COMMENTARY

Antifreeze protein hydration waters: Unstructured unless bound to ice

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How do fish, insects, and other organisms survive in frigid polar environments? They do so with the help of remarkable molecules known as antifreeze proteins (AFPs), which suppress freezing and associated cell death despite being present at concentrations of less than 1 wt % (1). In contrast, automotive antifreeze needs roughly 20 to 50 wt % of the additive to function (2). AFPs are able to suppress freezing at such low concentrations because, unlike antifreeze, they do not rely on altering the inherent structure of water; instead, they bind to nascent ice nuclei and prevent them from growing (3). Thus, being able to recognize and preferentially bind ice in a vast excess of water is the key to AFP function (1). However, in the absence of any chemical differences between water and ice, how do AFPs discriminate between them? Moreover, both water and ice are composed of a tetrahedral network of hydrogen bonds, so even the structural differences between them are subtle. Indeed, how AFPs are able to perform what has been touted as one of the most challenging molecular-recognition tasks in all of biology has long been a source of amazement and intrigue (1, 4). In PNAS, Hudait et al. (5) clarify important aspects of the ice-recognition puzzle by using molecular simulations to study *Tm*AFP, a hyperactive insect AFP.

What makes this puzzle even more fascinating is that a wide array of organisms, ranging from bacteria to insects and fishes, has independently evolved AFPs that display substantial differences in their sequences, structures, and ice-binding sites (IBS) (6, 7). In other words, there is not one, but a diversity of motifs that can confer AFPs with their ice-binding abilities. What, then, are the characteristic features of IBS, and how do they enable AFPs to bind ice? Early suggestions focused on hydrophilic moieties, which could hydrogen-bond with ice; the rationale was that if the hydrophilic groups on the IBS were spaced to match the lattice spacing of ice, the binding between the AFP and ice would be particularly favorable (1, 8). In other words, a complementarity in the spacing of hydrophilic groups on the AFP surface and the crystal planes of ice was believed to enable AFPs to bind, and integrate themselves into, the ice lattice.

However, as numerous AFPs from diverse organisms were discovered, and their crystal structures solved, it became clear that hydrophobic residues featured prominently on the IBS of AFPs (7). Even hydrophilic residues, such as threonine, had hydrophobic groups ($-CH_3$), which protruded outward from the IBS (Fig. 1*B*). The presence of hydrophobic groups on the IBS suggested that although hydrogen bonding and lattice matching could help AFPs bind ice, they were not sufficient by themselves. It also raised the question that, given that hydrophobes do not interact favorably with water or with ice, what role might they have in facilitating the binding of AFPs to ice? The hydration waters of a small hydrophobic group are constrained by having to hydrogen-bond around the hydrophobe, which is unfavorable from an entropic standpoint, so it was suggested that the release of such constrained waters from hydrophobic groups on the IBS might facilitate AFP–ice binding (1).

Thus, both hydrophilic and hydrophobic groups seem to be important in conferring AFPs with an affinity for ice. Mutagenesis studies have further shown that a single mutation, whether it replaces a hydrophobic group with a hydrophilic one or vice versa, can result in the loss of AFP function (4). Such seemingly whimsical behavior has also been observed in molecular-simulation studies, which probe the "ice philicity" of extended surfaces by interrogating their propensities for nucleating ice. In particular, Patey and coworkers found that kaolinite, a clay mineral, is only able to nucleate ice if it has the right amount of flexibility (9); Molinero and coworkers observed that the roughness of a graphitic surface influences its ice philicity (10); and Michaelides and coworkers showed that neither lattice matching (to ice) nor surface hydrophilicity alone is sufficient to confer ice philicity, but that appropriate values of both are needed (11). Collectively, these studies suggest that the affinity of an AFP surface for ice depends not only on its ability to hydrogen-bond with ice but also on

