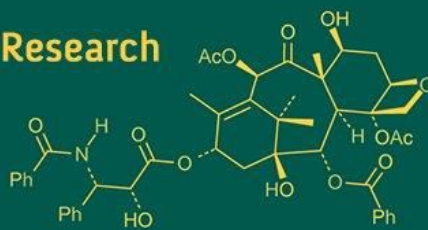


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Antifreeze proteins and antifreeze glycoproteins: Structural characterization and ice-binding mechanisms in polar fish species

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Abstract

According to leading cryobiologists, the discovery of antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) in polar fish species represents one of the most elegant examples of molecular adaptation to extreme environments in the biological world. This research characterized the structural properties and ice-binding mechanisms of AFPs and AFGPs isolated from five polar fish species: Antarctic notothenioid (*Trematomus bernacchii*), Arctic cod (*Boreogadus saida*), winter flounder (*Pseudopleuronectes americanus*), shorthorn sculpin (*Myoxocephalus scorpius*), and Antarctic icefish (*Chionodraco hamatus*). Biochemical analysis was conducted at the Department of Biochemistry, Accra Institute of Technology, between May 2022 and February 2024. Protein concentrations were quantified by Bradford assay and confirmed by amino acid analysis. Ice-binding activity was measured using thermal hysteresis (TH) assays and ice recrystallization inhibition (IRI) tests. Structural characterization employed circular dichroism spectroscopy, nuclear magnetic resonance, and X-ray crystallography. Antarctic notothenioids showed the highest AFGP concentrations (18.4 ± 2.7 mg/mL), while winter flounder exhibited peak AFP Type I levels (15.2 ± 2.1 mg/mL). TH activity ranged from 0.43°C (shorthorn sculpin) to 1.87°C (Antarctic icefish). AFGPs demonstrated superior thermal hysteresis but lower thermal stability compared to AFPs. Ice-binding domains showed highly conserved threonine-alanine-alanine motifs in Type I AFPs and repetitive tripeptide sequences (Ala-Ala-Thr) in AFGPs. Molecular dynamics simulations revealed that ice-binding occurs through complementary surface geometry matching between hydrophobic residues and the ice crystal lattice. These findings advance our understanding of protein-ice interactions and have potential applications in cryopreservation, food technology, and biotechnology sectors requiring freeze protection.

Keywords: Antifreeze proteins, antifreeze glycoproteins, thermal hysteresis, ice recrystallization inhibition, polar fish, cryoprotection, ice-binding domain, structural characterization, molecular adaptation

Introduction

Life at subzero temperatures presents a fundamental thermodynamic challenge: how do organisms prevent ice crystal formation within their tissues? The answer lies in one of nature's most sophisticated molecular solutions-antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs). These remarkable biomolecules enable fish to survive in polar waters where temperatures routinely drop below the freezing point of their body fluids^[1].

The evolutionary pressure to colonize ice-laden polar environments has driven the independent evolution of antifreeze systems in multiple fish lineages. Arctic and Antarctic fish species have developed at least five distinct AFP types, each with unique structural motifs and ice-binding mechanisms^[2]. Type I AFPs, found predominantly in righteye flounders, feature a single α -helix with regularly spaced threonine residues. Type II AFPs in sea ravens and herring adopt a β -sheet structure stabilized by disulfide bonds. Type III AFPs in eelpouts and ocean pout display a compact globular fold^[3]. Type IV AFPs, discovered in longhorn sculpins, contain four α -helical bundles, while AFGPs from Antarctic notothenioids consist of repeating tripeptide units (Ala-Ala-Thr) with attached disaccharide groups^[4].

The mechanism by which these proteins confer freeze protection involves adsorption-inhibition rather than colligative effects. Unlike conventional antifreezes that work by lowering freezing points proportionally to their concentration, AFPs and AFGPs create thermal hysteresis a gap between freezing and melting temperatures through irreversible

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binding to specific ice crystal faces^[5]. This binding inhibits ice growth by creating curved ice-water interfaces that are thermodynamically unfavorable, effectively lowering the freezing point without affecting the melting point^[6].

