Polypentagonal ice-like water networks emerge solely in an activity-improved variant of ice-binding protein

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Polypentagonal water networks were recently observed in a protein capable of binding to ice crystals, or ice-binding protein (IBP). To examine such water networks and clarify their role in ice-binding, we determined X-ray crystal structures of a 65-residue defective isoform of a Zoarcidae-derived IBP (wild type, WT) and its five single mutants (A20L, A20G, A20T, A20V, and A20I). Polypentagonal water networks composed of ~50 semiflavate waters were observed solely on the strongest A20I mutant, which appeared to include a tetrahedral water cluster exhibiting a perfect position match to the (1010) first prism plane of a single ice crystal. Inclusion of another symmetrical water cluster in the polypentagonal network showed a perfect complementarity to the waters constructing the (2021) pyramidal ice plane. The order of ice-binding strength was A20L < A20G < WT < A20T < A20V < A20I, where the top three mutants capable of binding to the first prism and the pyramidal ice planes commonly contained a bifurcated γ-CH\textsubscript{3} group. These results suggest that a fine-tuning of the surface of Zoarcidae-derived IBP assisted by a side-chain group regulates the holding property of its polypentagonal water network, the function of which is to freeze the host protein to specific ice planes.

Significance

This study expands our knowledge of protein hydration, which is highly related to the macromolecular antifreeze property of proteins. We examined a polypentagonal network formation of waters for a series of artificial variants of a 65-residue ice-binding protein. The polypentagonal waters were created solely on the surface of an activity-improved variant, which appeared to contain two sets of water clusters exhibiting a perfect position match to the waters constructing the first prism and pyramidal ice planes. These data suggest that a minute structural change in a protein organizes the surface waters into a polypentagonal arrangement, which merges with the intrinsically disordered ice surface and freezes to specific ice crystal planes.

Proteins are generally surrounded by a hydration shell composed of a number of water molecules in the vicinity of their surface. This has been known to play a critical role in the structural construction and ligand interactions of proteins (1, 2). Sun et al. (3) reported that a 33-kDa ice-binding protein (IBP) named “Maxi” locates an extremely unique shell composed of more than 400 waters forming a polypentagonal network. Maxi is folded into a four-helix bundle with this network, and a part of it extending outward was thought to work for interaction between the protein and an ice crystal surface. Water arrangement in a single ice crystal is hexagonal but not pentagonal, so that Maxi’s polypentagonal water network was thought to merge with, and freeze to, an intrinsically disordered water layer creating the ice surface (3, 4). In the insect IBPs, contiguous troughs uniquely created on their molecular surface are known to trap the waters and rank them at regular intervals, which exhibited a perfect position match with the hexagonal waters (5–7). Such organized waters on the protein were named anchored-clathrate waters (ACWs), which have been assumed to combine the host protein with specific ice-crystal planes (8). Less is known, however, about how a polypentagonal water network emerges on a protein and works for protein–ice interactions.

Supercooled water freezes in two steps. First, a nucleation of single ice crystals occurs, and then these crystals grow to form ice blocks (9, 10). IBPs are capable of binding to each single ice crystal to inhibit the ice block formation (11). The mechanism of ice-binding is not fully understood. A single ice crystal consists of water molecules forming a hexagonal unit defined by $a_1$–$a_3$ and $c$ axes, whereas a set of the water molecules forming an ice plane is represented by Miller–Bravais indices (12). For example, indices (0001), (1010), and (2021) represent a basal plane normal to the $c$ axis, a primary prism plane parallel to the $c$–axis, and a pyramidal plane defined for a sloped slice inclined by 14.9° to the $c$–axis, respectively. IBPs are unique macromolecules that are capable of binding to one or more ice planes (5) and creating a convex ice front on the plane between the bound IBPs through a Gibbs–Thomson effect (13). Such fronts are energetically unfavorable for further adsorption of bulk water molecules, leading to termination of growth of the ice crystal (14). When the growth of prism and/or pyramidal planes into facets is terminated, the ice crystal is changed into a hexagonal bipyramid or hexagonal trapezohedron (15). The ice-binding ability of IBP also depresses the nonequilibrium freezing point ($T_f$) and slightly elevates the nonequilibrium melting point ($T_m^\text{neq}$). The resultant difference between $T_f$ and $T_m^\text{neq}$ termed “thermal hysteresis” (TH), determines the ability of ice-growth-inhibition of IBP (13–16). IBPs exhibiting TH values of 3 °C–6 °C and 1 °C–2 °C are termed “hyperactive” and “moderately active” species, respectively (6). For IBPs that cannot perfectly arrest the ice crystal growth, ice-growth speed ($\mu$m/min) is used as an alternative measure of ice-growth inhibition (17).

