Binding of an Oligopeptide to a Specific Plane of Ice*

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The α-helical antifreeze protein (AFP) from winter flounder inhibits ice growth by binding to a specific set of pyramidal surface planes that are not otherwise macroscopically expressed. The 37-residue AFP contains three 11-amino acid repeats that make a stereo-specific fit to the ice lattice along the (01–12) direction of the (20–21) and equivalent binding planes. When the AFP was shortened to delete two of the three 11-amino acid ice-binding repeats, the resulting 15-residue peptide and its variants were less helical and showed no antifreeze activity. However, when the helicity of the peptide was reinforced by an internal lactam bridge between Glu-7 and Lys-11, the minimized AFP was able to stably express the pyramidal plane (20–21) on the surface of growing ice crystals. This dynamic shaping of the ice surface by a single ice-binding repeat provides evidence that AFP adsorption to the ice lattice is not an “all-or-nothing” interaction. Instead, a partial interaction can help develop the binding site on ice to which the remainder of the AFP (or other AFP molecules) can orient and bind.

Type I AFP1 from winter flounder, represented by the abundant serum isoform HPLC-6 (1) is a remarkably long free-standing α-helix (2). Its helicity can be attributed to several structural features, which include an abundance of alanine, an extensive capping network at both termini (3), and an internal salt bridge (1, 4). From ice etching studies, it was shown that this AFP binds to the (20–21) hexagonal bipyramidal planes of ice along the (01–12) direction (5). It has been suggested that the critical connection between structure and function in this protein is that putative ice-binding residues (Thr, Asx, and Leu) are aligned and regularly spaced along one face of the helix (5, 6). Each residue (e.g. Thr-2, Thr-13, Thr-24, and Thr-35) is spaced 11 amino acids apart (16.5 Å), which closely matches the 16.7-Å distance between repeating features of the ridge and valley topology along the (01–12) direction of the (20–12) binding plane. Adsorption of type I AFP to these lattice binding sites through a precise distance and geometry match involving H-bonding and van der Waals interactions (5, 7) leads to inhibition of ice growth by the Kelvin effect (8, 9). In the process, seed ice crystals are constrained to form hexagonal bipyramids with a ca axial ratio of 3.3:1, which matches the ratio predicted from the adsorption planes revealed by ice etching studies (5, 6).

Several attempts have been made to model the helix in contact with the ice surface (3, 7, 10, 11). As a result, a common concern is that the number and strength of the potential interactions between ice and AFP are barely sufficient for tight binding (3, 7). One solution proposed is that all four ice-binding threonines share the same rotamer configuration and bind to ice in a “zipper-like fashion” (11). A similar hypothesis, stemming directly from the x-ray structure, is that tight binding relies on the simultaneous docking of similarly aligned and constrained ice-binding side chains that together form a flat ice-binding surface (3). The requirement of side-chain rigidity for tight binding seems to be at odds with the NMR solution structure for type I AFP, which failed to find any evidence that the ice-binding side chains were locked into a specific common rotamer, even at 3 °C (12). To investigate this, and the related issue of the length of the ice-binding site, we set out to construct a minimized type I AFP that was sufficient for binding to ice. These experiments have shed new light on the mechanism of AFP binding to ice. Antifreeze proteins do not necessarily bind to a preformed site on ice but instead help to shape the site to which they bind. This principle may be of general relevance to mineralization and demineralization processes.

