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Corresponding Author	Family Name	**Kristiansen**
	Particle	
	Given Name	**Erlend**
	Suffix	
	Organization	NTNU University Library
	Address	Trondheim, Norway
	Email	erlendkr@ntnu.no
Abstract	Antifreeze (glyco)proteins, AF(G)Ps, are defined by their shared ability to prevent ice crystals from growing in supercooled solutions. They are categorized as being either moderately active or hyperactive. The distinct difference in antifreeze potency between these two categories is accompanied by distinct shapes of the ice crystals that are being stabilized in their presence; moderately active AF(G)Ps cause bipyramidal crystals to develop, a shape that only exposes a single crystal plane to the surrounding solution. In the presence of hyperactive AF(G)Ps, ice crystals express several crystal planes. A number of different factors affect their potency as antifreeze agents, from large organic macromolecules to inorganic ions. This chapter outlines current understanding of the modus operandi of AF(G)Ps. Attempts are made to provide some simple explanations to the antifreeze potency of AF(G)Ps, including their characteristics as moderately active or hyperactive, and how their antifreeze potency is affected by different factors. The different potencies of moderately and hyperactive AF(G)Ps are ascribed to differences in their adsorption habits. Effects of additives or molecular size on their potencies are ascribed to variations in protein solubility, induced by variations in molecular size or evoked by the presence of additives. Experimental proof of concept is discussed in the context of basic solubility theory. Some characteristics of ice-nucleating agents (INAs) in relation to AF(G)Ps and their relevance in cold tolerance is also briefly examined.	
Keywords (separated by '-')	Thermal hysteresis - Antifreeze activity - Kelvin effect - Critical radius - Hyperactive - Hyperactivity - Protein size - Protein solubility - Hofmeister series - Additive - Enhancement - Adsorption - Desorption - Heterogeneous nucleation - Ice nucleator - Ice nucleating agent	

Chapter 6
Thermal Hysteresis

Erlend Kristiansen

6.1 Introduction

Thermal hysteresis refers to the phenomenon where antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs) cause a separation of the freezing and melting temperature of existing ice crystals in solution. This ability to separate the melting and freezing temperature of ice is limited in that on sufficient cooling the ice crystal undergoes a sudden and rapid ice growth. Ramsay (1964) when studying a mechanism of water reabsorption in the beetle *Tenebrio molitor* first reported the phenomenon. In a footnote, he states:

> When small ice crystals are observed under the microscope, as in the freezing-point method of Ramsay and Brown, one notices that large crystals grow at the expense of small ones and that the edges of the crystals are rounded—the natural consequences of surface tension at the water-ice interface. The change of state between solid and liquid is perfectly temperature-reversible. . .. . .By contrast, the crystals which appear in fluid from the anterior perinephric space tend to have jagged outline and large crystals do not grow at the expense of smaller ones. Furthermore, the system is not temperature-reversible. As the temperature is raised the crystals decrease in size, but as the temperature is lowered they do not increase in size. After the temperature has been lowered by a few degrees the crystal suddenly begins to grow rapidly. On occasion undercooling of the order of 10 °C was observed (in the continued presence of small crystals) and then suddenly the whole sample appeared to solidify instantaneously.

The temperature interval between the melting and freezing temperatures is referred to as the hysteresis gap, and the lower temperature where rapid ice growth is initiated is termed the hysteresis freezing point. The quantitative difference between the melting temperature and the hysteresis freezing point is termed the hysteresis activity, or antifreeze activity.

E. Kristiansen (✉)
NTNU University Library, Trondheim, Norway
e-mail: erlendkr@ntnu.no

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Thermal hysteresis reflects the role of AF(G)Ps as protectors against ice nucleation in the supercooled body fluids of freeze avoiding animals. Their presence enables hypoosmotic fish to occupy ice-laden polar waters (DeVries 1971, 1982; Raymond and DeVries 1977), and allow terrestrial arthropods, such as insects, spiders, and collembolans, to remain year-round in the cold temperate and polar areas. The body temperatures of such terrestrial animals may in some cases drop well below –30 °C in winter (Zachariassen and Husby 1982; Duman 2001; Duman et al. 2004; Graham and Davies 2005). Within the animal, AF(G)Ps are known to act by inactivating structures in the body fluids that could initiate freezing, so-called ice-nucleating agents (INAs), and by preventing ice from penetrating through the body wall (Olsen and Duman 1997a, b; Olsen et al. 1998; Duman 2002).

AF(G)Ps are categorized as being moderately active or hyperactive, based on the hysteresis activity they cause at equimolar concentrations. This distinct difference in antifreeze potency is accompanied by distinct shapes of the ice crystals that form in their presence; moderately active AF(G)Ps cause bipyramidal crystals to develop, a shape that only exposes a single crystal plane to the surrounding solution. In the presence of hyperactive AF(G)Ps, ice crystals express several crystal planes, usually in the form of hexagonal discs. A number of different factors affect the hysteresis activity, including their size and the addition of large organic macromolecules and inorganic ions. This chapter outlines current understanding of the modus operandi of AF(G)Ps. An attempt is made to provide some simple explanations to the antifreeze potency of AF(G)Ps, including their characteristics as moderately active or hyperactive, and how their antifreeze potency is affected by their size and by different additives. Some characteristics of INAs and their relevance in cold tolerance are also examined briefly.

6.2 A Hysteresis Mechanism: The Kelvin Effect

The vapor pressure of bulk ice is lower than that of water. Thus, below the melting point a net transfer of water molecules from the bulk water to ice occurs and the ice mass grows. However, it follows from the observable fact that ice crystals in the presence of AF(G)Ps remain unchanged within a temperature interval, that the AF(G)Ps somehow causes vapor pressure equilibrium between ice and water at all temperatures within the hysteresis gap. This must be so, since the rate by which water molecules adds onto the crystal surface must equal the rate by which they leave. Otherwise, net transfer of water molecules would result, from solution to ice or vice versa and the crystal would visibly change volume. AF(G)Ps do not lower the vapor pressure of water any more than other solutes do (Westh et al. 1997). Thus, they must act by elevating the vapor pressure of the ice to correspond to the higher vapor pressure of the surrounding solution. The difference between the vapor pressure of water and ice increases with temperature departure below the equilibrium melting temperature. Thus, the effect of the AF(G)Ps on the vapor pressure of ice

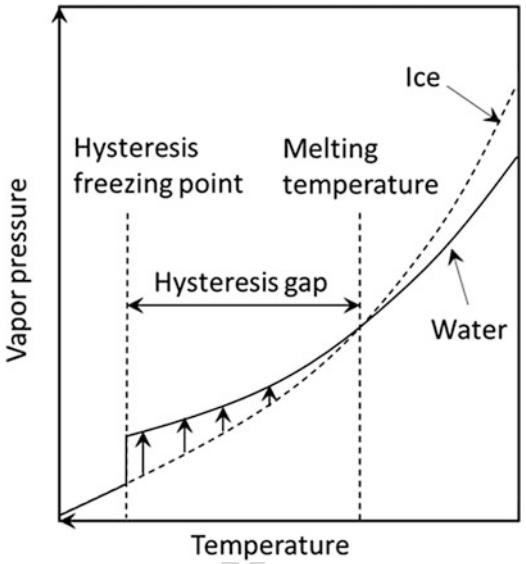

Fig. 6.1 Vapor pressure equilibrium within a temperature interval near the melting temperature. For the ice crystal to be stable within the hysteresis gap, the AF(G)Ps must elevate the vapor pressure of the ice surface to correspond to that of the surrounding supercooled solution. This elevation of the vapor pressure must increase with decreasing temperature. Adapted from Kristiansen and Zachariassen (2005)

must be temperature dependent and increase with decreasing temperature, see Fig. 6.1.

