



Inhibition of ice recrystallization with designed twistless helical repeat proteins

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Given the repetitive structure of crystalline ice, it is unsurprising that highly active ice-binding proteins (IBPs), often with beta-roll structures, also have repeating motifs. Here, we introduce a de novo designed family of ice-binding twistless alpha-helical repeat (iTHR) proteins. Each iTHR protein comprises two planar layers of parallel alpha-helices connected by loops—a structural topology not seen in native IBPs. The ice-binding helices contain an ordered array of TXXXAXXXAXX motifs, precisely spaced to complement the pyramidal {201} and secondary prism {110} planes of the ice lattice, with a designed 98.2° residue turn angle that orients all threonines uniformly toward the ice surface. iTHR proteins show high solubility, thermostability, and produce varied ice crystal morphologies depending on their intended target facet. Crucially, iTHR proteins exhibit ice recrystallization inhibition (IRI) at critical concentrations comparable to those of many native globular IBPs. Extensive site-specific mutagenesis shows that ice-binding activity in iTHR proteins is robust, remaining largely unaffected by changes in chemical composition. Variation in the repeat number reveals a nonmonotonic relationship to IRI activity. X-ray crystal structures of two designs confirm the intended orientation of threonines, uniformly pointing toward the ice surface. The iTHR family provides a versatile platform to systematically investigate the complex structure–activity relationships underlying protein–ice interactions.

protein design | ice binding proteins | ice recrystallization

Given that the structures and interactions of liquid water and solid ice are so similar, it is remarkable that nature has evolved molecules that bind to ice with high affinity, and often with specificity for particular crystal faces (1, 2). Ice-binding proteins are an important class of ice binders which have activities ranging from suppressing the growth of small ice crystals (3), lowering the freezing point of liquid water (4), to promoting the nucleation of ice crystals (5). Ice crystals possess a highly regular repetitive structure, which is bound by many native ice-binding proteins with geometrically complementary repetitive structures, in particular beta-roll structures (6). However, many other ice-binding proteins do not have repetitive structures. Additionally, the existence of many ice-binding glycopeptides (7, 8) illustrates that having a precisely folded structure is not a prerequisite for ice binding.

When designing proteins to bind crystalline surfaces, such as ice, using repeat proteins dramatically reduces the design space to that of a small repeating subunit. This allows large designs to be defined by a small number of degrees of freedom (9–12). This approach has been used to design helical repeat proteins with controlled assembly on the mica (001) surface (13), as well as helical repeat proteins that promote the nucleation of calcite (14) or zinc oxide (15). Recently, it was shown that by a judicious choice of the parameter values, it is possible to specifically design helical repeat proteins with extended flat surfaces, dubbed Twistless Helical Repeat, or THR proteins (16). As they contain highly flat repetitive surfaces, similar to those used to bind other crystals, we reasoned that THR proteins should be able to be engineered to bind ice.

Previously, we have shown that the helical TXXXAXXXAXX ice-binding motif, found in ice-binding proteins such as the winter flounder antifreeze protein (wfAFP) (17), can be incorporated in designed Twist-constrained Ice-binding Proteins, or TIPs (18). These proteins contain an ice-binding alpha-helix featuring TXXXAXXXAXX repeats, two flanking helices which constrain the turn angles of the ice-binding helix, and two loops which connect the helices into a monomeric three-helix bundle (18). TIPs showed Ice Recrystallization Inhibition (IRI) activity which was maximized when the consecutive motif threonine residues are precisely aligned in the same direction pointing toward the

Significance

Ice-binding proteins (IBPs) enable organisms to survive in freezing environments by controlling ice growth, yet the relationship between their structure and function remains poorly understood. We present a family of de novo designed IBP-ice-binding twistless helical repeat (iTHR) proteins featuring a flat architecture that is structurally distinct from natural IBPs. These proteins bind ice with high precision, matching specific ice crystal planes using rationally spaced threonine residues. Despite lacking sequence homology to natural IBPs, iTHR proteins exhibit potent ice recrystallization inhibition (IRI) activity and high stability. Their modular design enables systematic exploration of how protein structure affects ice interaction, offering valuable insights for designing synthetic IBPs tailored to cryopreservation, food preservation, and other applications requiring precise control over ice formation.

Competing interest statement: R.J.d.H., I.K.V., and R.d.V. are co-inventors in a patent filing WO2024076237, covering twist-constrained alpha-helical ice-binding proteins described in this work.

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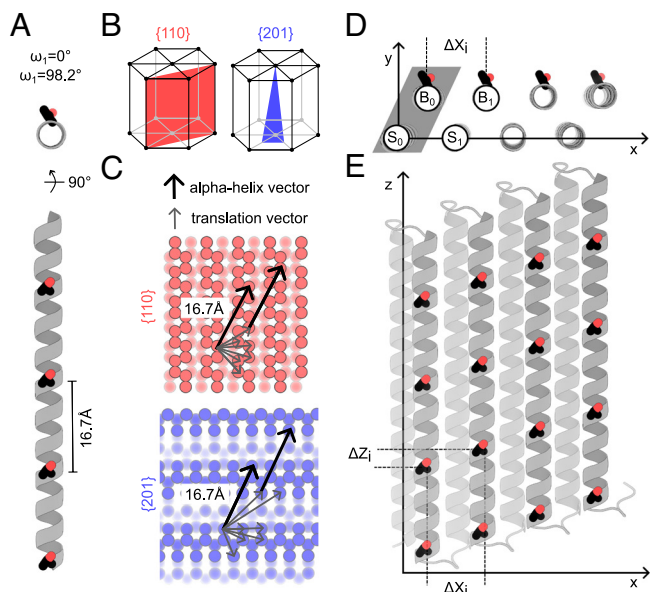


Fig. 1. Parametric design of expandable, twist-constrained iTHR proteins with threonine matched to oxygen lattice on ice crystal facets. (A) A 44 residue straight ($\omega_0 = 0^\circ$) ice-binding helix alpha-helix with 11-mer repeat sequence (TXXXAXXXAXX)₄ and undertwisted minor helical twist $\omega_1 = 98.2^\circ$. Threonine residues (sticks) are in perfect alignment, and spacing between each threonine on the 11-mer is spaced 16.7 Å. (B) Examples of ice crystal facet planes (red, blue) on hexagonal ice crystals that have 16.7 Å oxygen-oxygen spacing in plane: the secondary prismatic plane {110} and the pyramidal plane {201}. (C) Illustrative crystal slabs of ice facets {110} and {201} with oxygen-oxygen spacing of 16.7 Å indicated. Additional vectors indicate some potential translations for additional ice-binding helices to multiple rows of oxygen atoms in the plane. (D and E) Lattice-matched translations in ΔX and ΔZ are applied for i number of repeat modules consisting of one or more pairs of stabilizing (S_i) and ice-binding helices (B_i).

ice surface, by enforcing a helical turn angle of 98.2° in the ice-binding helix (Fig. 1A). Similarly to native type-I ice-binding proteins, such as helical wAFP (17) and ssAFP (19), TIPs shape ice into bipyramidal crystals, presumably by binding the pyramidal {201} plane. This behavior of type-I ice binders has been explained by the observation that the threonine residues in the ice-binding (TXXXAXXXAXX)_N helices are spaced approximately 16.7 Å, a distance that is also present between water oxygens on the pyramidal plane (20–22). However, this distance also occurs between oxygens on the secondary prism plane {110}, as shown in Fig. 1B and C.

