of the three isoforms could be ordered as *TisAFP8* > *TisAFP7* > *TisAFP6* (highest to lowest). *TisAFP7* shares 91% and 87% sequence identity with *TisAFP8* and *TisAFP6*, respectively, which supports its intermediate position among the isoforms. Based on the

TH value, *TisAFP7* is an intermediately active AFP. The denaturation profile monitored by CD spectra from 20 °C to 70 °C is shown in Supplementary Fig. S3c. Denaturation temperature (T_m) was estimated as a midpoint of the profile as 47.5, 50.0, 53.5 °C for *TisAFP7*, *TisAFP8*, and *TisAFP6*, respectively. Besides, defective mutants of *TisAFP7* exhibited no change in their T_m , as shown in Supplementary Fig. S3d. Our current result for T_m shows that the order of the thermal stability is not correlated with that of TH values. Contrary to our results, higher T_m was reported for moderately active *LeIBP*²¹ (61.0 °C) and *EfcIBP* (Mangiagalli, *et al.*, 2017) (66.4 ± 2.7 °C), and lower T_m was reported for hyperactive *FfIBP*²¹ (56.4 °C) and *IBPv* (Wang, *et al.*, 2016) (53.5 °C). On the other hand, thermal denaturation experiments for chimeric proteins for *LeIBP* and *FfIBP* (Do, *et al.*, 2014) demonstrated that denaturation temperature of microbial AFP might be affected by various structural segments including capping head regions, which cover both ends of β -helix. Therefore, the authors of the referenced paper 21 suggested that T_m is not directly related to antifreeze activity. For *TisAFP6*, most of the replaced residues (7 residues out of 11) from *TisAFP7* and *TisAFP8* are situated in the capping head regions, which seems to originate the highest T_m among the isoforms.

2.3.2. *TisAFP7* exhibits a concentration-dependent ice-bursting pattern

Figure 2.1b presents microscopic images of a cooled ice crystal used for TH measurements. At 0.23 mM *TisAFP7* (TH 0.9 °C), the single ice crystal retained its original size below T_m (Fig. 2.1b, panel 1). Once the temperature reached the non-equilibrium T_f , the ice crystal rapidly grew (burst) perpendicular to the *c*-axis, with a hexagonal-stellar pattern with dendritic branches (Fig. 2.1b, panel 2), which is typically observed for hyperactive AFPs (Cheng, *et al.*, 2016; Vance, *et al.*, 2018). The observed restriction of ice growth along the *c*-axis could be ascribed to AFP binding to the basal plane of ice crystal, as proposed by Scotter *et al.* (2018), a hallmark of hyperactive AFP. We showed that *TisAFP7* bound to the basal plane at high concentration (0.23 mM). At a relatively low concentration (0.01 mM), the seed ice crystal changed shape to a hexagonal bipyramid with truncated tips in the TH gap, and then grew rapidly, maintaining the bipyramidal shape (Fig. 2.1b, panels 3 and 4). This indicated that *TisAFP7* affects the ice morphology and ice-bursting pattern in a concentration-

dependent manner. The ice morphology at a low protein concentration revealed that the *c*-axis growth was not as inhibited as at a high protein concentration. The known hyperactive AFPs inhibit *c*-axis ice growth, with a hexagonal dendritic bursting pattern even at a low protein concentration. For example, the hyperactive isoform *TisAFP8* supports a hexagonal pattern burst at high and low concentrations (0.12 mM and 0.01 mM), as shown in Fig. 2.1b, panels 5, 6, 7, and 8. A similar bursting pattern was reported for *sbwAFP* (*Choristoneura fumiferana*)³⁴ and *SfIBP_1*²³. On the other hand, *TisAFP6* supported ice bursting along the *c*-axis even at a high protein concentration (0.1 mM; Fig. 2.1b, panels 9 and 10), which has also been reported for typical moderately active AFPs, including fish type III (Takamachi, *et al.*, 2007) and type II (Nishimiya, *et al.*, 2008) proteins. Comparison of the ice-bursting pattern of *TisAFP7* with those of the known AFPs suggests that it exhibits intermediate activity, positioned between those of hyperactive and moderately active AFP species.

2.3.3. FIPA analysis reveals that *TisAFP7* adsorbs to entire planes of ice crystal

To visualize the AFP-bound planes of ice crystal (Fig. 2.2), we prepared fluorescently-labeled *TisAFP7* and evaluated it by FIPA analysis. Figure 2.2a shows a fluorescent image of a grown ice hemisphere mounted on the cold finger parallel to the *c*-axis of the ice crystal. The orange fluorescence of the entire hemisphere indicated that *TisAFP7* was incorporated in the entire ice structure, i.e., that all ice planes, including the basal plane, were covered with *TisAFP7*. Similar observations have been reported for hyperactive AFPs, including *TisAFP8* (Cheng, *et al.*, 2016), *ColAFP* (Hanada, *et al.*, 201) *MpAFP_RIV* (Garnham, *et al.*, 2016), *sbwAFP* (*C. fumiferana*) and *TmAFP* (*Tenebrio molitor*) (Basu, *et al.*, 2014). Some moderately active AFPs, when assayed at a relatively high concentration (0.1 mg/ml), also exhibit whole-hemisphere binding, e.g., *BpAFP* (Mahatabuddin, *et al.*, 2017) and *AFPII* (Tsuda, *et al.*, 2020) (*Brachyopsis rostratus*), with a concentration-dependent multiple-plane binding. In the current study, FIPA analysis of *TisAFP7* was performed using a low protein concentration (0.007 mg/ml); the protein's multiple-plane binding ability was higher than those of the typical moderately active AFPs. The ability to bind the basal plane has been also reported for moderately

active AFPs, such as *TisAFP6* (Kondo, *et al.*, 2012) and *LpIBP* (Middleton, *et al.*, 2012) (*Lolium perenne*), but with the basal and primary prism planes covered by separate hemisphere protein patches, which was different from the whole-plane binding observed for *TisAFP7*. Based on the above, we propose that *TisAFP7* binds all ice planes, including the basal plane, with an intermediate binding strength, positioned between those of hyperactive and moderately active AFP. This is a unique ice-binding property, reflecting the sequence identity shared by the hyperactive and moderately active *TisAFP* isoforms.

2.3.4. Crystal structure of *TisAFP7*

Diffraction data for wild-type *TisAFP7* was collected using a crystal grown as a thin plate-like shape. The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit cell parameters of $a = 57.12 \text{ \AA}$, $b = 62.99 \text{ \AA}$, and $c = 101.21 \text{ \AA}$, with two molecules in an asymmetrical unit. The crystal structure of *TisAFP7* was determined at $1.54 \text{ \AA}$ resolution by a molecular replacement method. The statistics for data collection and refinement are summarized in Table 2.1. The final structure was refined with R factor of 0.195 and Free R factor of 0.229, and was composed of 446 (223×2) residues and 746 solvent molecules. The refined *TisAFP7* structure exhibited the root-mean-squared deviation (RMSD) of $0.37 \text{ \AA}$ and $0.34 \text{ \AA}$ from C α atoms of *TisAFP6* and *TisAFP8*, respectively, reflecting high sequence identities shared by the isoforms.

Figure 2.3 shows a schematic representation of *TisAFP7*. The structure is dominated by a right-handed β -helical domain, a typical DUF3494 structure (Fig. 2.3a). The β -helical domain is composed of 184 residues with six helical coils ($\beta 1$ – $\beta 6$), which constitute the N-terminal portion of the β -helix (Ile16–Gly73) and C-terminal portion of the β -helix (Ser98–Lys223). The helical coils are ordered in the sequence $\beta 1$ – $\beta 6$ – $\beta 5$ – $\beta 4$ – $\beta 3$ – $\beta 2$, which is unusual for typical β -helical proteins, and unique for DUF3494. Further, $\beta 1$ is composed of 17 residues (Ile16–Gly32); $\beta 6$ is composed of 18 residues (Arg206–Lys223); $\beta 5$ is composed of 18 residues (Val188–Gly205); $\beta 4$ is composed of 21 residues (Lys167–Gly187); $\beta 3$ is composed of 28 residues (Thr139–Ala166); and $\beta 2$ is composed of 22 residues (Pro117–Ser138). The non-uniform numbers of residues in each coil (18–28 residues) result in a slightly swelled backbone structure at an end of the domain, akin

to a semi-pear shape. An additional long α -helix (Thr74–Arg97) is situated parallel to the β -helix. The N-terminal segment (Ala1–Leu8) is extended in an antiparallel orientation with respect to the α -helix. Four short segments of 3_{10} helices ($\alpha 1$, Gly9–Asn13; $\alpha 2$, Gly41–Phe43; $\alpha 4$, Ile105–Gly108; and $\alpha 5$, Ala166–Asn168) connect the β -strands and loop segments. Figure 2.3b shows *TisAFP7* viewed along the β -helix axis. A triangular cross section is seen, with three flat surfaces (a-, b-, and c-face) composed of parallel β -sheets. The a-face is covered with a long α -helix and the N-terminal segment, while the b- and c-faces are exposed to the solvent. In Fig. 2.3c, the hydrophobic residues facing the inside of the β -helix and the interface between the a-face and long α -helix are shown, and form the hydrophobic cores of the molecule.

