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**Study on the structure and function of
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cold-adapted microorganisms**

by

Yuichi Hanada

CONTENTS

LIST OF ABBREVIATIONS	5
LIST OF ORIGINAL PUBLICATIONS	7
 Chapter 1: General introduction	 8
1.1. Introduction to antifreeze proteins (AFPs).....	9
1.1.1. Life at extreme environments	9
1.1.2. Ice-binding proteins (IBPs)	9
1.1.3. Function of AFPs	10
1.1.4. Structure of AFPs.....	12
1.1.5. Classification of AFPs	17
1.1.6. Evolutional background of AFPs.....	19
1.1.7. Structure of ice.....	20
1.1.8. Ice-binding mechanism of AFPs	22
1.2. Aims and objectives	23
 Chapter 2: Characterization of ice-binding property of TisAFP	 35
2.1. Introduction.....	37
2.2. Materials and methods	38
2.2.1. Expression	38
2.2.2. Purification	38
2.2.3. Measurements of antifreeze activity.....	39
2.2.4. Preparation of solutions for TH measurements	39
2.2.5. FIPA analysis	39
2.3. Results.....	40
2.3.1. Expression and purification.....	40
2.3.2. TH activity and ice growth pattern of TisAFP6	41
2.3.3. Effect of pH on the activity of TisAFP6.....	41
2.3.4. Influence of annealing time on the activity of TisAFP6.....	42
2.3.5. Effect of pH on the ice-binding affinity of TisAFP6.....	42
2.4. Discussion	43
2.4.1 TH activity and ice crystal burst pattern.....	44
2.4.1. Biological implications of the pH dependence.....	45
2.4.3. Effect of annealing time on the activity.....	45
2.4.4. Effect of pH on the ice-binding specificity	47

2.5. Conclusions	47
2.6. Supplementary data	48
2.6.1. Characterization of TisAFP purified from the natural source	48
Chapter 3: X-ray crystal structure and mutational analyses of ColAFP	56
3.1. Introduction	58
3.2. Materials and methods	59
3.2.1. Expression and purification of ColAFP and its mutants	59
3.2.2. Thermal hysteresis measurements and ice crystal morphology	60
3.2.3. FIPA analysis	60
3.2.4. Crystal structure analysis	61
3.2.5. Docking models of ColAFP to ice	61
3.3. Results	61
3.3.1. Antifreeze activity of recombinant ColAFP	61
3.3.2. FIPA analysis	62
3.3.3. Crystal structure	62
3.3.4. Structural features of the putative IBS of ColAFP	63
3.3.5. CD spectroscopy	64
3.3.6. Mutagenesis on the putative IBS of ColAFP	64
3.3.7. Docking study	66
3.4. Discussion	66
3.4.1. Concentration dependence of the TH activity	66
3.4.2. Ice crystal burst and macroscopic ice hemisphere	67
3.4.3. Possible interactions of ColAFP with ice	68
3.5. Conclusions	70
3.6. Supplementary data	72
3.6.1. Characterization of ColAFP expressed in the yeast <i>P. pastoris</i>	72
3.6.2. Effect of dilution to the activity of ColAFP	74
3.6.3. FIPA analysis of ColAFP with nfeAFP mixing	75
3.6.4. Comparison of the 3-D structures between type I IBPs	75
Chapter 4: Functional and structural analysis of NagAFP and comparison with its homologs	95
4.1. Introduction	97
4.2. Materials and methods	98
4.2.1. Expression of NagAFP8 and V220C mutant	98

4.2.2. Purification of NagAFP8	99
4.2.3. Measurements of TH activity	99
4.2.4. Effect of pH on TH activity	100
4.2.5. Effect of salt concentration and pH on TH activity	100
4.2.6. FIPA analysis	100
4.2.7. Homology modeling and molecular dynamics	100
4.3. Results	101
4.3.1. Expression and purification of NagAFP8	101
4.3.2. Antifreeze activity of NagAFP8	101
4.3.3. Effect of pH and salt on the activity of NagAFP8 and ColAFP	102
4.3.4. FIPA analysis of NagAFP8	102
4.3.5. Homology-guided structure of NagAFP8	102
4.4. Discussion	103
4.4.1. Antifreeze activity of NagAFP8	103
4.4.2. pH and salt-dependent property of NagAFP8	104
4.4.3. FIPA analysis of NagAFP8 and its implications for the IBS	104
4.4.4. Possible IBS of NagAFP8 and comparison with its homologs	105
4.5. Conclusions	106
Chapter 5: Effect of mixing distinct types of antifreeze proteins on their activity	116
5.1. Introduction	118
5.2. Materials and methods	119
5.2.1. Preparation of AFP solutions	119
5.2.2. Measurements of antifreeze activity	119
5.2.3. FIPA analysis	119
5.3. Results	120
5.3.1. TH activity of TisAFP6 and nfeAFP6 mixture	120
5.3.2. Ice crystal morphology in TisAFP6 and nfeAFP6 mixture	120
5.3.3. Ice-binding specificity of TisAFP6 and nfeAFP6	121
5.4. Discussion	122
5.4.1. Enhancement of TH activity	122
5.4.2. Ice crystal morphology	123
5.5. Conclusion	123
Chapter 6: General discussion	127
6.1. Summary	128

6.2. Conclusions	131
References	132
Acknowledgements	145

LIST OF ABBREVIATIONS

AFP	antifreeze protein
AFGP	antifreeze glycoprotein
Afp1	AFP from <i>Glaciozyma antarctica</i> PI12
BMMY	buffered methanol-complex medium
BMGY	buffered glycerol-complex medium
CBB	Coomassie Brilliant Blue
Cn-AFP	AFP from <i>Chaetoceros neogracile</i>
ColAFP	AFP from <i>Colwellia</i> sp. strain SLW05
CfAFP	AFP from <i>Choristoneura fumiferana</i>
DcAFP	AFP from <i>Daucus carota</i>
DAFP	AFP from <i>Dendroides canadensis</i>
FcAFP	AFP from <i>Fragilariopsis cylindrus</i>
FIPA	fluorescence-based ice plane affinity
FfIBP	IBP from <i>Flavobacterium frigoris</i> PS1.
GTA	3,3-dimethylglutaric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hyp-type I	hyperactive fish type I AFP
IBP	ice-binding protein
ISP	ice structuring protein
INP	ice nucleation protein
iwAFP	AFP from inchworm
IAS	ice-active substance
IAP	ice-affinity purification
I _h	unit structure of an ice crystal at 1 atm
IBS	ice-binding site
IPTG	isopropyl β-D-1-thiogalactopyranoside
KOD	DNA polymerase from <i>Thermococcus kodakarensis</i>
LpIBP	IBP from <i>Lolium perenne</i>
LeIBP	IBP from <i>Leucosporidium</i> sp. AY30
LB	Lysogeny Broth/Luria-Bertani
MpAFP	AFP from <i>Marinomonas primoryensis</i>
MD	molecular dynamics
MWCO	molecular-weight cutoff
MES	2-Morpholinoethanesulfonic acid

Mw	molecular weight
NMR	Nuclear Magnetic Resonance
nfeAFP	type III AFP from notched-fin eelpout
NagAFP	AFP from <i>Navicula glaciei</i>
PBS	phosphate buffered-saline
PVC	polyvinyl chloride
RiAFP	AFP from <i>Rhagium inquisitor</i>
RMSD	root-mean-square deviation
Sc	shape correlation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sfAFP	AFP from snow flea
THP	thermal hysteresis protein
TH	thermal hysteresis
TisAFP	AFP from <i>Typhula ishikariensis</i>
T_f	freezing point
T_m	melting point
TmAFP	AFP from <i>Tenebrio molitor</i>
Tris	tris(hydroxymethyl)aminomethane
TE	Tris-EDTA buffer
T_{burst}	the temperature at which an ice crystal starts to grow explosively
YPDS	Yeast Extract Peptone Dextrose medium with Sorbitol

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following published papers, which are referred to in the text by the roman numerals.

- i) **Hanada, Y.**, Nishimiya, Y., Miura, A., Tsuda, S., and Kondo, H. (2014) Antifreeze protein from an Antarctic sea ice bacterium *Colwellia* sp. exhibits hyperactivity with compound ice-binding site without structural regularity. *FEBS J.* **281**, 3576-3590.
- ii) Xiao, N., **Hanada, Y.**, Seki, H., Kondo, H. Tsuda, S. and Hoshino, T. (2014) Annealing condition influences thermal hysteresis of fungal type ice-binding proteins. *Cryobiology* **68**, 159-161.
- iii) Singh, P., **Hanada, Y.**, Singh, S. M., and Tsuda, S. (2014) Antifreeze protein activity in Arctic Cryoconite bacteria. *FEMS Microbiol. Lett.* **351**, 14-22.
- iv) Fukami, D., **Hanada, Y.**, Cheng, J., Tsuda, S., and Kondo, H. (2013) Functional Analysis of Antifreeze Protein from Ascomycete. *Cryobiology and Cryotechnology* **59**, 157-160.
- v) Tsuji, M., Fujiu, S., Xiao, N., **Hanada, Y.**, Kudoh, S., Kondo, H., Tsuda, S., and Hoshino, T. (2013) Cold adaptation of fungi obtained from soil and lake sediment in the Skarvsnes ice-free area, Antarctica. *FEMS Microbiol. Lett.* **346**, 121-130.
- vi) Yamanouchi, T., Xiao, N., **Hanada, Y.**, Kamijima, T., Sakashita, M., Nishimiya, Y., Miura, A., Kondo, H., and Tsuda, S. (2013) Dependence of freeze-concentration inhibition on antifreeze protein. *Low Temperature Science* **71**, 91-96.
- vii) Kondo, H. **Hanada, Y.**, and Tsuda, S. (2012) Fungal antifreeze protein consists of a unique β -solenoid structure. *Photon Factory Activity Report Part A*, 58-59.
- viii) Kondo, H., **Hanada, Y.**, Hoshino, T., Garnham, C.P., Davies, P.L., and Tsuda, S. (2012) Ice-binding site of snow mold fungus antifreeze protein deviates from structural regularity and high conservation. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 9360-9365.
- ix) Xiao, N., Inaba, S., Tojo, M., Degawa, Y., Fujiu, S., **Hanada, Y.**, Kudoh, S., and Hoshino, T. (2010) Antifreeze activities of various fungi and Stramenopila isolated from Antarctica. *North American Fungi* **5**, 215-220.
- x) Kondo, H., Sugimoto, H., Noro, N., Xiao, N., **Hanada, Y.**, Hoshino, T., Miura, A., and Tsuda, S. (2008) Crystal structure of an antifreeze protein from a snow mold fungi. *Acta Crystallographica Section A* **64**, C369.

Chapter 1: General introduction

1.1. Introduction to antifreeze proteins (AFPs)

1.1.1. Life at extreme environments

The life existing under extreme environments possesses particular biomolecules to achieve adaptation. In general, hot springs (high temperature), polar regions (low temperature), salt lakes (high salinity), deep sea (high pressure), and high acidic/basic water are known as representatives for extreme environments ([Warton, 2002](#)). Some archaea and eubacteria living in hot springs express enzymes which are enough active in high temperature around 100°C. Those organisms are therefore referred to as "thermophiles" ([Koga, 2012](#)). On the other hand, the term "psychrophile" is used to indicate the creatures thriving under harsh cold environments ([Storey and Storey, 2013](#)). They produce enzymes activated under low temperatures and constitute cell membranes having a high mobility even in sub-zero conditions. These are known to be the prerequisites for maintaining life at low temperatures. In addition, those creatures have to face growing ice crystals inside/outside of the cells at sub-zero conditions. Liquid water is the indispensable element for life, whereas solid water (ice) is unavailable for the solvent of biological reactions or rather deleterious for the creatures. Growing ice crystals disrupt cells mechanically and push away solutes into an unfrozen part, which results in a concentration of solute outside of the cells. Then osmotic pressure generated by a higher concentration of solutes induces the dehydration of water inside of cells, leading to the critical damage for the cells. Therefore, controlling ice growth is rather important for the life at sub-zero temperatures.

1.1.2. Ice-binding proteins (IBPs)

Ice-binding proteins (IBPs) are known to be a set of proteins that interact with ice specifically. They play important roles in biological adaptations to icy cold environments. So far at least three IBP subclasses are documented; ice nucleation proteins (INPs), ice structuring proteins (ISPs) and antifreeze proteins (AFPs). INPs, a group of membrane associated protein identified from bacteria, allow water to freeze at high sub-zero temperatures by avoiding supercooling of the solution ([Warren and Wolber, 1991](#)). Ice crystals in a solution tend to become larger at the expense of smaller ones. This phenomena is known to be general in most of crystals and termed as "Ostwald ripening", or particularly "ice recrystallization" for ice. ISPs inhibit the process of ice recrystallization by retaining the interface of individual ice grains ([Clarke](#)

et al., 2002). AFPs prevent the growth of ice crystals in supercooled solutions by covering their surface (Jia and Davies, 2002).

"IBP-ice" interactions provide a captivating and exquisite instance of the relationship between the structure and the function of proteins. For example, some AFPs display regularly spaced side-chains of amino acid on one face of the molecular surface, which mimics almost completely the spacing of oxygen atoms in a plane of ice (Steffen et al., 2000; Liou et al., 2000). IBP binding to ice indicates interactions occurred between nano meter-scaled biomolecules and solid, crystalline material of ice. The protein-crystal interactions have been observed in the process of "biomineralization" (Addadi and Weiner, 1985) and in pathological conditions such as stone formation (Bertrand et al., 1996). The biomineralization is known to as common phenomena in which organisms generate compound materials of inorganic and organic substances in their body, resulting in producing of shells for shell fishes, exoskeletons for invertebrates, bones and teeth for vertebrates, and so on. It is, therefore, considered that the techniques for controlling the shape and size of crystalline materials would serve in medical- and nano-technologies.

A recent study revealed that the interacting partner of AFPs are not restricted to ice alone, but also do bind naturally to nucleoside crystals (Wang et al., 2012). This finding has expanded the possibility of use of AFPs for applications without altering their active sites. AFPs, therefore, can be utilized for controlling the size and the shape of various crystals.

1.1.3. Function of AFPs

By binding to the surface of ice crystals, AFPs halt their further growth in supercooled solutions. This results in a depression of the freezing point (T_f) of the solution with a slight increment of the melting point (T_m). The temperature difference generated between the depressed T_f and the increased T_m is defined as thermal hysteresis (TH) and widely utilized for the measure of AFPs' activity (antifreeze activity) (Raymond and DeVries, 1977), which is schematically shown in Fig. 1-1. Technically, the increased range of T_m is termed as the "melting hysteresis", and the depressed range of T_f is termed as the "freezing hysteresis" (Celik et al., 2010). The exact TH values produced by an AFP is thus defined the sum of melting hysteresis and freezing hysteresis. In this context, AFPs are also called as thermal hysteresis proteins (THPs) (Barrett, 2001).

It has been so far argued that whether AFP binding to ice is reversible or irreversible (Knight and Devries, 1994; Fletcher et al., 2001). If AFPs bind to ice reversibly, it can be assumed that water molecules surrounding the ice rush to the sites where AFPs are absent. This would cause a sudden growth of ice. However, the idea in which two-interacting partners bind irreversibly without covalent bonds is not readily accepted, since diffusions might occur even in a receptor-ligand interaction with an extremely low dissociation constant (K_d). A recent study demonstrated experimentally that AFPs bind to ice irreversibly, based on the observations using a temperature-controlled microfluidic device in which the solvent surrounding ice crystals can be replaced (Celik et al., 2013).

The inhibitory effect of AFPs on ice growth is implicated in the modification of crystal morphologies due to the specific binding of AFPs. The change in the shape of ice crystals can be observed microscopically and exploited to detect the presence of AFPs (Strom et al., 2005; Bar-Dolev et al., 2012). Shape-modified ice crystals attain their defined forms within the TH window and grow suddenly at the temperature cooled below the T_f of the solution. This sudden and explosive growth of ice is referred to as "burst" and is a reproducible feature of the type of AFP present (Scotter et al., 2006). Recently, it was found that some AFPs fail to inhibit the growth of ice crystals completely, but only can modify their growing shape (Takamichi et al., 2009). The detailed observation in this study revealed that the AFP actually prevents the burst growth of ice. This leads to a novel definition of AFPs' activity; some AFPs depress the temperature at which ice crystals start to grow explosively (T_{burst}), while not producing TH activity. In this case, the activity of AFPs is defined as the temperature difference between the T_m and the T_{burst} .

The presence of TH in biological fluids was firstly reported by Ramsay from the blood of an insect *Tenebrio molitor* (Ramsay, 1964). The prevailing theory on how TH arises is known as the so-called "adsorption inhibition mechanism" (Fig. 1-2) (Raymond and Devries, 1977). According to this model, AFP molecules adsorb onto the surface of ice irreversibly and then, the ice surface grows out between the adsorbed molecules to form convex fronts. This local growth is energetically unfavorable due to the Kelvin effect (Kristiansen and Zachariassen, 2005). The further growth of ice is therefore restricted depending on the radius between adsorbed AFPs, resulting in a depression of the T_f in the solution.

1.1.4. Structure of AFPs

To date dozens of AFP molecules with distinct structures have been found a wide range of organisms, including fish, plants, invertebrates, algae, fungi, bacteria. A remarkable feature of these AFPs is the structural diversity despite they share a common function (i.e., ice-binding). The molecular feature of those are summarized as follows:

Fish AFPs

About a half century ago, polar researchers found that Antarctic fish avoid their body fluid from freezing by expressing a high concentration of an antifreeze substance (DeVries and Wohlschlag, 1969). Currently the substance is known as antifreeze glycoprotein (AFGP) and the very first molecule documented as an AFP. AFGP is made up by a simple tri-peptide repeat (Ala-Ala-Thr) and expressed as a set of molecularly heterogeneous group sized around 3 to 24 kDa (Vandenhede et al., 1972). The solution structure of this monomer unit has been analyzed (Tachibana et al., 2004). Most of fish AFPs show TH of 1°C or less and therefore known as the moderately active AFPs (mentioned below).

Type I AFP has been discovered from winter flounder (*Pseudopleuronectes americanus*) and known as the smallest molecule (Mw, ~4 kDa) among AFPs (Duman and Devries, 1974). The first X-ray structure of AFPs was reported on this Ala-rich protein (Yang et al., 1988; Sicheri and Yang, 1995), which clarified an amphipathic, long (~50 Å) rod-like single α -helix (Fig. 1-3A). A recent study (Marshall et al., 2004b) revealed that the winter flounder also expresses another AFP, which displays TH in excess of 2°C. The AFP is designated as hyp-type I AFP where "hype" stands for hyperactive (Graham et al., 2008). This TH value is almost comparable to those produced by AFPs from terrestrial arthropods. Moreover, a very recent study (Sun et al., 2014) showed the homodimeric, four helix-bundled structure of an elongated (5 times longer than the normal one) type I AFP variant (Fig. 1-3B) determined by X-ray crystallography. Remarkably, the bundle made up of four monomer units (ca. 15 kDa alone) is stabilized by numerous hydration water molecules, not by the typical hydrophobic interactions.

Type II AFP is further classified by the dependence on Ca^{2+} ion to its activity. The Ca^{2+} -independent species was firstly isolated from sea raven (*Hemitripterus americanus*) (Slaughter et al., 1981). On the other hand, Ca^{2+} -dependent species was identified in a smelt (*Osmerus mordax*) and an Atlantic herring (*Clupea harengus harengus*) simultaneously (Ewart and Fletcher, 1990). Both solution NMR and X-ray crystal structures were determined (Gronwald et al., 1998; Nismimiya et al., 2008),

revealing that the scaffold of the protein is highly similar to that of the carbohydrate recognition domain of C-type lectin (Ng and Hew, 1992) (Fig. 1-3C).

Type III AFP was firstly found in ocean pout (*Macrozoarces americanus*) (Hew et al., 1984). This type is known to have an unusual variation of isoforms (Nishimiya et al., 2005). Moreover, naturally dimerized variant was also isolated from the blood plasma of Antarctic eel pout (*Lycodichthys dearborni*) (Wang et al., 1995). X-ray and NMR structural analyses revealed that type III AFP comprises several short β -strands connected by an α -helical turn (Jia et al., 1996) (Fig. 1-3D).

Type IV AFP was discovered in the blood plasma of longhorn sculpin (*Myoxocephalus octodec imspinosis*) and characterized to be a 12.3 kDa protein composed of 108 amino acid residues (Deng et al., 1997). The longhorn sculpin is also known to produce type I AFP sufficient to avoid its blood from freezing by itself. Since the vast majority of AFP in the fish is type I, it has been realized that detecting type IV AFPs is extremely difficult. Therefore it was concluded that type IV AFP has not been selected for the antifreeze substance in this fish (Gauthier et al., 2008).

Arthropod AFPs

AFPs from terrestrial arthropods have been well characterized equally to those from fish. To date, at least five of distinct types of AFPs were isolated and characterized at the molecular level. These AFPs shows a potent TH activity and known as hyperactive AFPs, reflecting their habitat environments in which they are exposed to harsh cold air temperature ($\sim -30^{\circ}\text{C}$) (Graether and Sykes, 2004).

The first AFP (*TmAFP*) from arthropods was documented in the yellow mealworm *T. molitor* (Graham et al., 1997). The mealworm is known as a live food for birds and reptiles. This Thr- and Cys-rich AFP with a Mw of 8.4 kDa exhibits TH up to 5.5°C and folds into a right-handed β -helix structure (Liou et al., 2000) (Fig. 1-4A). In each β -strand on one side of the helix, *TmAFP* displays the Thr-X-Thr (T-X-T) pattern as the ice-binding site (IBS).

A moth, spruce budworm (*Choristoneura fumiferana*) produces a 9 kDa AFP (*CfAFP*) characterized by a rich content of Thr- and Cys residues (Tyshenko et al., 1997; Gauthier et al., 1998), similar to *TmAFP*. The solution structure of *CfAFP* revealed the left-handed β -helix structure of this protein (Graether et al., 2000) (Fig. 1-4B). The T-X-T pattern can be also observed in the IBS of *CfAFP*.

An inchworm, the larva of a moth *Campaea perlata*, produces both 3.5 and 8.3 kDa of AFP (*iwAFP*) isoforms which shows a quite strong TH activity of 6.4°C (Lin et

al., 2011). A predicted structural model has been proposed for the 8.3 kDa isoform, showing the AFP is folded into a flat silk-like β -helix.

Snow fleas are known to be a group of primitive arthropods where they prefer to live in the surface of snow. One of them, *Hypogastrura harveyi*, displays a TH activity of 5.8°C in its body extract (Graham and Davies, 2005). The biochemical analyses revealed that the activity is derived from two types of AFP (sfAFP) isoforms with the Mw of 6.5 and 15.7 kDa, respectively. The X-ray structure of the small isoform has been determined, which showed that the protein is made up of six antiparallel left-handed polyproline II-like helices (Pentelute et al., 2008) (Fig. 1-4C).

The longhorn beetle, *Rhagium inquisitor*, survives at a cold environment in the winter season of Siberia, which is due in part to the presence of an AFP (RiAFP) (Kristiansen et al., 1999). RiAFP, a 13 kDa molecule without isoforms, shows TH of around 6.5°C which is one of the highest measured among all AFP categories (Kristiansen et al., 2011). Recently, the X-ray crystal structure of RiAFP was reported, revealing its flat, silk-like β -helical (solenoid) structure similar to the model structure of wiAFP (Fig. 1-4D).

Plant AFPs

AFPs from animals have been summarized in the above text. AFPs are also widely found in a range of over-wintering plants. However, the physiological role of plant AFPs is fundamentally different to that of animals. Freezing is unavoidable for plants during winter. Ice crystals are formed in intercellular spaces (apoplast) and they cause freezing injury due to the dehydration of intracellular spaces (Pearce, 2001). Moreover, ice recrystallization also occurs in such conditions, where the expanded ice crystals disrupt the cells of plants mechanically. To minimize all of those critical damages induced by ice formation, it has been believed that plants make use of AFPs (Griffith and Yaish, 2004). Because of this, most of cold-adapted plants are known as "freeze tolerance". In contrast, animals having AFPs described above are known as "freeze avoidance", in which freezing of the body fluids itself is critical for their survival. In fact, TH levels measured in freeze tolerant-plant AFPs are substantially lower than those obtained by freeze avoidant-animal AFPs. Instead, the ice recrystallization inhibition activity exhibited by plant AFPs is typically higher than that of animal AFPs, reflecting their strategies for cold environments (Griffith and Yaish, 2004; Yu et al., 2010). Currently, at least two types of plant AFPs are characterized at the molecular level and summarized below.

A common vegetable, carrot (*Daucus carota*) produces an N-glycosylated, Leu-rich AFP (DcAFP) with a Mw of 36 kDa (Worrall et al., 1998). DcAFP is composed of 24-amino acid tandem repeats. The 3D structural model of DcAFP has been proposed based on this repetitive feature, showing β -helical structure displaying conservative Asn residues on one side as it is predicted to be the ice-binding site (IBS) (Zhang et al., 2004).

A forage grass, perennial ryegrass (*Lolium perenne*) produces an extremely heat-stable AFP (LpIBP) composed of 118 amino acid residues with a Mw of 11 kDa (Sidebottom et al., 2000). The recent X-ray crystallographic analysis reported LpIBP folds as left-handed β -roll structure (Middleton et al., 2012) (Fig. 1-5A). Although the high heat-stability of this protein is quite unusual, the mechanism is still remains to be investigated.

Bacterial AFPs

Similar to plants, most of AFP-producing bacteria are known to be freeze tolerant (Xu et al., 1998; Gilbert et al., 2005). The existence of AFPs in bacteria was firstly reported by Duman and Olsen (1993) on two species, *Rhodococcus erythropolis* and *Micrococcus cryophilus*. Subsequently, an AFP with a Mw of ca. 164 kDa was found to be produced by the plant growth promoting rhizobacterium, *Pseudomonas putida* GR12-2 (Sun et al., 1995; Xu et al., 1998). A carbohydrate moiety accounts for the 92 kDa part of this AFP and removal of this portion had no effect of its activity. Also this AFP is unusual because it shows ice nucleation activity, too. The TH activity of this AFP is quite low (0.1°C). Another bacterial AFP with low TH activity (0.1°C) was also isolated from *Moraxella* sp (Yamashita et al., 2002). This is a 52 kDa protein with a lipid moiety. Additionally, *Pseudomonas fluorescens* was reported to produce a 80 kDa AFP with a TH activity of 0.059°C (Kawahara et al., 2004).

A novel bacterial AFP (MpAFP) which displays a high TH activity (2°C) has been recently isolated and characterized from an Antarctic lake bacterium, *Marinomonas primoryensis* (Gilbert et al., 2005; Garnham et al., 2008). MpAFP (34 kDa by itself) is a part of exceptionally large (1.5 MDa) protein localized in the cell surface of the bacterium (Guo et al., 2012). The X-ray crystal structure of the 34 kDa part of MpAFP has been solved, revealing the β -helical fold containing xGTGND repeat motif in every turn as the IBS (Fig. 1-5B) (Garnham et al., 2011). The function of the entire part (1.5 MDa) of MpAFP was suggested to be anchoring the host cell onto the surface ice of the lake, where nutrients and oxygen are much more abundant than the bottom, rather than preventing the growth of ice (for freeze avoidance) or inhibiting the recrystallization of

ice (for freeze tolerant) (Guo et al., 2012). This is the recently emerging novel function of AFPs. Bacterial AFPs with potent TH activity have been also reported on *Colwellia* sp. SLW05 (i) and six strains of Arctic bacteria (iii). Both of which are known to belong to the group of "type I IBPs" described below.

Other microbial AFPs: type I and type II IBPs

Duman and Olsen (1993) also reported the presence of AFPs in several species of fungi. Snider et al. (2000) reported that antifreeze activities were detected in a culture medium of many snow mold fungi. The characterization of fungal AFPs at the molecular level has been achieved for the first time by Hoshino et al (2003a). The fungal AFP (*TisAFP*), which was isolated from a snow mold fungus, is composed of 223 amino acid residues with a Mw of 23 kDa and shows a TH activity of just under 1°C at a protein concentration of 20 mg/ml. To our knowledge, *TisAFP* was the first microbial protein with an appreciable TH activity. Subsequently, Janech et al. (2006) reported that they isolated IBPs from Antarctic sea ice diatoms, *Navicula glaciei* and *Fragilariopsis cylindrus*. In this paper, the authors prefer to use the word "IBPs" instead of "AFP", since the primary function of these proteins are considered to cause pitting and deformation on the surface of ice crystals. Surprisingly, it was found that the IBPs have similar molecular sizes (~25 kDa) and amino acid sequences (~50% identity) to those of *TisAFP*. To date so many homologues with an ice-binding activity have been identified in bacteria (Raymond et al., 2007; 2008; Do et al., 2012), unicellular algae (Raymond and Kim, 2012), a copepod (Kiko, 2010), and other fungi (Raymond and Janech, 2009; Lee et al. 2010) and diatoms (Gwak et al., 2010; Raymond and Morgan-Kiss, 2013; Jung et al., 2014). Although the exact route by which all these organism acquired the common gene encoding this type of AFPs/IBPs is still remains to be understood, horizontal transfer from bacterial origin has been proposed (Bayer-Giraldi et al., 2010; Sorhannus, 2011; Raymond and Kim, 2012). Raymond and Morgan-Kiss (2013) designated this type of AFPs/IBPs as "type I IBP". The first crystal structure of type I IBP has been reported by us and another group simultaneously on *TisAFP* (viii) and *LeIBP* (Lee et al., 2012) (Fig. 1-5C). Very recently, an AFP (designated as *Afp1* by the authors) from a psychrophilic yeast *Glaciozyma antarctica* PI12 was shown to have a sequence identity (~30%) at amino acid level to that of *TisAFP* (Hashim et al., 2013). *Afp1* is, however, composed of 177 amino acid residues in the mature form. This is relatively short considering that the average amino acid length of 220~250 in the homologs. Indeed, *Afp1* shows only a low TH activity of around 0.1°C. This might be ascribed to the lack of an important part for exhibiting the

antifreeze activity. Only a partially structure of Afp1 determined by NMR is available to date (Shah et al., 2012) (Fig. 1-5D).

Another line of microbial IBPs has been isolated from an Antarctic snow alga, *Chloromonas* sp (Raymond et al., 2009b). This IBP contains four isoforms with Mw of 36.2–37.1 kDa. Interestingly, the repetitive TXT motif is observed in this IBP through the entire sequence. Although the 3D structure of the IBP has not determined yet, it was predicted that the motif may form the IBS of this protein (Raymond et al., 2009b). Raymond and Morgan-Kiss (2013) designated this type of IBPs as "type II IBP".

1.1.5. Classification of AFPs

Functional difference

So many distinct types of AFP molecules have been isolated from a wide range of organisms, vertebrate animals to eubacteria, and characterized functionally and structurally as summarized above. Those are classified into two major groups, the moderately active and the hyperactive, based on their TH activity at the molar concentration (Scotter et al., 2006; Bar-Dolev et al., 2012) (Fig. 1-6). The moderately active AFPs are characterized by TH values around 0.5°C to 1.0°C at millimolar concentrations. On the other hand, the TH activity of hyperactive AFPs exhibit similar ranges to those of the moderately active AFPs at a hundredth of protein concentrations, reaching up to 6°C at a maximum. The beetle TmAFP was firstly documented as the hyperactive AFP (Graham et al., 1997). To date, most of arthropod AFPs including TmAFP (Graham et al., 1997), CfAFP (Tyshenko et al., 1997), iwAFP (Lin et al., 2011), sfAFP (Graham and Davies, 2005), and RiAFP (Kristiansen et al., 1999), a fish AFP (hyp-type I AFP, Marshall et al., 2004b), three bacterial AFP (MpAFP, Garnham et al., 2008; FfIBP, Do et al., 2012; ColAFP, i), a fungal AFP (TisAFP8, Xiao et al., 2010), and a diatom AFP (NagAFP, ii) have been reported to be hyperactive.

The difference between these two classes of AFPs is not only observed in their TH activity, but also in the patterns of ice crystal burst in their presence. The unit structure of an ice crystal (I_h) is defined as the hexagonal system with the vertical axis (c -axis) and horizontal axis (a -axis) (see details in Chapter 1.1.7.). Ice crystals formed in the presence of moderately active AFPs grow rapidly into the c -axis of ice when the temperature exceeded the maximum TH range (Raymond et al., 1989). In contrast to this pattern, the burst in the presence of hyperactive AFPs occurs as a dendritic expansion into the directions normal to the c -axis (Graether et al., 2000). These

phenomena are ascribed to the difference in the plane(s) at which AFPs bind. Hyperactive AFPs bind to both the prism and basal planes, while moderately active AFPs have no affinity to the latter ([Pertaya et al., 2008](#)).

The avidity of AFP molecules for ice has not been considered to be involved critically in producing the hyperactivity. In fact, irreversible binding of AFPs to ice has been claimed for the both classes as the mechanism by which they can halt ice crystals from growing even in the presence of an excessive amount (≈ 55 M) of water molecules ([Raymond and Devries, 1977](#); [Knight and Devries, 1994](#); [Celik et al., 2013](#)). In a previous research using type III AFP (moderately active) and CfAFP (hyperactive), it was clarified that the amount of AFP molecules incorporated into ice is almost identical to each other, despite their huge difference in TH activity ([Marshall et al., 2004a](#)). From these reasons, the affinity toward the basal plane of ice, therefore, has been widely recognized the key to the hyperactivity of AFPs.

Structural difference

Most of hyperactive AFPs structurally characterized to date have highly regular β -helical structures (TmAFP, [Liou et al., 2000](#); CfAFP, [Graether et al., 2000](#); iwAFP, [Lin et al., 2011](#); RiAFP, [Hakim et al., 2013](#); MpAFP, [Garnham et al., 2011](#)) and recognized as an useful protein scaffold to design and construct the molecules with an affinity toward various target crystalline materials ([Bar et al., 2008](#)). As exceptions to this rule, it is known that two hyperactive, sfAFP and hyp-type I AFPs are folded into the six antiparallel left-handed polyproline II-like helices ([Pentelute et al., 2008](#)) and four helix-bundled structure ([Sun et al., 2014](#)), respectively. The β -helical structures, however, have been also documented in several moderately active AFPs (LpAFP, [Middleton et al., 2012](#); DcAFP, [Zhang et al., 2004](#); LeIBP, [Lee et al., 2012](#); TisAFP, [viii](#)).

The IBSs of those hyperactive AFPs are characterized by repetitive sequence motifs regardless of the overall fold (β -helices or exceptions). TmAFP and CfAFP display a highly ordered 2D array of Thr residues, which is known as the T-X-T motif ([Liou et al., 2000](#); [Graether et al., 2000](#)). Extended T-X-T motifs are observed in iwAFP and RiAFP, where the T-X-T is duplicated (T-X-T-X-T-X-T) in each β -strand ([Lin et al., 2011](#); [Hakim et al., 2013](#)). MpAFP constitutes the IBS made up by the motif of X-G-T-G-N-D ([Garnham et al., 2011](#)). The repetitive motif of SfAFP is known to be G-X-X ([Mok et al., 2010](#)). Interestingly, the periodic residues (Thr/Ile, i+4 Ala, i+8 Ala) of hyp-type I AFP can be seen in the inward-pointing surfaces where the four monomer units are stacked each other ([Sun et al., 2014](#)). The repetitive sequence motifs listed

above are, obviously, pointing to solvent to interact with the surface of ice, except for that of hyp-type I AFP. It is considered that those periodic residues in hyp-type I AFP has an impact on organizing water molecules surrounding the tetrameric protein into an ice-like form, which binding the AFP indirectly to ice (Sun et al., 2014). This is so-called "anchored clathrate water" (Garnham et al., 2011) and will be mentioned below in detail. Taken together, all hyperactive AFPs have repetitive sequence motifs as their ice-binding residues to recognize the crystalline surfaces of ice which are composed of periodically arranged water molecules.