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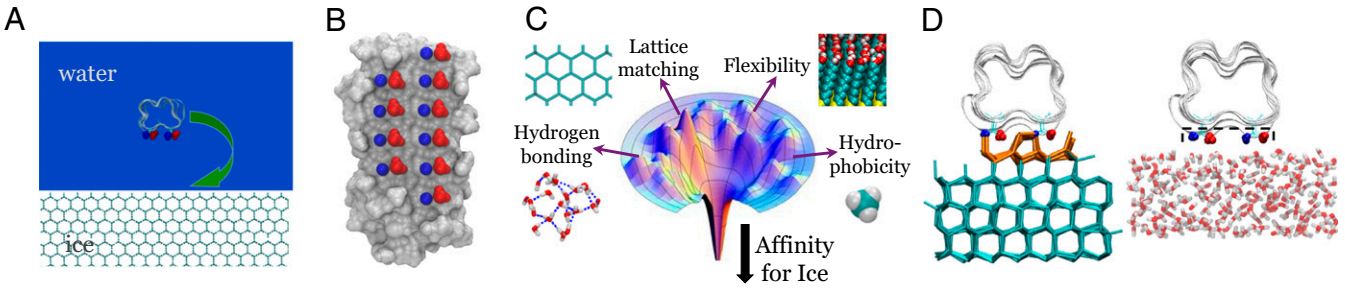

Fig. 1. (*A*) AFPs have evolved to recognize and bind ice in a vast excess of water. (*B*) The IBS of typical AFPs display both hydrophilic (blue) and hydrophobic (red) groups (7). (*C*) The affinity of a surface for ice depends sensitively on a number of surface properties, including its ability to hydrogen-bond with ice, correspondence with the ice lattice, flexibility, and the presence of nonpolar groups (4, 9–11). Adapted from ref. 24. (*D*, *Left*) Although *Tm*AFP binds ice through the AC motif, (*D*, *Right*) it does not preorder water in solution. Adapted with permission from ref. 5.

many other properties, such as its curvature, roughness, and flexibility, in a nontrivial manner (9–11) (Fig. 1*C*).

Although no clear unifying features appear to enable AFPs to preferentially bind ice, could the affinity of an AFP for ice be reflected in certain properties of its hydration waters? Using molecular simulations of diverse AFPs, Sharp and coworkers showed that the orientational distribution of water molecules in the vicinity of IBS display certain key differences, which persist across different classes of AFPs (12–14); Nutt and Smith reported a subtle increase in tetrahedral order in the hydration shell of a hyperactive insect AFP (15); and both molecular simulations and terahertz spectroscopy measurements have reported slower relaxation dynamics of the AFP hydration waters (16–18). In each case, the waters in the AFP hydration shell seem to, in one way or another, bear some resemblance to those in ice. Do such seemingly ice-like waters enable AFPs to bind to ice and, if so, how?

The crystal structure of a bacterial AFP, *Mp*AFP, solved by Davies and coworkers, provided an important clue by presenting, for the first time, a clear molecular picture of the AFP–ice binding interface (6, 19). Although AFP hydration waters can be resolved in the X-ray structure, they are usually perturbed by protein–protein contacts in the crystal and tend not to be characteristic of waters that mediate AFP–ice binding. However, *Mp*AFP crystallizes in a unique manner with its IBS well removed from any protein–protein contacts, so that the AFP–ice interface is clearly visible in the crystal structure (6, 19). In what Davies and coworkers called the anchored clathrate (AC) motif, the AFP uses both hydrophobic and hydrophilic groups on its IBS to bind ice (19). In the AC motif, water molecules adopt a highly ordered structure, forming a clathrate-like shell around the hydrophobic groups of the IBS and participating in hydrogen bonds with its hydrophilic groups (Fig. 1*D*). Based on the clear evidence of structured waters at the AFP–ice interface, and the fact that the IBS hydration waters are somewhat ice-like (in solution), it was suggested that a highly structured layer of clathrate-like waters exists at the IBS of some AFPs, which then enables the proteins to recognize and bind ice (6, 19).