2.3.5. Ice-plane specificity of the loop region and β -sheet of IBS.

Figure 2.4 shows the putative IBS of *TisAFP7*, which is located on the flat b-face of the β -helix. IBS consists of 26 residues of a six-stranded parallel β -sheet and 21 residues of the adjacent loop region connected to the a-face of the β -helix. Only five residues differentiate IBS of *TisAFP7* from the hyperactive *TisAFP8* isoform, all of which are located in the loop region. To assess their role in ice binding, Thr20 in the loop region, reported to be one of the residues key for *TisAFP8* hyperactivity²², was replaced with Tyr. The crystal structure of *TisAFP7* T20Y was determined at 1.72 Å resolution. The crystal was isomorphous with the wild-type crystal, and the final structure was refined with R-factor of 0.208 and Free R of 0.246, containing 446 residues and 554 solvent molecules. Another sampled substitution site was Ala214, located at the center of the IBS β -sheet. The two sites are shown in Fig. 2.5a. We predicted that the substitutions would interfere with the ice binding because of the steric hindrance of the bulky side chain of Tyr. Indeed, both substitutions resulted in a reduced TH activity (0.4 °C at 0.25 mM T20Y mutant and 0.45 °C at 0.27 mM A214Y mutant), equivalent to a 60% and 50% reduction from wild-type *TisAFP7*, accordingly (Fig. 2.5b). In the TH gap of the T20Y mutant, very small ice tips protruded from the seed ice crystal (Fig. 2.5c, panel 1). At the moment of freezing, the ice crystal rapidly grew in two directions along the *c*-axis (Fig. 2.5c, panel 2). A similar growth pattern was observed for wild-type *TisAFP7* at a low protein concentration

(0.01 mM, Fig. 2.1b, panels 3 and 4) and *TisAFP6* (Fig. 2.1b, panels 9 and 10), which suggested that the T20Y substitution reduced the protein's ability to bind the basal plane. In the case of the A214Y mutant, the ice crystal was modified to form a rounded hexagonal plate below T_m (Fig. 2.5c, panel 3) and expanded maintaining this shape (Fig. 2.5c, panel 4). Hence, the A214Y mutant retained the affinity for the basal plane. The TH of a double T20Y/A214Y mutant was lower than those of single mutants, and the mutant exhibited an impaired ice-growth inhibition (Fig. 2.5c, panels 5 and 6). On the other hand, a mixture of equal concentrations of the two single mutants recovered the TH to 68% that of the wild type (Fig. 2.5b). Furthermore, ice morphology in the mixture was the same as that for wild-type *TisAFP7* (Fig. 2.5c, panels 7 and 8).

Next, the single mutants T20Y and A214Y, and double mutant T20Y/A214Y were fluorescently labeled and subjected to FIPA analysis (Fig. 2.2b, panels 1, 3, and 5). The samples were mounted on the cold finger in the same orientation as that for wild-type *TisAFP7* analysis shown in Fig. 2.2a. In this orientation, the basal plane was illuminated in the center zone of the hemisphere, and the perimeter zones corresponded to the primary or secondary prism planes with a hexagonal symmetry, as schematically shown in Fig. 2.2e (upper panels). In Fig. 2.2b, panels 2, 4, and 6, the hemispheres were mounted with the primary prism plane perpendicular to the cold finger. Figures 2.2d and e (bottom panels) show a schematic overview of the corresponding regions of the prism, pyramidal, and basal planes in the equator, middle latitude, and polar region of the hemisphere, respectively. As shown in Fig. 2.2b, panels 1 and 2, the T20Y mutant covered only the perimeter zone of the hemisphere, corresponding to the secondary prism planes. The reduced coverage of ice planes by the T20Y mutant indicated lost ice-binding affinity for the basal, primary prism, and some pyramidal planes. On the other hand, the A214Y mutant retained the ability to bind the basal and secondary prism planes, whereas it was devoid of the ability to bind the primary prism and some pyramidal planes. Hence, the replacement of specific IBS residues impaired the protein's binding to different ice planes. These observations suggest that residues T20 and A214 play different roles in basal-plane binding, which might affect the AFP hyperactivity. The T20Y/A214Y double mutant lost the ability to bind the basal, primary prism, and all pyramidal planes, except for the secondary prism planes, as shown in Fig. 2.2b, panels 5 and 6, similar to the T20Y mutant. Further, the mixture of T20Y and A214Y mutants appeared to cover nearly the entire

hemisphere, incompletely recovering the ability to bind to the primary prism plane, as shown in Fig. 2.2c. This observation, together with the partial recovery of the TH value shown in Fig. 2.5b, suggests that the two mutants preferably adsorb to discrete ice planes via their IBS, to partially compensate for their defective activity. It has been proposed that in the hyperactive *ColAFP*²⁰ and *TisAFP8* (Cheng, *et al.*, 2016) proteins, the loop region of compound IBS is involved in basal-plane binding. The findings of the current study offer additional experimental evidence in support of this notion. Also, partial recovery of ice-binding ability observed for the mixture of the defective mutants exhibits that both T20 and A214 are required for binding to entire planes of ice crystal while they are proposed to play different roles in basal-plane binding. This view also suggests that the loop and sheet region of compound IBS should adsorb together to the ice plane to exert the original antifreeze activity of *TisAFP7*. The spacing between the adsorbed AFP molecules on the ice plane was estimated as 70-350 Å for *TmAFP*-GFP (Drori, *et al.*, 2015). Here, we assume that *TisAFP7* also adsorbs to ice in a similar interval with *TmAFP*. Also, If we assume that the loop and sheet IBS individually adsorbs to the ice plane in the mixture of the defective mutants, these two IBS might be too distant to cover entire ice planes, which might bring about the partial recovery of ice-binding ability. Therefore, the compound IBS situated in the same molecule should be essential for the full antifreeze activity of *TisAFP*. From this point of view, we speculate that the loop and sheet region of IBS of *TisAFP* might simultaneously bind to a target ice plane although the surfaces of the loop and sheet IBS does not make a flat plane and intersect with approximately 150°.

2.3.6. Network structure of water molecules bound to IBS

In one asymmetric unit, two *TisAFP7* molecules were surrounded by 388 and 358 bound water molecules, respectively. The hydrated waters on IBS for each *TisAFP7* molecule were organized in a specific manner, and could be superposed, indicating that the structure of the bound water molecules is not affected by crystal packing. Among the approximately 54 waters molecules on IBS (Supplementary Fig. S1), six water molecules were aligned in a small trough formed by the inward-facing hydrophobic residues (Val22, Val213, Val195, Val177, Leu150, and Val126) at the β -sheet region of IBS. The trapped

water molecules were situated in a line, at an average interval of 4.6 Å. Typically, the distances between the water molecules 3 and 4, and 5 and 6 were 4.36 Å and 4.99 Å, respectively. The interval between water molecules 3 and 6 was 14.51 Å, which closely matched that between the water molecules in a prism ice plane (4.6 Å and 14.7 Å). The positional similarity with the prism plane may assist *TisAFP7* in recognizing the prism plane via the β -sheet region. The trapped water molecules aligning at regular intervals in the IBS trough are also observed in *TisAFP6* and *AnpIBP1a*, where they have been proposed to facilitate prism-plane binding (Yamauchi, *et al.*, 2020). That notion was also supported by the reduced binding of the prism plane by the A214Y mutant (Fig. 2.2b, panels 3 and 4), where the row of water molecules may be disturbed by eliminating water molecules 2 and 3.

To survey the bound water structure in the IBS loop region in detail, we next compared the structure of *TisAFP7* with those of *TisAFP6* and *TisAFP8*. In all these isoforms, 12 water molecules in corresponding positions formed a zigzag-pattern network with branches, shown by red spheres in Fig. 2.6a. The distance from each water molecule to the proximal water molecule ranged from 2.58 Å to 3.67 Å, with an average of 2.86 Å (Supplementary Fig. S1c). The angles between three adjacent water molecules were 105–126°, with an average of 116° (Supplementary Fig. S1d). Figure 2.6b shows the superposition of the water molecule network on a set of ice water molecules in the basal plane (RMSD of 0.77 Å). Close geometrical similarity between the consecutive water molecules on IBS and the ice basal plane appeared to be essential for the ability of *TisAFP* isoforms to bind the basal plane. However, only *TisAFP8* retained a water molecule network extending toward the outer regions of IBS and involving additional water molecules, shown by blue spheres in Fig. 2.6a. The additional portion of the network was less similar to the ice basal plane than the zigzag waters, but it covered a wider area of IBS than *TisAFP7*. The comparison of the water network was also illustrated in Supplementary Fig. S4. Water networks have been identified on various AFP structures, including those of the hyperactive *MpAFP_RIV* (Garnham, *et al.*, 2011) and Maxi (Sun, *et al.*, 2014) proteins, and that of a moderately active NfeAFP (Mahatabuddin, *et al.*, 2018) protein. They form an ice-like structure in the vicinity of IBS, that could merge with the quasi-liquid water layer near the ice surface, as previously proposed (Davies, *et al.*, 2014; Nutt, *et al.*, 2008), thus inducing ice-binding by the proteins.