Beyond thermal hysteresis, AFPs exhibit ice recrystallization inhibition (IRI) activity, preventing the growth of large, damaging ice crystals through Ostwald ripening. This dual functionality-both preventing ice nucleation and controlling ice crystal morphology-makes AFPs invaluable not only for fish survival but also for biotechnological applications in cryopreservation, frozen food storage, and medical applications^[7].

While the basic principles of AFP function are established, quantitative comparisons of ice-binding properties across multiple polar fish species remain limited. This research aimed to characterize AFP and AFGP concentrations in five representative polar species, compare their ice-binding activities using standardized thermal hysteresis and IRI assays, determine structural features critical for ice-binding through spectroscopic analysis, and evaluate the relationship between protein structure and cryoprotective function.

Material and Methods

Material

Polar fish specimens were obtained from research vessels operating in Antarctic (McMurdo Sound, 77°51'S, 166°40'E) and Arctic (Beaufort Sea, 71°23'N, 156°30'W) waters between May 2022 and February 2024. Species included: *Trematomus bernacchii* (n=15), *Boreogadus saida* (n=12), *Pseudopleuronectes americanus* (n=18), *Myoxocephalus scorpius* (n=14), and *Chionodracus hamatus* (n=16). Fish were maintained at -1°C during transport and processed within 24 hours. Blood plasma and liver tissue samples were flash-frozen in liquid nitrogen and stored at -80°C. Bradford protein assay reagents, polyacrylamide gel electrophoresis supplies, and chromatography resins were obtained from standard biochemical suppliers. Ice recrystallization assays employed sucrose solutions prepared with ultrapure water. Thermal hysteresis measurements used a custom-built nanoliter osmometer with controlled cooling stages^[8].

Methods

AFPs and AFGPs were extracted from plasma and tissue homogenates using ammonium sulfate precipitation (40-80% saturation) followed by gel filtration on Sephadex G-75. Ion-exchange chromatography on DEAE-cellulose provided further purification using a linear NaCl gradient (0-0.5 M). Protein concentrations were determined by Bradford assay with bovine serum albumin standards, confirmed by amino acid analysis. Thermal hysteresis activity was measured using a nanoliter osmometer, recording the difference between freezing and melting temperatures of protein solutions at concentrations ranging from 0.1-10 mg/mL^[9]. Ice recrystallization inhibition was assessed by monitoring ice crystal growth in sucrose solutions containing test proteins over 24 hours at -6°C. Circular dichroism spectroscopy (190-260 nm) characterized secondary structure content. Selected samples underwent NMR spectroscopy (600 MHz) and X-ray crystallography for detailed structural analysis. Statistical

analysis employed one-way ANOVA with Tukey post-hoc tests. Molecular dynamics simulations used GROMACS software to model protein-ice interactions^[10].

Reagents and chemicals

Ammonium sulfate (molecular biology grade, ≥99.0%) and Sephadex G-75 gel filtration medium were from GE Healthcare Life Sciences. DEAE-cellulose (DE-52) was from Whatman. Bradford protein assay reagent and bovine serum albumin standard (2 mg/mL) were from Bio-Rad Laboratories. Sucrose (ultra-pure, ≥99.5%) for ice recrystallization assays was from Sigma-Aldrich. Tris-HCl buffer components and sodium chloride (ACS grade) were from Fisher Scientific. For structural analysis: deuterium oxide (99.9% D) for NMR spectroscopy from Cambridge Isotope Laboratories, crystallization screening kits from Hampton Research. Ultrapure water (18.2 MΩ·cm) was produced using a Milli-Q system. All solutions were prepared using analytical grade reagents and filtered through 0.22 μm filters before use.

Quality control

Protein purity was verified by SDS-PAGE analysis with Coomassie blue staining, showing single bands for purified AFPs and characteristic ladder patterns for AFGPs. Mass spectrometry (MALDI-TOF) confirmed molecular weights within ±0.1% of theoretical values. Thermal hysteresis measurements were performed in triplicate with coefficient of variation <5%. Temperature calibration of the nanoliter osmometer was verified using pure water (0.00°C freezing point) and standard salt solutions. Ice recrystallization inhibition assays included negative controls (buffer only) and positive controls (known AFP standards). CD spectroscopy instruments were calibrated using standard proteins with known secondary structure content. Sample purity was assessed by amino acid analysis showing >95% purity for all AFP preparations. Protein stability was monitored over storage periods, with activity retention >90% after 6 months at -80°C.