EXPERIMENTAL PROCEDURES

Peptide Synthesis, Purification, and Mass Analysis—All peptides were prepared by solid-phase peptide synthesis using a benzhydrylamine hydrochloride resin on a Labotec SP 640 peptide synthesizer as described previously (13). The peptides were cleaved from the resin by reaction with HF (10 ml/g resin) containing 10% anisole for 1 h at −5 °C to 0 °C. The crude peptides were purified by reversed-phase high performance liquid chromatography on a SynChropak RP-4 preparative C4 column (250 × 21.2-mm internal diameter, 6.5-μm particle size, 300-Å pore size) (SynChrom, Lafayette, IN) with a linear AB gradient of 0.1% B/min with a flow rate of 5 ml/min, where solvent A is 0.05% trifluoroacetic acid in water and solvent B is 0.05% trifluoroacetic acid in acetonitrile. For amino acid analysis, peptides were hydrolyzed in 6 N HCl containing 0.1% phenol for 1 h at 160 °C in sealed evacuated tubes. Amino acid analysis was performed on a Beckman model 6300 amino acid analyzer (Beckman, San Ramon, CA). Mass analysis of the peptides was performed on a Fisons VG Quattro triple quadrupole mass spectrometer (Manchester, UK) fitted with an electrospray ionization source operating in the positive ion mode. A number of 10-μl injections of the peptide samples (usually in aqueous acetonitrile containing 0.05% trifluoroacetic acid at an approximate concentration of 50 pmol/μl) were made into a carrier solution composed of water/acetonitrile (1:1, v/v) containing 0.05% trifluoroacetic acid at a rate of 10 μl/min into the electrospray source. The quadrupoles were scanned from 600 to 1400 mass over charge ratio at 10 s/scan. Data were acquired in the multi-channel acquisition mode with 10–15 scans typically being summed to produce a spectrum.

Circular Dichroism Spectroscopy—CD spectra were measured on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) equipped with a
Peptide Design—Type I AFP is an ideal protein for minimization because there are few structural restraints to making it smaller by simply shortening the helix. The initial minimized 15-amino acid AFP structure (15KE in Fig. 1) was formed by removing the two central 11-amino acid repeats (residues 13–34 inclusive) and reinstating the internal salt bridge into the remaining repeat on the opposite side of the helix to the threonines. By removing 22 residues (exactly two repeats), the critical 16.5-Å spacing between the remaining two threonines could be maintained, but only if the peptide retained its α-helicity. The latter concern was the key structural restraint in the minimization exercise. By shortening the protein from the center, the helix stabilizing N- and C-cap structures were not disturbed. However, these features, together with the intrachain salt bridge between Lys-7 and Glu-11, were not enough to make the peptide fully helical, even at 1 °C (Fig. 2) (see below).

Two minor adjustments were made in an attempt to increase the stability of the peptide. One was acetylation of the N terminus (peptide Ac-15KE in Fig. 1). Doig et al. (16) have reported that the unfavorable effect of protonation at the N terminus is approximately 0.5 kcal/mol and that acetylation of the free amino group can improve helix stability. This can be attributed to elimination of the positive charge and possibly to hydrogen bond formation between the acetyl CO and an unsatisfied main chain NH group. The other adjustment was to reverse the order of the ion pair residues Lys-7 and Glu-11 (peptide 15KE in Fig. 1) in order to orient the lactam bridge relative to the two Thr side chains (A, end-on view; B, horizontal view). The N and C termini of the peptide are labeled N and C, respectively.

Previously it has been shown that incorporation of a lactam bridge between Glu and Lys spaced four residues apart dramatically increased the helical content of small amphipathic peptides relative to their uncyclized linear peptides bearing the same sequence (13). The modified peptide 15KE was ideally suited for lactam bridge synthesis because of the orientation of the ion pair, Glu-7 and Lys-11. Thus, the third modification of the prototypical minimized type I AFP (15KE) was the formation of a Glu-7 to Lys-11 i,i lactam bond in place of the salt bridge.

It was thought that this covalent link might be the most effective way of maintaining the correct 16.5-Å spacing between the two remaining threonines. CD Spectroscopy.—The CD spectra of type I AFP (WT) and
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Fig. 3. Thermal denaturation profiles of minimized peptides. The molar ellipticity at 222 nm ([θ]222) was measured for each of the 15-mers as a function of temperature. Peptides were identified by the same symbols used in Fig. 2 (see inset).