With this in mind, we designed a family of Ice-binding Twistless Helical Repeat (iTHR) proteins, which contain multiple parallel alpha-helices displaying the TXXXAXXXAXX motif spaced to geometrically complement either the {201} or {110} ice surface. A supporting layer of helices constrains the turn angle of the ice-binding helices to 98.2° to align the threonines toward the ice (Fig. 1A), and loops connect both sets of alpha-helices into a structure containing tandem repeats. As we will show, the additional design features of the iTHR proteins, as compared to the TIP family of designed ice-binding proteins, allows for designs with more than 1 order of magnitude higher activity than that of the TIP family of ice-binders, having IRI activities that rival that of many native ice-binding proteins.

Results

Parametric Design of Expandable Twist-Constrained iTHR. We reasoned that a more active family of ice-binding proteins could be obtained if we have arrays of parallel (TXXXAXXXAXX)_N ice-binding helices (Fig. 1D and E). If we assume the axes of

the alpha-helices are along the z -axis and the ice crystal planes to which we want to bind are normal to the y -axis, such arrays are characterized by the translations ΔX and ΔZ that relate one ice-binding helix to the next one in the array (Fig. 1C and D). We intend to design ice-binding iTHR proteins consisting of two layers of parallel alpha-helices. The top layer contains a first set of binding helices (“B”) that binds to ice and a second set of supporting helices (“S”) that constrain the ice-binding helices to have a turn angle of $\omega_1 = 98.2^\circ$ per amino acid (Fig. 1D). Hence, further parameters characterizing the iTHR designs are those that relate the position and orientation of the binding helices (B_i) to those of the supporting helices (S_i).

To design iTHR ice binders we started by compiling a set of translations ΔX and ΔZ that allow for threonines on multiple parallel ice-binding helices to interact with repeated sites on the {110} or {201} crystal facets of hexagonal ice (Fig. 1C). A set of possible translations ΔX and ΔZ for the ice-binding helices that geometrically match the {110} and {201} facets is given in SI Appendix, Table S1. We restricted ourselves to values for ΔX and ΔZ within realistic ranges for the design of THR proteins. For example, ΔX values that are too large or too small will not allow for good packing of neighboring helices.

We decided to design with ice-binding helices of either 22 or 44 residues, with 2 or 4 repeats of the putative ice-binding TXXXAXXXAXX sequence. Ice-binding helices B_i with $i = 0, 1, 2, 3$, generated by ΔX and ΔZ translations, are constrained by corresponding supporting S_i helices that allow for fixing a helical turn of $\omega_1 = 98.2^\circ$ per amino acid in the ice-binding helices B_i (Fig. 1D and E).

For a first set of parametric backbone designs, the repeat unit of the iTHR proteins consists of a single ice-binding helix “B”, and its corresponding supporting helix “S” (gray parallelogram; Fig. 1E). For a second set of designs, the repeat unit contains two ice-binding helices and two corresponding supporting helices. This increases diversity in input backbones for improved sampling. Loops of 3 to 4 residues connecting helices within and between repeats are designed using RosettaRemodel (23). Because each repeat is structurally identical and has the same positional constraints (including the ΔX and ΔZ translations), the arrangement can be propagated to generate designs with any number of repeats.

Next, ProteinMPNN (24) is used to design amino acid sequences to populate the looped parametric backbones, while maintaining internal symmetry by tying the identity of residues at the same position within each repeat. The putative ice-binding residues are constrained to follow the TXXXAXXXAXX motif during sequence design, while all other residue identities are chosen by ProteinMPNN. Residues exposed to the solvent at the capping helices are designed to enhance solubility. Finally, AlphaFold2 (25) and RoseTTAFold (26) are used to select sequences that are predicted to fold into the designed structures. Designs were selected for experimental screening if they had a mean AlphaFold2 pLDDT score greater than 85, a C α rmsd of less than 2.5 Å between the AlphaFold2 prediction and the parametric backbone, and a C α -rmsd of less than 5 Å between the AlphaFold2 and RoseTTAFold predictions.

Biochemical Characterization of iTHR. For a first experimental screening of the selected design, a total of 36 designs were cloned with Golden Gate assembly from e-blocks, and proteins were expressed in 4 mL *Escherichia coli* cultures and purified via immobilized metal affinity chromatography (IMAC). 32/36 designs yielded protein bands by sodium dodecyl sulfate–polyacrylamide gel electrophoresis after purification. Out of these, two {110} binding designs (a-iTHR-110, b-iTHR-110) and two

{201} binding designs (a-iTHR-201, b-iTHR-201) were selected for further characterization because they appeared to have high yield, high purity, and the expected molecular weight in SDS-PAGE analysis. Importantly, these repeat proteins contained four 44-residue ice-binding helices, each containing four copies of the TXXXAXXXAXX motif. Translations and other design parameters for these four designs are provided in Table 1.

For the four selected iTHR designs, AlphaFold2 models were nearly identical to the initial parametrically designed structures, including the location and orientation of the ice-binding threonine residues (SI Appendix, Fig. S1). All designs contained four threonines per ice-binding helix, except for b-iTHR-110, which had three threonines. The missing threonine was redesigned during loop remodeling to facilitate packing of the loop.

All iTHR proteins were purified in larger 50 mL cultures and found to be monomeric in Size Exclusion Chromatography (SEC), eluting at the expected retention volumes (Fig. 2B). Furthermore, all iTHR proteins showed the expected high alpha-helical content by circular dichroism (CD) and were thermostable (Fig. 2 C and D). Only the a-iTHR-110 design shows gradual unfolding at higher temperatures, but completely refolds after cooling (SI Appendix, Fig. S2). Mass spectrometry confirmed that all four iTHR proteins had the expected molar mass (SI Appendix, Table S2).

Characterization of Ice-Binding Activity. Native antifreeze proteins bind to ice facet planes, causing the ice crystals to form distinct shapes. For an initial screening of ice-binding activity, we performed an ice-shaping experiment at high protein concentrations (113 μM, 26 μM, 58.8 μM, and 101.7 μM). An ice-shaping experiment involves nucleating ice crystals in PBS + 20% sucrose containing ice-binding proteins, then slowly cooling the solution further and using microscopy to monitor how the proteins interact with different ice facets to influence the shape and growth of the crystals. We observed a range of distinct crystal shapes, indicating ice-binding activity: a-iTHR-110 formed bipyramidal shapes, b-iTHR-110 produced rectangular and hexagonal crystals, a-iTHR-201 exhibited bipyramidal shapes, and b-iTHR-201 generated blunt-ended bipyramidal crystals (Fig. 2E). Full views of the shaping experiment are shown in SI Appendix, Fig. S3. Compared to the native wfAFP (Fig. 2G), a-iTHR-201 showed most similar bipyramidal shapes (Fig. 2E).