In contrast, the IBS of most moderately active AFPs are known to be lacking in the periodicity. Fish type II and type III AFPs are globular molecules in which their IBSs are made up of loop regions with heterogeneous amino acid residues (Jia et al., 1996, Liu et al., 2007). A plant LpIBP has a β -helical fold similar to hyperactive AFPs, while the ice-binding residues are varied over the face (Middleton et al., 2012). The same periodic pattern (Thr/Ile, i+4 Ala, i+8 Ala) of amino acid residues as hyp-type I is observed in fish type I AFP (Baardsnes et al., 1999). The TH activity of normal type I AFP, however, is known to be around 1°C (Patel and Graether, 2010).

1.1.6. Evolutional background of AFPs

The remarkable divergence in 3D structures of AFPs has been explained as a result of convergent evolution in which the organisms evolved their own AFPs independently from so many different origins in response to the threat of cold climate (Scott et al., 1986). In sea level, geological records describe that the appearance of ice dates back 10-30 million years ago in the southern hemisphere and 1-2 million years ago in the northern hemisphere (Kerr, 1984; Shackleton et al., 1984). At least for fish AFPs, it has been claimed that they were evolved as a direct response to those recent emergence of ice in ocean (Cheng, 1998; Fletcher et al., 2001). In bacteria, brackish environments where the organisms encounter the drastically fluctuated temperature are considered to be the key to the evolution of AFPs since there was no evidence show the presence of AFP-producing bacteria in fresh water (Gilbert et al., 2004).

Moreover, ice, the interacting partner of AFPs, displays many different planes in which the arrangement of oxygen atoms varies (see details in Chapter 1.1.7.). In fact, different AFPs are shown to recognize the different set of oxygen atoms (Basu et al., 2014). Therefore it has been proposed that the diversity of ice structure also makes AFPs diversified (Fletcher et al., 2001; Jia and Davies, 2002).

1.1.7. Structure of ice

Hexagonal ice crystal

It is generally known that ice exists in numerous crystalline forms depending on the temperature and the pressure in which ice crystals are formed. Of these, hexagonal ice (I_h) is known to be stable under ordinary pressures at around 0°C. The structure of I_h is shown in [Fig. 1-7](#) to facilitate understanding. Here, the "*c*-axis" is defined as the hexagonal symmetrical axis and the three "*a*-axes" are perpendicular to the *c*-axis. These brief features of ice are summarized by Hew and Yang (1992).

There are numerous planes defined by Miller indices in crystalline materials. However, a limited number of planes is known to emerge during growth in ice crystals. The different planes of ice have a specific array of water molecules, which makes their surface structures fundamentally varied. In the next section, ice planes involving in AFP interactions will be listed ([Table 1-1](#)). Those surface structures and arrangements of oxygen atoms are shown in [Fig. 1-8](#).

Determination of ice planes to which AFPs bind

For the very first time Raymond and his colleagues reported the direct evidence that AFPs become incorporated into ice during the growth ([Raymond and DeVries, 1977](#)). Subsequently, Raymond and his colleagues presented the evidence of specific binding by AFPs to several planes of ice ([Raymond et al., 1989](#)). In this paper, the authors described that a macroscopic single crystal of ice with known orientation fixed on a glass slide were soaked in an AFP (AFGP) solution and observed its growing direction at the temperature below T_f . It was found that AFGP prevent ice growth from *a*-axis direction by binding to the prism and pyramidal planes of ice. Due to the lack of an affinity toward the basal plane, AFGP was predicted to allow ice crystals to grow into the *c*-axis direction. After these findings, a simple but robust method for determining the crystallographic planes of ice at which AFPs bind was developed and designated as the "ice-etching technique" ([Knight et al., 1991](#)). Here they applied macroscopic single crystals of ice, similar to the method of [Raymond et al., 1989](#), but the crystals were grown to form a hemispherical shape in a cylindrical insulated cup containing diluted AFPs. The ice surface was scratched to remove residual AFP molecules taken over from the solution after growing of the ice hemisphere. Then the ice hemispheres were left at a freezer to allow evaporation of ice. As a result,

AFP-bound regions were visualized as etched patterns (Fig. 1-9 A). In that way they succeeded to determine, at the crystallographic level, the bound planes of ice by type I AFP variants from a couple of fish.

Decades later, a new method designated as the fluorescence-based ice plane affinity (FIPA) analysis replaced by the traditional ice-etching method was established by Garnham et al (2010). Although the concept and the principle of this method is fundamentally identical to that of the traditional ice-etching technique, the FIPA analysis makes use of fluorescently-labeled AFPs to increase the detectability of bound molecules on ice planes. Naturally, the observations of resulting ice hemispheres created by FIPA analysis are performed under an UV light in a cold dark place. The clearer pattern of AFP-bound regions on ice can be obtained compared to those produced by the traditional ice-etching techniques. An example of FIPA analysis is shown in Fig. 1-9 B for clarity of the difference between these two method. Taken together, the ice-etching techniques, followed by the FIPA analyses have so far revealed the ice planes to which different types of AFPs recognize. All those known data are compiled and shown in Table 1-1.

Ice-shaping ability of AFPs

AFPs do not only inhibit the growth of ice crystals, but also affect their morphology due to the specific binding of AFPs to ice planes. For instance, ice crystals tend to be modified into bipyramidal shapes in the presence of fish AFPs (Chao et al., 1995). The characteristic shape of ice crystals in a solution containing a certain AFP can be observed even at very low protein concentrations (Deluca et al., 1996). Ice crystals produced by hyperactive AFPs are varied. TmAFP modifies ice crystals into a lemon-like shape (Graham et al., 1997), while a rice grain-like ice crystals is known to be observed in the presence of sfAFP (Graham and Davies, 2005).

In general, bipyramidal ice crystals in fish AFP solutions are created and can be observed as those crystals are growing at the temperatures below the T_f . However, a recent study has pointed out that shaping of ice crystals in hyperactive AFP solutions are observed during they are melting (Bar-Dolev et al., 2012). In other words, ice-shaping ability of hyperactive AFPs becomes obvious under the melting course. This has been newly recognized as another difference between hyperactive and moderately active AFPs, in addition to the structural regularity at their IBS and the ice planes bound by themselves.

1.1.8. Ice-binding mechanism of AFPs

Hydrogen bonding

As Raymond and DeVries (1977) proposed several decades ago, the "adsorption inhibition" model so far has been generally accepted as the mechanism by which AFPs depress the T_f of solution and inhibit the growth of ice crystals. This theory, however, fails to explain how AFP molecules bind to ice at the molecular level. The first mechanistic insight into the antifreeze phenomena was provided by the X-ray structure of type I AFP determined at 2.5 Å resolution (Yang et al., 1988). In this paper, the authors described that the first, the dipole moment provided by the straight helical structure of type I AFP determines the alignment of the peptide along the c-axis of ice; second, regularly spaced-polar side chain groups (mainly Thr) well match the spacing of the prism plane of ice; third, hydrogen bonding mediated by those polar groups contribute to the stability of the "type I AFP-ice" complex. Later on, the refined crystal structure of type I was reported at 1.5 Å resolution (Sicheri and Yang, 1995). This further supported the model proposed by Yang et al. (1988) by a series of docking models between the AFP and ice based on the refined AFP structure.

Van der Waals, hydrophobic interactions and the surface planarity

The mechanism by which AFPs interact with the ice lattice through hydrogen bonding was called in question by the mutagenesis experiments where the central two Thr residues of type I AFP had been substituted into Val/Ser residues (Chao et al., 1997; Haymet et al., 1998). They found that the Val mutant had almost comparable activity to that of wild type, while the Ser mutant was virtually inactive. The side chain of Val is composed of two methyl (-CH₃) groups, whereas that of Ser is comprised of a methyl group and a hydroxyl (-OH) group. In the case of Thr, the both methyl and hydroxyl groups constitute the side chain. Therefore they hypothesized that the hydrophobic, Thr methyl groups of type I AFP play an important role in its ice-binding, rather than hydrophilic groups. In this scenario, AFP-ice interactions are stabilized by van der Waals contacts and hydrophobic effects. The latter force is derived from the entropic gain by releasing constrained water molecules on the IBS of an AFP upon ice-binding.

Meanwhile, DeLuca et al. (1998) proposed that the surface planarity of the IBS is deeply involved in ice-binding of type III AFP to exert the surface complementarity to the ice lattice, based on the numerous IBS mutants and X-ray structure of the protein. Subsequently, Leinälä et al. (2002a) concluded that AFP binding to ice is substantially based on the surface complementarity by introducing the Sc (shape correlation

statistics) calculation of AFP-ice interfaces. The SC is built-in the program suite of CCP4 ([Winn et al., 2011](#)) and originally invented by Lawrence and Colman ([1993](#)). The Sc value 1 indicates a perfect match between two interacting molecules. Here, they hypothesized that CfAFP binds to the primary prism plane of ice through the geometrical fit between the AFP and the ice plane ([Leinala et al., 2002a](#)). They found that the hydroxyl and methyl groups of the ice-binding Thr residues well match the small cavities of the ice plane with a relatively good Sc score of 0.7-0.76.

Anchored clathrate water

The following, up-to-date mechanism of AFP-ice interactions was proposed by Nutt and Smith ([2008](#)) and experimentally established by Garnham et al. ([2011](#)). In the paper ([Garnham et al., 2011](#)), the authors termed their new theory the "anchored clathrate water" mechanism. In this scenario, the function of the IBS of an AFP is understood as the area in which water molecules are organized into an ice-like arrangement. The water molecules with an ice-like arrangement on the IBS are thought to be directly incorporated into an ice plane with a similar water arrangement. In other words, AFP binding to ice is mediated by hydration water molecules on the IBS. Here, the hydrophobic property so far observed as a characteristic for AFPs' IBS play an important role in ordering waters into an-ice-like arrangement and those waters are stabilized by hydrogen bonding mediated by hydrophilic residues on the IBS. Currently this model is generally accepted and believed to be true ([Sharp, 2011](#); [Hakim et al., 2013](#); [Sun et al., 2014](#)).

1.2. Aims and objectives

AFPs constitute a unique class of biomolecules that interact with ice crystals specifically and inhibit their growth to reduce injury caused by freezing at supercooled conditions. It has been therefore considered that AFPs play important roles in adaptation to cold environments for various organisms ranging from animals to unicellular microbes. Although AFPs from fish and arthropods are well characterized, yet relatively little is known about those from microbes. The first structural characterization of microbial AFPs was carried out on MpAFP ([Garnham et al., 2011](#)), which had been isolated from an Antarctic lake bacterium ([Gilbert et al., 2005](#)). As the second and third

cases, the crystal structures of TisAFP (viii) and LeIBP (Lee et al., 2012) were determined. Both of which are sharing the sequence identity at amino acid level, therefore thought to be homologous to each other. Their homologous proteins have been discovered in a wide variety of microorganisms including fungi (Raymond and Janech, 2009), diatoms (Janech et al., 2006; Gwak et al., 2010), copepods (Kiko, 2010) and bacteria (Raymond et al., 2007; 2008). These proteins were recently designated as type I IBP (Raymond and Morgan-Kiss, 2013). Those homologous AFP/IBP sequences were compiled and aligned for comparison in Fig. 1-10.

As shown by the X-ray structures of TisAFP and LeIBP, they share a common protein scaffold of the β -helix along with an α -helix (Fig. 1-5 C). Despite this, it has been gradually realized that the antifreeze activities exhibited by the individual AFPs/IBPs are varied fundamentally. In other words, the class of type I IBP contains both hyperactive and moderately active AFPs despite they share the common protein scaffold. TH activities reported on this type of AFPs/IBPs are summarized in Table 2. Remarkably, it was found that the putative ice-binding residues identified in TisAFP are not conserved among the homologs (Fig. 1-10). It is therefore strongly expected that the origin of proteins' ice-binding ability would be elucidated by revealing what structural feature determines an AFP hyperactive or moderately active. The accurate information on the main attributes of ice-binding function of AFPs would enable us to depict the nature of interactions between proteins and crystals, which leads to the development of techniques controlling the size, the shape, and the texture of crystalline materials. Thus, the protein architecture of type I IBP is thought to be an ideal example for those purposes considering their functional diversity exerted through the common β -helical structure.

The goal of the present thesis is to shed light on the structure-function relationship of type I IBPs, thereby understanding what structural characteristics of their IBS generate the huge difference in their antifreeze activity. Type I IBPs from a snow mold fungus *Typhula ishikariensis* (TisAFP), an Antarctic sea ice bacterium *Colwellia* sp. SLW05 (ColAFP), and an Antarctic sea ice diatom *Navicula glaciei* (NagAFP) have been selected and characterized thoroughly in the present study as the author's preliminary study (the Master's thesis) showed their large functional difference. The specific objectives in each chapter are as follows:

Chapter 2. To examine the ice-binding property of TisAFP. Thorough functional analyses including TH activity measurements as a function of AFP concentration, solution pH, and annealing time and FIPA analyses were performed.

Chapter 3. *To clarify the molecular basis of the hyperactivity of ColAFP.* The crystal structure of ColAFP was determined. Site-directed mutagenesis was carried out to identify the IBS.

Chapter 4. *To understand the functional difference between naturally expressed one and recombinant version of NagAFP.* An isoform of NagAFP was expressed and characterized functionally. The putative IBS was predicted, based on the homolgy-guided 3-D model structure and the results of FIPA analysis.

Chapter 5. *To gain experimental insights into the possibility of use of AFPs to control the shape of ice crystals.* Different AFP types (fish type III AFP and TisAFP) were mixed and assayed for their TH activity and the crystals shape in their presence.

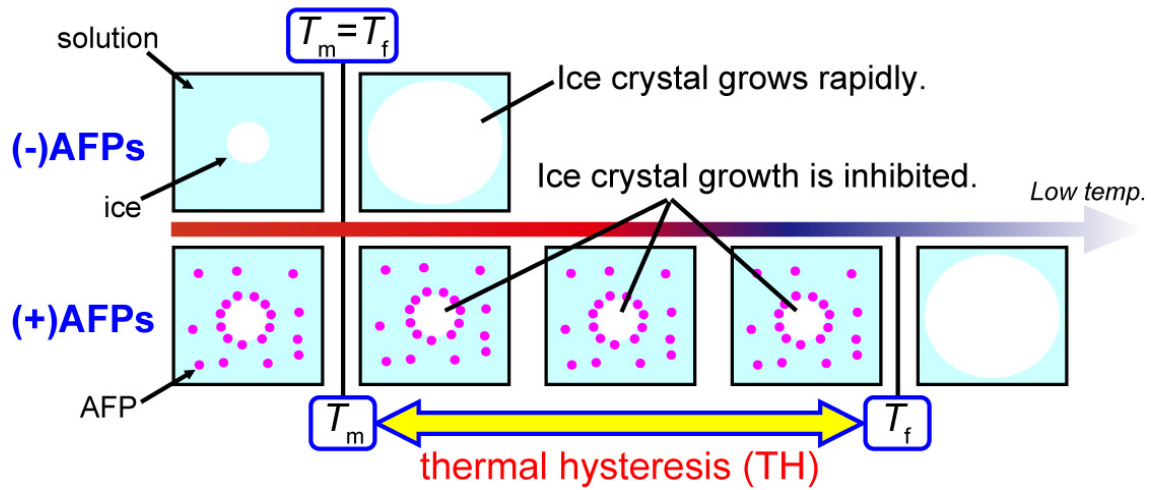


Fig. 1-1. Schematic representation of antifreeze activity. In the absence of AFPs (upper column), the T_m of solution corresponds to the T_f . Therefore, ice crystals grow rapidly at the temperature just below the T_f (T_m). In the presence of AFPs (shown as pink particles in lower column), they accumulate on the surface of the ice. AFP binding to ice prevent the growth of ice. As a result, the depressed T_f can be detected. The difference between the T_f and the depressed T_f is defined as thermal hysteresis (TH).

A



B

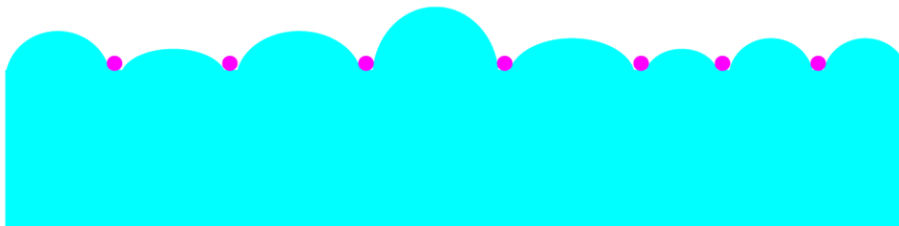


Fig. 1-2. Schematic diagram illustrating the "adsorption inhibition mechanism".

(A) AFP molecules (pink) adsorb onto the surface of ice (light blue) at the temperature near to the T_m ($\approx 0^\circ\text{C}$). At this stage, the interface between ice and water is assumed to be flat. (B) The local growth of ice at the temperature well below the T_m causes curvatures between adsorbed AFP molecules, leading the depression of the T_f due to the Kelvin effect. This figure is created by reference to the two-dimensional schematic of ice growth inhibition displayed in a previous paper ([Knight and DeVries 1994](#)).

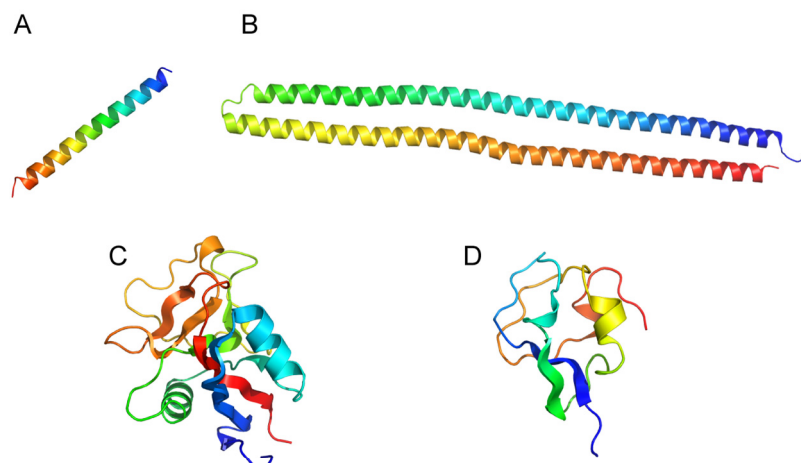


Fig. 1-3. 3-D structures of fish AFPs. (A) the X-ray structure (PDB code: **1WFB**) of type I AFP from the winter flounder. (B) the X-ray structure (**4KE2**) of hyperactive, type I AFP (hyp-type I AFP) from the winter flounder. (C) the X-ray structure (**2PY2**) of Ca^{2+} -dependent type II AFP from herring. (D) the X-ray structure (**1HG7**) of type III AFP from the ocean pout. These cartoon representations were created by the PyMOL.

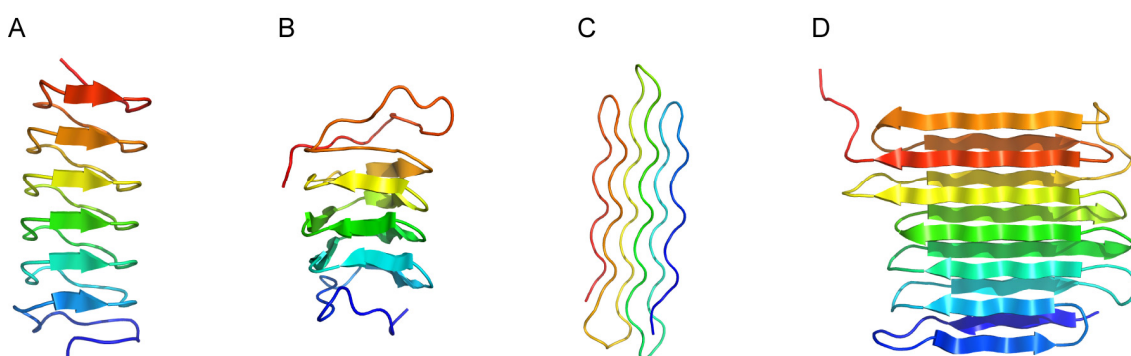


Fig. 1-4. 3D structures of arthropod AFPs. (A) the X-ray structure of TmAFP (PDB code: **1EZG**). (B) the NMR solution structure of CfAFP (**1N4I**). (C) the X-ray structure of sfAFP (**2PNE**). (D) the X-ray structure of RiAFP (**4DT5**). These cartoon representations were created by the PyMOL.

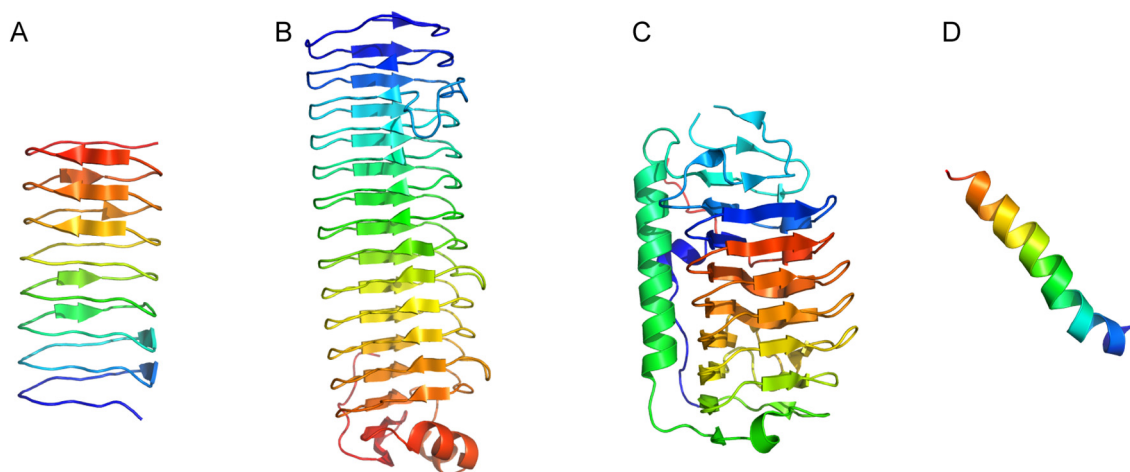


Fig. 1-5. 3D structures of other AFPs. (A) the X-ray structure of LpIBP (PDB code: **3ULT**). (B) the X-ray structure of MpAFP (**3P4G**). (C) the X-ray structure of LelBP (**3UYV**). (D) the NMR solution structure of a fragment of Afp1 (**2LQ0**). These cartoon representations were created by the PyMOL.

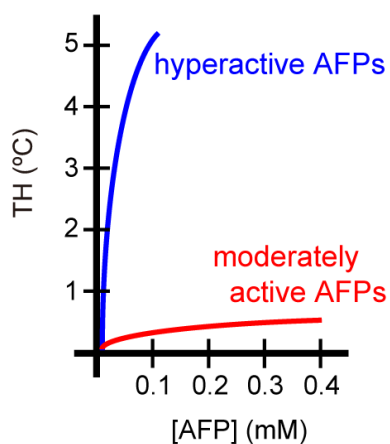


Fig. 1-6. Schematic representation of a TH graph showing the difference in TH between two classes of AFPs. The blue line shows TH of hyperactive AFPs, and the red line for moderately active AFPs.

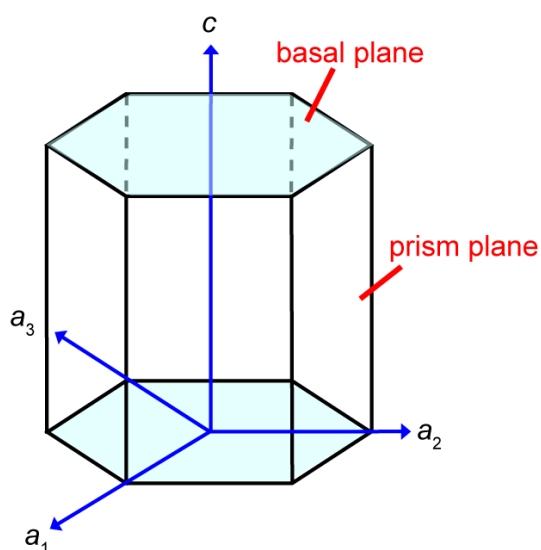


Fig. 1-7. A diagram illustrating the cell structure of an ice lattice (I_h). The c - and a axes, the basal plane with 2-folds symmetry and the six-equivalent prism planes are shown.

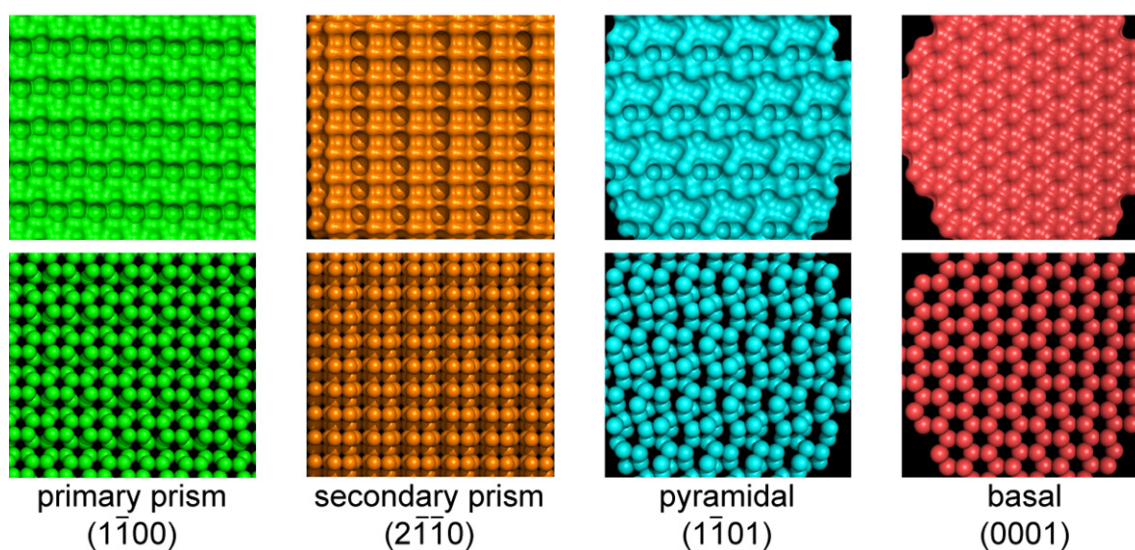


Fig. 1-8. Representative ice planes involving in AFP interactions. The surface (upper column) and the space-filling (lower column) structures are shown. The ice planes were created by VESTA ([Momma and Izumi, 2008](#)).

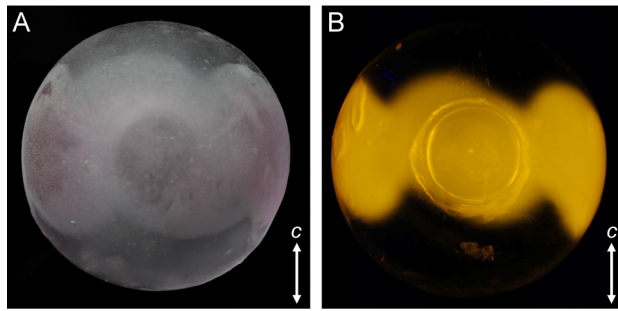


Fig. 1-9. Comparison of the traditional "ice-etching" technique and the "FIPA" analysis. The ice-etching (A) and the FIPA (B) patterns produced by a type III AFP (nfeAFP11 V9Q/V19L/G20V/I41V mutant). The *c*-axis direction is shown an arrow diagram within the figures. The experiments were performed by Christopher P. Garnham from Queen's University (Canada) and conducted in our laboratory. A similar, but not identical image is available in the published paper by [Garnham et al, 2012](#).

Tis	AGPSAVPLGTAGNYVILAS	***	TGVSTVPQSVITGAVGVSP-GTAASL----	TGFSLILSGTG	55
Led	AGPAAVNLGTAGNYAILAKS	***	GISTVPEIISGNIGVSPISTTAF----	TGFSETLDSTG	55
Leu	QRDLSELGVASNFAL---	***	AKAGISSVPDSAILGDI-GVSPAAATYIT	TGFGLTQDSS	55
Nag	AEQSAVDLGTAGDFA----	***	VLSKAGVSTTGPTTEVTGDIGTSPIASTALT	TGFALIKDSS	55
Fpo	-GPAPVLLGKAENFAILSET	***	GVSNVLDSSVNCIDIGVSPIGASGV----	TGFSLTGDSGG	54
Col	AGPYAVELGEAGTFTIL---	***	SKSGITDVYPSTVTGNVG-----	TSP---ITGAA	43
Ste	AEQSAVDLGTAGDFA----	***	VLSKAGVSTTGPTTEVTGDIGTSPIASTALT	TGFALIKDSS	55
	TFSTSSQVTGQLTGADYGT	***	PTTSILTT-----AIGDMGTAYINAATRSGPDFLEIYTGA	109	
	KFATSQQVVGELFAASFAAPT	***	PTTLTT-----AVSDMQTAF-NDATGRV-TPDFTNLGG	107	
	TYATSPQVTGLIYAADYST	***	PTPNYLA-----AVANAETAYNQAAAGFVDPDFLELGAGE	109	
	TFSTSSLVTGKIYAADYTAPT	***	PSKMTT-----AISDMSTAFTDAAGRSDPDFLELGAGS	109	
	SFSTSKQVTGRVYASTYGDPT	***	PASLTT-----AVFDMENAYKDAQERIDPDFTNLHTGA	108	
	LLLNCEVTDGAMYTVDGAPL	***	PCSINSPYLLELAVSDMGIAYNDAAGRVPADHTELGTGE	103	
	TFSTSSLVTGKIYAADYTAPT	***	PSKMTT-----AISDMSTAFTDAAGRSDPDFLELGAGS	109	
	L--GGTLLPGLYKWT-SVGASADFTISGTSTDWIFQ	***	IIDGLDVATGKQITLVGGAQ-	165	
	GELGGLVLTPGLYKWTGAVSVNSTGVTTAGTPLDHFIFQ	***	IPATLGFAAASRVTLVGGIP-	166	
	L--RDQTLVPLGLYKWT-SVSVPTDLTFEGNGDATWVFQ	***	IAGGLSLADGVAFTLAGG---	163	
	I--EGETLVAGLYK-WGTDVSFTSSLVFDGSATDVWILQ	***	VAKDFIVGNGAQMYLTGT---	163	
	L--GGAILVPLGLYKFTT-GVSITADLVLTGGPTDTYIFQ	***	IAGTSLAAGVKINLVGGLLP	165	
	I--GGLTLEPG-VYKWSSDVNISTDVTFNGTMDDVWIMQ	***	ISGNLNQANAKRVTLTGGA-	159	
	I--EGETLVAGLYK-WGTDVSFTSSLVFDAAATDVCILQ	***	VAKDFIVGNGAQMYLTAT---	163	
	AKNII-----WVVGAVNIEVGAKFEGTILAKTAVTFK	***	TGSSLNGRILAQ-----TSVAL	215	
	ASNIV-----WAATSVVTAGAGSHIEGVVLAKTAVTLET	***	GATMNGRILAQ-----TFVAL	216	
	ANSTNIAFQVGDDVTVGKGAHFEVLLAKRFVTLQTGSS	***	LNGLRVLSQTEVALQKATVNSP	223	
	AKAENIFIQVSGAVNIGTTAHVEGNILSATAIALQTGSS	***	LNGLKALSQTAI-----TLDS-	217	
	ANVVW-----AVADSVTVAAT-SSFQGILLGKTQVVVN	***	TNASVEGRILAQ-----TAVVL	214	
	AKNIF-----WQVAGYTAGTYASFEGIVLSKTLISVNT	***	TGTTVNGRLLAQ-----TAVTL	209	
	AKAENIFIQVSAAVNIATTAHVEANILTATAIAMQTGSS	***	LNGLKALSQTAI-----TLDS-	217	
	Q-----ATIVEK-----	223	identity		
	Q-----AT-VIG-----	223	50%		
	FVPAPVQKRSNARQWL---	241	48%		
	-----VTI-----	220	47%		
	QK-ATVIVPGVCGA-----	227	46%		
	QK-----NTINAPTEQYEEAPL	226	42%		
	-----VTIVS-----	222	41%		

Fig. 1-10. Alignment of amino acid sequences of the type I IBPs. Gaps have been inserted to improve alignment. Conserved residues are shaded by yellow. The putative ice-binding residues defined in TisAFP6 are shown as asterisks within red frames. Identities of these proteins to TisAFP6 are shown in the end of the alignment. Abbreviations in this figure are as follows: Tis, an AFP (TisAFP6) from *Typhula ishikariensis*; Led, an IBP from a shiitake mushroom *Lentinula edodes*; Leu, an IBP (LeIBP) from *Leucosporidium* sp. AY30; Nag, an AFP (NagAFP8) from *Navicula glaciei*; Fpo, an IBP from a enoki mushroom *Flammulina populicola*; Col, an AFP (ColAFP) from *Colwellia* sp. SLW05; Ste, an AFP from a sea ice copepod *Stephos longipes*.

Table 1-1. Ice planes preferentially bound by individual AFPs. AFP-binding planes of ice determined are indicated by the symbol of (+). All planes are marked from TmAFP to NagAFP8, which shows the uniform binding of those AFPs to ice hemispheres observed in FIPA analyses.

AFP type	1st prism	2nd prism	pyramidal	other	basal	references
fish type I (wf)			+			Knight et al., 1991
fish type I (ss)		+				Knight et al., 1991
fish type II (Ca ²⁺ -in)	+					Liu et al., 2007
fish type II (Ca ²⁺ -de)		+				Nishimiya et al., 2008
fish type III	+		+			Garnham et al., 2010
fish type IV				+		Deng et al., 1998
fish AFGP	+					Knight et al., 1994
LpIBP	+				+	Middleton et al., 2012
LeIBP	+				+	Park et al., 2012
TisAFP6				+	+	(viii)
TmAFP	+	+	+	+	+	Middleton et al., 2012
CfAFP	+	+	+	+	+	Pertaya et al., 2008
sfAFP	+	+	+	+	+	Mok et al., 2010
RiAFP	+	+	+	+	+	Hakim et al., 2013
MpAFP	+	+	+	+	+	Garnham et al., 2011
ColAFP	+	+	+	+	+	(i)
NagAFP8	+	+	+	+	+	this thesis

Abbreviations used in this table: wf, winter flounder; ss, shorthorn sculpin; Ca²⁺-in, Ca²⁺-independent; Ca²⁺-de, Ca²⁺-dependent.

