

A peek at ice binding by antifreeze proteins

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What is the toughest recognition problem in biology? Arguably, it is that faced by antifreeze or thermal hysteresis proteins. These proteins provide a broad range of organisms with protection against freezing damage by depressing, in a noncolligative manner, the freezing point of water. They do this by binding to nascent ice nuclei and inhibiting their growth (1). In other words, antifreeze proteins (AFPs) must distinguish one phase of water, ice, from another phase, liquid. Moreover, the latter is present in great excess at 55 M. There are no chemical differences to key off, just the subtle structural differences, still poorly characterized, that exist between the surface of ice nuclei and liquid water. In addition to the intrinsic interest of the ice recognition problem, AFPs have application to cryopreservation of tissues and organs (2) and to the food industry (3). The work of Davies and colleagues (4) in PNAS represents a significant step forward in understanding the detailed mechanism of AFP action (i.e., a first look, if you will, at an AFP in the act of discriminating between different structural states of water). To put this work into perspective, it is useful to have some background on the study of AFPs, which has a number of paradoxical elements.

First, there is the sheer exuberance of structural motifs found in AFPs. AFPs have been isolated and structurally characterized from a wide range of organisms from bacteria, through insects to arctic fish. Fig. 1 illustrates just 3 of the 12 or so different structural motifs discovered in AFPs to date. Each is oriented so that the ice-binding surface (IBS) is uppermost. Reading from the top, there is a small globular protein from sea pout, a single α -helix from winter flounder, and a stack of left-handed PP-II helices from the snow flea. The work of Garnham et al. (4) in PNAS gives a fourth example. *MpAFP* from the Antarctic bacterium *Marinomonas primoryensis* adopts a right-handed, parallel β -helix. Discovery of a previously undescribed AFP quite often reveals a unique protein motif. A case in point is the snow flea AFP, discovered by Graham and Davies (5). Structure determination, as described by Pentelute et al. (6), revealed the unique motif in Fig. 1. In a fascinating side note, this was made possible through generation of racemic protein crystals using total chemical synthesis. This simplified the crystallo-

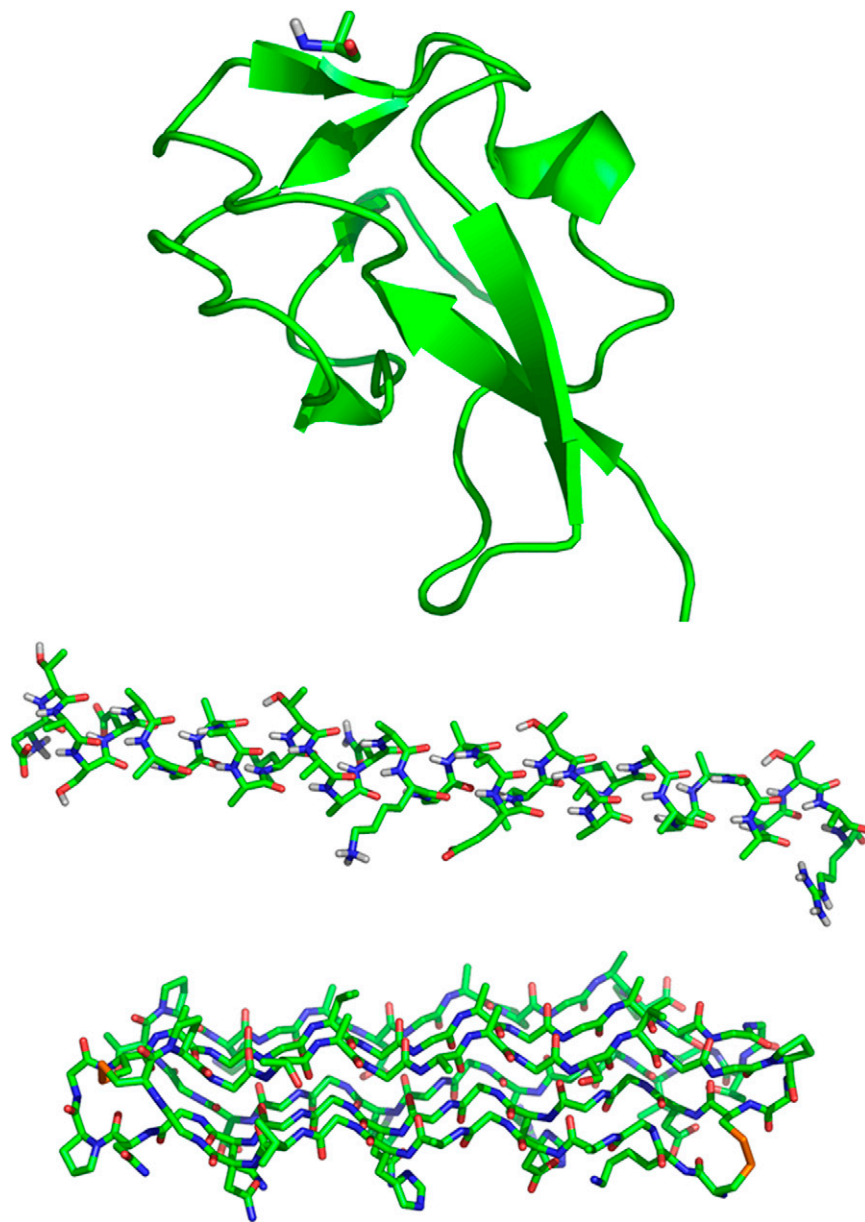


Fig. 1. Three AFP structural motifs. The IBS is oriented uppermost. (Top) Type III AFP from ocean pout (PDB ID code 1MSI) (18). Alanine 16, a key residue in the IBS, is indicated in stick representation. (Middle) Type I AFP from winter flounder (PDB ID code 1WFA) (19). (Bottom) AFP from the snow flea (PDB ID code 2PNE) (6).