Ice crystal shaping is thought to reflect preferences of binding to specific ice crystal planes (27). However, the relation is not straightforward since ice crystal shapes are sensitive to many experimental parameters. Nevertheless, if proteins bind to and stabilize the {110} face one would expect to see rectangular or hexagonal shapes, whereas blunt-ended or bipyramidal crystal shapes as observed for wfAFP are expected for {201} binders (2). While b-iTHR-110, a-iTHR-201, and b-iTHR-201 align with the observed trends, a-iTHR-110 does not follow this trend. This deviation suggests that the a-iTHR-110 design does not preferentially bind its target facet. Several factors which could contribute to this phenomenon will be explored in the discussion section.

Next, for an initial screening of IRI activity, dispersions of small ice crystals with and without a fixed concentration ranging from 26 to 113 μM of the iTHR ice binders in PBS + 40% sucrose were kept at -8 °C while monitoring the growth of the ice crystals. Optical micrographs of the ice crystal dispersions at 0 and 60 min show that ice crystals remain smaller in all iTHR samples than in the control sample without iTHR or the native wfAFP (Fig. 2F). To quantify and compare IRI activity, we determined the rate of ice crystal growth for various concentrations of each of the iTHR designs and wfAFP (Fig. 2H).

A key parameter for assessing IRI efficacy is the inhibitor concentration at which the transition occurs from fast recrystallization to slow (if even detectable) recrystallization, marking the curve's inflection point (3). We find an inhibitor concentration of $C_i = 5.6 \mu\text{M}$ for the a-iTHR-110 design, while $C_i = 11.4 \mu\text{M}$ for b-iTHR-110 is approximately twofold higher, indicating inferior performance. Notably, b-iTHR-110 contains one fewer threonine residue per repeat compared to a-iTHR-110 (Fig. 2A). In comparison, the iTHR-201 designs demonstrated enhanced mid-point concentrations: a-iTHR-201 with $C_i = 1.4 \mu\text{M}$ and b-iTHR-201 with $C_i = 0.97 \mu\text{M}$. Both of these designs significantly outperform the wild-type antifreeze protein (wfAFP), which has a $C_i = 4.6 \mu\text{M}$, and is reported to bind the {201} facet (17).

As our designed iTHR proteins have four ice-binding alpha-helices, while wfAFP has one, one may also compare their activities normalized by the number of ice-binding helices. Multiplying the C_i measurements of the iTHR proteins, gives values of 22.4 μM, 45.6 μM, 5.6 μM, and 3.9 μM, for a-iTHR-110, b-iTHR-110, a-iTHR-201, and b-iTHR-201, respectively. Therefore, in terms of the activity per ice-binding helix, only b-iTHR-201 shows a slightly higher activity compared to wfAFP.

Importance of Residue Identity and Geometric Complementarity on Activity. An outstanding question in the literature on ice-binding proteins is to which extent ice binding is determined by geometric complementarity to the ice crystal surfaces and to which extent by chemical recognition (28, 29). Our designed iTHR proteins are a unique testing ground to address this issue. We designed steric knockout mutants of a-iTHR-110 in which either the threonine in position 1 of the 11-mer ice-binding motif (TXXXAXXXAXX) (a-iTHR-110-KO.Thr), or the alanine at position 5 (a-iTHR-110-KO.Ala) was mutated to a glutamic acid (Fig. 3A). In native antifreeze proteins, often a single or a few steric mutations on the ice-binding surface can significantly disrupt ice-binding activity (30) and result in a complete loss of TH activity (31, 32). Here, 12 Ala to Glu or 16 Thr to Glu mutations on the ice-binding site reduce IRI activity approximately threefold, but surprisingly, significant activity remains for both knockouts: The C_i was 14.6 μM for a-iTHR-110-KO.Ala, and 20.2 μM for a-iTHR-110-KO.Thr (Fig. 3B). This suggests that geometric complementarity alone can drive ice binding to some extent.

A comparison of a-iTHR-201 and b-iTHR-201 allows for assessing the importance of the surface residues on the non-ice-binding

Table 1. Translations of twist-constrained de novo helical repeat protein designs

Design	helices per repeat	$\Delta X (B_0 \rightarrow B_1) (\text{\AA})$	$\Delta Z (B_0 \rightarrow B_1) (\text{\AA})$	$\Delta X (B_0 \rightarrow B_2) (\text{\AA})$	$\Delta Z (B_0 \rightarrow B_2) (\text{\AA})$	Target facet
a-iTHR-110	4	13.831	9.256	27.662	1.902	{110}
b-iTHR-110	2	-10.375	2.828	-20.751	5.656	{110}
a-iTHR-201	2	12.396	-5.516	24.792	-11.032	{201}
b-iTHR-201	2	12.396	-5.516	24.792	-11.032	{201}

The internal symmetric repeat consists of 1 or 2 ice-binding helices. Translations shown are relative to the first ice-binding helix (B_0).