Table 1-2. The basic property of type I IBPs.

specific name	organism	TH (°C) [AFP conc]	Mw (kDa)	locality	reference
TisAFP	<i>Typhula ishikariensis</i> BRB-1	1 [1 mM]	23	snow-covered soil in Arctic region	Hoshino et al. 2003a
-	<i>Coprinus psychromorbidus</i>	1 [1 mM]	22	snow-covered soil in Arctic region	Hoshino et al. 2003a
NagIBP	<i>Navicula glaciei</i>	3.2 [1.6 mM] ⁽ⁱⁱ⁾	25	sea ice in Antarctica	Janech et al., 2006
FcAFP	<i>Fragilariopsis cylindrus</i>	0.9 [350 µM] ¹	28	sea ice in Antarctica	Janech et al., 2006
ColAFP	<i>Colwellia</i> sp. SLW05	3.8 [0.14 mM] ⁽ⁱ⁾	26	sea ice in Antarctica	Raymond et al., 2007
IBPv	unidentified bacteria	n.d.	54	ice core in Vostock	Raymond et al., 2008
-	<i>Lentinula edodes</i> (shiitake)	n.d.	28	supermarket	Raymond et al., 2009
-	<i>Flammulina populicola</i> (enoki)	n.d.	25	supermarket	Raymond et al., 2009
TisAFP8	<i>T. ishikariensis</i>	2°C [180 µM]	23	(recombinant)	Xiao et al., 2010
Cn-AFP	<i>Chaetoceros neogracile</i>	0.8 [40 µM]	29	Antarctic ocean	Gwak et al., 2010
St-AFP	<i>Stephos longipes</i>	3.8 [n.d.]	22	sea ice in Antarctica	Kiko, 2010
LeIBP	<i>Leucosporidium</i> sp. AY30	0.35 [370 µM] ²	25	pond in Arctic	Lee et al., 2010
Afp-1	<i>Glaciozyma antarctica</i>	0.08 [10 mM] ³	18	sea ice in Antarctica	Shah et al., 2012
FfIBP	<i>Flavobacterium frigoris</i> PS1	2.5 [50 µM]	28	sea ice in Antarctica	Do et al., 2012
PgAFP	<i>Pyramimonas gelidicola</i>	0.6 [6 mM]	27	Antarctic ocean	Jung et al., 2014
NagAFP8	<i>N. glaciei</i>	1 [0.3 mM]	22	(recombinant)	this thesis
TisAFP6	<i>T. ishikariensis</i>	0.5 [0.3 mM]	23	(recombinant)	this thesis

Abbreviations here; n.d., no data; ¹Bayer-Giraldi et al., 2011; ²Lee et al., 2012; ³Hashim et al., 2013

Chapter 2: Characterization of ice-binding property of *Tis*AFP

Preface

In this chapter the author describes the contents of an unpublished paper entitled " An unusual ice-binding property of a fungal antifreeze protein" by Yuichi Hanada, Jing Cheng, Yoshiyuki Nishimiya, Ai Miura, Sakae Tsuda, and Hidemasa Kondo. The authors' contributions are as follows: the manuscript was written by Y.H. with a significant input from H.K; Y.H., S.T. and H.K. designed research; Y.H., J.C., Y.N., and A.M. performed experiments.

Abstract

A fungal antifreeze protein (*TisAFP*) from *Typhula ishikariensis*, which was recently shown to fold into a novel irregular β -helical structure, represents a newly characterized group of ice-binding proteins from diverse microorganisms. *TisAFP* contains seven isoforms which share 82-99% sequence identity at amino acid level. Here, we have examined the effects of protein concentration, solution pH and annealing time on the activity of one of the isoforms, *TisAFP6*. *TisAFP6* inhibited the growth of seed ice crystals to 0.5°C (thermal hysteresis, TH) below the melting point of solution. Ice crystals obtained in *TisAFP6* solutions grew rapidly along the *c*-axis at the freezing point. The behaviors drastically changed when ice crystals were annealed at a constant temperature within the TH range. The TH level was enhanced up to 3-fold, and ice crystals grew explosively into the *a*-axes directions. Fluorescence-based ice plane affinity analysis on *TisAFP6* at various pH conditions revealed that it tended to exhibit a higher affinity toward the basal plane at pH above 6, while at pH below 5 no or less affinity for that was detected. The present study represents the new findings that certain experimental conditions, which seem to reflect those in nature, significantly affect on the activity of AFPs.

2.1. Introduction

A snow mold fungus, *Typhula ishikariensis*, secretes AFP (TisAFP) into the extracellular space in snow-covered soil (Hoshino et al., 2003a). The purified TisAFP was shown to be composed of seven isoforms (Fig. 2-1). One of them, denoted TisAFP8, exhibited a potent TH around 2°C (Xiao et al., 2010). Our previous study also revealed that another isoform, TisAFP6, is folded into a β -helical structure resembling “semi-pear” shape (viii). The β -helix is a well-characterized structural motif in the hyperactive AFPs including TmAFP (Liou et al., 2000), CfAFP (Graether et al., 2000), MpAFP (Garnham et al., 2011), and RiAFP (Hakim et al., 2013), except for sfAFP (Pentelute et al., 2008) and a fish hyperactive AFP (Sun et al., 2014). The hyperactive AFPs have in common an extremely flat ice-binding site (IBS) consisting of regular arrays of conserved amino acid residues (Thr and/or Asn). It was found, however, that the IBS of TisAFP6 is lacking of regularity and repetitive motifs, similar to a β -helical, moderately active lpIBP (Middleton et al., 2012). It was also reported that LeIBP, a homologue of TisAFP, has the same protein scaffold (Lee et al., 2012), confirming that the irregular β -helical structure is common to the numerous homologues. Yet, the actual TH activity of TisAFP6 remains to be determined because of the difficulty in producing it in an *Escherichia coli* expression system without GST as a fusion partner (Kondo et al., 2012). In order to understand the ice-binding mechanism underlying irregular β -helical AFPs, it is important to correlate the structure with their functional properties.

In the present chapter, the author produced the recombinant TisAFP6 without any affinity tags in the methylotrophic yeast *Pichia pastoris*. TisAFP6 showed a TH activity that comparable to the moderately active AFPs. The ice crystals formed in the presence of TisAFP6 grew rapidly along the *c*-axis. Annealing of an ice crystal in TisAFP6 solution effectively enhanced the TH activity over 2-fold. Surprisingly, the annealed crystal for 2 h expanded explosively into the directions normal to the *c*-axis. FIPA analysis demonstrated that TisAFP6 binds to the multiple planes of ice including the basal plane at pH 6, whereas it shows less affinity toward the basal plane at pH 3. Taken together, these findings indicate that TisAFP6 exhibits a different specificity for ice planes depending on the experimental conditions. To our knowledge, this is a quite unusual property never seen in any known AFPs.

2.2. Materials and methods

2.2.1. Expression

TisAFP consists of a set of seven isoforms. In the present study, one of them, designated *TisAFP6* (DDBJ accession no. AB109744), was used. The cDNA encoding *TisAFP6* was amplified by PCR with a primer set (forward, AAA ACT CGA GAA AAG AGC TGG TCC CTC TGC TGT C; reverse, CGG CCG CCT ACT TTT CCA CAA TG), then ligated to the pGEM T easy vector (Promega). After digesting with *XhoI* and *NotI*, the DNA fragments were cloned into the corresponding sites of the pPICZ α vector (Invitrogen). The resulting plasmids were transformed into *E. coli* strain JM109 (Toyobo) and selected on Luria-Bertani agar plates containing ZeocinTM (25 μ g/ml). The plasmid with a correct sequence of *TisAFP6* was prepared, then linearized with *PmeI* and transformed into a yeast *P. pastoris* strain X-33 using the Pichia EasyComp Kit (Invitrogen). Positive transformants were grown in a 100 mL culture of BMGY (buffered glycerol complex medium, 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4 x 10⁻⁵% biotin and 1% glycerol) at 30°C for 24 h. Cells were harvested by centrifugation and resuspended in a 1 L of BMMY (buffered methanol-complex medium, BMGY supplemented with 0.5% methanol instead of 1% glycerol), and again incubated at 15°C for 96 h. During growth, cell density was monitored using a spectrophotometer at 600 nm every 24 h. After incubation, cells were removed by centrifugation, leaving recombinant *TisAFP6* to be purified from the medium.

2.2.2. Purification

The spent culture media containing recombinant *TisAFP6* was dialyzed against 25 mM glycine-HCl buffer (pH 3.0) and applied to a MacroPrep High S column (Bio-Rad). Active fractions were eluted with the same buffer containing 0.1 M NaCl and dialyzed against 25 mM ammonium bicarbonate. The dialysates were further purified through a MacroPrep High Q column (Bio-Rad) and unbound materials including mainly *TisAFP6* were collected. The flow-through fraction from the High Q column was again dialyzed against 25 mM glycine-HCl buffer (pH 3.0) and loaded onto a Mono S column (GE Healthcare). The column bound-AFP was eluted with a linear NaCl gradient (0–50 mM) using the same buffer containing 1 M NaCl. The purity of *TisAFP6* was checked by SDS-PAGE, followed by silver staining.

2.2.3. Measurements of antifreeze activity

TH activity of the purified *Tis*AFP6 was evaluated using our in-house photomicroscope system equipped with a temperature-controlled stage and a CCD camera (Takamichi et al., 2007). Briefly, a 0.8 μ L of sample solution was applied into a capillary tube and then set on the cooling stage of the photomicroscope. The sample solution was frozen completely at 20°C and then warmed immediately until an ice crystal was apparent. The T_m of the solution was measured as the lowest temperature in which melting of ice crystals get started. Then the sample solution was cooled at a constant rate (0.1°C/min) until the ice crystal initiates to grow (the T_f). Determinations of T_m and T_f were repeated at least three times, and the averaged values were used for the TH activity ($TH=|T_f-T_m|$) of AFP solutions. Annealing time-dependence of TH activity was examined in a way similar to the procedure described above, but after incubating an ice crystal at a constant temperature ($T_m - 0.2^\circ\text{C}$) for a couple of hours the sample solution was started to cool. The directionality of the ice crystals prepared in AFP solutions was determined based on the crystallographic symmetry (bursting with two-fold symmetry indicates a crystal growth along the *c*-axis, and with six-fold symmetry indicates a crystal growth in directions normal to the *c*-axis).

2.2.4. Preparation of solutions for TH measurements

The purified *Tis*AFP solution was concentrated using a centrifugal filter device, Amicon Ultra-4 (Millipore) at 25 mM MES-NaOH buffer solution (pH 6.0) and diluted to each concentration with the same buffer, then measured TH activity as a function of AFP concentration.

The pH of solutions was adjusted by diluting 1 mM of *Tis*AFP6 dissolved in 1 mM ammonium bicarbonate with 25 mM GTA pH 2.0, 25 mM Glycine-HCl pH 3.0, 25 mM sodium acetate pH 4.0, 25 mM MES-NaOH pH 6.0, 25 mM Tris-HCl pH 8.0, 25 mM Glycine-NaOH pH 9.0, 25 mM Glycine-NaOH pH 10.0 to obtain 0.1 mM solution of *Tis*AFP6. The pH of the resulting solutions were measured and plotted as actual values for each point.

2.2.5. FIPA analysis

Single ice crystals were prepared following the method described by Garnham et al. (2010). Briefly, degassed ultra pure water (MilliQ) was poured into a stainless steel pan bearing PVC pipes with a tiny notch on the bottom that floats in a temperature-controlled bath and supercooled at -0.5°C . The supercooled water was then seeded with an ice particle. The ice needle that is supposed to be a single crystal entered

into the PVC pipes through the tiny notch. Consequently, single ice crystals were grown in the PVC pipes and checked their quality through a polarizing filter.

A 1 mL of purified *TisAFP6* solution (2.2 mg/ml; 100 μ M) was dialyzed against 100 mM NaHCO₃ pH 8.5. The resulting dialysate was recovered in a microtube containing 50 μ L of 10 mg/ml 6-(tetramethylrhodamine-5-(and-6)-carboxamido) hexanoic acid, succinimidyl ester (product code T6105, Invitrogen), then allowed to rotate at room temperature for 3 h. To remove the unreacted dye, the AFP solution was dialyzed against 1 mM ammonium bicarbonate. In this study, FIPA analysis was carried out in different pH solutions (pH 3.0 – 8.0). For each condition, pH was adjusted by diluting fluorescently-labeled *TisAFP6* solution with 35 mL of 10 mM glycine-HCl pH 3.0, 10 mM sodium acetate pH 4.0, 10 mM sodium acetate pH 5.0, 10 mM MES-NaOH pH 6.0, 10 mM HEPES-NaOH pH 7.0, 10 mM Tris-HCl pH 8.0 to give a final AFP concentration of *ca.* 1 or 0.25 μ M. The pH of the solutions was checked and then used for the analysis.

The single ice crystals were mounted on the brass cold finger with the direction parallel to the *c*-axis and grown in degassed ultra pure water at -5°C to produce a hemispherical shape. Subsequently, the degassed water was replaced with a buffer solution containing fluorescently-labeled *TisAFP6* and allowed to grow at -10°C for 3-4 h. After growing, the ice hemisphere was removed and photographed under UV light box at a cold room (-1°C).

2.3. Results

2.3.1. Expression and purification

To characterize the activity of *TisAFP6*, the recombinant protein was produced by using a yeast *P. pastoris* wild type strain X-33. The plasmid vector pPICZ α bearing α -factor signal sequence was used to secrete *TisAFP6* into the culture medium of *P. pastoris*. The activity of *TisAFP6*, however, was undetectable in the culture medium after cultivating for 96 h at 30°C . Additionally, the culture medium resulted in numerous bands on the SDS-PAGE gel (Fig. 2-2, lane 1). Since it is known that proteases derived from *P. pastoris* are released into the culture medium, *TisAFP6* might be degraded after 96 h when expressed at 30°C . The temperature effect was subsequently tested to optimize the expression level of *TisAFP6* at 15°C and 30°C . It was found that lower

temperatures increased the yield of *TisAFP6* markedly and allowed us to identify the band corresponding to it on the SDS-PAGE gel (Fig. 2-2, lane 2). We then determined the optimal condition for the expression of *TisAFP6* in *P. pastoris* to be 96 h at 15°C.

The recombinant *TisAFP6* was purified by three-step procedure starting with the supernatant of the culture medium, essentially following the method described by Hoshino et al. (2003a) with a slight modification. *TisAFP6* was firstly subjected to cation-exchange chromatography to concentrate it (Fig. 2-2, lane 3). Subsequently, fractions showing the activity was passed through anion-exchange chromatography to remove impurities including brownish substances derived from the medium (Fig. 2-2, lane 4). The active fractions were further purified by the second round of cation-exchange chromatography to eliminate any remaining impurities (Fig. 2-2, lane 5 and 6). The yield of purified *TisAFP6* from a 1 L culture was estimated to be 7.4 mg.

2.3.2. TH activity and ice growth pattern of *TisAFP6*

The purified *TisAFP6* solution was tested for TH activity as a function of its concentration in a buffer adjusted to pH 6.0 (Fig. 2-3A). The TH activity of *TisAFP6* was gradually increased with a hyperbolic relationship as the protein concentration increased. *TisAFP6* produced TH of 0.5°C at a concentration of 6.6 mg/ml (0.3 mM). Even at a very low concentration (0.01 mg/ml=0.45 μ M), *TisAFP6* exhibited TH of around 0.1°C.

The ice crystals formed in the presence of *TisAFP6* retained their original shape just after melting (Fig. 2-3B) within the TH gap, rather than being modified into a specific shape like the bipyramid observed in fish AFP solutions (Chao et al., 1995). It was observed that the ice crystals in the *TisAFP6* solution grew rapidly along the *c*-axis when cooled below the T_f .

2.3.3. Effect of pH on the activity of *TisAFP6*

TH activity of *TisAFP6* was examined at various pH ranging from 2 to 10 (Fig. 2-4A). Although two peaks of activity were observed at pH 4 and 9, the difference in TH activities at each pH appears to be a little (0.38°C at pH 4 vs. 0.28°C at pH 2). It is known that the TH activity of *TisAFP8* fluctuates greatly with pH changes, and is maximized at pH 5 (2.0°C) and minimized at pH 2 (0.6°C) (Xiao et al., 2010).

Unexpected results were obtained through observations of the ice growth pattern in *TisAFP6* solutions at different pH values (Fig. 2-4B). The ice crystals grown in *TisAFP6* solutions with pH below 4 were modified into the bipyramidal shape, as seen in solutions containing fish AFPs (Chao et al., 1995). In *TisAFP6* solutions with pH above

6, however, the ice crystals maintained their shapes immediately after preparing as well described above. In both pH conditions, the ice crystals elongated rapidly along the *c*-axis at the temperatures below the T_f .

2.3.4. Influence of annealing time on the activity of TisAFP6

TH activity of *TisAFP6* was measured as a function of annealing time (0-2 h) after preparing a seed ice crystal at two pH conditions (Fig. 2-5A). It is known that annealing of an ice crystal significantly enhances TH activity of AFPs (Takamichi et al., 2007). Annealing was performed at a constant temperature ($T_m-0.2^\circ\text{C}$) and a fixed protein concentration (0.1 mM). At pH of 6, *TisAFP6* had a TH of around 0.3°C in a condition with 0 h of annealing. Even after 0.5 and 1 h of annealing, TH values were virtually identical to that obtained without annealing. In both conditions, the annealed ice crystals for 0.5 and 1 h grew into the *c*-axis direction. These are similar to that observed in a *TisAFP6* solution being not subjected to annealing. Interestingly, *TisAFP6* showed an increased TH of around 0.4°C and 0.7°C after 1.5 h and 2 h of annealing, respectively. During annealing, the ice crystals formed in a *TisAFP6* solution were gradually modified into the bipyramidal shape that evokes the crystals obtained in acidic conditions (Fig. 2-5B and C). More surprisingly, the ice crystals obtained after 2 h of annealing expanded explosively into the directions perpendicular to the *c*-axis (Fig. 2-5C), while those obtained after 1.5 h grew in the same way obtained with 0–1 h of annealing.

At pH of 3, *TisAFP6* exhibited a TH of around 0.3°C in a condition without annealing. After 0.5 h of annealing, TH value was enhanced approximately 1.5-fold and did not change over the annealing periods (0.5-2 h) tested here. Ice crystals produced in *TisAFP6* solutions at pH 3 were immediately modified into the bipyramidal shape even in conditions without annealing, then sustained the shapes stably within the TH range (Fig. 2-5B). Unlike the condition of pH 6, the annealed ice crystals elongated along the *c*-axis at the temperatures below the T_f even after 2 h. Thus, *TisAFP6* seemingly has no ability to bind to the basal plane at acidic conditions.

2.3.5. Effect of pH on the ice-binding affinity of TisAFP6

In order to determine the ice planes to which *TisAFP6* binds at different pH conditions, we performed a series of FIPA analysis (Garnham et al., 2010; Basu et al., 2014) (Fig. 2-6). Single crystal ice hemispheres were immersed in buffer solutions with pH ranging from 3.0 to 8.0 containing 1 μM of *TisAFP6* labeled with tetramethylrhodamine. The ice hemispheres were then allowed to grow for 3-4 h to

obtain a diameter of 4 cm. This process allows *TisAFP6* to be incorporated into the growing ice hemisphere and enable us to visualize directly the ice planes bound by the AFP under UV illumination. In solutions with pH above 6, *TisAFP6* bound to the ice hemispheres uniformly including the basal plane but with slight separate patches (for pH 6), consistent with our previous observation (viii). In contrast to this pattern, the ice hemispheres grown in solutions with pH below 5 resulted in no or less fluorescent patch on the basal plane.

The effect of AFP concentrations on FIPA pattern was then tested at solutions with pH 3 and 6 as the representatives of acidic conditions and neutral conditions, respectively. Ice hemispheres grown in a diluted *TisAFP6* solution (0.25 μ M) with pH 3 further indicated that it had no ability to bind to the basal plane at acidic conditions. In the solution with pH 6, however, *TisAFP6* turned the ice hemispheres broadly fluorescent including the area corresponding to the basal plane with jagged pattern. These results clearly demonstrated that *TisAFP6* binds to the multiple planes of ice including the basal plane at neutral conditions, while not at acidic conditions.

2.4. Discussion

There are a few studies available on the structure–function relationship of a group of newly discovered AFPs, which were isolated from numerous cold-adapted organisms such as fungi (Hoshino et al., 2003a; Raymond et al., 2009a; Xiao et al., 2010), yeast (Lee et al., 2010), diatoms (Janech et al., 2006; Gwak et al., 2010), algae (Raymond and Kim, 2012), copepods (Kiko, 2010), and bacteria (Raymond et al., 2007, 2008; Do et al., 2012). Just last year, the X-ray crystal structures of this type of AFPs were reported simultaneously by us for *TisAFP6* (viii) and another group for LeIBP (Lee et al., 2012), revealing their common structural feature characterized by an irregular β -helical structure along with a long α -helix. Of all the homologous AFPs functionally characterized to date, both moderately active (Lee et al., 2010, Gwak et al., 2010, Kiko et al., 2010, Bayer-Giraldi et al., 2011) and hyperactive AFPs (Xiao et al., 2010, Do et al., 2012) have been identified (Table 1-2). However, the activity of *TisAFP6* has not been well understood since our earlier studies (Hoshino et al., 2003a; viii) had not described it in detail. Besides, we pointed out previously that few residues that make up the putative IBS are conserved among the homologues (viii). A detailed structural and

functional comparison of these homologues might reveal the main attributes that allow an AFP to be either hyperactive or moderately active.

2.4.1 TH activity and ice crystal burst pattern

Analysis of TH activity as a function of the protein concentration showed that *Tis*AFP6 produced TH of 0.5°C at a concentration of 6.6 mg/ml (0.3 mM) (Fig. 2-3A). This value is comparable to those obtained with the moderately active AFPs, including most of fish AFPs (Chao et al., 1997; Nishimiya et al., 2008a; DeLuca et al., 1998b), a plant *Lp*AFP (Middleton et al., 2012), and *Le*IBP (Park et al., 2012). The ice crystals formed in the presence of *Tis*AFP6 grew rapidly along the *c*-axis at temperatures below the T_f . The same phenomena, namely bursting into the *c*-axis direction, have been extensively documented in fish AFPs and known as a characteristics of the moderately active AFPs. These results are inconsistent with our earlier observation on the FIPA analysis of *Tis*AFP6, which showed a circular patch on the basal plane surrounded by six equivalently spaced patches on the prism plane (viii). The binding affinity toward the basal plane of ice is generally accepted to be the hallmark of hyperactive AFPs (Scotter et al., 2006; Pertaya et al., 2008). For an exception to the rule, a moderately active, *Lp*AFP binds to not only the primary prism plane, but also the basal plane (Middleton et al., 2012). However, ice crystals grown in a *Lp*AFP solution expands along the *a*-axes at temperatures below the T_i , consistent with its affinity for the basal plane.

Previously, we reported that a high TH of around 2°C was obtained with another isoform, *Tis*AFP8 (Xiao et al., 2010). Also, *Tis*AFP8 showed a dendritic ice growth pattern at the end of TH range, as observed in the presence of hyperactive AFPs (Scotter et al., 2006). Type I AFPs are so far known to be the only one in which one particular molecule exhibits hyperactivity, whereas the others have a moderate activity (Marshall et al., 2004b). In this case, however, there is a remarkable difference in their molecular size (32 kDa for the hyperactive one and 3–4 kDa for the others) (Graham et al., 2008). It has been generally observed that AFPs with a wider IBS tend to have a greater TH (AFGP, Wu et al., 2001; CfAFP, Leinala et al., 2002b; *Tm*AFP, Marshall et al., 2004c; type III AFP, Nishimiya et al., 2003; sfAFP, Mok et al., 2010). They make up their wider IBSs by simply increasing the repeat sequences or dimerizing. Given that the two isoforms share 81.6% sequence identity at amino acid level and have the identical length of the polypeptide chains (Fig. 2-1), the difference in their activities seems to be outstanding. Structural determination of *Tis*AFP8 would be beneficial for understanding of the molecular mechanism underlying the difference.

2.4.1. Biological implications of the pH dependence

The effect of pH on the activity of AFPs have so far received relatively little attention. A few studies have examined the pH-dependence of the activity of AFPs (type III AFP, [Chao et al., 1994](#); type IV AFP, [Deng and Laursen, 1998](#); *Ri*AFP, [Kristiansen et al., 2005](#); *Cf*AFP, [Gauthier et al., 1998](#)). These AFPs apparently act as an inhibitor of the ice growth in the body. The pH of body fluids must be maintained at a constant value, therefore it is considered that the pH-dependence indicates the protein's biochemical property for these types of AFPs. *Tis*AFP and other microbial AFPs, however, are secreted and act in the extracellular space. Thus, the pH-dependence profile of microbial AFPs seems to be indicative of their physiological adaptations to environments.

*Tis*AFP6 was fully active at various pH ranging from 2 to 10. In contrast, the activity of *Tis*AFP8 is remarkably sensitive to the solution pH where TH was maximized at pH 5 and minimized at pH 3, respectively ([Xiao et al., 2010](#)). These observations suggest that the pH range in which *Tis*AFP8 is stable and active might be narrow compared to that of *Tis*AFP6. Another study showed that *T. ishikariensis* grows actively between pH 4.0 and 9.0 with a plateau-shaped growth curve and a slight peak at pH 6.0 ([Wu and Hsiang, 1999](#)). Although *Tis*AFP8 is obviously a strong inhibitor of ice growth, it should be inactivated when *T. ishikariensis* is exposed to pH levels beyond its optimum growth range. In this situation, *Tis*AFP6 might play a role in the survival of the fungus. Further studies will be required to reveal the relationship between the optimum pH for the activity of secretory microbial AFPs and the pH condition of their environments, but the pH-dependence of microbial AFPs might be the rule rather than the exception.

2.4.3. Effect of annealing time on the activity

Previously, it was reported that annealing of an ice crystal for a couple of hours enhanced the TH activity of fish type III AFP ([Takamichi et al., 2007](#)). It is thought that an elongated period of annealing provides a long time lapse of cooling on the ice crystal. A faster cooling rate results in a decrease in the TH activity of fish AFPs ([Fletcher et al., 2001](#)). In general, cooling rates used *in vitro* assays might be faster than those occurring in nature. Annealing of an ice crystal in AFP solutions, therefore, could offer an insight into how the AFPs behave in nature. Hence we tested whether annealing periods (up to 2 h) could affect the activity of *Tis*AFP6.

Annealing experiments in *Tis*AFP6 solutions were performed at both pH 3 and 6 within the TH gap ($T_m-0.2^{\circ}\text{C}$) ([Fig. 2-5](#)). The experiments showed that annealing of an

ice crystal for 2 h significantly enhanced the TH activity of *Tis*AFP6 and the annealed ice crystal expanded rapidly along the *a*-axis direction at pH of 6. By contrast, a slight increase in TH value was detected at pH 6 after 1.5 h and at pH 3 after 0.5–2 h of annealing, respectively, and the ice crystals grew into the *c*-axis direction. In solutions with pH 6, the ice crystals were gradually modified into the bipyramidal shape during the time course of annealing. Again conversely, the bipyramidal ice crystals were immediately produced at pH 3.

In bipyramidal ice crystals, the exposed area of the basal plane to supercooled water should be minimized. Since there is no evidence that *Tis*AFP6 binds to the basal plane sufficiently at acidic pH range (Fig. 2-6), the site seems to be a weak point in the inhibitory effect on ice growth. Modifying ice crystals into the bipyramidal shape, therefore, would be favorable for preventing their growth. Given that the inability of *Tis*AFP6 to bind to the basal plane at acidic pH range, it is likely to induce bipyramidal ice shapes, as fish AFPs do. Hence, the subtle increases in TH activity detected over a range of the annealing periods at pH 3 might be attributed to the minimization of the exposed area of the basal plane.

On the other hand, *Tis*AFP6 is predicted to bind gradually to the basal plane at neutral pH range. This binding would inhibit the initial growth of seed ice crystals along the *c*-axis, thereby preventing the modification of the crystals into the bipyramidal shape. However, until *Tis*AFP6 binds to the basal plane sufficiently, the site is poorly protected and can be a weak point in the inhibitory effect on ice growth. This process in which the basal plane is fully covered by *Tis*AFP6, therefore, appears to be time-consuming. Similarly to the acidic conditions, a slight increment in TH obtained after 1.5 h of annealing can presumably be explained by its ice crystal morphology. Although the bipyramidal shape was produced immediately at acidic conditions, it seems that the modification process at neutral conditions finished after 1–1.5 h (Fig. 2-5C). Only after completing the modification, an enhanced TH could be detected. These results suggest that *Tis*AFP6 requires substantial time (approximately 2 h) to accumulate onto the basal plane enough to prevent the ice growth along the *c*-axis.

As Garnham et al. stated (2011), anchored clathrate waters on the IBS may be involved in ice-binding of *Tis*AFP6. Although we previously identified several water molecules that likely match the distance between oxygen atoms seen in certain planes of ice (viii), they were much less in number and less organized compared to those of *Mp*AFP (Garnham et al., 2011). It is therefore considered that ordered water molecules on the IBS of *Tis*AFP6 has a rather short lifetime. This seems to explain, in part, the time dependent nature of the activity of *Tis*AFP6.

2.4.4. Effect of pH on the ice-binding specificity

A simple and robust method of determining the ice planes at which AFPs bind was devised over 20 years ago ([Knight et al., 1991](#)) and has recently been improved by using fluorescent dyes or tags ([Garnham et al., 2010](#)). We previously reported that the ice hemisphere grown in a diluted *TisAFP6* solution showed a fluorescent spot on the basal plane surrounded by the six equivalent patterns on the prism plane ([viii](#)). The present study, however, has revealed that the unusual ice-binding property of *TisAFP6* in which not only the TH activity but also the ice growth pattern depend on experimental factors such as solution pH and annealing time. To relate the activity of *TisAFP6* observed through the TH measurements to its ice-binding specificity, we carried out FIPA analysis at various pH levels ranging from 3 to 8.

It was found that there was a remarkable difference in the FIPA patterns between pH values of 3–5 and 6–8: *TisAFP6* illuminated all surface of the ice hemispheres at pH above 6, whereas a significantly reduced fluorescence intensity on the basal plane was observed at pH below 5 ([Fig. 2-6](#)). The decreased affinity toward the basal plane at acidic pH range was further confirmed with the ice hemispheres grown in a 10–times diluted solution of *TisAFP6*. Since we have observed that an appreciable time was required for inhibiting ice growth along the *c*-axis in *TisAFP6* solutions, binding to the basal plane can be considered as a slow process. However, the present study also showed that the FIPA patterns produced by *TisAFP6* at pH above 6 were virtually indistinguishable from those obtained with the hyperactive AFPs ([Pertaya et al., 2008](#); [Mok et al., 2010](#); [Garnham et al., 2011](#); [Hakim et al., 2012](#)). This is likely attributed to the experimental condition: the long period (3–4 h) of ice growth in FIPA analysis allows a sufficient amount of *TisAFP6* molecules to adsorb onto the basal and also, naturally, to the prism planes, enabling us to detect them on the entire surface of ice hemispheres. These results clearly demonstrated that *TisAFP6* possesses a limited ability to bind to the basal plane of ice, depending on both solution pH and time.

2.5. Conclusions

A recently characterized, one of a new group of β -helical IBPs (type I IBP), *TisAFP6* was shown to have a TH that comparable to the moderately active AFPs. The

TH activity of *Tis*AFP6 was dramatically elevated by annealing of an ice crystal for 2 h, as seen in a fish type III AFP solution (Takamichi et al., 2007). Through observations of the pattern in which ice crystals grow in the presence of *Tis*AFP6 when solutions cooled below the T_f , we found that bursts occur as a rapid expansion all round the seed ice in the plane of the *a*-axes after 2 h of annealing; however, the ice crystals obtained with no annealing elongate along the *c*-axis. A series of FIPA analyses exhibited that *Tis*AFP6 binds to multiple planes of ice under circumstances where the solution pH is around 6. These findings provide a new insight into the activity of AFPs, particularly of a group of the β -helical IBPs. Parameters such as solution pH and annealing time might affect on the ice-binding specificity of AFPs. This concept is potentially quite important, given that these most likely reflect the behavior of AFPs in nature.

2.6. Supplementary data

2.6.1. Characterization of *Tis*AFP purified from the natural source

Purification of *Tis*AFP from the culture medium of *T. ishikariensis*

T. ishikariensis strain BRB-1 was aseptically inoculated onto the potato dextrose broth (PDB) and cultured statically for 6 months at -1°C. Then the culture was harvested, centrifuged, and filtrated to remove any insoluble materials. The supernatant was dialyzed against 25 mM acetate-sodium acetate buffer (pH 4) and loaded to a High-S column with a 30 ml bed-volume. The fractions showing antifreeze activity were eluted with the buffer containing 50 mM NaCl. The eluate was dialyzed against 25 mM ammonium bicarbonate and loaded to a High-Q column. The passed-through solution was recovered as active fraction. Finally the solution was again dialyzed against 25 mM acetate-sodium acetate buffer (pH 4) and loaded to a High-S column with a 5 ml bed-volume. *Tis*AFP was eluted with a liner gradient of NaCl (0-50 mM). In this final step of purification four peaks appeared (Fig. S2-1), which all showed antifreeze activity.

pH-dependent activity of *Tis*AFP peaks

Sample preparation- The solvent of purified *Tis*AFP peaks were replaced with water and concentrated up to 200 μ M. The solution pH of AFP solutions at each point

was adjusted by mixing 0.2 M GTA (see below section) buffer pH ranging from 1 to 10 at the same volume to give a final AFP concentration of 100 μ M.

GTA buffer- 3,3-dimethylglutaric acid, tris (hydroxymethyl) aminomethane, and 2-amino-2-methyl-1,3-propanediol were dissolved in water to give a final concentration of 100 mM each (the resulting solution was defined as 0.3 M GTA). To prepare solutions with desired pH, 2 M NaOH or 1 M HCl was added to 0.3 M GTA and then the solutions were diluted with water up to 0.2 M.

Results- As shown in [Fig. S2-2](#), TH activity of peak 1 and 2 were almost independent to the solution pH. In contrast, that of peak 3 greatly fluctuated over a range of pH tested: the highest activity was obtained at pH 6 and it was minimized at pH 2-4, and 10. Also in peak 3, TH values with a large deviation were measured at pH 7 and 8. Peak 4 showed a maximum TH with a large deviation at pH 6.

Ice crystal morphology- peak 1 produced so-called “arrowhead” shaped ice crystals described in the paper of Hoshino et al ([2003a](#)). In that paper, the AFP solution tested was a mixture just before divided into the four peaks, which may reflect the abundance of peak 1 ([Fig. S2-1](#)). The ice crystals observed in peak 2 solution was more like “lemon” shape rather than the arrowhead. As described above, the present study has shown the ice crystal morphology created in the presence of one isoform (TisAFP6) which had been isolated from peak 2 ([Fig. 2-3B](#)), turning out to be irregular shapes at pH above 6 and bipyramidal shapes at pH below 5. The lemon-shaped ice crystal seems to not resemble neither case in recombinant one. This could suggest that two or more isoforms were still contained in peak 2 and the result was considered as the sum of their effect. Peak 3 produced an arrowhead one but with expanded shape in the *a*-axes. Ice crystals observed in peak 4 were closely similar to that in another fungal AFP from *Coprinus psychromorbidus*. ([Hoshino et al., 2003a; 2003b](#))

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TisAFP6 AGPSAVPLGTAGNYVILASTGVSTVPQSVITGAVGVSPGTAASLTGFSLLISGTGTFSTS
TisAFP4 AGPSAVPLGTAGNYVILASTGVSTVPQSVITGAVGVSPGTAASLTGFSLLISGTGTFSTS
TisAFP5 AGPSAVPLGTAGNYVILASTGVSTVPQSVITGAVGVSPGTAASLTGFSLLISGTGTFSTS
TisAFP3 AGPSAVPLGTAGNYVILASTGVSTVPQSVITGAVGVSPGTAASLTGFSLLISGTGTFSTS
TisAFP2 AGPSAVPLGTAGNYVILASTGVSTVPQSVITGAVGVSPGTAASLTGFSLLISGTGTFSTS
TisAFP7 AGPTAVPLGTAGNYAILASTAVSTVPQSAITGAVGISPAAGTFLTGFSLTMSGTGTFSTS
TisAFP8 AGPTAVPLGTAGNYAILASAGVSTVPQSVITGAVGLSPAAGTFLTGFSLTMSSTGTGTFSTS
***:*****.*****:*****.*****:***.:.: ***** :*.*****

SQVTGQLTGADYGTPTPSILTTAIGDMGTAYINAATRSGPDFLEIYTGALGGTTLLPGLY
SQVTGQLTGADYGTPTPSILTTAIGDMGTAYINAATRSGPDFLEIYTGALGGTTLLPGLY
SQVTGQLTGADYGTPTPSILTTAIGDMGTAYINAATRSGPDFLEIYTGALGGTTLLPGLY
SQVTGQLTGADYGTPTPSILTTAIGDMGTAYINAATRSGPDFLEIYTGALGGTTLLPGLY
SQVTGQLTGADYGTPTPSILTTAIGDMGTAYINAATRSGPDFLEIYTGALGGTTLLPGLY
TQVTGQLTAADYGTPTPSILTTAIGDMGTAYTNGATRSGPDFLEIYTGALGGTTLLPGLY
TQVTGQLTAADYGTPTPSILTTAIGDMGTAYVNAATRSGPNFLEIYTGALGGKILPPGLY
:*****.*****.*****.*****.*****.*****.*****.*****.*****.*****
*****.*****.*****.*****.*****.*****.*****.*****.*****.*****

KWTSSVGASADFTISGTSTDWIFQIDGTLDVATGKQITLVGGAQAKNIIWVVAGAVNIE
KWTSSVGASADFTISGTSTDWIFQIDGTLDVATGKQITLVGGAQAKNIIWVVAGAVNIE
KWTSSVGASADFTISGTSTDWIFQIDGTLDVATGKQITLVGGAQAKNIIWVVAGAVNIE
KWTSSVGASADFTISGTSTDWIFQIDGTLGGAAGKKITLVGGAQAKNIIWVVAGAVNIE
KWTSSVGASADFTISGTSTDWIFQIDGTLGGAAGKKITLVGGAQAKNIIWVVAGAVNIE
KWTSSVGASADFTISGTSTDWIFQIDGTLGGAAGKKITLVGGAQAKNIIWVVAGAVNIE
KWTSSVGASADFTISGTSTDWIFQIDGTLGGAAGKKITLVGGAQAKNIIWVVAGAVNIE
KWTSPVGASADFTIIGTSTDWIFQIAGTLGGAAGKKIILAGGAQAKNIIWVVAGAVNIE
****.*****.*****.*****.*****.*****.*****.*****.*****.*****
****.*****.*****.*****.*****.*****.*****.*****.*****.*****

VGAKFEGTILAKTAVTFKTGSSLNGRILAQTSVALQSATIVEK identity (%)
VGAKFEGTILAKTAVTFKTGSSLNGRILAQTAVALQSATIVEK 99
VGAKFEGTILAKTAVTFKTGSSLNGRILAQTAVALQSATIVEK 99
VGAKFEGTILAKTAVTFKTGSSLNGRILAQTAVALQSATIVEK 99
VGAKFEGTILAKTAVTFKTGSSLNGRILAQTAVALQSATIVEK 99
AGAQFEGVILAKTAVTLKTGSSLNGRILAQTSVALQSATVVQK 90
AGAKFEGVILAKTAVTLKTGSSLNGRILSQTAVALQKATVVQK 85
.***:***.*****:*****.*****.*****.*****.*****.*****.*****

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Fig. 2-1. Alignment of TisAFP isoforms at amino acid. The meanings of each symbol used are: *, completely conserved residues; “.”, well conserved residues, “:”, partially conserved residues. Each sequence is available under the accession code of [Q76CE6](#) for TisAFP6, [Q76CE5](#) for TisAFP5, [Q76CE3](#) for TisAFP3, [Q76CE4](#) for TisAFP4, [Q76CE2](#) for TisAFP2, [Q76CE7](#) for TisAFP7, and [Q76CE8](#) for TisAFP8.

