STRUCTURE-FUNCTION STUDIES OF A
TRUNCATED ICE-NUCLEATION PROTEIN

By

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Abstract

Bacterial ice-nucleation proteins (INPs) initiate water freezing at high subzero temperatures. Despite having an opposite function to antifreeze proteins (AFPs), INPs and AFPs both have significant similarities in sequence and structure. Based on these common features, I hypothesize that INPs also organize water molecules on their surface into ice-like geometries, as suggested for AFPs. If this is true, an undefined structural characteristic must differentiate ice-nucleation. While AFPs are generally small (<40 kDa) monomeric proteins in vivo, INPs form large (>1 MDa) aggregates on the outer-membrane surface. Therefore, I propose that the distinguishing feature of ice nucleation is a relatively large water-organizing template.

To test this hypothesis, I set out to engineer an AFP from a small INP fragment. A 26.5-kDa recombinant protein was designed by joining two sections of the Pseudomonas syringae InaV INP, one encoding P141-A220 and the other encoding G1021-E1196. CD spectroscopy of this recombinant protein indicated it had β-rich secondary structure that was increased by detergent. The construct shaped ice into a hexagonal bipyramid at high concentrations, but evaluation of antifreeze activity was complicated by trace AFP impurities leaching from shared equipment. The truncated INP in a zwitterionic detergent did show small but significant potentiation of ice nucleation. Taken together, these results are inconclusive about the relationships between INPs and AFPs but point the way to more definitive studies.
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<th>Description</th>
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<tr>
<td>ACW</td>
<td>Anchored clathrate water</td>
</tr>
<tr>
<td>AFP</td>
<td>Antifreeze protein</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>FIPA</td>
<td>Fluorescent ice-plane affinity</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FP</td>
<td>Freezing point</td>
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<tr>
<td>GFP</td>
<td>Green-fluorescent protein</td>
</tr>
<tr>
<td>HG</td>
<td>N-Hexyl β-D-glucopyranoside</td>
</tr>
<tr>
<td>IBP</td>
<td>Ice-binding protein</td>
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<tr>
<td>IBS</td>
<td>Ice-binding site</td>
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<tr>
<td>INP</td>
<td>Ice-nucleation protein</td>
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<tr>
<td>INA</td>
<td>Ice-nucleation activity</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose-binding protein</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular dynamics</td>
</tr>
<tr>
<td>MTAB</td>
<td>Myristyltrimethylammonium bromide</td>
</tr>
<tr>
<td>NLS</td>
<td>Sodium N-lauroylsarcosinate</td>
</tr>
<tr>
<td>OTG</td>
<td>N-Octyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>SB314</td>
<td>3-(N,N-Dimethylmyristylammonio)propanesulfonate</td>
</tr>
<tr>
<td>TH</td>
<td>Thermal hysteresis</td>
</tr>
<tr>
<td>TW20</td>
<td>Polysorbate 20 (or Tween 20)</td>
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CHAPTER 1: INTRODUCTION

1.1 Ice-Binding Proteins and Antifreeze Proteins

Ice-binding proteins (IBPs) from polar- and temperate-zone-residing organisms serve a number of different roles in nature (Figure 1.1). Of these, antifreeze proteins or AFPs (Figure 1.1A) are the best characterized to date. First discovered nearly a half century ago in Antarctic Nototheniidae blood plasma (1), AFPs from a number of species have since been isolated, including fish, insects, plants, and microorganisms (2–5). Generally, AFP-producing organisms are those that encounter subzero temperatures at some time during the year. By adsorbing to small ice crystals, AFPs minimize structural damage that would occur with continued freezing.

1.2 AFP Function Described by Absorption-Inhibition

In the presence of ice, antifreeze proteins depress the freezing temperature of water below the equilibrium freezing point. This phenomenon can be explained by an adsorption-inhibition mechanism (6). AFPs bind the surface of a growing ice crystal, restricting its growth to unbound regions. This adsorption results in a localized positive curvature that is thermodynamically opposed to growth, as described by the Gibbs-Thomson effect. Rapid, 'burst'-like growth will occur at a reproducible point during further supercooling.
Due to the irreversibility of the AFP–ice interaction, ice crystal melting produces a negative surface curvature between bound AFPs that results in melting hysteresis (7, 8). The combined temperature difference between the non-equilibrium melting and freezing points is termed the thermal hysteresis (TH) gap. The ability to produce TH is the defining feature of AFPs (Figure 1.2). Freezing point depression is the main contributor to TH activity.

Figure 1.1 – Roles of confirmed IBPs in nature. (A) Antifreeze. A circulatory channel (white) enclosed by a capillary wall (thick green line), with AFPs appearing as red dots. Inset shows AFPs bound to an ice crystal surface (blue). (B) Ice recrystallization inhibition. Ice crystal grains are indicated as hexagons of various colour and geometry. Inset shows AFPs (red dots) again bound to the ice crystal surface, producing either positive (top) or negative (bottom) surface curvature. (C) Ice structuring. AFPs produced by a microorganism (‘Alga’, green ellipse) are secreted into extracellular fluid (white) and bind to surrounding ice (blue). (D) Ice adhesion. An ice-binding adhesin on the bacterial cell surface (yellow ellipse) binds to the underside of lake ice (blue bar at top). Regions of the ice adhesin are indicated by Roman numerals (I to V). This image was generously provided by Davies (2).
Figure 1.2 – AFPs produce thermal hysteresis. Left of the temperature scale shows a single ice crystal in the absence of AFPs. Melting and freezing points are in thermal equilibrium. Right of temperature scale shows an ice crystal in the presence of AFPs. AFP-bound ice becomes faceted, freezes at a lower temperature, and melts at a higher temperature. The temperature gap produced by AFP-ice adsorption is termed thermal hysteresis. This image is courtesy of Davies (2).

1.3 Ice-Crystal Geometry and Relevance to AFP Function

Under atmospheric pressure and temperature, water freezes into a hexagonal prism with six-fold rotational symmetry (Figure 1.3). The c-axis runs perpendicular to the basal plane, while the three a-axes run through each vertex of the basal face.
Figure 1.3 – Ice-crystal geometry. (Left) Schematic representation of hexagonal ice (thick solid lines). The $c$- and $a$-axes are indicated as thin dashed lines with arrows at either end. (Right) Hexagonal ice crystals, with basal planes (top in red), primary prism planes (middle in blue), and secondary prism planes (bottom as dashed lines) indicated. The $c$-axis orientation for each ice crystal on the right is included.

AFPs can be one of two activity types: hyperactive or moderately active. These groups are distinguishable by their specific activities: hyperactive AFPs produce TH at least an order of magnitude greater than moderately active AFPs at the same concentration (9). The molecular explanation for this phenomenon is the difference in ice-plane affinity between hyperactive and
moderately active AFPs. Generally, hyperactive AFPs bind both the basal and prism planes of ice while moderately active AFPs bind only the prism or pyramidal planes (6, 10). This has several implications for the ice crystal (Figure 1.4). First is the ice-crystal shaping: ice bound by hyperactive AFPs is shaped into a hexagonal prism upon melting whereas ice bound with moderately active AFPs is shaped into a hexagonal bipyramid during freezing (11). Second is the ice-crystal burst orientation upon supercooling: ice bound by hyperactive AFPs bursts perpendicular to the c-axis while ice bound with moderately active AFPs bursts along the c-axis (12, 13).