IBPs have been isolated from cold-adapted fishes, insects, plants, and microorganisms, and they display a remarkable

\[ \text{hydration shell} \mid \text{ice-binding protein} \mid \text{crystal structure} \mid \text{water cluster} \mid \text{polypentagonal waters} \]
diversity in their amino acid sequences and structures (5). A species of *Zoarcidae, Macrozoarces americanus* (ocean pout), contains a total of 12 isoforms of IBPs, which contain 65–66 amino acid residues and are monomeric globular proteins (molecular weight: 6.5–7.0 kDa) (18). These IBP isoforms are also known as type-III antifreeze proteins. The X-ray crystal structure of two isoforms denoted HPLC5 and HPLC12 has been determined (19–21). Both isoforms comprise many twisted loops folded into triple-strand β-sheets, the N-and C-terminal parts of which are related with a local twofold axis of symmetry. A compound ice-binding site (IBS) was postulated on one side of this structural motif, composed of two adjacent planar surfaces forming an angle of 150° (22, 23). One of the surfaces contributed by Q9, P12, N14, T15, A16, and N44 is thought to adsorb to the (2021) pyramidal plane, and the other containing T18, L19, and V20, to the (1010) first prism plane. They were named pyramidal-plane–binding IBS (denoted “pyramidal IBS” here) and prism-plane–binding IBS (“prism IBS”), respectively. The residues on the pyramidal IBS are well conserved in this protein family, while the residues on the prism IBS are frequently replaced (18). Computer simulations have suggested the existence of ordered water molecules around this compound IBS, which was thought to work for ice binding (24, 25). The insect IBPs consist of repetitive amino acid sequences form 2D arrays of ice-binding residues to create the contiguous troughs (5). The ice-binding residues of *Zoarcidae*-derived IBPs, however, are not aligned on the protein surface.

A midlatitude fish, *Zoarces elongatus* Kner (Notched-fin eelpout, NFE), is known to contain at least 13 isoforms of this type of IBS (26). As a model protein to observe the polypentagonal ice-like water networks on its surface, we selected a 65-residue defective isoform of this IBS denoted NFE6, which only slows but cannot perfectly terminate the ice crystal growth (17). The ice-binding ability of NFE6 was improved dramatically by the replacement of P19 and A20 with Leu and Val, respectively (denoted P19L/A20V-mutant) (22). The ice-binding ability of another defective isoform (NFE11) also improved with the V9Q/V19L/G20V triple mutation (23). Similar engineering results of this type of IBS allowed us to speculate that amino acid replacement of the 20th residue of NFE6 drastically changed its ice-binding property. We hence prepared a wild type (WT) of NFE6 and its five single mutants of A20L, A20G, and WT solutions, while the growth is inhibited in A20T, A20V, and A20I solutions. Their detailed time-dependent data (Fig. 1B), measured at the Tm = 0.05 °C with 0.2 °C/min of cooling, showed that each ice bipyramid grows in proportion with the annealing time. The ice-growth rates for A20L, A20G, WT, A20T, A20V, and A20I were evaluated at 26.5, 10.6, 7.9, 0.8, 0.3, and 0.2 μm/min, respectively. Larger growth rates imply weaker inhibition of ice growth, so that the strength order of ice-growth inhibition is A20L < A20G < WT < A20T < A20V < A20I. Maximal thermal hysteresis activity for A20I was evaluated at ~1.2 °C. FIPA analysis (Fig. 1) showed that the weakest A20L and A20G mutant represses less ice crystal growth than WT, while the A20T, A20V, and A20I mutants have a significant inhibitory effect on ice crystal growth (Fig. 1B). The size of the ice crystal is modulated according to the sample’s affinity to an ice crystal plane. (E) Actual FIPA patterns observed for A20L, A20G, A20T, A20V, and A20I mutants. (Scale bars, 1 cm.)
binds to an ice hemisphere only partly, whereas the strongest A20I binds to a substantial area of both pyramidal and first-prism planes. The size of the IBP-bound area on an ice hemisphere, therefore, is well correlated with the strength of ice-growth inhibition. It is significant that only one amino acid replacement at the 20th residue causes such a systematic change of the inhibitory action of ice crystal growth.