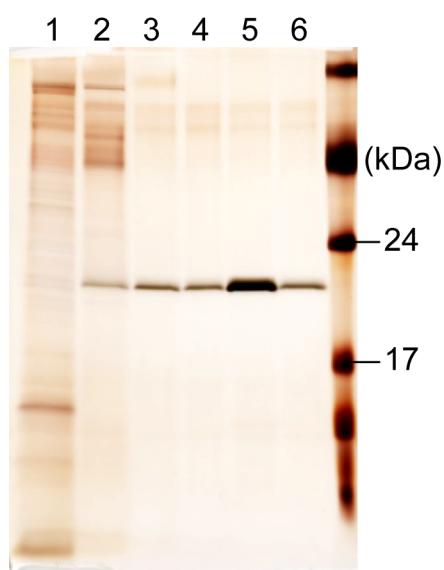


Fig. 2-2. SDS-PAGE pattern at each step of expression and purification of TisAFP6. Lane 1, culture of *P. pastoris* expressing TisAFP6 at 30°C for 96 h; lane 2, the same as lane 1 but at 15 °C; lane 3, High-S bound fraction; lane 4, High-Q passed fraction; lane 5, Mono S bound fraction; lane 6, another Mono S bound fraction.

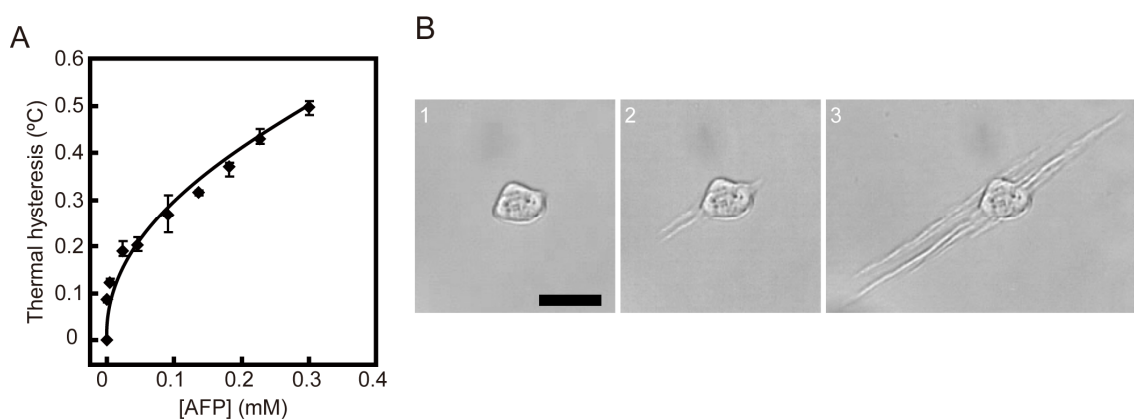


Fig. 2-3. Antifreeze activity of TisAFP6. (A) TH values were plotted as a function of AFP concentration (mM). Each reading was taken three times and averaged. Standard deviations are shown as vertical bars. Measurements were performed in 25 mM MES-NaOH buffer pH 6.0. (B) Ice crystal morphology and growth pattern in the presence of TisAFP6 at 0.23 mM (5 mg/ml). The scale bar represents 31 μm. Frame 1, crystal retained its original shape after preparing with the TH range; 2, temperature cooled below the T_f ; 3, crystal grew rapidly into the *c*-axis direction.

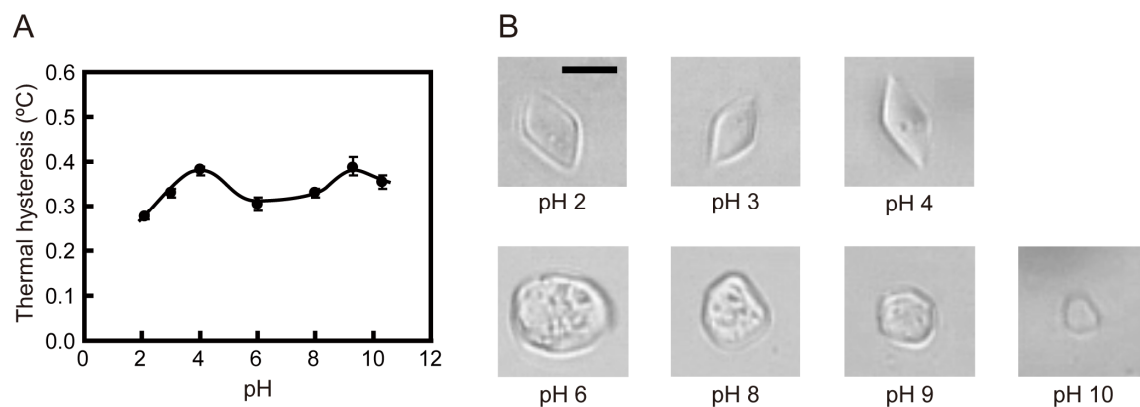


Fig. 2-4. Effect of pH on the activity of *TisAFP6*. (A) TH activity was plotted as a function of solution pH. The concentration of AFP was fixed at 0.1 mM. Each reading was taken three times and averaged. Standard deviations are shown as vertical bars. (B) Ice crystal morphologies observed at different pH conditions. The scale bar represents 15 μm .

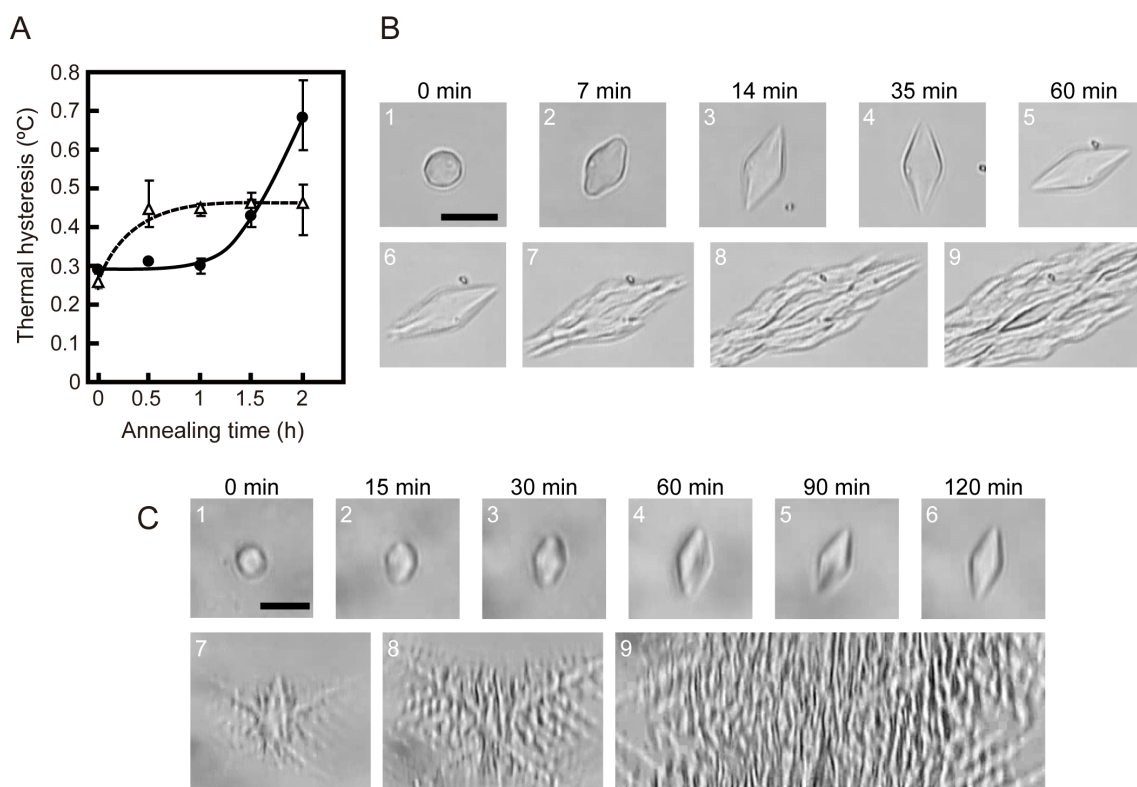


Fig. 2-5. Effect of annealing on the activity of *TisAFP6*. (A) TH activity was plotted as a function of annealing time. closed circles with solid line indicates the condition at pH 6, opened triangle indicates the condition at pH 3. The concentration of AFP was fixed at 0.1 mM in both pH conditions. Each reading was taken three times and averaged. Standard deviations are shown as vertical bars. (B) Time course change in the morphology of an ice crystal under annealing at the $T_m-0.2^\circ\text{C}$ (frame 1~5) and the ice growth pattern (6~9) at the T_f in pH 3 solution. The scale bar represents 11 μm . (C) Time course change in the morphology of an ice crystal under annealing at the $T_m-0.2^\circ\text{C}$ (frame 1~6) and the ice growth pattern (7~9) at the T_f in pH 6 solution. The scale bar represents 16 μm .

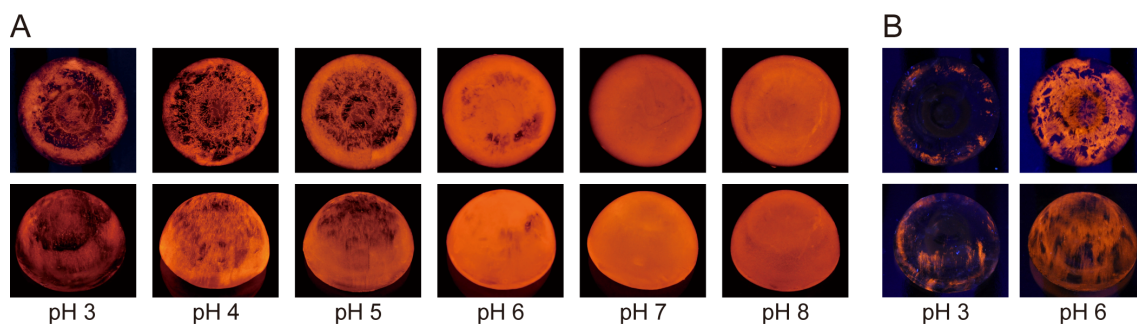


Fig. 2-6. Ice-binding specificity of *TisAFP6* at various pH conditions. (A) FIPA patterns created by tetramethylrhodamine labeled-*TisAFP6* at a protein concentration of 1 μM . The ice hemispheres were mounted to the cold-finger parallel to the *c*-axis. The upper pictures were taken from the top of the hemispheres. The lower pictures shows an oblique view of the same hemispheres. (B) Same as in (A), here the concentration of *TisAFP6* was adjusted at 0.25 μM .

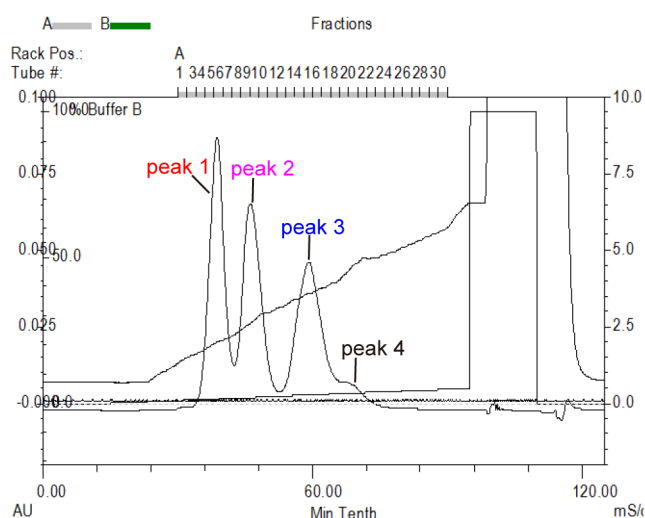


Fig. S2-1. Ion-exchange chromatography of *TisAFP* from natural source. This is a chromatogram obtained in the final step of purification. *TisAFP* isoforms are divided into four peaks with a liner gradient of NaCl (0-50 mM) through High-S, as indicated in arrows.

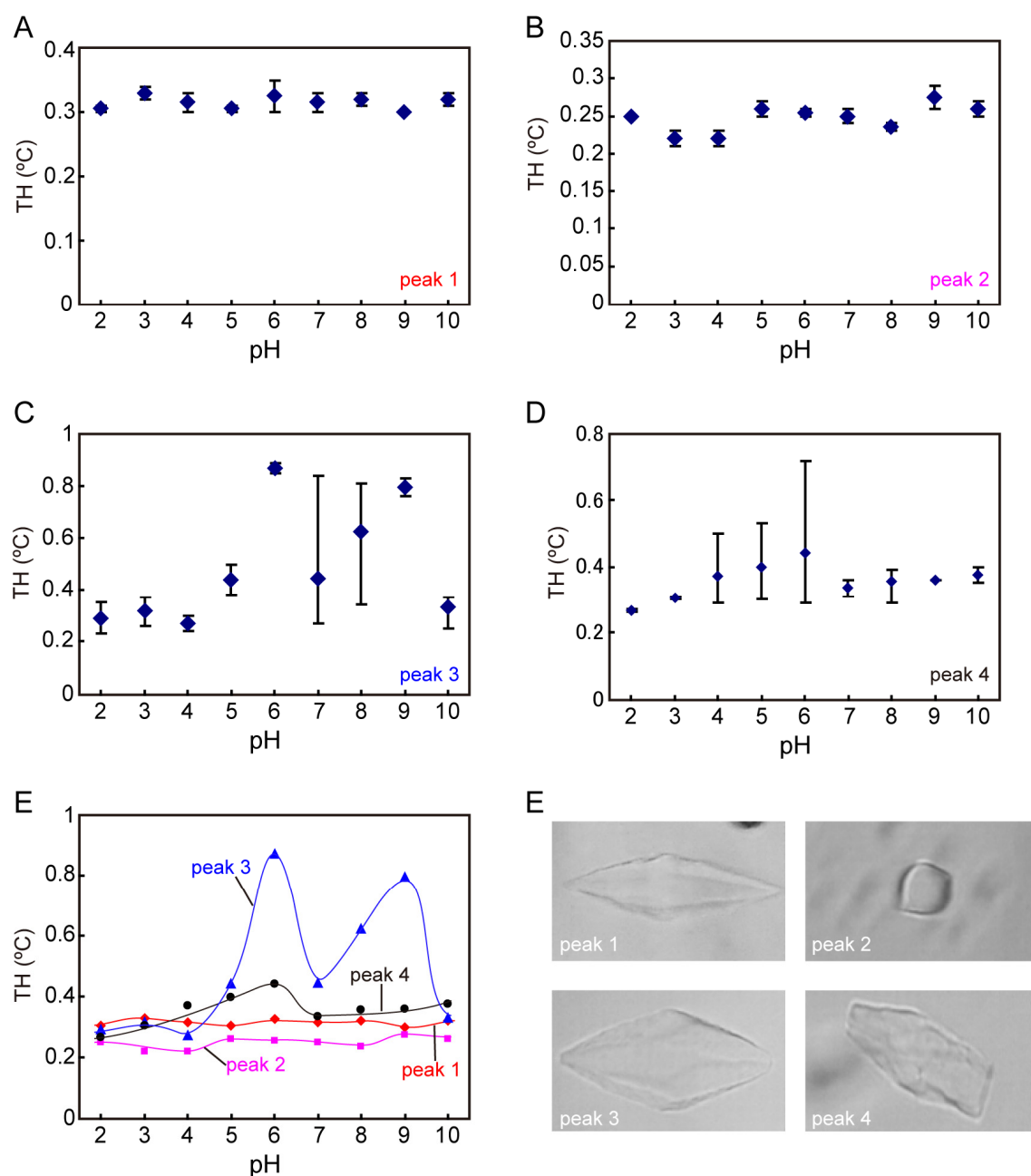


Fig. S2-2. Antifreeze activity of each TisAFP peak. (A)~(D) TH activity of peak 1(A), peak 2 (B), peak 3(C), and peak 4 (D). Protein concentrations for each peak were fixed at 100 μ M. The vertical bars represent standard deviation calculated from three-duplicated measurement at each data point. (E) All profiles are compiled in a graph without error bars for ease of comparison. (E) Ice crystal morphologies produced in each peak at low AFP concentrations.

Chapter 3: X-ray crystal structure and mutational analyses of ColAFP

Preface

In this chapter the author describes the contents of a published paper entitled "Hyperactive antifreeze protein from an Antarctic sea ice bacterium *Colwellia* sp. has a compound ice-binding site without repetitive sequences" by Yuichi Hanada, Yoshiyuki Nishimiya, Ai Miura, Sakae Tsuda, and Hidemasa Kondo. The Authors' contributions are as follows: Y.H. S.T. and H.K. planned experiments and analyzed data.; Y.H., Y.N., A.M., and H.K. performed experiments; Y.H. and H.K. wrote the paper.

Abstract

Antifreeze proteins (AFPs) are structurally diverse macromolecules that bind to ice crystals and inhibit their growth to protect organism from injuries caused by freezing. An AFP identified from an Antarctic bacterium, *Colwellia* sp. strain SLW05 (*Col*AFP) is homologous to AFPs from a wide variety of psychrophilic microorganisms. To understand the antifreeze function of *Col*AFP, we have characterized the antifreeze activity and determined the crystal structure of this protein. The recombinant *Col*AFP exhibited thermal hysteresis activity of approximately 4°C at a concentration of 0.14 mM and induced a rapid growth of ice crystals in the hexagonal direction. Fluorescence-based ice plane affinity analysis showed that *Col*AFP binds to multiple planes of ice including the basal plane. These observations classify *Col*AFP as a hyperactive AFP. The crystal structure of *Col*AFP determined at 1.6-Å resolution revealed an irregular β -helical structure, which is similar to known homologs. Mutational and molecular docking studies clarified that *Col*AFP binds to ice through a compound ice-binding site (IBS) located at a flat surface of the b-helix and the adjoining loop region. The IBS of *Col*AFP lacks structural regularity which has been recognized as a characteristic of hyperactive AFPs. These results conclude that *Col*AFP exerts antifreeze activity by utilizing its compound IBS, which is distinct from the characteristic feature of IBS shared by other hyperactive AFPs. This study represents a novel aspect for protection from freezing achieved by AFPs in psychrophilic microorganisms.

3.1. Introduction

In a previous study, Raymond *et al.* reported that the Gram-negative bacterium *Colwellia*, strain SLW05 from an Antarctic sea ice produces an extracellular protein with ice-binding activities (*ColAFP*), as determined using an ice-pitting assay (Raymond *et al.*, 2007). *ColAFP* shares about 40% identity in amino acid sequence with a number of microbial AFPs from fungi (Hoshino *et al.*, 2003a; Raymond *et al.*, 2009a; Lee *et al.*, 2010), bacteria (Raymond *et al.*, 2008), diatoms (Janech *et al.*, 2006; Bayer-Giraldi *et al.*, 2010; Gwak *et al.*, 2010) and copepods (Kiko, 2010). It has been proposed that horizontal transfer introduces these AFP genes from bacteria, because of their broad distribution among a wide variety of microorganisms (Raymond and Kim, 2012). The potential importance of AFPs in diverse microorganisms, therefore, leads us into close examination of their structure-function relationships.

We reported the crystal structure of an isoform of AFP from a snow mold fungus, *Typhula ishikariensis* (*TisAFP6*) (viii), which is the homolog of *ColAFP*. *TisAFP6* folds into a right-handed β -helical structure comprised of nine turns without repetitive motifs, exhibiting a “semi-pear-like” shape. Site-directed mutagenesis revealed that IBS of *TisAFP6* lies on a relatively flat surface of one side of the β -helix. Water molecules bound in the troughs within the IBS appeared to improve the planarity of the IBS. Although β -helices are a well-characterized structural motif found in hyperactive AFPs including *TmAFP* and *MpAFP* as mentioned above, the IBS of *TisAFP6* lacks regularity and repetitive motifs, suggesting that *TisAFP6* interacts with ice crystals via a distinct mechanism that differs from other known β -helical AFPs. An additional crystal structure of homologous AFPs has been reported for the IBP from an Arctic yeast *Leucosporidium* sp. AY30 (*LeIBP*) (Lee *et al.*, 2012), which is composed of the same β -helical scaffold as *TisAFP6*, confirming that irregular β -helical structures are widely shared among the numerous homologs. Raymond *et al.* proposed that *ColAFP* protects the cell membrane from freezing damage by inhibiting ice recrystallization (Raymond *et al.*, 2007). However, the TH activity and the molecular basis underlying the ice-binding ability of *ColAFP* remain unresolved.

Here we report the antifreeze activity and crystal structure of *ColAFP*. The results showed that *ColAFP* is a novel member of the β -helical hyperactive AFP family without repetitive structure. A series of site-directed mutational analyses introduced in the surface residues, along with docking simulations with ice planes, revealed that the IBS of *ColAFP* is composed of two adjacent regions. From these observations, we describe

how *Col*AFP likely exerts its hyperactivity by binding to multiple planes of ice through the compound IBS without regularity.

3.2. Materials and methods

3.2.1. Expression and purification of *Col*AFP and its mutants

DNA encoding the mature region of *Col*AFP (DDBJ accession no. DQ788793) was commercially synthesized by Life Technologies Japan (Tokyo, Japan). The *Col*AFP DNA was amplified by PCR and inserted into an expression vector pET-20b(+) (Novagen, Madison, WI, USA). The vector was then transformed into *Escherichia coli* BL21 (DE3). Transformants were plated onto Luria–Bertani (LB) agar supplemented with 100 µg/ml ampicillin. A colony was used to inoculate 50 mL LB medium, and the cells grown for 12 h at 37 °C. The culture was then transferred into 1 L of LB medium and cultured at 30 °C. The expression of recombinant *Col*AFP was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside when the optical density at 600 nm was 0.4–0.8. After further culturing for 24 h at 15 °C, the cells were harvested by centrifugation and resuspended in 20 mM Tris–HCl pH 7.4, 100 mM NaCl and 1 mM EDTA. All mutagenesis was performed using the KOD-plus mutagenesis kit (Toyobo, Osaka, Japan) and confirmed by DNA sequence analysis. The primer sequences used for both wild type and are presented in [Table 3-1](#).

The resuspended cells were disrupted by sonication and cell debris were removed by centrifugation. The disruption was duplicated three times using precipitated cell debris ([Fig. 3-1A](#)). The supernatant was dialyzed against 10 mM glycine pH 3.0 by packing it into a dialysis tube with molecular weight cut-off (MWCO) of 14000 ([Fig. 3-1B](#)). During dialysis, vast majority of impurities derived from *E. coli* cells were insolubilized due to low pH ([Fig. 3-1C, D](#)). To eliminate those insolubilized materials, the dialyzate was centrifuged ([Fig. 3-1E](#)) and filtrated with a 0.22 µm pore-sized filter ([Fig. 3-1F](#)). The resulting solution containing *Col*AFP was loaded onto an Econo-Pac High S column (Bio-Rad, Hercules, CA, USA) equilibrated with the same buffer and eluted with a 0–150 mM NaCl linear gradient over five column bed volumes ([Fig. 3-1G](#)). Fractions displaying TH activity were pooled and confirmed for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) ([Fig. 3-1H](#)).

3.2.2. Thermal hysteresis measurements and ice crystal morphology

The TH of wild-type and mutant *Col*AFPs was measured by using a photomicroscope system equipped with a temperature controller, according to the published procedure by Takamichi *et al.* (2007). Initially, a 0.8 μ L AFP solution was frozen completely by cooling to -20°C . The frozen sample was then thawed gradually to prepare a seed ice crystal in the solution. The melting point was determined as the temperature in which the ice started to melt. The sample was cooled again at a constant rate of $0.1^{\circ}\text{C}/\text{min}$, and the temporal change of the crystal shape was captured by a CCD camera. The non-equilibrium freezing point of the solution was determined as the temperature in which the ice crystal grows rapidly. Serial dilutions of wild-type and mutants *Col*AFP were prepared in 25 mM MES–NaOH pH 6.0. Measurements of the freezing and melting points were repeated at least three times. The TH value for each AFP concentration was determined as the averaged difference between the freezing and melting points.

3.2.3. FIPA analysis

Single ice crystals were prepared according to the procedure developed by Garnham *et al.* (2010) (see Chapter 2).

Ten milliliters of *Col*AFP (0.1 mg/ml) was dialyzed against 100 mM NaHCO_3 (pH 8.5) and then concentrated using an Amicon Ultra 4 centrifugal filter device (10,000 MWCO, Millipore, Billerica, MA, USA) to a final volume of 1 ml.

The fluorescent-labeled *Col*AFP was prepared by reacting 1 ml of 1.0 mg/ml *Col*AFP in 100 mM NaHCO_3 pH 8.5 with 50 μ l of 10 mg/ml Pacific BlueTM succinimidyl ester (Life Technologies Japan Ltd., Tokyo, Japan). The solution was mixed in a tube rotator for 3 h at room temperature. The unreacted reagent was removed by dialyzing against distilled water.

A single ice crystal was mounted onto a brass cold-finger cooled at -0.5°C and immersed in approximately 30 ml of a diluted solution of fluorescent-labeled *Col*AFP (0.025 mg/ml [1 μ M]) in an insulated cup. The temperature of the cold-finger was immediately lowered to -5°C and the ice was allowed to grow in a hemisphere shape for 4–5 h. The ice hemisphere was removed from the solution when it had grown to 5 cm in diameter and wiped with a disposable tissue to remove nonspecifically bound materials. The fluorescence from the ice hemisphere was photographed under a UV light in a cold room of -1°C .

3.2.4. Crystal structure analysis

ColAFP was crystallized using the hanging drop vapor-diffusion method (McPherson, 1990) at 20 °C using 20 mg/ml *ColAFP*. The initial condition of crystallization was screened using CrystalScreen, CrystalScreenII, Index (Hampton Research, Aliso Viejo, CA, USA) and Wizard (Rigaku Reagents, Bainbridge Island, WA, USA), and further optimized. The diffraction data of the *ColAFP* crystal were collected on NW-12 at the Photon Factory, KEK, Japan with 1.0000 Å radiation and processed using MOSFLM (Leslie and Powell, 2007) and CCP4 (Winn et al., 2011). The structure of *ColAFP* was determined by molecular replacement methods with the program PHENIX (Adams et al., 2010) using the coordinates of *TisAFP6* (PDB ID: 3VN3) as a search model. The model of *ColAFP* was built using the program COOT (Emsley et al., 2010) and refined using CNS (Brünger et al., 1998, Brünger, 2007) and REFMAC (Murshudov et al., 1997).

3.2.5. Docking models of ColAFP to ice

Coordinates without the hydrogen atoms of four types of ice planes (primary prism, secondary prism, pyramidal and basal planes) were generated by the program VESTA (Momma and Izumi, 2008). The docked model of *ColAFP* and each ice plane with the minimum overall docking score was searched with the program HEX 6.3 (Ritchie and Venkatraman, 2010), using the default parameters except for the Correlation Type = Shape + Electrostatics, post processing = MM, and Minimisation and Final Search = 30. The shape complementarities of the AFP-ice interfaces for the resulting models were evaluated using the program SC (Lawrence and Colman, 1993) in the CCP4 program suite.

3.3. Results

3.3.1. Antifreeze activity of recombinant ColAFP

Recombinant *ColAFP* was purified from *E. coli* cellular lysate in two steps. The majority of the impurities, including *E. coli* proteins and other substances, were precipitated during dialysis at pH 3.0. The supernatant of the dialyzate was further fractionated by cation-exchange chromatography. The purified *ColAFP* appeared as a

single band on a silver-stained SDS-PAGE (Fig. 3-1G). The total amount of purified ColAFP from a 1 L culture was 2.2 mg.

Fig. 3-2A shows the TH activity of ColAFP measured at various concentrations. The TH increased as a function of ColAFP concentration in a hyperbolic relationship to reach 3.8 °C at 0.14 mM (3.3 mg/ml). During the TH measurements, ice crystals grown in the ColAFP solution did not show a distinct facet (Fig. 3-2B upper row). Typically, the seed ice crystal did not change its original size and shape within the TH window. At the freezing point, the ice crystal grew rapidly (“burst”) in six directions, expanding in a dendritic pattern. Clear hexagonal symmetry identified in the grown ice showed that the crystals grew perpendicularly to the *c*-axis of the crystal. A similar growth pattern has been observed for hyperactive insect and bacterial AFPs (Scotter et al., 2006). At a very low concentration (1.4 μM), ColAFP produced a rugged, rock-like ice crystal (Fig. 3-2B lower row). At the freezing point the ice crystal started to grow slowly and enlarged while keeping its original shape.

3.3.2. FIPA analysis

The FIPA analysis visualized the ColAFP-binding site on the ice crystal. Fig. 3-3 shows UV-illuminated images of a single ice crystal hemisphere grown in the presence of fluorescence-labeled AFP. The *c*-axis of the ice crystal is normal to the equatorial plane of the hemisphere. Blue fluorescence was observed over the entire hemisphere in ColAFP (Fig. 3-3A and B), as observed in the hyperactive AFPs (Pertaya et al., 2008; Mok et al., 2010; Garnham et al., 2011; Hakim et al., 2012). In contrast, for fish type III AFP, fluorescence patches were observed in the equatorial region of the hemisphere with hexagonal symmetry (Fig. 3-3C and D).

3.3.3. Crystal structure

The crystal of ColAFP was grown in plate- and needle-like shapes under 0.1 M Tris-HCl pH 8.5, 0.2 M sodium acetate and 30% polyethylene glycol 4000. The diffraction data of the crystal were collected to 1.6 Å resolution. The crystal belongs to the orthorhombic space group of C222₁ with unit cell parameters of $a = 90.25$ Å, $b = 106.95$ Å and $c = 44.08$ Å. The asymmetric unit contains one ColAFP molecule, giving a V_M of 2.26 Å³ Da⁻¹ and a solvent content of 45.6% (Matthews, 1968). Structure determination by the molecular replacement method gave a clear solution and a distinct electron density for the majority of the molecule including the loop region. Two residues at the C-terminus were not visible in the electron density map and not included in the current model. The final model of ColAFP contains 224 amino acid residues out

of 226 and 205 water molecules with an *R* factor of 0.198 and a Free *R* factor (Brünger, 1992) of 0.242. The coordinates were deposited to the Protein Data Bank (PDB) as ID: 3WP9. Statistics of data collection and refinement are summarized in Table 3-2.

The overall structure of *Col*AFP is illustrated in Fig. 3-4A. *Col*AFP is composed mainly of β -helical domain and additional single α -helix. The β -helical domain is composed of 203 residues (N-terminus to Asn⁶⁹ and Arg⁹¹ to C-terminus) and folds into a left-handed helix with triangle intersections and six parallel β -sheets. The long α -helix composed of 21 residues (Ser⁷⁰ to Gly⁹⁰) is located in the middle of the sequence and aligned in parallel to β -helix.

The root mean square distance of the corresponding C α atoms between *Col*AFP and known AFPs was determined to be 0.75 Å and 0.79 Å for *Tis*AFP6 (PDB ID: 3VN3) and *Le*IBP (PDB ID: 3UYU), respectively, indicating that the overall structure and topology of *Col*AFP, especially in β - and α -helices, are nearly identical to these AFPs. Slight deviations are identified only in the loop region, which caps the end of the N-terminal half of the β -helix. In this loop, *Col*AFP lacks six residues, instead forming an intramolecular disulfide bond between Cys⁴⁸ and Cys⁶⁶.

3.3.4. Structural features of the putative IBS of *Col*AFP and comparison with *Tis*AFP6

The previous report proposed that the IBS of *Tis*AFP6 lies on one side of the β -helix, which is composed of 30 residues in a five-stranded parallel β -sheet. The location of the IBS for *Le*IBP was also proposed to be the same as *Tis*AFP6. Fig. 3-4B and C show the molecular surface and IBS of *Col*AFP and *Tis*AFP6, respectively. No regularly arranged residues are found in their IBSs, reflecting the absence of repeat motifs in their amino acid sequences. There are five parallel β -sheets that are composed of six residues, in which four side chains face toward the solvent, and two residues face into the interior of the β -helix, thereby giving an out-out-in-out-in-out pattern (Fig. 3-4D and E). In *Col*AFP, the IBS outward pointing residues in the fourth and sixth positions form a tiny trough, which traverses the IBS in parallel to the β -helical axis. The trough is occupied by four bound water molecules aligned with a constant interval of approximately 4.75 Å, which is equivalent to the distance between the β -sheets. In the center of the IBS of *Col*AFP, there is a hydrophobic patch formed by Leu¹⁸⁸ and Tyr¹⁷⁰, whereas there is a tiny trough, which accommodates seven water molecules for *Tis*AFP.