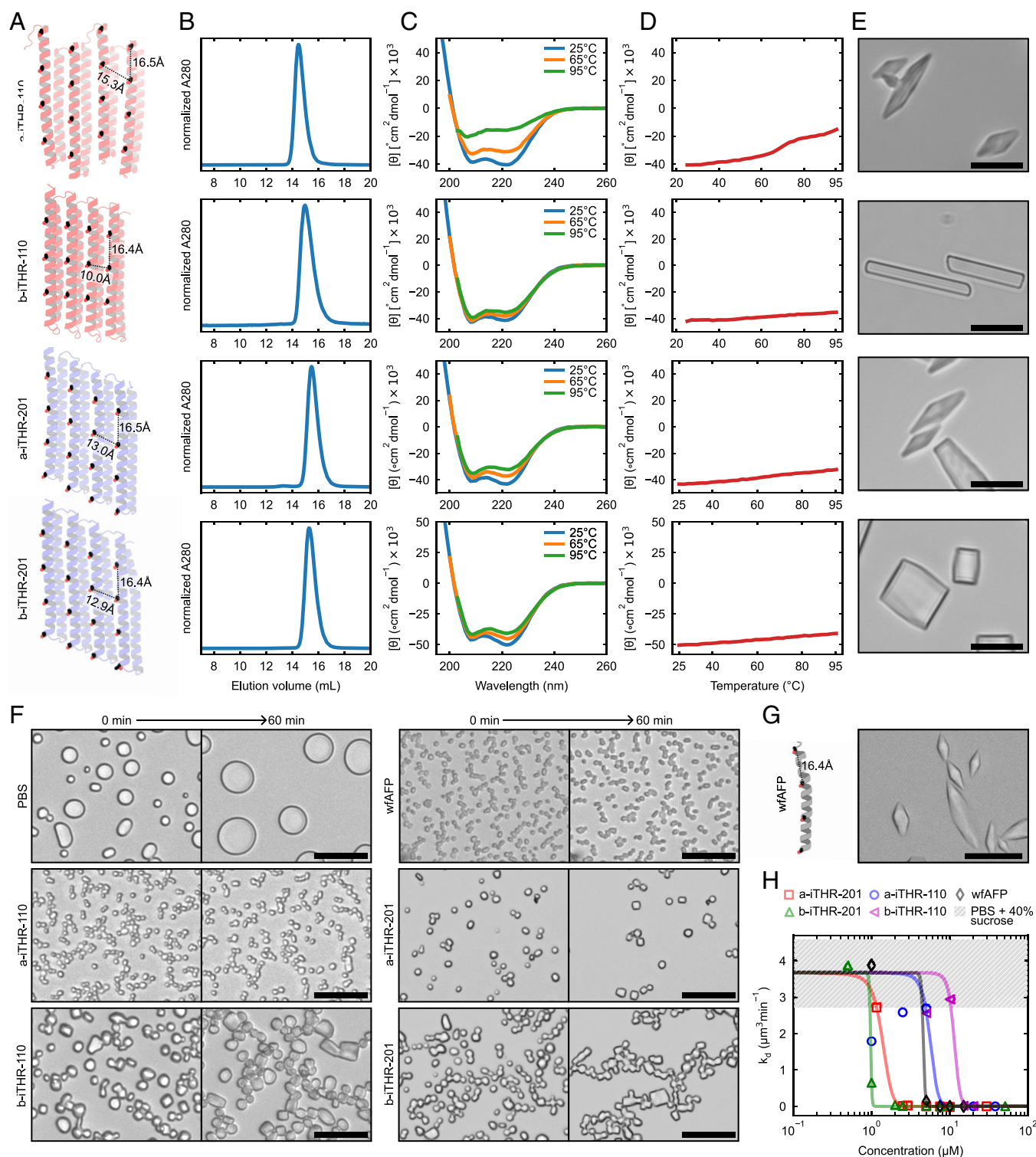


Fig. 2. Biochemical characterization and ice-binding activity of iTHRs. (A) AlphaFold2 models for iTHR designs. Ice-binding threonine residues shown as sticks. The average threonine C β -C β distances are annotated (not the same as translation vectors). (B) Size-exclusion chromatography of iTHR proteins on Superdex 200 Increase 10/300 column in PBS. (C) Circular Dichroism spectra in PBS buffer, showing molar residue ellipticity $[\theta]$ over wavelength. (D) Heat ramp at 222 nm, showing molar residue ellipticity $[\theta]$ over temperature. (E) Ice crystal shaping at 113.3 μ M a-iTHR-110, 26.0 μ M b-iTHR-110, 58.8 μ M a-iTHR-201, and 101.7 μ M b-iTHR-201. (F) IRI screening. Stills at 0 min. and 60 min are shown after incubation at -8°C in PBS + 40% sucrose. Protein concentrations are 38 μ M (a-iTHR-110), 26 μ M (b-iTHR-110), 29.4 μ M (a-iTHR-201), 50.9 μ M (b-iTHR-201), and 15.0 μ M (wAFP). (Scale bars: 50 μ m.) (G) wAFP (PDB ID: 1WFA) structure (Left) and ice crystal shaping at 20.0 μ M wAFP (Right). (Scale bar: 25 μ m.) (H) Quantitative IRI: ice crystal growth rate (k_d) vs. protein concentration (C_i). The inflection concentration (C_i) are 5.6 μ M (a-iTHR-110), 11.4 μ M (b-iTHR-110), 1.4 μ M (a-iTHR-201), 1.0 μ M (b-iTHR-201), and 4.5 μ M (wAFP). The mean growth rate in PBS buffer alone was $3.7 \pm 0.9 \mu\text{m}^3/\text{min}$ ($n = 3$ measurements), with the height of the box representing one SD above and below the mean.

side of the proteins: Although the two structures exhibit identical backbone displacements in the ΔX and ΔZ directions (Table 1), they feature different loops and distinct protein sequences. The

alignments of their AlphaFold2 models are very close, with a Ca-rmsd = 2.0 $\text{\AA}$ (over 372 atoms). The ice-binding helices on the ice-binding site (IBS) share a high degree of similarity, with a

