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Salt-induced enhancement of antifreeze protein activity: A salting-out effect ☆

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ABSTRACT

Antifreeze proteins are a structurally diverse group of proteins characterized by their unique ability to cause a separation of the melting- and growth-temperatures of ice. These proteins have evolved independently in different kinds of cold-adapted ectothermic animals, including insects and fish, where they protect against lethal freezing of the body fluids. There is a great variability in the capacity of different kinds of antifreeze proteins to evoke the antifreeze effect, but the basis of these differences is not well understood. This study reports on salt-induced enhancement of the antifreeze activity of an antifreeze protein from the longhorn beetle *Rhagium inquisitor* (L.). The results imply that antifreeze activity is predetermined by a steady-state distribution of the antifreeze protein between the solution and the ice surface region. The observed salt-induced enhancement of the antifreeze activity compares qualitatively and quantitatively with salt-induced lowering of protein solubility. Thus, salts apparently enhance antifreeze activity by evoking a solubility-induced shift in the distribution pattern of the antifreeze proteins in favour of the ice. These results indicate that the solubility of antifreeze proteins in the solution surrounding the ice crystal is a fundamental physiochemical property in relation to their antifreeze potency.

Antifreeze proteins (AFPs) are defined by their unique capacity to separate the melting- and growth-temperatures of ice in aqueous solutions. This phenomenon is known as thermal hysteresis and is manifested in the body fluids of many organisms inhabiting ice-laden environments, including polar fish and terrestrial insects, spiders and collembolans [10,25,29,38]. The antifreeze activity varies from about 1 °C in fish blood [29] to 5–7 °C in insect hemolymph [38]. Thermal hysteresis is assumed to reflect the role of AFPs as protectors against lethal freezing of the body water [27,28,38]. There is a great variability in the capacity of different kinds of AFPs to evoke the antifreeze effect, but the basis of these differences is not understood. This study attempts to elucidate an origin of these differences.

AFPs are thought to produce the antifreeze effect by becoming irreversibly adsorbed onto ice crystal surfaces. This mechanism of action is referred to as the adsorption–inhibition mechanism [29] and is supported by the remarkable structural fit between the ice-binding sites of AFPs and the arrangement of water molecules in specific ice crystal planes [13,17,20]. According to this view, as the temperature is lowered, the ice surface grows out as convex interfaces between the adsorbed AFPs. The convexity of these growth zones elevates the ice vapour pressure to that of the surrounding solution, i.e. the Kelvin effect, and ice-water vapour pressure equilibrium persists within a temperature interval [22]. At some lower temperature, the hysteresis freezing point, the antifreeze effect is abruptly terminated, and the sample rapidly freezes. The antifreeze activity is defined as the difference between the melting temperature and hysteresis freezing point, and increases with decreased spacing between the AFPs adsorbed on the ice surface [22,29].

Several authors have reported that the antifreeze activity is elevated in the presence of various solutes of low molecular mass. Kerr et al. [19] and Caple et al. [4] reported that a number of sugars and polyols enhanced the antifreeze activity of different kinds of fish AFPs. The enhancement effect was solute-specific and increased in a linear fashion with the concentration of the additive to between 0.13 °C and 0.31 °C at a concentration of 80 mg/ml of the additive. The presence of a certain concentration of an additive also caused an approximately constant addition to the antifreeze activity irrespective of the concentration of AFP [4]. In an extensive study by Li et al. [24], the antifreeze activity of AFPs from the beetle *Dendroides canadensis* was reportedly enhanced in the presence of a range of salts, amino acids, sugars and polyols. The effect was solute-specific and increased with solute concentration. In particular, in the presence of 1 M tri-sodium citrate the antifreeze activity was elevated almost sixfold, from 1.2 °C to 6.8 °C, whereas a number of the other solutes tested elevated the antifreeze activity approximately four-fold and three-fold. For several compounds the antifreeze activity increased in an approximately linear fashion with solute concentration. More recently, Evans et al. [11] tested the effect of tree salts on the antifreeze activity of several types of fish AFPs. They showed that the salts caused a linear increase in the antifreeze activity as a function of salt concentration and also that

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the presence of a given concentration of salt resulted in an approximately constant increase in the antifreeze activity irrespective of the concentration of AFP.

The observation that such a diverse group of compounds enhance the antifreeze activity in such a qualitatively similar fashion suggests that the additives share a common mode of operation as enhancers. Since the effects of the additives on the antifreeze activity are qualitatively similar for so many different kinds of AFPs they presumably act on a physiochemical property shared by all AFPs that govern their antifreeze potency. Thus, understanding the mechanism by which these solutes operate as enhancers may give insight into the origin of the antifreeze potency of AFPs. The existing explanations for the enhancement effect of the various additives are quite diverse but all seem to fall short of revealing the mechanism by which they operate. The enhancement effect has been proposed to have a colligative origin, resulting either from an association between the AFP and the additive [4,19] or from the additive binding free water [11]. However, no abnormalities in the melting points of the solutions were reported. In their extensive study, Li et al. [24] suggested that the solutes may act by optimizing the higher-order structure of the AFPs for binding to ice, but concluded that the mechanism remains unknown. Evans et al. [11] also proposed that salts may prevent the formation of aggregates of AFPs, thereby increasing the concentration of monomeric AFP molecules in solution available to adsorb to the ice. However, this would imply that fish AFPs are present as large aggregates at physiological salt concentrations, since the effect continued to increase up to a concentration of 1 M salt.

