Antifreeze Protein-induced Morphological Modification Mechanisms Linked to Ice Binding Surface*

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The mechanisms by which the antifreeze protein (AFP) modifies the ice morphology are identified precisely as surface poisoning by the ice binding surface (IBS) of insect AFPs and as bridge-induced surface reconstruction by the IBS of fish AFPs and antifreeze glycoproteins. The primary surfaces of hexagonal ice have predetermined face indices. The “two-dimensional” insect type IBS has regularly spaced binding intervals in two directions. It causes surface poisoning by matching and reinforcing simultaneously intersecting strong bonding directions on the primary ice surfaces. The secondary ice surfaces have variable face indices. The “one-dimensional” and “irregular” IBS variants of fish AFPs and antifreeze glycoproteins are either linearly extended with regular ice binding intervals or have ice binding sites lacking spacing regularity. These variants can bridge transversely lattice periods or shorter oxygen–oxygen distances between parallel adjacent strong bonding directions that do not intersect. Thus, one-dimensional and irregular IBS variants induce supplementary bridges cross-wise on selected secondary surfaces by mimicking strong bonding directions that are not present in the ice structure. These proteins cause surfaces with variable face indices, which in the absence of the AFPs would not grow flat, to appear in the morphology. Whereas for the primary ice surfaces it is only the morphological importance that is determined by the experimental conditions, for the secondary ice surfaces it is the face indices themselves that become adjusted in the process of maximizing the AFP-substrate interaction through attainment of the best structural match. The growth morphology of the AFP-ice system is derived from various factors, including the face indices, surface molecular compositions, relative growth rates, and the mechanisms responsible for that morphology. The theoretical formulation agrees with experiments over a wide range and resolves these, to date, unexplained phenomena.

Freezing is a process of ice crystallization from supercooled water. Ice should first experience ice nucleation, followed by growth (1). Whether or not freezing takes place is determined to a large extent by ice nucleation (2, 35). There is evidence that fish antifreeze proteins bind to and reduce the efficiency of heterogeneous nucleation sites rather than bind to embryonic ice nuclei (2, 35). Similar phenomena were obtained and explained in Refs. 3–5. The inhibition of ice nucleation is important in any antifreeze process. The antifreeze action of the AFP1 is actually first to inhibit nucleation by terminating the relevant kinetics (3–5). When the inhibition of ice nucleation fails, the AFP proceeds to inhibit the growth of ice.

In this work the growth morphology is derived to first-order by taking into account the AFP-ice system. For the first time, the face indices, the surface molecular compositions, and the assessment of relative growth rates of the ice crystals as well as the AFP-induced morphological modification mechanisms are predicted, based on the AFP-ice system, by the periodic bond chain (PBC) theory developed by Hartman and Perdok (cf. some of the original references, Refs. 6–8, 36–39, and an extensive list of references in Ref. 9). According to that theory, the observed morphology is determined as a composite effect of a progression of internal and external factors. A zero-order basic theoretical morphology depends solely on the ice structure. The influence of the environment causes a morphological modification. The environment is usually the surrounding liquid, which often contains influential molecular species. Such species may exert an even stronger morphological effect than the liquid itself, as is the case with the AFP.

In a first-order approximation, the AFP action, whether direct or mediated through the interfacial liquid, is the major external factor affecting the morphology, because its role outweighs by far the role of the surrounding phase. In this work, the engagement of the AFP on the ice lattice is analyzed correctly in the context of the approximate AFP-ice system, and this analysis delivers a comprehensive explanation for the observed important features of the AFP-induced morphological modifications. A second-order morphological effect would involve the growth (not the equilibrium) processes of the AFP-ice-water system, which is also an approximate system. A third-order morphological effect would involve the growth processes of a realistic scenario including AFP, ice, water, and molecular species. Both of these effects are outside the present scope.

Initially, in trying to understand the AFP-induced morphological modifications, researchers carried out docking experiments with the AFP on the bare ice substrates, mainly focusing on the adsorption of antifreeze protein molecules on specific surfaces of ice (2, 10, 11, 35). They relied on the “hydrogen bond theory” to explain the antifreeze action of the fish AFP in terms of hydrogen bond formation between protein residues and lattice water molecules. Subsequent mutant-AFP experiments proved that hydrogen bonding could not be the primary cause of the freezing inhibition and that Van der Waals forces were

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1 The abbreviations used are: AFP, antifreeze protein; IBS, ice binding surface; PBC, periodic bond chain; TmAFP, Tenebrio molitor AFP.
equally unable to provide a complete explanation. As a reaction to these results, some of the erroneous conclusions that were obtained were attributed to the approximation derived from considering the AFP-ice system in isolation and neglecting the role of the ice-water interface. This work will show that part of the reason for erroneous conclusions was overreliance on incorrect assumptions.

In later works, researchers abandoned the AFP-ice system and carried out molecular dynamics simulations on the AFP-ice-water system (12). However, molecular dynamics simulations such as those carried out by those authors can only deliver information on the equilibrium molecular distribution of the simulated faces, provided, moreover, that the simulations are done correctly. Noteworthily, crystal growth is not an equilibrium process, for which reason the molecular dynamics results could say nothing about the relative growth rates that are needed to explain the morphology and/or its AFP-induced modification. The molecular dynamics results merely confirmed that hydrogen could not be the primary factor of the freezing inhibition. In studying the morphology or the morphological modification, one is forced to deal with the mechanisms producing or modifying the face orientations and the surface molecular compositions as well as the relative growth rates. Examples of such mechanisms include the formation of kinks and steps (if the faces are essentially flat) on the ice crystals, the motion of steps, and the transport of impurities and heat. If the AFP is involved, the adsorption of the AFP on the ice surface and the impact of the kink and step integration kinetics should be included. These are definitely not equilibrium processes but growth mechanisms.