The water network of *TisAFP7* was less extensive than that of *TisAFP8*. This prompted us to propose that the antifreeze activity of *TisAFPs*, typically reflecting the affinity toward the ice basal plane, is correlated with the water coverage on IBS via the extended network of water molecules, as observed in the current study. This notion was supported by the truncated water network in the T20Y mutant (Fig. 2.6d). In the mutant, the bound water molecules near residue 20 were removed by introducing the side chain of Tyr, thus truncating the zigzag pattern.

Further, on the top edge of the IBS loop of *TisAFP7*, the hydrophobic residue Phe43 was surrounded by 10 water molecules connected in a ring-like manner, as shown in Fig. 2.6c. The distance between the adjacent water molecules ranged from 2.5 Å to 3.02 Å, with an average of 2.74 Å. The average distance between the comprising water molecules and the nearest carbon atom of the interior aromatic side chain was 3.4 Å, which implies that these water molecules were constrained to engage in hydrophobic interactions with the Phe43 side chain. In the crystal structure of the hyperactive *TisAFP8* protein, no water ring was apparent because of the molecular interactions at Phe43 in the crystal. Nevertheless, a high sequence identity of the IBS residues in the vicinity of residue 43 in *TisAFP8* and *TisAFP7* might suggest that *TisAFP8* coordinates ring water molecules similar to *TisAFP7*. In addition, in the hyperactive *ColAFP* protein, the corresponding Phe residue is situated in the same position and conformation as that in *TisAFP7* and *TisAFP8*. On the other hand, in the moderately active *TisAFP6*, Phe is replaced with Ser in this position, and no water ring is observed in the crystal structure, even though IBS is exposed to the solvent area in the *TisAFP6* crystal. Based on these observations, we suggest that the hydrophobic hydration on IBS enhances the affinity of *TisAFP7* and *TisAFP8* for ice, as proposed earlier (Sönnichsen, *et al.*, 1996). The water coverage on IBS for *TisAFP* isoforms was also assessed as water residence time on the protein surface by using the molecular dynamics (MD) simulation. Table 2.2 shows water residence time on IBS residues, non-IBS residues, and all residues for *TisAFP* isoforms. For *TisAFP7* and *TisAFP8*, water residence time on IBS was longer than that on other areas, suggesting that IBS of *TisAFP7* and *TisAFP8* retain more water molecules. On the other hand, for *TisAFP6*, non-IBS residues exhibited longer residence time than IBS, which might be attributable to the substituted residues located in the capping head regions, similarly to higher thermal stability estimated by CD measurement. For the bound water molecules

on IBS residues, hyperactive *TisAFP8* exhibited the longest residence time and *TisAFP6* exhibited the shortest, which is the same order for their antifreeze activities. Our current MD calculation offers a simple estimation of the relationship between the water coverage on IBS and its ice plane affinity. Therefore, discussion with the careful calculation should be necessary in the separated report.

Detailed analysis of water-molecule dynamics might be required for further elucidation of the relationship between the bound-water structure and ice-binding property of AFP. The unique hydration structure of surface water of AFP has been reported by utilizing molecular dynamics simulation (Chakraborty, *et al.*, 2018), terahertz spectroscopy (Meister, *et al.*, 2013), and sum frequency generation spectroscopy (Meister, *et al.*, 2014). The current study provides basic insights that can be utilized in these experimental approaches.

To conclude, we here showed that the different activities of *TisAFP* isoforms are associated with the bound water structure around IBS. The IBS residues act like a platform on a β -helical scaffold, organizing the hydration waters. This is an astounding example of evolution of the antifreeze properties of microbial AFPs, namely, tuning the hydration structure, as a means to adapt to the microbial habitat.

Data availability

The atomic coordinates have been deposited in the Protein Data Bank under the accession numbers ID 7DC5 and 7DDB for the wild-type *TisAFP7* and *TisAFP7* T20Y, respectively.

Table 2.1 Data collection and refinement statistics for *TisAFP7* and *TisAFP7* T20Y

Crystal	<i>TisAFP7</i>	<i>TisAFP7</i> T20Y
Data Collection		
Space group	$P2_12_12_1$	
Unit cell parameters (a , b , c),	57.12, 62.99, 101.21	56.79, 63.14, 102.09
Beam line	Photon factory BL-1A	
Wavelength (Å)	1.1000	
Resolution range (Å)	49.75–1.54	49.68–1.72
$R_{\text{merge}}^{a,b}$	0.131 (1.167)	0.192 (1.137)
Observed reflections	689607	530653
Independent reflections	54671	41109
Completeness ^a (%)	99.8 (99.0)	99.8 (99.9)
Multiplicity ^a	12.6 (13.1)	12.9 (12.6)
$\langle I/\sigma(I) \rangle^a$	12.3 (2.3)	8.6 (2.3)
Refinement		
R factor ^{a,c}	0.195 (0.457)	0.208 (0.398)
Free R factor ^{a,c,d}	0.229 (0.467)	0.246 (0.440)
R.M.S. bond length (Å)	0.013	0.010
R.M.S. bond angle (°)	1.709	1.633
Residues	446 (223×2)	446 (223×2)
Number of non-hydrogen		
Protein atoms		
Protein	3131	3120
Water	746	554
Other	30 ($\text{SO}_4^{2-} \times 6$)	3 ($\text{Mg}^{2+} \times 3$)
Ramachandran plot ^e (%)		
Residues in favored regions	96.4	97.5
Residues in allowed regions	3.6	2.5
Residues in outlier regions	0	0
Average B factor (Å ²)	11.0	12.0

^a Values in parentheses are for the highest-resolution shell. ^b $R_{\text{merge}} = \sum_j \sum_i |I(h)_i - \langle I(h) \rangle| / \sum_j \sum_i \langle I(h) \rangle$, where $\langle I(h) \rangle$ is the mean intensity of a set of equivalent reflections.

^c R factor = $\sum ||F_{\text{obs}}(h)| - |F_{\text{calc}}(h)|| / \sum |F_{\text{obs}}(h)|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. ^d For the calculation, 5% of the

data were randomly chosen and the free R factor calculated (Brunger, *et al.*, 1992). ^e

Statistics were obtained from MolProbity (Williams, *et al.*, 2018).

Table 2.2 Water residence time for *TisAFP* isoforms on the molecular surface.

	IBS	non-IBS	whole surface
<i>TisAFP7</i>	79.43 ps	68.37 ps	69.90 ps
<i>TisAFP8</i>	85.98 ps	66.93 ps	70.13 ps
<i>TisAFP6</i>	73.16 ps	79.41 ps	78.20 ps

The values were estimated for water molecules making hydrogen bonds with IBS residues (positions 19 – 25, 38 – 45, 123 – 129, 147 – 153, 174 – 180, 192 – 198, and 210 – 216), and non-IBS residues, and all residues.

Figures and legends

Figure 2.1. Comparison of the antifreeze properties of *TisAFP7* and two other *TisAFP* isoforms, the moderately active *TisAFP6* and hyperactive *TisAFP8*. **(a)** Plot of thermal hysteresis values for the three isoforms at various protein concentrations. Purple, *TisAFP7*; red, *TisAFP8*; and green, *TisAFP6*. The values are means and the error bars are standard deviations from three independent observations at each protein concentration. The values for *TisAFP8* and *TisAFP6* are adopted from reference (Cheng, *et al.*, 2016). The curves were fitted for ease of interpretation. **(b)** Microscopic images of ice crystal morphology in solution of AFP isoforms at melting (T_m) and non-equilibrium freezing temperature (T_f). High and low concentrations denote 0.23 mM and 0.01 mM for *TisAFP7*; and 0.12 mM and 0.01 mM for *TisAFP8*. For *TisAFP6*, ice crystals grown at 0.1 mM are shown. The *c*-axis direction of the ice crystal is shown in the figure as a circle or an arrow. The images are representative of three observations under each condition.

