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Ice restructuring inhibition activities in antifreeze proteins with distinct differences in thermal hysteresis ☆

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ABSTRACT

Antifreeze proteins (AFPs) share two related properties: the ability to depress the freezing temperature below the melting point of ice (thermal hysteresis; TH); and the ability to inhibit the restructuring of ice into larger crystals. Since the 'hyperactive' AFPs, which have been more recently discovered, show an order of magnitude more TH than previously characterized AFPs, we have now determined their activities in ice restructuring inhibition (IrI) assays. IrI activities of three TH-hyperactive AFPs and three less TH-active AFPs varied over an 8-fold range. There was no obvious correlation between high TH activity and high IrI activity. However, the use of mutant AFPs demonstrated that severe disruption of ice-binding residues diminished both TH and IrI similarly, revealing that that the same ice-binding residues are crucial for both activities. In addition, bicarbonate ions, which are known to enhance the TH activity of AFPs, also enhanced their IrI activity. We suggest that these seemingly contradictory observations can be partially explained by differences in the coverage of ice by TH-hyperactive and non-hyperactive AFPs, and by differences in the stability of AFP-bound ice under supercooled and recrystallization conditions.

Introduction

Antifreeze proteins (AFPs) are found in certain fishes, insects, plants and micro organisms that live at sub-zero temperatures where uncontrolled ice growth can be lethal. AFPs are used as part of a freeze-avoidance strategy in some fish and insects [6,9], or to enhance freeze tolerance in some plants, bacteria and other insects [1,13]. This apparent incongruity is explained by the two properties that all AFPs possess: thermal hysteresis (TH) activity and ice recrystallization inhibition (IRI). TH, or non-colligative freezing point depression, enables polar fish and some insects to lower the freezing temperature of body fluids (blood plasma or hemolymph) and thus help protect against inoculative freezing [6,9]. Ice recrystallization is a process that occurs in frozen solutions at high sub-zero temperatures, in which the growth of larger ice crystals at the expense of smaller ones is thermodynamically favourable [16]. Thus, IRI activity in freeze-tolerant organisms that produce AFPs is thought to reduce freezing-related tissue damage.

A working definition of AFPs is that they are ice-binding proteins with TH activity. Since proteins with TH activity all appear to also have IRI activity, this supports the hypothesis that both TH and IRI activities derive from a single underlying property of AFPs: their ability to act at the ice-water interface to halt or modify the growth of ice crystals. AFPs are structurally diverse [14,22] and differ considerably in their TH levels. As more AFPs are discovered, it has become clear that they can be placed in at least two categories. Those from polar fishes, where the serum freezing point need only be lowered to just below that of sea water, typically have TH activities of 1–2 C° at millimolar concentrations. These can be classified as moderately active AFPs and include AFP types I, II and III, and the antifreeze glycoproteins [4]. Plant AFPs such as that from the perennial ryegrass, *Lolium perenne* [26], have TH activities at the low end of this range (~0.5 C° at half millimolar concentrations). In contrast, AFPs from arthropods, such as *Cf*AFP from the spruce budworm moth (*Choristoneura fumiferana*), *Tm*AFP from the yellow mealworm beetle (*Tenebrio molitor*), *Dc*AFP from the fire-coloured beetle (*Dendroides canadensis*) and sfAFP from the snow flea have TH activities of 4–6 C° at sub-millimolar concentrations [7,11,12,19,28]. Based on their much higher TH activity at lower protein concentrations, these AFPs were termed "hyperactive"

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[12]. Hyperactive AFPs have since been found in bacteria and fish, specifically *Mp*AFP from the Antarctic bacterium (*Marinomonas primoryensis*) and hyperactive type I AFP from the winter flounder (*Pseudopleuronectes americanus*) [11,19].

A distinguishing feature between the moderate and hyperactive AFPs, aside from the order of magnitude difference in their TH activities, is the way in which they direct ice crystal growth when TH is exceeded. At freezing temperatures, surface-adsorbed AFPs are no longer able to prevent ice growth, which occurs explosively due to supercooling. Moderately active AFPs show spicular ice growth along the *c*-axis, whereas all hyperactive AFPs appear to direct growth along the *a*-axes [25]. The dependence of TH activity on concentration is well established, both for moderately active and hyperactive AFPs. However, little is known regarding the relationship between TH and IRI for a given AFP. Here we set out to investigate if the elevated TH activity of hyperactive AFPs is accompanied by superior IRI activity.

Considerable progress has been made towards defining some of the physical features required for the adsorption of AFPs to ice. Despite their extreme structural diversity, AFPs all have an ice-binding site that is relatively flat and hydrophobic, that occupies a large proportion of the AFP's surface area, and that makes a 'snug' fit to the plane(s) of ice to which they bind [3,5]. These principles have been established by disturbing the ice-binding face by mutations. It is therefore reasonable to suggest that the ice-binding site that gives an AFP its TH activity is also responsible for IRI. If so, mutations that reduce TH activity should also reduce IRI activity.