Raymond and DeVries (1977) proposed that the AF(G)Ps act by changing the microscopic growth pattern of the ice surface. Since this is achieved by the AF(G)Ps becoming irreversibly adsorbed onto the ice surface, they coined the mechanism the adsorption–inhibition mechanism. Since then, several investigators have had similar approaches to explaining the phenomenon by irreversible adsorption, including Wilson (1993) and Kristiansen and Zachariassen (2005).

Using fluorescently tagged AFPs, Celik et al. (2013) exchanged the slightly supercooled solution surrounding an ice crystal. The ice surface of the supercooled crystals remained fluorescent following the exchange of the surrounding solution, showing that AFPs were adsorbed onto the crystal surface. Further, the removal of AFPs in the surrounding solution by the exchange process did not weaken the hysteresis effect. These observations provide the most unequivocal evidence to date to show that AF(G)Ps become irreversibly adsorbed onto the ice surface and that the phenomenon is caused only by the surface-bound AF(G)Ps. Also, Chao et al. (1995) and DeLuca et al. (1998) found that AF(G)Ps principally operate as monomeric units.

Elevation of the vapor pressure of the ice by the changed microscopic surface growth pattern could occur by the so-called Kelvin effect. In the following, a brief historical outline of the Kelvin effect is provided. This is followed by a description of how the Kelvin effect is thought to operate at the ice surface.

6.2.1 The Kelvin Effect: Vapor Pressure at a Curved Interface

In 1871, Prof. William Thomson, later to become first Baron Kelvin, pointed out that the vapor pressure of water at a concave and a convex surface must be lower and higher, respectively, than at a plane surface of the water (Thomson 1871). This was deduced by considering the rise and fall of liquids in a capillary tube as a function of the curvature of the meniscus; in an atmosphere saturated with vapor, the vapor pressure decreases with height above the surface of a liquid. Consequently, since a concave interface in a capillary causes the liquid to come to rest at some fixed height above the liquid body, Thomson deduced that the vapor pressure at the elevated concave meniscus is reduced relative to the vapor pressure at the lower plane surface and must correspond to the lowered saturated atmospheric vapor pressure at that height. Otherwise, a perpetual net directional motion of water molecules would develop, as there would be continuous net evaporation at the elevated meniscus and consequently net condensation at the lower plane surface. Such perpetual motion of water molecules would violate the fundamental law of thermodynamics. Convex interfaces must have the opposite effect on the vapor pressure, as such an interface comes to rest below the plane liquid body where the saturated vapor pressure is higher. The effect of a surface curvature on the vapor pressure has since become known as the Kelvin effect.

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6.2.1.1 The Critical Radius of Curvature

A decade later, Prof. John Henry Poynting (1881) recognized that the effect of a surface curvature on the resultant vapor pressure in Thomson's capillary is caused by a change in the bulk pressure in the water in the capillary; a concave interface evokes a lower pressure inside the liquid water, as evident from the rise in the capillary, and hence to a lower vapor pressure, and vice versa for a convex interface. Thus, the underlying cause of the changing vapor pressure with changing curvature of an interface is an accompanying curvature-induced change in bulk pressure within the curved volume.

Poynting applied his reasoning to the melting temperature of ice. He inferred that if the bulk pressure of ice alone was elevated, then the resultant elevated vapor pressure of the ice would depress the temperature at which the vapor pressures of ice and water coincides, i.e., a pressure-induced depression of the melting temperature of the ice surface. By extension, since the pressure-elevating effect of a convexity increases with decreasing radius, there must be a convexity with a radius small enough to cause a pressure great enough for ice/water vapor pressure equilibrium to develop at any temperature below the normal melting point. The radius of this convexity at a specific temperature is referred to as the critical radius of curvature at that temperature.

6.2.2 The Kelvin Effect at the Ice Surface

It follows from the above paragraphs that AF(G)Ps that are irreversibly adsorbed onto the ice surface could evoke the Kelvin effect by causing the ice surface to grow out as many tiny convex interfaces between them. These convex interfaces would elevate the vapor pressure of the ice surface and, hence, eliminate the difference between the vapor pressures at different temperatures, as illustrated in Fig. 6.1.

The Kelvin effect implies that, at any temperature below the normal melting temperature, the growth of the convex surface zones between the adsorbed AF(G)Ps will halt when they obtain a curvature with a radius corresponding to the critical radius at that temperature. Thus, at any temperature where the phenomenon is expressed, the surface of the entire ice crystal is covered by spherical growth regions with identical convexities, i.e., identical local vapor pressures. This causes the entire ice crystal surface to be in vapor pressure equilibrium with the surrounding supercooled solution, and hence the crystal surface is at its melting temperature, see Fig. 6.2. A. Such a crystal could in principle remain unchanged indefinitely. Crystals in supercooled solutions of AF(G)Ps have been observed for many days without expressing any visible growth (DeVries 1971; Raymond and DeVries 1977; Graether et al. 2000; Fletcher et al. 2001).

As the temperature is lowered further, the many tiny surface zones expand until their convex interfaces again cause vapor pressure equilibrium with the surrounding

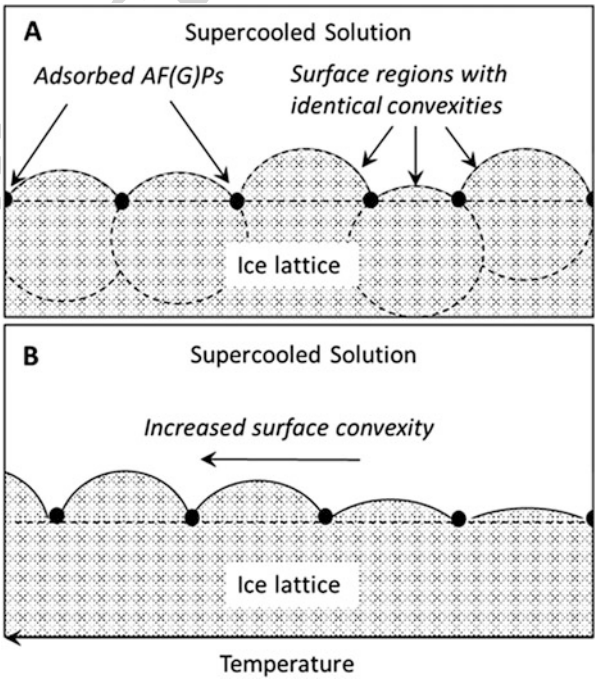

Fig. 6.2 The convexities of the growth zones within the hysteresis gap. (**a**) All growth zones must have the same convexity at a specific temperature within the hysteresis gap. (**b**) The convexities increases with decreasing temperature and elevates the vapor pressure of the ice surface in a temperature-dependent manner, as seen in Fig. 6.1. Adapted from Kristiansen and Zachariassen (2005)

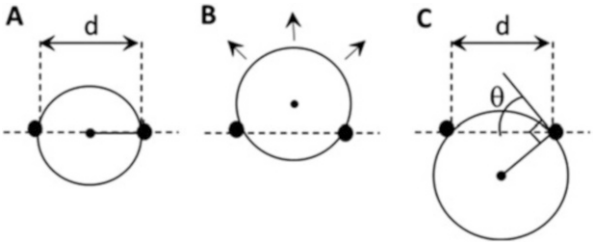

Fig. 6.3 At the hysteresis freezing point. (**a**) When one of the convexities has reached the shape of a half-sphere it has reached its maximum convexity. (**b**) Any further growth of this structure will cause the convexity to decrease and cause spontaneous growth. (**c**) The relation between adsorbent spacing, *d*, and the angle, *θ*. Adapted from Kristiansen and Zachariassen (2005)

solution. In this manner ice/water vapor pressure equilibrium is maintained across a temperature interval, the hysteresis gap, see Figs. 6.1and 6.2b.

There is a limit to how much such a crystal can be cooled, i.e., how convex the tiny curved interfaces may become; no surface zone can become more convex than that of a half-sphere. Once such a shape is reached, then any further cooling will result in the convexity of the structure to decrease on growth. The resultant drop in vapor pressure due to the reduced convexity will result in spontaneous growth. This is illustrated in Fig. 6.3a and b. This temperature is the hysteresis freezing point.

