A model for binding of an antifreeze polypeptide to ice

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ABSTRACT A model is proposed, based on recent peptide analog and ice crystal etching studies, whereby an alanine-rich, α-helical antifreeze polypeptide (AFP) from the winter flounder inhibits the growth of ice crystals by hydrogen bonding of Thr, Asn, and Asp side chains in a specific pattern to the $\{20\bar{2}1\}$ hexagonal bipyramidal planes of ice. It is further suggested that this mode of binding is unidirectional, maximizing opportunities for packing of AFPs on the ice surface, and that ice crystal growth inhibition occurs by a two-step mechanism involving hydrogen bonding and hydrophobic interpeptide interactions.

INTRODUCTION

Antifreeze polypeptides (AFPs), synthesized by polar fishes, lower the plasma freezing point by arresting the growth of ice nuclei down to about −1.9°C, the freezing point of seawater (1, 2). Several classes of AFPs exist, but the simplest and best studied are the alanine-rich, α-helical AFPs (M_r 3000–4000) typified by those from the winter flounder, *Pseudopleuronectes americanus* (2). These AFPs exist as α-helical rods having a regularly spaced array of polar amino acids (e.g., Thr, Asn, Asp) along one face of the helix. It has been suggested by several investigators (3–6) that the polar residues (in particular, Thr) bind to ice, thereby presenting the relatively hydrophobic opposite face of the AFP to liquid water and inhibiting growth of the ice lattice. Up to now, however, the details of the AFP–ice interaction have not been well described.

Some important new clues as to the nature of this interaction have come from results of Knight et al., (3), who concluded from studies of etched single crystals of ice that binding of an α-helical AFP is along the direction $\langle 01\bar{1}2\rangle$ on the $\{20\bar{2}1\}$ hexagonal bipyramidal planes of ice, and who noted that the 16.7-Å spacing along this direction closely matches the 16.6-Å spacing of Thr residues along the helix axis of the AFP. In addition, we have recently shown (7) that systematic rearrangement of the Thr, Asn, and Asp residues in synthetic analogs of the winter flounder AFP, designated S00, results in moderate to complete loss of antifreeze activity, depending on the extent of modification (Table 1). From these results we have concluded that Asn/Asp residues are probably at least as important as Thr for binding. In this study we also found evidence for a two-step process of ice crystal growth inhibition: at low AFP concentrations, ice crystals grow slowly as hexagonal bipyramids with a constant c-axis to a-axis ratio of about 3.3, which is close to the ratio of 3.26 calculated for a hexagonal bipyramid composed of twelve planes $\{20\bar{2}1\}$; above a critical concentration, which is probably related to the binding constant of the AFP for ice, crystal growth ceases until the freezing point is reached. At the freezing point, in concentrated AFP solutions, ice crystals grow rapidly along the c-axis to form characteristic needles (4). In the present communication, we present a model for AFP binding that incorporates the foregoing observations and gives new insights into the mechanism of AFP activity.

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THE MODEL

The details of our proposed binding mechanism are given in Figs. 1 and 2. Because it cuts through the ice lattice obliquely to the c-axis, the plane $(20\bar{2}1)$ has a stepped surface with ridges and valleys (Fig. 1 *B*) that potentially can match an AFP having regularly spaced protrusions along the helix axis. In this respect, the $\{20\bar{2}1\}$ surfaces differ from the prism $\{10\bar{1}0\}$, secondary prism $\{2\bar{1}\,\bar{1}0\}$ and basal (0001) planes, which have many closely spaced water oxygens in the same plane. The latter planes also have fewer potential hydrogen bonding partners, and $\{10\bar{1}0\}$ and (0001) have no 16.7 Å spacings. Molecular modeling and energy minimization studies show that AFP S00 can bind to $(20\bar{2}1)$ along $[\bar{1}102]$[1] very comfortably,[2] with each Thr and each Asn

[1] The hexagonal bipyramid, as represented in Figs. 1 *A* and 3, has twelve equivalent surfaces $\{20\bar{2}1\}$ and twelve corresponding AFP alignment positions $\langle 01\bar{1}2\rangle$, one of which is $[\bar{1}102]$. The specific alignment direction used in this discussion, $[\bar{1}102]$, is that which is appropriate for the specific plane $(20\bar{2}1)$.