One of the challenges for comparing TH and IRI activities of the different AFPs has been the qualitative nature of IRI activity assays [15]. To improve the measurement of IRI activity, we previously developed a capillary method that gives reproducible values for the endpoint obtained by serial dilutions [27]. However, the use of IRI to describe this assay has been disputed because the mechanism by which ice crystals grow larger has not been proven to be grain boundary migration. It has been suggested that the growth of larger ice crystals might be driven by the release of strain on the snap-frozen samples in the capillaries, or that it might be the result of new ice nucleation. Whereas in the more two-dimensional splat assay it is possible to follow the change in individual ice grains, as some shrink and others grow, this is not easily done in three-dimensions with samples in the capillaries. Although we hypothesize that the growth of ice crystals in the capillary assay [27] is due to recrystallization we have elected to call it restructuring. This term fits with the use of 'ice structuring proteins' [29,30] used to refer to antifreeze proteins used in foodstuffs like ice cream to keep ice crystals small. Therefore, the capillary method measures ice restructuring inhibition (IrI), which may or may not be IRI. The IrI endpoint is defined in this method as the lowest AFP concentration that is still able to suppress ice restructuring under defined conditions.

This assay has allowed us to compare here the IrI and TH activities of AFPs from fish, insects, plants and bacteria to determine if TH hyperactivity correlates with increased IrI activity, and if genetically engineered decreases and increases in TH activity are mirrored by changes in IrI activity.

Materials and methods

Antifreeze proteins

Wild-type recombinant sea raven type II AFP, ocean pout type III AFP (QAE isoform HPLC-12), *Cf*AFP (isoform 337) and *Tm*AFP (isoform 4–9) were prepared as previously described [25]. Recombinant bacterial *Mp*AFP was expressed in *Escherichia coli* [10]. Recombinant AFP of the perennial ryegrass, *Lolium perenne,* (*Lp*AFP) and four mutants (V45Y, T53Y, T62Y and T67Y) were produced as previously described [21]. Type I AFP (winter flounder isoform HPLC-6) and all four Ala to Leu variants (A17L, A19L, A20L and A21L) were made by solid-phase peptide synthesis [2].

Preparation of AFP solutions

Stock solutions of most AFPs were prepared in 0.1 M ammonium bicarbonate (pH 7.9), and also in TBS (Tris-buffered saline; 150 mM NaCl and 10 mM Tris–HCl pH 7.5). The calcium-dependent *Mp*AFP was dissolved in 5 mM Tris–HCl (pH 8.0) containing 2 mM $CaCl_2$, and *Lp*AFP and its mutants were made up in 100 mM NaCl, 50 mM Tris–HCl (pH 7.6), 1 mM EDTA. In each case, serial 2-fold dilutions of the AFP stocks were prepared in the appropriate buffer/saline solutions from 0.4 mM stock solutions over the range 0.2 mM to 0.4 nM. The concentrations of stock solutions were determined spectrophotometrically in relation to extinction coefficients determined from amino acid analyses (Advanced Protein Technology Centre, Hospital for Sick Children, Toronto, ON, Canada). In addition, protein concentrations of both the wild-type type I and *Lp*AFP and their mutants were verified by TH measurements in comparison with the previously published TH activity-concentration curves [2,21].

Capillary method for IrI assay

IrI activity was determined by the capillary method [27]. IrI endpoints were measured on at least four separate dilution series for each protein. Samples were loaded into 10 μL glass capillaries (51 mm in length, 1 mm outer diameter; Drummond Scientific, Broomall, PA, USA) by capillary action. A set of capillaries containing an AFP dilution series and buffer/saline controls were sealed at both ends using high vacuum grease (Dow Corning, Midland, MI, USA), aligned and placed in a locally-fabricated sample holder. The set of samples was snap frozen in 95% ethanol cooled to approximately –60 °C with dry ice. After snap freezing, the samples were immersed in a jacketed beaker (Allen Glass Scientific, Boulder, CO, USA) filled with 50% clear ethylene glycol (BioShop Canada, Burlington, ON, Canada) at –6 °C, and maintained at this temperature by circulating 50% clear ethylene glycol through the apparatus from a refrigerated bath (model RTE-7, NESLAB, Portsmouth, NH, USA). Samples were incubated at –6 °C overnight (approximately 16 h). Microscopy and image capture were done as previously described [27].

Type III AFP at a concentration of 1.6 μM was used as a positive control in each IrI assay because this AFP is well-characterized, and it is well above its IrI endpoint at this concentration [27]. Since 0.1 M ammonium bicarbonate was used for the 2-fold serial dilution for five of the six samples, this salt solution was used as a negative control. The exception was *Mp*AFP; due to its Ca^{2+} requirement for activity, it was prepared in 5 mM Tris–HCl (pH 7.5) containing 2 mM $CaCl_2$ to prevent the loss of Ca^{2+} through the formation of insoluble calcium carbonate. Samples were routinely taken from new stock solution aliquots to avoid possible loss of activity resulting from the repeated freezing and thawing of stock AFP solutions.

Splat IRI assay

Ice recrystallization inhibition was measured using the splat assay with minor modifications [15]. Briefly, aqueous sample (15 μL) was dropped approximately 1.5 metres onto a glass slide that had been equilibrated on an aluminium block held at −80 °C. Each sample froze instantly into a thin disc approximately 1 cm in diameter. Serial dilutions of type III AFP and of *Tm*AFP ranging from 1–1000 nM were used in the assay along with BSA (20 μg/mL) as a