Most of the above studies suffer from the same drawback, i.e. the ice substrates (13) considered were produced by cutting the hexagonal ice structure randomly and juxtaposing the AFP ice binding surface to that planar crystal cut (examples are a “different (201) cut” (in Ref. 28) or a “(201) cut 2,” which cannot be a valid substrate, and a computer simulation concluding that there is the “likely occurrence” of the (111) ice surface on the morphology (22), whereas (111) is not a valid surface because it is roughened). The crystallographic validity of the randomly obtained planar cut surfaces was not called into question. However, crystallographically valid flat surfaces need not be planar cut slices of the structure; in fact, these surfaces often exhibit heavily undulating and corrugated surface boundaries (9). Both the AFP-ice interaction and the molecular equilibrium distribution in the AFP-ice-water system depend crucially on the detailed definition of the simulated ice crystal substrates. Whenever those substrates are invalid, so are the results.

The appearance of pyramidal forms on ice crystals grown under the influence of the AFP has thus far been explained (2, 14, 35) as the occurrence of an intermediate orientation between the basal face and the primary prism. For example, it is held in Refs. 2 and 35 that reduction of the (100) prismatic growth rate could give rise to a pyramid; it is also held in Ref. 14 that the (201) pyramid is the result of an intersection between the basal face and the primary prism when the RD3 AFP attaches one terminus of its ice binding surface (IBS) to the basal face (001) and the other terminus to the prism (100). However, crystallographic face orientations cannot be averaged. Consider two valid growth layers, \((h_1 k_1 l_1)\) and \((h_2 k_2 l_2)\), that are adjacent; none of the remaining valid growth layers is intermediate, i.e. located in between these two. Depending on the respective growth rates, one of the following two occurrences will take place: (i) either \((h_1 k_1 l_1)\) or \((h_2 k_2 l_2)\) will suppress the other if its growth rate is dominantly lower; or (ii) both \((h_1 k_1 l_1)\) and \((h_2 k_2 l_2)\) will appear on the crystal, their relative importance being reflected in their relative growth rates. The following occurrence, i.e. “crystallographic averaging”, will never occur; some intermediate growth layer, which, by the above definition, is not a valid growth layer because it is adjacent to both \((h_1 k_1 l_1)\) and \((h_2 k_2 l_2)\), will never appear on the crystal as a “crystallographic average” between \((h_1 k_1 l_1)\) and \((h_2 k_2 l_2)\) regardless of the respective growth rates of \((h_1 k_1 l_1)\) and \((h_2 k_2 l_2)\). If averaging were a legitimate crystallographic operation, in the course of many repeatedly applied averaging operations we would hardly be observing any faceted crystals!

Additionally, the following phenomena received insufficient attention in previously related studies. A broad variety of observed pyramidal ice forms has been reported repeatedly (15). Because one may not expect the variation in pyramidal shapes to result from a variation in the lattice constants of hexagonal ice, that variation must be based on a strong variation of the pyramidal face indices. This exceptional occurrence of variable face orientations in AFP-modified ice has not been dealt with.

The unusual behavior of the AFP of one insect (Tenebrio molitor) (13) in producing pyramidal ice crystallites, as opposed to hexagonal disk-shaped ones, has puzzled the literature because, as will be shown in this work, previous studies failed to distinguish the predetermined face indices of the primary pyramid from the variable face indices of the secondary pyramids (17). The superior activity of the insect AFPs (13) in depressing the freezing point of water has also remained unexplained; it can, however, be attributed to the difference between the surface poisoning mechanism on one hand and the mechanism of bridge-induced surface reconstruction on the other. In experimental observations, the diversity of AFP structures capable of inhibiting freezing is accompanied by a remarkable uniformity of the morphological modification resulting from the AFP action. The unifying structural elements that underlie the antifreeze activity have not been identified.

Protein activity in inhibiting freezing is largely measured in terms of thermal hysteresis. The freezing inhibition is brought about by adsorption of the AFP binding sites on ice substrates. Freezing inhibition is always accompanied by a modification of the morphology of the ice crystallites. The converse does not hold true; a morphological modification of ice does not necessarily imply that the freezing point has been depressed, because a change in morphology can have different causes and can be accompanied by total protein inactivity (cf. Refs. 13, 16, and 17). The particular ice morphology observed is a direct consequence of the structural details related to the adsorption mechanism of the AFP on the ice surfaces, such that different modes of adsorption trigger different crystal habits consistently and predictably.

The structure of the ice binding surface of the AFP is the instrument of freezing inhibition. The known ice binding surfaces of all AFPs are fairly flat and rigid (18) and are classified as the two types shown in Fig. 1. The two-dimensional “insect-type” IBS, typical of the insect AFPs such as those of the spruce budworm and T. molitor, is a rigid, planar, and repetitive IBS with regularly spaced binding intervals in two directions (Fig. 1a). The “fish-type” IBS, typical of fish AFPs and antifreeze glycoproteins, comprises two variants as follows: the “one-dimensional” IBS variant is linearly extended with regularly arranged binding intervals (Fig. 1b); the “irregular” IBS variant is two-dimensional and may well (but not necessarily) be planar in order to function but lacks regularity in the spatial arrangement of its binding sites (13) (Fig. 1c).