[2] To assess the relative goodness of fit of AFP molecules bound to the ice surface in various orientations, the energy of the AFP-ice system was compared to the sum of the energies of the AFP and the ice alone: the better the binding, the greater should be the differences. This was done by first making the best visual fit of the AFP on the ice lattice on the Silicon Graphics workstation monitor. The Thr hydroxyl and Asp carboxyl oxygen and Asn nitrogen atoms were then constrained in their "optimal" hydrogen bonding positions, and the AFP and ice lattice were energy minimized together (using CHARMm; ST2 water model; steepest descent method; dielectric constant = 1.00), keeping the ice lattice rigid. The constraints were then released and the system was reminimized to give a total energy (E_B) for the bound AFP. Energies were also calculated for the ice lattice alone (E_I) and the peptide alone (E_P). The difference in energies (ΔE_B) between the interacting and noninteracting systems was calculated as $\Delta E_B = E_B - E_I - E_P$. The ΔE_B values for binding of S00 to the $(20\bar{2}1)$ surface along $[\bar{1}102]$, $[0\bar{1}12]$ (the reflection of $[\bar{1}102]$) and $[1\bar{1}0\bar{2}]$ (180° rotation of $[\bar{1}102]$ are −307, −260, and −248 kcal, respectively, which is consistent with most

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TABLE 1 **Amino acid sequences and activities of synthetic antifreeze peptides**

Peptide	Amino acid sequence	Relative activity	Hydrogen bonds
		%	
	1 12 23 34		
S00	D TASDAAAAAAL TAANAKAAAEL TAANAAAAAAA TAR	100	13
S04	D TASDAAAAAAT LAANAKAAAEL TAANAAAAAAA TAR	65	11
S11	D TASDAAAAAAL TAANAKAAAEL TAAAAANAAAA TAR	65	11
S23	D TASDAAAAAAL NAATAKAAAEL TAAAAANAAAA TAR	17	7
S22	D TASDAAAAAAN LAATAKAAAEL TAAAAANAAAA TAR	30	7–8
S21	D TASADAAAAAN LAANAKAAAEL TAAAAANAAAA TAR	0	6
S40	D TASDAAAAAAA TAANAKAAAEA TAANAAAAAAA TAR	67	13

The winter flounder AFP (S00) and six analogs (S04–S40) were synthesized and assayed for antifreeze activity (7), which is defined here as the freezing point depression of a 1 mM solution relative to that of S00. Circular dichroism data showed that all peptides were essentially 100% α-helical (7). Gaps have been placed so as to emphasize the 11-residue repeat sequences. In S00 the Asn (N), Asp (D), and Thr (T) residues, which form the polar face (see Fig. 1 *C*) of the α-helix, are in boldface. All of the mutations (*underlined*) are exchanges of two or three amino acids, except for S40, where the Leu residues are replaced by Ala. The bound peptide was energy-minimized as described in Fig. 2, and the computed hydrogen bonds were counted.

making two hydrogen bonds, but Asp only one (Fig. 2 *B*). Unexpectedly, on energy minimization of S00 on ice, the two Leu side chains migrated down into valleys (Fig. 2), suggesting a possible gain of binding energy due to van der Waals interactions with the ice lattice itself. This idea is strengthened by the observation that replacement of the Leu residues by Ala in S40 results in a loss of about one-third the activity (Table 1). The other analogs, depending on the extent of mutation, can make fewer hydrogen bonds, resulting in lower antifreeze activity. Analog S23, which has the identical sequence to S11, except for the interchange of residues Thr[13] and Asn,[16] has only about 25% the activity of S11, indicating that the T- and N-sites are not equivalent. Modeling shows that no hydrogen bonds can be formed with these residues in S23, except by distorting the helix backbone. On the other hand, further rearrangement, as in S22, puts Asn in a Leu position in a valley, where it appears to be able to form one weak hydrogen bond. Perhaps for this reason, S22 is nearly twice as active as S23. S00 can also be fit to the surface of the secondary prism plane (3), but fewer hydrogen bonds appear to form and the overall congruence of peptide and ice surface is not as good as for $(20\bar{2}1)$.