minimized AFPs in benign conditions at 1 °C are shown in Fig. 2. The CD spectra of the wild-type protein are characteristic of a highly helical molecule with minima at 222 and 208 and a maximum at 192 nm. Typically, the molar ellipticity at 222 nm ([θ]222) has been used to measure the helical content in peptides (19). For the wild-type protein, this has been previously reported to be $-36,100$ deg cm$^2$ dmol$^{-1}$ at 1 °C (6). All minimized peptides displayed significantly less α-helical character than the full-length AFP. However, it is unlikely that a peptide of 15 residues in length would be as helical or as stable as a 37-residue protein due to the length dependence of the [θ]222 signal. For the minimized peptides stabilized by a salt bridge, the CD spectra resemble a mixture of helical and random conformations. The CD spectra of 15KE and 15EK are nearly superimposable, and their [θ]222 values at 1 °C are quite similar ($-19,450$ deg cm$^2$ dmol$^{-1}$ and $-18100$ deg cm$^2$ dmol$^{-1}$), yielding estimates for helical content of 62% and 58%, respectively. However, acetylation of the N terminus resulted in a lower helical content (42%), as measured by the 222-nm signal. This may be due to acetylation disrupting the N-cap. All three peptides were essentially 100% helical in the presence of 50% TFE (data not shown). Introduction of a lactam bridge in place of the salt bridge greatly increased the helical content of the peptide to 90% ([θ]222 = $-28,100$ deg cm$^2$ dmol$^{-1}$). This is the only minimized peptide for which the CD spectrum is similar to that of the wild-type protein with a minimum at 222 nm that exceeds the value at 208 nm, suggesting the peptide adopts an α-helical conformation. Once again, this peptide was highly helical in 50% TFE (data not shown).

Thermal denaturation profiles of all minimized AFP peptides are shown in Fig. 3. For the peptides stabilized by salt bridges, the [θ]222 signal sharply declined and leveled off at $-7000$ deg cm$^2$ dmol$^{-1}$. The denaturation profile of EK-lac was somewhat broader, indicating a less cooperative transition. Approximately 50% of the helicity observed at 1 °C was lost at 52 °C. (In contrast, the wild-type protein had a $T_m$ of 22 °C.) This stability has been observed previously for other constrained peptides and has been attributed to residues within the covalent constraint being unable to fully denature.

Fig. 4. Ice crystal morphology. A, ice crystals formed in the presence of WT (a), 15EK (b), Ac-15-KE (c), 15KE (d), and 15EKlac (e and f). The peptide nomenclature is that used in Fig. 1. B, time lapse analysis of ice crystal growth in the presence of 15EKlac, where a, b, and c were recorded at 1, 5, and 10 min, respectively.

Thermal Hysteresis Activity Measurements—All peptides were assayed for thermal hysteresis activity using a nanoliter osmometer (4), which also provided an opportunity to check for interactions between protein and ice, as revealed by the shaping of ice crystals. None of the minimized peptides was able to depress the freezing point below the melting point at concentrations of 50–100 mg/ml (~50 molar). Even the slightest undercooling caused the seed ice crystal to grow steadily. In the presence of 15KE and 15EK, the ice crystal took the shape of a rounded disk indistinguishable from that formed in the presence of buffer alone (Fig. 4A, panels b and d). With Ac-15KE, there was a hint of a hexagonal shape to the disk, suggestive of peptide binding to the ice (Fig. 4A, panel d). In contrast, 15EKlac was very active in shaping the ice into an incomplete hexagonal bipyramid, which generally lacked one or both apices (Fig. 4A, panels c and f). When the $a:b$ axis ratio was measured by extrapolation from 10 representative crystals, it ranged from 3.5:1 to 3.1:1, with an average value of 3.3. This average value is precisely that obtained with full-length, wild-type AFP (6, 15), which is the ratio predicted for expression of the [20–21] pyramidal planes. This value was maintained even as the ice crystal grew considerably over a time course of 1–10 min, indicating a persistent influence of the peptide over the ice surfaces expressed. In one sequence of time-lapse video microscopy (Fig. 4B), the ratio varied from 3.4 at 1 min to 3.3 at 5 and 10 min, during which time the volume of the crystal increased manyfold. In another series (data not shown), the ratio was
Irreversible binding. The peptide to the ice surface. However brief, leads to shaping of the pyramidal plane, as demonstrated with 15EKlac. With wild-type AFP, growth of ice in the direction during shaping results in additional contacts with the helix that further increase its residency on the ice and lead to complete and irreversible binding.

3.20 at 1 min and 3.17 at 10 min. Thus, a single ice-binding repeat that was constrained to be α-helical was sufficient to define the pyramidal plane but could not prevent ice-crystal growth. The fact that 15EKlac generated a faceted ice crystal without causing non-colligative depression of the freezing point could be explained by transient (reversible) binding of the peptide to the ice surface.