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2 See also the Antifreeze Protein Type I (AFPI) page at http://pout.cwru.edu/~frank/afpi1/.
thereby causing a transition in the ice morphology between pyramidal and prismatic (29). Antifreeze glycoproteins trigger the growth of secondary prisms with indices as high as \((120)\) or \((410)\) that give way to the primary prism \((100)\) at increased AFP concentrations (30). Ref. 31 reports a prismatic morphology, which might possibly be the \((100)\) prism observed in fish AFPs.

Whereas fish AFPs trigger a hexagonal prismatic or pyramidal morphology, most (but not all!) insect AFPs (e.g. the spruce budworm AFP) trigger a disk-type morphology, i.e. hexagonal plates consisting of the basal face \((001)\) and the hexagonal prism \((100)\). Nevertheless, the insect \(T.\ molitor\) AFP, despite the results of docking experiments showing an increased affinity for the basal face \((001)\) and the primary prism \((100)\), persistently produces stubby, lemon-shaped hexagonal ice pyramids that, judging from Fig. 3 in Ref. 27 and Fig. 7a in Ref. 19, seem to maintain constant face indices such as those of the \((101)\) pyramid face as depicted in Fig. 2.

In this study we will conclude that crucial properties of the ice substrates on which the AFPs act have, in the past, been overlooked or misunderstood. Consequently, some important differences in the way different AFPs operate have not been recognized, and the respective mechanisms by which these AFPs modify the ice morphology have been misinterpreted.

The Hartman-Perdok theory of crystal growth and morphology (6–9, 36–39) provides a first-principles approach to predicting crystal morphology and morphological modification. Crystal growth is governed by two-dimensional network formation consisting of periodic chains of strong bonds. The orientations and compositions of all of the possible surfaces that could grow in a flat mode are determined theoretically. Morphological modifications can be predicted when the influence of the growth environment is known. Morphological modification mechanisms are surface poisoning, e.g. (32), surface reconstruction, e.g. (33), and other factors.

**THEORY**

PBC analysis of the growth morphology of ice in the presence of the AFP leads, in a natural way, to two AFP-induced morphological modification mechanisms associated with AFPs that have, effectively, a two-dimensional IBS and AFPs that lack a regular two-dimensional character. The latter IBS type is effectively one-dimensional or spatially irregular. A general exposition is provided in this work. Detailed explanations and supporting experimental evidence of the morphological processes related to AFP, i.e. substrate interaction as regards the above two IBS types, will be the subject matter of forthcoming publications.

**Hartman-Perdok Theory of Periodic Bond Chains and Crystal Morphology**

The occurrence of crystallographic faces on a crystal cannot be arbitrary; it is controlled by the growth kinetics of the...
specific faces. According to PBC theory, the observed crystal morphology consists of a basic or structural morphology for which the structure is responsible and, often, a morphological modification effect due to external factors. A crystal is normally bounded by “flat” crystal faces (6–9, 36–39). The lower the growth rate of a given flat face, the larger its surface area and the more important the face. By applying the PBC theory we can determine the structural morphology and, to a large extent, also predict the influence of external factors on the growth fronts if we are given information on the growth conditions. The PBC theory gives no information on the absolute size of the grown crystals.

The growth of the most stable faces is controlled by a layer-by-layer mechanism. The structural characteristics of flat faces can be explained by the existence of networks of strong bonds parallel to the growing surface \((hkl)\). Strong bonds are bonds between growth units (usually molecules) in the first coordination sphere. These surface configurations are actually networks of intersecting (nonparallel) PBCs.

A PBC is, by definition, an uninterrupted chain (or bundle of chains) of strong bonds in the first coordination sphere that has a unique and well-defined crystallographic orientation \([uvw]\). The molecular content of a PBC is at most the entire unit cell content. Naturally, PBCs come in families of infinitely many identical parallel chains generated by lattice translations. By its very definition, a valid PBC may not contain lattice translations (between identical growth units) that diverge from its own direction \([uvw]\).

Two global categories of surfaces exist. These are surfaces that have the capacity to grow slowly while maintaining a well-defined surface orientation that is manifested in a flat form, and surfaces that do not have this capacity.

When a surface parallel to two or more intersecting PBCs satisfies the flatness condition (7, 37, 38), it is a primary surface, meaning that it is capable of growing as a flat face. Its growth is governed by two-dimensional nucleation or spiral growth due to the non-zero step free energy in two nonparallel growth directions, i.e., directions containing PBCs, is quite limited.

Surfaces parallel to PBCs in only one direction or having no PBCs at all are incapable of growing as flat faces (6, 9, 36). When a surface is parallel to PBCs in only one direction there can be no two-dimensional nucleation barrier on that face, and deviations from that face parallel to the PBC cannot be suppressed. Thus, the growth of that face would be fast, and the face will soon disappear from the growth form even if it did occur at the beginning. Thus, a face parallel to only one PBC direction is, in principle, unstable. The number of strong bonding directions, i.e., directions containing PBCs, is quite limited. Although a surface possesses infinitely many geometrical directions, it need not possess any PBCs. The above conclusion referring to kinetic instability holds by major reason for a surface possessing no PBCs at all. There exist infinitely many faces parallel to a single direction. Therefore, when a surface is constrained to be parallel to only one strong bonding direction, its orientation cannot be predetermined and its face indices can vary. In this paper, surfaces with indeterminate orientations are called secondary surfaces.

An essential property is the crystallographic condition of “flatness” (6, 7, 9, 36–38). The requirement for a surface to be flat does not arise from the geometrical concept of a growth layer being bounded by planar cut surfaces. Rather, it means that the basic block, having a content equivalent to that of the unit cell and generating the growth layer, should not contain lattice translations between identical molecules oblique to the \((hkl)\) face. This crystallographic flatness condition is essential to the slow, layer-by-layer crystal growth that is responsible for the emergence of flat surfaces in crystals.