6.3 Hysteresis Activity

In the following paragraphs, an attempt is made to explain what fundamentally determines the hysteresis freezing point, based on the theory outlined above. This explanation is then extended to incorporate the characteristic difference in activity between moderately active and hyperactive kinds of AF(G)Ps.

6.3.1 The Largest Intermolecular Adsorbent Gap Determines Hysteresis Activity

If only a single one of all the tiny growth zones that protrude out at the crystal surface should fail, then the hysteresis phenomenon is terminated. Hence, the hysteresis freezing point is determined by the single growth zone that reaches the shape of a half-sphere at the highest temperature. Any further growth of this single growth zone, i.e., any further cooling, will only result in a reduction in its convexity and, consequently, the phenomenon is terminated.

Since all the surface growth zones have the same convexity, it will be the single one growth zone with the widest diameter that will reach the shape of a half-sphere at

the highest temperature. Thus, the hysteresis freezing point, and therefore the hysteresis activity, is determined by the single largest intermolecular adsorbent spacing between AF(G)Ps that comprise a single growth zone at the crystal surface. Mathematically, the hysteresis activity (ΔT) as a function of the largest such adsorbent spacing, d, may be expressed as (Kristiansen and Zachariassen 2005):

$$\Delta T = \frac{4\gamma T_{\mathrm{E}} \sin\theta}{\Delta H \mathrm{d}}; \tag{6.1}$$

where d is the spacing in units of cm, γ is the ice/water interfacial tension (taken to be 32 ergs/cm^2), T_E is the normal melting temperature for a plane interface (units of K), and ΔH is the heat of fusion of water ($3.3 \cdot 10^9$ ergs/cm^3). θ is an angle describing the situation if a curvature fails before reaching the shape of a half-sphere. For a half-sphere, θ is 90° and, hence, the term (sin θ) is 1. See Fig. 6.3. C for an illustration of the angle θ.

6.3.2 *Moderately Active and Hyperactive AF(G)Ps*

There is a great difference in the hysteresis activities caused by different AF(G)Ps. Based on their activities at equimolar concentrations and the shape of the crystals they form in solution, they fall into two categories: hyperactive and moderately active.

Marshall et al. (2004a) found that moderately and hyperactive AFPs accumulate in ice to a similar extent. Also, experimentally determined estimates of average adsorbent spacings between AF(G)Ps on the surface of ice crystals are quite similar in the case of moderately and hyperactive AF(G)Ps; Drori et al. (2015) estimated the average adsorbent distance between hyperactive TmAFP to 7.6–35.2 nm at concentrations ranging from 31.4 to 0.4 μM. Comparable results were obtained by Celik et al. (2013) for the same protein. For the moderately active type III AFP, Drori et al. (2015) estimated the average adsorbent distance to be 8.7 to 24.7 nm at concentrations ranging from 19.8 to 1.2 μM. Others have estimated similar values for moderately active AF(G)Ps (Wilson et al. 1993; Grandum et al. 1999; Zepeda et al. 2008). Thus, the principal cause of the great difference in the activities of moderately and hyperactive AF(G)Ps do not seem to be due to differences in their preference for ice. Rather, it is likely that the distinct difference between them is the result of the single largest adsorbent gap at the ice surface for some reason is much larger in the case of moderately active AF(G)Ps.

6.3.2.1 Moderate or Hyperactive: Caused by Plane Specificity and Adsorption Pattern?

Moderate Activity A characteristic feature of moderately active AF(G)Ps is that they only adsorb onto a single crystal plane in the ice structure. Notably, none of the moderately active AF(G)Ps adsorb onto the basal plane of crystals, only onto a single prism or pyramidal plane (Knight and DeVries 1988; Knight et al. 1991). This plane-specific adsorption is apparently a consequence of structural features of their ice-binding sites (IBSs), that restricts these AF(G)Ps to only become irreversibly adsorbed onto a single plane and orientation. Laursen et al. (1994) showed this by observing that the moderately active chiral L-AFP I and D-AFP I variants resulted in adsorption on mirror image directions on the ice surface. The result of such a specific preference for a single crystal plane is a crystal that only expresses this single protected crystal plane toward the surrounding supercooled solution. Consequently, in the presence of moderately active AF(G)Ps crystals obtain a bipyramidal shape, as this is the only possible crystal shape whose entire surface consists of a single plane. At the hysteresis freezing point, these bipyramidal crystals freeze out from their apexes (Raymond and DeVries 1977; Jia and Davies 2002). The fact that they characteristically grow out of their apexes at the hysteresis freezing point strongly suggests that the antifreeze potency of moderately active AF(G)Ps are limited by a large intermolecular spacing at the apex of the bipyramidal crystal (Jia and Davies 2002). This must arise from the fact that these proteins only adsorb onto a single crystal plane.

The surface area involved in determining the hysteresis activity for moderately active AF(G)Ps is only that miniscule fraction of the total surface area of the crystal that comprises the two apexes of the bipyramid. Consequently, the hysteresis activity in the presence of moderately active AF(G)Ps should not be much affected by changing the total surface area of the ice. Consistent with this, the hysteresis activity of moderately active AF(G)Ps are reportedly rather insensitive to the amount of ice present in the sample; large variations in the ice content, i.e., large variations in total ice crystal surface area, does not appreciably affect the hysteresis activity, see Fig. 6.4 (Hansen et al. 1991; Wöhrmann 1996; Sørensen and Ramløv 2001).

Hyperactivity In contrast to the moderately active AF(G)Ps, the hyperactive AF(G)Ps have been shown to adsorb to several crystal planes that differ greatly in their orientation, such as both prism and basal planes (Graether et al. 2000; Liou et al. 2000). Structural studies have shown that hyperactive AFPs have IBS that afford the protein freedom to adsorb in different orientations and on different planes. Their ability to adsorb onto multiple crystal planes, and most notably the basal plane, is a feature that separates them from the moderately active AF(G)Ps. Basal plane adsorption has been implicated as a key feature that causes them to be hyperactive (Graether et al. 2000; Liou et al. 2000; Pertaya et al. 2008). Because of their ability to adsorb onto multiple planes, crystals formed in the presence of hyperactive AF(G)Ps expresses multiple planes to the surrounding supercooled solution and usually take the form of hexagonal discs (Graether et al. 2000; Liou et al. 2000).

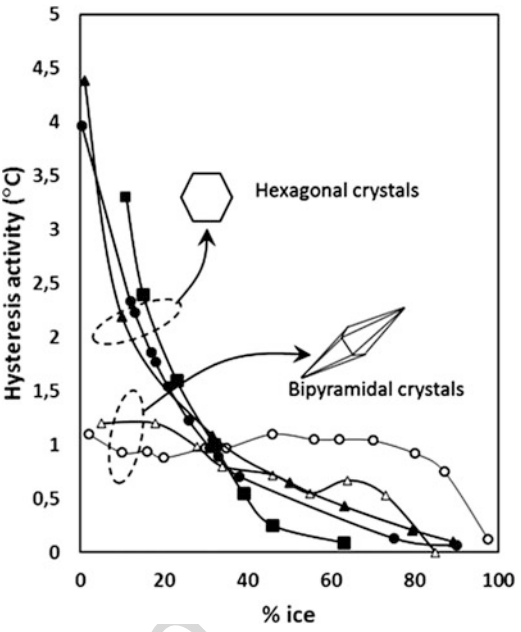

Fig. 6.4 The dependency of the hysteresis activity on the % ice in the sample. Filled symbols: hyperactive AF(G)Ps. Open symbols: moderately active AFGP. (Filled square) PAGP, a hyperactive AFGP from the notothenid *Pleuragramma Antarcticum* (Wöhrmann 1996). (Filled circle) Hemolymph from *Tenebrio molitor* (Hansen and Baust 1988). (Filled triangle) Hemolymph from *Rhagium inquisitor* (Zachariassen et al. 2002). (Open circle) Serum from *P. antarcticum*. (Open triangle) AFGP from *P. antarcticum* (Wöhrmann 1996). For explanation, see text

Because of their ability to adsorb onto different planes and at different orientations, hyperactive AFPs likely become spread out across the crystal surface in a rather random adsorption pattern. Such a random pattern should, by chance alone, result in the largest adsorption gap increasing with increasing surface area. Consequently, the hysteresis activity of hyperactive AF(G)Ps should decrease with increasing crystal surface area. Consistent with this, several investigators have reported strong dependence of hyperactive AF(G)Ps on the amount of ice present in the sample, see Fig. 6.4 (Zachariassen and Husby 1982; Hansen and Baust 1988; Wöhrmann 1996). As can be seen from the figure, "hyperactivity" is apparently a consequence of using small ice crystals in the experiment, since hyperactive AF(G)Ps have a lower hysteresis activity than their moderately active counterparts at higher contents of ice in the samples.