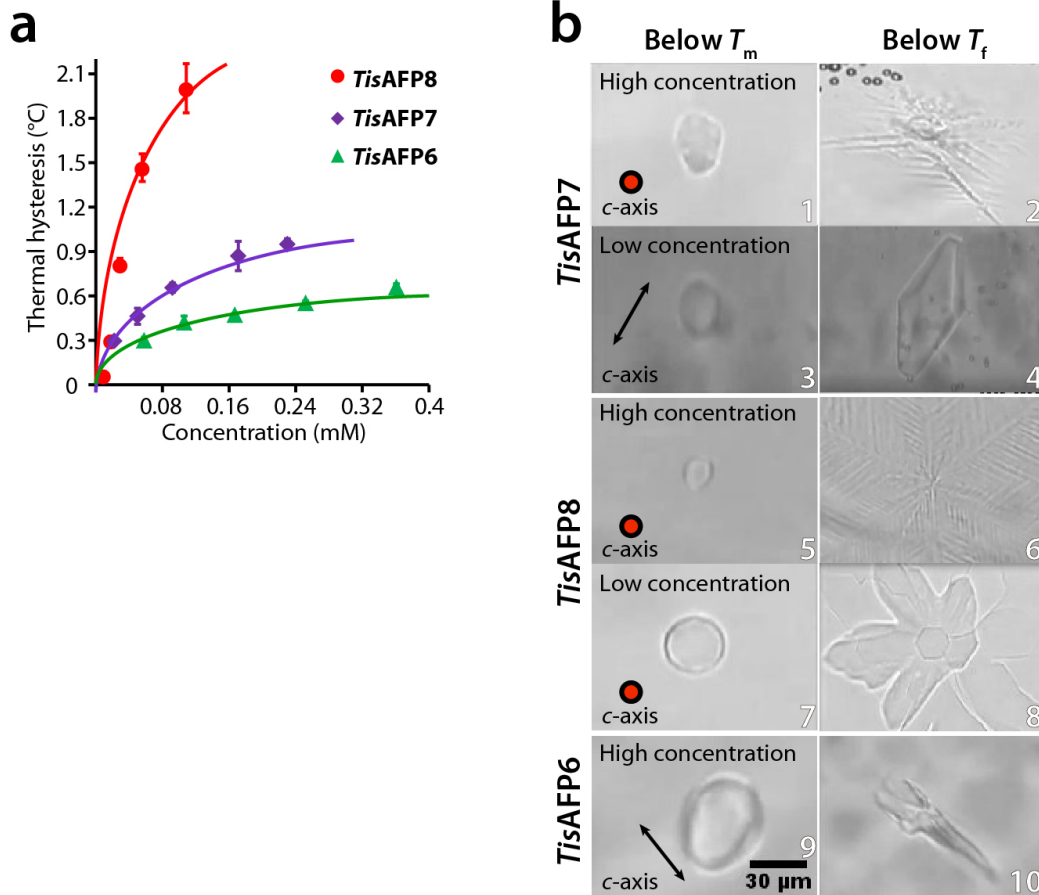


Figure 2.2. Ice plane specificity of wild-type *TisAFP7* and its mutants, as determined by FIPA analysis. The concentration of protein solution was 0.007 mg/ml in all experiments. **(a)** Fluorescence image of a single ice-crystal hemisphere grown in a solution of wild-type *TisAFP7* labeled with an orange fluorescent dye. The single ice crystal was mounted on the cold finger perpendicular to the basal plane. The *c*-axis direction of the ice crystal is shown in the figure as a circle. **(b)** Fluorescence from single ice-crystal hemispheres grown in solutions of *TisAFP7* T20Y, A214Y, and T20Y/A214Y mutants labeled with a green fluorescent dye. Upper panels, the hemispheres were mounted in the same orientation as in **(a)**; lower panels, the hemispheres were mounted with a primary prism plane perpendicular to the cold finger. The *c*-axis direction is shown as an arrow. **(c)** A single ice crystal grown in the solution containing equal concentrations of T20Y and A214Y mutants (0.0035 mg/ml of each). **(d)** Schematic illustration of ice planes mapped onto a hemispherical-shaped ice crystal. The polar regions correspond to the ice basal plane. The equator and middle latitude zones correspond to the prism and pyramidal planes, respectively. **(e)** Schematic illustrations of fluorescent patches corresponding to the primary and secondary prism planes on the ice hemisphere in known orientation. When the ice crystal is mounted with the basal plane perpendicular to the cold finger, the primary (1st) prism plane appears between the a_1 -, a_2 -, and a_3 -axes with a hexagonal symmetry, as shown by the upper images. The secondary (2nd) prism plane is situated on the region pierced with the a_1 -, a_2 -, and a_3 -axes. When the ice crystal is mounted with the primary prism plane perpendicular to the cold finger, three patches are observed on the equator of the hemisphere for the primary prism plane, whereas two patches are seen for the secondary prism plane.

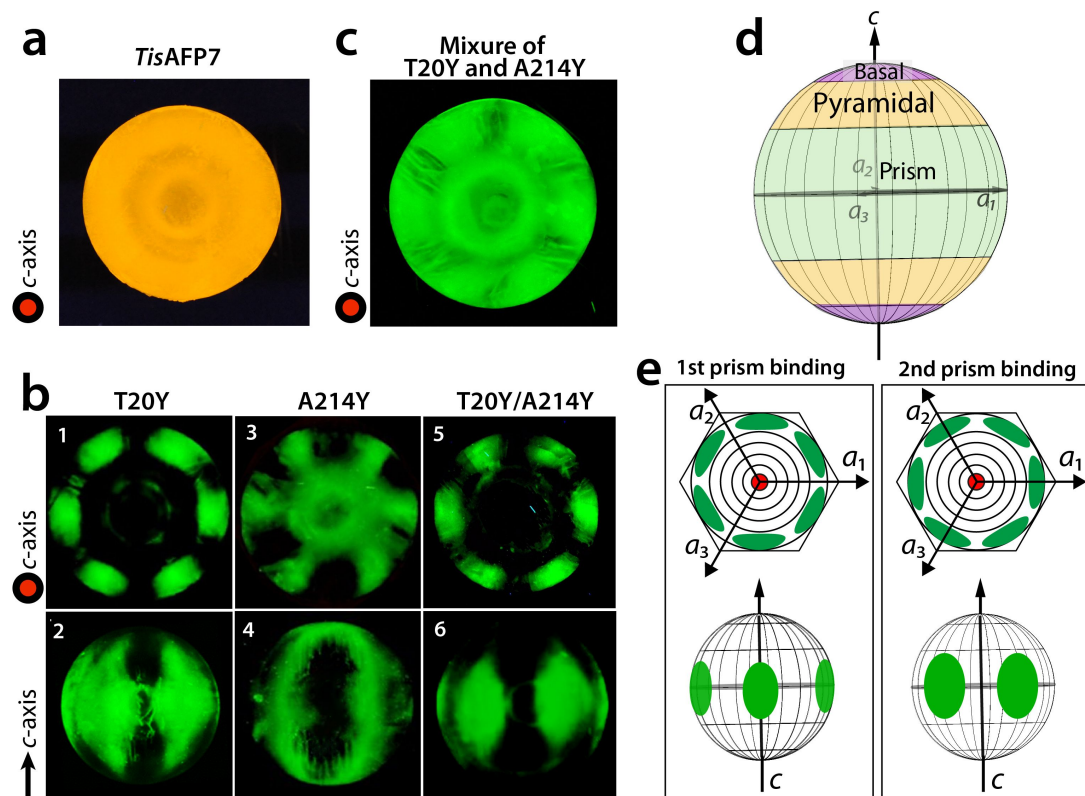


Figure 2.3. Schematic illustration of the *TisAFP7* crystal structure. **(a)** View along the normal to the β -helical axis with the N- and C-termini denoted in blue and red, respectively (the helical axis runs in parallel to the page, from top to bottom, and the molecule is viewed along the axis perpendicular to the helical axis). Helical coils of irregular length are denoted as β -1, and β -6 to β -2, from N-terminal half of the solenoid. The long α -helix is denoted as α -3, with additional 3_{10} helices numbered α -1, α -2, α -4, and α -5. **(b)** View along the β -helical axis upon 90° rotation of the view in **(a)**, showing a triangular cross-section (the helical axis is perpendicular to the page). The molecular faces of β -helix are denoted as the a-, b-, and c-faces, accordingly. Here, the c-face is a putative ice-binding site. **(c)** Hydrophobic core formed within the β -helices, and between the a-face and α -3 helix, drawn as a stick model. The *TisAFP7* structures presented in this and other figures were prepared using UCSF Chimera (Pettersen, *et al.*, 2004).