Should more than one primary surface molecular composition be admissible in the same face orientation \((hkl)\), then the composition with the largest slice energy (the amount of energy contained in that growth layer) or, equivalently, the lowest attachment energy is expected to prevail (6, 8, 9, 36, 39). The attachment energy is the amount of energy released when a new growth layer becomes attached to the crystal. The larger the slice energy, the lower the attachment energy, considering that the sum of these two must be the crystal energy, which is a constant.

The more important the face, the lower its growth rate. It is empirically known that the lower the attachment energy, the lower the growth rate. A rough impression of the structural morphology is obtained by assuming that the growth rates of all primary surfaces are proportional (6, 36) to the respective minimum attachment energies and by accounting for the geometrical factors. Whether or not a primary surface will actually appear on the growth form and to which extent it will dominate the morphology depend on the relative growth rates of the neighboring primary surfaces.

Normally, surfaces lacking the ability to grow as flat faces are either absent from the growth form due to their high growth rate or, if they occur, have a molecularly roughened appearance lacking a perfectly well defined and unique surface.
orientation. Such surfaces are either kinetically unstable due to a lack of intersecting strong bonding directions, meaning that they have only one strong bonding direction or none at all, or they are roughened surfaces of which the molecular compositions contain lattice translations in directions oblique to the face, meaning that they violate the "flatness condition."

Surfaces lacking intersecting strong bonding directions are unstable because they lack a two-dimensional nucleation barrier. Secondary surfaces having only one strong bonding direction could, in exceptional cases, become stabilized under the influence of an external environmental factor. Secondary surfaces with large amounts of slice energy could, under certain circumstances, grow as flat faces.

Violation of the flatness condition results in roughened growth, according to which growth units are deposited in subsequent layers before the underlying layers can be completed. Because the lattice translations in such surfaces are not restricted to being parallel to the face, crystal growth is not restricted to lateral, face-parallel directions. Such surfaces cannot strictly follow a layer-by-layer growth mechanism. Interlayer growth becomes possible and inevitable. The surface grows randomly away from the face, giving rise to roughened growth.

Predictions of the Structural Morphology of Ice Based on the PBC Theory

For the PBC analysis of hexagonal ice, the reader is referred to “Appendix.” Here we utilize the results of that analysis and the tabulated energy properties to explain the surface orientations and molecular compositions of the ice crystals and to construct the structural morphology.

**PBCs and F Faces of Hexagonal Ice**—Each PBC is a chain of strong oxygen–oxygen bonds, has infinite extent, and actually represents a family of infinitely many parallel identical PBCs. Two or more intersecting PBCs thus form a network of strong bonds defining the molecular composition of a valid growth layer. Fig. 4 shows the important PBC directions and the primary surfaces in a combined prismatic-pyramidal form; in the hexagonal (100) prism the horizontal edge is along PBC [010], the vertical edge is along PBC [001], and the remaining oblique edge is along PBC (100), which is symmetrically identical to [010]. In the (101) pyramid the baseline is along the (010) PBC (or the [100] PBC), and the edge is along the [−1 0 1] direction. The basal face (001) is the horizontal plane, which is not visible in Fig. 4. The molecular compositions of the primary surfaces (001), (100), and (101) (7, 37, 38) are shown edge-on in a projection that is perpendicular to the [010] direction, i.e. the 6-axis in Fig. 5. The molecular composition of the basal growth layer has thickness $d_{002} = d_{003}/2$ (as determined by the extinction conditions of the space group), that of the prism (100) has thickness $d_{100}$, and that of the pyramid (101) has thickness $d_{101}$. Three growth layers for each surface orientation are shown in Fig. 5, with straight lines marking their boundaries. Note that the growth layers $d_{002}$ of the basal face (001) alternate between two different molecular compositions related by space group symmetry, each of which contains two oxygen atoms, that is, half the unit cell content. Fig. 5 also shows face-on the primary surface configuration of the basal face (001). The molecular compositions of the primary prism (100) and the primary pyramid (101) are shown face-on in Figs. 7 and 8.

**Structural Morphology of Ice**—The structural morphology of hexagonal ice is the morphology for which the structure is responsible, i.e. in the absence of any external influence produced by the experimental conditions. It is constructed from the primary surfaces of Table I combined with the attachment energies tabulated in Table III under “Appendix.” Following the rule of thumb prescription of taking the growth rates of the various theoretical flat faces proportional to their respective attachment energies (6, 36) and taking into account geometrical factors, we can construct schematically (not to scale) the structural growth form of ice as shown in the lower portion of Fig. 6. We found that the (101) primary pyramid (normalized growth rate 3 in Table III) does not appear on the growth form.
because it happens to lie just below the threshold of appearance by a small amount. The threshold of appearance of (101) lies high for a geometric reason and for a reason related to the relatively high attachment energy of (101); the latter has a value of 3 as compared with a value of 2 for (001) or (100) in Table III. Therefore, (001) and (100) dominate the morphology. Should some external factor cause a reduction in the growth rate of (101) to bring it below the theoretical threshold, then we may observe the primary pyramid (101) on the growth form of ice.

RESULTS AND DISCUSSION

According to the PBC theory, the growth environment can cause morphological modifications depending on the experimental situation. Two of the several possible types of morphological modification are surface poisoning (e.g. Ref. 32) and surface reconstruction (e.g. Ref. 33).