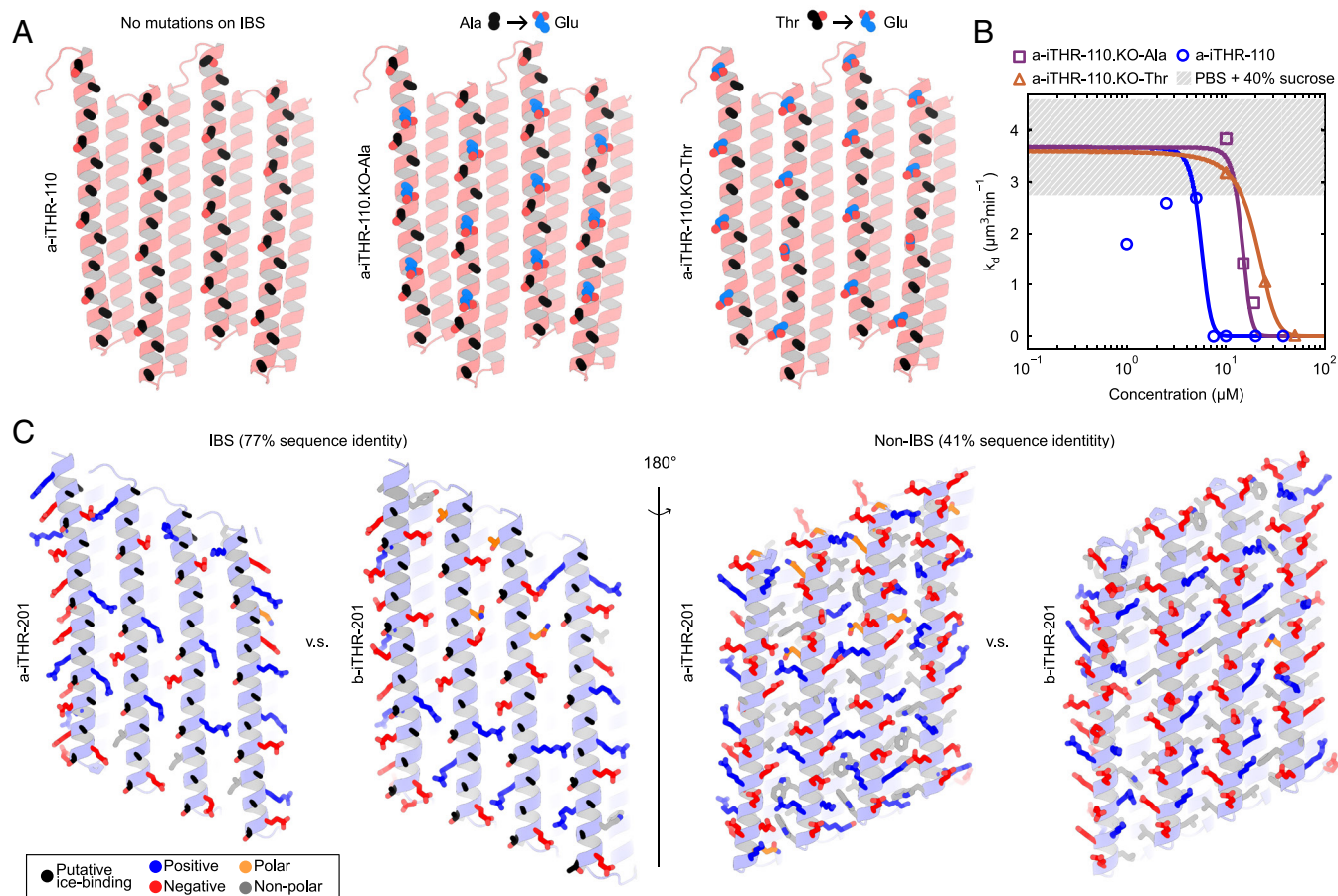


Fig. 3. iTHR ice-binding and non-ice-binding surface chemistry. (A) AlphaFold2 models of a-iTHR-110 and knockout mutants a-iTHR-110.KO-Ala and a-iTHR-110.KO-Thr with Ala to Glu or Thr to Glu mutations, respectively. (B) Quantitative IRI: ice crystal growth rate (k_d) vs. protein concentration for a-iTHR-110, a-iTHR-110.KO-Ala, and a-iTHR-110.KO-Thr. The inflection concentrations are 5.6 μM (a-iTHR-110), 14.6 μM (a-iTHR-110.KO-Ala), and 20.2 μM (a-iTHR-110.KO-Thr). (C) AlphaFold2 models of the putative ice-binding surface (IBS) and the non-IBS for a-iTHR-201 and b-iTHR-201. Both {201} binders have identical rigid body translations and similar C_i (1.4 and 1.0 μM). The surface exposed residues on the ice-binding surface (IBS) shows a high degree of sequence similarity of 77%, while sequence identity of surface residues on the non-IBS side was only 41%. Colors of side chains indicate negative (Glu and Asp in red), positive (Lys and Arg in blue), polar (Asn, Gln, Thr, and Ser in orange), and hydrophobics (Ala, Val, Ile, Leu, Phe, and Trp in gray) residues. The putative ice-binding residues shown in black.

sequence identity of 77% at the ice-binding surface. In contrast, the surface residues on the back sides of IBS show considerable variation, with only about 41% sequence identity (Fig. 3C). Given that the C_i for both proteins is relatively similar (1.4 μM for a-iTHR-201 and 1.0 μM for b-iTHR-201), this suggests that the identity of the back side residues is likely not crucial for IRI activity.

Dependence of Activity on iTHR. A key feature of repeat proteins that bind at solid–liquid interfaces is that one can modulate binding affinity by simply changing the number of repeats. This was observed for designed DHRs that adsorb to mica and CaCO_3 surfaces (13, 14).

To test the impact of the number of repeats on the activity of the iTHR, we prepared versions of the a-iTHR-201 and a-iTHR-110 proteins with, respectively, twice as many helical repeats (a-iTHR-110x2 and a-iTHR-201x2) and half as many helical repeats (a-iTHR-110x0.5 and a-iTHR-201x0.5) (Fig. 4A). IRI activity curves for different iTHR lengths are shown in Fig. 4B and C. For the a-iTHR-201 series, the IRI-activity C_i of proteins with increasing repeat numbers are 1.4 μM , 5.6 μM , and 1.9 μM . For the a-iTHR-201 series, the C_i of proteins with increasing repeat numbers are 0.92 μM , 0.97 μM , and 2.2 μM . For both series we find that increasing the repeat number or IBS did not markedly improve the IRI activity (33).

Crystallographic Structure Determination. Finally, to validate experimentally that the iTHR proteins indeed fold precisely into the designed target structures, we attempted to crystallize and solve crystal structures for all four iTHR designs. High-resolution structures were obtained for b-iTHR-110 and a-iTHR-201. The a-iTHR-201 structure matches very well both with parametrically designed backbone structure (rmsd = 1.1 Å, Fig. 5C and D). The b-iTHR-110 crystal structure is slightly twisted, but otherwise resembles the parametric design backbone (rmsd = 2.5 Å, Fig. 5A and B). In both structures, the orientations of the ice-binding threonine and alanine residue are preserved and the ice-binding helix retains the designed alpha-helical turn angle of $\omega_1 = 98.2^\circ$ per amino acid of the ice-binding helix. This suggests that our computational design method is able to yield, with high accuracy, the target geometric spacings and residue orientations required for ice binding. The rotamers of most residues at the core of the protein closely match the design model (Fig. 5B and D), demonstrating high accuracy at the sidechain level as well.

Discussion

We have demonstrated how to design iTHR proteins, a new topology of ice-binding proteins containing parallel alpha-helices presenting TXXXAXXXAXX motifs, which have IRI activities comparable to those of native IRI-active ice-binding proteins

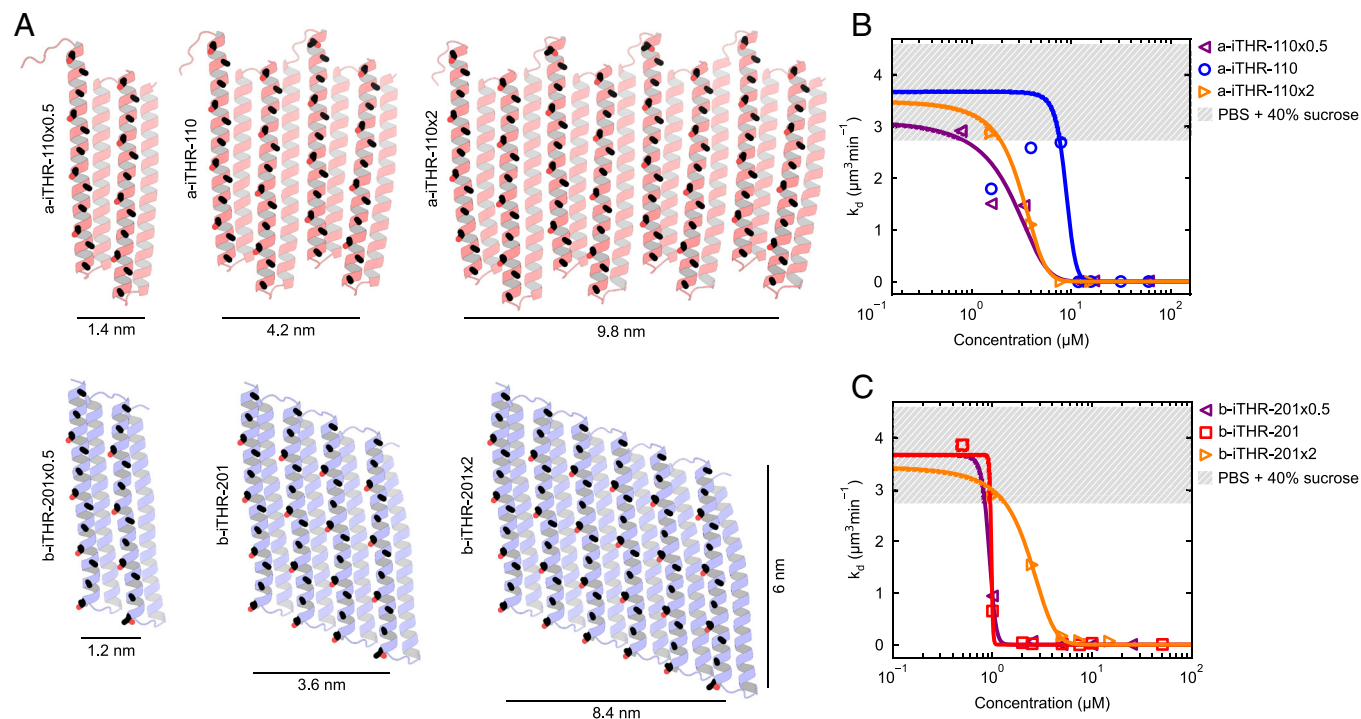


Fig. 4. iTHR extension series. (A) AlphaFold2 models with 0.5 $\times$ and 2 $\times$ repeats of a-iTHR-110 and a-iTHR-201. The width of the protein is shown in the figure. Putative ice-binding residues shown in black. The approximate ice-binding surface is 8 nm², 25 nm², and 59 nm² for a-iTHR-110 series, and 7 nm², 22 nm², and 50 nm² for a-iTHR-201 series. (B) Quantitative IRI: ice crystal growth rate (k_g) vs. protein concentration for the a-iTHR-110 and a-iTHR-201 extension series. The inhibitory concentrations (C_i) in full protein units are 1.4 μ M, 5.6 μ M, and 1.9 μ M for a-iTHR-110x0.5, a-iTHR-110, a-iTHR-110x2, respectively. (C) Quantitative IRI for the a-iTHR-201 series the C_i in full protein units are 0.92 μ M, 0.97 μ M, and 2.2 μ M for a-iTHR-201x0.5, a-iTHR-201, a-iTHR-201x2, respectively.