1.4 AFPs Bind Ice by an Anchored Clathrate Water Mechanism

Despite their diverse tertiary structures, all AFPs exhibit TH activity as a result of their surface adsorption to ice. The structural reasoning for adsorption is that the AFP’s ice-binding site (IBS) organizes waters into a "quasi-liquid layer" that organizes water molecules into ice-like arrangements. This has been demonstrated by molecular dynamic (14) and X-ray crystallographic (15, 16) studies. The anchored clathrate water (ACW) bound to the IBS is able to fuse to the quasi-liquid layer of a growing ice crystal (Figure 1.5) in a loose receptor-ligand interaction (14).
Figure 1.4 – Differences between moderately active and hyperactive AFPs. (A) Ice crystal shaping during melting (more positive to the right) for moderately active (top) and hyperactive (bottom) AFPs. Concentrations used are indicated to the right of the AFP name. A 10-μm scale bar for both sets of images is shown at the top right. (B) Ice crystal morphology during supercooling (more negative to the right) for hyperactive (top) and moderately active (bottom) AFPs. Concentrations used are indicated beneath the AFP name. A 250-μm scale bar for all images is shown at the bottom right. Ice-crystal orientation for each 'burst' image is indicated by a black arrow. Temperatures are those recorded when the image was captured. (A) is adapted from Bar-Dolev et al. (11) and (B) from Graether et al. (10).
Figure 1.5 – Ice-like supermolecular assembly of water on the IBS facilitates the binding of AFP to ice. The ice-like waters (cyan layer) of the growing ice crystal (grey) and those organized by the AFP (teal ribbon) fuse, forming new ice with continued supercooling. Image originally produced by Nutt and Smith (14).

Generally the AFP IBS is a flat, predominantly hydrophobic surface of the protein (Figure 1.6). Hydrate cages assume ice-like geometry around nonpolar moieties and are “anchored” to the protein via nearby hydrogen-bond donors and acceptors. These ice-binding motifs are often repeated consecutively over the length of the IBS, organizing anchored waters into regularly structured clathrate superstructures. The anchored clathrate water hypothesis underlies AFP interaction with both ice and gas hydrates (17).
Figure 1.6 – Surface models of AFP ice-binding sites. Labels are as follows: (A) sfAFP, (B) LpAFP, (C) TmAFP, (D) RiAFP, (E) MpAFP, (F) CfAFP. In each case, the IBS is shaded blue. A 10-Å scale bar for each image is shown at the middle right. Image generously provided by Hakim et al. (18).
Ice-nucleation proteins (INPs) are a subset of IBPs that increase the probability of water freezing. A homogenous water sample will freeze at approximately –40 °C (19); INPs can raise the temperature of freezing up to –2 °C (Figure 1.7). This most potent form of ice-nucleation activity (INA) is termed Type I activity; less-active Type II and III nucleators elevate the freezing point to approximately –5 and –8 °C respectively (20, 21).

INPs have been isolated from a number of Gram-negative phytopathogenic bacteria from *Pseudomonas*, *Xanthomonas*, and *Erwinia* genera (22–24). The protein serves two purposes for these organisms. First, inducing freezing at higher subzero temperatures promotes nutrient accessibility by more readily damaging the epidermal layer of plant leaves and exposing underlying tissue (25, 26). Second, bacteria enter the water cycle as aerosols and are distributed over widespread areas through precipitation (27, 28).
Figure 1.7 – Ice nucleators and the freezing point of water. The dashed horizontal line indicates 0 °C. Left of temperature scale shows that in the absence of ice nucleators (here INP), water freezes around –40 °C. Right of temperature scale shows that the addition of INP to water raises the likelihood of a freezing event, with some freezing occurring at subzero temperatures as high as –2 °C.
INPs are the most potent ice nucleators known (5), making them primary candidates for freeze-inducing applications:

- INPs produced in edible yeast could be used to improve the preparation and preservation of frozen foods due to freezing temperature elevation and altered texture of the formed frozen product (29).
- INPs can be used to ensure the quality of freeze-sensitive goods. For example, while shipping medications there is a fine balance between keeping the sample cold but unfrozen. FREEZEmarker® freeze event indicators (TempTime Corp.) change colour below a certain temperature, marking a compromised package.
- Snomax® (SMI Snow Makers AG) and similar products use INPs to produce artificial snow and ice for recreational purposes at ski and snowboard resorts and ice rinks.

One particularly attractive idea is for INPs is to replace silver iodide in cloud seeding. INPs are both more effective at nucleation (30) and more environmentally friendly than silver iodide, currently the most used heterologous nucleator. The crystal-lattice geometry of ice and silver iodide closely match (31). Thus, waters and silver iodide molecules could form a regular hexagonal lattice and, moreover, a sufficiently large silver iodide crystal could act as a template with which waters could interact. When the temperature is sufficiently low, a critical number of water molecules will be of low enough kinetic energy to simultaneously bind the silver-iodide seed crystal, initiate ice formation, and be pulled towards the Earth as precipitation.
*Pseudomonas* and *Erwinia* sp. have been isolated from different snow and cloud water samples (32, 33), suggesting atmospheric INPs already have an important role in promoting precipitation.

### 1.7 INP Domain Architecture and Function

Ice-nucleation proteins identified to date are between 1,034 and 1,567 amino acids in length and, based on sequence comparison, each contains three domains: A non-repetitive N-terminal domain, a repetitive central domain, and a non-repetitive C-terminal domain (N, R, and C domains respectively) (Figure 1.8).

![INP Domain Architecture](image.png)

**Figure 1.8 – Schematic representation of INP domain architecture.** N (red) and C (blue) are the non-repetitive N- and C-terminal domains respectively, while R (green) is the central domain containing between 52 and 81 tandem hexadecamer repeats. Repeat boundaries within the R domain are denoted as black vertical lines.

The N domain is required for cell secretion and surface display on the outer membrane (34, 35). Outer-membrane association is the mechanism by which INPs form MDa-scale clusters at the cell poles (20, 36–39). The secretory mechanism can accommodate relatively long
polypeptide chains (40, 41), making the N domain a viable option for cell-surface display of large fusion proteins.

The C domain is required in its entirety for ice-nucleation activity (INA) in vivo (42). It was previously implicated in membrane association (43). However, more recent reports suggest its role is solely as a capping structure for the C-terminal end of the R domain, as suggested by the potential acid-base complementarity of these regions (Figure 1.9).

The R domain contains between 52 and 81 16-aa tandem repeats (44) and is necessary for INA (45). Repeat composition is similar amongst the INPs (Figure 1.10). Circular dichroism (CD) spectroscopy (46, 47) and molecular dynamics (MD) simulation (48–50) experiments propose that the sequence forms a beta-solenoid similar to those found in hyperactive insect AFPs (10, 51).

Another feature the most recently modeled INP (50) shares with many arthropod AFPs is outward orientation of threonine side chains in the canonical TxT ice-binding motif (where 'x' represents any residue). As this sequence occupies a single planar surface of the protein, it coincides with experimental data that suggests bacterial ice nucleation uses a protein template for water organization.
Figure 1.9 – C-terminal amino-acid sequences from INPs. The sequence segments are from (top) InaZ from *Pseudomonas syringae* pv. *syringae*, (middle) IceE from *Erwinia herbicola*, and (bottom) InaX from *Xanthomonas campestrini* pv. *translucens*. Residue boundaries are noted to the right of the gene name. The first 10 residues at the N-terminal end of each segment are identical. The R domain repeats are aligned and have a green background while the C domain residues have a blue background. Polar and nonpolar residues are denoted in black, while acidic and basic residues are coloured red and blue, respectively.
Figure 1.1 – Three INPs' R domain sequence logos. The R domains of (top) InaZ from *Pseudomonas syringae* pv. *syringae*, (middle) IceE from *Erwinia herbicola*, and (bottom) InaX from *Xanthomonas campestrini* pv. *translucens* are used. Residue boundaries are noted beside the gene name. Each repeat position is denoted in the x axis, and bit score in the y axis. A greater number of bits corresponds to higher conservation. Nonpolar residues are denoted in black, polar residues in green, acidic residues in red, and basic residues in blue.
INPs, like AFPs, most likely use ACWs to organize water into an ice-like pattern. Indeed, in addition to their shared structural features, modeled waters on the putative INP IBS form a regular geometry similar to the crystallographic waters on an AFP’s IBS (Figure 1.11). This ice-nucleation mechanism is analogous to that of silver iodide, except that it makes use of a protein template.

Figure 1.11 – AFP–INP IBS comparison. Top-down perspective of the IBSs from spruce budworm AFP (sbwAFP on left, purple, PDB 1M8N chain A), *Pseudomonas borealis* INP (PbINP in middle, green), and *Tenebrio molitor* AFP (TmAFP on right, light blue, PDB 1EZG chain A). Nitrogen atoms are coloured blue, and oxygens coloured red. Modeled waters (red spheres) on the putative PbINP IBS show a similar hydrogen-bonding (black dashed lines) pattern to those from crystallized sbw and TmAFPs. Image courtesy of Garnham *et al.* (50).