The Shape of the Bipyramidal Apexes When bipyramidal crystals form in the presence of moderately active AF(G)Ps, the ice crystal grows out from the basal planes. Once this bipyramidal shape is formed the crystal stops growing and it remains stable within the hysteresis gap. What is the physical shape of the apex interfaces? Since the moderately active AF(G)Ps do not adsorb onto the basal plane, is the apex a tiny unprotected flat basal plane? If so, then one could envision two-dimensional curved interfaces protruding out only in the direction of the prism planes that form the surrounding edge of the exposed apex basal plane (Raymond and DeVries 1977). The effect of these 2D curvatures that are in the prism plane direction must then also elevate the vapor pressure beyond the base of the curvature toward the center of the flat basal plane in order for vapor pressure

equilibrium to persist between the flat apex interface and the surrounding solution. Another, and perhaps simpler, approach is to assume that the apexes are three-dimensional spheres protruding out in the basal plane direction. In any event, it is these areas of the bipyramidal crystal that apparently determines the hysteresis activity of the moderately active AF(G)Ps.

6.4 Factors That Affect the Hysteresis Activity

In the above paragraphs the categorization of AF(G)Ps into moderately active and hyperactive were ascribed to consequences of irreversible adsorption to the ice surface that arises from features of their IBS. In the following, differences in hysteresis activity within each of these categories will be ascribed to the situation that exist prior to the AF(G)Ps becoming irreversibly adsorbed. It will be argued that, while the ice crystal is held at the equilibrium melting temperature, AF(G)Ps acquire an equilibrium distribution between the crystal surface melting region and the surrounding solution. Then, following a cooling event, AF(G)Ps within this surface region freeze onto the solidifying crystal surface and, hence, become irreversibly adsorbed (Kristiansen and Zachariassen 2005). Any change in this distribution pattern prior to the cooling event will result in changes in the surface density of irreversibly adsorbed AF(G)Ps after the cooling event and, hence, to changes in the observed hysteresis activity. Differences in hysteresis activity among hyperactive or among moderately active AF(G)Ps, may be attributed to differences in the solubility of the AF(G)Ps in the solution; a lowered solubility results in a shift in the distribution of the AF(G)Ps toward the ice surface region prior to the cooling event, and hence, to increased hysteresis activity (Kristiansen and Zachariassen 2005; Kristiansen et al. 2008).

6.4.1 The Factors

Several investigators have reported that the size of the AF(G)Ps can have a profound effect on their capacity to cause thermal hysteresis. For structurally similar isoforms, their potency reportedly increases with molecular size for both moderately active AFGPs (Schrag et al. 1982; Chao et al. 1996; Miura et al. 2001; Baardsnes et al. 2003; Nishimiya et al. 2003) and hyperactive AFPs (Leinala et al. 2002; Marshall et al. 2004b; Liu et al. 2005; Mok et al. 2010; Friis et al. 2014). Synthetic oligomers of moderately active AFPs also reportedly have increased potency (Nishimiya et al. 2005; Holland et al. 2008; Can and Holland 2011, 2013; Stevens et al. 2015). In all the cases mentioned above, the increased size is accompanied by an increased IBS or the addition of multiple IBSs. Other investigators have reported that AFPs are potentiated by ligation to, or interaction with, large non-ice binding structures (Deluca et al. 1998; Hakim et al. 2013; Wu and Duman 1991, Wu et al. 1991;

Horwath et al. 1996; Wang and Duman 2005, 2006). In these cases, the IBS is unchanged.

In addition to the effect of molecular size, several authors have reported that the hysteresis activity is also elevated in the presence of various low-mass co-solutes. These low-mass solutes include sugars, polyols, salts, amino acids, salts of polycarboxylates, and NADH. The effect has been reported for both moderately active AF(G)Ps (Kerr et al. 1985; Caple et al. 1986; Evans et al. 2007; Gong et al. 2011) and hyperactive AFPs (Li et al. 1998; Kristiansen et al. 2008; Amornwittawat et al. 2008; Wang et al. 2009a, b; Amornwittawat et al. 2009; Wen et al. 2011; Liu et al. 2015).

There is one thing that variations in molecular size and additives have in common; they change the solubility of proteins in solution. Moreover, they reportedly enhance the hysteresis activity in manners predicted by their general effects on protein solubility. In the following section, the potential importance of the solubility of AF(G)Ps to their antifreeze potency is briefly explored.

6.4.2 The Solubility of the AF(G)Ps: A General Concept to Explain Variability?

Several authors have in various ways implicated protein solubility as a relevant factor in antifreeze potency (Kristiansen and Zachariassen 2005; Evans et al. 2007; Kristiansen et al. 2008; Wang et al. 2009a). Solubility of AF(G)Ps have also inadvertently been implicated in the manner the AF(G)Ps are thought to orient toward the ice; these proteins are somewhat amphipathic, were the more hydrophobic side that contains the IBS orient toward the ice (Yang et al. 1988; Sönnichsen et al. 1996; Haymet et al. 1998, 1999). In other words, the less soluble side of the molecule orients toward the ice whereas the more soluble side orients toward the water. The logical extension of this is that a less soluble AFP would have a greater affinity toward the ice surface than a more soluble AFP. In the following paragraphs, a brief examination of the significance of this common denominator, the solubility of the AF(G)Ps, to their potency is presented.

6.4.2.1 The AF(G)P/Ice Interaction Is Temperature Dependent

The ice surface in equilibrium with surrounding liquid water is not distinct but a transition region where the configuration of the water molecules changes from the ordered crystal structure of the ice lattice to the random distribution of the bulk water in the surrounding solution. This change occurs across a 1–2 nm deep region called the interfacial region or the melting/freezing region (Hayward and Haymet 2001).

As stated in the introductory quote by Ramsay (1964), AF(G)Ps act at temperatures below the equilibrium melting temperature of the ice, not at temperatures above

it, i.e., the ice crystal does not grow below this temperature but melts above it (but see also next section concerning superheating of ice crystals). This suggests that the AF(G)Ps are irreversibly adsorbed onto the ice crystal surface only at temperatures below the melting temperature. A simple explanation to this is that AF(G)Ps freeze onto the crystal surface as the temperature is lowered to within the hysteresis gap and then melt off the ice when the temperature is raised to the melting temperature (Kristiansen and Zachariassen 2005). Such a temperature-dependent behavior of freezing onto (adsorption) and melting off (desorption) would explain why ice crystals in the presence of AF(G)Ps typically melt at the equilibrium temperature irrespective of any colligative variation in this temperature. It also provides an intuitive and simple explanation to the long-standing conundrum of the origin of the necessary bond strength to achieve irreversible adsorption (Wen and Laursen 1992; Knight et al. 1993; Chao et al. 1995); the bond strength between the irreversibly adsorbed AF(G)P and the ice surface corresponds to those between water molecules in bulk ice at that temperature. Recently, Garnham et al. (2011a) showed that the hydration water of a hyperactive AFP has a clathrate-like configuration and is firmly embedded by extensive H-bonds to the backbone of the protein. Hence, this crystalline-like water at the IBS appears to be prone to fuse together with the solidifying crystalline interface once the temperature is lowered and melt off when the interface disintegrates into chaos on warming to the equilibrium melting temperature. Molecular dynamics studies support this contention (Chakraborty and Jana 2019; Zanetti-Polzi et al. 2019).