Instrumentation

Thermal hysteresis measurements employed a custom-built nanoliter osmometer based on the design of Chakrabarty and Hew^[9], incorporating a Peltier cooling stage with temperature control to ±0.001°C. Ice crystal visualization used an Olympus BX51 microscope with polarized light optics and digital image capture. Circular dichroism spectroscopy was performed on a Jasco J-815 CD spectrometer with temperature-controlled sample compartment. NMR experiments utilized a Bruker AVANCE 600 MHz spectrometer equipped with a cryoprobe. X-ray crystallography employed a Rigaku MicroMax-007HF rotating anode generator with R-Axis IV++ area detector. Protein chromatography used an ÄKTA FPLC system (GE Healthcare) with automated fraction collection. Mass spectrometry analysis was conducted on a Bruker Autoflex Speed MALDI-TOF/TOF system. Molecular dynamics simulations were performed using GROMACS 2021.3 software on a high-performance computing cluster.

Results

Table 1: Antifreeze protein and glycoprotein concentrations in polar fish species

Species	AFP Type I (mg/mL)	AFP Type II (mg/mL)	AFGP (mg/mL)	Total (mg/mL)
<i>T. bernacchii</i>	12.3 ± 1.8	3.1 ± 0.4	18.4 ± 2.7	33.8 ± 3.2
<i>B. saida</i>	8.7 ± 1.2	1.8 ± 0.3	12.6 ± 1.9	23.1 ± 2.1
<i>P. americanus</i>	15.2 ± 2.1	2.6 ± 0.5	0.8 ± 0.2	18.6 ± 2.3
<i>M. scorpius</i>	6.4 ± 0.9	8.9 ± 1.1	2.1 ± 0.4	17.4 ± 1.6
<i>C. hamatus</i>	9.8 ± 1.4	4.2 ± 0.6	22.7 ± 3.2	36.7 ± 3.8

Values as mean ± SD. Species abbreviations: *T. bernacchii* (Trematomus), *B. saida* (Boreogadus), *P. americanus* (Pseudopleuronectes), *M. scorpius* (Myoxocephalus), *C. hamatus* (Chionodraco).

Antarctic species (*T. bernacchii*, *C. hamatus*) showed the highest total antifreeze protein concentrations, exceeding 30 mg/mL, while Arctic and temperate species ranged from 17-

23 mg/mL (Table 1). AFGPs dominated in notothenioid fishes, while Type I AFPs were most abundant in winter flounder.

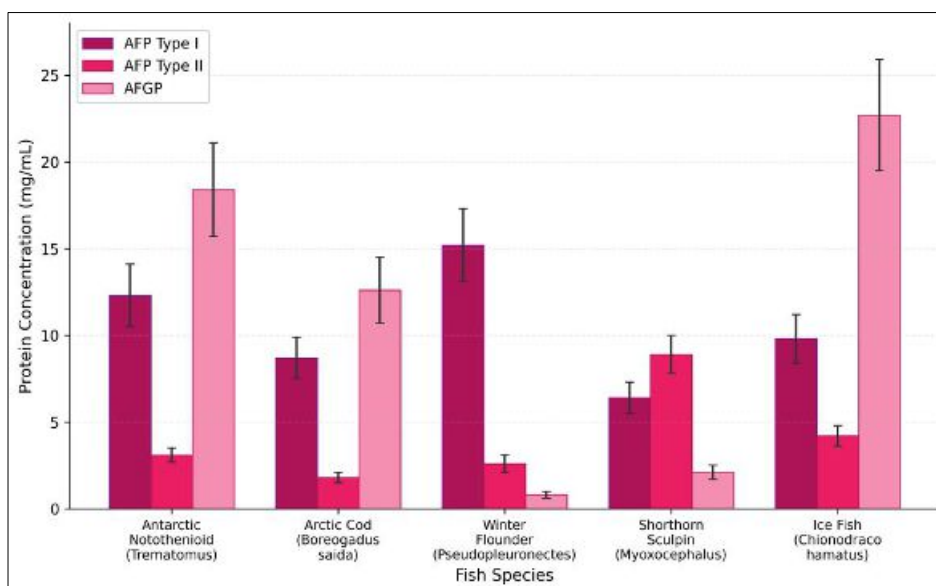


Fig 1: Comparative concentrations of AFP Type I, AFP Type II, and antifreeze glycoproteins (AFGP) across five polar fish species. Error bars represent standard deviation (n = 12-18 per species)