Poor ice-growth inhibition was reported previously for WT (17). This is verified by both the FIPA pattern and the fast ice-growth rate in our study (Figs. 1 and 2). The ice-growth inhibition ability of WT was decreased by the A20G mutation, in which the side-chain group of the 20th residue became smaller (CH₃ → H). The ice-growth inhibition ability of A20G was further decreased by the A20L mutation, in which the side-chain group became larger [H → -CH₂-CH-(CH₃)₂]. The ice-binding ability is therefore not correlated with the size of the side-chain group. Indeed, A20I containing the side-chain CH(CH₃)-CH₂-CH₃ group, the molecular weight of which is exactly the same as that of A20L, behaved in an opposite fashion to A20L (Fig. 1). Both A20I and A20V exhibited superior ice-binding ability and contain bifurcated β-carbon located in the γ-CH₃ group. Another mutant A20T containing a γ-CH₃ group exhibited similar ability to A20I and A20V. It is therefore speculated that the location of the γ-CH₃ group at the 20th residue plays a crucial role in the ice-binding ability of this IBP to both the first prism and the pyramidal ice planes. The γ-CH₃ group of V20 is contained in the HPLC12 isoform that also exhibits superior ice-binding ability, which might be further evidence of the significance of this side-chain group location in this series of IBP.

**Structures of the Mutants Are Highly Identical.** We prepared 75–200 mg/mL of the protein solutions and mixed 1–4-μL samples of these solutions with an equal amount of the reservoir solution (0.1 M sodium citrate, pH 3.5, with 2.0–2.5 M ammonium sulfate) (29), which generated the protein crystals of A20V, A20I, A20L, and A20T. The WT sample was crystallized into two different forms belonging to the space groups C222₁ and P₂₁2₁₂₁, denoted WT1 and WT2, respectively. Structures of WT1, WT2, A20V, A20I, A20L, and A20T were determined by the molecular replacement method utilizing the coordinates of HPLC3 [Protein Data Bank (PDB) ID 1OPS] and/or HPLC12 (PDB ID 1HG7). The structural refinement of these molecules was successively performed using 33,361–167,949 reflections to achieve 0.97–1.19 Å of high resolution with 0.11–0.13 of fine crystallographic R-factor (SI Appendix, Table S1). Every structure was characterized by an internal-dyad motif with a local twofold axis of symmetry contributed by many short twisted loops (Fig. 3), which is the well-known structural property of Zoaridace-derived IBPs (19–21). Averaged values of interatomic distances between HPLC3 and the determined structures of the mutants are highly identical. We prepared 75–200 mg/mL of the protein solutions and mixed 1- to 4-μL samples with an equal amount of the reservoir solution (0.1 M sodium citrate, pH 3.5, with 2.0–2.5 M ammonium sulfate) (29), which generated the protein crystals of A20V, A20I, A20L, and A20T. The WT sample was crystallized into two different forms belonging to the space groups C222₁ and P₂₁2₁₂₁, denoted WT1 and WT2, respectively. Structures of WT1, WT2, A20V, A20I, A20L, and A20T were determined by the molecular replacement method utilizing the coordinates of HPLC3 [Protein Data Bank (PDB) ID 1OPS] and/or HPLC12 (PDB ID 1HG7). The structural refinement of these molecules was successively performed using 33,361–167,949 reflections to achieve 0.97–1.19 Å of high resolution with 0.11–0.13 of fine crystallographic R-factor (SI Appendix, Table S1). Every structure was characterized by an internal-dyad motif with a local twofold axis of symmetry contributed by many short twisted loops (Fig. 3), which is the well-known structural property of Zoaridace-derived IBPs (19–21). Averaged values of interatomic distances between HPLC3 and the determined structures of the mutants are highly identical.
structures were evaluated, with root-mean-square deviation (rmsd) of only 0.35 Å (Fig. 3B). This indicates that the backbone conformations of all samples are highly identical to each other. All of the mutants had a 10-20th loop segment on their surface, and a region encircled by this loop was assigned to a compound IBS consisting of the prism and pyramidal IBSs (Fig. 3B) (25). The former is contributed by the putative ice-binding residues of T18, P19, and A20, and the latter by those of Q9, P12, N14, T15, A16, and Q44 (Fig. 3C). Overall, alteration of the ice-binding property originated solely from a limited structural change near the 20th residue by the present mutations.