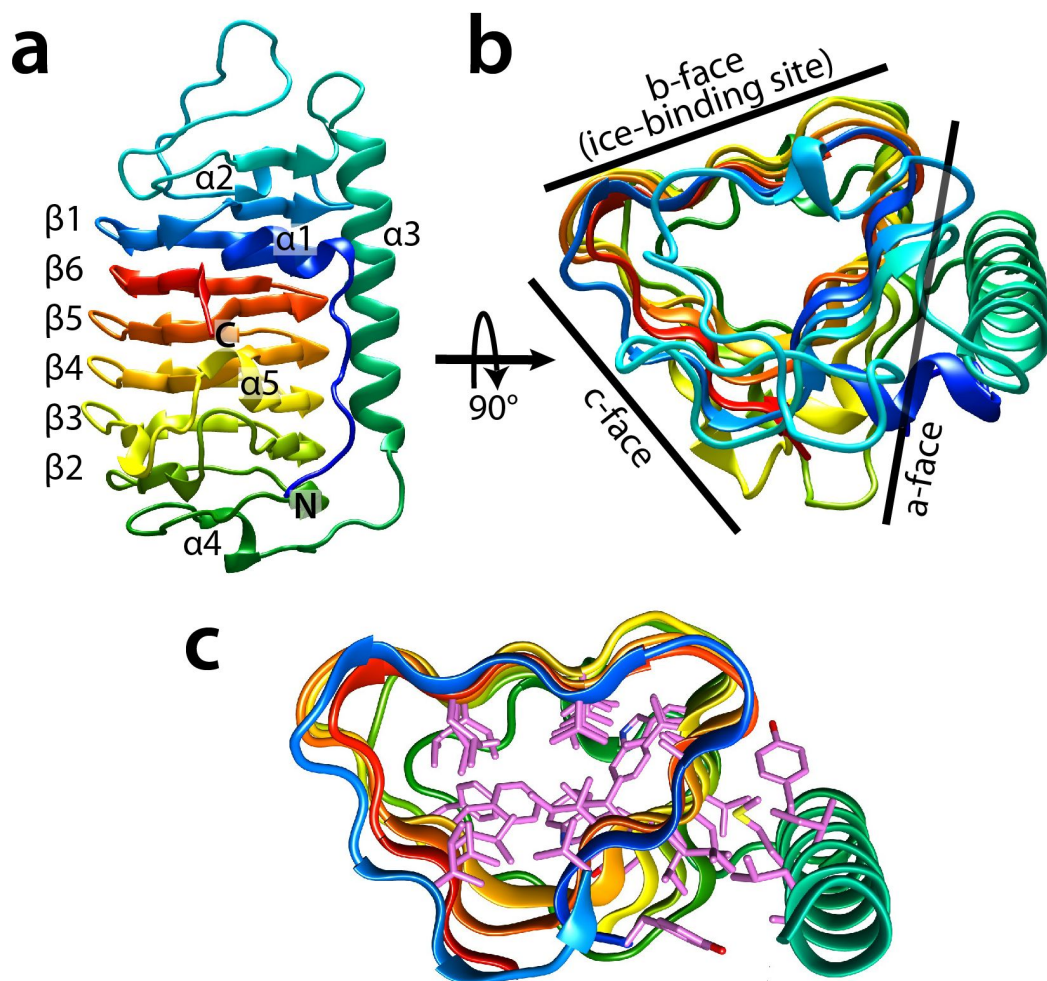


Figure 2.4. Front view of the putative ice-binding site (IBS) of *TisAFP7*, viewed from the b-face. The loop and sheet regions of the compound IBS are enclosed by red and blue boxes, respectively. In (a), the side chains of residues composing the putative IBS are drawn as a stick model. In (b), amino acids at corresponding positions in IBS are shown. Upper and lower letters indicate outward- and inward-facing residues, respectively. Five residues that are substituted from IBS of *TisAFP8* are indicated by red letters.

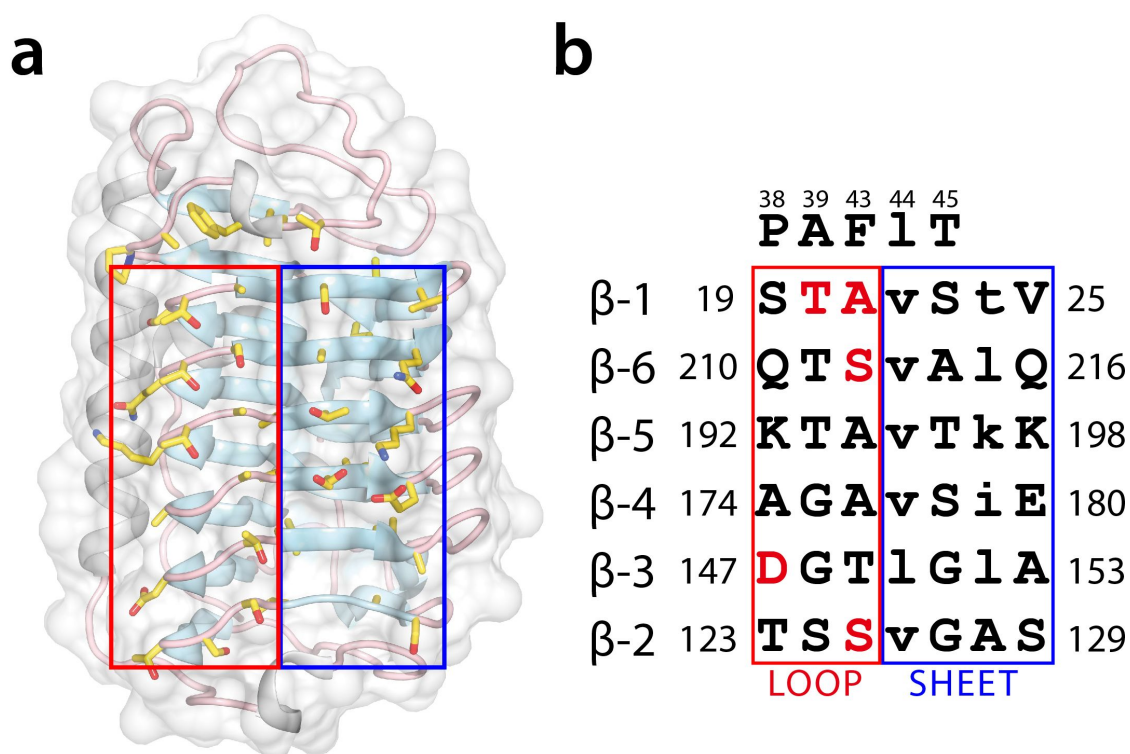


Figure 2.5. Effect of amino acid substitution on ice-binding site (IBS) in *TisAFP7*. **(a)** Locations of the T20Y and A214Y point substitutions in the loop and sheet regions of IBS, respectively. **(b)** Thermal hysteresis profiles as a function of protein concentrations. The values are means and the error bars are standard deviations from three independent observations at each protein concentration. **(c)** Microscopic images of single ice crystals in the sample solutions. Upper panels, ice crystal morphology in the thermal hysteresis gap (below the melting point). Lower panels, ice crystal morphology below the non-equilibrium freezing point. The *c*-axis direction of the ice crystal is shown in the figure as a circle or an arrow. The images are representative of three observations under each condition.