Pertaya et al. (2008) reported on the fluorescence associated with an ice crystal in a solution containing fluorescently tagged AFP. When slowly melting a crystal at a temperature just above that of equilibrium the crystal showed no fluorescence, indicating no adsorbed AFPs. When cooled to within the hysteresis gap the crystal surface became fluorescent, indicating irreversible adsorption. Similar results were reported by Pertaya et al. (2007), who used a technique of photo-bleaching of fluorescently tagged AFPs to study the AFP/ice association at the crystal surface at temperatures within, and just above, the hysteresis gap. Bleached AFPs at the surface were not replaced within the hysteresis gap but were replaced at temperatures just above, showing that the AFPs were irreversibly adsorbed within the hysteresis gap and desorbed off the ice at the melting temperature.

While in the desorbed state, at the melting temperature of the crystal surface, there must be a distribution of AF(G)Ps between the melting/freezing region and the bulk solution. It is this distribution pattern that presumably becomes affected by changes in the solubility of the AF(G)Ps; a lowered protein solubility means that the AF(G)P has an increased tendency to move away from the solution and toward the melting/freezing region. This results in more AF(G)P molecules being at the ice/water interfacial region and available to freeze onto the solidifying crystal surface the instant the temperature is lowered. Consequently, lowered solubility of an AF(G)P should result in greater surface density of the AF(G)P below the melting temperature and, hence, to greater hysteresis activity (Kristiansen and Zachariassen 2005).

Superheating of Ice Crystals Several investigators have reported that ice crystals in solutions of AF(G)Ps may superheat slightly (Celik et al. 2010; Cziko et al. 2014). Celik et al. (2010) reported that tiny ice crystals became superheated by 0.04 °C and 0.44 °C in the presence of several hyperactive AFPs. In the case of moderately active AFPs, superheating up to 0.02 °C was reported at high AFP concentrations. The observed superheating reflects the presence of concave surface regions developing between irreversibly adsorbed AF(G)Ps at temperatures above the equilibrium temperature (Knight and DeVries 1989). These observations potentially contradict the notion of an equilibrium distribution of AF(G)Ps developing between the solution and the ice surface region at the equilibrium temperature, as outlined above.

The samples that expressed this superheating also expressed hysteresis activities ranging from 1.7 °C to 4.1 °C. The hysteresis activity increases approximately as a function of the square root of the surface density of AF(G)Ps (Raymond and DeVries 1977; Kristiansen and Zachariassen 2005). Thus, apparently only a small fraction of the AFPs that was originally frozen onto the surface and caused these high hysteresis activities was subsequently involved in the comparatively much lower superheating. That is, most AFPs melted off the ice surface.

The superheating phenomenon requires a cooling event to occur; when Celik et al. (2010) melted out ice in solutions with high concentrations of moderate AFPs or low concentrations of hyperactive AFPs, they observed that the many small crystals decreased uniformly in size. If the melting process was briefly halted, then the crystals began to show slight superheating. This change in melting behavior following a brief cooling event suggests that AFPs in solution do not adsorb irreversibly to the ice surface unless there is a cooling event, i.e., the adsorption is a freezing of the AFPs onto the ice surface. The subsequent desorption as the temperature is raised is for some of the adsorbed AFPs a delayed process.

Why do some of the AF(G)Ps not simply melt off the surface as the temperature is raised to the melting point? The freezing of the AF(G)Ps onto the ice surface imply that the hydration water at the IBS becomes part of the crystal lattice. Above its equilibrium melting temperature, ice melts from its surface, as lattice water molecules are released to the fluid hydrogen-bonding network of the surrounding solution. However, if no liquid water is in contact with the lattice that is to be melted, e.g., in the interior of a crystal, the lattice structure may superheat extensively before a melting nucleation event occurs (Turnbull 1950; Chalmers 1964; Lu and Li 1998). Consequently, if the crystalline water at the IBS of an adsorbed AFP is shielded from the surrounding liquid solution, then the melting process at the IBS is prevented and the AFP will remain adsorbed onto the crystal surface at temperatures above the melting point. The distinct difference in the capacities of moderately and hyperactive AFPs to cause superheating reported by Celik et al. (2010) presumably reflect differences in their respective capacities to shield the crystalline water at the IBS from the surrounding liquid water when adsorbed onto the ice. They observed that in the presence of hyperactive AFPs, crystals sporadically disappeared over time up to 4 h, showing that this situation can be quite stable if it develops. Since the phenomenon is very weak compared to the hysteresis activity, it might be that only those AF

(G)Ps with certain rare orientations at the crystal surface is able to postpone the initiation of the melting process at the IBS.

6.4.3 Basic Concepts in Solubility Theory

The solubility of a protein in water reflects its energetic state in water (Reynolds et al. 1974). Once present in the water, the solubility of a protein is determined by two opposing effects acting on structural features of the protein; favorable attractive forces such as van der Waals- and dipole-type forces lower the energy state of the protein and therefore increase its solubility. This is opposed by an energetic cost associated with occupying a cavity within the water that increases its energetic state and therefore lowers its solubility (Uhlig 1937; Tolls et al. 2002). In the latter case, the presence of the protein in the water effectively adds additional high-energy water surface at the water/protein boundary of the cavity occupied by the solute. The presence of nonpolar surface regions of the protein restricts hydrogen bond formation between water molecules in the surface boundary, and consequently reduces the freedom of these local water molecules to orientate. This structuring of water at the protein/water boundary is known as the hydrophobic effect.

According to Uhlig (1937), the solubility (S) of a dissolved molecule may be expressed as:

$$RT \ln(S) = -A\gamma + E \tag{6.2}$$

where R and T are the universal gas constant and the absolute temperature, respectively. The first term on the right side of Eq. (6.2), $A\gamma$, represents the "hydrophobic" effect that lowers the solubility of a molecule. This effect is a function of the nonpolar surface area, A, of the molecule in contact with water, and the energetic state of the water at this surface, expressed as the water surface tension, γ. This hydrophobic effect is opposed by the second term on the right-hand side of Eq. (6.2), the favorable "electrostatic" effect, E, that raises the solubility of the dissolved molecule (Reynolds et al. 1974; Melander and Horváth 1977).

Changing the size of the AF(G)Ps, for instance by adding or removing repetitive peptide segments, inadvertently also changes the nonpolar surface area, A, of the protein and consequently its solubility. Also, for structurally similar isoforms of different size, their nonpolar surface areas, and hence, their solubility, correlate with their size. The small mass solutes that reportedly enhance the hysteresis activity, such as salts, sugars, polyols, and amino acids are known to elevate the surface tension, γ, of water (Washburn 1929; Melander and Horváth 1977; Kaushik and Bhat 1998; Landt 1931; Matubayasi and Nishiyama 2006; Bull and Breese 1974). Thus, their reported enhancement effect may simply be the result of the solutes lowering the solubility of the AF(G)P by elevating γ at the protein/water interface.

The basic framework outlined above may be useful when interpreting natural and induced variations in the antifreeze potency among moderately active and among

hyperactive AF(G)Ps. In the following paragraphs, standard solubility theory will be applied to examine some of the reported effects small co-solutes and variations in size have on hysteresis activity.

6.4.4 *Low-Mass Additives, Solubility, and Antifreeze Potency*

The effects of salts on the hysteresis activity in relation to solubility theory will be exemplified by the effects of salts on the hyperactive AFP, RiAFP, from the cerambycid beetle *Rhagium inquisitor* (Kristiansen et al. 2008). Wang et al. (2009a) also had a quite similar approach to this issue. It will be shown that these effects are entirely consistent with being caused by salt-induced lowered solubility of the RiAFP molecules. To support this claim, the nonpolar surface area and the dipole moment of RiAFP is derived from the effects of salts on its antifreeze potency.