Molinero and coworkers explore this tantalizing suggestion in a series of recent articles (5, 20, 21). In ref. 20, the authors studied a number of hyperactive AFPs derived from insects and found that AFP binding to ice can occur not only through the AC motif but also through other similar motifs, wherein structured waters bridge the AFP surface and ice. Given the diversity of IBS observed across different AFPs, this result makes sense. Although both experiments and simulations highlight the presence of structured waters at the AFP–ice interface, does this structuring precede binding, and, if so, could the preordering of waters by a surface be used as a signature of its affinity for ice? To answer these questions, Hudait et al. (5) chose to study *Tm*AFP, a hyperactive insect AFP, which displays the best lattice matching with hexagonal ice and may thus be the most likely to structure waters into the AC motif in solution. Using molecular simulations with three different water models, and multiple ways of characterizing the structuring of IBS hydration waters, the authors conclusively show that the IBS does not display ice-like or AC-like order in solution; rather, the AC motif forms only after the AFP has moved next to the ice surface and aligned itself in an orientation that is optimal for binding to ice (5). These results suggest that a preordering of their hydration waters is not needed for ice recognition by AFPs. Support for these findings is also provided by NMR studies, wherein a fast (subnanosecond) exchange of waters is observed between the hydration shell of *Tm*AFP and the bulk (22); by contrast, AC waters ought to relax much more slowly, akin to those in ice. More support comes from earlier work by Molinero and coworkers, which showed that poly(vinyl alcohol), a flexible polymer that does not preorder water, nevertheless binds ice (21). As more AFPs belonging to different classes are studied, and as even more AFPs are discovered, it will be interesting to see if an AFP capable of preordering its hydration waters will be found.

Ice-nucleating proteins (INPs) represent another class of proteins that are ice-philic; although they are structurally and chemically similar to AFPs, INPs are much bigger in size and can even cluster, thereby providing large surfaces capable of nucleating ice. Although *Tm*AFP does not structure its waters in solution, might the larger INPs or their clusters be able to do so? To answer this question, Hudait et al. (5) use model AFP-like surfaces spanning a range of sizes; INP monomers are represented by rectangular surfaces with a fixed nanoscopic width and increasing lengths, whereas INP clusters are mimicked using square surfaces. The authors find that large rectangular surfaces do not structure their hydration waters in solution, but that as the size of the square surfaces is increased, they become more effective at ordering water. These findings suggest that it is not easy for surfaces to structure their hydration waters into ice-like or clathrate-like order; only large extended surfaces, like the ones assembled by INP clusters, may be successful in doing so. To facilitate experimental validation of their findings, the authors also used their simulations to predict vibrational spectra of the OH stretch of water; however, they find that neither infrared nor Raman spectra can discriminate between AC waters at the AFP–ice interface and the hydration waters of the AFP in solution (5).

Despite the remarkable progress made in understanding AFPs, their most sought-after secrets remain elusive for now—both what

the molecular signatures of surface ice philicity are and how they enable AFPs to bind ice continue to mystify. However, the work of Hudait et al. (5) shows that the preordering of waters in solution, whether in the AC motif or otherwise, is not necessary for AFPs to bind ice. Importantly, the authors demonstrate how molecular simulations can build upon the wealth of knowledge provided by structural studies, and how they are beginning to make meaningful contributions in furthering our understanding of AFPs. With such synergistic advances, we are hopeful that it will not be long before the secrets of AFPs are uncovered. A fundamental understanding of ice philicity could also have far-reaching practical implications and may lead to the discovery of synthetic molecules and materials that are even more potent at binding ice than AFPs; such materials would find use in diverse contexts, ranging from the preservation of organs for transplant to increasing the freeze tolerance of crop plants and the transportation of frozen foods (23).

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