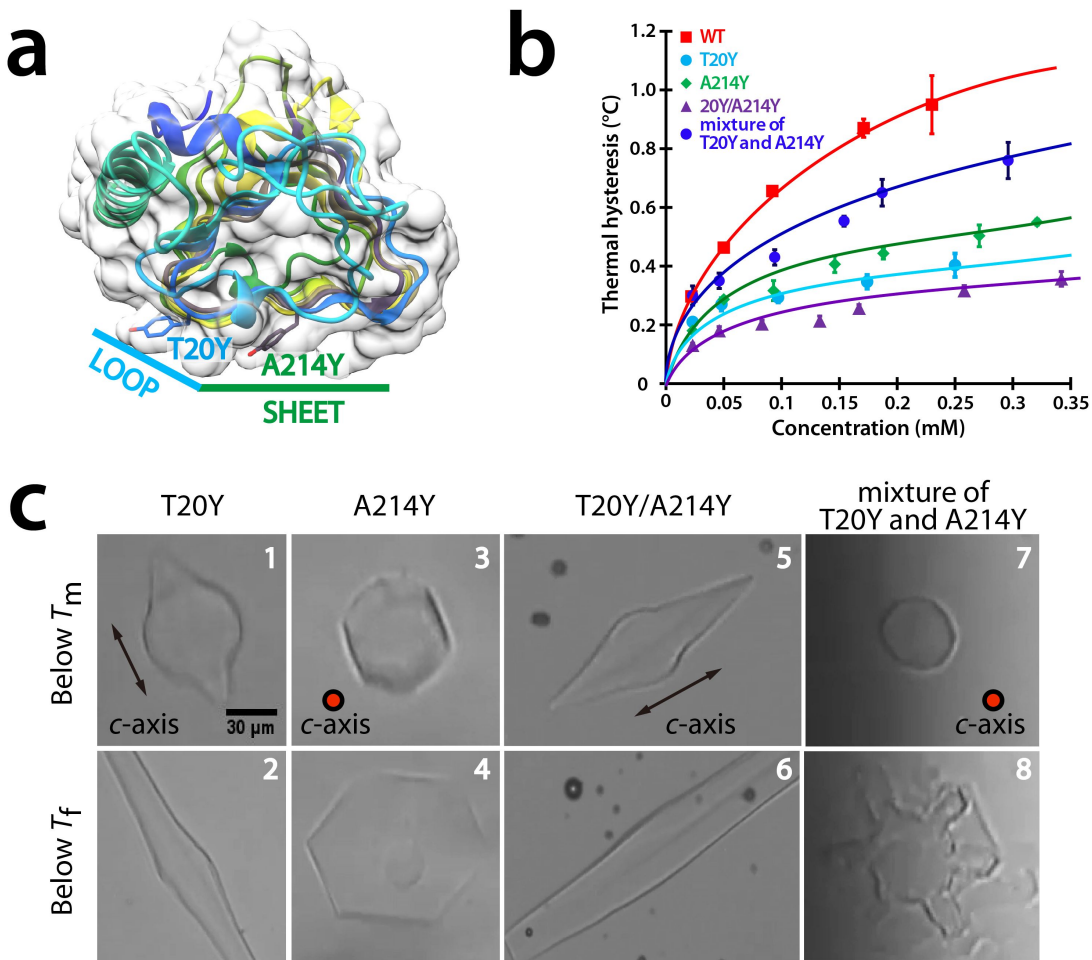


Figure 2.6. Bound water network and superposition on the basal ice-plane water. **(a)** Surface structure of *TisAFP7*, with bound water molecules in the loop region of ice-binding site (IBS) colored in yellow. The bound water molecules in *TisAFP7*, *TisAFP8*, and *TisAFP6* are drawn as spheres in red, blue, and green, respectively. Proximal water molecules, within 3.7 Å, are connected by solid lines. **(b)** Eleven bound water molecules at the IBS loop of *TisAFP7* superposed on the basal plane water molecules of an ice crystal with root-mean-squared deviation of 0.77 Å. **(c)** Crystal structure of the T20Y mutant with bound water molecules on its surface. The altered site (Tyr20) is indicated in yellow. IBS water molecules are denoted in red and other bound water molecules in pink. **(d)** Magnified view of Phe43 surrounded by a water ring composed of 10 water molecules drawn in cyan. Each water molecule located within 3.5 Å of each other is connected by a dashed line.

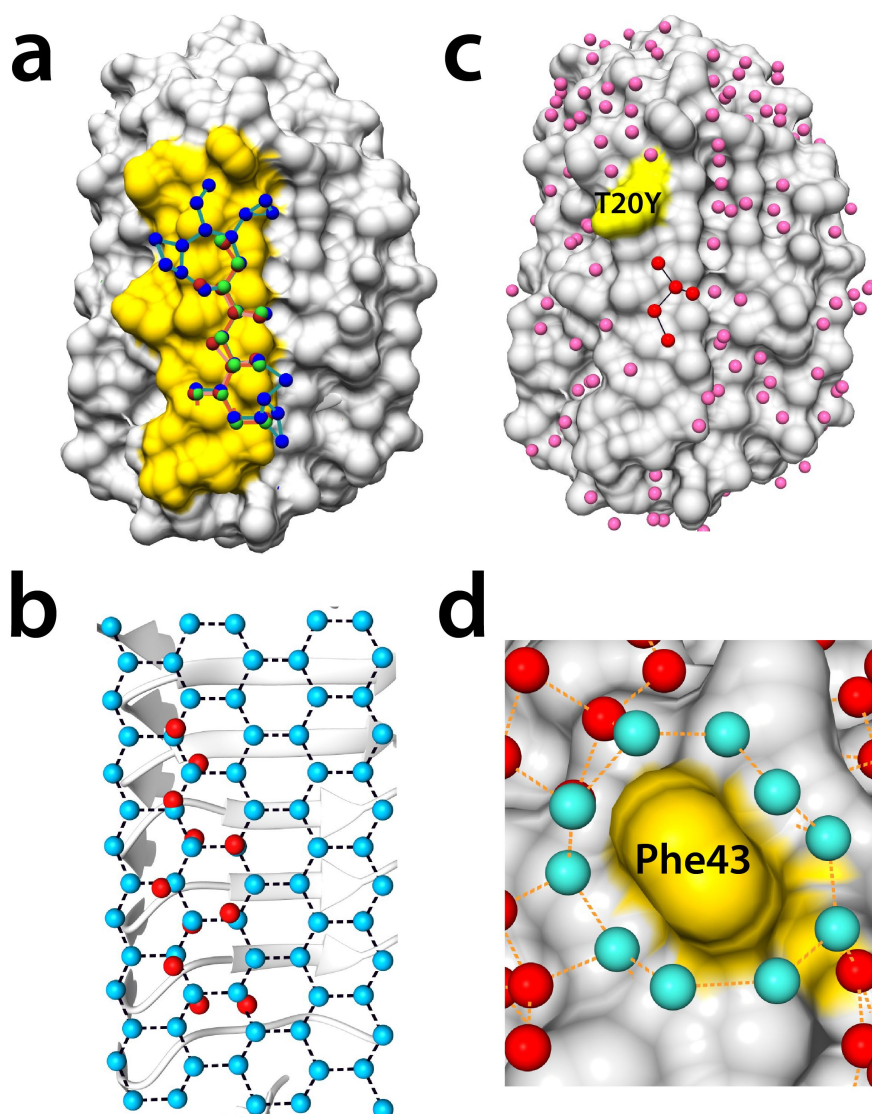
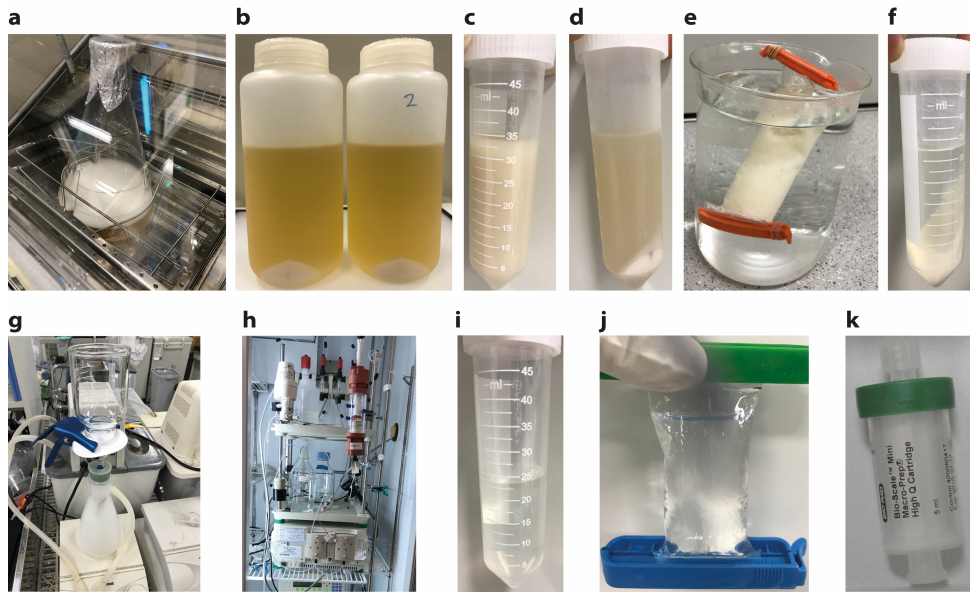


Figure 2.7. Detail process of recombinant *TisAFP7* purification. (a) 1.0 L culture of *E. coli* cell at 15 °C for 24 hours. (b) Collection of cells by centrifugation. (c) Crude solution after cell lysis (d) Centrifuged to separate cell debris. (e) Dialysis of supernatant solution from d. (f) Centrifuged after dialysis. (g) Filtration of supernatant of f. (h) Chromato-chamber. (i) Solution to subject for High-S column. (j) Dialysis of High-S fraction containing AFP. (k) Cartridge of High-Q column used for 2nd and last chromatography for purification.