6.4.4.1 The Salting-Out Constant, K_s

As mentioned above, salts are known to lower the solubility of proteins. This effect is termed "salting-out." The salting-out effect is qualitatively similar for different kinds of proteins and different kinds of salts in that the solubility of the protein changes in a log-linear manner with the concentration of salt (Cohn 1925; Melander and Horváth 1977):

$$\ln(S) = \beta - K_s m \tag{6.3}$$

where S is the solubility of the protein (mg/ml), β is the solubility of the protein in the absence of salts (mg/ml), m is the concentration of the salt (molal), and K_s is known as the salting-out constant (molal^{-1}). K_s is an expression of the sensitivity of the solubility of a particular protein to the presence of a particular salt. The value of K_s depends on both the salt and the protein and is experimentally determined as the slope of the linear relationship between ln(S) and m.

6.4.4.2 Obtaining Salting-Out Constants from Measurements of Hysteresis Activity

Since the presence of salts increases the hysteresis activity, adding salts is equivalent to increasing the concentration of the AF(G)P. Since solubility is in units of concentration, Eq. (6.3) should describe the salt-induced apparent changes in the concentration of AF(G)P. Thus, the salting-out constant, K_s, in the presence of a particular salt may be obtained from the hysteresis measurements as follows; the actual concentration of AF(G)P in the samples is kept unchanged during the

procedure. An "apparent" concentration of AF(G)P in the presence of different concentrations of salts is then obtained by converting the observed enhanced hysteresis activity in the presence of salts to the equivalent concentration of AF(G)P needed to cause this activity in the absence of salt. The value of K_s for that salt is then obtained simply as the slope of the linear relationship obtained by plotting the natural logarithm of the "apparent" concentration of AF(G)P in the samples as a function of the concentration of salt.

Kristiansen et al. (2008) used this method to determine K_s for each of ten different salts from the salt-induced enhancement of the hysteresis activity for RiAFP. As predicted by Eq. (6.3) all "apparent" concentrations were log-linear functions of the concentrations of the different salts tested.

6.4.4.3 The Hofmeister Series and Its Linearity

An experimentally determined salting-out constant, K_s, is an expression of the two opposing effects acting on structural features of the protein, the favorable "electrostatic" effect that increases the solubility of the protein and the unfavorable "hydrophobic" effect that lowers its solubility, as outlined in Eq. (6.2). The net observed salting-out constant, K_s, in Eq. (6.3) is given by (Melander and Horváth):

$$K_s = \Omega\sigma - \Lambda \tag{6.4}$$

where Ω is a protein-specific intrinsic salting-out constant (cm dyn^{-1}), σ is the molal surface tension increment of the salt (10^{-3} dyn g/cm mol), and Λ is a protein-specific intrinsic salting-in constant (molal^{-1}).

By arranging salts according to their ability to lower the solubility of proteins, the so-called Hofmeister series of salts is obtained. The arrangement of different salts in the Hofmeister series may be understood from Eq. (6.4); for a specific protein, the molal surface tension increment, σ, is the only variable in the equation. Thus, for any single protein the arrangement of salts according to their ability to lower the solubility of that protein is similar for all proteins and dictated by the molal surface tension increment, σ, of the different salts. For example, the following eight salts listed in descending order according to their ability to lower protein solubility form the Hofmeister series as (value for σ in parenthesis): $Na_3C_6H_6O_7$ (3.12) > $(NH_4)_2SO_4$ (2.16) > NaCl (1.64) > KCl (1.40) > NH_4Cl (1.39) > $NaNO_3$ (1.20) > NaI, (1.09) > $N(CH_3)_4Cl$ (0.76). Since the value of σ is actually the surface tension increment of the water/air interface and not the protein/water interface, there are slight differences between the predicted and observed Hofmeister series. However, this general arrangement of salts varies little for different proteins. Hence, if the salt-induced enhancement of the hysteresis activity is caused by salt-induced reduction in the solubility of RiAFP, then the enhancement-effect of the different salts should reflect the Hofmeister series. In the case of RiAFP the experimentally determined K_s values were arranged as (value for σ in parenthesis): $Na_3C_6H_6O_7$ (3.12) > $(NH_4)_2SO_4$ (2.16) > NaCl (1.64) > KCl

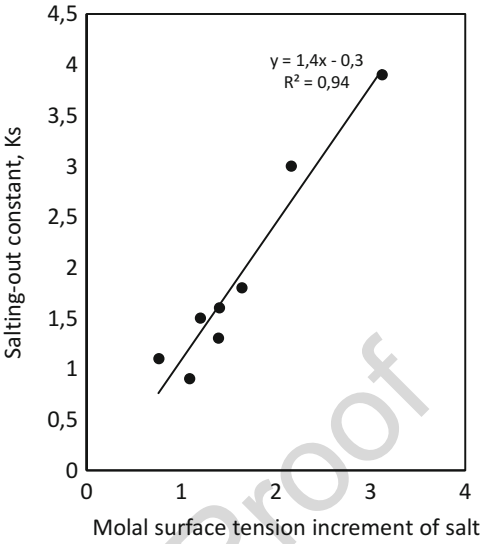

Fig. 6.5 The linearity of the Hofmeister series. Different salting-out constants, K_s, determined from the hysteresis activity in the presence of different kinds of salts, versus the surface tension increment of the salts used. The slope of the linear line is the intrinsic salting-out constant, Ω, for the protein. The intercept value is the intrinsic salting-in constant, Λ, for the protein. These two protein-specific constants may be used to determine the nonpolar surface area and the dipole moment of the protein. Adapted from Kristiansen et al. (2008)

(1.40) > $NaNO_3$ (1.20) > NH_4Cl (1.39) > $N(CH_3)_4Cl$ (0.76) > NaI (1.09). This arrangement is in close agreement with the Hofmeister series. An examination of the results of Wang et al. (2009a) also shows that arrangement of the salts according to their ability to enhance a hyperactive AFP, DAFP, from the beetle *Dendroides canadensis* corresponds well with the Hofmeister series. For those salts where the value for σ is known, they are listed as (value for σ in parenthesis); NaCl (1.64) > KCl (1.40) > KBr (1.31) > NaBr (1.32) > KI (0.84) > NaI (1.09) > $NaClO_4$ (0.55). In their extensive study, Li et al. (1998) found that, among all the different compounds tested, citrate was the strongest enhancer of the antifreeze potency of DAFP. Citrate has among the highest known surface tension increments (σ of 3.12) and was also the strongest enhancer in the case of RiAFP. Evans et al. (2007) did not find differences in the efficacies of LiCl, NaCl, and KCl to enhance the antifreeze activity of different kinds of fish AF(G)Ps. This is consistent with the fact that the molal surface tension increments of these salts are very similar, 1.63, 1.64, and 1.40, respectively.

Since both Ω and Λ of Eq. (6.4) are constant features of the protein, the K_s values obtained for that protein will be a linear function of the molal surface tension increment, σ, of the different salts. Figure 6.5 shows that the salting-out constants of RiAFP, determined from the salt-induced enhancement of the hysteresis activity, vary as a linear function of σ, consistent with this prediction of Eq. (6.4). Thus, the linear relationship depicted in Fig. 6.5 is a quantitative representation of the Hofmeister series for RiAFP.

6.4.4.4 Quantitative Predictions of Protein Properties from Salt-Induced Enhancement

The protein-specific salting-out constant, Ω, and the protein-specific salting-in constant, Λ, of Eq. (6.4) reflect physicochemical properties of the protein (Melander and Horváth 1977). Thus, if the concept of solubility-induced enhancement is correct, then it should be possible to use the information derived from the antifreeze measurements to predict features of the protein that are reflected by these constants.

