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

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Poly-pentagonal 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). Poly-pentagonal water networks composed of ~50 semicathrate 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 poly-pentagonal 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 -CH₃ 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 poly-pentagonal water network, the function of which is to freeze the host protein to specific ice planes.

hydration shell | ice-binding protein | crystal structure | water cluster | poly-pentagonal waters

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 poly-pentagonal 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 poly-pentagonal 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 poly-pentagonal 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, where 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) (16). The resultant difference between T_f and T_m , 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 ($\mu\text{m}/\text{min}$) 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

Significance

This study expands our knowledge of protein hydration, which is highly related to the macromolecular antifreeze property of proteins. We examined a poly-pentagonal network formation of waters for a series of artificial variants of a 65-residue ice-binding protein. The poly-pentagonal 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 poly-pentagonal arrangement, which merges with the intrinsically disordered ice surface and freezes to specific ice crystal planes.

Author contributions: S.M., Y.N., and S.T. designed the research; S.M., D.F., T.A., Y.N., R.S., and C.S. performed the research; H.K. and M.A. analyzed the data; and S.M. and S.T. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID codes: 5XQN, 5XQP, 5XQR, 5XQU, 5XQV, and 5XR0).

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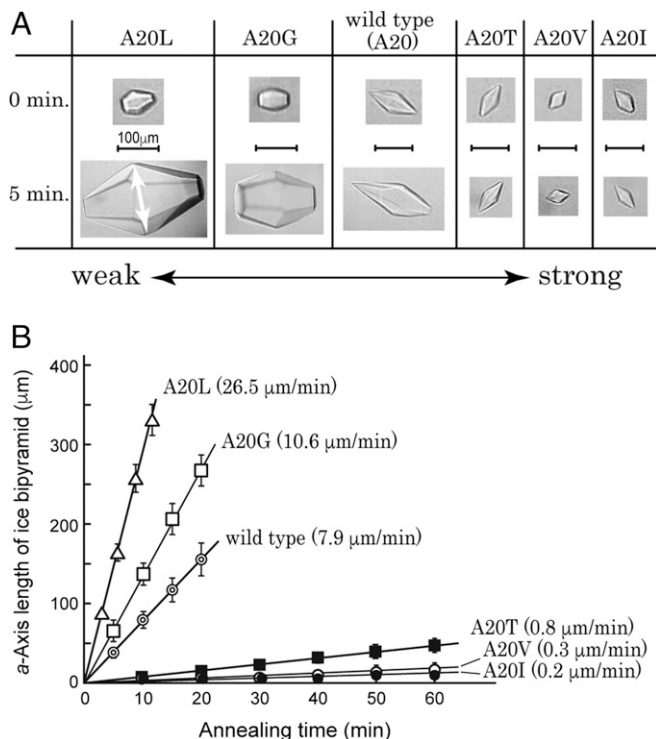


Fig. 2. Comparison of the strength of ice-growth inhibition between WT and mutant proteins. (A) Images of bipyramidal ice crystals captured for A20L, A20G, WT, A20T, A20V, and A20I mutants before and after 5 min of annealing time. The arrow indicates the length of the *a* axis. (B) Dependence of the length of the *a* axis on the annealing time measured at the $T_m - 0.05^\circ\text{C}$ with $0.2^\circ\text{C}/\text{min}$ of cooling. The larger growth rate ($\mu\text{m}/\text{min}$) implies a weaker ability for the ice to inhibit growth.

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 ($\text{CH}_3 \rightarrow \text{H}$). The ice-growth inhibition ability of A20G was further decreased by the A20L mutation, in which the side-chain group became larger [$\text{H} \rightarrow -\text{CH}_2-\text{CH}(\text{CH}_3)_2$]. The ice-binding ability is therefore not correlated with the size of the side-chain group. Indeed, A20I containing the side-chain $\text{CH}(\text{CH}_3)-\text{CH}_2-\text{CH}_3$ 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 - CH_3 group. Another mutant A20T containing a γ - CH_3 group exhibited similar ability to A20I and A20V. It is therefore speculated that the location of the γ - CH_3 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 - CH_3 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- to 4- μ L 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 $C22_1$ and $P2_12_12_1$, 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. 34), which is the well-known structural property of *Zoarcidae*-derived IBPs (19–21). Averaged values of interatomic distances between HPLC3 and the determined