Surface Poisoning in the AFP-Ice System—The natural morphology of ice when it grows out of water is an example of surface poisoning by water molecules. The exact growth form depends on the level of supercooling and likely deviates from the structural morphology shown in Fig. 6. Any enhancement of specific surfaces, in comparison with the structural morphology, is attributed to an increased interaction with water molecules. This increased interaction could in turn be caused, for example, by the circumstance that non-crystallographic surface sites are more readily accessible to water molecules on the enhanced surfaces (see below). From the absence of the primary pyramid (101) in the natural morphology of ice we also conclude that the interaction between the (101) primary pyramid and liquid water molecules cannot be sufficiently strong so as to enable that pyramid to overcome its geometrical and energetic barrier and appear on the growth form.

As a rule, surface poisoning refers as to the primary surfaces. Molecular species of any size can affect the morphology by interacting with some of the growing surfaces. The interaction between a growing surface and the adjacent species tends to a maximum attraction. The stronger the interaction of a crystal surface with the molecular species in the adjacent liquid, the more pronounced the resulting growth rate reduction. The change in the relative growth rates modifies the morphology.

A macromolecule such as an AFP can block simultaneously several surface sites accessible to water molecules. As a first example, a drastic growth rate reduction of the basal plane (001) relative to the primary prism (100), in either the natural or the structural morphology depicted in Fig. 6, should lead to flat hexagonal plates by enhancing (001); this has been observed in the action of most insect AFPs such as the spruce budworm AFP (cf. Figs. 3 and 6). Because the prism and the basal face are open forms, we can never expect to observe either one of them by itself. We may also expect to observe, and do observe (29), the prism or the basal face in combination with each other and/or in combination with a pyramid.

As a second example, the (101) pyramid lies below the geometrical and energy threshold of appearance in the structural morphology and in the naturally grown ice because its growth rate is too high. A reduction in its growth rate by an AFP may cause the (101) face to appear in combination with the (100) prism and/or the (001) basal face. But if that reduction is drastic enough, the primary pyramid (101), which is a closed form, might completely dominate the morphology even to the extent of suppressing the primary prism and/or the basal face. In a forthcoming publication we will argue that the pyramidal ice crystal form grown in the presence of the TmAFP is the primary pyramid (101), which is quite distinct from the range of other pyramidal shapes produced by the fish AFPs. A schematic drawing of such a crystallite is included in Fig. 2 for comparison with the image of the crystallite produced by the TmAFP, which is shown in Fig. 3. The reader is referred to the images in Fig. 3b of Ref 27 and Fig. 7 of Ref. 19 and other sources in which these crystallites are reported as having a typical lemon shape with a constant apical angle. The rounding off of the (101) pyramid observable in these images (cf. Fig. 3) could be due to roughening. But it is equally possible that the (101) pyramid appears in combination with a weak prism and/or a weak basal face, both of which are primary surfaces.

The criteria for the occurrence of surface poisoning are as follows: (i) the appearance of primary surfaces on the growth form; (ii) the constancy of the observed face indices on such surfaces; and (iii) the availability of the two-dimensional IBSs possessing regular binding intervals in two directions matching the periods of the ice lattice. The insect-type IBSs provide a structural match with surface molecular compositions containing intersecting strong bonding directions so as to minimize the AFP-substrate interaction. These criteria agree with the known properties of insect AFPs described earlier.

The morphological modification caused by surface poisoning is associated with primary surfaces that have predetermined orientations. The observed face indices, namely (001) and (100) in the hexagonal disk-type morphology and (101) in the pyramid morphology, show no variation in the experiments. In sharp contrast to the range of crystal pyramids produced by the fish AFPs, the crystal pyramids produced by the TmAFP show consistently a constant shape.

The most effective mechanism for reducing the growth rates of the primary surfaces is to engage the surface along its strong bonding directions. To match the lattice translations of at least two existing intersecting PBCs simultaneously, the IBSs of the AFP must possess some periodicity properties in the form of regularly spaced binding intervals in two directions. The two-dimensional insect type IBSs are best suited for that purpose. Fish-type IBSs are not equipped for this task, either because they have regularly spaced binding intervals in only one direction or because they have binding intervals that lack spacing regularity. Because the IBSs of insect AFPs are planar and regular, they are able to engage the primary surfaces through surface poisoning. Indeed, the morphological modification caused by the insect AFPs is consistently associated with the predetermined face indices of the primary surfaces.

The morphological modification mechanism of surface poisoning occurs when the AFPs become statistically distributed in a particular preferred orientation over the entire ice substrate. The predominant alignment is associated with the well
Additional bonding can also take place in ways other than the present in the bulk structure. In this way, additional bonding combine to form additional (usually strong) bonds that are not i.e. secondary surface configurations exist, induced surface reconstruction is foreshadowed by the energy bonding directions. The fundamental reason for the bridge-ary surface configurations.

Surfaces are only marginally more optimal than some second-least energetically the molecular compositions of the primary

quantities of the low index secondary surface configurations, the following important conclusion (7, 37, 38). The energy the bonding strength of the various ice surfaces, they lead to energy quantities of that table offer only global indications of

true that the one-dimensional IBSs of the fish AFPs could also conceivably cause surface poisoning by blocking accessible lat-ice sites to approaching water molecules. However, the two-dimen-sional IBSs of the insect AFPs, of which the binding intervals match the PBC periods, are much more effective energetically. The interaction between the protein structure and the surface structure of ice is maximized most efficiently by reinforcing the strength of all (or perhaps just the strongest) pair of existing PBCs on that surface.