The protein-specific salting-out constant, Ω, of Eq. (6.4) represents the hydrophobic properties of the protein and is a function of its nonpolar surface area, ϕ. The numeric value of ϕ, in units of square Ångstrøm, may be obtained from Ω as (Melander and Horváth 1977):

$$\phi = 411\Omega - 12 \tag{6.5}$$

According to Eq. (6.4), Ω is given by the slope of the linear relationship depicted in Fig. 6.5. Using the value of 1.4 cm dyn^{-1} for Ω in Eq. (6.5) gives a value for ϕ for RiAFP of 563 $Å^2$. This is about 20% of the total surface area of the protein (Kristiansen et al. 2008). According to Melander and Horváth (1977), ϕ is typically between 20% and 40% of the total surface area of proteins. The sensitivity of RiAFP to become enhanced by salts therefore seems to correspond well with the expected salt sensitivity of a protein of its size.

The intrinsic protein salting-in constant, Λ, of Eq. (6.4) reflects the favorable electrostatic forces acting to enhance the solubility of the protein and is a function of its dipole moment, μ. The dipole moment, μ, may be numerically obtained in units of Debye from Λ using the formula (Melander and Horváth 1977):

$$\mu = -578\Lambda \tag{6.6}$$

According to Eq. (6.4), the value of Λ is given by the intercept of the linear relationship depicted in Fig. 6.5 and has the value of −0.3 $molal^{-1}$. This gives a predicted dipole moment for RiAFP of 173 Debye. Since the original study was published (Kristiansen et al. 2008), the crystal structure of RiAFP has become available (Hakim et al. 2013). The structure file (PDB 4DT5) contains two molecules, A and B, which, when submitted to the online Protein Dipole Moments Server (Felder et al. 2007) has predicted dipole moments of 182 Debye and 125 Debye, respectively. It is noteworthy that the dipole moment of RiAFP, derived from its molecular structure, coincides within a few percentage points with the dipole moment derived from the effects of salts on the antifreeze potency of the protein.

Considering the above presented relations, it appears obvious that salts enhance the antifreeze potency by lowering the solubility of AF(G)Ps. Since the other small mass solutes known to enhance the antifreeze potency of AF(G)P, i.e., polyols, amino acids, sugars etc., act on protein solubility in a manner similar to that of salts, they are all likely to operate by the same mechanism.

6.4.5 Molecular Size, Solubility, and Antifreeze Potency

Several explanations are provided for the effect of size on the potency of AF(G)Ps. In those cases where the IBS does not vary with the size of the protein, the size effect is ascribed to the larger AFP–macromolecule complex covering a larger surface area than the AFP alone. This larger coverage effectively reduces the intermolecular adsorbent gap between adsorbed AFPs at the ice surface, thereby displacing the hysteresis freezing point to a lower temperature (Wu et al. 1991). When the variation in molecular size of the protein involves changes in the size of the IBS, then the effect has additionally been ascribed to various aspects of their ice-binding ability (Leinala et al. 2002; Mok et al. 2010; Chao et al. 1996; Liu et al. 2005). The increased potency reported for a natural and several synthetic intramolecular multimers of AFPs is ascribed to an overall greater likelihood of successful adsorption due to the presence of multiple IBSs (Miura et al. 2001; Nishimiya et al. 2003) or to increased overall ice-binding area (Baardsnes et al. 2003).

Although some, or even all, of these explanations may contribute to some extent to the observed effect, there are nevertheless problems associated with their applicability. For instance, Marshall et al. (2004b) pointed out that, explanations relying on differences in interaction energies at the IBS are not likely to be correct, since AF(G)Ps are irreversibly adsorbed onto the ice surface, i.e., it is an all-or-none situation. As alluded to above (Sect. 6.4.2), if the AF(G)Ps become irreversibly adsorbed by freezing onto the interface, then they are as strongly adsorbed to the ice as any piece of ice is to the surface of ice. Thus, changing the size of the IBS, or the like, should not make any difference. In the case of the added surface cover explanation provided by Wu et al. (1991), it is intuitively logical and could well be a satisfactory explanation. However, as pointed out by the original authors, experimentally there is no correlation between the size of the enhancer and the enhancement effect (Wu and Duman 1991). The enhancers, identified by Wu and Duman (1991), range according to efficiency as 70 kDa (endogen enhancer) > 70 kDa (protein ice nucleator) > 800 kDa (lipoprotein ice nucleator) > 150 kDa (antibody) > gelatin (80–375 kDa) > agar (average 120 kDa). The effectiveness of all these enhancers is surpassed by a 28 kDa endogenous enhancer (Wang and Duman 2006). Also, Horwath et al. (1996) reported that an efficient endogenous enhancer from the beetle *Tenebrio molitor* was 12 kDa, about the same size as the AFP. Thus, there seem to be little experimental support for the otherwise logical contention that the enhancement effect of size arises from added surface cover of the adsorbent complex.

Equation (6.2) provides a general explanation to the size effect; variations in size is inevitably accompanied by variations in the nonpolar surface area, A, of the protein and probably also variations in the electrostatic forces, E, acting between the protein and the solution. Such size-induced differences in solubility is consistent with the gradual increase in antifreeze potency with size that are reported for structurally similar variants of both hyperactive and moderately active AF(G)Ps. This approach also provides an explanation as to why there is no correlation between antifreeze potency and size for macromolecules that are very different; if the

structures are different, then differences in their nonpolar surface areas and the strength of the electrostatic forces acting between the structure and the surrounding water do not vary with size. In other words, the solubility of structurally different compounds does not vary with molecular size. This would explain why a 28 kDa protein is a far more efficient enhancer than a protein of 800 kDa; the smaller is simply less soluble.

Some complicated and intriguing findings have been reported that ties in well with the concept of solubility-induced enhancement; Wang and Duman (2005) found that certain of the isoforms of hyperactive AFPs, DAFPs, from *D. canadensis* interact, and the association results in greater activities. This greater activity may be ascribed to a reduced solubility due to the overall larger nonpolar surface area, ϕ, of the complex (Eqs. 6.2, 6.4 and 6.5). But further, they found that the additive glycerol only acted as an enhancer if the isoforms interacted. This may also be understood from Eqs. (6.4) and (6.5); the sensitivity of a protein to some additive increases with increased nonpolar surface area, ϕ. It should be noted that several of the polyols, glycerol included, actually reduces the surface tension of water. Nevertheless, Gekko and Timasheff (1981) found that glycerol lowered the solubility of proteins by the same mechanism as salts, i.e., polyols act differently at the air/water interface than at the protein/water interface.

Amornwittawat et al. (2008) found that many carboxylates enhanced DAFPs and ascribed the effect to aggregation of DAFPs. As in the case with Wang and Duman (2005), such aggregation results in lowered solubility due to increased overall nonpolar surface area, which could explain the increased activity. Wang et al. (2009b) identified the binding sites for these carboxylates to be specific arginine residues in the DAFP structure, since blocking these residues abolished the effect. With intact such residues the monomeric DAFP aggregated in the presence of carboxylates and the complex was more sensitive to other additives. This situation is similar to that of Wang and Duman (2005) described above, i.e., the greater nonpolar surface area, ϕ, of the complex makes the overall complex more sensitive to additives than the monomers alone (Eqs. 6.2, 6.4 and 6.5).

As have been outlined above, ascribing variations in antifreeze potency to variations in protein solubility explain many aspects of hysteresis activity, including the significance of size and how additives enhance AF(G)Ps. This approach also explains why interactions between isoforms cause enhancement and the increased sensitivity to additives when isoforms interact. Ascribing variability of antifreeze potency to variations in protein solubility give a plausible explanation to the natural variability reported among AF(G)Ps that are either hyperactive or moderately active. Both qualitative and quantitative agreements with predictions based on established theory support this approach.