(Fig. 2H). The iTHR proteins were designed to target the {110} or {201} facets of ice, and three of the four ice crystal shapes are as expected (SI Appendix, Fig. S3). The remaining design, a-iTHR-110, which appears to stabilize the off-target {201} facet (SI Appendix, Fig. S3 A and E), has the largest spacing between helices and is the least thermostable design (Fig. 2C), perhaps indicating that the off-target binding is due to poorer core packing and greater flexibility. Variants of the designs were used to show that geometric complementarity to ice crystal surfaces alone is sufficient to obtain significant IRI activity (Fig. 3). We observed

that increasing the size of the ice-binding surface does not translate into a higher IRI activity (Fig. 4). The IRI activity of flexible antifreeze glycoproteins and polyvinyl alcohol polymers increases with increasing chain length (27), however, for native ice-binding beta-roll repeat proteins it has been established that the relation between ice binding and number of repeats is nonmonotonic, with a clear optimal length (33, 34). Consistent with these prior reports, we find that reexpressing activity as a function of ice-binding unit concentration (SI Appendix, Fig. S6) does not reveal any systematic multivalent gain. This pattern likely reflects

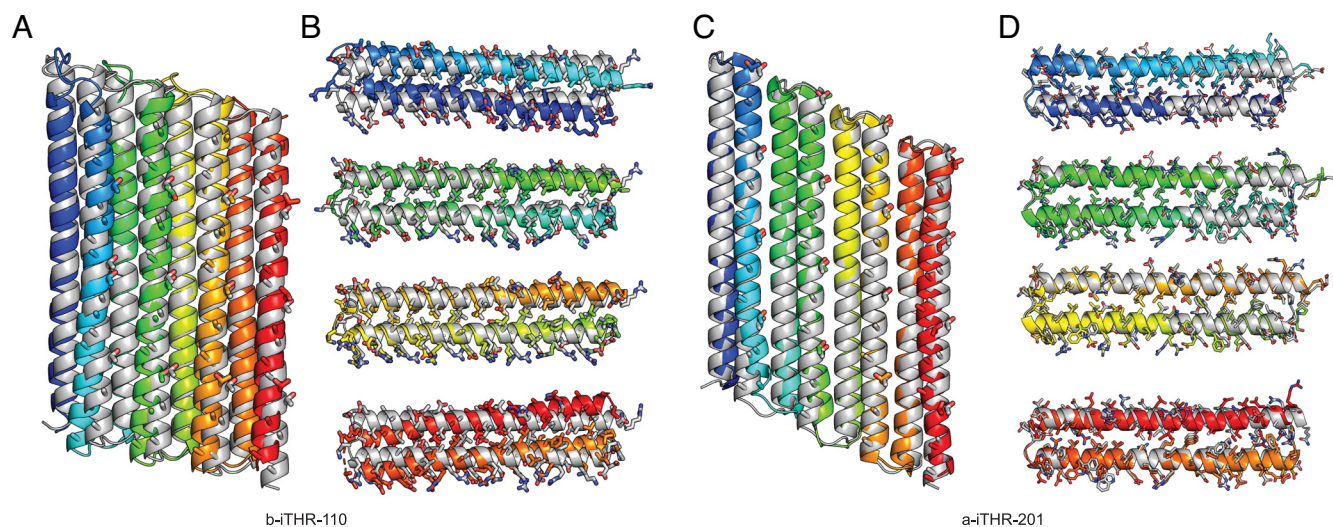


Fig. 5. Crystal structures of iTHR aligned to parametric designed models. (A) Overlay of the b-iTHR-110 (PDB ID: 9MG8) crystal structure (colored) and the parametric designed model (gray). C_α -rmsd = 2.5 Å (over 368 atoms). The putative ice-binding threonine and alanine residues are shown as sticks. (B) Side views of individual b-iTHR-110 repeats showing all side chains as sticks. (C) Overlay of the a-iTHR-201 (PDB ID: 9D01) crystal structure (colored) and the parametric designed model (gray). C_α -rmsd = 1.1 Å (over 370 atoms). The putative ice-binding threonine and alanine residues are shown as sticks. (D) Side views of individual a-iTHR-201 repeats showing all side chains as sticks.

geometric or structural constraints—e.g., registry mismatch to the ice lattice, helix curvature, or local misfolding—rather than multivalent affinity effects.

The IRI activities of these iTHR are more than 1 order of magnitude higher than the previously designed TIP ice-binders (18). Many (but not all) natural IRI-active proteins also have thermal hysteresis (TH) activity (1, 2); in fact, some of the most potent IRI-active proteins display little or no TH activity. We have found that some of the iTHR variants have very low but measurable TH activity of ~ 0.1 °C, at submillimolar concentrations (*SI Appendix, Fig. S5 and Table S5*). Currently, the design of proteins with TH activity is challenging, since it is not yet clear what physical properties are required for proteins to show TH activity. Superresolution microscopy results suggest that TH activity requires long residence times of ice-binding proteins on the ice surface (35), whereas proteins with short residence times only show IRI activity. Performing similar superresolution microscopy experiments on the designed TIP and iTHR proteins may inform the design of TH-active proteins. Additionally, as the iTHR proteins are highly stable—especially compared to many native ice-binding proteins—we can systematically vary the interfacial “X” residues in the TXXXAXXXAXX motifs and the geometry and chemistry of the non-IBS surface and measure how these changes affect IRI and TH activity.

The present iTHR designs may offer advantages for applications of ice-binding proteins that are currently being explored such as cryopreservation of cells, tissues, and organs (36), since they are thermostable and express with high yield. The highly regular and modular structures of these iTHR may allow further engineering of the ice–water interface by incorporating additional protein–protein interfaces (16) to form large protein assemblies that drive ice nucleation, or protein nanocages that encapsulate ice crystals. Additionally, recent advances in deep-learning methods, such as RFdiffusion (37), may enable the design of ice-binding sites and motifs that are not seen in nature.

Materials and Methods

Computational Design. An ice-binding helix (“B” helix) with $N = 2$ or 4 copies of the putative ice-binding motif TXXXAXXXAXX with a minor helical pitch of $\omega_1 = 98.2^\circ$ per residue was generated using a python script that incorporates Watson–Crick parameters (18, 38). The “B” helix was aligned to the z-axis by its helical axis and centered at the origin in PyMOL. Next, the “B” helix was rotated about the z-axis such that the $C\alpha$ atom to $C\beta$ atom vector of the threonine is pointing parallel to the y-axis. Subsequently, the phase of the “B” helix is sampled in 18 increments of 6° to create diversity in the orientation of the ice-binding residues facing the solvent.