If indeed both AFPs and INPs organize water molecules into ice-like arrangements at their surface, what feature distinguishes ice-nucleation activity from antifreeze? In terms of structure, AFPs are typically small (3-20 kDa), soluble, single-domain proteins (2); in contrast, bacterial INPs are 50x larger, outer-membrane-bound, and form MDa-scale clusters on the
surface of their host (37, 45, 52). The most obvious difference then between these two groups of IBPs is their size.

I hypothesize that the antagonistic functions of AFPs and INPs are distinguished by the respective sizes of their IBSs (Figure 1.12). With antifreeze proteins, a small number of water molecules are organized at a given time, allowing the protein to fuse only with discrete points of an existing ice crystal. Alternatively, ice-nucleation proteins, structured in a rigid, 2D array, might organize sufficient waters to serve as a seed ice crystal.

![Schematic representation of differences in AFP and INP structure-function.](image)

Figure 1.12 – Schematic representation of differences in AFP and INP structure-function. The functional INP IBS (upper left) is much larger than that of any AFP (upper right), and organizes many more waters (blue dots). The functional size of the INP IBS is also increased by anchoring to the outer membrane and localizing at bacterial poles (lower left). In contrast, AFPs (lower right) act individually in the extracellular space. Cell components are labeled where relevant. Image created by collaboration with Koli Basu from our lab.
The Gibbs-Thomson effect relates the melting temperature of a curved surface to its radius in a reciprocal manner. To elaborate, Liu et al. found that the melting point depression relates to the radius by the following equation \( \Delta T = -\frac{50 \text{ nm} \cdot ^{\circ}\text{C}}{r} \) (53). Thus, for a given supercooling, there is a critical radius of curvature at which smaller crystals will melt while bigger crystals will grow. Assuming that ice-like water forms on the surface of either the AFP or the INP IBS, this can serve as a nucleus only if it is bigger than the critical radius for a given amount of supercooling (49). Thus, while INP aggregates a hundred-nanometer in diameter might nucleate ice after only a few degrees of supercooling, AFPs with a size of a few nanometers will nucleate ice at temperatures closer to the homogeneous nucleation point.

1.9 Objective: Engineering an AFP from an INP Fragment

Accepting the hypothesis that AFPs and INPs are distinguished by their respective sizes implies that small INPs might behave as AFPs and, conversely, that AFPs made large enough might become INPs. Evidence for this hypothesis was provided by Kobashigawa et al. (46), who found that a 9.2-kDa recombinantly-produced central domain fragment of the *Pseudomonas syringae* INP had weak antifreeze activity, and by Wilson et al. (54), who found exceptionally high concentrations of type I AFP could promote ice nucleation.

Neither of these examples provides convincing evidence with respect to the "size begets function" hypothesis. With this in mind, I set out to engineer an INP R domain fragment with TH activity. There are a few complications with this approach. First, if indeed the R domain is partially responsible for aggregation as proposed by Garnham et al. (50), then a high number of
GYGS motifs could complicate isolation of monomeric protein. Second, regions of the N and C domains are well conserved amongst INPs, and are important to overall function (42). These segments are likely important for excluding solvent from the minimal hydrophobic core and preventing end-to-end aggregation similar to amyloid plaque deposition in Alzheimer's and Jakob-Creuzfeldt diseases (55). Finally, the N domain may mediate proper folding of the β solenoid. This effect could be direct (56) or indirect, as membrane localization may provide the environment necessary for proper folding of the protein.

To investigate the link between AFPs and INPs, an INP R-domain fragment was engineered that circumvented these challenges. Highly conserved portions of the N and C termini were retained, and terminal repeats used to minimize GYGS content. The recombinant INP$^{aR}$ was larger than that tested previously (46), containing a small N-terminal domain region, eleven central domain repeats, the entire C-terminal domain, and a hexahistidine tag. Experiments were conducted with co-supplementation of INP$^{aR}$ with various detergents, to replicate the membrane-like environment. This technique has been used successfully to restore activity in purified ice-nucleation protein preparations (37, 52, 57, 58).

After iterative preparations, I have determined that the current INP construct does not produce measurable TH activity or ice-plane affinity. A few earlier preparations were shown to have TH and ice binding properties more characteristic of hyperactive AFPs; however, I subsequently determined that these samples were contaminated by trace amounts of hyperactive antifreeze protein that leached from a communal chromatography column. Later preparations performed using purification materials that had never seen another ice-binding protein showed no activity. These results emphasize the importance of using purification materials uncontaminated by antifreeze proteins. Additionally, ice-nucleation assays using INP$^{aR}$ with
detergent reported small but statistically significant INA potentiation with the zwitterionic additive. Taken together, these results could suggest either that the current INP construct does not accurately demonstrate the same water-organization abilities as the native INP, or that there is some unique feature of ice nucleation that distinguishes it from thermal hysteresis.
CHAPTER 2: METHODS

2.1 INP\textsuperscript{trR} Expression and Purification

The INP\textsuperscript{trR} construct was designed with input from Tianjun Sun in our lab, inserted into pET24a vector, and cloned into \textit{Escherichia coli} ArcticExpress competent cells (Agilent Technologies) by Sherry Gauthier. The construct contains two ligated sections of the \textit{Pseudomonas syringae} InaV (PsInaV) ice-nucleation protein: one encoding amino acids P141-A220 and the other encoding amino acids G1021-E1196 (Figure 3.1B). The nucleotide sequence was codon optimized for \textit{E. coli} expression (GeneArt Gene Synthesis, Life Technologies).

Cells containing the INP\textsuperscript{trR} plasmid were streaked from a frozen stock onto an LB-agar plate containing 100 µg/mL kanamycin, and colonies were grown overnight at 37 °C. A single colony was added to 20 mL of LB media supplemented with 100 µg/mL kanamycin and grown overnight at 37 °C with shaking at 210 rpm. Starter cultures were used to inoculate 1-L preparations of the above media, but with an additional 30 µg/mL gentamycin. Cells were cultured at 37 °C with shaking at 225 rpm until an OD\textsubscript{600} = 0.7, at which point cultures were placed in a water-ice slurry for 15 min before expression of INP\textsuperscript{trR} was induced with 1 mM IPTG. Cells were grown for an additional 48 h at 10 °C.

To harvest cells, cultures were centrifuged at 4,000 rpm for 20 min at 4 °C in a JS–4.2 series rotor (Beckman Coulter). The resultant pellet was re-suspended in N buffer (50 mM Tris–HCl, 10 mM imidazole, 0.5 M NaCl, 2% (M/M) glycerol at pH 7.6) supplemented with an
EDTA-free protease inhibitor cocktail tablet (Roche Applied Sciences) and either subjected to sonication or flash frozen for use at a later date.

Cells were lysed by sonication with a Sonic Dismembrator 500 Model (Fisher Scientific). Insoluble material was pelleted at 4 °C by centrifugation for 40 min at 16,000 rpm in a J2–21M centrifuge (Beckman Coulter). The supernatant was syringe-filtered through a 0.45-mm filter (Qiagen GmbH) and mixed with 5 mL pre-equilibrated Ni$^{2+}$-NTA agarose beads. The slurry was incubated at 4 °C with gentle agitation for 1.5 h before being loaded into a column. Unbound material was eluted and the beads were washed with at least 20 column volumes of N buffer prior to the elution step.

To elute the target protein, beads were mixed with four column volumes of NI buffer (50 mM Tris–HCl, 1 M imidazole, 0.5 M NaCl, 2% (M/M) glycerol at pH 7.6) and the mixture was gently agitated for 1.5 h before re-addition to the column. Beads were washed with 2 column volumes of NI buffer to elute residual protein.