In general, IBPs tend to crystallize so as to locate their ice-binding surface face-to-face in an asymmetric unit because of their relatively flat and hydrophobic nature (30). Such a preferred protein–protein contact has been thought to displace the surface waters from their original positions. For example, no ordering of water molecules was detected in the crystal structure of an insect IBP, whereas rings emerge if the waters on the multiple structures in an asymmetric unit are merged onto one structure (7). The distance between the merged waters in the rank showed an excellent match to that of waters constructing an ice plane, so they are assumed to anchor the IBP–ice complex formation. To forcibly expose an ice-binding surface to solvent and to intentionally observe ice-like waters, Sun et al. (31) crystallized the HPLC12 isomorph as a fusion molecule with a maltose-binding protein. They detected no robust network of clathrate waters on this synthesized molecule, but revealed several waters located on the pyramidal IBS. Here, it appeared that one molecule of A20T, A20L, and A20I, two molecules of A20V and WT1, and four molecules of WT2 were crystallized in an asymmetric unit of each protein crystal (SI Appendix, Fig. S1). The compound ice-binding site of all proteins was exposed to the solvent regardless of their crystal packing, and formation of ice-like waters was comparable between the molecules.

Waters Construct Polypentagonal Networks on A20I. Computer simulations have shown that an interfacial region between bulk waters and bulk ice consists of a 10- to 15-Å thick, intrinsically disordered quasi-liquid layer, which is more ordered than bulk waters, but less ordered than that of an hexagonal ice lattice (32, 33). The pentagonally arranged waters located outside of a four-helix-bundle structure of Maxi were thought to merge with those in the disordered layer in conjunction with the ice growth (3, 4). The A20I mutant (PDB ID 5XQU) similarly located ~50 semicladethrate waters consecutively jointed to form 18 polypentagonal networks (Fig. 3C and SI Appendix, Fig. S2). For the other mutants, pentagonal waters exist only dispersively and do not construct any robust network. These ice-like waters of A20I are mostly situated along the side-chain atoms of the ice-binding residues, which are roughly divided into two groups according to their locations. The first group, composed of seven pentagons (1–7 in SI Appendix, Fig. S2) with 19 semicladethrate waters, is located near the boundary of the two ice-binding sites contributed by Q9, P12, T15, A16, T18, P19, I20, M21, and Q44 (Fig. 3C). Five waters of 19 are hydrogen-bonded to either the e-amino group (denoted NE2) of Q9, the γ-hydroxyl group (OG1) of T15 and T18, or the backbone N and O atoms of A16, which strengthen this network formation. The second group consisting of 10 pentagons (8–18) with ~25 waters propagates from a region containing P12 to the end of the pyramidal-plane-binding IBS contributed by L10, I13, T47, and P48 (SI Appendix, Fig. S2). Hydrogen bondings between 7/25 waters and L10 (N, O), I13 (N, O), T47 (OG1), and K51 (N) strengthen the network formation of this second group. Before this study, five pentagonal ice-like water clusters were observed in crystals of crombin (PDB ID 1CRN), a 46-residue seed storage protein, as a result of intermolecular packing (34). Water polygons (pentagons or hexagons) were also created on hydrophobic regions formed between the helices of tetrameric malate dehydrogenase from the bacterium Chloroflexus aurantiacus (Ca MalDH) (PDB ID 4CL3) (35). Since its ortholog from a halophilic bacterium Salinibacter ruber (PDB ID 3NEP) contains no organized waters, acidic residue substitutions are thought to disrupt the pentagonal water formation. It was speculated from these results that surface waters tend to organize themselves as polygons with their nearest stable waters on a hydrophobic surface, not always related to the ice-binding ability.

Polypentagonal Network Contains Two Water Clusters. A neutron diffraction study has revealed the location of a tetrahedral water cluster in the first hydration layer of the HPLC12 isoform (PDB ID 3OF6) (36), the position of which matched those of the waters constructing the (1010) first prism plane. This water cluster is constructed from four surface waters and one γ-hydroxyl group of T18, where the average distance between the central water and the four vertices is 2.77 ± 0.02 Å, and the average H–O–H angle is 114.5 ± 1.3°. Here, we prepared a water coordinate file of the first prism plane with a size of 86 × 70 × 2 Å by employing a software VESTA (jp-minerals.org/vesta/jp) (37), and manually docked it onto the A20I structure (PDB ID 5XQU) to overlap a selected oxygen atom in the prism plane onto that in the polypentagonal waters. The coordinate file consists of only first and second layers of the prism plane, which avoided congestion of waters during the docking process and facilitated our position-match evaluations. Since a water cluster should be symmetrically located on the protein, we manually rotated this prism plane coordinate around the selected water to x, y, and z axes to achieve multiple superpositions as possible. The best-fit result was verified with evaluation of rmsd between the distances of all of the superimposed waters. As a consequence, we identified a water cluster prism (Fig. 4A, Left) in the first group of the polypentagonal network of the A20I mutant, the geometry of which is highly similar to that of the tetrahedral waters on the HPLC12 isoform. The identified waters were labeled 103–105 and 114, in which 103 and 104 are hydrogen-bonded to T18-OG1 and Q9-N2E2, respectively. The rmsd between the oxygen atoms of the water cluster prism and those of the first prism plane was only 0.14 Å, indicating their perfect position match. Significantly, this water cluster prism is located over the water cluster consisting of five water molecules labeled 103, 105, 104, 106, and 107, which are situated on the latter IBS. The second best mutant A20V locates this water cluster prism in the same position, although it has no polypentagonal network. In WT1, WT2, A20T, and A20L mutants, however, at least one of the waters appeared to be displaced from the tetrahedral geometry described above, as shown in Fig. 4B. In A20L, for example, four corresponding waters were displaced toward the 20th residue position without keeping the tetrahedral formation. This is probably due to a lack of a γ-CH3 group that creates a space to pull these waters inside, disrupting the water arrangement necessary to bind to the ice prism plane.