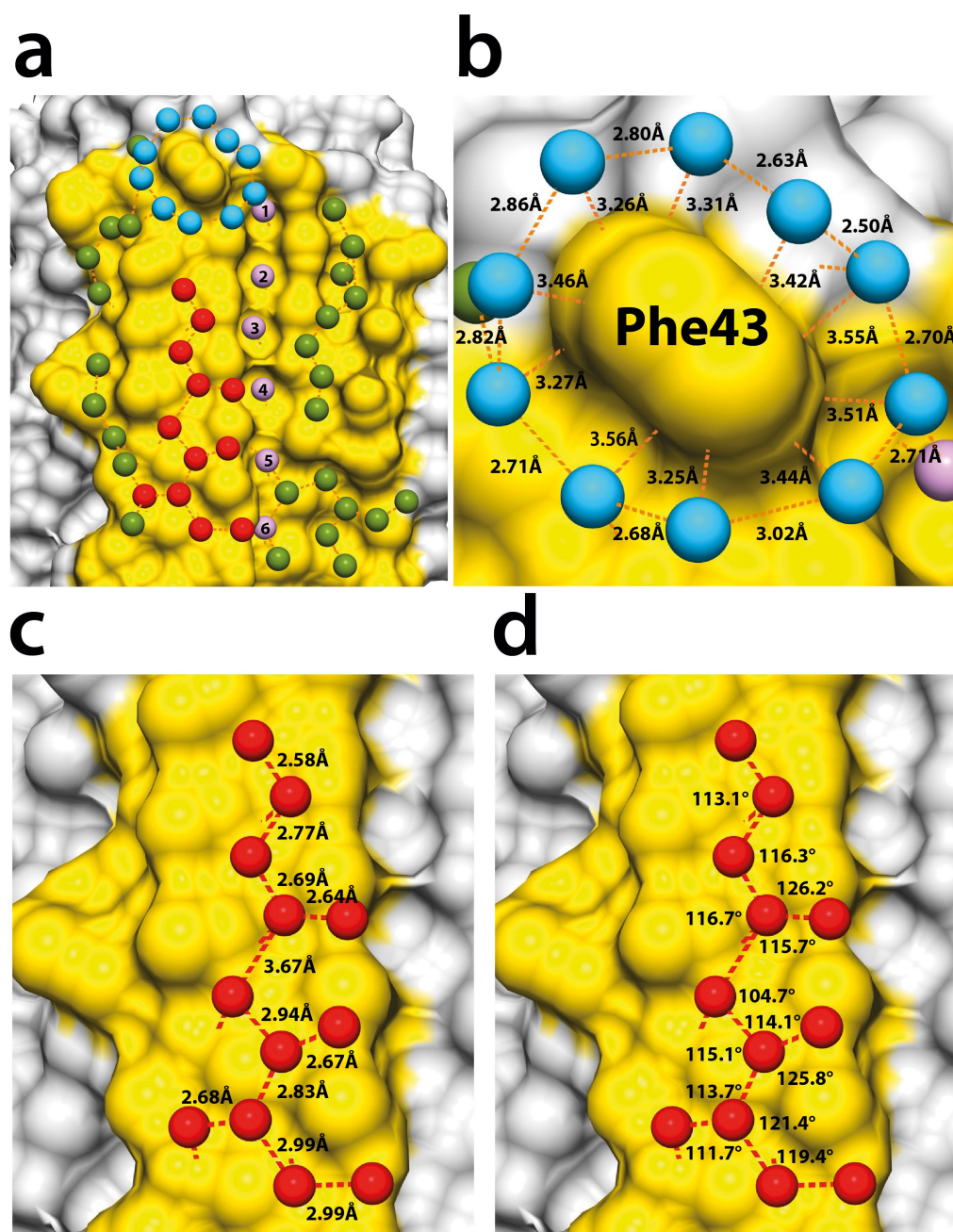
2.3.7. Supplementary Information

Supplementary Table S1 Sequence of primers used for the construction of *TisAFP7* mutants

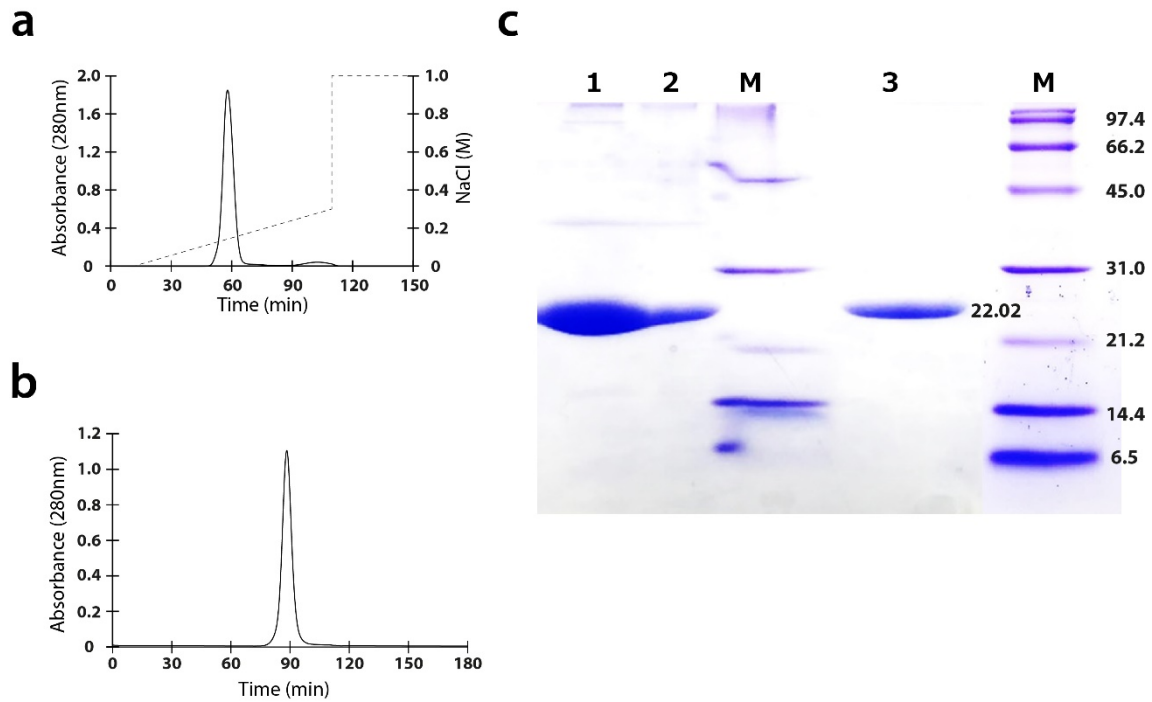
Protein	Primer sequence
T20Y	5'– TAT GCCGTTAGCACCGTTCCGAGA–3'
	5'–GCTGGCCAGAAATGGCATAATTACCTG–3'
A214Y	5'– TAT CTGCAGAGCGCCACCGTGGTG–3'
	5'–AACGCTGGTCTGGGCCAGAAATGCG–3'

The nucleotide positions corresponding to each mutation site are shown in bold.

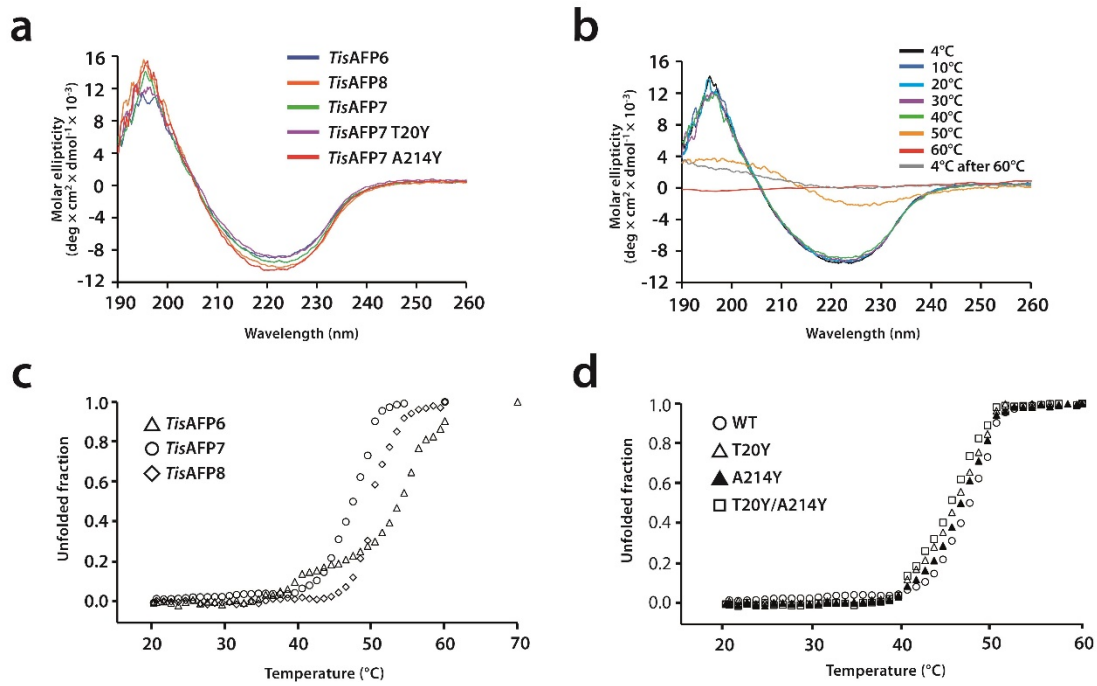
Supplementary Figure S1. Network of bound water molecules on the ice-binding site (IBS) of *TisAFP7*. **(a)** Overview of the surface area of IBS (denoted in yellow), with the water molecules drawn as spheres. Six water molecules aligned in a trough on IBS are denoted in pink and numbered 1–6. Bound water molecules on IBS loop forming a zigzag pattern are denoted in red. Other water molecules on IBS are denoted in green. **(b)** A water ring surrounding Phe43 side chain, denoted in cyan. Interaction between water molecules and side chain atoms are shown, with the distance between the proximal water molecules and carbon atoms indicated. **(c)** Distances between the zigzag water molecules. **(d)** Angles between three adjacent water molecules.