Surface Reconstruction—In general, a surface is considered to become “reconstructed” when dangling bonds on the surface combine to form additional (usually strong) bonds that are not present in the bulk structure. In this way, additional bonding enhances that surface by increasing its effective slice energy. Additional bonding can also take place in ways other than the fusion of dangling bonds on the surface, as will be shown in this work when the AFP is involved.

In the case of fish AFPs, surface reconstruction occurs by the ability of the AFP to induce bridges across parallel strong bonding directions. The fundamental reason for the bridge-induced surface reconstruction is foreshadowed by the energy quantities of Table III under “Appendix.” Even though the energy quantities of that table offer only global indications of the bonding strength of the various ice surfaces, they lead to the following important conclusion (7, 37, 38). The energy quantities of the low index secondary surface configurations, e.g. (110), (120), (102), and (201), are very similar to those of the primary surface configurations. The first two of these faces are parallel to the strong bonding direction [001], and the last two are parallel to the strong bonding direction [010]. Hence, at least energetically the molecular compositions of the primary surfaces are only marginally more optimal than some second-ary surface configurations.

This finding means that, in the case of ice, two families of secondary surface configurations exist, i.e. one parallel to the [001] PBC and the other parallel to the [010] PBC, comprising energetically significant surface molecular compositions. In other words, the extra energy cost needed for secondary sur-faces to predominate above the primary surfaces in the hexag-onal ice morphology is not large. Therefore, the main impediment to the appearance of secondary surfaces in the growth form of ice can hardly arise from small energy deficiency.

Because of their nature, these energetically significant sec-ondary surface configurations are composed, so to speak, of incomplete networks because they contain one (or more) PBC families in one and only one direction. Thus, the main impediment to the appearance of a secondary surface on the growth form of ice is the absence of a valid full-fledged network of strong bonds in at least two intersecting directions as is required to deliver the kinetic stability and slow growth rate of a surface capable of growing as a flat surface. In other words, what is necessary is the introduction of a two-dimensional nucleation barrier parallel to the only existing PBC on the face. As explained under “Appendix,” the essential PBC directions present in the low index secondary surfaces are [001] and [010] (identical to [100]).

The most frequently observed secondary prism is (110). Fig. 7 shows face-on the molecular composition of the primary prism on (100) at the center. On either side, two symmetrically distinct molecular PBC compositions parallel to [001] are drawn on the secondary prism face (110). These compositions are labeled A and B in Table II under “Appendix.” Two adjacent parallel chains are depicted for the molecular composition A, and two are depicted for the molecular composition B. The most frequently observed secondary pyramid is (201). The right side of Fig. 8 shows face-on the molecular composition of the primary pyramid (101). The left side of Fig. 8 shows the only available molecular PBC composition parallel to [010]; two adjacent parallel chains are depicted.

Interactions between the surface and large foreign particles like the AFPs could induce cross-linkages between these parallel PBCs, mimicking two or more intersecting PBCs. The additional links brought about by the action of macromolecules would be important, not so much because they might add to the slice energy of the layer but because they induce bridges on a surface that, in the absence of the AFP, would not be capable of growing flat; such additional links cause the surface to grow as a flat surface. The mechanism bringing about this effect is the introduction of a new strong bonding direction along a lattice translation that intersects the single preexisting PBC direc-tion. Because this type of transverse bonding is missing from the secondary surface configurations, such macromolecules would, in effect, complete the network formation and activate, as it were, the growth mechanism of a flat, primary surface.

Surface reconstruction takes effect by means of transverse supplementary bonding; such bonding is characterized by the variety of observed face indices of the ice crystals and the one-dimensional or irregular binding pattern of the IBS AFP types I, II, and III. These characteristics are in good agreement with the observations reported in the literature.

The morphological modification caused by surface recon-struction is associated with secondary surfaces. These surfaces belong to families of infinitely many members, each family being parallel to only one strong bonding direction. In contrast to the primary surfaces, the secondary face indices are not predetermined, and, hence, they are variable. For that reason, the observed face indices of the secondary (hk0) prisms and secondary (h0l) pyramids show a broad variation in the experiments. The variation of the secondary pyramids is more easily observable than the variation of the secondary prisms because the pyramid is a closed form and its shape variation is easily seen in angles or ratios between edges (cf. Fig. 2). The most commonly observed secondary pyramid in ice crystals produced
by fish AFPs is (201), but extremely elongated secondary pyramids with indices up to (501) or higher have been reported (cf. Fig. 3a and Ref. 15). Secondary prismatic faces have been reported with indices (110)–(410) (30).

Fish AFPs induce surface reconstruction on secondary pyramids and prisms by structure matching in two steps. First, matching of the AFP binding intervals with one of the (many available) lattice periods or shorter oxygen–oxygen distances transverse to the single PBC direction takes place. This matching is necessary in order to define a group of candidate face orientations (among the many available) of the secondary surface configuration that could be engaged for reconstruction by the peptide. Second, matching of the ice binding surface of the AFP with the structures of the candidate surfaces takes place. This second type of binding is necessary in order to make a final selection of that surface orientation for which the AFP-substrate interaction is as large as possible. Indeed, reports on the alignment of the α-helix of a fish AFP on the (201) pyramid mention the lattice translation [2 0 0] as the direction of alignment (16). To date, there is no evidence of alignment of the helix along the important single PBC directions [001] (in the case of secondary prisms) or [010] (in the case of secondary pyramids). This lack of evidence is hardly surprising; if the one-dimensional IBS of the AFP aligned itself along the single PBC direction, surface reconstruction could not take place! A detailed treatment of the AFP-induced surface reconstruction will follow in a forthcoming publication.