The eluted material was diluted five-fold with Q buffer (50 mM Tris–HCl, 1 mM EDTA, 1 mM 2ME at pH 7.6). If the total volume after this step exceeded 150 mL, then the eluted fraction was concentrated to 30 mL using an Amicon Ultra 15 centrifugal filter (GE Healthcare) with a 10-kDa MWCO prior to dilution. A Q Sepharose anion-exchange column (Amersham Biosciences) was pre-equilibrated by washing first with 2 column volumes of QS buffer (50 mM Tris–HCl, 1 M NaCl, 1 mM EDTA, 1 mM 2ME at pH 7.6), followed by the same volume of Q
buffer, and then loaded with the protein solution at a flow rate of 3 mL/min using an AKTA FPLC (Amersham Biosciences).

Unbound protein was removed from the column by washing with 70% Q buffer, 30% QS buffer until the pre-run baseline absorbance was reached; at which point, target protein was eluted using 30% Q buffer, 70% QS buffer. Fractions shown to contain target protein by SDS-PAGE were pooled, concentrated as described above, and dialyzed against working buffer (20 mM Tris–HCl, 2 mM DTT at pH 7.6) for 24 h at 4 °C.

2.2 MBP-INP<sup>trR</sup> Expression and Purification

The INP<sup>trR</sup> nucleotide sequence was excised from its pET24a vector by 1.5-h digestion with NdeI and XhoI restriction enzymes. The INP<sup>trR</sup> insert was separated by agarose gel electrophoresis and solubilized using a protocol outlined in the QIAquick Gel Extraction kit (Life Technologies). The pMal vector containing an N-terminal maltose-binding protein (MBP) construct, supplied to our lab by Cynthia Wolberger's group, was cut using the same restriction enzymes as above and separated from its insert by size. The pMal vector and INP<sup>trR</sup> insert were ligated together overnight at 14 °C.

Success of the ligation reaction was tested by transformation of the product into DH5α competent cells. A single resultant colony was used to inoculate LB media (3 mL) containing 100 µg/mL kanamycin for growth overnight at 37 °C with shaking at 210 rpm. From the resultant culture, 2 mL was used for plasmid DNA preparation according to the protocol outlined in the GeneJET Plasmid Miniprep kit (Thermo Scientific). Plasmid DNA was digested with NdeI
and XhoI for 1.5 h at 37 °C, and the product screened for proper sizes of cleaved DNA. The remaining 1 mL from cultures that yielded proper ligation products was used to make a frozen stock by adding 7% (v/v) DMSO and storing at −80 °C.

Expression and purification of the MBP-INP\textsuperscript{trR} fusion protein were done as described above for INP\textsuperscript{trR}.

### 2.3 \textsuperscript{NC}-GFP Design and Expression

The INP\textsuperscript{NC}-GFP construct produced with assistance from Adam Hassen was designed to contain residues M1-A176 and T1149-E1200 from \textit{P. syringae pv. syringae} InaQ INP, a thrombin protease cut site, and enhanced green-fluorescent protein (Figure 2.2). The GeneArt-optimized nucleotide sequence contained N-terminal \textit{NdeI} and C-terminal \textit{XhoI} restriction sites for cloning, tandem \textit{HindIII} and \textit{EcoRI} cut sites after the A176 codon for protein insertion, and \textit{BamHI} on the N- and C-terminal sides of the CTD so it could be removed in other constructs.

INP\textsuperscript{NC}-GFP DNA was digested with \textit{NdeI} and \textit{XhoI} for 1.5 h at 37 °C and separated by 1% agarose gel electrophoresis for subsequent recovery (Life Technologies). INP\textsuperscript{NC}-GFP DNA was ligated into pET24α plasmid and transfected into DH5α cells as described for INP\textsuperscript{trR}. Plasmid DNA was extracted as before, and the insert screened by restriction enzyme digestion and direct sequencing (London Region Genomics Center, London ON). Plasmid DNA with the correct insert was used to transform \textit{E. coli} ArcticExpress cells (Agilent Technologies).
INP^{NC}-GFP expression is identical to that of INP^{HR}, except that 0.1 mM IPTG was used for induction. Cultures were harvested as above and resuspended in 20 mL PBS buffer (pH 7.6).

Figure 2.1 – Schematic representation of the INP^{NC}-GFP construct. N (red) is the InaQ ice-nucleation protein N-terminal domain (Accession No.: B0FXJ3), C (blue) is the C-terminal domain from the same protein, and GFP is the superfolder GFP (59). Orange denotes a thrombin protease cut site and yellow denotes a hexahistidine affinity tag. Restriction enzyme cut sites present at the DNA level are marked in the relevant positions and are listed in order from 5' (top) to 3' (bottom).

2.4 INP^{N}-GFP Design and Expression

Recovered INP^{NC}-GFP DNA was digested with BamHI to remove the C-terminal domain (InaV aa. 1148-1196) and religated at room temperature for 3 h. The resultant plasmid was transformed into DH5α and ArcticExpress. INP^{N}-GFP expression was identical to INP^{NC}-GFP, except that 0.01 mM IPTG was used for induction instead of 0.1 mM.
2.5 INP\textsuperscript{IR} Concentration Measurements and Reconstitution

To supplement pre-existing INP\textsuperscript{IR} solutions with detergent, 2x detergent stock solutions were prepared (20 mM Tris–HCl, 2 mM DTT, 2% detergent (M/M) at pH 7.6). Nonionic (OTG, HG, TW20) and ionic (SB314, MTAB, NLS) detergents were tested (Figure 2.1). The resultant solution was left in the cold room for 2 h with gentle rocking. Periodically, detergent would precipitate in the tube; in these instances, the sample would be warmed until once again transparent.

All protein concentrations were measured using the A\textsubscript{280} reading of denatured protein. Approximately 150 μg protein was added to >6 M guanidine hydrochloride to a final volume of 600 μL and left at room temperature with gentle agitation for 1 h. The A\textsubscript{280} was measured in triplicate by a Multiskan GO spectrophotometer (Thermo Scientific) and subtracted by basal absorbance of working buffer alone diluted by the same amount of guanidine hydrochloride.

2.6 Circular Dichroism Spectroscopy of INP\textsuperscript{IR}

Samples were prepared by adding equal volumes of purified INP\textsuperscript{IR} in 10 mM Tris–HCl and 10 mM Tris–HCl with 2% (M/M) of the indicated detergent's stock solution. The result was INP\textsuperscript{IR} in 10 mM Tris–HCl and 1% (M/M) detergent. Protein concentrations of these samples were individually measured after preparation, and all treatments fell between 21.3 and 30.2 μM. During data acquisition, each condition was measured six times and the average subtracted by the buffer baseline. Data files written using Proviewer Software (Applied Photophysics) were deconvoluted by OLIS SpectralWorks (Online Instruments).
Figure 2.2 – Chemical structures of detergents used with INP\textsuperscript{trR}. The nonionic detergents used are listed in the top row and include from left to right: N-Octyl β-D-1-thioglucopyranoside or OTG, N-hexyl β-D-glucopyranoside or HG, and polysorbate/Tween 20 or TW20. In the TW20 structure specifically, $w + x + y + z = 20$ subunits (denoted by black arrows). The ionic detergents used are listed in the bottom row and include, from left to right: 3-(N,N-dimethylmyristylammonio)propanesulfonate or SB314, sodium N-lauroylsarcosinate or NLS, and myristyltrimethylammonium bromide or MTAB. All images are courtesy of Sigma-Aldrich.

2.7 Ice-Affinity Purification of INP\textsuperscript{trR}

IAP was performed as described previously (60). Briefly, cells were lysed in 20 mM Tris–HCl, 1 mM DTT, 1 mM EDTA at pH 7.6 and the supernatant was made up to 100 mL with 1% (M/M) detergent (either NLS or OTG). A pre-cooled cold finger with a thin ice layer was submerged into a solution-filled beaker. Temperature was depressed linearly from −1.0 °C to −8.0 °C over 24 h. Formed ice was subsequently melted at room temperature with gentle shaking. The remaining liquid and ice fractions were analyzed by SDS-PAGE.
2.8 Antifreeze Activity of the Truncated INP

Thermal hysteresis and ice crystal morphology studies on INP^{trR} were performed as described previously (61) with minor modifications. Briefly, single droplets of protein sample were flash frozen at -40 °C. Gradual melting produced a single ice crystal that was held at its melting temperature for 10 min to allow protein accumulation on its surface (62). Then, temperature was depressed at a rate of ~20 mOsm per min (1 Osm = 1.86 °C) until the non-equilibrium freezing point was reached. Images were taken with a Panasonic WV-BL200 digital camera at a rate of 30 fps. TH values for three concentrations were measured in triplicate from two separate protein preparations.