Next, a stabilizing helix (“S” helix)—with $\omega_1 = 100^\circ$ per residue—was generated using the BundleGridSampler (39) with 96 varieties of geometry defined by a subtag of the mover: Helix r0=“10” delta_omega0_min=“52” delta_omega0_max=“68” delta_omega0_samples=“3” z0_offset_min=“6” z0_offset_max=“10” z0_offset_samples=“4” invert=“true” delta_omega1_min=“1” delta_omega1_max=“96” delta_omega1_samples=“8”. For each “B” helix and “S” helix pair, rigid bodies are copied and translated using the RigidBodyTransMover in PyRosetta following the translations in *SI Appendix, Table S1*. Residues that were not “T” or “A” motif residues were mutated to “G” to simplify tracking of residue identities downstream.

The rigid body translated copies of the “B” and “S” helix pairs were then looped together with the ConnectChainsMover in RosettaScripts. Because this looped unit now contains i) two copies of each helix, ii) all the necessary loops built for a repeat protein, and iii) the intended transformation between repeat units, a repeat protein model can be built from this unit that can be extended arbitrarily with the RepeatPropagationMover (16); in this case, we chose to move forward with four repeat units so that the interior two repeats could be identical in sequence, representing the potentially duplicatable interface between repeats.

At this point, all residue identities in the models are either “T” or “A” if they are part of the ice-binding motif, or “G” if they are a nonmotif part of the helix,

or “V” if they were built with the ConnectChainsMover. ProteinMPNN (24) was used to design all of the residues on these structures except for positions that were already “T” or “A”; and each of the 4 repeats was required to design the same sequence as the other repeats. “C” was the only prohibited residue identity. All residues between each repeat (asymmetric unit) were linked, except for solvent-accessible residues at the capping repeats to avoid forming hydrophobic caps.

Subsequently, structures were predicted from the designed sequences using both AlphaFold2 (25) and RoseTTAFold (26). Designs with <2.5 Å $C\alpha$ rmsd between the AlphaFold2 model and the parametrically generated backbone and <5 Å rmsd between the AlphaFold2 and RoseTTAFold predictions were selected for experimental characterization.

Synthetic Gene Bacterial Protein Expression and Purification. Synthetic gene fragments encoding 36 iTHR designs were codon optimized for expression in *E. coli* were acquired from IDT and cloned into a vector pET-29(b+)-derived vector, using golden gate assembly. The constructs were transformed into BL21 (DE3) *E. coli* competent cells.

Transformants were cultured in 4×1 mL Terrific Broth medium supplemented with 200 mg mL^{-1} kanamycin in deep 96-well plates. Expression, under the control of a T7 promoter, proceeded for 24 h at 37°C using Studier autoinduction (40). Cell pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 30 mM Imidazole, with 1 mg of DNaseI) and chemically lysed by addition of BugBuster® (Sigma-Aldrich). The soluble fraction, clarified by centrifugation, underwent purification via Ni^{2+} IMAC using Ni-NTA resin. The resin with bound cell lysate was washed with ten column volumes of wash buffer (50 mM Tris, pH 8.0 + 300 mM NaCl, 30 mM imidazole) followed by elution with 50 mM Tris pH 8.0 + 300 mM NaCl, 300 mM imidazole. The soluble fractions were subjected to SDS–polyacrylamide gel electrophoresis analysis.

Expression cultures were scaled up to 0.5 L for further characterization, with expression again proceeding for 24 h at 37°C using 1 mM Isopropyl β -D-1-thiogalactopyranoside at OD 0.6 before harvesting by centrifugation and purification in similar conditions. After purification proteins were loaded on a SEC Superdex Increase 200 10/300 gL (Cytiva) column in running buffer phosphate-buffered saline (PBS) pH 7.4. Purified protein was checked for correct mass via Liquid Chromatography–Mass Spectrometry (*SI Appendix, Table S3*).

Liquid Chromatography–Mass Spectrometry Measurements (LC–MS). Molecular weights of each protein were determined by reverse-phase LC–MS with an Agilent G6230B time-of-flight instrument on an AdvanceBio RP-Desalting column. The solvents used were water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). The spectra were then deconvoluted with a total entropy algorithm in BioConfirm.

MW-Assisted Solid-Phase Peptide Synthesis (MW-SPPS). The wAFP was synthesized on TentaGel™ S-RAM resin (loading 0.25 mmol/g , $100 \mu\text{m}$), following the Fmoc/t-Bu strategy under nitrogen atmosphere. The sequence elongation was performed on a 0.1 mmol scale with a fivefold molar excess of Fmoc-AA-OH on a microwave-assisted solid-phase peptide synthesizer (Liberty Blue, CEM, Matthews, NC, U.S.A.). Activation was achieved with DIC and Oxyma Pure at 90°C . The reaction temperatures were monitored by an internal fiber-optic sensor and deprotection and coupling reactions were performed in a Teflon vessel, applying microwave energy under nitrogen bubbling. The peptide elongation was performed by repeating the MW-SPPS cycle for each amino acid, using a modified protocol based on the CARBOMAX method provided by CEM.

wAFP sequence: *H*-DTASDAAAAAA~~LT~~AANAKA AA~~EL~~TAAANAAAAA~~AT~~AR-NH₂. In short, the peptide was elongated with the following cycles. Fmoc deprotection was performed using a 20% (v/v) piperidine solution in DMF in four steps: 25°C , 2 W, 30 s, $\Delta T = 5^\circ\text{C}$; 75°C , 125 W, 20 s, $\Delta T = 1^\circ\text{C}$; 90°C , 5 W, 5 s, $\Delta T = 0^\circ\text{C}$; 90°C , 20 W, 90 s, $\Delta T = 0^\circ\text{C}$, followed by washing with DMF ($3 \times 4 \text{ mL}$). Coupling was performed with Fmoc-AA-OH (2.5 mL, 0.2 M in DMF, 0.5 mmol, 5 eq), DIC (1 mL, 1.0 M in DMF, 1 mmol, 10 eq), and Oxyma Pure (0.5 mL, 1.0 M in DMF, 0.5 mmol, 5 eq) in 4 steps: 40°C , 5 W, 30 s, $\Delta T = 5^\circ\text{C}$; 75°C , 163 W, 12 s, $\Delta T = 1^\circ\text{C}$; 90°C , 5 W, 9 s, $\Delta T = 0^\circ\text{C}$; 90°C , 20 W, 120 s, $\Delta T = 0^\circ\text{C}$, followed by a DMF wash step (4 mL). All couplings after β -branched amino acids (in bold) used prolonged coupling times of 240 s for the final step.