Fig. 3-4F and G show loop regions (colored in slate) adjacent to the IBS for *Tis*AFP (G) and *Col*AFP (F). In *Col*AFP, six water molecules situated on the loop form

hydrogen bonds to carbonyl oxygen atoms of the main chain with a constant interval. The waters appear to be embedded in a shallow trough made up of two ranks of residues (P³⁸-K¹⁹-Q²⁰⁴-K¹⁸⁶ and T⁴⁰-I³⁹-S²⁰-T²⁰⁵-T¹⁸⁷). This feature could not be detected in the corresponding area of *Tis*AFP6 and suggests that the loop region also plays a role in the ice-binding of *Col*AFP. In *Tis*AFP6, 20 of the outward pointing residues are identified to be involved in ice-binding (shown in yellow, Fig. 3-4C). Three imperfect rows of ordered water molecules are found within the IBS of *Tis*AFP6 (shown in cyan, Fig. 3-4C). The central trough, including seven water molecules, is made up of two ranks of amino acid residues with smaller side chains (G²¹-S²¹²-A¹⁹⁴-A¹⁷⁶-T¹⁴⁹-S¹²⁵ and S²³-A²¹⁴-T¹⁹⁶-N¹⁷⁸-D¹⁵¹) in *Tis*AFP6. Compared with this pattern, there is no trough region in the center of the putative IBS of *Col*AFP (Fig. 3-4B). Since the two ranks of residues (G²¹-A²⁰⁶-L¹⁸⁸-Y¹⁷⁰-N¹⁴³-D¹¹⁹ and T²³-T²⁰⁸-S¹⁹⁰-A¹⁷²-N¹⁴⁵-N¹²¹) include relatively long hydrophobic groups, they seem to stack against each other to embed completely the central trough region of *Col*AFP. This makes a hydrophobic patch at the center of the putative IBS for *Col*AFP. Thus, a trough with five constrained water molecules can be found on the right side of the putative IBS of *Col*AFP (Fig. 3-4B), which is clearer than that observed for *Tis*AFP6.

3.3.5. CD spectroscopy

The CD spectra of *Col*AFP mutants were measured to confirm their protein folding properties. It was found that the patterns of CD spectra obtained by all mutants were very similar to that of wild type (Fig. 3-5), indicating they are properly folded. They typically showed a minimum ellipticity at around 218 nm and a maximum at 199 nm, which are known to be the characteristic spectrum for β -sheet-rich proteins.

3.3.6. Mutagenesis on the putative IBS of *Col*AFP

In order to explore the structural basis for the hyperactivity of *Col*AFP, a series of mutants focusing on the protein surface around the putative IBS were prepared and evaluated for their TH activity. The IBS of the microbial AFPs (*Tis*AFP6 and *Le*IBP) were identified to be on one side of the β -helix (iiiiv; Lee et al., 2012). AFP binding to ice requires an intimate surface complementarity between proteins and ice planes. Therefore, it has been shown that substitutions of ice-binding residues to amino acids with bulkier side chains disrupt the ice-binding capabilities of AFPs (Garnham et al., 2008; Marshall et al., 2002). Here, we focused on the residues located on the putative IBS (S¹⁹⁰ and A²⁰⁶) and the adjacent loop region (T¹⁸⁷, T²⁰⁵, and T²⁰⁸). All of these residues were replaced with tyrosine and only T²⁰⁵ was also replaced with lysine. Each mutant

was purified and assayed for their TH activity to compare results with the wild-type *Col*AFP. As shown in [Fig. 3-6A](#), S190Y exhibited virtually identical TH activity when compared with that of the wild-type protein. Another mutant made in T²⁰⁸, which is located on the same rank as S190, showed a decreased TH activity (0.62°C) by 83% compared to that of wild type (3.8°C) at a concentration of 0.14 mM. Mutations T187Y and T205Y resulted in significant losses of activity by 80% and 50% at a concentration of $\approx$ 0.14 mM, respectively. Both residues are located within the loop region and not at the putative IBS proposed for *Tis*AFP6 and *Le*IBP, suggesting that not only the β -sheet but also this loop might play a role in the ice-binding of *Col*AFP, unlike them. Interestingly, T205K showed an increased TH when compared with wild-type data. As reported recently, an insect hyperactive AFP from *Rhagium inquisitor* (*Ri*AFP) was found to have highly regular IBS composed of TXTTXT motifs ([Hakim et al., 2013](#)). An outward-pointing Lys residue is located on the same face as the IBS of *Ri*AFP, which apparently seems to reduce the surface complementarity between the AFP and ice. It is known, however, that *Ri*AFP shows an extremely high TH above 6 °C at concentrations below 0.1 mM ([Kristiansen et al., 2011](#)). Therefore, it can be considered that hydrophilic residues surrounding the IBS of an AFP may contribute to strengthen the ice-binding activity. The TH activity of A206Y decreased dramatically. A206Y formed an ice crystal that resembled an "arrowhead" ([Fig. 3-6B](#)), similar to that produced in the presence of *Tis*AFP ([Hoshino et al., 2003a](#)). This suggests that the area surrounding A206 is particularly important for the interaction of *Col*AFP with the surface of ice planes. To further test the importance of the both β -sheet and loop region on the activity, a double mutant (T187Y/A206Y) was prepared and examined for its activity. T187Y/A206Y had only 2.6% (0.1 °C) TH activity when compared with the wild-type activity (3.8 °C) at around 0.14 mM. This critical loss of activity is likely due to the disruption of both IBSs. These observations strongly indicate that two adjacent protein faces composed of the β -sheet and the loop account for the ice-binding activity of *Col*AFP ([Fig. 3-6C](#)).

Moreover, these mutants were examined by FIPA analysis to evaluate the effect of the mutagenesis on their ice-binding specificity ([Fig. 3-6D](#)). Almost all mutants could produce the identical pattern to that of wild type where the ice hemispheres become fluorescent entirely. However, a significant difference was observed in the pattern produced by T187Y/A206Y double mutant: no fluorescent pattern was detected on the area corresponding to the basal plane. The results indicate that the mutagenesis introduced on those residues primarily affects on an affinity toward the basal plane.

3.3.7. Docking study

In order to gain further insight into the interactions of *Col*AFP with ice, a molecular docking technique using HEX 6.3 was employed. The primary prism, secondary prism, pyramidal and basal planes were selected to be possible interacting partners of *Col*AFP. Since it is known that some hydration water molecules on the IBS are directly involved in binding to ice in certain AFPs (Nishimiya et al., 2008a; Garnham et al., 2011; Hakim et al., 2013), a series of *Col*AFP structures, in which those water molecules were gradually deleted, were prepared and used for the docking study. The resulting docking patterns showed that *Col*AFP exhibits plausible interactions with four of all the ice planes tested. Moreover, it was predicted that *Col*AFP recognizes all of the ice planes through two different faces of the protein, suggesting that *Col*AFP has two IBSs (Fig. 3-7). One IBS (sheet IBS) is composed primarily of a β -sheet component consisting of the putative IBS area and many water molecules (Fig. 3-7 E-H), whereas the other IBS (loop IBS) is composed of a loop region adjacent to the sheet IBS (Fig. 3-7 A-D). For the latter, there were no hydration water molecules at the interface between the AFP and the planes. A row of 10-residues (A⁴²-A⁴³-G²¹-A²⁰⁶-L¹⁸⁸-Y¹⁷⁰-N¹⁴³-D¹¹⁹) aligned parallel to the helical axis appear to be the central spine that is shared by both of the IBSs. The interaction models were validated by calculating shape correlation (Sc) values and contact surface areas (Table 3-3). Interaction between the sheet IBS including water molecules and the four ice planes scored lower Sc values (0.49–0.53) with relatively large contact areas (1242–1635 Å²), whereas the loop IBS produced significantly higher Sc values (0.57–0.61) with smaller contact areas (909–1037 Å²).

3.4. Discussion

3.4.1. Concentration dependence of the TH activity; comparison with other microbial AFPs

In the present study, recombinant *Col*AFP had a TH of approximately 4 °C at a concentration of 0.14 mM (Fig. 3-2), whereas Raymond *et al.* reported that the spent culture medium of *Colwellia* sp. strain SLW05 exhibited a TH of < 0.1 °C (Raymond et al., 2007). In this study, the concentration of *Col*AFP secreted in the medium was not described, which is likely to be very low when compared with our study. TH values of

various homologous AFPs have been reported and summarized in [Table 1-2](#). The present study shows that *Col*/AFP exhibits the highest TH value among the known homologous AFPs. In addition, typical moderate active AFPs from fish are known to exhibit maximum TH values of 0.6 °C at 1 mM (7 mg/ml) ([Nishimiya et al., 2005](#)). The high TH value of *Col*/AFP measured in our study shows that the antifreeze activity of *Col*/AFP is clearly distinguishable from the moderate active AFPs. Hyperactive *Tm*AFP produces a maximum TH of 4 °C at 180 µM (1.5 mg/ml) ([Marshall et al., 2004c](#)). The TH value of *Col*/AFP corresponds to TH values observed for hyperactive AFPs. The present study revealed that *Col*/AFP possesses an antifreeze activity comparable to hyperactive AFPs.

3.4.2. Ice crystal burst and macroscopic ice hemisphere

Observation of ice crystals using our in-house microscopic system ([Takamichi et al., 2007](#)) showed that *Col*/AFP caused an explosive burst of ice growth in a direction normal to the *c*-axis when cooled below freezing point, suggesting an affinity of the AFP towards the basal and prism plane of ice. This phenomenon of ice crystal bursts in a direction normal to the *c*-axis is a key characteristic of all hyperactive AFPs ([Scotter et al., 2006](#)), and consistent with the observed superior TH activity of *Col*/AFP.

To determine which ice planes are bound by *Col*/AFP, FIPA analyses ([Garnham et al., 2010](#); [Basu et al., 2014](#)) were performed. Macroscopic single crystal ice hemispheres grown in the presence of fluorescent labeled-*Col*/AFP directly demonstrated that this AFP adsorbs to all surfaces of the ice crystal, including the prism and basal planes. Similarly, uniform binding to multiple planes of ice has been visualized in hyperactive AFPs from the spruce budworm ([Pertaya et al., 2008](#)), snow flea ([Mok et al., 2010](#)) and a bacterium *M. primoryensis* ([Garnham et al., 2011](#)). On the other hand, there is no evidence of basal plane binding by non-hyperactive AFPs from the results of ice etching experiments (fish type I, [Knight et al., 1991](#); fish type IV, [Deng et al., 1998](#); fish type III, [Antson et al., 2001](#)), except for the case of a grass IBP (LpIBP, [Middleton et al., 2012](#)). Although LpIBP shows a TH activity comparable to those of moderately active AFPs, it is capable of binding to the basal plane of ice in addition to the six-equivalent prism planes. Ice crystals formed in the presence of the grass AFP are modified into the bipyramidal shape ([Middleton et al., 2012](#)), despite the binding affinity toward the basal plane. These previous observations evoke the situation surrounding A206Y mutant in the present study. The mutant produced ice crystals with two elongated tips ([Fig. 3-6B](#)) which were totally different to those obtained by *Col*/AFP wild type. It was found that, however, the mutant still retained an affinity toward the

basal plane of ice (Fig. 3-6D). Interestingly, FIPA analysis on T187/A206Y, the least active mutant, showed that the basal plane affinity of ColAFP could be abolished by the mutagenesis (Fig. 3-6D). Therefore it appears that a double mutation made in both IBS could actually spoil the basal plane affinity of ColAFP, while a single mutation made in one IBS may not. Thus, we propose that the binding of ColAFP to the basal plane of ice with a high affinity is the basis for its hyperactivity.

Of all homologous microbial AFPs functionally characterized, both non-hyperactive (Gwak et al., 2010; Kiko, 2010; Lee et al., 2012) and hyperactive (Xiao et al., 2010; i, ColAFP) AFPs have been identified. Although it is unclear what structural features make an AFP hyperactive, an extensive comparison of these homologs with regard to their ice-binding structures could reveal attributes that allow an AFP to produce excellent TH activity at low protein concentration.

3.4.3. Possible interactions of ColAFP with ice

In ice, there are numerous crystallographic planes defined by Miller indices. Only a few of them; however, are known to be the planes to which AFPs bind (Wathen et al., 2003); i.e., primary prism ($1 \bar{1} 0 0$), secondary prism ($2 \bar{1} \bar{1} 0$), pyramidal ($2 0 \bar{2} 1$) and basal ($0 0 0 1$) planes. To clarify possible interactions between ColAFP and ice, docking simulations were carried out using HEX 6.3 (Ritchie and Venkatraman, 2010) based on the crystal structure of ColAFP and individual ice planes created by VESTA (Momma and Izumi, 2008). The result of this docking study showed that ColAFP interacts with all four ice planes through the IBSs consisting of the β -sheet and the loop, and this result is consistent with the results obtained by FIPA and mutational analyses.

The IBS of AFPs is typically characterized by its hydrophobicity and flatness (Jia and Davies, 2002). The flatness is considered to provide an ideal geometrical fit to certain ice planes (Leinala et al., 2002a) and hydrophobicity plays a role in ordering hydration water molecules into ice-like arrangements (Nutt and Smith, 2008; Garnham et al., 2011). The degree of geometrical fit between two interacting partners can be evaluated by a shape correlation (Sc) value using the program SC (Lawrence and Colman, 1993). An Sc value of 1.0 indicates a perfect match between the two binding molecules. Previous studies reporting Sc values calculated between AFPs and ice showed that AFP-ice interactions typically yield scores above 0.5 and below 0.8 (Hakim et al., 2013; Nishimiya et al., 2008a; Leinala et al., 2002a). In the present study, docking of non-AF proteins to the ice primary prism plane was also simulated as a reference. The proteins include lysozyme (PDB ID: 3A3Q) and glutathione S-transferase (1M99) as representatives of globular proteins, and a β -helical carbonic anhydrase (1THJ),

which was noted as a structural analog of *Tis*AFP6 (viii) and *Col*AFP also. The Sc values of the resulting docking models were calculated to be 0.25 for 3A3Q, 0.38 for 1M99 and 0.23 for 1THJ, respectively. These are obviously much less than those produced by AFP-ice interactions. Our simulation studies showed that *Col*AFP binds to the ice planes through the IBS composed of the β -sheet with Sc values of 0.44 for $(1 \bar{1} 0 0)$, 0.39 for $(2 \bar{1} \bar{1} 0)$, 0.46 for $(1 \bar{1} 0 1)$ and 0.47 for $(0 0 0 1)$. Interestingly, these values were significantly improved by including the hydration water molecules in the Sc calculations, as follows: 0.51 for $(1 \bar{1} 0 0)$, 0.49 for $(2 \bar{1} \bar{1} 0)$, 0.52 for $(1 \bar{1} 0 1)$ and 0.53 for $(0 0 0 1)$. In this binding mode, hydration water molecules on the β -sheet IBS may directly interact with ice. Moreover, it was found that the extensive β -sheet region provides larger contact surface areas of 1242 $\text{\AA}^2$ to $(1 \bar{1} 0 0)$, 1635 $\text{\AA}^2$ to $(2 \bar{1} \bar{1} 0)$, 1280 $\text{\AA}^2$ to $(1 \bar{1} 0 1)$ and 1316 $\text{\AA}^2$ to $(0 0 0 1)$ than those obtained with the loop IBS mentioned below. These larger values likely compensate for the relatively low Sc values.

Binding of *Col*AFP to the ice planes through the loop IBS produced Sc values of 0.59 for $(1 \bar{1} 0 0)$, 0.57 for $(2 \bar{1} \bar{1} 0)$, 0.61 for $(1 \bar{1} 0 1)$ and 0.59 for $(0 0 0 1)$. These values are higher than the values observed for the IBS composed of the β -sheet. In these cases, there were no constrained water molecules at the interface between the AFP and the ice planes. In addition, most of the constrained water molecules on this IBS were found to cause steric hindrance with the ice surfaces when Sc values were calculated in their presence. Releasing hydration water molecules anchored on the IBS upon binding to ice was suggested to be entropically favorable and could be a driving force for AFP-ice interactions (Nishimiya et al., 2008a; Sönnichsen et al., 1996; Chao et al., 1998). This concept was further supported recently by a combination of NMR and molecular dynamics studies [59]. Surface contact areas to the ice planes in this binding mode were calculated to be 1037 $\text{\AA}^2$ to $(1 \bar{1} 0 0)$, 936 $\text{\AA}^2$ to $(2 \bar{1} \bar{1} 0)$, 953 $\text{\AA}^2$ to $(1 \bar{1} 0 1)$ and 909 $\text{\AA}^2$ to $(0 0 0 1)$. Although these values are smaller than those produced by the β -sheet IBS, a better surface complementarity seems to make up the deficiency in the size of the binding area.

The idea in which AFPs should have a compound IBS was originally proposed in a study on a type III AFP (Garnham et al., 2010). In this case, a different IBS was considered to bind to a different ice plane, respectively. In the case of *Col*AFP; however, a series of the simulation studies suggested that both IBSs account for binding to multiple planes of ice. In other words, *Col*AFP is likely to have two IBSs that are capable of producing full activity independent of each other. Having multiple ice-binding active sites within a molecule would be beneficial under conditions of

selective pressure. Low-temperature environments are known to be a hot spot for microbial evolution (Kiko, 2010; Anesio and Bellas, 2011). In the case of *Col*AFP, when one IBS was inactivated by mutation, the antifreeze activity of the protein would still be maintained owing to the presence of the second active IBS.

3.5. Conclusions

AFPs from bacteria were first documented by Duman and Olsen two decades ago (1993). Since then, a growing number of bacterial AFPs have been isolated and characterized from diverse species (Raymond et al., 2007, 2008; Sun et al., 1995; Xu et al., 1998; Gilbert et al., 2005; Wilson et al., 2006; Kawahara et al., 2007). As to creatures having freezing-avoidance strategies, such as fish and terrestrial invertebrates, AFPs should play a critical role in preventing their organs, tissues and cells from freezing. In contrast, little is known about the physiological significance of AFPs from organisms that tolerate freezing, including plants and microbes. Of all bacterial AFPs, the most extensive research has been carried out on *Mp*AFP, revealing not only its functional and structural properties (Garnham et al., 2008, 2011) but also the physiological role (Guo et al., 2012). A recently characterized novel class of AFPs (or IBPs), including *Col*AFP (Raymond et al., 2007), *Tis*AFP (Hoshino et al., 2003a), *Le*IBP (Lee et al., 2010) and other related proteins (Raymond et al., 2008, 2009a; Janech et al., 2006; Gwak et al., 2010), have been isolated mainly from microorganisms such as bacteria, fungi, diatoms and unicellular algae. Although most of them are thought to be freeze-tolerant, the locations and environments in which they live are totally different. A common gene (*afp*) encoding this type of AFP appears to have been spread by horizontal transfer (Raymond and Kim, 2012). As a previous study (Bayer-Giraldi et al., 2010) showed, the *afp* genes are widely distributed not only in organisms from polar or subpolar regions, but also in organisms living in temperate regions. This finding suggests the possibility that protein products of the *afp* genes are multifunctional. Given the functional diversity, it can be hypothesized that these proteins, including AFPs, have evolved independently from a common ancestral gene acquired by horizontal transfer in response to different environments. *Colwellia* sp. SLW05 was isolated from brine channels in sea ice (Raymond et al., 2007). In sea ice, IBPs are thought to play a role in preserving a liquid environment that is critical for the survival of organisms (Raymond,

2011). As shown in the present study, *Col*AFP exhibits an extraordinarily high TH activity when compared with its homologs, and perhaps is the most active IBP among the IBPs produced by sea ice organisms. Only a small amount of *Col*AFP secreted into the extracellular space may support not only the bacterium itself but also other coexisting organisms within a sea ice environment.

To summarize, in the present study the TH activity of *Col*AFP has been tested and the crystal structure of the protein determined. The results showed that *Col*AFP exhibits a potent TH, reaching up to 4 °C at 3.3 mg/ml. *Col*AFP turned single crystal ice hemispheres uniformly fluorescent, further confirming that the basal plane affinity is the key to the hyperactivity of AFPs (Scotter et al., 2006). The crystal structure analysis revealed a semi-pear-shaped β -helical structure characterized by irregularly spaced amino acid residues throughout the entire surface of the molecule, which is remarkable considering that all hyperactive AFPs structurally analyzed have regularly aligned repetitive sequence motifs on the flattest face of the proteins for their IBSs. Our mutational studies focusing on the putative IBS suggested that two adjacent faces composed of the β -sheet and loop regions account for the activity of *Col*AFP. The likelihood of a compound IBS in *Col*AFP was further supported by molecular docking studies with ice, which showed feasible interactions between *Col*AFP and four distinct ice planes, including the basal plane. One major implication stemming from these findings is that the hyperactivity of an AFP can be exerted through even an IBS lacking any regularity. A potential low ice-affinity due to the structural irregularity of *Col*AFP might be compensated for by the compound IBS with relatively extensive contact areas, enabling it to bind irreversibly to multiple planes of ice as all known hyperactive AFPs do. This also raises the possibility that irregularly arranged side chains of the ice-binding residues appears to provide a less constrained, nonspecific geometrical fit to the surface of multiple ice planes. The rugged, rock-like ice crystals formed in a diluted *Col*AFP solution (Fig. 3-2) can be partially explained by this unusual interaction between *Col*AFP and ice. Moreover, the rugged shaped-ice crystals were also observed in several related-AFPs (Hoshino et al., 2003a; Lee et al., 2010). Thus, the partially nonspecific binding to ice might be a general mechanism among the related-AFPs, because they share a common protein scaffold. Further understanding of their functional and structural features may lead us to a new mechanism that describes how this group of AFPs recognize the surface of ice crystals.

3.6. Supplementary data

3.6.1. Characterization of ColAFP expressed in the yeast *P. pastoris*

Expression and purification

The cDNA encoding ColAFP (DDBJ accession no. DQ788793) was synthesized artificially by and purchased from Invitrogen. The ColAFP sequence was amplified by polymerase chain reaction (PCR) from the supplied vector using a forward primer (5'-AAAACTCGAGAAAAGAGCAGGCC CCTATGCGGTA-3'), which contains an *Xho*I site and a reverse primer (5'- AAGCGGCCGCTTAT AGAGGAGCTTCTTC -3'), which contains a *Not*I site and then cloned into the TA cloning vector, pGEM-T Easy Vector System (Promega) and transformed into *Escherichia coli* JM109. Following digestion with *Xho*I and *Not*I from the TA vector, the ColAFP sequence was ligated with a plasmid pPICZ α at the corresponding sites immediately downstream of the yeast α -mating factor signal sequence without any affinity tag. The ligation mixture was transformed into *E. coli* JM109. The plasmid DNAs prepared from resulting transformants were sequenced to ensure the fidelity of the sequence encoding ColAFP. The plasmid with a correct insert was digested with *Pme*I to obtain linearized DNA. The linearized DNA was transformed into *Pichia pastoris* X-33 strain, following a method described in the EasySelect *Pichia* Expression Kit manual (Invitrogen, available on the web: http://tools.invitrogen.com/content/sfs/manuals/easyselect_man.pdf). Briefly, chemically competent cells prepared with the *Pichia* EasyComp Kit (Invitrogen) were used. Transformed cells were incubated on YPDS agar plates (1% of yeast extract, 2% each of peptone, glucose and agar, 1 M sorbitol) containing 100 μ g/ml of a antibiotic, Zeocin (Invitrogen) at 28°C for 2-3 days and selected by increasing the Zeocin concentration up to 2000 μ g/ml. Finally, colonies grown on YPDS agar plate containing 2000 μ g/ml of Zeocin were used in subsequent experiments. Single colony from selected cells was inoculated into buffered complex glycerol medium (BMGY, 1% each of yeast extract and glycerol, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4 x 10⁻⁵% biotin) and cultured at 28°C with shaking until OD₆₀₀ reached at 2-6. Cells were harvested by centrifugation and resuspended in buffered complex methanol medium (BMMY, BMGY without glycerol supplemented with 0.5% methanol) and growth was continued at 15°C for 96 hours. After growing, cells were removed by centrifugation, leaving a supernatant containing the recombinant ColAFP.

The recombinant ColAFP was purified from the culture medium using our modified ice affinity purification (Kuiper et al., 2003) method. This detailed procedure will be described in elsewhere (Hanada et al., in preparation). Briefly, the cell-free crude supernatant was dialyzed against distilled water and then incubated at subzero temperatures to facilitate freezing of the solution. The resulting ice fraction was removed from excluded liquid fraction and washed with ice-cold distilled water. Purity was checked by SDS-PAGE with Coomassie brilliant blue (CBB) staining.

A recombinant mature form of ColAFP was successfully expressed in the culture medium of the methylotrophic yeast *P. pastoris* by incubating at a low temperature (15°C). Expression at a low temperature increased the yield of ColAFP dramatically and decreased the amount of non-AFP substances released from the host cells. After centrifugation, cell-free supernatant was subjected to two rounds of our modified ice affinity purification (Hanada et al., in preparation). A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that two major proteins were preferentially incorporated into the ice fraction in ice affinity purification (Fig. S3-1). Incorporation of proteins into the ice clearly indicates that these proteins are the ice-binding proteins (i.e., AFPs). It was confirmed that the upper band is partially glycosylated molecules by glycoprotein staining and binding capacity to the Con A-sepharose 4B beads (GE healthcare) that specifically recognize N-linked carbohydrate moiety. In nature, there is no evidence that ColAFP is subjected to glycosylation during biosynthesis (Raymond et al., 2007). After separating the mixture into each molecule (with and without carbohydrate moiety) using the Con A-sepharose, the recombinant ColAFP was examined for TH activity at a same concentration to confirm the influence of glycosylation on its ice-binding ability. The result showed each ColAFP molecule exhibited identical TH activities to each other (data not shown). Therefore, we used the ice affinity-purified recombinant ColAFP in subsequent experiments without dividing each molecule.

TH activity

The purified ColAFP was tested for TH activity as a function of its concentration in 25 mM MES-NaOH buffer pH 6.0, showing the overall shape of TH activity against AFP concentration was a hyperbolic relationship (Fig. S3-2). ColAFP produce TH value of 2.0°C at a concentration of 70 μ M (1.57 mg/ml). At this concentration, typical fish AFPs would only produce about 0.4°C of TH, whereas hyperactive AFPs produce

TH values several times greater than those of fish AFPs. Even at a concentration of 0.36 μM (8 $\mu\text{g/ml}$), 0.1°C of TH was measured.

Mutational study

In order to roughly locate the IBS of ColAFP, mutations were introduced to at each enter of the three molecular surfaces (Fig. S3-3A)., which include an α -helical portion, one side of β -sheet, and another side of β -sheet represented by T86, A206, and N215, respectively. The results clearly indicated that only one mutant (A206Y) decreased the activity significantly, suggesting the importance of the β -sheet encompassing A206, which is equivalent to the so-called “b-face” of TisAFP6 and LeIBP (viii, Lee et al., 2012).

Then, the roles of respective residues at the IBS were explored by mutagenesis focusing on D¹¹⁹, S¹⁹⁰, and A²⁰⁶ (Fig. S3-3B). Interestingly, S190Y mutant had 1.5 times higher TH activity than that of wild type. D119Y showed a comparable activity to that of wild type. However, the double mutant D119Y/S190Y exhibited a decreased TH, and the entire shape of activity curve was sigmoidal. Other double mutants D119Y/ A206Y and S190Y/A206Y displayed very weak activities. A206 seems to play a crucial role in ice-binding of ColAFP, and might be the epicenter of its IBS.

Finally, the role of the S-S bond at the loop region capping the β -helix was examined by replacing the C⁶⁶ with Ala. The result showed that the activity curve of C66A changed slightly, but the activity was still rather high and comparable to that of wild type. Therefore it can be concluded that the S-S bond has no significant role in producing TH activity.

3.6.2. Effect of dilution to the activity of ColAFP

To test the least concentration at which ColAFP could produce any appreciable TH level and how TH increase at low protein concentrations, serial dilutions were made from 10 μM to 1 μM (Fig. S3-4A). When a solution was diluted from 10 μM to 5 μM , TH value dropped to 61% (0.84°C to 0.52°C). A further diluting the solution by half (5 μM to 2.5 μM) resulted in 30% loss of TH (0.52°C to 0.36°C). At concentrations below 2.5 μM , three conditions (1.4, 1.25, and 1 μM) were prepared and those produced TH of 0.18, 0.13, and 0.11. respectively.

As shown in Fig. 3-2B, the growing behavior of an ice crystal in the presence of ColAFP at a low concentration was described. It was recently reported that the characteristic ice-shaping ability of hyperactive AFPs could be observed during melting the ice crystals not in growing (Bar-Dolev et al., 2012). In line with the

newly-recognized phenomena, the “melting shape” of an ice crystal in ColAFP solution was tested. The ice crystal initially had a rounded shape (Fig. S3-4B, frame 1). As the ice crystal was melting, a defined shape with hexagonal symmetry appeared at around from frame 5, which is similar to the patterns observed in hyperactive AFP solutions (Fig. 4 of the paper by Bar-Dolev et al., 2012).

3.6.3. FIPA analysis of ColAFP with nfeAFP mixing

In order to make the difference in ice-binding affinity between ColAFP and type III AFP (nfeAFP6) clearer, FIPA analysis on their coexisting condition was carried out (Fig. S3-5). The result clearly showed that ColAFP binds to the entire surface of the ice hemisphere including the basal plane, while type III AFP does not.

3.6.4. Comparison of the 3-D structures between type I IBPs

To date, four X-ray structures of type I IBPs are available; TisAFP6 (viii), LeIBP (Lee et al., 2012), FfIBP (Do et al., 2014), and ColAFP (i). TisAFP6 and LeIBP are known to be moderately active, and FfIBP and ColAFP are hyperactive. To differentiate the structural characteristic of one class from another's, structure-based comparison was carried out (Fig. S3-6). At first, these IBP structures were superimposed to see the difference in the overall fold (Fig. S3-6A). The R.M.S.D. of FfIBP, LeIBP, and TisAFP6 from ColAFP were calculated to be 0.385, 0.540, and 0.544, respectively. In fact, the α -traced structures of ColAFP and FfIBP matched well (Fig. S3-6A, left panel), similarly in TisAFP and LeIBP (Fig. S3-6A, middle panel), while not in ColAFP and TisAFP (Fig. S3-6A, right panel). The difference was supposed to be mainly derived from the capping loop structure of β -helix where an S-S bond is formed in FfIBP and ColAFP.

In Fig. S3-6B, amino acid residues on their IBS are shown. Also, sequence alignment of these residues were performed (Fig. S3-6C). In these ice-binding residues, 9 of the 36 (25% identity) were found to be conserved among the four IBPs. Within a class of hyperactive AFPs (ColAFP and FfIBP), 20 of the 36 (55% identity) were conserved. Similarly, 16 of the 36 (44% identity) were conserved in moderately active AFPs (TisAFP6 and LeIBP). When the sequence comparison made for their entire amino acids, the hyperactive class shares 53% identity, while that of the moderately active class is 48%. Therefore the difference in their IBS seems to be insignificant considering their entire sequence. Among them ColAFP has a feature where a Tyr residue (Y¹⁷⁰), which usually interferes “AFP-ice” interactions, is located on near the center of its IBS. Although LeIBP does also (F²¹⁴), the feature in this protein appears to

partially explain its low TH activity. ColAFP is remarkable also in the hydrophobic patch located on the center of its IBS, which are mainly made up by a row of residues A²⁰⁶, L¹⁸⁷, and Y¹⁷⁰. This could play an important role in organizing water molecules into an ice-like structure.

Do et al. (2014) describes that FfIBP has an ice-binding motif of (T-A/G-X-T/N). The motif, however, is detected in only the strands of β 1, β 6, and β 5. Therefore the idea in which the motif can be the structural feature making FfIBP hyperactive is hard to grasp when compared to the corresponding insect AFPs' where the T-X-T residues align on one side of the protein. Also, there is no such pattern in the corresponding ColAFP's site despite they share a similar degree of activity. From all those perspectives, one fascinating knowledge provided by their ice-binding structures is that repetitive sequence motifs seen in known hyperactive AFPs seems to be not important for type I IBPs. The structural attributes that divide activity class in type I IBPs are remains to be determined. The more structural information on this type of IBPs being provided, the better to understand this captivating example of protein architecture.

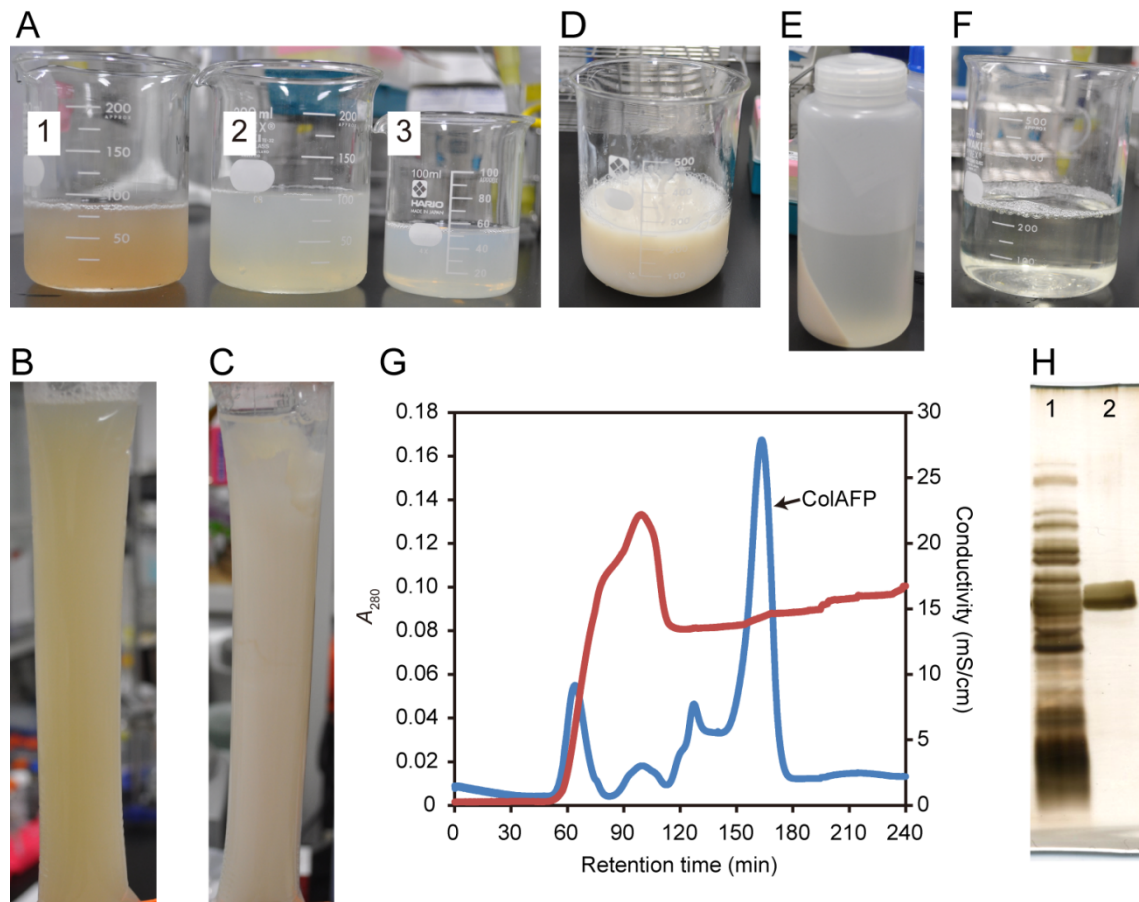


Fig 3-1. Purification of ColAFP. (A) *E. coli* cells lysate. The disruptions of cells were repeated three times (1~3 in the panel). (B) and (C) Before and after dialysis against glycine-HCl (pH 3) buffer. (D) the dialyzate including numerous insolubilized materials. (E) a solution just after centrifugation of (D). (F) the supernatant of (E). (G) A representative chromatogram of ColAFP purification with High-S resin. Blue, absorbance at 280 nm; red, conductivity. ColAFP was detected in a peak indicated by an arrow. (H) SDS-PAGE analysis of purified ColAFP followed by silver staining. Lane 1, the supernatant (F) of the dialyzate against the buffer pH 3; lane 2, the peak indicated by an arrow in (G) where ColAFP appears as a single, dense band.