To search for additional water clusters, we manually docked a water coordinate of the (1000) basal and the (2021) pyramidal planes in the size of 86 × 70 × 2 Å onto the polypentagonal waters of the A20I mutant to superpose a selected oxygen atom of each plane onto another selected atom from the network waters. By employing the procedure utilized to find the water-cluster prism, we manually rotated each ice plane around the selected water to x, y, and z axes to achieve multiple superpositions of the other water molecules (SI Appendix, Fig. S3). The final docking model was evaluated by calculation of an average value of rmsd for all superimposed waters. Although no clustering waters corresponded to the (1000) basal plane, another water cluster consisting of five water molecules labeled 103, 105, 106, 107, and 113 (Fig. 4A, Right) was detected. These exhibited a perfect position match to the waters constructing the (2021) pyramidal plane, with only 0.28 Å rmsd, and were named “water
Among them, four water molecules, 103, 106, 107, and 113, are symmetrically located around the central water (105) to form four identical isosceles triangles, where the average distance between the four vertices and the center is 2.76 ± 0.03 Å. The waters 103 and 105 are also the constituents of the water cluster prism, and 103 and 107 are hydrogen-bonded with T18 (OG1) and A16 (O), respectively. This water cluster pyramid is constructed over the prism and pyramidal IBs (Fig. 3), in which only 106 and 107 are located on the latter.

For the A20V, A20T, WT1&2, and A20L mutants, at least one of the waters is shifted from the ideal symmetrical geometry, as shown in Fig. 4B. The difference between the ice-binding area (Fig. 1) and the strength (Fig. 2) of the variants is therefore ascribable to the degree of perfection of the water cluster prism and water cluster pyramidal constructed in each molecule, but not to the construction of the poly-pentagonal water network.

Polypentagonal Networks Merge with the Quasi-Liquid Layer and Freeze to Ice Lattice. Molecular dynamics calculation of the growth kinetics of the ice-water interface suggests that IBPs initially present an intermediate state in the quasi-liquid layer (Fig. 5) to dynamically associate with both mobile and restricted waters on the ice surface (38). To recognize such a disordered water layer, the hydration waters are thought to form a preconfigured structure (39). Our detection of a polypentagonal water network on an activity-improved variant of IBP is consistent with these previous indications; these organized waters in the hydration shell assist the A20I mutant in immersing into the quasi-liquid layer, which leads to both ice-binding sites being located in close proximity to the waters constructing the solid ice lattice (Fig. 5A). This facilitates successive binding of this activity-improved variant to both the (1010) prism and the (2021) pyramidal ice planes through the water cluster prism and water cluster pyramidal composing the polypentagonal network (Fig. 5B and C). The involvement of both ice-like semiclathrate waters and perfect lattice-match waters in the network waters must be effective for these two ice-binding steps to occur. Known examples of such space-match waters are seven linearly aligned waters on a hyperactive IBP from the beetle Tenebrio molitor (40) and the waters constructing extensive arrays on another hyperactive IBP from the bacterium Marinomonas primoryensis (41), which are thought to connect these proteins to an ice crystal plane through the ACW mechanism. The present mutants are also capable of binding to the ice planes through this mechanism with their constituents of the water cluster prism and water cluster pyramidal. Inclusion of an additional water cluster corresponding to the basal plane in the polypentagonal network may combine the host protein with the multiple ice planes of an ice crystal.
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