DISCUSSION

According to our model, there is only one favorable orientation of the AFP on the plane $(20\bar{2}1)$, namely along $[\bar{1}102]$ with the amino-terminus oriented towards the apex of the hexagonal bipyramid (Fig. 3). Rotation of the AFP by 180° would place Asn in a T-site and Thr in an N-site, which is unfavorable because of steric interaction of the Thr methyl with the surface. It is possible to construct a model for binding along the reflected direction $[0\bar{1}12]$, but the interactions are not so favorable, as the potential N- and T-sites are now only 4.5 Å apart, and the Leu side chains are forced to occupy sites above the ice surface. This proposed selectivity of binding helps us to understand why Knight et al., (3) saw no evidence for binding along the reflected direction $[0\bar{1}12]$. Further, it follows that if only a single orientation is allowed, the opportunities for packing of AFP molecules on the surface are increased, as indicated in Fig. 1, resulting in maximum coverage of the surface and increased activity. It is noteworthy that the packing arrangement shown in Fig. 1 *A* is the only one that allows close association of AFP chains. Because the helix axis and the Thr/Asx axis are skew (Fig. 1 *C*), placing two molecules side-by-side in any other arrangement will put them either too close or too far apart to make favorable van der Waals contact. It is also interesting, and perhaps not coincidental, that in the packing arrangement shown in Fig. 1 *A* the termini of the AFP molecules can be aligned along $[\bar{1}\,\bar{1}26]$, which is the same direction as an edge in the hexagonal bipyramid.

We envisage a two-step mechanism for binding: at low AFP concentrations, relatively few molecules bind (probably reversibly) to the ice surface, and the observed slow growth of the hexagonal bipyramidal ice crystals is permitted (Fig. 3 *B*). At sufficiently high concentrations the AFP molecules begin to pack together (Figs. 1 *A* and 3 *A*), greatly enhancing binding as well as antifreeze activity by cooperative intermolecular interactions. This would explain the rather sharp transition from the slow-growth to the no-growth stage (7). In other words, at low concentrations, activity depends primarily on the hydrogen bonding of the AFP analog to the ice surface, but

favored binding (by about 60 kcal) along $[\bar{1}102]$, as proposed by Knight et al. (1991). Of course it must be realized that these calculations, which do not include solvent, ignore the energy cost of removing solvent water molecules from the AFP before binding to ice. However, this contribution should be the same for all orientations, and subtracting it should reduce the magnitude of ΔE_B values, but greatly increase the degree of difference between them.