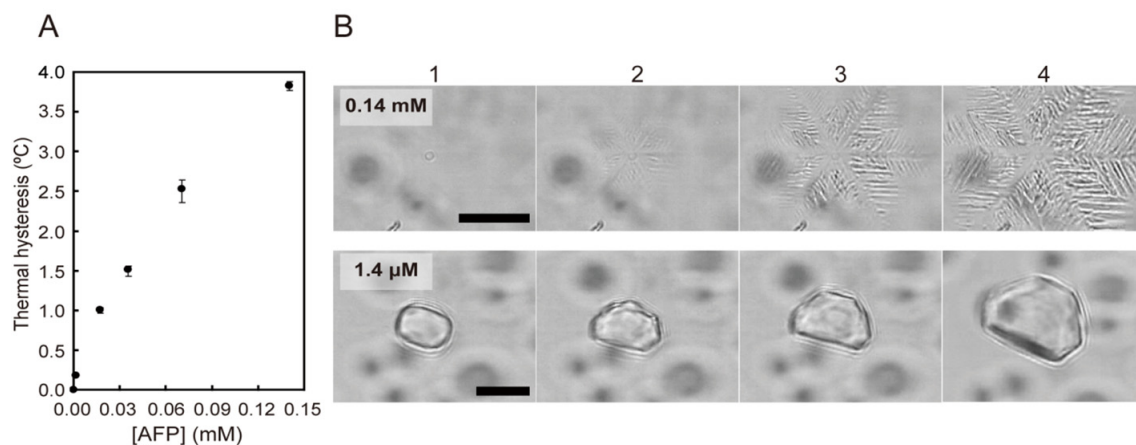


Fig 3-2. Antifreeze activity of *ColAFP*. (A) The TH value is plotted as a function of protein concentration. Vertical bars represent the standard deviation. (B) Ice crystal morphologies and growth patterns in *ColAFP* solutions. Upper row shows a rapid ice crystal growth (burst) observed in the presence of 35 μ M *ColAFP*. Images were taken at 0.1 s intervals from the initiation of the burst. The scale bar represents 30 μ m. Lower row shows a progressive slow growth of an ice crystal observed in the presence of 1.4 μ M *ColAFP*. Images were taken at 4 s intervals from the initiation of the ice growth. The scale bar represents a length of 30 μ m.

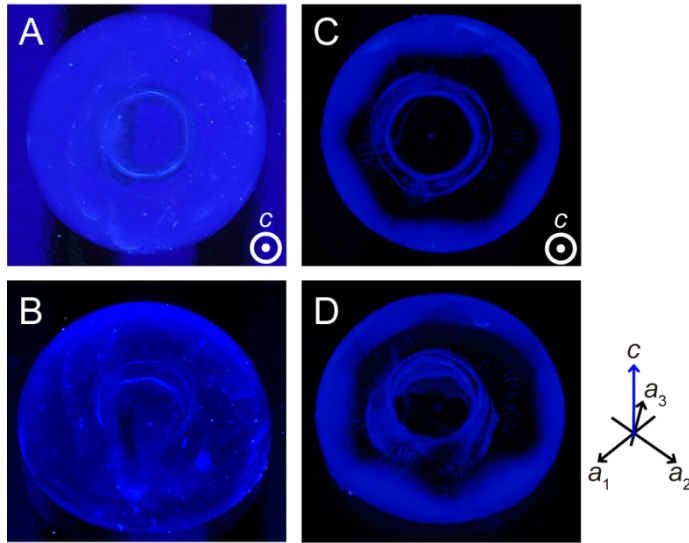


Fig 3-3. Ice hemisphere grown in the presence of Pacific Blue-labeled *Col*AFP and type III AFP (*nfe*AFP8). (A) A top-down view of an ice hemisphere grown in a *Col*AFP solution (1 μ M). The ice hemisphere was mounted with a basal plane oriented perpendicular to the cold-finger. The direction of the *c*-axis is indicated. (B) An oblique view of the same ice hemisphere shown in (A). An arrow diagram illustrates the relative orientation of the *c*- and *a*-axes of the ice hemispheres for (B) and (D). (C) and (D) Top down and side views of an ice hemisphere grown in a fish type III AFP solution as a comparison.

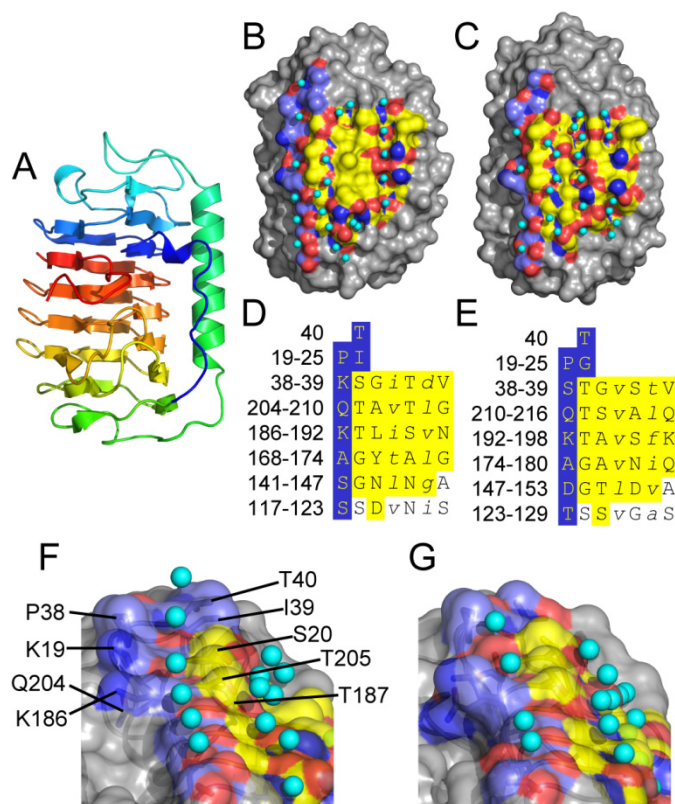


Fig 3-4. Crystal structure and molecular surface of *ColAFP* compared with *TisAFP6*. (A) Schematic presentation of crystal structure of *ColAFP*. (B) Front view of the putative IBS of *ColAFP*. The area corresponding to the IBS proposed in *TisAFP6* (PDB: 3VN3) is shown in yellow for carbon, red for oxygen and blue for nitrogen. The loop region adjacent to the putative IBS is shown in slate for carbon, red for oxygen and blue for nitrogen. Partially ordered water molecules are shown in cyan. (C) Front view of the IBS for *TisAFP6*. The color scheme is the same as (B). (D) and (E) Amino acid residues equivalent to the area indicated in (A) and (B). Letters filled by yellow and by deep blue are the putative IBS and the loop region, respectively. Small italic letters indicate the residues with inward-pointing side chains. (F) and (G), magnification of the loop region for *ColAFP* (F) and *TisAFP6* (G) focusing on the ordered water molecules. Color scheme is the same as (B).

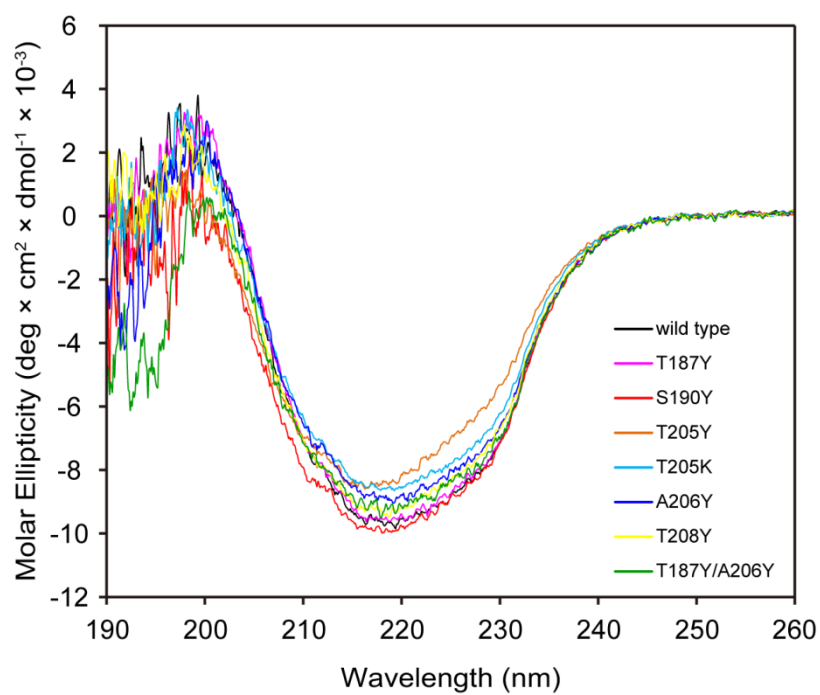


Fig 3-5. Circular dichroism (CD) spectra of ColAFP wild type and the mutants. The eight spectra were colored individually for ease of identification (shown in the inset).

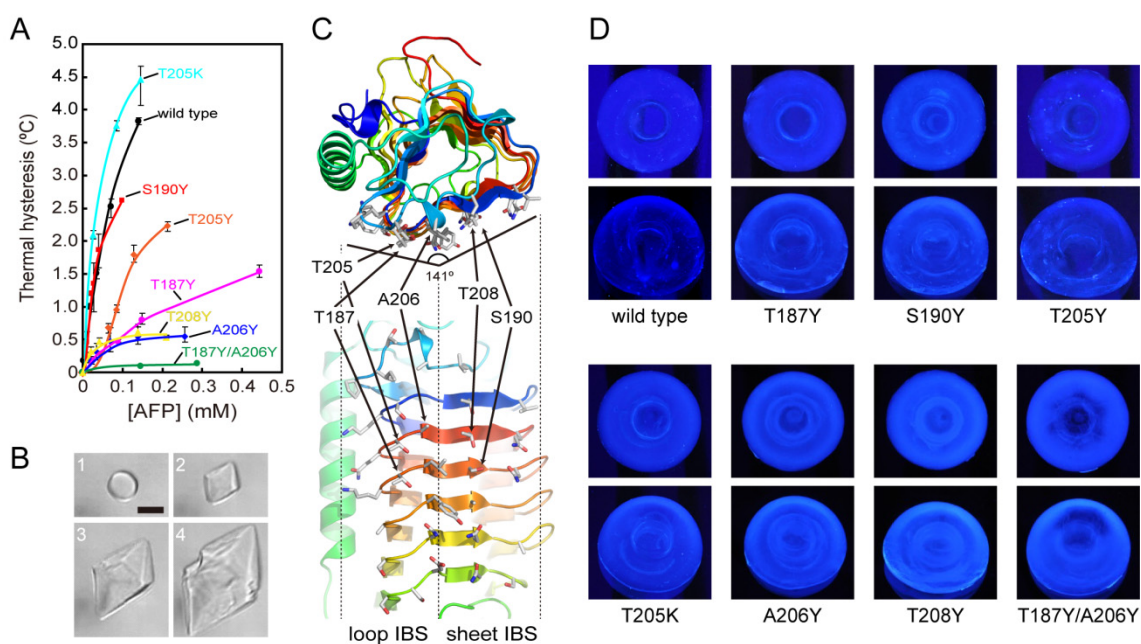


Fig 3-6. TH activity and ice-binding specificity of ColAFP mutants and location of the compound IBS. (A) TH activity curves for ColAFP wild-type and the mutants. (B) Growth pattern of a microscopic ice crystal in the presence of ColAFP A206Y (17 μM). The scale bar represents 22 μm. (C) The two IBSs of ColAFP predicted from the mutational study. The upper panel shows a top down view along the helical axis of the β-helix. The lower panel shows a front view of the IBS. Putative ice-binding residues are shown in sticks with a color scheme as follows: nitrogen atoms, blue; oxygen atoms, red; carbon atoms, white. Mutated residues are indicated by arrows. (D) FIPA analysis of ColAFP mutants and comparison to wild type. The upper panels show the ice hemispheres viewed along the c-axis and the lower panels show an oblique view of the same hemisphere.

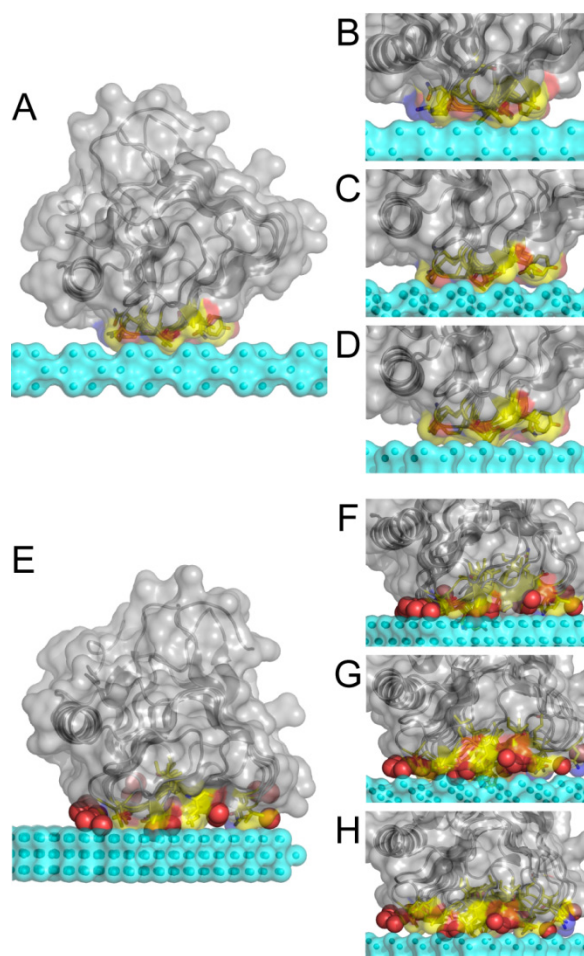


Fig 3-7. Docking of *Col*AFP to the multiple planes of ice. (A)-(D) docking of *Col*AFP to the primary prism (A), secondary prism (B), pyramidal (C) and basal planes (D) via the loop IBS. The overall structure is shown only in (A) as a representative for this binding mode. Ice-binding residues are highlighted by sticks with colors. The color scheme is the same as Fig. 3. (E)-(H) docking of *Col*AFP to the primary prism (E), secondary prism (F), pyramidal (G) and basal planes (H) via the β -sheet IBS. The overall structure is shown only in (E) as a representative for this binding mode. Bound water molecules that contribute to create a flat face are shown as red spheres.

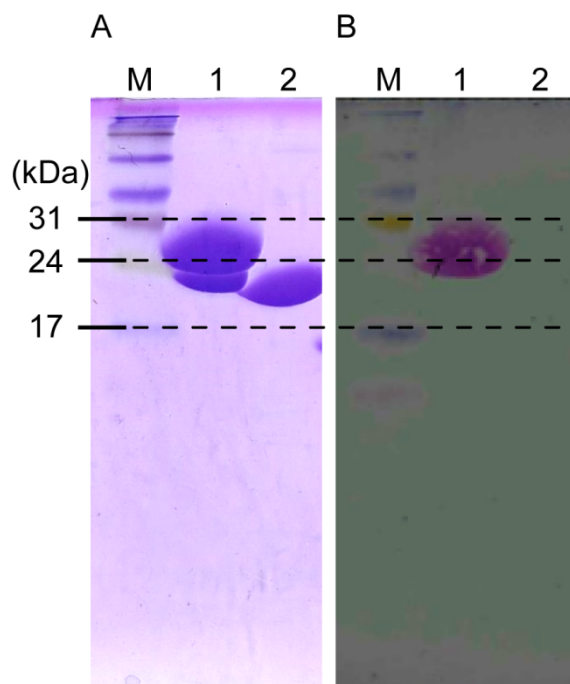


Fig. S3-1. SDS-PAGE analysis and glycoprotein staining. (A) SDS-PAGE with 16% acrylamide gel and CBB staining. (B) Glycoprotein staining on the blotted membrane from the same SDS-PAGE gel. Lane M, molecular mass markers (RPN756E, GE Healthcare); lane 1, the ice affinity-purified recombinant ColAFP (2.4 mg/ml); lane 2, diatom *Navicula glaciei* AFP (2.0 mg/ml) as a negative control.

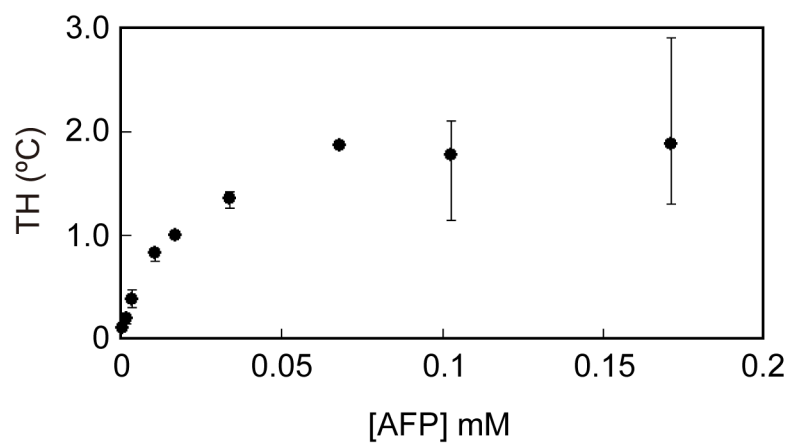
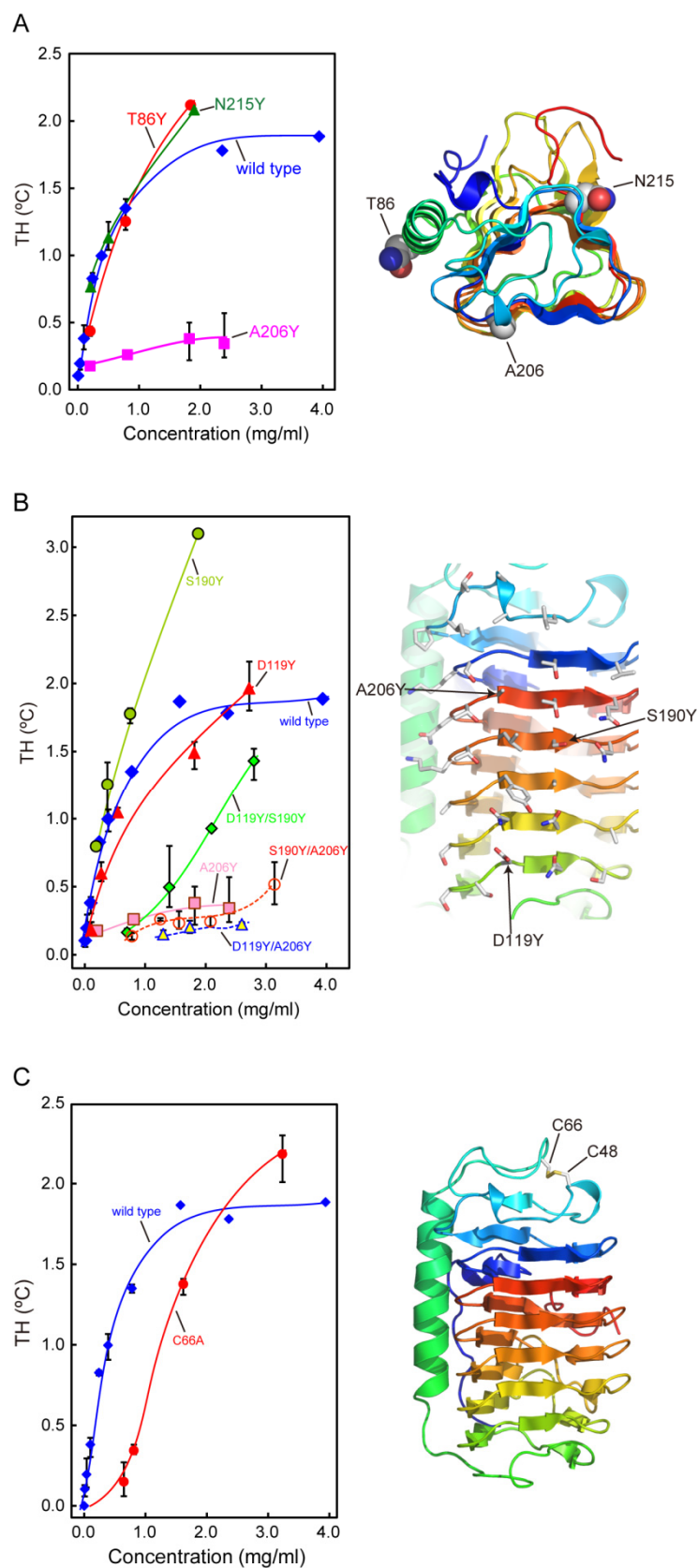


Fig. S3-2. Antifreeze activity of ColAFP. TH value was plotted as a function of protein concentration. Three replicates of each concentration were measured and averaged. Vertical bars represent the standard deviation.



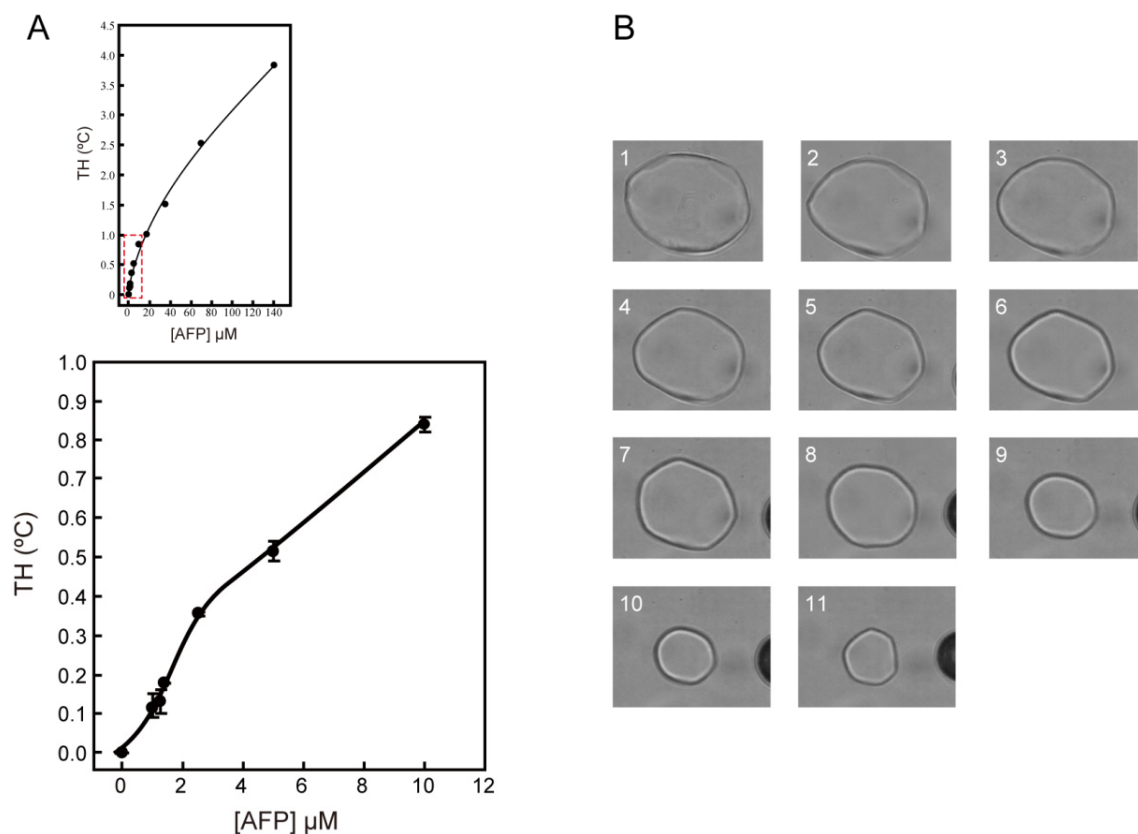


Fig. S3-4. Antifreeze activity of ColAFP (produced by *E. coli*) at low concentrations.

(A) TH activity of ColAFP at low concentrations. The part indicated by red broken lined-box in the upper panel is magnified in the lower panel. (B) The melting behavior of an ice crystal formed in the presence of ColAFP at 5 μM . The ice crystal was kept at the T_m of the solution and melted gradually from frame 1 to 11.

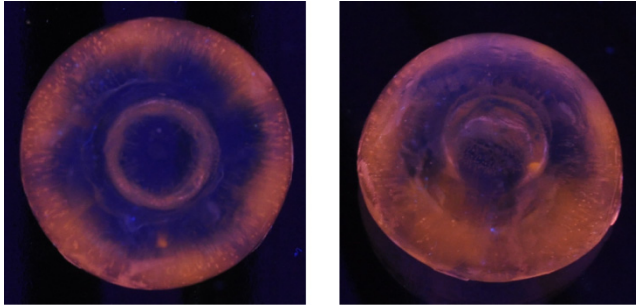
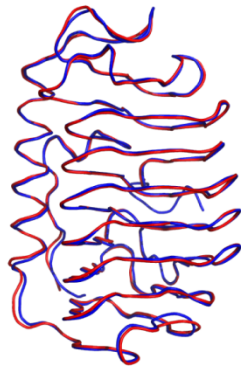
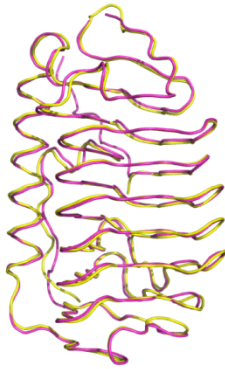


Fig. S3-5. FIPA analysis on ColAFP (blue) with type III AFP (orange). The right panel shows the top down view of the ice hemisphere. The c -axis is parallel to the plane of the paper. The left panel is the same hemisphere viewed with an angle.

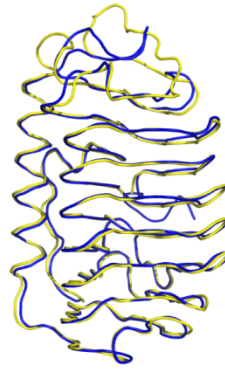
A



ColAFP (blue) and FfIBP (red)

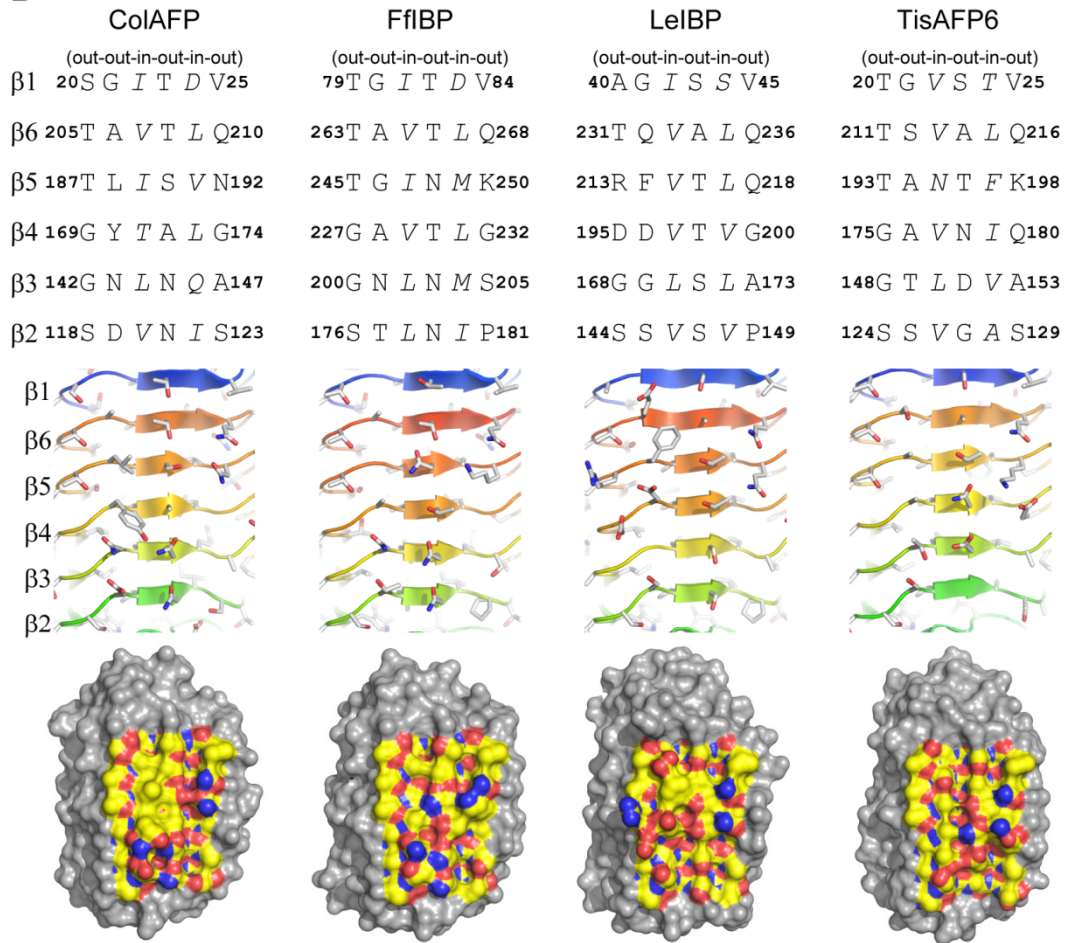


TisAFP6 (yellow) and LeIBP (pink)



ColAFP (blue) and TisAFP6 (yellow)

B



C



Fig. S3-6. Extensive structural comparison among the type I IBPs. The experimentally determined X-ray structures of ColAFP (PDB ID: 3WP9), FfIBP (4NU2), LeIBP (3UYV), and TisAFP6 (3VN3) were used for comparison. (A) The superposition of IBPs shown in the C α -trace. Blue, ColAFP; red, FfIBP; LeIBP, pink; TisAFP6, yellow. (B) Comparison of the amino acid residues located on their IBS. In the top panel, residues are indicated with their numbers. The parenthesis indicates the pointing directions of these residues, “in” stands for inward-pointing (to the core of protein) and “out” stands for outward-pointing (to the solvent). Also, inward-pointing residues are shown in *Italic*. In the middle, the corresponding sites are shown in ribbon diagram with sticks. In the bottom, the surface structures are shown with colored IBS region. (C) Amino acid sequence alignment of ice-binding residues. Entirely/partially conserved residues are shaded by yellow, light blue (ColAFP and FfIBP), and orange (LeIBP and TisAFP6).

Table 3-1. Primer sequences used for construction of wild type and the mutants of *ColAFP*. The nucleotide positions corresponding to each mutation site are highlighted in bold letters.

name	primer sequence
wild type	5' -GCAGGCCCTATGCGGTAGA-3'
	5' -CATATGTCTTTTCTCGAGAGATACCCCTTC-3'
T187Y	5' - TACT TGATTTCTGTTAATACTGGAACCACT-3'
	5' -TTTACTGAGCACGATACCTTCAAAAGAT-3'
S190Y	5' - TAT GTTAATACTGGAACCACTGTTAACGGG-3'
	5' -AATCAAGGTTTTTACTGAGCACGATACCTTC-3'
T205Y	5' - TAC GCTGTCACATTGCAAAAAAACACGATT-3'
	5' -TTGCGCCAATAATCTCCCGTTAACAGT-3'
T205K	5' - AAG GCTGTCACATTGCAAAAAAACACGATT-3'
	5' -TTGCGCCAATAATCTCCCGTTAACAGT-3'
A206Y	5' - TAC GTCACATTGCAAAAAAACACGATTAAC-3'
	5' -AGTTTGCGCCAATAATCTCCCGTTAACAGT-3'
T208Y	5' - TACT TGCAAAAAAACACGATTAACGCGCCA-3'
	5' -GACAGCAGTTTGCGCCAATAATCTCCCGTT-3'

Table 3-2. Data collection and refinement statistics for ColAFP

Data collection	
Space group	$C222_1$
Unit-cell parameters (a, b, c), (Å)	90.25, 106.95, 44.08
Number of molecules in asymmetric unit	1
Beam line	Photon Factory AR-NW12
Wavelength (Å)	1.0000
Resolution range (Å)	69.0–1.6 Å
$R_{\text{merge}}^{*,\dagger}$	0.060 (218)
Observed reflections	603,990
Independent reflections	27057
Completeness (%) [*]	99.8 (100.0)
Multiplicity [*]	21.2 (13.8)
$\langle I/\sigma(I) \rangle$ [*]	7.5 (3.5)
Refinement	
R factor ^{*,‡}	0.198 (0.282)
Free R factor ^{*,‡,§}	0.242 (0.366)
R.M.S bond lengths (Å)	0.021
R.M.S bond angles (°)	2.124
Residues	224
Number of non-hydrogen atoms	
Protein	1635
Solvent	205
Ramachandran plot (%) [¶]	
Residues in favored regions	95.5
Residues in allowed regions	3.6
Residues in outlier regions	0.9
Average B factor (Å ²)	24.06

^{*} Values in parentheses are for the highest resolution shell (1.69–1.6 Å for data collection and 1.64–1.6 Å for refinement)

[†] $R_{\text{merge}} = \sum_j |\langle I(h) \rangle - I(h)_j| / \sum_j \langle I(h) \rangle$, where $\langle I(h) \rangle$ is the mean intensity of a set of equivalent reflections.

[‡] 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.

[§] A randomly chosen 5.1% of the data were used to calculate the free R factor (Brünger, 1992).

[¶] Statistics were obtained from MolProbity (Chen et al., 2010).

Table 3-3. Sc values calculated between proteins and several ice planes.

protein	ice plane	Sc value
lysozyme	1-100	0.25
GST	1-100	0.38
carbonic anhydrase	1-100	0.23
β -sheet IBS w/o waters	1-100	0.44
	2-1-10	0.39
	1-101	0.46
	0001	0.47
β -sheet IBS with waters	1-100	0.51
	2-1-10	0.49
	1-101	0.52
	0001	0.53
loop IBS	1-100	0.59
	2-1-10	0.57
	1-101	0.61
	0001	0.59

Chapter 4: Functional and structural analysis of NagAFP and comparison with its homologs

Preface

In this chapter, the author describes the contents of an unpublished paper entitled "Characterization of a recombinant antifreeze protein from a sea ice diatom *Navicula glaciei*" by Yuichi Hanada, Yoshiyuki Nishimiya, Ai Miura, Sakae Tsuda, and Hidemasa Kondo. The Authors' contributions are as follows: Y.H. S.T. and H.K. planned experiments and analyzed data.; Y.H., Y.N., A.M., and H.K. performed experiments; Y.H. and H.K. wrote the paper.

Abstract

Antifreeze proteins (AFPs), also known as ice-binding proteins (IBPs), represent a structurally diverse class of macromolecules, showing a specific function of inhibiting the growth of ice by binding to. The structure of type I IBPs is characterized by a unique β -helical structure. In the present study, one of type I IBP (NagAFP) from a sea ice diatom *Navicula glaciei* was produced recombinantly and examined for the activity. NagAFP was found to halt the growth of ice crystals up to 1.7°C (thermal hysteresis, TH) well below the melting point of the solution at a 0.24 mM protein concentration. When the solution cooled below the freezing point, the ice crystals grew explosively along the *a*-axes. The TH activity of NagAFP was further tested as a function of solution pH. NagAFP showed a maximized TH level at pH 4, which was 2-fold greater than that in neutral conditions. Although NagAFP wild type, whose Lys residues were labeled, failed to produce reasonable patterns in fluorescence-based ice plane affinity (FIPA) analysis, V220C mutant labeled by Cys-attaching reagent demonstrated the protein's affinity toward entire surface of ice including the basal plane. The homology-guided structure of NagAFP suggested in conjunction with the results of FIPA analysis that it has a unique ice-binding centering a Lys residue.

4.1. Introduction

An AFP isolated from algae was firstly reported on that from *Navicula glaciei*, an Antarctic sea ice diatom, by Janech et al. (2006). Sea ice represents one of the most extreme environment for organisms, in which they are exposed to low temperatures, high salinity, and low light intensity (Thomas and Dieckmann, 2002). Diatoms, which are known to be dominant among organisms thriving in sea ice, start to grow up on the underside of the sea ice and reach at a maximum of their population from November to December (Grossi et al., 1987). The growth of diatoms is significantly reduced by snow-covering on the surface of sea-ice, since snow decay the intensity of light from the sun (Grossi et al., 1987). In addition to diatoms, many different types of microscopic creatures are living in sea ice and they in total construct an ecological system. The environment inside of sea ice is schematically shown in Fig. 4-1.