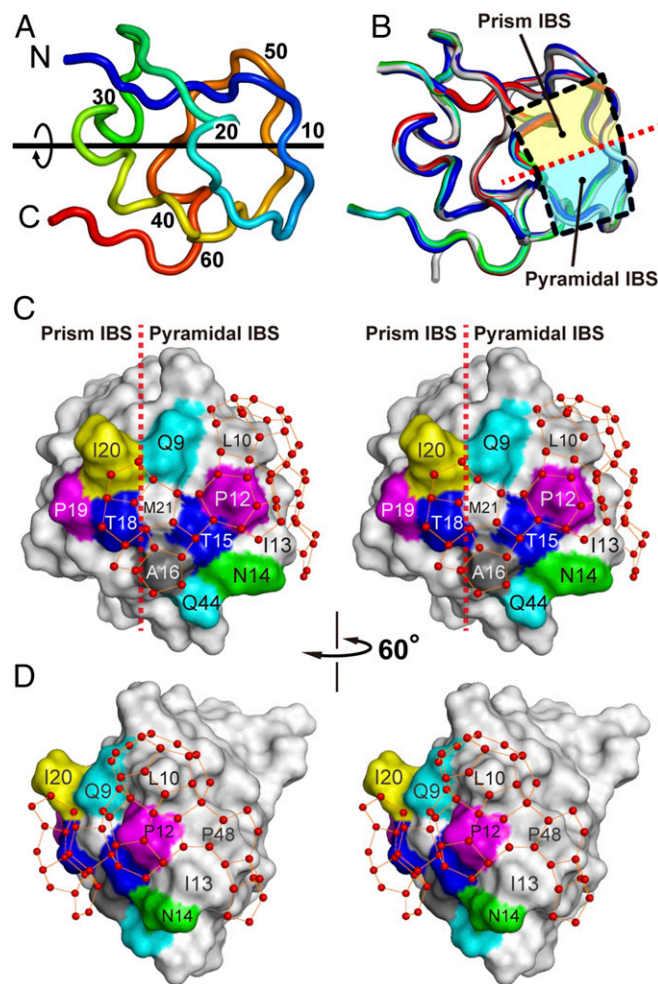


Fig. 3. Construction of polypentagonal water networks on the A20I mutant. (A) Loop representation of the backbone structure of WT1 (PDB ID 5XQN) characterized with an internal-dyad motif with a local twofold axis of symmetry typical for *Zoarcidae*-derived IBPs. (B) Overlay of the backbone structures of WT1, WT2, A20L, A20T, A20V, A20I, and HPLC3 (PDB ID 1OPS). Two squares separated by a broken line indicate the location of two IBSS: one is the first prism plane (prism IBS, yellow) and another the pyramidal plane (pyramidal IBS, cyan). (C) Stereoview of the A20I structure with ~50 semicathrate waters constructing the polypentagonal network. A broken line indicates the boundary of the two IBSS. (D) A 60°-rotated image of the A20I structure showing the side of the polypentagonal networks that surround edge of the pyramidal IBS.

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) (22). 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 ranks 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 isoform 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 semiclathrate 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 semiclathrate 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 ϵ -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 crambin (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 3QF6) (36), the position of which matched those of the waters constructing the (10 $\bar{1}$ 0) 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 $114.5 \pm 1.3^\circ$. Here, we prepared a water coordinate file of the first prism plane with a size of $86 \times 70 \times 2$ 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 on 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-NE2, 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 first prism plane IBS and the pyramidal plane IBSs; the water 103 is situated on the boundary, 114 on the former IBS, while 104 and 105 are 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 (20 $\bar{2}$ 1) pyramidal planes in the size of $86 \times 70 \times 2$ 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 (20 $\bar{2}$ 1) pyramidal plane, with only 0.28 Å rmsd, and were named “water

Materials and Methods

Measurement of Ice-Growth Rate and TH Activity. We used a Leica DMLB100 photomicroscope system equipped with a Linkam THMS 600 temperature controller to examine the morphology of a single ice crystal and its growth rate. A 0.7- μ L droplet of the IBP solution was placed near the center of a capillary tube ($\phi = 0.92$ mm, $l = 10$ mm), the termini of which were sealed with mineral oil. This capillary tube was inserted into a disk-shaped holder ($\phi = 17$ mm, thickness = 2.4 mm), which was placed on the cooling stage of the photomicroscope system (28). The temperature of the protein solution in the capillary tube was controlled with an accuracy of ± 0.2 °C. The tubes were frozen entirely at once by lowering the temperature to -25 °C to form a multicrystalline state and melted back to near 0 °C to carefully leave one single ice crystal with a size of 10–20 μ m, which enabled the melting point (T_m) to be determined. The single ice crystal in the protein solution was modified into a bipyramid by lowering the temperature at a constant cooling rate (0.1 °C/min), which started off bursting ice crystal growth at a certain temperature. This observation allowed us to determine both T_{burst} and the ice-growth speed (μ m/min). The values of T_m , T_{burst} , and the ice-growth speed were measured at least three times, and their averaged value was used for comparison.

X-Ray Crystallography. All proteins were crystallized at 277 K by the hanging-drop vapor-diffusion method using EasyXtal 15-Well Tool (Qiagen). Before crystallization, the solutions of all mutants were dialyzed against 20 mM

Tris-HCl buffer (pH 8.0) and concentrated by ultrafiltration using Amicon ultra 3000NMWL (Merck). The crystallization droplets were equilibrated to 500 μ L of the reservoir solution. The X-ray diffraction data were obtained at 100 K in Photon Factory (BL5A, 6A, and 17A) and processed with the program HKL2000 (42). It should be noted that both the crystal and hydration structures of HPLC12 determined at 110 K (PDB ID 1HG7) (21) and 277 K (PDB ID 1MSI) (19) were not significantly different; their backbone structures exhibited only 0.32 Å of rmsd. The crystallographic refinements of the protein structures were performed with the program PHENIX (43). When the structure was refined by replacing one of the polypentagonal waters with an Na⁺ ion, a negative density of the difference Fourier map at the replaced position was observed. The replacements also increased the B-factor of that position, implying that the Na⁺ ion is not positioned in the water network. The deposited PDB codes (<https://www.rcsb.org/>) of each sample are 5XQN (WT1), 5XQP (WT2), 5XQR (A20V), 5XQU (A20I), 5XQV (A20L), and 5XR0 (A20T). Statistics of obtained diffraction data and crystallographic parameters are listed in *SI Appendix, Table S1*.

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