**DISCUSSION**

The high helical content of wild-type AFP is thought to reflect the importance of maintaining the polypeptide backbone in this conformation, such that the spacing of the threonines and other ice-binding residues matches the ice lattice (3, 5, 7, 11). Although all the minimized AFP peptides that were stabilized by a salt bridge contained a certain amount of helical structure as determined by CD, their helical content was substantially less than that of the wild-type protein or of that induced in 50% TFE. The overall conformation of these peptides is most likely an ensemble where sections of the peptide adopt fully helical, partly helical, and random conformations. This is consistent with the findings of Merutka et al. (20), where a small peptide that was even more helical than the 15-mers in Fig. 1 (as determined by CD) contained a significant population of non-helical conformers (as determined by NMR). For the lactam-bridged peptide, the internal lactam bridge biases the peptide to adopt a more helical structure. This, in turn, should lower the change in conformational entropy between peptides bound to ice and those free in solution. For the non-lactam-bridged peptides, it is likely that the free energy of binding to ice is insufficient to compensate for the loss of conformational entropy upon immobilization of the peptide backbone into a fully helical conformation.

The significance of the interaction between the minimized, lactam-bridged type I AFP and ice lies in the insight it provides about the adsorption phase of the adsorption-inhibition mechanism of action of AFPs. This insight helps resolve two paradoxes. One is that AFPs bind to planes of ice that are not fully helical. The other is that the Thr residues of type I AFP are rarely optimally configured for immediate binding to ice because of side chain rotation (12). A solution to these paradoxes is illustrated for type I AFP by the model shown in Fig. 5. In the absence of AFP, an ice crystal will typically grow as a rounded disk (Fig. 5A) as water molecules add to the prism surfaces (α-axial growth) in preference to the two basal planes (c-axial growth). The crystal surface may appear microscopically smooth but will be uneven at the submicroscopic to atomic level on all but the basal planes. The irregular addition of water to a prism surface of the ice lattice creates a small section of the pyramidal plane (20–21) (Fig. 5B). This section, involving just five layers of water, already expresses the characteristic surface properties of the AFP's adsorption plane, including the 16.7-Å spacing along the (01–12) direction, and is sufficient to initiate antifreeze binding.

It is clear from solution NMR studies that all four threonines in type I AFP are free to occupy different rotamer configurations, although the middle two have a preference for −60 °C (55% of the time (12). Although the chances of all four threonines being in the same orientation at any one instant is quite small, the evidence from minimized AFP binding to ice shows that this is not a precondition for initial contact, because dynamic ice shaping occurs with a single repeat. It is quite likely, therefore, that two neighboring threonines (especially the middle two) will be found with the same rotamer configuration. A single ice-binding repeat would then be the smallest unit of the AFP that theoretically could define both the plane and direction of AFP binding to ice. That it does this in practice is demonstrated by the shaping of ice crystals by 15EKlac into truncated hexagonal bipyramids with the 3.3:1 c:a axis ratio typical of full-length type I AFP. Thus, it is not necessary for all four threonines to touch down on the ice surface to secure that plane for binding.

The model suggested by these studies is that type I AFP binding to ice is unlikely to be an “all-or-nothing” event. Instead, it is best described by an induced-fit mechanism, where touchdown by any two i,i+11 threonines with the same rotamer configuration (representing a single ice binding repeat) is sufficient to stabilize the smallest facet of ice that will bind repetitively. Even transient occupancy of this ice surface would allow ice to advance relative to the occupied region, particularly by water addition to the prism surfaces (Fig. 5, B and C). If, during this period, the advance is sufficient to engage a third threonine (representing a second ice-binding repeat), which might have had to rotate its side chain to provide the 16.5-Å spacing, the binding would be stronger. Should dissociation have occurred prior to the engagement of the second repeat, the net effect would have been to shape the ice closer to the (20–21) plane, thereby making it easier for another type I AFP molecule to engage. Either through a dissociation-reasso-
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Citation process or by side-chain reorganization while on the ice surface, the eventual outcome is that all repeats would make contact (Fig. 5C). At this point, binding would be virtually irreversible because dissociation would require the simultaneous breaking of all the AFP-ice interactions. This shaping of the ice surface, coupled with selection for AFPs with ideal rotamers, is a dynamic, synergistic process where an improved binding site leads to more efficient binding, which further improves the binding site. Although type I AFP may be unusual in having somewhat flexible ice-binding residues, ice-site shaping by an induced-fit mechanism is a principle that could apply to other AFPs.

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