2.9 Ice-Plane Affinity of the INP^{trR}

To examine ice-plane affinity, INP^{trR} (3 mg) was labeled with fluorescein isothiocyanate (FITC) in sodium bicarbonate buffer (pH 9.0). FITC-labeled INP^{trR} was isolated by size-exclusion chromatography, resuspended in 30 mL of pre-cooled buffer solution (10 mM Tris–HCl, 80 mM OTG, 700 mM sucrose, and 1 mM DTT), and incubated with a single ice crystal hemisphere as previously described (63). Images of INP^{trR} binding were captured by excitation of the fluorochrome at 495 nm and examination of the ice crystal (flat side down) with a Canon EOS 50D digital camera equipped with a green emission filter.

2.10 Ice-Nucleation Assays of INP^{trR}

Ice-nucleation assays on INP^{trR} were performed as previously described (64). A 2x detergent stock solutions was added to an equal-volume sample 2 h prior to the experiment, and
incubated in the cold room (~8 °C) with gentle rocking. Ten 2-μL drops per sample were pipetted onto a nitrocellulose film that was subsequently placed onto a walled metal plate within a 50% ethylene glycol cold bath. The temperature was depressed to –20 °C using a VWR digital temperature controller at a rate of ~0.2 °C per min, with images being captured at 1-min intervals using a Canon PowerShot SX110 IS9 digital camera (9 Mpx) equipped with a polarizer.

From these images, the temperatures at which individual droplets had frozen was recorded. For the twelve treatment groups, the temperature where 50 and 90% of samples had frozen was determined. To assess significance of the measured differences between sample groups, average freezing temperature and standard deviation for each treatment was also calculated. Twenty to forty droplets were used per treatment, so a t-distribution was assumed. ANOVA and Student's t-tests were performed to determine significant difference between groups within a single buffer condition.
CHAPTER 3: RESULTS

3.1 INP_trR Construct Design

The INP construct tested here (denoted INP_trR) fused two segments from *P. syringae* INP InaV encoding P141-A220 and G1021-E1196 (Figure 3.1A). Ligation of these two sections retained eleven central repeats while removing forty-nine (Figure 3.1B). The retained central repeats preserve fundamental positions of the repeat (Figure 3.1C), yet do not closely conform to the overall consensus for the repeat region (Figure 3.1D). Although the better conserved repeats could potentially organize waters with high regularity, these repeats were not selected due their increased risk of recombining in the bacterial host. Also, their potential to dimerize and perhaps aggregate, a function attributed to the outward-facing, regularly spaced tyrosines (50) would complicate many aspects of the study.

N- (P141-A172) and C- (R1149-E1196) terminal segments necessary for ice nucleation *in vivo* (42) were included in the construct. These regions do not contain high tyrosine content, and thus would not be expected to dimerize. In summary, the INP_trR construct contained an initiator methionine, an N-terminal segment, three N-terminal repeats, eight C-terminal repeats, the entire C-terminal domain, and a C-terminal (His)_6 tag (Figure 3.2). This construct was larger than that previously shown to have slight TH activity (46), but smaller than that which had lost INA activity (45). While only the three N-terminal hexadecamer repeats closely match the consensus, all thirteen retain the most highly conserved residues S(4n), S(12n), and L(14n) (where *n* is an integer number denoting the repeat).
Figure 3.1 – Schematic representation of the INP<sup>trR</sup> construct design. (A) The Pseudomonas syringae InaV INP, accession no. O33479, was used to direct INP<sup>trR</sup> construct design. The 172-residue N-terminal domain is indicated in red, the 952-residue R domain is indicated in green with the 60 hexadecamer repeats indicated by vertical black lines, and the 48-residue C terminal domain is indicated in blue. Domain boundaries are denoted above the figure. (B) The INP<sup>trR</sup> construct was designed by ligating the portions of the InaV INP indicated by dashed lines. Residue boundaries are noted with respect to InaV INP. Briefly, INP<sup>trR</sup> contains an initiator methionine, 80 residues from the N-terminal end of the R domain (32 residues from the N domain and three N-terminal repeats), and 175 residues from the C-terminal end (eight C-terminal repeats and the entire C domain). A C-terminal (His)<sub>6</sub> tag was added to assist with purification. Domain boundaries for this construct are denoted below the figure. (C) The sequence logo for the InaV INP, and (D) for INP<sup>trR</sup>. For both (C) and (D), the position of the repeat is represented in the x axis, while conservation is represented in the y axis (higher bit score indicates higher conservation). In these sequence logos, nonpolar residues are coloured black, polar residues coloured green, basic residues coloured blue, and acidic residues coloured red. Finally, the putative ice-binding sequence motif (IBS) within each repeat is indicated by a thick black underline.
Figure 3.2 – Protein sequence of the INP\textsuperscript{trR} construct. Repeat periodicity is used for showing the N and R domain segments. The N domain segment is in red lettering; R domain segments in green lettering (if from the N-terminal side, dark green; if from the C-terminal side, light green), the C domain segment in blue lettering, and the (His)\textsubscript{6} tag in yellow lettering. Repeats that could potentially organize waters are underlined. (MW) The theoretical molecular weight is calculated; (*) - Assumes no posttranslational modification.

3.2 Expression and Purification of INP\textsuperscript{trR}

INP\textsuperscript{trR}, purified by Ni\textsuperscript{2+}-NTA Agarose affinity chromatography and anion-exchange chromatography, appeared as a single band on SDS-PAGE with an apparent molecular weight just below 35 kDa (Figure 3.3), which is somewhat larger than the calculated mass of 26.5 kDa (Figure 3.2). Atypical migration on SDS-PAGE is a characteristic of some beta-solenoid AFPs (65), but might conceivably be due to posttranslational modification (66, 67).
Figure 3.3 – INP\textsuperscript{trR} expression and purification. MW: Molecular weight standards and their sizes (indicated in the left margin). In the uppermost row, each labeled bracket indicates the purification step from which the fractions were taken. Left to right indicates the order of procedures. SN: Soluble material from the lysate supernatant used for purification. (For Ni\textsuperscript{2+}-affinity chromatography) Ld: sample loaded onto the Ni\textsuperscript{2+}-NTA Agarose affinity column. FT: flow-through from this column. El: eluted fraction. (For anion-exchange chromatography) Ld: sample loaded onto the anion-exchange column. FT: flow-through from this column. El: eluted fraction. The same volume of sample (10 μL) was loaded into each well after mixing with loading dye (5 μL). The INP\textsuperscript{trR} label in the right margin indicates the migration position of the purified product.

3.3 INP\textsuperscript{trR} secondary structure sensitive to Detergent Supplementation

Phospholipid- or detergent-supplementation has been reported to restore INA in purified full-length INPs (37, 52, 57, 58). Thus, the effect of ionic (SB314, NLS, MTAB) and nonionic (TW20, OTG, HG) detergents on INP R domain secondary structure was examined by circular dichroism spectroscopy with INP\textsuperscript{trR} (Figure 3.4). All spectra, even that without detergent, have a
distinct shape compared to the spectrum of INP\textsuperscript{trR} denatured in urea, suggesting the protein was folded to some degree.