The presence of a 1 molal solution of sodium citrate has the effect on the hysteresis activity of RiAFP equivalent to elevating its concentration 50-fold (Kristiansen et al. 2008). Thus, aside from the categorization into hyperactive and moderately active, which are consequences of structural aspects of their IBS, the physicochemical property of solubility is probably the most dominant determinant of AF(G)P potency.

6.5 AF(G)Ps and Ice Nucleation

The convex surface zones that grow out at the ice surface within the hysteresis gap are developing ice nuclei with their critical radius at that temperature. At the hysteresis freezing point, the phenomenon is terminated by a surface nucleation event, as one of these surface nuclei initiates nucleation. Apart from causing thermal hysteresis by controlling the development of nuclei at the ice surface, AFPs also interact with structures in the body fluids that can trigger an ice nucleation event. Such a structure is known as an ice-nucleating agent, INA, and the nucleation process triggered by INAs is referred to as heterogeneous nucleation. This is to distinguish this kind of nucleation from that which occur by spontaneous ordering of water, so-called homogenous nucleation. Evidence suggest that the ice nucleation sites of INAs are structurally related to the IBSs of AFPs. Thus, the mechanism of ice nucleation by INAs may be very similar to the mechanism of adsorption of AFPs to ice.

6.5.1 Biological Relevance of INAs

Freeze-avoiding species die if their body fluids freeze out. Consequently, they rely on extensive supercooling of their body fluids to survive subfreezing temperatures. Any incidental INAs in the body fluids of such an organism would therefore be potentially lethal. Freeze-avoiding insects are known to remove or reduce the amount of such incidental INAs that could pose a threat (Neven et al. 1986; Olsen and Duman 1997a, b). In addition, AFPs prevent incidental INAs from initiating freezing by physically interacting with such structures (Olsen and Duman 1997a, b; Duman 2002). By removal of INAs from their body fluids and by producing high concentrations of AFPs, the supercooling points of freeze avoiding larvae of the pychroid beetle *Dendroides canadensis* changes from about −7 °C in the summer to below −30 °C during winter (Olsen and Duman 1997a, b).

Freeze-tolerant species, that adaptively allow their body fluids to freeze out, often produce INAs and allocate them to the extracellular fluid. The principal function of such adaptive INAs in freeze tolerance is to prevent harmful cellular freezing by initiating a preemptive nucleation event outside the cells at a temperature above the nucleation temperature of any incidental harmful cellular INAs (Zachariassen and Hammel 1976). Since solutes are excluded from the growing ice mass, the extracellular freezing event causes the remaining unfrozen extracellular fluid fraction to become increasingly concentrated. This in turn initiates a concomitant osmotic efflux of water out from the cells. The extracellular freezing process and consequent efflux of cell water continues until the melting point of the remaining unfrozen fluid fraction is colligatively depressed to the environmental temperature, at which point the danger of harmful cellular freezing is eliminated.

6.5.2 *Overall Structural Aspects of INAs*

It is vital for the functionality of adaptive INAs found in freeze-tolerant species that their nucleation temperature is above that of any incidental harmful cellular INAs. The efficiency of INAs to initiate nucleation depends on their size. This may be understood from Eq. (6.1); the larger the diameter of the INA, d, the less supercooling, ΔT, is required to initiate nucleation. Consistent with this, adaptive INAs found in freeze tolerant species are very large structures. It is likely that the great potency of biologically adaptive INAs results from association between monomeric INA molecules; it has been shown that an adaptive 800 kDa INA from freeze-tolerant larvae of the cranefly, *Tipula trivittata*, form long chains of 800 kDa monomers, akin to pearls-on-a-string, and that two such chains align side by side into extended dimers (Yeung et al. 1991). This association apparently relies on the presence of phosphatidylinositol, PI, at the surface of the INA, as enzymatic removal of PI depressed the nucleation temperature (Neven et al. 1989). PI has also been shown to anchor highly active bacterial INAs to the bacterial membrane (Kozloff et al. 1991) and thereby possibly causing them to cooperate. The proposed structure of a large repetitive segment of the 123 kDa INA from *Pseudonomas borealis* suggests that the operating INA consists of at least two monomers (Garnham et al. 2011b).

AFPs are known to physically interact with INA molecules (Wu and Duman 1991). A simple explanation to how AFPs depress the nucleation temperature of INAs would be if they act by preventing them from forming larger associations, analogous to the effect of reducing the diameter of growing surface nuclei at the ice surface (Eq. 6.1). A peculiar aspect of this AFP/INA association is that it apparently does not involve the IBS of the AFP (Duman 2001). This is evident from the fact that the hysteresis activity, which requires the IBS to be free to adsorb onto the ice surface, is enhanced by the AFP/INA interaction (Wu and Duman 1991). It remains unclear if AFPs contain some secondary functional surface-site outside the IBS dedicated to the interaction of structures other than ice (Duman 2001).

Although the details of how INAs trigger freezing is not entirely identified, it is likely that they do so by structuring their hydration water to mimic that of ice. It has been shown that the hydration water at the IBS of β-helical hyperactive AFPs are clathrate-like, and this structured water has been implicated in the process of adsorption (Garnham et al. 2011a). Large internal repetitive parts of several bacterial INAs have been modeled to fold into β-helixes (Graether and Jia 2001; Garnham et al. 2011b). The structural similarity between the IBS of the β-helical AFPs and the suspected nucleation sites of the INAs suggest they share a similar mode of operation. Supporting this contention, Kobashigawa et al. (2005) reported that a recombinant protein corresponding to an internal part of one of these bacterial INAs shape ice crystals into hexagonal bipyramids. Similar results were also reported by Xu et al. (1998), who found that a 164 kDa molecule with INA activity shaped ice crystals into hexagonal bipyramids. Apparently, these bacterial INAs have some kind of internal IBS. It is not clear if the part of the INA responsible for the observed

structuring of the ice, an IBS, corresponds to the site that causes nucleation. Another aspect is the shape of the ice crystals in the presence of the INAs reported by Kobashigawa et al. (2005); these INAs are those of the species *Pseudonomas syringae*, the same INA modeled as a β-helix by Graether and Jia (2001). The ice crystals in the presence of all known β-helical AFPs express multiple ice crystal planes, e.g., in the form of hexagonal discs. These INAs, on the other hand, shape ice into hexagonal bipyramids, as seen in the presence of the monoplane-specific AF(G)Ps of fish.

If the IBS of AF(G)Ps is structurally comparable to the nucleation sites of INAs, then why are AF(G)Ps not INAs? The explanation may in part rely on differences in the structure of the hydration water at the IBS/nucleation site and in part be due to the large difference in size of AFPs and INAs. What is clear is that β-helical insect AFPs do not act as INAs within the supercooling range of the freeze avoiding insects, i.e., down to about −30 °C, or even below.

6.6 Conclusions

This chapter has dealt with the modus operandi of AF(G)Ps. The characteristic prevention of ice growth within the hysteresis gap is explained by ice/water vapor pressure equilibrium being maintained by the Kelvin effect as the ice surface grows out as microscopic curvatures between adsorbed AF(G)Ps. The different potencies of moderately and hyperactive AF(G)Ps are ascribed to differences in their adsorption habits, whereas variations in antifreeze potencies within each of these categories are ascribed to variations in their solubilities. In the latter case, experimental proof of concept is discussed in the context of basic solubility theory. Some characteristics of ice-nucleating agents (INAs) in relation to AF(G)Ps and their relevance in cold tolerance was also briefly examined.

AF(G)Ps as a group are defined by their shared capacity to prevent ice in solution from growing at temperatures below the melting point. However, another widespread trait observed for many of these proteins when at very low concentrations occurs at the melting temperature; they inhibit the spontaneous process by which larger ice crystals grow at the expense of smaller crystals. This trait is not an exclusive property of AF(G)Ps but are also found among non-antifreeze proteins and organic solutes. This fascinating phenomenon of recrystallisation inhibition is both biologically and commercially important and is the topic of the next chapter.

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