graphic solution. Because AFPs recognize an achiral “substrate,” they are one of the few proteins in which both enantiomers would have identical biological activity. Fig. 1 also shows that there is little uniformity of AFP structure within broad classes of organism: Two very different structures come from ocean fish. Thus, the identical function, depression of the freezing point, can be manifested by

widely different protein motifs. This raises a number of interesting questions. Do these AFPs all work by the same mechanism? Do they bind to the same plane(s)

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of the ice crystal? Can a particular AFP bind to more than one ice plane? The paradox of these structural studies is that although they provide a detailed look at various AFPs, and individual water molecules are often resolved in the crystals, they show little about the relevant interactions with water. In almost all cases, the IBS of the protein is in close contact with another copy of the protein. Hydrating waters lie in narrow spaces between protein molecules. Thus, any structural features of the hydration are likely to be heavily influenced by lattice contacts. This is not the case for the crystals of *Mp*AFP. As Garnham et al. (4) note, there are “two areas in the unit cell where the IBS was completely solvent-exposed, ensuring specific protein:solvent interactions free from crystal-packing artifacts.”

A long-standing question about the AFP mechanism is what kind of interactions mediate ice binding. Because ice crystal surfaces are bristling with OH groups, and early studies on type I AFPs revealed a regular array of Thr side chains on the IBS (Fig. 1, *Middle*), H-bonding interactions were a logical first choice (7). Type III AFPs (Fig. 1, *Top*) clearly do not have such an array of H-bonding groups, however. In fact, the IBS of ocean pout has an essential hydrophobic residue, Ala, at its center. This and other work suggested that hydrophobic groups were key (8, 9), along with a flat-binding surface (10). The contribution of mutation studies to this debate was another paradox. For example, making the mutation Thr→Ala in the IBS decreases thermal hysteresis activity in type I AFP (9), whereas the opposite mutation, Ala→Thr, decreases activity in type III AFP (11). Either different proteins are using different types of residues or the recognition event is more subtle, involving more than one kind of interaction. Use of exclusively H-bonding groups or exclusively apolar groups to drive binding also raises a problem I call the affinity-specificity paradox. If one uses H-bonding groups alone, these would provide good affinity by making strong H-bonds with

water. It would be difficult to achieve specificity for ice, however, because these groups prefer less ice-like water structures. Conversely, use of purely apolar groups would confer specificity, because such groups prefer a more ice-like solvation structure. There would be little affinity, however, because they are, well, hydrophobic. An obvious suggestion is that AFPs use both interactions but that structural evidence is lacking. The analysis of Garnham et al. (4) clearly implicates both hydrophobic and H-bonding groups.

A final paradoxical element in the study of AFPs is the contrast between the simplicity of the assay for function and the difficulty of studying the “active complex.” The assay is depression of freezing point, which basically requires a temperature measurement. Study of the active complex of an AFP is fraught with difficulties. The usual tools of enzyme-substrate and protein-ligand analysis are absent: no inhibitors, competitive or otherwise, and no substrate analogs. The task of cocrystallizing the relevant complex for X-ray studies is daunting: One would have to crystallize an AFP bound to an ice nucleus in the presence of water under constant but nonequilibrium (i.e., supercooled) conditions. Even leaving aside the protein, our understanding of the structural differences between the ice surface and liquid water is still imperfect and basic issues, such as the number of H-bonds involved, are subject to debate (12, 13). “Structural” studies of binding have been largely limited to ice etching and analysis of ice crystal morphology changes (14). These studies have identified which planes of ice a particular AFP binds to and have established that similar AFPs may bind different faces. How the growth of ice crystals is inhibited after binding occurs is a separate problem and is largely unknown. Two mechanisms are straightforward steric occlusion of the growing plane (15) and remodeling of the ice surface (16). Given the difficulties of studying the actual AFP-ice nucleus complex, the structure of *Mp*AFP described here takes

us as close as we might hope. There is a large region of solvent adjacent to the IBS that is resolved into individual water molecules. Moreover, there are two non-crystallographic symmetry related (i.e., independent) copies in the crystal structure, showing very similar hydration structure. The “anchored clathrate” in the title of Garnham et al.’s article (4) summarizes well the principal features of the IBS hydration they see. The water adopts a more ice-like structure surrounding hydrophobic groups. This structure is anchored at the edges by H-bonds to nearby polar groups. The answer is “yes” to the question, “Do AFPs use H-bonding or apolar groups to bind ice?” The resulting hydration structure has good spacing for binding to both basal and primary prism faces of ice, indicating that binding to multiple ice sites is possible for this AFP. Ice-etching studies could presumably test this. Moreover, by inducing a more ice-like structure than in the surrounding bulk water, the AFP may be performing its binding site. This also suggests a third possible binding mechanism: The AFP brings its own “ice” with it, similar enough to bind the ice nuclei but different enough to slow crystal growth once attached. Speculating even further, if the AFP left this hydration ice behind on dissociation, multiple binding events could enable it to poison growth over a larger area than its actual IBS, acting in a quasi-symmetrical manner.

Are the hydration structure of *Mp*AFP and the putative binding mechanism common to other AFPs? Molecule dynamics simulations of type III AFP, which is probably as different in size and structure as any known AFP, suggest so. Its IBS hydration is remarkably similar. Clathrate-like water is found around an apolar IBS residue, anchored at its edges by interaction with the immediately surrounding polar groups (17). New crystal structures of other AFPs with similar large regions of solvent around their IBSs may tell us more.

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