**Figure 3.4 – INP\textsuperscript{trR} circular dichroism spectral changes in detergent.** INP\textsuperscript{trR} dissolved in 20 mM Tris–HCl (pH 7.6) and 1% (M/M) of the indicated detergent unless otherwise noted. Protein spectra are the average of six measurements subtracted against a buffer baseline. The red spectrum is for INP\textsuperscript{trR} (26.5 μM) with OTG; orange for INP\textsuperscript{trR} (29.9 μM) with HG; yellow for INP\textsuperscript{trR} (21.4 μM) with TW20; green for INP\textsuperscript{trR} (26.7 μM) with SB314; blue for INP\textsuperscript{trR} (24.0 μM) with NLS; and violet for INP\textsuperscript{trR} (21.3 μM) with MTAB. The black spectrum denotes INP\textsuperscript{trR} (30.2 μM) in 20 mM Tris–HCl without detergent and the dashed black spectrum is INP\textsuperscript{trR} (44.6 μM) with 8 M urea.

Collected spectra were deconvoluted for comparison of secondary structure content between treatments (**Table 3.1**). Nonionic detergents aside from OTG had little effect on the secondary structure compared to the control with no detergent; OTG measurably increased the \(\beta\) content of this construct. Of the ionic detergents, only the complete spectrum for SB314, the
zwitterionic additive, could be collected. When the SB314 spectrum was analyzed quantitatively, it showed a slight increase in helical structure content. The effect of the other ionic detergents, NLS and MTAB, appear similar to SB314 based on the available data.

Table 3.1 – Secondary structure content of INP^frR in various additives. CD spectra for the OTG, HG, TW20, SB314, and no-additive treatments were sufficient for quantitative analysis. Spectra for NLS, MTAB, and urea were unavailable for deconvolution due to poor signal-to-noise resolution at the wavelengths <200 nm. Aside from the control, all detergents were 1% (M/M). The α group is the sum of α and distorted helices; the β group is the sum of β and distorted strands, as well as β turns.

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<td>SB314</td>
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3.4 Ice-affinity purification of INP^frR

Ice-affinity purification specifically isolates proteins with affinity for ice (60). Other proteins and solutes are not incorporated into the growing ice fraction, and remain in solution. Subsequent melting of the ice yields the enriched IBPs, and furthermore, can be used to isolate properly folded forms of the protein (68).
Polycrystalline ice attached to a cold finger was placed into a INP$_{\text{TR}}$ solution with either 1% OTG or 1% NLS present, as both have been used to restore INA in purified INPs in the previous reports (52, 58). Ice was allowed to grow by slow depression of the cold finger temperature. At subzero temperatures, OTG precipitated out of solution, yielding ice with a cloudy appearance and a white precipitate on its surface (Figure 3.5A). When this ice was melted, detergent was reintroduced into solution. Conversely, NLS did not precipitate during IAP (not shown). Analysis of melted ice and unfrozen solution for both IAPs showed INP$_{\text{TR}}$ in both liquid and ice fractions (Figure 3.5B and C). Unknown proteins were also found in both fractions, suggesting they were incorporated into the ice-bound precipitate; as a result, IAP failed to enrich INP$_{\text{TR}}$. 
Figure 3.5 – Ice-affinity experiments of INP\textsuperscript{trR} and detergent. (A) Ice crystal hemisphere following IAP with INP\textsuperscript{trR} in 1% OTG solution. The precipitate was reintroduced into solution during ice crystal melting. (B) Left: IAP with 1% OTG. For this gel; L: Liquid fraction from this IAP with 1% OTG; I: Ice fraction from this IAP. MW: Molecular weight standards, with sizes indicated in the central column. The position to which INP\textsuperscript{trR} migrated is labeled in the left margin. Right: IAP with 1% NLS. MW: Molecular weight standards. L: Liquid fraction from this IAP with 1% OTG; I: Ice fraction from this IAP. Liquid and ice fraction lanes (15 μL total) contain equal volumes from the IAP (10 μL) stained with Coomassie Blue (5 μL). As before, the migrated position of INP\textsuperscript{trR}, is labeled in the right margin.
3.5 Thermal Hysteresis Experiments

To test the hypothesis that size of the water-organizing surface determines ice-binding protein function, INP\textsuperscript{trR} was used in ice-crystal shaping and thermal hysteresis experiments. All treatments contain protein reconstituted into working buffer (20 mM Tris–HCl, 2 mM DTT at pH 7.8). For one treatment, no other additions were made (Figure 3.6A). For other treatments, 1% (M/M) detergent was also present, either OTG, HG, or SB314 (Figure 3.6B, C, and D, respectively). These groups correspond to those seen in CD spectroscopy studies. Additionally, commercially available P. syringae INP (Ward's Natural Science) were also used as an additive (Figure 3.6E). Finally, to control for the fact that removed sections of the INP N-terminal domain might act as a structural cap or promote folding of the R domain, an MBP–INP\textsuperscript{trR} protein fusion was generated (Figure 3.6F). None of these treatments imparted detectable TH activity to the INP\textsuperscript{trR} construct. Some conditions were able to shape ice into a hexagonal geometry upon melting; however, these crystals could not be maintained for the 10-min time span where temperature was 'held' constant. Further, when the hold duration was shortened to one minute, no activity with INP\textsuperscript{trR} was detected. Thus, it is unlikely that INP\textsuperscript{trR} in its current form can act as an AFP.
Figure 3.6 – Influence of purified INP\textsuperscript{trR} protein on ice-crystal morphology. (A) INP\textsuperscript{trR} (500 μM) in solution of working buffer (10 mM Tris–HCl (pH 7.8), 1 mM DTT). Other treatments used for separate experiments contained the same concentration of INP\textsuperscript{trR} in working buffer with either 1% (M/M) (B) OTG, (C) HG, or (D) SB314. (E) INP\textsuperscript{trR} (500 μM) in working buffer with commercially available \textit{P. syringae} (1 mg/mL). The buffer alone did not produce the same shaping (not shown). (F) MBP-INP\textsuperscript{trR} (500 μM) in working buffer. In the bottom left-hand corner, a scale bar denotes 20 μm, and the c-axis orientation for all ice crystals is indicated.
In a few preparations, INPtrR produced TH and ice-crystal shaping resembling a hyperactive AFP. The ice-crystal burst pattern seemed unique amongst AFPs (Figure 3.7A – D), and was highly dendritic at elevated concentrations (Figure 3.7C). Also, shaping occurred during melting (Figure 3.7E and F) as it does for hyperactive AFPs. This preliminary result suggested that INPtrR was somewhat less active than other hyperactive AFPs but much more active than moderately active AFPs at a similar concentration (Table 3.2).

INPtrR preparations with apparent TH activity were examined for their ice-plane affinity. INPtrR was labeled with FITC, resuspended in solution with detergent, and allowed to adsorb onto a single ice-crystal hemisphere. The INPtrR appeared to bind uniformly over the ice crystal (Figure 3.8A). The presence of detergent affected the appearance of the ice crystal in comparison with experiments without detergent (Figure 3.8E and F, respectively). However, the presence of detergent did not adversely affect protein binding to the ice crystal as demonstrated by repeating the ice-plane affinity experiments with a moderately active AFP (Figure 3.8B).

To complete the preliminary activity profile (Figure 3.9), the protein was concentrated to >10 mg/ml and its TH activity examined. The previous levels of freezing point (FP) depression could not be replicated. It was noted that in the highly active samples, MpAFP had been purified on the Q Sepharose anion-exchange column prior to INPtrR purification. MpAFP is highly active, and could produce the observed TH measurements at 0.3% of the sample protein. Further, these proteins run at about the same size on the gel further complicating detection. Because these data could not be reliably reproduced, it was determined that the result was a false positive.
Figure 3.7 – Ice crystal morphology influenced by select INP<sup>trR</sup> preparations. A 20-μm scale bar is included in each image, as well as an indication of c axis orientation. (A) The 'burst' pattern of a single ice crystal formed by INP<sup>trR</sup> (0.03 mM) in OTG buffer (10 mM Tris-HCl (pH 7.8), 80 mM n-octyl-beta-D-thioglucopyranoside, 700 mM sucrose, 13 mM DTT), viewed down the c axis. (B) The same as (A) except with 0.15 mM INP<sup>trR</sup>. (C) The same as (A) except with 0.30 mM INP<sup>trR</sup>. (D) Single ice crystal burst from a sample with protein concentration and conditions identical to (B), but with the ice crystal viewed down one of the a axes. (E) Ice crystal shape formed during melting in the presence of INP<sup>trR</sup> (0.15 mM) viewed down the c axis. (F) The same as (E) except with 0.30 mM INP<sup>trR</sup>. For comparison, the initial ice crystal shaping for (G) MpAFP RIV upon melting and (H) type III AFP upon freezing are shown, courtesy Garnham <i>et al.</i> (15).
Table 3.2 – TH activity comparison between select INP^{trR} preparations and other IBPs.