The presence of an ice-active substance (IAS) in sea ice diatoms was demonstrated for the first time by Raymond and his colleagues (1994). Then, the wide distribution of IASs among Antarctic sea ice diatoms and their partial molecular backgrounds were shown and discussed that IASs are closely associated with diatoms (Raymond, 2000). Later on, an IAS from *Navicula* sp. was found and tested for its ice-recrystallization inhibition activity and ice-binding specificity (Raymond and Knight, 2003). Also the authors suggest in this paper that the IAS has cryoprotective ability during freeze-thaw cycles. An AFP (NagAFP) from *N. glaciei* has been eventually isolated molecularly via these precedent findings (Janech et al. 2006). As described here and elsewhere in the present thesis, NagAFP is known to be a member of type I IBPs which are a unique class of AFPs/IBPs shared among a wide variety of microbes (Raymond and Morgan-Kiss, 2013). Currently, three type I IBPs from polar diatoms have been isolated and characterized at the molecular level (NagAFP, Janech et al. 2006; ii; Cn-AFP, Gwak et al., 2010; FcAFP, Janech et al. 2006; Bayer-Giraldi et al., 2011).

Recently, Xiao et al. (ii) reported that TH activity of NagAFP reached 2°C at a low protein concentration (*ca.* 0.2 mM), which is comparable to those of hyperactive AFPs (Scotter et al., 2006). The sample used in the study was the mixture of NagAFP isoforms obtained from the culture of *N. glaciei* itself. It is known that NagAFP contains at least five isoforms which are available online. The amino acid sequence alignment of the isoforms are shown in Fig. 4-2. Here it is questioned that the hyperactivity measured in the mixture of NagAFP isoforms is ascribed to the cooperative effect between the isoforms. The cooperative effects between AFP isoforms have been documented in the cases of type III AFP (Nishimiya et al., 2005; Takamichi et al., 2009) and an insect AFP

(Wang and Duman, 2005). In the present chapter, the author describes the TH activity, ice-binding property, and structural model of an isoform of NagAFP (denoted as NagAFP8). Moreover, the effect of the presence of salt (NaCl) on the TH activity of NagAFP was tested in order to understand its actual activity in nature. Since it is known that sea ice contains up to 150 g/L of salts (cf. 35 g/L at sea water) (Thomas and Dieckmann, 2002), it can be imagined that the high level of salinity might affect the protein's biochemical properties. In fact, Bayer-Giraldi et al. (2011) reported that approximately 3-folds higher TH was obtained by addition of 60 g/L salt to FcAFP solutions. The data on NagAFP8 obtained through those experiments will be compared to that of ColAFP, which was also isolated from a bacterium inhabiting in an Antarctic sea ice environment (Raymond et al., 2007). Also, the author predicts where the putative IBS of NagAFP8 is located from the homology-guided 3D structural model, which will be compared to those of homologs structurally characterized to date (ColAFP, i; TisAFP6, viii; LeIBP, Lee et al., 2012).

4.2. Materials and methods

4.2.1. Expression of NagAFP8 and V220C mutant

DNA encoding the mature sequence of NagAFP8 (DDBJ ID: DQ062563) was synthesized by and purchased from Invitrogen. The NagAFP DNA fragment was amplified from the supplied vector by PCR using a primer set, AAA ACT CGA GAA AAG AGA GCA AAG CGC TGT CGA C for forward (containing an *Xho*I site) and AAG CGG CCG CTT AAC TGA CAA TGG TAA C for reverse (containing a *Not*I site). The Expand High Fidelity^{PLUS} PCR system (Roche) was used for this PCR. The PCR product was purified by the QIAquick® PCR Purification Kit (QIAGEN) and cloned into a TA cloning vector using the pGEM®-T Easy Vector System (Promega). The plasmid DNA was extracted and purified by QIAquick® Spin Miniprep Kit (QIAGEN) from the transformants obtained from the TA cloning, and then digested with *Xho*I and *Not*I. The DNA fragment digested by *Xho*I and *Not*I was excised and purified by QIAquick® Gel Extraction Kit (QIAGEN) from the corresponding band on an agarose gel. The resulting DNA fragment with cut sites of the both restriction enzymes was ligated into the corresponding site of the pPICZα vector (Invitrogen), and then transformed into *E. coli* JM109. The transformants were inoculated onto a LB medium

supplemented with 20 µg/ml Zeocine (Invitrogen). The plasmid pPICZα bearing NagAFP8 gene was extracted and purified from the *E. coli* transformants and digested with *Pme*I. The linearized plasmid was purified through a DNA extraction from the corresponding band on an agarose gel and transformed into *P. pastoris* X-33 strain (Invitrogen) following the procedure of the *Pichia* EasyCompTM Kit (Invitrogen). The resulting transformants of *P. pastoris* was used for the expression of recombinant NagAFP8.

The pre-culture of *P. pastoris* was performed in a 80 mL of BMGY (Buffered glycerol-complex medium, 2% peptone; 1% yeast extract; 1 M potassium phosphate pH 6.0; 10% glycerol) for 24 h at 28°C. Then the cells were harvested by centrifugation, and resuspended in a 1 L of BMMY (Buffered methanol-complex medium, BMGY supplemented with 0.5% methanol instead of 10% glycerol). The growth of cells in BMMY was continued for 96 h at 15°C and a 5 ml of 100% methanol was added to the medium every 24 h to induce the expression of recombinant NagAFP8.

V220C mutant was prepared by PCR using a primer set, the same forward primer as that for wild type and AAG CGG CCG CTT AAC TAC AAA TGG TAA C for reverse (the underlined triplet shows the mutation site).

4.2.2. Purification of NagAFP8

The supernatant of BMMY culture after 96 h was dialyzed against distilled water by using a cellulose tube with the molecular weight cut-off (MWCO) of 14000 (EIDIA Co., Ltd. Tokyo, Japan). The dialyzate was filtrated by a filter with the pore size of 0.22 µm and subjected into ice-affinity purification (Kuiper et al., 2003). The ice-affinity purified sample was concentrated through a anion-exchange chromatography at pH 8 using an Econo Pack High-Q Column (Bio-Rad) and eluted with 0.5 M NaCl. The active fractions were collected and further concentrated by ultrafiltration using the Amicon Ultra-4 (Millipore). The purity of NagAFP was checked by SDS-PAGE followed by silver staining.

4.2.3. Measurements of TH activity

TH activity of NagAFP was measured by an in-house system utilizing a photomicroscope equipped with a temperature-controlled stage (Takamichi et al., 2007). Serial dilutions up to 240 µM of purified NagAFP were prepared in 25 mM MES-NaOH pH 6.0 and used for measurements. The measurements were repeated at least three times and averaged.

4.2.4. Effect of pH on TH activity

The TH activity of NagAFP was tested for its pH-depending property at pH values of 2.0, 3.0, 4.0, 6.0, 8.0, 9.0, and 10.0. A concentrated NagAFP8 solubilized in distilled water was diluted by each pH-adjusted buffer of 50 mM GTA pH 2.0, 25 mM Glycine-HCl pH 3.0, 25 mM sodium acetate pH 4.0, 25 mM Tris-HCl pH 8.0, 25 mM Glycine-NaOH pH 9.0, and 25 mM Glycine-NaOH pH 10.0 to give a final AFP concentration of 50 μ M. The measurements were repeated at least three times and averaged.

The recombinant ColAFP was prepared as previously described (see Chapter 3). The final concentration of ColAFP was adjusted to 5 μ M by each pH-adjusted buffer of 50 mM GTA pH 2.0, 25 mM Glycine-HCl pH 3.0, 25 mM sodium acetate pH 4.0, 25 mM MES-NaOH pH 6.0, 25 mM Tris-HCl pH 8.0, and 25 mM Arginine-NaOH pH 11.

4.2.5. Effect of salt concentration and pH on TH activity

A concentrated NagAFP8 solubilized in distilled water was diluted by each pH-adjusted buffer of 25 mM Glycine-HCl pH 3.0, 25 mM MES-NaOH pH 6.0, and 25 mM Tris-HCl pH 8.0 to give a final AFP concentration of 100 μ M. These pH-adjusted AFP solutions were diluted by the same volume of 100 mM, 200 mM, and 600 mM of NaCl to give final concentrations of 50 μ M AFP, 50 mM, 100 mM, and 300 mM NaCl, respectively.

For ColAFP, the final protein concentration was fixed at 5 μ M.

4.2.6. FIPA analysis

Ice single-crystals were prepared as previously described (see Chapter 2). NagAFP8 wild type was labeled by 50 μ l of 10 mg/ml tetramethylrhodamine (product code T6105, Invitrogen) at 100 mM NaHCO₃ pH 8.5. NagAFP V220C mutant was labeled by the 100 μ l of 1.25 mM C₅-maleimide (Invitrogen) at phosphate buffered-saline (PBS). The labeled protein solutions were then dialyzed against distilled water to remove unreacted reagents.

4.2.7. Homology modeling and molecular dynamics

Modeling the 3-D structure of NagAFP8 was performed by the Modeller 9.12 (Martí-Renom et al., 2000), based on its homology to TisAFP6 (viii, PDB ID: 3VN3) at the amino acid level. The resulting structural model was energetically optimized by Gromacs v4.5.4 (Van Der Spoel et al., 2005). Firstly, the energy minimization of the structure was carried out using pdb 2gmx, OPLS-AA/L as the force field, hbonds as the

constraints, steep as the integrator, spc216 as the water structure. Subsequently, full molecular dynamics for the energy-minimized structure was performed using the GROMOS 43a1 as the force field for 2 ns.

4.3. Results

4.3.1. Expression and purification of NagAFP8

An isoform of NagAFP (NagAFP8) was produced recombinantly by the *P. pastoris* expression and purified from the spent culture medium by two-round of IAP. The SDS-PAGE analysis followed by silver staining of each step at IAP was shown in Fig. 4-3. As shown in Fig. 4-3A, there were many impurities in the starting material (lane 1), while the band assumed to be NagAFP8 can be observed clearly (indicated by arrow). Although the purity of NagAFP8 was significantly increased after the 1st round of IAP (lane 3, Fig. 4-3A), the remaining impurities were also detected. It was found that those impurities were almost completely removed after the 2nd round of IAP (lane 3, Fig. 4-3B). After IAP, ice fractions were collected and concentrated through an anion exchange chromatography. The yield of NagAFP8 from a 1 L culture medium was estimated to be around 1 mg.

4.3.2. Antifreeze activity of NagAFP8

TH activity of NagAFP8 was measured at 25 mM MES-NaOH pH 6.0 and compared to that of ColAFP (Fig. 4-4A). It is generally known that AFP concentration and TH activity are characterized by a hyperbolic relationship (Scotter et al., 2006). NagAFP8, however, exhibited a unique overall shape of activity curve: once TH increased rapidly at 0.1 mM and then elevated moderately at 0.17 mM. After 0.17 mM, TH again increased exponentially at around 0.24 mM. It is known that ColAFP produces TH of 1°C at 17 μ M (i), while 10 times higher concentration (0.17 mM=170 μ M) was necessary for NagAFP8 to obtain the same degree of TH.

Ice crystal burst patterns at both high (B) and low (C) AFP concentrations were shown in Fig. 4-4. At a high AFP concentration (0.24 mM), ice crystals grew explosively and rapidly with a dendritic growth pattern. At a low AFP concentration (0.024 mM), ice crystals expanded from all side of the initial crystals and several defined crystal facets were observed.

4.3.3. Effect of pH and salt on the activity of NagAFP8 and ColAFP

TH activities of NagAFP8 and ColAFP were measured as various pH conditions ranging from 2 to 10 or 11 (Fig. 4-5A). TH of NagAFP8 was maximized at pH 4 and minimized at pH 2. A slight increment in TH was also detected as around pH 9. In sharp contrast to this pattern, TH of ColAFP tended to be greater as pH decreased. Finally, 4-times higher TH was obtained at pH 2 compared to that at neutral conditions (pH 6-8).

Addition of NaCl to AFP solutions effectively enhanced the TH activity at the pH conditions tested (Fig. 4-5B and C). Remarkably, TH of NagAFP8 in the presence of 0.3 M NaCl was highest, despite that the reduced TH was obtained at pH 3 without NaCl. For ColAFP, TH at each pH condition increased regularly depending on the concentration of NaCl.

4.3.4. FIPA analysis of NagAFP8

Firstly, NagAFP8 wild type was labeled by tetramethylrhodamine which specifically attaches to Lys residues of proteins and subjected to FIPA analysis. The resulting FIPA pattern showed faint fluorescent patches on the area corresponding to the basal and high-ordered pyramidal planes (Fig. 4-6A), inconsistent with the ice burst pattern shown in Fig. 4-4B. Because it has been widely observed that AFPs showing dendritic ice growth pattern into the a-axis direction turn ice hemispheres uniformly fluorescent (Mok et al., 2010; Garnham et al., 2011; Hakim et al., 2013; i). One could imagine that labeling Lys residues by the fluorescent molecules would cover the IBS of NagAFP8. Then the C-terminal residue of V220 was replaced by Cys and the resulting V220C mutant was labeled by C₅-maleimide which specifically to Cys residues. There is no Cys residues on the entire sequence of NagAFP8. Consequently, it was found that the C₅-maleimide-labeled NagAFP8 V220C bind to ice hemisphere uniformly (Fig. 4-6B), consistent with the ice burst pattern. It was considered that these results suggest the potential importance of Lys residues in the activity of NagAFP8.

4.3.5. Homology-guided structure of NagAFP8

To facilitate structure-based discussion of the antifreeze activity of NagAFP8, 3-D structural model was constructed by referencing to TisAFP6 based on their sequence homology at the amino acid level (viii). The initial model created by the Modeller was subjected to a MD simulation for 2 ns with the program GROMOS. The resulting structural model is shown in Fig. 4-7A, in addition to the model before MD as a comparison. The large differences in the structures before and after MD were detected

in an α -helix and a loop region which caps the top of the β -helix. It seems that the α -helix slightly bent and projected toward the inside of the protein and the loop region was compressed, thereby making a more compact structure.

Based on this structure, possible location of Lys residues is indicated in Fig. 4-7B. Lys residues were found to be located on the opposite side of the putative IBS proposed in TisAFP6 (viii) and LeIBP (Lee et al., 2012) and the adjacent region composed of loops and β -strands.

4.4. Discussion

4.4.1. Antifreeze activity of NagAFP8

The present study revealed that the TH activity of NagAFP8 reaches *ca.* 1.7°C at a protein concentration of 0.24 mM (Fig. 4-4A), which is well below that produced by ColAFP. ColAFP could produce the same level of TH at the one tenth concentration. A previous paper (ii) described that NagAFP obtained from the diatom culture, which might contain several isoforms, exhibits approximately 2°C of TH at around 0.25 mM. Although the TH values obtained by an isoform NagAFP8 seems to be slightly lower than that of the isoform mixture, the difference may not be overwhelming. Considering the high identity (~95%) at amino acid sequence among the isoforms, it is difficult to predict that one isoform could show a higher activity (Fig. 4-2). Also, the relationship between TH and the concentration of NagAFP8 seems to be irregular (Fig. 4-4A). It is known that the isoform mixture of NagAFP, however, shows a normal hyperbolic relationship between them. One plausible explanation for this difference is that there might be some cooperative effects among the isoforms. The isoforms could help one another to show the sufficient level of activity at a lower protein concentration range.

A high concentration (0.024 mM) of NagAFP8 directed ice crystals burst into *a*-axis direction with a dendritic pattern (Fig. 4-4B), which is known to be the general phenomena seen in the solution containing hyperactive AFPs (Scotter et al., 2006). Also, the burst patterns observed in NagAFP8 solutions were indistinguishable from those of the isoform mixture (ii). In a solution with low protein concentration (0.024 mM), the shape of ice crystals were modified as they were growing at the temperature below the T_f (Fig. 4-4C). Although this behavior evokes the one observed in a ColAFP solution at very low concentration (Fig. 3-2B), but the both are not identical and distinguishable.

Recently, it has been argued that the modification of ice microstructure by AFPs/IBPs was rather important for sea ice organisms than producing high TH levels to prevent ice growth (Bayer-Giraldi et al., 2011; Raymond, 2011; Raymond and Morgan-Kiss 2013). The difference in the shape of ice crystals formed in the presence of AFPs might have a distinct impact on the modification of ice microstructure. Therefore it can be considered that ColAFP and NagAFP8 modify ice microstructure in a distinct manner, thereby contributing to generate a habitable environment for each organism (a bacterium *Colwellia* sp. SLW05 and a diatom *N. glaciei*).

4.4.2. pH and salt-dependent property of NagAFP8

TH activities of NagAFP8 and ColAFP were tested for their pH-dependent property (Fig. 4-5A). As describe in Chapter 2, it might be important to see their functional relevance to conditions of solution such as pH and salts. ColAFP showed the highest activity (2°C) at pH 2, which was 4-folds larger than that produced at pH 6 (0.5°C). Also, ColAFP was sufficiently active in a wide range of pH from 2 to 11, which implies the structure of ColAFP is stable at any pH conditions. On the other hand, the highest activity (1.2°C) for NagAFP8 was obtained at pH 4, which was 2-times greater than that at pH 6 (0.6°C). A sudden decrease in the activity of NagAFP8 was observed in pH 3 and the activity abolished almost completely at pH 2. It is known that pH of sea ice tends to become higher (up to pH 11) since inorganic carbon sources are exhausted by photosynthesis of algae (Thomas and Dieckmann, 2002). Normal sea water is generally maintained at around pH 8. Therefore, the optimum pH for AFPs from sea ice organisms is likely to be around 8 to 11. The highest TH values measured for ColAFP and NagAFP8 in solutions with pH 2 or 4 seems to be contradicting to the possible optimum pH and remain to be solved.

4.4.3. FIPA analysis of NagAFP8 and its implications for the IBS

The FIPA analysis on NagAFP8 wild type resulted in a faint fluorescent pattern at around the top of ice hemispheres (Fig. 4-6A). This was inconsistent with the speculation in which AFPs inducing the ice crystal burst into *a*-axis could adhere the entire surface of ice hemispheres. Since the fluorescent reagent tetramethylrhodamine recognize specifically and adsorb to the side chain of Lys residues, it can be predicted that the molecules hamper the ice-binding ability of NagAFP8 by covering its IBS. Because of this reason, the V220C mutant was prepared, labeled by C₅-maleimide which attaches to the side chain of Cys residues, and tested for its ice plane affinity. As a result, the ice hemisphere turned uniformly fluorescent (Fig. 4-6B), as seen in those

grown in the presence of most hyperactive AFPs (Mok et al., 2010; Garnham et al., 2011; Hakim et al., 2013). These two results strongly suggest that Lys residues are involved in the ice-binding of NagAFP8.

4.4.4. Possible IBS of NagAFP8 and comparison with those of the homologs

The FIPA analysis of both NagAFP8 wild type and the V220C mutant implied that the potential importance of Lys residues for the activity of NagAFP8. In order to predict the locations of Lys residues in NagAFP8 structure, the homology-guided model structure was created (Fig. 4-6B). In total, there are eight Lys residues in the entire sequence of NagAFP8. Three of them were assumed to be involved in the ice-binding of NagAFP8, since another five were located in the back side of the putative IBS (shown in black line in Fig. 4-7B-D). The back side region contains bulging loops and extended β -strands (Fig. 4-7B, right panel). Therefore the region seems to be irrelevant to the activity of NagAFP8. Interestingly, there is only one Lys residue (K149) in the edge of the putative, common IBS identified in TisAFP6 (viii), LeIBP (Lee et al., 2012), FfIBP (Do et al., 2014). Instead, two Lys residues (K19 and K121) are located in the central part of the adjacent face of the putative IBS (Fig. 4-7D, shown in pink line). It can be considered that if NagAFP8 binds to ice through the common IBS shown in black line, there might be few effect on its ice-binding activity when the fluorescent molecules attached to the K149. On the other hand, if NagAFP8 binds to ice through the adjacent face shown in pink line, labeling of K19 and K121 by the fluorescent molecules would significantly reduce its ice-binding activity. Thus, the IBS of NagAFP8 appears to be the adjacent region of the common IBS, which is made up of an elongated α -helix and loops forming the edge of β -helix (Fig. 4-7D).

To obtain a further evidence on that the region may account for the activity of NagAFP8, the corresponding sites of its homologs' structures were shown and compared to each other (Fig. 4-8). The location of Lys residues in this face has been highlighted by circles in the figure, which clearly showed that Lys residues are also located on the central area of the face in TisAFP6 and ColAFP. These two AFPs, however, are enough active to produce an uniform fluorescent pattern in FIPA analysis even after Lys residues were labeled by dye (Chapter 2, 3). On the other hand, NagAFP8 failed to bind to the entire surface of ice hemispheres when Lys residues were labeled by the fluorescent dye (Fig. 4-6A). From these observations, it can be concluded that NagAFP8 is most likely to have a unique IBS which is different to any other homologs.

4.5. Conclusions

The present study further revealed the diversity of activity among type I IBPs. A series of experiment including FIPA analysis which helped to identify the IBS of NagAFP8 and structural modeling suggested that those homologous protein even more have different ice-binding active sites. Once the organisms acquired a common gene encoding type I IBP, one can predict that they have evolved the gene suitable for their purpose. This hypothesis can partially explain the diversity of their activity.

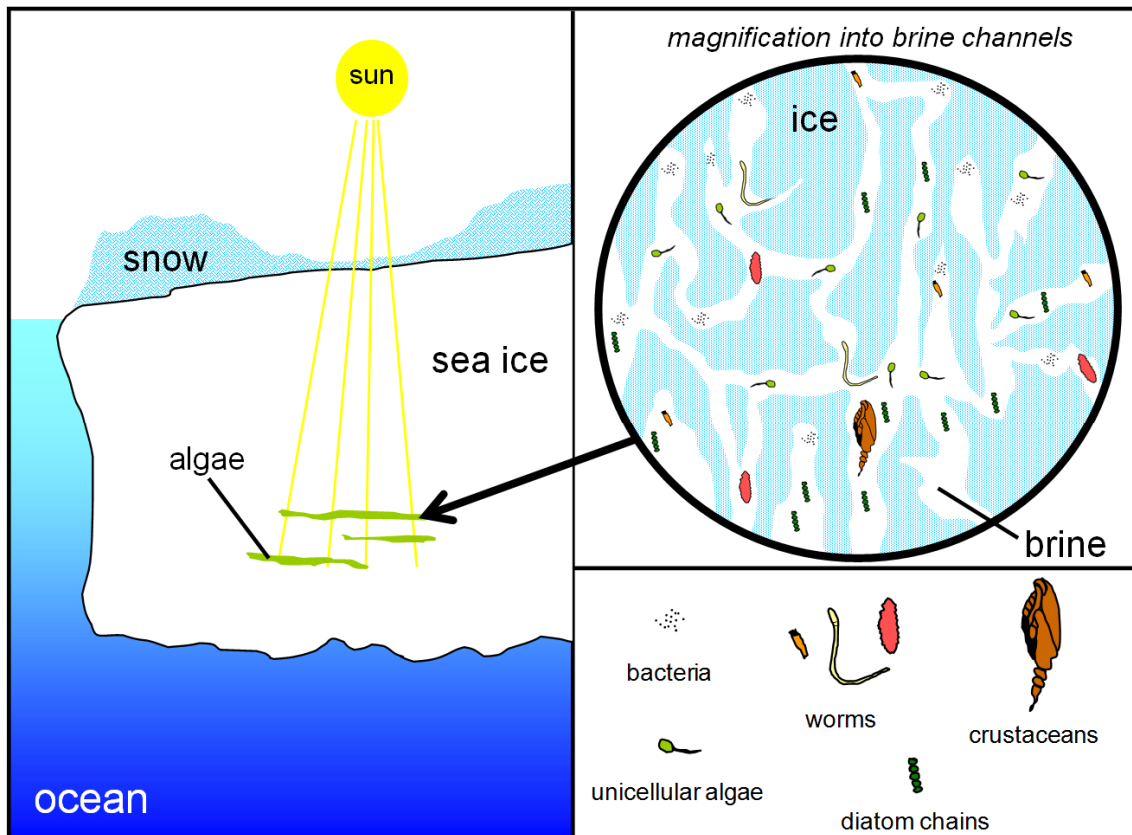


Fig. 4-1. Sea ice as a habitat for microorganisms. Sea ice indicates huge blocks of ice floating on the polar oceans. Algae, mainly composed of diatoms, are known to be the primary producer in that environment. The sunlight transmits to the bottom of the ice where algae live through surface areas with thin-covered snow (left panel). Small flow channels between ice grains (right panel) are called as "brine" which contains concentrated salts and nutrients stemmed from sea water. This figure was created by me referencing to a web page of National Oceanic and Atmospheric Administration (http://www.arctic.noaa.gov/essay_krembsdeming.html).

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IBP-8  AEQSAVDLGTAGDFAVLKAGVSTTGPTTEVTGDIGTSPIASTALTGFALIKDSSNTFSTS 60
IBP-10 -----
IBP-9  AEQSAVDLGTAGDFAVLKAGVSTTGPTTEVTRDIGTSPIASTALTGFALIKDSSNTFSTS 60
IBP-3  AEQSAVDLGTAGDFAVLKAGVSTTGPTTEVTGDIGTSPIASTALTGFALIKDSSNTFSTS 60
IBP-13 AEQSAVDLGTAGDFAVLKAGVSTTGPTTEVTGDIGTSPIASTALTGFALIKDSSNTFSTS 60
*****

SLVTGKIYAADYTAPTPSKMTTAISDMSTAFTDAAGRSDPDFLELGAGSIEGETLVAGLY 120
-----MTTAISDMSTAFTDAAGRSDPDFLELGAGSIEGETLVAGLY 41
SLVTGKIYAADYTAPTPSKMTTAISDMSTAFTDAAGRSDPDFLELGAGSIEGETLVAGLY 120
SLVTGKIYAADYTAPTPSKMTTAISDMSTAFTDAAGRSDPDFLELGAGSIEGETLVAGLY 120
SLVTGKIYAADYTAPTPSKMTTAISDMSTAFTDAAGRSDPDFLELGAGSIEGETLVAGLY 120
*****

KWGTDVSFTSSLVFDGSATDVWILQVAKDFIVGNGAQMYLTGTAKAENIFIQVSGAVNIG 180
KWGTDVSFTSSLVFDGSATDVWILQVAKDFIVGNGAQMYLTGTAKAENIFIQVSGAVNIG 101
KWGTDVSFTSSLVFDGSATDVWILQVAKDFIVGNGAQMYLTGTAKAENIFIQVSGAVNIG 180
KWGTDVSFTSSLVFDGSATDVWILQVAKDFIVGNGAQMYLTGTANAENIFIQVSAVNIG 180
KWGTDVSFTSSLVFDAAATDVCILQVAKDFIVGNGAQMYLTATAKAENIFIQVSAVNIA 180
*****

TTAHVEGNILSATAIALQTGSSLNGKALSQTAITLDSVTIVS 222 identity (%)
TTAHVEGNILSATAIALQTGSSLNGKALSQTAITLDSVTIVS 143 100
TTAHVEGNILSATAIALQTGSSLNGKALSQTAITLDSVTI-- 220 99
TTAHVEGNILSATAIALQTGSSLNGKALSQTAITLDSVTIVS 222 99
TTAHVEANILTATAIAMQTGSSLNGKALSQTAITLDSVTIVS 222 95
*****

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Fig. 4-2. Amino acid sequence alignment of NagAFP isoforms. The alignment was performed by the ClustalW (<http://clustalw.ddbj.nig.ac.jp/>). Positions where the sequence is not conserved are shaded by yellow. Conserved residues among the five isoforms are indicated by asterisks. In this figure, IBP-8 is equivalent to NagAFP8.

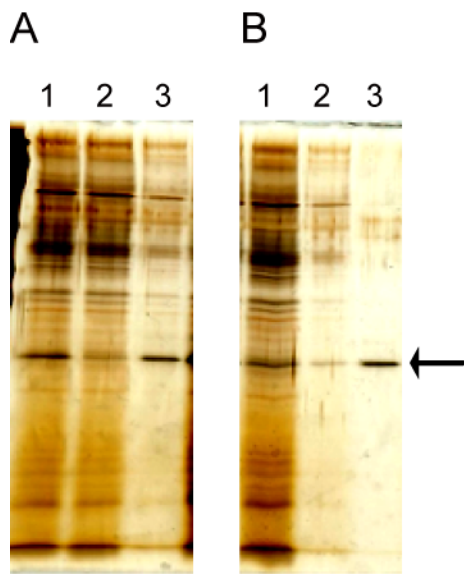


Fig. 4-3. SDS-PAGE showing the purification of NagAFP8. (A) The 1st round of ice-affinity purification (IAP). lane 1, crude culture; lane 2, liquid fraction; lane 3, ice fraction. (B) The 2nd round of IAP. lane 1, liquid fraction; lane 2, wash; lane 3, ice fraction. A black arrow indicates NagAFP8 (Mw of 22 kDa).

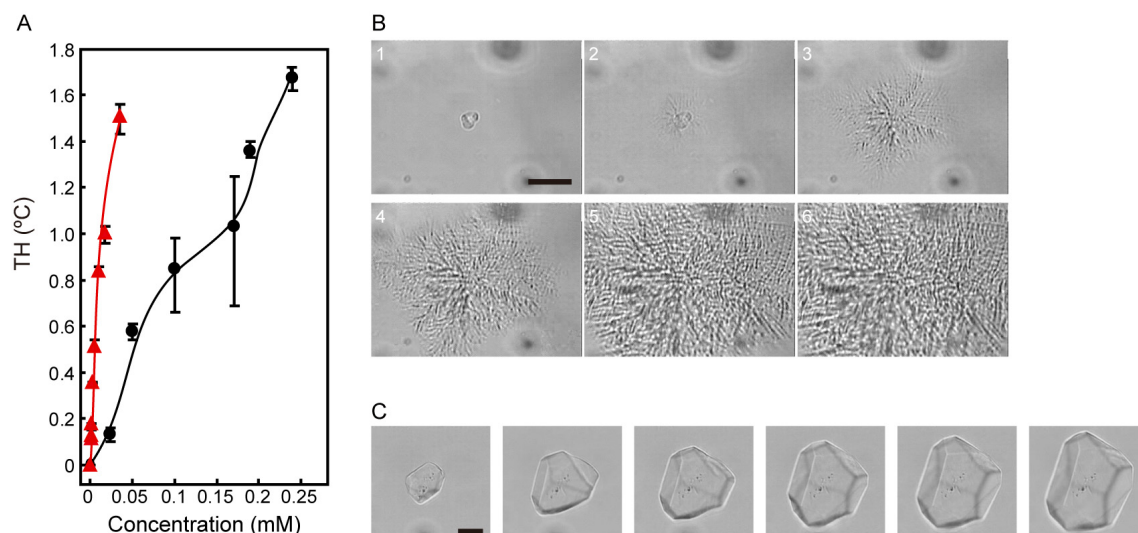


Fig. 4-4. Antifreeze activity and ice growth pattern of NagAFP8. (A) TH activities of NagAFP8 (black circle) and ColAFP (red triangle) as a function of AFP concentration. The TH of ColAFP is shown up to 40 μ M. (B) Ice crystal burst pattern at the end of TH in a 0.24 mM NagAFP8 solution. Pictures were taken at 0.1 s intervals. The scale bar represents 30 μ m. (C) Ice crystal burst pattern at the end of TH in a 0.024 mM NagAFP8 solution. Pictures were taken at 2 s intervals. The scale bar represents 30 μ m.

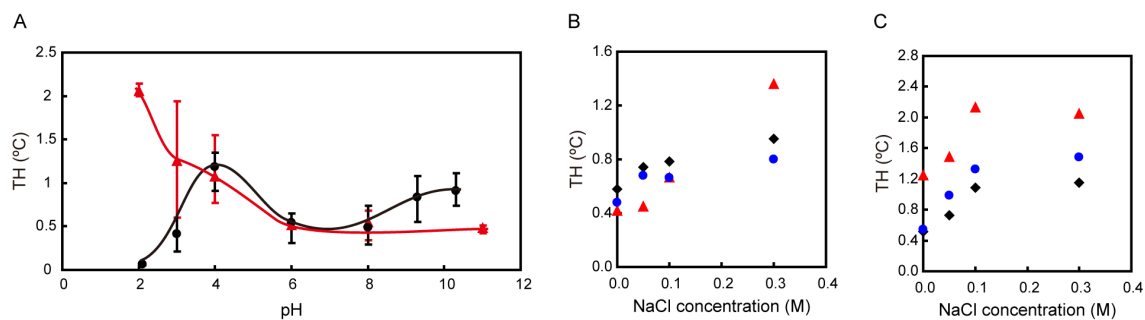


Fig. 4-5. The pH dependence profile of antifreeze activity of microbial AFPs. (A) TH activity of NagAFP8 (black) and ColAFP (red) as a function of solution pH. The protein concentrations of NagAFP8 and ColAFP were adjusted at 50 μ M and 5 μ M, respectively. (B) and (C) Effect of salt (NaCl) at different pH conditions on the activity of NagAFP8 (B) and ColAFP (C). Red triangles, pH 3; black diamond, pH 6; blue circles, pH 8. Protein concentration for each AFP are the same as (A).

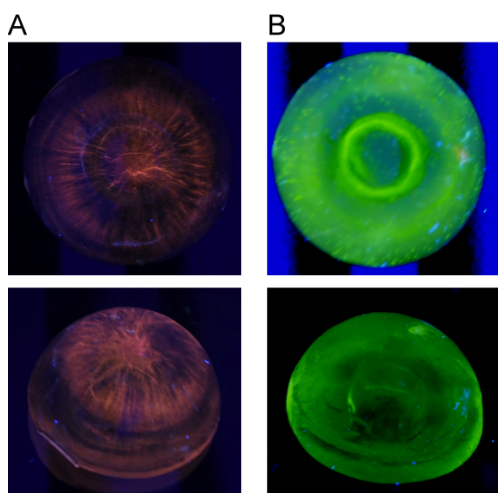


Fig. 4-6. FIPA patterns produced by NagAFP8 wild type and the mutant V220C. Ice hemispheres grown in the presence of tetramethylrhodamine-labeled NagAFP8 wild type (A) and C₅-maleimide-labeled NagAFP8 V220C (B). The ice hemispheres were mounted to the cold-finger parallel to the *c*-axis. The upper pictures were taken from the top of the hemispheres. The lower pictures shows an oblique view of the same hemispheres.