Kristiansen and Zachariassen [22] proposed that the solubility of the AFPs in the solution surrounding the ice crystal form an important basis of the difference in the antifreeze potency of AFPs. When an ice crystal is at its equilibrium melting temperature, its surface zone is a melting/freezing region several nm in depth, the so-called ice/water interfacial region [15]. Thus, while the ice crystal is at this temperature it does not have a clearly defined rigid surface structure onto which the AFPs can become irreversibly adsorbed [7,15,21]. Kristiansen and Zachariassen [22] pointed out that irreversible AFP–ice adsorption likely develops as a consequence of the solidification of the interfacial region when the temperature is lowered below the equilibrium melting temperature of the ice crystal. This implies that while the ice crystal is at its equilibrium melting temperature the AFPs attain a steady-state distribution between the solution and the ice/water interfacial region, as outlined in Fig. 1. According to Kristiansen and Zachariassen [22], while the ice crystal is at its equilibrium melting temperature, factors that lower the solubility of the AFPs in the solution surrounding the ice crystal are likely to cause a shift in their steady-state distribution in favour of the ice/water interfacial region. This shift in the distribution pattern of the AFPs towards the interfacial region results in increased surface density of irreversibly adsorbed AFPs at the ice surface once the interfacial region solidifies upon cooling. Thus, according to this view, lowered AFP solubility in the solution surrounding the ice crystal should result in increased antifreeze activity. In accordance with this view, all the low-mass solutes identified as enhancers of the antifreeze potency of AFPs are also known to affect the solubility of proteins in solution, including salts [16], polyols [18] and amino acids [35].

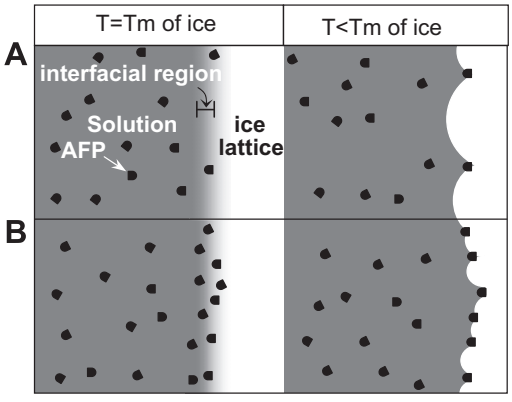

Fig. 1. An extension of the adsorption-inhibition hypothesis. (A) The AFPs become irreversibly adsorbed to the ice surface as a consequence of the solidification of the interfacial region following cooling below the equilibrium melting temperature of the ice. While at the equilibrium melting temperature the AFPs are in a steady-state distribution between the solution and the interfacial region. (B) Lowered solubility of the AFPs in the solution surrounding the ice crystal shifts their steady-state distribution in favour of the interfacial region, causing enhanced antifreeze activity.

Another possible mechanism by which these solutes may operate as enhancement agents of the antifreeze activity is by improving the chance of individual AFPs to become successfully adsorbed onto the ice surface within a certain time period. Although the AFP–ice adsorption has traditionally been attributed to hydrogen-bond formation [8,20,21], the ice-binding sites of several AFPs are reported to be quite hydrophobic compared to the rest of their surface [2,32]. Hydrophobic surfaces are water structuring, and exclusion of the ice-binding site of these AFPs from the water phase therefore represents an entropic gain for the solvent. This effect has been proposed to be a driving force for the AFP–ice adsorption process for these AFPs [2,32]. Salts are known to elevate the protein/water interfacial tension at the hydrophobic part of the protein surface [26]. Thus, salts may act as enhancers by strengthening this hydrophobic effect.

This study proposes a mechanism by which salts act as enhancement agents of antifreeze activity based on the effect of salts on the antifreeze activity caused by a hemolymph AFP from the longhorn beetle *Rhagium inquisitor*.

Materials and methods

Determination of the antifreeze activity

Using a Clifton nanoliter osmometer (Clifton Technical Physics, USA), small sample droplets (~10 nl if not otherwise stated) were submerged in liquid paraffin and frozen by rapid cooling to −40 °C. The droplets were then heated to a preset temperature somewhat below the expected melting point of the sample. While observing the sample through a microscope the temperature was slowly raised (~0.5 °C/min) until only a single small crystal remained, with an approximate diameter of 7 μm. The temperature was then kept unchanged for 1 min to allow the crystal to stabilize, followed by slow cooling (~1 °C/min) until rapid ice growth was observed. This temperature of ice growth was taken as the hysteresis freezing point. The difference between the stabilization temperature and the hysteresis freezing point was taken as the antifreeze activity. The average antifreeze activity based on 5–7 measurements was taken as the antifreeze activity of the sample.

The hysteresis freezing point could in a few cases not be determined. This was due to the combined effect of the antifreeze activity and a strong colligative melting point depression in the presence of high salt concentrations, resulting in a hysteresis freezing point that was below the measuring range of the osmometer (−9.49 °C, i.e. 5100 mOsm).

Solutions and preparations of samples

The AFP used in this investigation was the 12.8 kDa RiAFPH4, abbreviated here to RiAFP. It was purified from the hemolymph of