**AFP-Ice-Water System**—In a second-order approximation to the ice morphology in the presence of the AFP, the interfacial water, assumed to be pure, enters the picture. This effect involves the additional interactions between water and the AFP and between water and ice. The relative growth rates are affected because two categories of water molecules will approach lattice-accessible positions (34). A proportion of the water molecules (the so-called crystallographic water) will arrive at crystallographic sites on the surface and become incorporated in the structure, requiring no dehydration and causing no delay in growth. Another proportion of the water molecules (the so-called non-crystallographic water) will arrive at water-accessible sites on the surface that are not lattice sites. Because non-crystallographic water must be dehydrated before crystal growth can continue, the non-crystallographic water will cause a growth delay. Thus, the relative growth rates and, hence, the morphology determined for the AFP-ice-water system will partly depend on the proportion of crystallographic to non-crystallographic water in the interfacial layer, as this applies to the various faces.

In a third-order approximation the real system is considered, including an assortment of organic molecular species in addition to the AFP-ice-water system. The desolvation or, more specifically, the dehydration process described above will change radically because all of these additional molecules arriving at accessible surface positions must become dehydrated, thus upsetting the balance between crystallographic and non-crystallographic water. The desolvation rate will depend specifically on the molecular composition of the particular face. The general trend that can be expected to be observed (34) is that the ice morphology will become modified so as to resemble less closely the morphology triggered by the pure aqueous solution and, more closely, the morphology based solely on the AFP-ice system.

**CONCLUSIONS**

Studies of the AFP-ice and the AFP-ice-water systems have often relied on incorrect ice substrates produced by cutting the hexagonal ice structure indiscriminately without regard to its crystallographic validity. Crystallographically valid, kinetically stable surface configurations capable of slow flat growth and having a well defined unique orientation must satisfy strict conditions; none of these conditions states that they need to be planar cut slices of the structure. On the contrary, many crystallographically valid surfaces are not planar cut, as they involve heavily undulating and corrugated surface boundaries.

Even in cases wherein the correct ice substrates were used for molecular dynamics simulations of the AFP-ice-water system, the results delivered not the growth morphology but the equilibrium molecular distribution at the interface. The processes taking place in crystal growth cannot be described by equilibrium processes.

It has been argued erroneously that the ability of AFP to engage the primary prism as well as the basal face could be the cause of the appearance of pyramidal faces as geometrically intermediate forms. The supposition that crystallographic face orientations might be averaged is inconsistent with both theory and experiment.

Ice pyramids produced by fish AFPs cover a broad range of shapes, but this variation passed unnoticed and remained unexplained. It is not plausible that the variation of shapes is caused by variation in the lattice constants of hexagonal ice, so it must be attributed to face indices capable of varying from (102) to higher than (501).

A correct analysis of the engagement of the AFP on the ice lattice led, for the first time, to a satisfactory first-order approximation of the growth morphology of ice in the presence of the AFP. The pyramidal and prismatic face index variation results from the morphological modification mechanism of surface reconstruction, according to which the secondary pyramid face indices are not predetermined but are selected by the AFP action. The primary surfaces (001) and (100) may, in theory, appear alongside the reconstructed secondary pyramidal and
prismatic surfaces, an occurrence that is occasionally observed experimentally.

The IBS of the fish-type AFP selects the appropriate surface orientations each time so as to maximize the AFP-substrate interaction. The secondary face indices \((hk0)\) and \((h0l)\) are selected by a two-step structure match between the structure of the IBS of the AFP and the structure of the ice surface. Thus, for example, the shape of the pyramid is expected to vary under the influence of parameters that effectively affect the structure of the IBS of the AFP and no other parameters.

Key points of agreement between the PBC-theoretic formulation and experimental observations include the correlation of the insect-type IBS with fixed primary face indices and the correlation of the fish-type IBS with variable secondary \((hk0)\) and \((h0l)\) face indices. These correlations are especially evident in the observed broad range of pyramidal shapes, of which \((201)\) is prevalent. Additional points, to be described in detail in forthcoming publications, are as follows: (i) the alignment of the insect-type IBS on PBC directions and the alignment of the fish-type IBS transverse to the \([001]\) and \([010]\) directions on the \((hk0)\) and \((h0l)\) surfaces, respectively; (ii) the appearance of combined primary and secondary surfaces on ice growth forms; and (iii) the decline in space group symmetry observed on secondary pyramids. Finally, the experimental absence of the \((111)\) bipyramid confirms the PBC-theoretic result that \((111)\) is molecularly roughened and, hence, a crystallographically invalid surface.

Despite the diversity of AFP structures capable of inhibiting freezing, the observed prevailing uniformity in the modified morphology is explained, because in both morphological modification mechanisms, namely surface poisoning and surface reconstruction, properties other than the goodness of fit between the IBS and the ice surface structure are, at best, of subsidiary relevance.

The structure of the insect TmAFP is a \(\beta\)-sheet with a two-dimensional IBS that exhibits superior activity, much like the remaining insect AFPs. Its seemingly deviant behavior in producing pyramidal ice crystallites, as opposed to hexagonal disk-shaped ones, had thus far defied explanation. In accordance with the morphological modification mechanism by surface poisoning that is triggered by all insect AFPs, the face indices of the ice crystals are primary surfaces with fixed orientations. The behavior of the insect TmAFP is not exceptional in producing the primary pyramid. This finding explains the constancy of the \((101)\) face indices as opposed to the variation of the pyramids produced by fish AFPs despite the similarity in experimental conditions. Because pyramidal morphology has been associated with inferior fish AFP potency to inhibit freezing, the superior activity of the insect TmAFP, while producing pyramidal ice crystals, had remained unexplained; there is no longer any contradiction in this respect.