Once the synthesis was complete, the resin was transferred into a syringe equipped with a filter, washed with DMF ($3 \times 4 \text{ mL}$), DCM ($3 \times 4 \text{ mL}$), and dried under nitrogen flow. Crude peptide was obtained after acidic deprotection and

cleavage from resin with TFA/TIS/H₂O (10 mL; 190/5/5; v/v/v) for 2 h while shaking, followed by precipitation in cold Et₂O (50 mL). Purification by reverse-phase high-performance liquid chromatography (linear-gradient 10 to 70% ACN in water with 0.1% TFA, 60 min, ReproSil Gold 120 C18, 10 μ m–240 $\times$ 20 mm, 5 mL/min) yielded wfAFP as a white powder after lyophilization in 8.59 mg (2.65 μ mol). HPLC-MS analysis was performed to validate purity, using a C18 Jupiter SuC4300A 150 $\times$ 2.0 mm column using miliQ water with 0.1% FA and acetonitrile with 0.1% FA, using a gradient of 5 to 100% ACN over 10 min, connected to a Thermo Fisher LTQ XL Linear Ion Trap Mass Spectrometer (rt = 3.25 min) (*SI Appendix, Fig. S4*). Exact mass calculated [C₁₃₃H₂₂₆N₄₄O₅₀ + H]⁺: 3241.6595; [C₁₃₃H₂₂₆N₄₄O₅₀ + 2H]²⁺: 1621.3334; [C₁₃₃H₂₂₆N₄₄O₅₀ + 3H]³⁺: 1081.2247; [C₁₃₃H₂₂₆N₄₄O₅₀ + 4H]⁴⁺: 811.1703.

Circular Dichroism Measurements. Proteins were diluted to 0.2 mg/mL in PBS (10 mM sodium phosphate pH 7.4, 140 mM NaCl) in a quartz cuvette with a 1 mm pathlength. On a JASCO J-1500 (JASCO Corporation), Spectral scans from 200 to 280 nm were performed at 25 °C, 65 °C, and 95 °C where temperature was ramped at rate of 1 °C/min and ellipticity was monitored at 222 nm.

IRI Assay. Samples were prepared by dilution of the protein to the indicated concentration in PBS + 40 wt% sucrose. Subsequently, a 1 μ L sample was applied on a 22 $\times$ 22 mm coverslip, and a second coverslip was lowered on top of the drop so that the sample was sandwiched between the coverslips and transferred to a Nikon ECLIPSE Ci-Pol optical microscope equipped with a Nikon L Plan 20 $\times$ (NA 0.45) objective and the Linkam LTS420 stage. This stage was controlled by the Linksys32 software. To measure the ice-recrystallization rates, the sample was first completely frozen by reducing the temperature to –40 °C with 20 °C/min. After freezing, the temperature was gradually increased to –10 °C with 10 °C/min and then further to –8 °C with 1 °C/min, upon which individual crystals could be observed and the sample was stabilized. Recrystallization was monitored by obtaining an image each minute. IRI rates were then analyzed using ImageJ and Python. In brief, the 8-bit images of the ice crystals were subjected to the bandpass filter, enhance contrast, and subtract background functions of imageJ. Subsequently, the bright signal at the edges and then the individual crystals were isolated by the autoThreshold function using the Otsu method, followed by the Convert to Mask function. “Analyze particles” was then used to obtain the area of each crystal. These data were imported into python, upon which the radius was calculated to determine the cubed number-average radius per image. Recrystallization growth rates were determined by applying a linear fit to the resulting cubed number-average radius as a function of time traces

Ice Shaping Assay. To monitor ice shaping in the presence of the various proteins, the samples were prepared similarly to the IRI assays, but diluted into PBS containing 20 wt% sucrose. The sample was stabilized around –4.3 °C, so that a few crystals were observed in the field of view. After stabilization, the samples were supercooled with decreasing temperature 0.2 °C/min, which forced the crystals to shape in presence of the proteins, or PBS buffer only. Samples were monitored with 1 or 5 s intervals using a Nikon 50 $\times$ ELWD objective. Stills that were used in the figures were taken after 1 min of supercooling (full views in *SI Appendix, Fig. S3*).

Thermal Hysteresis. Thermal hysteresis activity, defined as the temperature difference between the melting temperature and the temperature at which explosive ice growth was observed, was measured using an in-house-built cold stage based on the nanoliter osmometer (41). The sample temperature was controlled using custom-built software developed in-house and detected with an accuracy of 0.001 °C. A metal disk with six holes of 0.6 mm was placed onto the cold plate of the stage. Type B Cargille Immersion Oil was inserted into these holes, followed by a tiny droplet of the protein solution of interest in 20 mM TRIS buffer at pH 7.5. The six droplets were frozen by rapidly cooling to –35 °C and melted back until a single crystal was observed in the solution droplet. The melting temperature was determined as the temperature at which this single crystal shrank in size. The crystal was equilibrated at 0.05 °C below its melting temperature for 2 min, followed by cooling at a constant rate of 0.1 °C/min. Every 0.5 s, snapshots were captured with a Lumenera Infinity1 camera using an Olympus BX53M optical microscope with an Olympus LMPLFLN 50 $\times$ magnification objective (NA 0.50), resulting in a resolution of approximately 0.1 μ m per pixel. During these TH measurements, the crystals grew in size within the TH gap until a threshold temperature was reached at which explosive crystal growth occurred. This latter temperature was taken as the freezing temperature.

Crystallization. Crystallization samples were prepared by concentrating a-iTHR-201 protein to 52 mg/mL and b-iTHR-110 to 14.3 mg/mL in TBS (20 mM Tris-HCl pH 8, 150 mM NaCl). All crystallization experiments were conducted using the sitting drop vapor diffusion method. Crystallization trials were set up in 200 nL drops using the 96-well plate format at 20 °C. Crystallization plates were set up using a Mosquito from SPT Labtech, then imaged using UVEX microscopes and UVEX PS-600 from JAN Scientific. Diffraction quality crystals formed in 2.8 M Sodium acetate trihydrate pH 7.0 for a-iTHR-201 and 0.2 M Sodium chloride, 28% (w/v) MPD for b-iTHR-110.

Diffraction data were collected at the Advanced Light Source (ALS) HHMI beamline 8.2.1/8.2.2. X-ray intensities and data reduction were evaluated and integrated using x-ray detector software (42) and merged/scaled using Pointless/Aimless in the CCP4 program suite (42, 43). Starting phases were obtained by molecular replacement using Phaser (44) using the designed model for the structures. Following molecular replacement, the models were improved using phenix.autobuild (45). Structures were refined in Phenix (45). Model building was performed using COOT (46). The final model was evaluated using MolProbity (47). Data collection and refinement statistics are recorded in *SI Appendix, Table S4*. Data deposition, atomic coordinates, and structure factors reported in this paper have been deposited in the Protein Data Bank (PDB) (48), <http://www.rcsb.org/>, with accession code: 9D01 (a-iTHR-201) and 9MG8 (b-iTHR-110).

Data, Materials, and Software Availability. An example RosettaScripts script and input for generating iTHR proteins are provided at [10.5281/zenodo.17089482](https://zenodo.org/record/17089482) (49). The example script was confirmed to successfully run with PyRosetta version 4 as available at <https://pyrosetta.org> (ref. 50). Documentation for ProteinMPNN sequence design is available at <https://github.com/dauparas/ProteinMPNN> (ref. 24). Designs were filtered with AlphaFold 2 available at <https://github.com/google-deepmind/alphafold> (ref. 25). Crystallographic datasets with resolved side chains have been deposited in the Protein Data Bank (PDB ID: 9MG8 for b-iTHR-110 (51); PDB ID: 9D01 for a-iTHR-201 (52)). All data and design models are available in the main text or the *SI Appendix*.

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