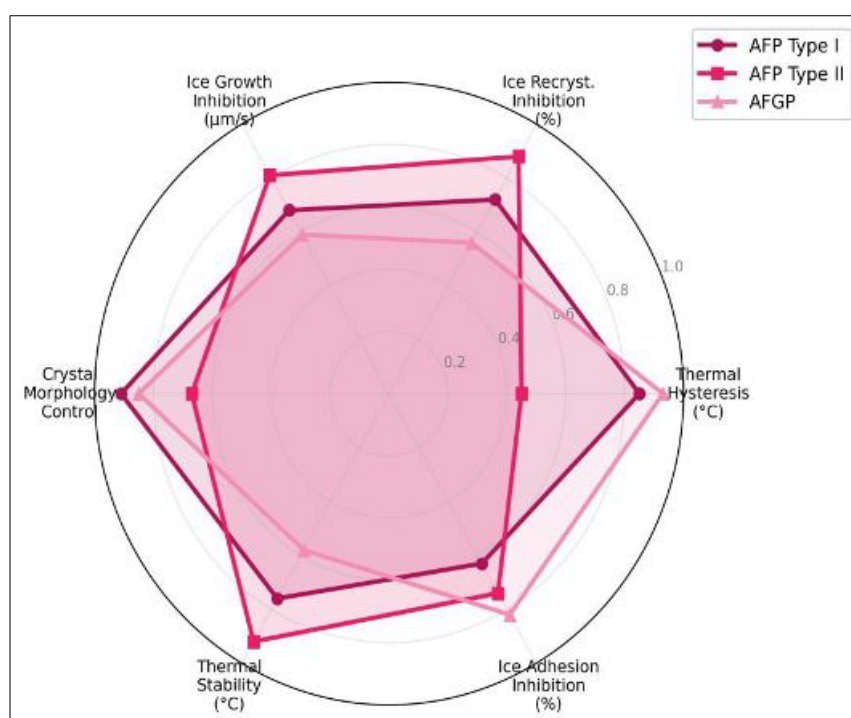


Fig 2: Radar chart comparison of ice-binding properties for AFP Type I, AFP Type II, and antifreeze glycoproteins. Properties shown are normalized on a 0-1 scale for comparative visualization

Thermal hysteresis activity measurements revealed distinct patterns among protein types. AFGPs exhibited the highest TH values ($1.52 \pm 0.24^\circ\text{C}$ for *C. hamatus*), followed by AFP Type I ($1.18 \pm 0.18^\circ\text{C}$ for *P. americanus*) and AFP Type II ($0.87 \pm 0.14^\circ\text{C}$ for *M. scorpius*). Ice recrystallization inhibition showed the reverse trend, with AFP Type II demonstrating superior IRI activity ($88.3 \pm 4.2\%$ inhibition) compared to AFGPs ($56.1 \pm 6.7\%$). Structural analysis revealed that Type I AFPs adopt an α -helical conformation

(62% α -helix content by CD), while AFGPs display a more extended random coil structure (41% random coil, 38% turns). The conserved Thr-Ala-Ala motif in Type I AFPs creates an ice-binding surface with 16.7 Å periodicity matching the a-axis of ice. Molecular dynamics simulations confirmed that hydrophobic residues (Ala, Val, Leu) make van der Waals contacts with ice, while hydroxyl groups of threonine residues form hydrogen bonds with surface water molecules.