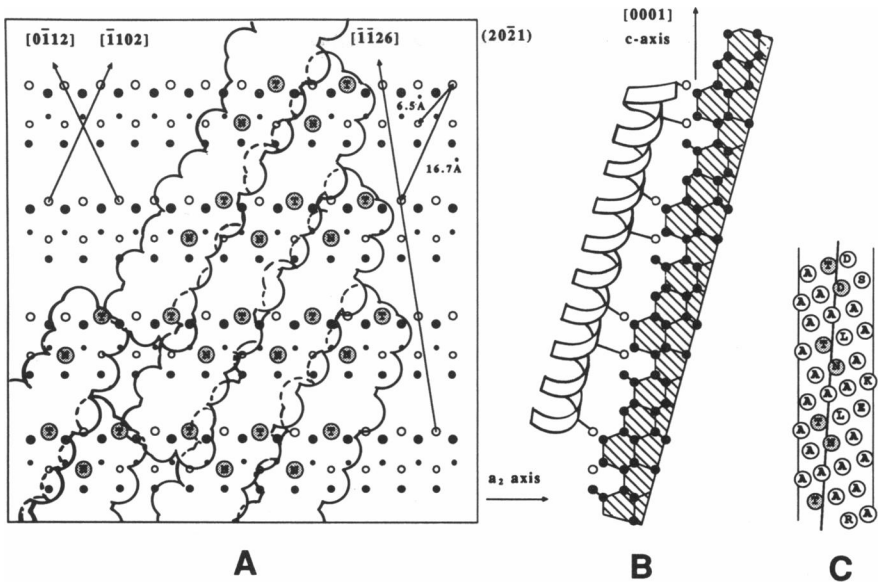

FIGURE 1 Proposed AFP-ice binding model. (*A*) Schematic view of ice normal to the hexagonal bipyramidal plane (20$\bar{2}$1). Filled circles represent surface water oxygens, where the largest circles are highest on the surface; open circles are water oxygen positions (and potential Thr (T) and Asn/Asp (N) binding sites) in the virtual ice layer above the ice surface; large open circles are T-sites located on ridges at the surface, and small open circles are N-sites at a lower elevation; shaded circles represent sites occupied by AFP molecules, whose van der Waals footprints are superimposed on the surface. The spacing between T- and N-sites is 6.5 Å, which closely matches that between the Thr hydroxyl oxygen and the Asn amide nitrogen in the Thr-X-X-Asx pairs on the AFP helix face (*C*), found by molecular modeling using the default parameters in the Quanta modeling program. The 16.7-Å spacing between respective T- and N-sites on [$\bar{1}$102] (equivalent to [01$\bar{1}$2] referred to by Knight et al. (3); see footnote 1) approximates the 16.6-Å Thr and Asn repeat distances on the AFP. Each T-site has two, and each N-site three potential hydrogen bonding partners (*filled circles*) in the ice lattice. The AFP footprints are shown in their most favorable potential packing arrangement, with interdigitated hydrophobic side chains and close contact between charged NH_2-terminal Asp and COOH-terminal Arg side chains. (*B*) Profile of the (20$\bar{2}$1) surface as viewed along the a_2-axis; filled circles represent water molecules in the ice lattice, and open circles the T- and N-sites. The N-site, being depressed relative to the T-site, can easily accommodate the more bulky Asn and Asp residues. (*C*) Helix net representation of AFP S00, showing the arrangement of Thr and Asn/Asp residues on the helix face. The axis through these residues is skew to the helix axis, which affects the packing arrangement shown in (*A*).

above some critical concentration, hydrophobic interpeptide interactions become more important. Complete coverage of the ice surface would be possible only if adsorption were freely reversible, thus allowing AFP molecules to reorganize themselves optimally. It seems more likely, however, that the AFPs bind in patches surrounding individual nucleating molecules laid down randomly on the surface (Fig. 3 *B*). Because the nucleating molecules would not necessarily be in perfect register, the patches could not coalesce to cover the surface completely. Feeney et al. (1) have shown that the antifreeze activity of certain antifreeze glycoproteins has a sigmoidal dependence on concentration, suggesting reversible binding and cooperativity. Such may also be the case

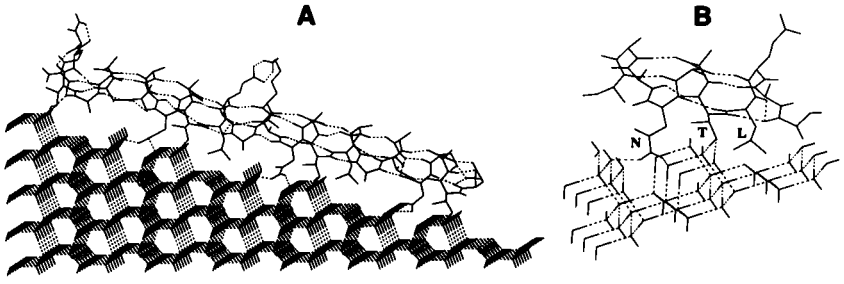

FIGURE 2 (*A*) Line model of an AFP molecule bound to the (20$\bar{2}$1) plane of ice, showing the hydrogen bonding pattern. The NH_2-terminus is at the right. This model was constructed by energy minimization along [$\bar{1}$102] on the ice surface using CHARMm, with the constraint that the Asn amide nitrogen, Asp carbonyl oxygen, and Thr hydroxyl oxygen must occupy respective N- and T-sites. Under these conditions, Asn and Thr formed two hydrogen bonds each, and the Asp one bond, and the energy of the system was lowered slightly, compared with the unconstrained system. The Leu side chains, particularly that of Leu,[23] are also seen as making van der Waals contact in a valley of the ice surface. (*B*) Detail of (*A*) showing contacts of Leu,[23] Thr,[24] and Asn.[27]