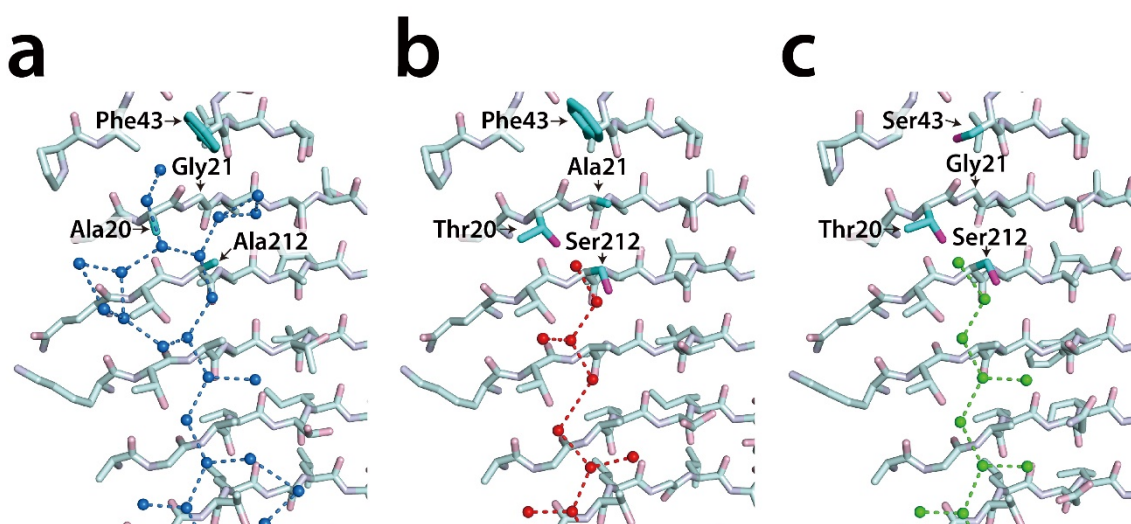
Supplementary Figure S2. Purification of *TisAFP7*. **(a)** Chromatogram of a cation exchange chromatography. Y-axis on the left side shows the optical absorbance at 280nm. A concentration of NaCl applied to elute the adsorbed sample is indicated in the right y-axis. **(b)** Chromatogram for analytical size-exclusion chromatography for checking the purity of the sample. **(c)** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified *TisAFP7*. Lane 1 and 2, fractions after a cation exchange chromatography. Lane 3, the flow-through fraction of anion exchange chromatography. *M*, molecular weight standard.



Supplementary Figure S3. Thermal denaturation of *TisAFP7*, T20Y, A214Y, T20Y/A214Y mutants, *TisAFP8* and *TisAFP6* estimated by CD spectrometry. **(a)** CD spectra of *TisAFP7*, its mutants (T20Y, A214Y), *TisAFP8*, and *TisAFP6* observed at 4 °C. **(b)** CD spectra change of *TisAFP7* upon heating. Measurement temperatures are indicated in the legend. **(c)** Thermal denaturation profiles of *TisAFP7*, *TisAFP8*, and *TisAFP6*. **(d)** Thermal denaturation profiles of *TisAFP7*, T20Y, A214Y, and T20Y/A214Y.



Supplementary Figure S4. The structure of the water network and *TisAFP* isoforms are aligned for comparison with three isoforms, (a) *TisAFP8*, (b) *TisAFP7* and (c) *TisAFP6*, viewing from the same orientation. Water molecules that are positioned within 3.7 Å from adjacent water are drawn as spheres and connected by the dotted lines. The protein structures are illustrated for the residues located in the ice-binding site as a stick model. The residues that are substituted between the isoforms are indicated by arrows. The figure was prepared using the software open-source PyMOL v. 1.5.0.3 (<http://pymol.org>)¹.



Chapter 3

General Discussion

Antifreeze protein was first discovered by Arthur L. DeVries in 1960s. Till to date AFPs are identified from fish, insects, plants, various microorganism including fungi, yeast, bacteria, diatom and so on. AFPs are potential guard for their host from cold damage. AFP molecules possess tiny size (3.5 kDa) to large molecule of 35 kDa. However, all these molecules bind on ice planes perfectly with their diversified ice-binding sites to shapes the ice crystal to bipyramid, lemon or hexagonal plate as well as inhibit their growth.

Early researchers suggested the binding of AFPs on ice surface through hydrogen bonds (DeVries, *et al.*, 1971; 1977; Knight, *et al.*, 1993) or by the hydrophobic effect (Sönnichsen, *et al.*, 1996; Garnham, *et al.*, 2011). Recent studies propose the binding mechanism via hydration waters, which are organized on IBS like waters of ice planes. These organized waters are termed as ice-like water which first interact with the quasi-liquid layer and finally stick on the ice planes (Mahatabuddin, *et al.*, 2018; Hudait, *et al.*, 2018). Hydration waters on IBS of AFP were found for MpAFP which are perfectly match with basal plane water as well as prism plane water. Anchored clathrate water (ACW) mechanism for ice-binding of AFPs was proposed by the authors according to their experimental findings. Their speculation was gone for all AFPs may bind on ice plane through ice-like water construct by the IBS of each AFP molecule (Garnham, *et al.*, 2011). For the special orientation of bound water on IBS, water molecules are ordered by the hydrophobic effect of the amino acid residues and bound on surface of the protein via hydrogen bonds.

It is a common feature for IBS from all AFP species to be flat, hydrophobic and extensive (Bar-Dolev, *et al.*, 2016). Microbial AFPs possess a common protein fold of β -helical structure that have a wide, flat and hydrophobic dominating face. On the other hand, it lacks the regularity and repeating motifs. However, many of these proteins show very high activity. Nevertheless, it is not clear that these proteins follow the anchored clathrate mechanism. Hanada *et al.* 2014 and Cheng *et al.* 2016 proposed compound ice binding sites for hyperactive AFP from bacterium ColAFP and for fungi *Tisphula ishkariensis*, TisAPF8. By docking studies, they proposed that these AFPs might organize hydration waters on IBS to form ice-like waters and bind on ice planes following the ACW mechanism (Cheng, *et al.*, 2016).

The limitation for experimental evidence to find the ice-like water formation in the crystal structure is the protein contact. Most of the AFPs come close contact through IBS in their crystal packing

which may replace the water structure constructed by IBS residues. In our crystallization trial *TisAFP7* wild type and a mutant *TisAFP7* T20Y both of them were packing in isomorphous space group keeping their IBS exposed to the solvent. These crystal packing provided us the water structure on IBS of the AFP molecules. Specific orientations of hydration water were observed on compound IBS sites, loop IBS and sheet IBS. An ice-like water network constructed by loop IBS that shows position matching with basal plane water of ice. In addition, a hydrophobic interaction by Phenylalanine43 residue organized ten water molecules on the top of the loop IBS as a ring-like manner. We assumed; these hydrophobic hydration waters enhance the basal plane binding of the molecule. When mutation on loop disrupt this ice-like network which observed in the crystal structure of *TisAFP7* T20Y (Fig. 2.6c) consequently this mutant lost its basal plane binding. This result supported by different experimental observation, for example, ice crystal morphology of this mutant which burst along the *c*-axis from the two tips of the crystal (Fig. 2.5c-1, 2). Fluorescence-based ice plane affinity analysis also showed the loss of basal plane binding (Fig. 2.2b-1, 2). Among the three isoforms, the hyperactive *TisAFP8* extended its network outer region of the IBS. It might be influenced by the hydrophobic residue Ala20 (Fig. S4a) like Phe43. For *TisAFP7* and *TisAFP6*, Ala20 is replaced by Threonine which support the concept of water ordering by hydrophobic effect. Another set of hydration water (6 water molecules) was trapped in a small trough formed by inward residues on the β -sheet IBS. These waters are fine-tuned by the IBS which can perfectly match with the prism plane of ice. The mutation on sheet A214Y might destroy these tuned waters causing deficiency for the mutant to bind on prism plane. For this mutant, the ice crystal morphology and FIPA show lack of prism plane binding (Fig. 2.2b-3, 4 & Fig. 2.5c-3, 4). In our research, another important finding is entire ice planes binding like hyperactive AFP by a moderate TH producing AFP, *TisAFP7*. The AFP molecule covered the entire ice hemisphere even at very low concentration 0.007 mg/ml.

In this thesis the findings are summarized as *TisAFPs* including other microbial AFPs possess compound IBS consists of loop and sheet IBS. Compound IBS organize bound water into different orientation to approach to different ice planes with variation in strength related to their activity. However, it is necessary to exist both IBS simultaneously in the same molecule for its activity. This might be reason of evolution of microbial AFPs to tune the hydration water structure to adapt the host to its cold environment.

Data availability

The atomic coordinates have been deposited in the Protein Data Bank under the accession numbers ID 7DC5 and 7DDB for the wild-type *TisAFP7* and *TisAFP7* T20Y, respectively.

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