It is well known that the insect AFPs, in engaging the primary pyramid or basal face with the prism, achieve a higher level of activity than do the fish AFPs engaging secondary pyramids or prisms. The thought may be plausibly entertained that the superior activity of the insect AFPs, which produce a hexagonal disk-type ice morphology, should be attributed to the fact that these AFPs engage two crystal forms, whereas the pyramid is a single crystal form. The reason for the superior activity of the TmAFP that produces pyramidal ice crystallites has remained unexplained. Nevertheless, experiment compels us to conclude that all insect AFPs reduce the growth rates of the ice surfaces much more severely than do the fish AFPs. Moreover, in this work the pyramid produced by the insect TmAFP is established to be the primary \((101)\) pyramid. The cause for the superior activity of the insect AFPs, including the TmAFP, should be sought not in the engagement of more than one crystal form but in the associated morphological modification mechanism that is surface poisoning. The reason for this conclusion can be seen heuristically, based on energy considerations. By means of surface poisoning, an insect AFP reinforces the bonds of two existing PBCs. By means of surface reconstruction, however, a fish AFP does not reinforce the bonds of any PBC but simply introduces previously non-existing transverse strong bonding directions. The former operation imparts, in effect, a larger amount of slice energy to the ice surfaces and therefore causes a larger reduction in the corresponding growth rates, leading to superior freezing inhibition.

Lastly, the statement on the “Fishy Proteins” web page,\(^3\) which says that the AFP “modifies the shape of the ice crystal, and that modification inhibits further crystal growth,” is incorrect. The present work has shown that morphological modification is not the cause of the freezing inhibition but merely its visible manifestation.

Acknowledgments—We are indebted to Dr. Li Dawei and Dr. Zhang Keqin for assistance in preparing the figures and to Du Ning for assistance with the submission of the manuscript.

APPENDIX

PBC Analysis of Hexagonal Ice

The Ice Structure—Hexagonal ice belongs to the space group \(P_6_3/mmc\) with lattice constants \(a = 4.519\) and \(c = 7.357\) Å. The unit cell contains four water molecules, which are considered to be represented by their respective oxygen atoms. The hydrogen atoms play no role in the PBC analysis. Oxygen–oxygen bonds are in tetrahedral coordination. Each oxygen has four bonds in the first coordination sphere with bond lengths 2.763–2.765 Å. Fig. 9 shows the four oxygen atoms in the first coordination sphere in the hexagonal unit cell of ice projected on a plane perpendicular to the \(b\)-axis.

Growth Layers as Networks of Intersecting PBCs—A PBC may consist of one or more chains containing the unit cell content or a fraction thereof. The endpoints are translationally related molecules, and lattice translations are forbidden in the interior of the chain. In the case of ice, the PBCs have a very simple form. The derivation of PBCs and theoretical flat surfaces is performed graph theoretically by the program FFACE (7, 37, 38). Table I lists, in summary, all of the directions in which chains were found together with the primary surface molecular compositions containing the PBC combinations admissible by “flatness.” The eight faces belonging to the \((111)\) family violate the flatness condition and are not listed. If such faces occur on the ice habit, they would be roughened. Infinitely many secondary surfaces exist. In Table I, the only symmetri-

\(^3\) See the Fishy Proteins web page at http://www.psc.edu/science/Madura/fishy_proteins.html.
Faces (7, 37, 38). Only the PBCs shown in Fig. 4 are listed in Table II.

The molecular compositions of the primary surfaces can be determined equally well by alternative combinations of different PBCs. Only the PBCs shown in Fig. 4 are listed in Table II. Additional PBCs, not shown here, feature in the primary surfaces (7, 37, 38).

### Table I

Summary of all chain directions [uvw] emerging from the program GFACE (7, 37, 38) (excluding opposite directions)

<table>
<thead>
<tr>
<th>Primary form</th>
<th>(hkl)</th>
<th>[uvw] (\cap (hkl))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prism</td>
<td>(100)</td>
<td>[010] [001] [011] [0 1 -1]</td>
</tr>
<tr>
<td></td>
<td>(010)</td>
<td>[001] [010] [1 0 -1]</td>
</tr>
<tr>
<td>Pyramid</td>
<td>(101)</td>
<td>[010] [1 0 -1] [1 1 -1]</td>
</tr>
<tr>
<td></td>
<td>(10 -1)</td>
<td>[010] [1 0 -1] [1 1 -1]</td>
</tr>
<tr>
<td></td>
<td>(01 -1)</td>
<td>[010] [0 1 -1] [1 1 -1]</td>
</tr>
<tr>
<td>Basal face</td>
<td>(001)</td>
<td>[100] [010]</td>
</tr>
</tbody>
</table>

### Table II

Some of the PBCs featured in the primary surfaces (7, 37, 38) that suffice to define the molecular compositions of (100), (101), and (001)

Each chain begins with an oxygen in the 0-cell and ends with an identical oxygen in cell [uvw], indicating the PBC direction. Chains in the same direction are grouped together.

(A): \(O_1-O_3-O_1\) [100]  
(B): \(O_2-O_4[100]-O_2[100]\)

### Table III

Slice and attachment energies of the primary and some secondary surfaces expressed in terms of bonds in the order of decreasing slice energy

<table>
<thead>
<tr>
<th>Face</th>
<th>(E_{\text{slice}}) norm</th>
<th>(E_{\text{att}}) norm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary forms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal face (001)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Prism (100)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Pyramid (101)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td><strong>Secondary forms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prism (110)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Prism (120)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Pyramid (102)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Pyramid (201)</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

### References