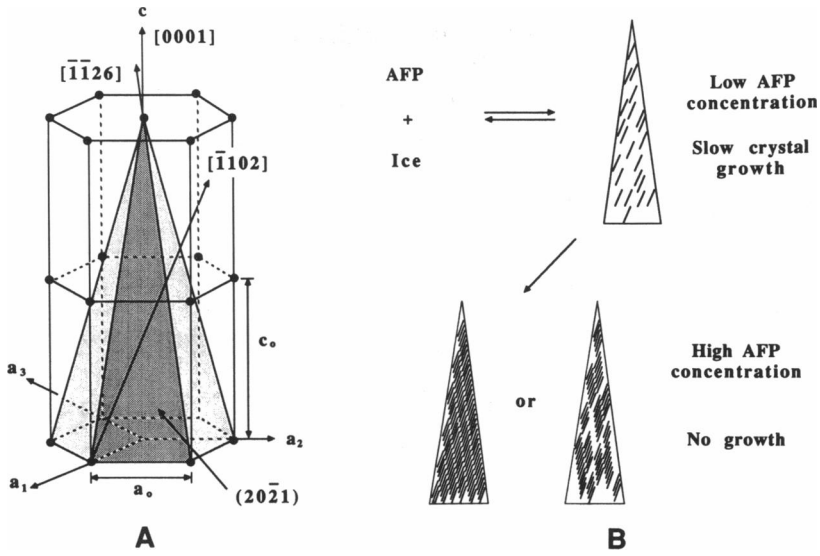

FIGURE 3 (*A*) Ice cell showing half of a hexagonal bipyramid (*shaded area*) with vector notations,[1] where c_o = 7.352 Å and a_o = 4.514 Å. (*B*) Representation of the mechanism of binding of AFPs to the (20$\bar{2}$1) surface at low and high concentrations. At low concentrations, AFP molecules bind randomly and, presumably, reversibly to the surface; at high concentrations, intermolecular interactions occur and the surface becomes covered, either completely or in a patchwork pattern, depending on how much reorientation of the AFP molecules is possible. Under these conditions, binding may be essentially irreversible.

with the α-helical AFPs, but more precise measurements of activity versus concentration are needed before this conclusion can be made.

This communication provides a detailed description, at the molecular level, of the binding of an AFP to an ice surface and of the implications such binding might have on the mechanism of antifreeze activity. Chou (5) has proposed a model based on the results of Knight et al. (3) for binding of AFPs by the Thr residues. However, this model does not take into account the Asn/Asp residues, which give the helix face asymmetry, and thus does not explain the apparent preferred orientation (3) of binding. Nor does it make any predictions about possible intermolecular interactions on the ice surface. An earlier proposal of Yang et al. (4), that AFPs can bind to ice with many orientations by virtue of the flexibility of the polar side chains, is also inconsistent with our hypothesis.

Our model can be tested by studying the activity of other analogs. For example, if our proposal is correct, then an α-helix composed of all D-amino acids should have the same activity as that of an all L-AFP, except that it would bind in the direction [0$\bar{1}$12] (Fig. 1 *A*), as could be determined by crystal etching studies (3). Similarly, analogs can be synthesized that should interfere with packing but not hydrogen bonding. This model does not explain, however, what happens in concentrated AFP solutions at or near the freezing point, where rapid growth along the *c*-axis ensues. Under these conditions, the system is far from equilibrium and other rules regarding binding surfaces may apply.

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