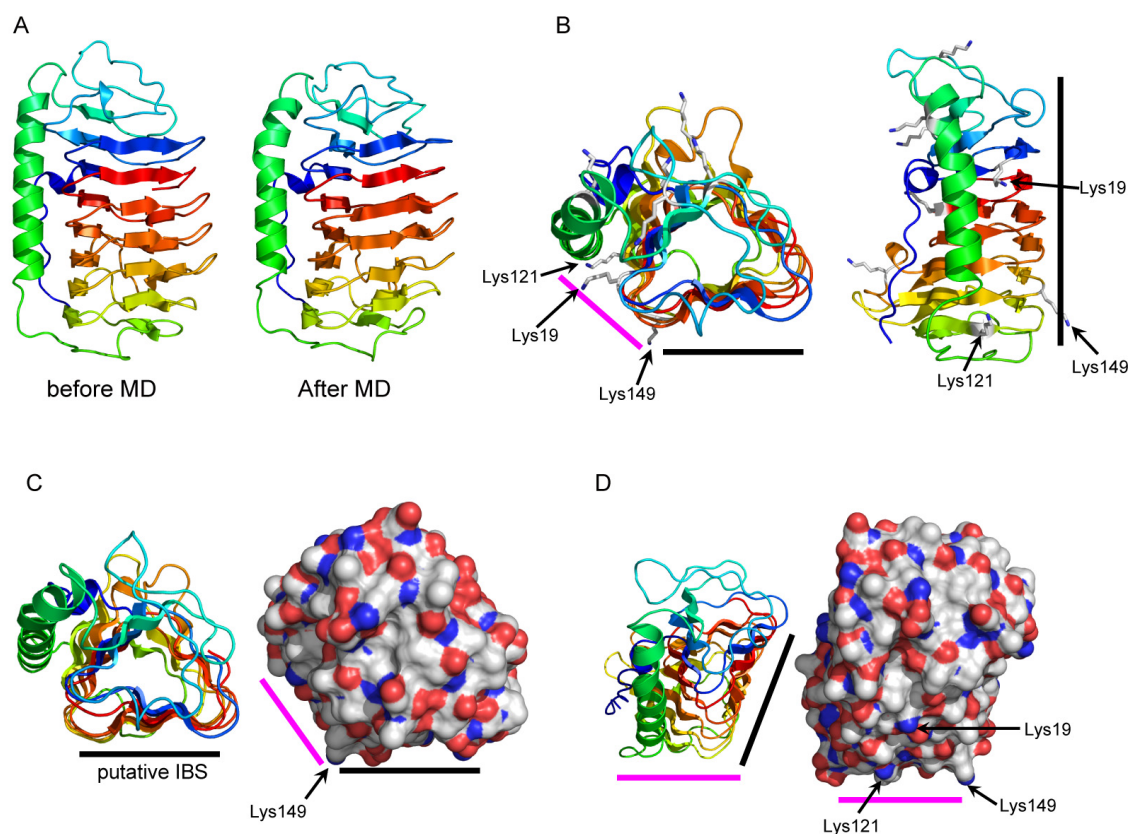


Fig. 4-7. Predicted structure of NagAFP8. (A) Cartoon representation of NagAFP8 model structure before and after 2 ns of molecular dynamics (MD). (B) Top down view along the helical axis of the NagAFP8 structure. The location of Lys residues are shown by sticks. In total, eight Lys are out there. Blue, nitrogen atoms; gray, carbon atoms. The black line indicates the putative IBS region deduced in TisAFP6. The pink line shows another candidate for the IBS. (C) Top down of the NagAFP8 structure, same as B. The surface structure viewed the identical direction to the left picture was shown. Blue, nitrogen atoms; gray, carbon atoms; red, oxygen atoms. (D) Cartoon and surface representations of another IBS candidate. The locations of Lys residues are shown by arrows.

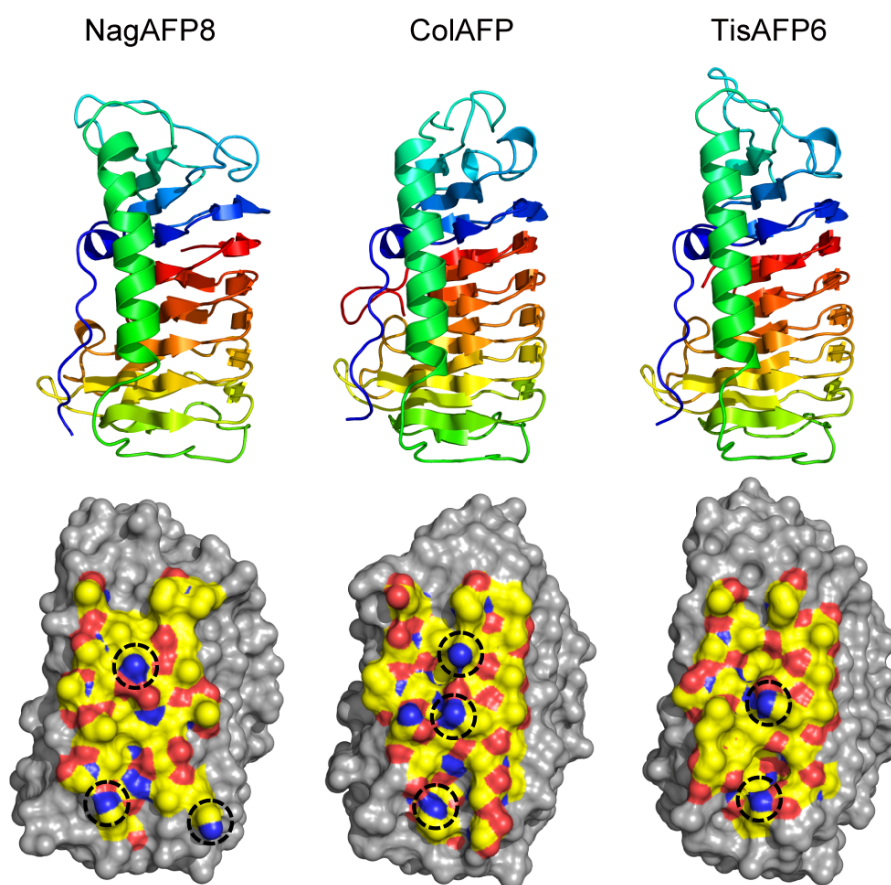


Fig. 4-8. Surface structures of the putative IBS of NagAFP8 and comparison to the corresponding sites of its homologs. Cartoon representations of NagAFP8, ColAFP (3WP9), and TisAFP6 (3VN3) (upper panels). The surface structures of the corresponding sites to the upper figures are shown in lower panels with colored putative ice-binding residues. Lys residues within the IBS are highlighted by dashed circles. Yellow, carbon atoms; blue, nitrogen atoms; and red, oxygen atoms.

Chapter 5: Effect of mixing distinct types of antifreeze proteins on their activity

Preface

In this chapter, the author describes the contents of an unpublished paper entitled "Effect of mixing distinct types of antifreeze proteins on their activity" by Yuichi Hanada, Mami Sakashita, Ai Miura, Sakae Tsuda, and Hidemasa Kondo. The Authors' contributions are as follows: Y.H. S.T. and H.K. planned experiments and analyzed data.; Y.H., M.S., and A.M. performed experiments; Y.H. and H.K. wrote the paper.

Abstract

Techniques controlling over the behavior of ice growth have a remarkable potential for profit in a certain area of industry such as preservation of frozen foods, donated bloods and organs, etc. Antifreeze proteins (AFPs), a class of biomolecules serving to protect organisms from freezing injuries by binding to ice, have been expected to be utilized in those technologies, although the mechanism of actions is incompletely established. In the present study, two distinct (fungal and fish type III) AFP types were mixed and examined for the activity in their coexistence to see how they act cooperatively. The fish AFP (nfeAFP6) tested in this study is so weakly active that it can't halt ice growth by itself. It was found that the fungal AFP (TisAFP6), showing a moderately active by itself, effectively helped nfeAFP6 to arrest ice growth. Observations of the shape of ice crystals grown in the mixture clearly indicated that they bind to ice simultaneously. Fluorescence-based ice plane affinity analysis also showed the direct evidence in which the both bind to ice without any competition. Mixing distinct AFP types can be applied to the techniques for producing desirable shape of ice crystals.

5.1. Introduction

AFPs have been realized to have a lot of potential for applications to industrial uses (Nishimiya et al., 2008b). Cryopreservation of cells (Kamijima et al., 2013) and food industries (Zhang et al., 2007), for examples, are known to be the major interest of AFPs' application. In addition, ice templating techniques has been recently recognized as another intended use of AFP application (Deville et al., 2006). Ice templating, also known as freeze-casting process, enable us to produce porous, layered materials just like nacre. In this method, ceramic suspensions are firstly frozen unidirectionally, followed by lyophilizing to remove the liquid portion.

When ice is growing, the expanded crystals disrupt mechanically the organized structure of water-containing materials (Mazur, 1984). AFPs can reduce the damages caused by ice growth through affecting the shape and the texture of those crystals (Knight and Duman, 1986).

The past three chapters (Chapters 2~4) in the present thesis, we have seen that three homologous AFPs (TisAFP6, ColAFP, and NagAFP8) had a different effect on the morphology of ice crystals. It is considered that those distinct feature could lead to a different ice growth habit. Thus, it is interesting to examine how different AFPs affect on ice growth. Moreover, one might conceive that variation of ice growth habit can be generated by mixing different AFP types in a solution. In the present chapter, ice growth habit and TH activity in the presence of both type III AFP and TisAFP6 were examined.

5.2. Materials and methods

5.2.1. Preparation of AFP solutions

TisAFP6 was produced recombinantly and purified as described previously (see [Chapter 2](#)). Purified, concentrated AFP solution was diluted into 2, 20, 50, 100, and 200 μ M at 10 mM MES-NaOH (pH 6.0).

Recombinant protein of type III AFP (nfeAFP6) from notched-fin eelpout was prepared, following the published method ([Nishimiya et al., 2005](#)). The cDNA encoding the mature nfeAFP6 sequence was ligated into the pET20b vector (Novagen, Madison, WI, USA) flanked by *Nde*I and *Xho*I, and transformed into *E. coli* JM109. The plasmid DNA was extracted from the transformants and further transformed into *E. coli* BL21 (DE3). The BL21 (DE3) transformants were cultured at 37°C until OD₆₀₀ reaches at around 0.5-0.8. Then 0.5 M of IPTG was added to the culture to give a final concentration of 0.5 mM and the growth was continued for 24 h at 28°C. Subsequently, the cells were cultivated by centrifugation, suspended by TE (10 mM Tris-HCl pH 8/EDTA) buffer, and disrupted by sonication. The resulting cellular lysate was dialyzed against 50 mM citrate-NaOH (pH 2.9) buffer. The dialyzate was centrifuged and filtrated to remove precipitated materials and loaded onto a cation-exchange chromatography using the Econo-Pack High-S Column (Bio-Rad). The fractions showing antifreeze activity were obtained by elution with a liner NaCl gradient (0-0.5 M). The active fractions were dialyzed against MillQ water and lyophilized. The lyophilized powder was dissolved at 10 mM MES-NaOH buffer to give a concentrations of 0.2 and 1 mM.

The both AFPs adjusted at desired concentrations were mixed at the same volume just before measuring the activity.

5.2.2. Measurements of antifreeze activity

Measurements of TH activity were performed using an in-house photomicroscope system as described in Chapter 2, essentially following the procedure of Takamichi et al ([2007](#)).

5.2.3. FIPA analysis

Purified TisAFP6 solution (1 mg/ml) was dialyzed against PBS (phosphate buffered-saline). Labeling of TisAFP6 was conducted by addition of 100 μ l of 1.25 mM C₅-maleimide (Invitrogen) to the 1 ml dialyzate. The reaction mixture was wrapped with an aluminum foil to avoid being exposed under light and incubated at room

temperature for 2 with rotating. The labeled protein solution was then dialyzed against distilled water to remove unreacted fluorescent molecules.

Purified nfeAFP6 solution (1 mg/ml) was dialyzed against 100 mM NaHCO₃ (pH 8.5). A 50 µl of 10 mg/ml tetramethylrhodamine (Invitrogen) was used for labeling.

5.3. Results

5.3.1. TH activity of TisAFP6 and nfeAFP6 mixture

The TH activities of TisAFP6, nfeAFP6, and their mixed solutions were compiled in a graph (Fig. 5-1). It has been realized that nfeAFP6 itself has no TH activity (Fig. 5-1, green line) but only shows ice-shaping ability in which crystals are modified into the bipyramidal morphology (Fig. 5-2A). In nfeAFP6 solutions, ice crystals grow gradually but slowly and a rapid crystal growth occurs out of the tips of the bipyramid at a certain temperature. This temperature is defined as T_{burst} (Takamichi et al., 2009). The TH activity of TisAFP6 reaches approximately 0.25°C at 90 µM (Fig. 5-1, black line). Surprisingly, TH activity was effectively enhanced in coexisting conditions of TisAFP6 and nfeAFP6 (100 µM). The TH values were exponentially increased by addition of only a 1 µM TisAFP6 to a 100 µM nfeAFP6 solution, and reached a plateau at 25 µM TisAFP6 added condition. The TH values obtained at the plateau were comparable to that produced by TisAFP6 at 90 µM. Moreover, 500 µM nfeAFP6 added TisAFP6 showed a higher TH of around 0.45°C at the entire concentration range tested.

5.3.2. Ice crystal morphology in TisAFP6 and nfeAFP6 mixture

Ice crystals morphologies created under TisAFP6 and nfeAFP6 mixtures at each concentration were monitored in order to see how they cooperatively affect on their morphology (Fig. 5-2). Fig. 5-2A shows that the ice crystals produced by themselves alone. In TisAFP6 solutions, ice crystals retained their original shapes just after melting over a range of TH (Fig. 5-2A, see also Chapter 2). In nfeAFP6 solutions, ice crystals are modified into the so-called bipyramidal shape (Fig. 5-2A, Takamichi et al., 2009). In contrast to those patterns, ice crystals formed in the presence of both AFPs exhibited unique morphologies; they changed depending on the ratio in mixture. A bipyramidal shaped-ice crystal with elongated tips was observed under 100 µM nfeAFP6 supplemented with 1 µM TisAFP6 (Fig. 5-2B). Crystals formed in a solution containing

100 μM nfeAFP6 and 10 μM TisAFP6 were modified into an unusual shape, resembling a boat. Ice crystals produced in 100 μM nfeAFP6 solution supplemented with 25/50 μM TisAFP6 tended to be small in size and showed a diamond shape, which evokes those obtained in TisAFP6 solutions at acidic pH conditions (Fig. 2-4B, Chapter 2). In solutions including both AFPs at the same concentration (100 μM), the morphological change of ice crystals was smaller, only a slight extension along the *c*-axis could be detected.

Fig. 5-2C shows that ice crystals produced in the presence of 500 μM nfeAFP6 and each concentration of TisAFP6. It seems nfeAFP6 with 1 μM TisAFP failed to halt the ice growth entirely because the ice expanded along the *c*-axis from the beginning to the end of the TH range. The behavior in which ice grows appeared to be different to that obtained in nfeAFP6 solutions. In nfeAFP6 solution ice crystals expand entirely, retaining their original shape and the constant *c*- and *a*- axes ratio. Except for this condition, in the solutions containing TisAFP6 above 1 μM , ice crystals showed a typical bipyramidal shape which is usually seen in moderately active AFP solutions (Bar-Dolev et al., 2012).

All crystals produced here grew along the *c*-axis when the temperature dropped below the T_f . These results describe above were the reproducible phenomena, not depending on the chance of measurements.

5.3.3. Ice-binding specificity of TisAFP6 and nfeAFP6

The ice planes to which each AFP binds were determined by Granham et al. (2010) for nfeAFP6 and by our group for TisAFP6 (viii, and see Chapter 2). In the present study, FIPA analysis was carried out on their coexisting condition to clarify their difference in ice-binding specificity more closely (Fig. 5-3). It has been originally demonstrated that type III AFP, a well characterized isoform HPLC12 from ocean pout, binds to both primary prism (1-100) and pyramidal (20-21) planes. Similarly, nfeAFP6 is supposed to bind to the identical planes but with less affinity and less coverage area (Fig. 5-3A). TisAFP6 binds to the basal and high latitude area with less affinity for prismatic planes (Fig. 5-3B). In a mixing condition of the both, the ice hemisphere became fluorescent uniformly by sum of the two AFPs (Fig. 5-3C).

5.4. Discussion

5.4.1. Enhancement of TH activity

There are many cases known on the enhancement of AFPs' activity by mixing of additives. One fascinating example is provided by the case of nfeAFP isoforms, nfeAFP8 and nfeAFP6 (Takamichi et al., 2009). A defective isoform of nfeAFP6, which has no TH activity itself but modifies the shape of ice crystals into bipyramidal, was found to exhibit an appreciable TH activity in the presence of only 1% of nfeAFP8 at the molar basis. The authors have hypothesized that nfeAFP8 presumably helps nfeAFP6 to bind to ice crystals irreversibly, since they assumed the latter's binding ability is scarcely irreversible (Takamichi et al., 2009). They also claimed that there was no "protein-protein" interaction in this system from the experimental result obtained by NMR spectroscopy. Another instance would be presented in a series of AFP (DAFP) isoforms isolated from a beetle *Dendroides canadensis* (Wang and Duman et al., 2005). A certain pair of isoforms was found to show a synergistically enhanced activity. In this case, an obvious interaction between isoforms has been detected by the yeast two-hybrid screening. The authors therefore claimed that the interaction between isoforms is essentially important for the enhancement of TH activity and the effect should be stemmed from the larger surface coverage provided by protein complexes than monomer's. As another line of experimental evidence for that the molecular bulkiness of AFP (complex) plays a main role to increase the TH activity, genetically engineered type III AFPs fused with non-antifreeze proteins were studied in late 1990's (DeLuca et al., 1998b). In that study thioredoxin and maltose-binding protein were utilized as fusion partners, which gave resulting molecular weight after fusing with type III AFP (*ca.* 7 kDa by itself) to approximately 20 kDa and 50 kDa, respectively. The measurements of activity for these variants showed that TH levels increased depending on the molecular weight.

In both cases, the processes in which the activity increases must occur naturally in their body fluid (the blood plasma of the fish and the hemolymph of the beetle) except for the last case of type III AFP-fusion proteins. In contrast, the enhanced activity obtained in the solutions containing TisAFP6 and nfeAFP6 is completely artificial. This enhancement effect seems to be synergistic rather than additive, because nfeAFP6 could show the full activity by addition of only a 1 μ M TisAFP6 (Fig. 5-1). In a previous study (Chao et al., 1995), it has been demonstrated that mixing different AFP type (I, II, and III) had no effect on the TH activity. Although the ice planes bound by those AFPs are different as shown in Table 1-1, they share a common ground to not have an affinity

toward the basal plane. In the present study, the basal plane affinity provided by TisAFP6 is most likely to contribute to the enhanced activity of the mixture.

5.4.2. Ice crystal morphology

Obviously, the two AFPs examined do not share the ice planes to be recognized: nfeAFP6 binds to the pyramidal and prism planes, while those targeted by TisAFP6 are basal and some high-order pyramidal planes (Fig. 5-3). Both AFPs could accumulate on the distinct ice planes individually, resulting in a wider coverage of the ice surface than that produced by themselves alone. The unique shapes of ice crystals obtained whole range of mixtures might reflect that multiple binding to ice.

5.5. Conclusion

As shown in Table 1-1, different AFPs have different specificity for ice planes, which might be stemmed from their structural difference. It is widely recognized that fish AFPs produce ice crystals with distinguishable bipyramidal shapes (Chao et al., 1995). Recently, a study has shown that hyperactive AFPs also modify ice crystals into characteristic shapes depending on the type of AFP present (Bar-Dolev et al., 2012). The present study clearly revealed that distinct AFP types can exist together and act independently, thereby generating cooperatively-enhanced activity. On the basis of the findings, one wants to test the effect of different combinations of AFPs on the behavior of ice growth. Such challenges are expected to lead to production of a wider variety of ice crystal shapes depending on the intended use.

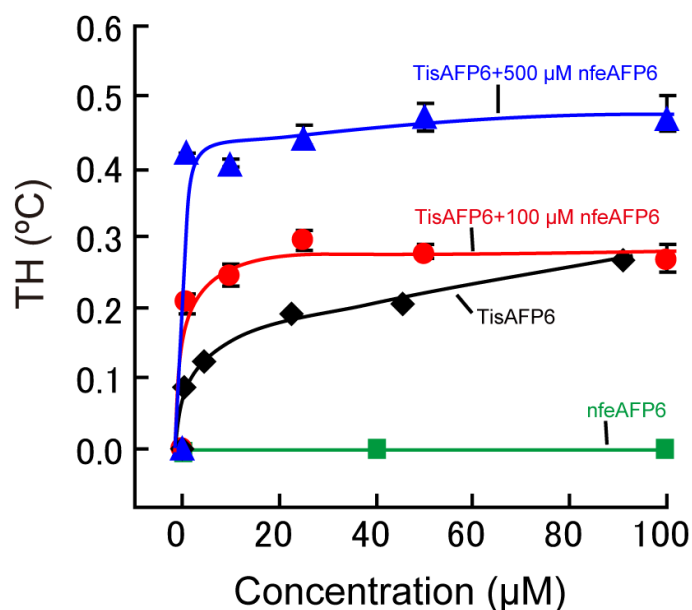


Fig. 5-1. TH activity of TisAFP6 in the presence of nfeAFP6. Effect of mixing different AFPs on their activities was tested in the mixture of TisAFP6 and nfeAFP6. TH activities of TisAFP6 and nfeAFP6 by themselves are shown in the black diamonds and green square, respectively. It is known that nfeAFP6 itself fails to produce any appreciable TH even at millimolar protein concentration (Nishimiya et al., 2005; Takamichi et al., 2009). Measurements of TH at those mixtures were conducted at five different concentrations of TisAFP6 (1 μM, 10 μM, 25 μM, 50 μM, and 100μM) and two fixed concentrations of nfeAFP6 (100 μM and 500 μM). Red circle, TisAFP6 in the presence of 100 μM nfeAFP6; TisAFP6 in the presence of 500 μM nfeAFP6.

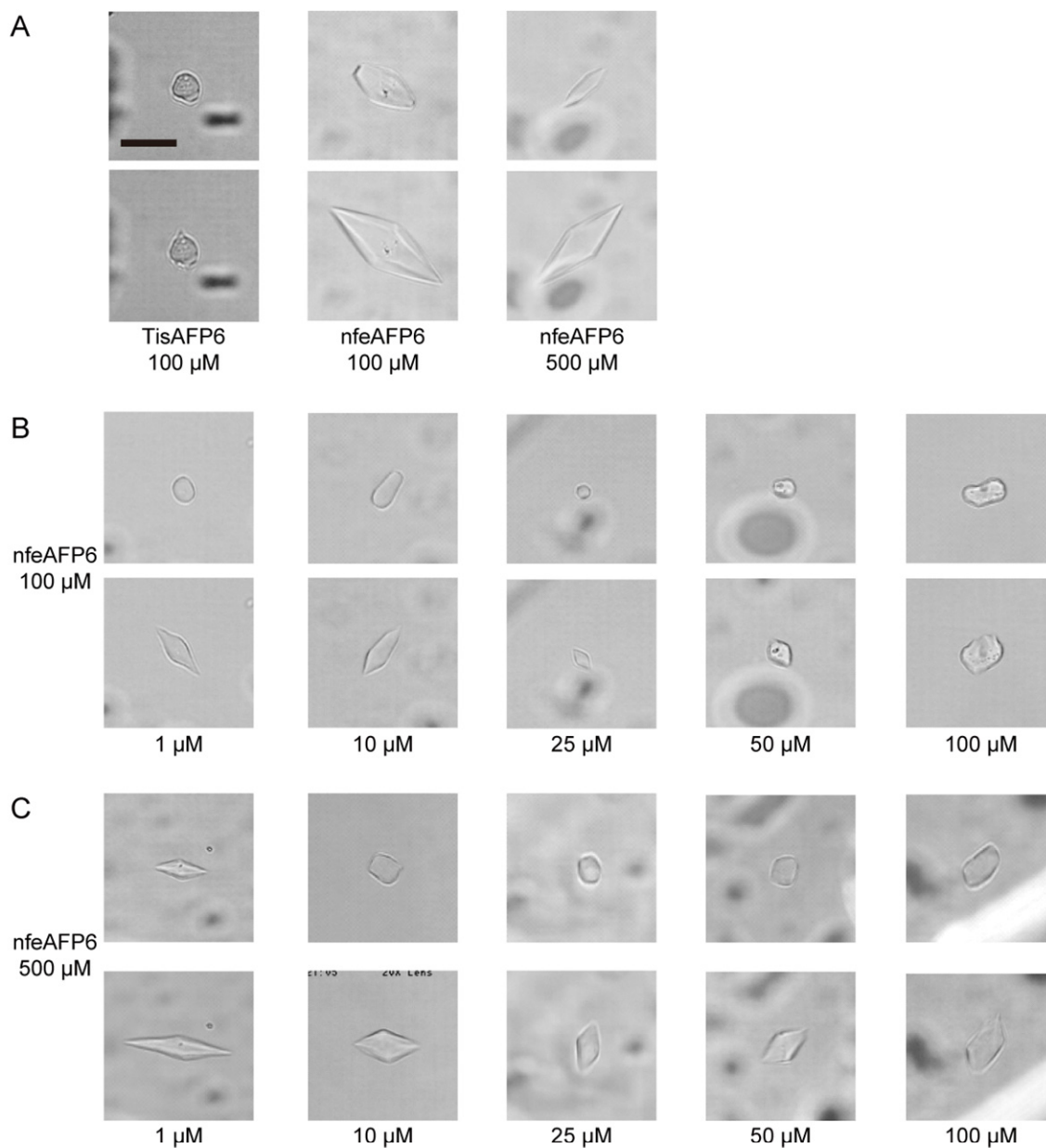


Fig. 5-2. Ice crystal morphology observed in the mixture of TisAFP6 and nfeAFP6.

(A) Ice crystal morphologies created by themselves alone. Typically, TisAFP6 produces ice crystals that do not change their shapes within the TH window. In contrast, the bipyramidal shaped-ice crystals are always observed in nfeAFP6 solutions. Upper pictures show the crystals just before starting to cool. Lower pictures show the crystals just before bursting. The scale bar indicated in the picture of TisAFP6 100 μ M shows 30 μ m in length. These schemes are common throughout this figure. (B) Ice crystals produced by the mixture of TisAFP6 and nfeAFP6 (100 μ M). The concentrations of TisAFP6 at each data point are shown beneath the photographs. (C) Ice crystals

produced by the mixture of TisAFP6 and nfeAFP6 (500 μ M). The concentrations of TisAFP6 at each data point are shown beneath the photographs.

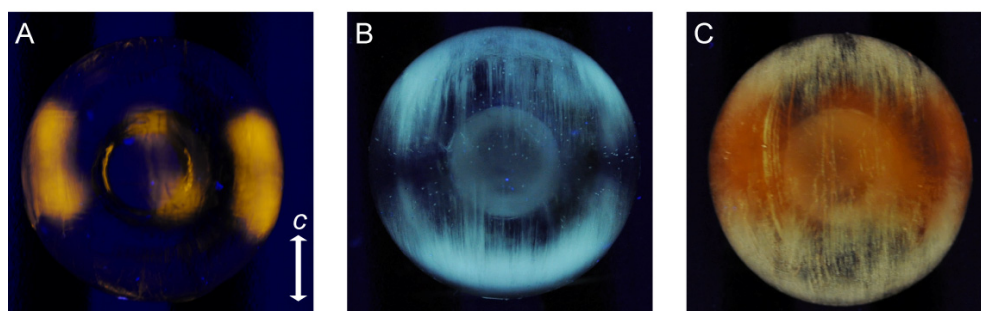


Fig. 5-3. FIPA analysis of TisAFP6 and nfeAFP6. (A) The pattern created by nfeAFP6 labeled by tetramethylrhodamine. This picture was taken by Maya Tamura, a former student of this laboratory. (B) The pattern created by TisAFP6 labeled by C₅-maleimide. (C) The pattern created by the both AFPs labeled by tetramethylrhodamine (for nfeAFP6, orange) and C₅-maleimide (for TisAFP6, light green). The *c*-axis direction of all ice hemispheres shown here is indicated by an arrow within the figure (A).

Chapter 6: General discussion

6.1. Summary

AFPs/IBPs significantly contribute to certain organisms to survive at low temperature environments from sea to land. Biologists, biochemists, and biophysists across the world have been trying to understand the mechanism of action by which AFPs interact with ice for the past decades. Those efforts led us to realize that there are so many distinct protein molecules are involved in and exerting their own ice-binding function. One of the major findings in the recent years might be the newly-developed hypothesis, “anchored clathrate water mechanism” (Garnham et al., 2011), which depicts how AFPs recognize the surface of ice crystals. On the basis of this mechanism, structural determination not only on the protein molecule itself, but also on its hydration waters has been realized to be much important for AFP works. As the authors suggested in that paper (Garnham et al., 2011), the hydration behavior of an AFP might affect on both the concentration dependence (DeLuca et al., 1996) and the time dependence (Takamichi et al., 2007) of AFPs’ activity. From this standpoint, it is interesting to measure the time dependent TH activity of several different AFPs. **Chapter 2** in the present thesis describes TisAFP6 was the least active among the three AFPs (TisAFP6, ColAFP, and NagAFP8). Despite this, it was found to produce a comparable pattern of FIPA to those of ColAFP and NagAFP8. The evidence for the hyperactivity of TisAFP6 became detectable at photomicroscopic level only after 2 hr of annealing. Through these observations, the author concluded annealing of an ice crystal in the AFP solution could provide a time enough to accumulate on the basal plane, which ultimately led to the explosive burst into *a*-axes directions. The series of experiments suggested the possibility in which some AFPs requires a substantial time (up to several hours) to organize water molecules in its IBS into a clathrate structure which merges with the basal plane of ice. In the first paper reported on the time dependent enhancement of AFP’s activity (Takamichi et al., 2007), the authors hypothesized that the AFP (type III AFP) molecules might undergo the second binding to the convex ice front of an ice crystal over a very slow time scale. The description “over a very slow time scale” is now considered as a time required to rearrange the structure of hydration water molecules on the IBS into the one which fits into the arrangement in the convex ice front. Another major finding obtained in the **Chapter 2** would be the pH dependent change in the basal plane affinity of TisAFP6: it binds to the basal plane at neutral pH range, while not at acidic pH. As far as the author knows, this is the first report on an AFP whose ice-binding specificity is affected by solution pH.

It is widely recognized that the development of rapid gene sequencing techniques enables us to easily determine the sequences of a whole mRNAs expressed in a given time and also the genome itself for a wider variety of creatures, especially for microbes. These efforts have greatly increased the number of putative proteins with unknown function in databases. Type I IBPs, which was firstly isolated from a fungus *T. ishikariensis* (TisAFP) (Hoshino et al., 2003), have a growing number of homologues with defined function (i.e., ice-binding), and also relatives with uncharacterized functions in databases. This led me to an idea in which they play an important roles in adapting to harsh cold environments for a wide range of microorganisms. TisAFP (viii) along with LeIBP (Lee et al., 2012) is also known as the proteins whose 3-D structures were solved experimentally for the first time among type I IBPs. Although the two are known to be moderately active, the first structure of a type I IBP showing hyperactivity was solved very recently for the bacterial FfIBP (Do et al., 2014). In order to gain further insight into the structural attributes which make an AFP hyperactive, the author addressed to solve the structure of another type I IBP from the sea ice bacterium *Colwellia* sp. strain SLW05 (ColAFP) in the **Chapter 3**. As expected, ColAFP was found to be folded into the β -helical structure as similar as its homologues. Through the mutational analysis aiming to identify the IBS, it was found that ColAFP has a compound IBS composed of the β -sheet and the adjacent loop area. The IBS of most hyperactive AFPs are generally characterized by repetitive amino acid residues with highly-ordered side chains, which enable them to organize anchored clathrate waters suitable for binding to multiple planes of ice (Hakim et al., 2013). Remarkably, the crystal structure of ColAFP revealed that there is no any repetitive sequences nor side-chains with a aligned rotamer in its IBS. However, a set of partially organized water molecules were found in the IBS of ColAFP. The author therefore concluded that even an AFP lacking any repetitive sequence exerts hyperactivity based on this protein scaffold (the β -helical structure). Fish type II and III AFPs are known to be moderately active, and their IBS is mainly made up by loop structure. To have a hyperactivity for AFPs, β -sheet components regardless of the presence of repetitive sequences seem to be essential. The constant interval of around 5 Å in main-chain provided by adjacent two strands of β -sheet may is more likely to be important, although some AFPs with β -helical (-solenoid) structures are moderately active (TisAFP6, **Chapter 2**; LeIBP, Lee et al., 2012; LpIBP, Middleton et al., 2012). It can be claimed that not every β -helical AFP shows hyperactivity, but all hyperactive AFPs must have β -helical structure. One captivating case deviating from this rule is seen in the structure of hyperactive sfAFP (Pentelute et al., 2008). SfAFP is folded into PPII-like helices structure. Interestingly,

the distance of C α atoms between two adjacent helix of SfAFP is approximately 5 Å, consistent with that of β -sheet. In fact, water molecules organized through backbone atoms are recently recognized to affect on the arrangement of external hydration waters, as Sun et al. (2014) proposed. Therefore, it can be hypothesized that to have backbone distances of *ca.* 5 Å is prerequisite for hyperactivity of AFPs. Yet, the determinant structural features that make an AFP hyperactive/moderately active remains to be investigated, since the case of ColAFP abolished the feasible idea of the structural basis for hyperactive AFPs: highly-repetitive structure is essential.

Sea is one of the world's largest ecosystem since it covers up to 13% surface area of the Earth (Thomas and Dieckmann et al., 2002). As illustrated in Fig. 4-1, microscopic networks of channels filled with brine are formed within sea ice and serve as the habitat of “sea-ice” organisms (Krembs et al., 2002). The abilities to resist and even thrive at harsh cold temperature down to -20°C and high salinity up to 150 g/L (15%) which is equivalent to four times normal sea water are prerequisite for sea-ice organisms. Therefore, AFPs/IBPs from sea-ice organisms can be consider to be halophilic. In fact, sea-ice organisms are most likely to be classified into the “moderate” halophiles which are defined as a preference of salt concentrations of 4.7 to 20% (Ollivier et al., 1994). In general, halophilic proteins are characterized by high contents of acidic amino acid residues (Asp/Glu) with less amount of basic residues (Paul et al., 2008). This tendency can be partially detected in type I IBPs: sea-ice organisms-produced ColAFP and NagAFP8 contain 21 acidic residues in entire amino acid length of 226 (Col) and 221 (Nag), respectively. In contrast, TisAFP6, which has been isolated from the soil-living fungus, has 11 acidic residues in 223. On the other hand, the number of their basic residues is 9 in common. These feature could confer the difference in their activity and be considered as the result of independent evolution from the ancestral gene spread by horizontal transfer. The homology-guided structure of NagAFP8 followed by MD analysis in **Chapter 4** provided an additional case of the structural diversity in the IBS stood on the identical protein scaffold in type I IBPs, which may confer the difference in activity.

As described here and elsewhere repeatedly, type I IBP constitutes a unique class of IBPs with different activities. Taking the case of fish type II AFPs as an example, they have homologous with no antifreeze activity (Loewen et al., 1998): a calcite-binding protein (lithostathine) widely distributed in mammals is known to be the one (Gerbaud et al., 2000). The functional difference among type II AFP-like proteins is considered to be the consequence of convergent evolution from a common gene. In an analogous way, it is possible that some of type I IBP-related proteins have unrelated

functions to ice-binding, considering the existence of widely-spread genes encoding them among various microbes. If the structure-based comparison between an IBP and a protein with no ice-binding activity could be performed, that would provide an informative insight into the structural attributes that bring a protein a specific ability of binding to ice.

6.2. Conclusions

The results presented here have provided remarkable information on structure and function of a new class of microbial AFPs, type I IBPs. As described in the General introduction, the present thesis have set its goal to elucidate the structure-function relationship of this type of AFPs, and thereby understanding what structural features make them diverse in activity. By using the three different AFPs (TisAFP6, ColAFP, and NagAFP8) with distinguishable activity, the goal has been partially achieved: TisAFP6 has a potential affinity toward the basal plane of ice, although it requires a substantial time to attain that binding (**Chapter 2**). The superior activity of ColAFP is considered to be derived from a compound IBS composed of two adjacent structural elements, an extensive parallel β -sheet and a turn connecting each β -strands (**Chapter 3**). NagAFP8 exerts an intermediate activity between the two formers through its own IBS made up of a turn including seven loops with a regular spacing of *ca.* 5 Å at the backbone level and a side of a long α -helix (**Chapter 4**). Taken together, the significant difference in their activity was concluded to be generated by the IBS located at distinct regions in each AFP.

As several lines of evidence ([Bayer-Giraldi et al., 2010](#); [Sorhannus, 2011](#); [Raymond and Kim, 2012](#)) suggested, type I IBPs are more likely to have evolved from a common ancestral gene introduced to the host organisms through horizontal transfer. So many studies including the present thesis so far have revealed their diversity in activity, as listed in [Table 1-2](#). It is, however, unclear what substitutions at amino acid in the evolutionary process confer the specified properties on individual proteins. Understanding of the structure and function relationship along with the evolution would provide not only the evolutionary background of type I IBPs at the molecular level, but also clue as to design artificial proteins with desired functions.

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