Measurements for the INP^{trR} were performed in triplicate using two separate preparations. INP96 TH activity was reported by Kobashigawa et al. (46), all other values were reported by Scotter et al. (9).

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<th>Conc. (µM)</th>
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<td><strong>Hyperactive AFPs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TmAFP</td>
<td>8.5</td>
<td>0.063</td>
<td>7.3</td>
<td>0.65-0.76</td>
</tr>
<tr>
<td>Winter flounder type-I-hyp</td>
<td>32</td>
<td>0.05</td>
<td>1.5</td>
<td>0.76-0.95</td>
</tr>
<tr>
<td>MpAFP</td>
<td>58</td>
<td>0.05</td>
<td>0.8</td>
<td>0.76-0.97</td>
</tr>
<tr>
<td>Snow flea</td>
<td>6.5</td>
<td>0.1</td>
<td>15.3</td>
<td>0.25-0.47</td>
</tr>
<tr>
<td>Spruce budworm isoform 501</td>
<td>12</td>
<td>0.075</td>
<td>6.2</td>
<td>0.56-0.72</td>
</tr>
</tbody>
</table>
Figure 3.8 – Ice-binding characteristics of hyperactive-AFP-like INP<sup>trR</sup>. (A) Single ice crystal hemispheres bound by FITC-labeled INP<sup>trR</sup> in OTG buffer (10 mM Tris-HCl (pH 7.8), 80 mM n-octyl-beta-D-thioglucopyranoside, 700 mM sucrose, 13 mM DTT), and (B) FITC-labeled Type III AFP in the same buffer. For comparison, single crystal hemispheres of (C) GFP-tagged MpAFP RIV and (D) Pacific blue-labeled Type III AFP are shown. (E) Non-fluorescent hemisphere incubated in OTG buffer and (F) in PBS, shown for comparison. All hemispheres are
ca. 5 cm in diameter with c-axes perpendicular to the figure plane. This apparent result was one that was observed only once.

Figure 3.9 – Preliminary activity profile for two INP^{trR} preparations exhibiting hyperactive-AFP character. For most points, measurements were performed in triplicate. The maximal TH of these preparations was not determined.
To determine whether INP\textsuperscript{trR} was simply made into a weaker ice nucleator, droplet freezing assays were performed. Treatment groups include INP\textsuperscript{trR} in working buffer (20 mM Tris–HCl, 2 mM DTT at pH 7.8), INP\textsuperscript{trR} in working buffer with 1% (M/M) OTG, INP\textsuperscript{trR} in working buffer with 1% (M/M) HG, INP\textsuperscript{trR} in working buffer with 1% (M/M) SB314. Controls used were each of these buffers alone and each of these buffers with dissolved \textit{P. syringae} (Ward's Science).

To visually compare ice-nucleation efficiency between treatments, the T\textsubscript{50} and T\textsubscript{90} temperatures are reported (Table 3.3). Activity was most potent in whole bacteria whereas values for INP\textsuperscript{trR} and buffer were similar. The larger detergents, OTG and SB314, slightly weakened the ice-nucleation activity of dissolved \textit{P. syringae} extracts.

Table 3.3 - Ice-nucleating temperatures T\textsubscript{50} and T\textsubscript{90} for buffer (left column), INP\textsuperscript{trR} (middle column), and \textit{P. syringae} (right column). T\textsubscript{50} and T\textsubscript{90} values are the average of at least two separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Buffer</th>
<th>INP\textsuperscript{trR} (0.5 mM)</th>
<th>\textit{P. syringae} (1 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T\textsubscript{50} (°C)</td>
<td>T\textsubscript{90} (°C)</td>
<td>T\textsubscript{50} (°C)</td>
</tr>
<tr>
<td>No Additives</td>
<td>-6.94</td>
<td>-7.39</td>
<td>-7.05</td>
</tr>
<tr>
<td>OTG</td>
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<td>-7.35</td>
<td>-6.35</td>
</tr>
<tr>
<td>HG</td>
<td>-7.2</td>
<td>-7.73</td>
<td>-7.35</td>
</tr>
<tr>
<td>SB314</td>
<td>-7.03</td>
<td>-7.53</td>
<td>-6.68</td>
</tr>
</tbody>
</table>

To assess the significance of the differences between buffer and INP\textsuperscript{trR} treatments, the T\textsubscript{avg} and standard deviation of these samples group was calculated. Between twenty-nine and
forty samples were used for each group, thus a Student's $t$-distribution was assumed. The difference in ice-nucleating temperature between INP$^{trR}$ and buffer was significant in the case of SB314 supplementation ($P < 0.01$) but not for any other treatment, even at a less stringent significance threshold ($P > 0.05$).

**Figure 3.10 – Ice-nucleation activity of INP$^{trR}$ with detergent supplementation.** The average freezing temperature ($T_{avg}$) was calculated for four differently supplemented INP$^{trR}$ samples (right bars; dark grey) and their corresponding buffers (left bars; light grey). The number of samples used for each dataset is included within the appropriate bar, at the top of the plot. Significant difference between datasets is indicated by an (*) (significance level $P < 0.01$).

3.8 Polar Localization is a Function of The N-Terminal Domain

The role of the INP N-terminal domain was investigated by visualizing the subcellular localization of GFP-fusion proteins. NC and N constructs were fused to superfolder GFP and
expressed in *E. coli* ArcticExpress at 10 °C. Protein expression was induced and aliquots of cell cultures fixed to microscope slides. Protein was visualized by fluorescence microscopy. In both INP^N^ and INP^{NC} fusions, most bacteria featured a GFP spot localized at either cell pole (Figure 3.11). This result suggests that the N domain alone is sufficient for localization. In some cells only one spot could be seen, and a few bacterial cells had no GFP at either pole; the latter case was more often observed in INP^{NC} which was induced with less IPTG. In the cases where cells had GFP spots at both poles, one was somewhat brighter than the other.

![INP^N−GFP and INP^{NC}−GFP](image)

**Figure 3.11** – INP^N− (left) and INP^{NC}−GFP (right) polar localization in *E. coli* ArcticExpress grown at 10 °C. Induced cultures (2 μL) were fixed to the microscope slide with DAKO mounting medium (5 μL). To visualize superfolder GFP, images were captured with excitation at 485 nm and emission at 510 nm. Whole cells are outlined by a dashed white line. The scale bar in the bottom left corner of each image indicates 2 μm under the microscope magnification.
CHAPTER 4: DISCUSSION

4.1 R Domain Structure

Despite INPs having been discovered over 40 years ago (23), details of the INP structure have remained elusive. Characterization is made challenging due to several factors, namely INP size, repetitiveness, membrane association, and propensity to aggregate. To aid protein preparation, the INP\textsuperscript{trR} construct was designed without sequence elements that might be responsible for some of these functions, such as the N domain and the higher identity central repeats. This approach however could have also compromised INP function. For example, in a recently discovered AFP, a conserved rank of seven tyrosines was predicted to make up the putative IBS (68). Thus, it is plausible that the N domain by itself is sufficient for INP multimerization, and that the conserved tyrosine of the INP hexadecamer repeat is actually a component of its water-organizing surface. It would follow that higher GYGS content would be necessary to produce a proper ice-binding protein.

Multiple reports have suggested that the INP R domain is rich in β sheet content (47, 69). The data herein agrees with this previous work, and establishes that the structure of INP\textsuperscript{trR} with or without detergent is rich in β–structure and distinct from the denatured protein. It is unclear whether the changes in secondary structure content are biologically relevant. However, a number of reports suggest this is likely, with reconstitution of INA in purified, full-length INP being dependent on phospholipid or detergent supplementation (37, 52, 57, 58, 68).