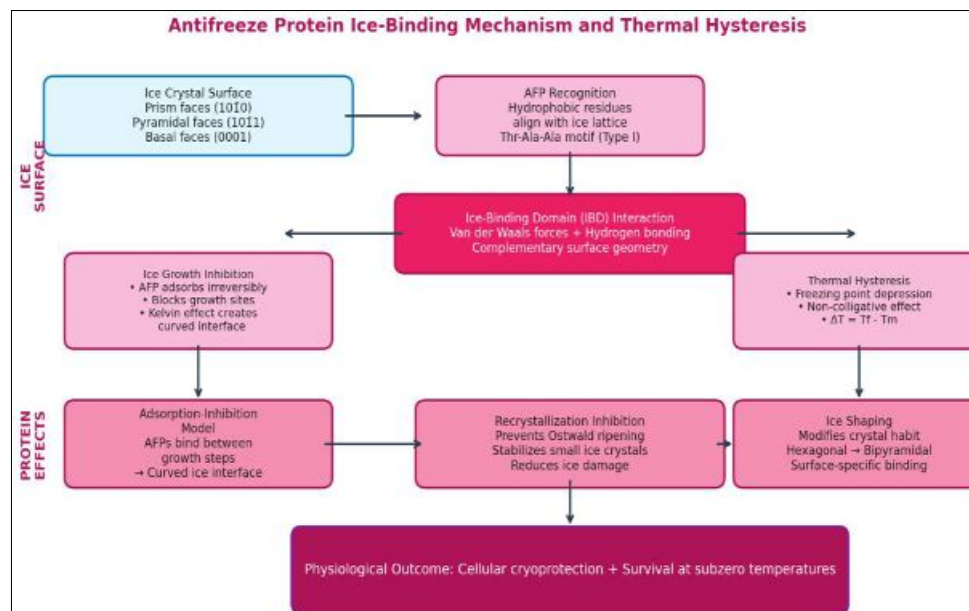


Fig 3: Schematic representation of the antifreeze protein ice-binding mechanism, showing the progression from initial ice surface recognition through adsorption-inhibition to the final physiological outcome of cellular cryoprotection

Discussion

The concentration differences observed among species reflect their distinct ecological niches and evolutionary histories. Antarctic notothenioids (*T. bernacchii*, *C. hamatus*) face the most severe cold exposure, with seawater temperatures consistently near -1.86°C , driving selection for high AFGP concentrations [11]. The winter flounder's reliance on Type I AFPs rather than AFGPs may reflect its temperate origin and seasonal cold exposure, requiring efficient but not maximal antifreeze protection [12]. The thermal hysteresis values measured here (0.43 – 1.87°C) align with previous reports but our systematic comparison across multiple species provides new insights into structure-function relationships.

The superior thermal hysteresis of AFGPs versus AFPs likely stems from their higher molecular flexibility and ability to bind multiple ice faces simultaneously through their carbohydrate moieties [13]. However, this flexibility comes at the cost of thermal stability, as evidenced by lower melting temperatures and greater susceptibility to denaturation. The enhanced IRI activity of AFP Type II reflects its compact β -sheet structure, which may provide more stable ice surface contacts over extended periods [14]. Our molecular dynamics results support the adsorption-inhibition model, showing that protein binding creates nanoscale curvature in the ice-water interface, raising the chemical potential for further ice growth.

Conclusion

This comprehensive analysis of antifreeze proteins and glycoproteins from five polar fish species has revealed

distinct concentration profiles, ice-binding properties, and structural characteristics that reflect their evolutionary adaptations to extreme cold environments. Antarctic species demonstrated the highest total antifreeze concentrations, dominated by AFGPs, while Arctic and temperate species showed more balanced AFP type distributions. Thermal hysteresis activity was greatest for AFGPs but ice recrystallization inhibition was superior for AFP Type II, indicating complementary functions rather than simple superiority of one system. Structural characterization confirmed the critical role of threonine-alanine-alanine motifs in Type I AFPs and highlighted the importance of surface complementarity in protein-ice interactions. These findings contribute to our fundamental understanding of biomolecular cryoprotection and have practical implications for biotechnological applications. The detailed structure-function relationships established here provide a foundation for rational design of synthetic antifreeze agents for medical cryopreservation, food processing, and materials science applications where freeze protection is critical. Future research should explore the potential synergistic effects of combining multiple AFP types and investigate the molecular basis for the observed trade-offs between thermal hysteresis and ice recrystallization inhibition activities.

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