The latest INP model (50), inspired by some of the most recently discovered hyperactive AFPs, is folded into a rectangular β solenoid. However, the modeled section was only eight
repeats long and it is assumed that this pattern continues throughout the central repetitive region. Modeling the structure as a dimer suggests that even if one flat surface is needed for membrane association a second identical flat surface would be available for organizing waters. If the role of the outer membrane is to structure a flat IBS, it might not be surprising that lower activity of purified preparations occurs due to twisting about the β-helical axis. It follows that detergent micelles and phospholipid vesicles might mimic this role of the membrane. Another concern is the discord between expected and calculated sheet content. Based on the Garnham model, the theoretical β content should be upwards of 80%. My CD analysis however places it is closer to 60%, which is the highest value reported for an INP to date. There are, however, a couple reconciliations to take into account: first, the lower identity repeats at the N- and C-terminal ends of the R domain might have a greater amount of unstructured character; second, the C-terminal domain is likely to have its own unique secondary structure.

4.2 N Domain Function

The fact that the N-terminal domain alone is sufficient for cell-pole localization on the outer membrane of Gram-negative bacteria implies that it is responsible for both secretion and localization. Little is known about the INP secretion mechanism; however Li et al. (35) found the first 18 amino acids to be essential for this activity. Consolidating information about the INP-lipopolysaccharide relationship (57, 66) and the low-temperature dependency of INP-cluster formation (20, 36), it is likely that under sufficiently cold conditions, the INP gene is expressed, protein is pre-fused to periplasmic vesicles, and exocytotic vesicles are targeted to outer-membrane poles. For plant biotechnologists, this secretion pathway might be a viable antibiotic
target to diminish the crop-damaging potential of *P. syringae*; furthermore, it may be effective against other Gram-negative human pathogens.

Capping structures are important to β-solenoid structures as they block end-to-end aggregation, protect the generally minimalist core, and in some cases direct protein folding (56). Thus, atomic structure determination of INP\textsuperscript{N} could be used to determine the necessary capping structure for the INP R domain, or direct engineering of soluble INPs. Solution-NMR with either bilayer nanodises or detergent micelles is the best-suited technique for this job due to the non-repetitive nature, relatively small size (<20 kDa) and outer-membrane association of the INP N domain.

4.3 INP Complex Size

Multiple reports have concluded that bacterial ice-nuclei are several MDa in size, and that larger ice nuclei generate greater INA (37, 70). Several groups have considered the molecular mechanism that drives formation of these superstructures. A couple of research groups have found that a low growth temperature is of primary importance in generating highly active type I ice-nuclei (39, 71). Other reports have suggested transition to more active preparations are glycerophospholipid- and carbohydrate-dependent (66, 67). A compromise might be that certain membrane constituents or lower temperatures promote large-scale assembly of INPs. For example, lipid raft formation or decreased membrane fluidity at lower temperatures could explain the temperature-dependency of INP clustering.
Although there are some data on the size of these superstructures, it is not known how they are organized at the molecular level or how they are oriented on the membrane in vivo. The size of these superstructures and the fact their activity is dramatically reduced when removed from their native environment makes the full-length INP an ideal candidate for cryoelectron microscopy. Another interesting possibility here is that the N-terminal domain might promote or facilitate end-to-end aggregation of INPs in order to achieve the necessary complex size, similar to engineered TmAFP fibrils produced by Peralta et al. (72).

4.4 Purification of IBPs

Results in this report are an important and unfortunate reminder of how readily IBP samples can be subject to contamination. A hyperactive AFP contaminant at <10 μM could produce a high degree of TH activity that would yield a false positive in the characterization of a novel AFP. Based on SDS-PAGE and marginal activity, this might be relevant to the previously characterized TH-active INP fragment (46). In my thesis research, one of the most potent hyperactives, MpAFP, is the culprit. This protein was used on the purification column preceding INPtrR purification. Based on data from Scotter et al. (9), the contaminant need not be present at high concentrations; 0.8 μM MpAFP (~ 0.3% of the total protein) could produce the maximal values observed herein. Also, MpAFP runs at ~35 kDa on SDS-PAGE, roughly the reported site of INPtrR, further complicating detection.

To circumvent the contamination problem, our lab is equipped with a Q Sepharose column specific for low-activity IBPs. I was made aware of this column after the initial observation of hyperactive-AFP-like activity, at which point previous results could not be
replicated. I propose new students be made aware of this column during their first week in the lab, and use it exclusively for INP purification. However, if a sample of unknown activity is run on this column and then later discovered to have hyperactive antifreeze activity, the column would have to be rigorously cleaned or replaced. Clearly, novel or low-activity IBPs require their own specific resources. Viable options include free anion-exchange beads (Streamline) or initial-capture anion-exchange columns (Bio-Rad). Only once a protein is determined to have TH activity can it be run on refining columns, as having a somewhat heterogeneous product would not interfere with this initial characterization.

4.5 The difference between antifreeze and ice-nucleation

The goal of this research was to elucidate the underlying difference in function between ice-nucleation and antifreeze activities. INPs and AFPs share several structures features; most intriguingly a repetitive, extended template with amino-acid composition that suggests the ability to organize waters into anchored clathrates on their surface. The most obvious difference in these two proteins is their functional size, with AFPs generally being monomers < 40 kDa in size and INPs being multimers of MDa-scale. My hypothesis was that this size difference manifests as a difference in the number of water molecules organized by these proteins. AFPs bind fewer waters, thus forming a small quasi-ice surface; conversely, INP aggregates form a quasi-ice surface sufficiently large to act as a seed crystal for further crystallization. It follows that AFPs made sufficiently large would be ice-nucleation active and INPs made small enough would have antifreeze activity.
The biggest shortcoming of this hypothesis is native, purified INPs and fragmented INPs have not shown convincing (> 0.1 °C) TH activity even at high concentrations. Moreover, my results from ice-nucleation assays with detergent suggest even truncated INPs could retain activity, which is similar to results of previous studies. For example, Green et al. (45) found that removal of thirty-four 16-aa repeats was sufficient for elimination of ice-nucleation activity. I could envision a couple of explanations for this phenomenon. The first is that INPs order water molecules with greater regularity than AFPs. In this model, surface waters are more ice-like than liquid, which makes them incapable of fusing to an existing ice crystal. This is supported by the AFP-inhibition of ice nucleation, where AFPs presumably bind to the INP seed crystal, thereby limiting its effective size (73, 64, 74). The second factor lies in the other major difference between AFPs and INPs: INPs are universally vesicle- (75) or membrane-associated (76). Our current understanding is that the membrane allows clustering of a sufficiently large number of INPs. However, in addition to localization, a two-dimensional membrane might also structure or even partially make up the ice-binding template. This would explain the large decrease in activity between purified INPs, which only show class III activity, and membrane-bound INPs, which show more potent class I or II activities. I found this effect could not be replicated by adding bacterial lysate to the INP<sup>trR</sup> solution; thus either the preexisting INP impeded INP<sup>trR</sup>-membrane interaction, or the association cannot happen when the two molecules are separated. An experiment where the INP<sup>trR</sup> or a similar truncation is fused to the N domain and assessed for both TH and ice-nucleation activities would elaborate on the membrane-structuring hypothesis.
4.6 Conclusions and Contributions

- INP$^{\text{hrR}}$ secondary structure is mostly β-sheet.
- INP$^{\text{hrR}}$ influences ice crystal morphology at high concentrations, but does not produce freezing point depression.
- Additives such as OTG and SB314 affect INP$^{\text{hrR}}$ secondary structure, but neither these nor bacterial membrane extracts promote freezing point depression.
- SB314 supplementation enhances ice-nucleation activity of INP$^{\text{srR}}$, as determined by the Student's $t$-test. The sample size for this experiment was relatively small (40), so additional experiments should be done to confirm this result.
- Low-activity IBPs should be isolated separately from hyperactive AFPs, perhaps with their own respective purification material.
- The N-terminal domain alone is sufficient for secretion to the extracellular environment and localization at the poles of the outer membrane. This role might be integral to INP R domain structuring.
BIBLIOGRAPHY


