Mixing antifreeze protein types changes ice crystal morphology without affecting antifreeze activity

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Abstract All three fish antifreeze protein types (I, II and III) inhibit the growth of ice to form hexagonal bipyramidal ice crystals of characteristic morphology. Mixtures of these different antifreezes produced ice crystals of hybrid shapes and dimensions, consistent with the different antifreeze types binding to the same ice surfaces. The activity of the mixtures was independent of the proportions of the iso-active antifreeze protein stocks present, indicating that the different antifreezes neither attenuated nor potentiated each other's activity. We suggest that antifreeze protein molecules are independently active and do not require protein–protein interactions for ice-binding.

Key words: Fish antifreeze protein; Freezing point depression; Ice-binding

1. Introduction

Fish antifreeze proteins (AFPs) and glycoproteins (AFGPs) form a group of structurally diverse macromolecules that provide freeze-resistance to teleost fishes living in ice-laden marine environments [1]. The fish AFPs are further classified as Types I, II and III AFP. Type I AFP is a single α-helix with a molecular weight of 3,300 [2]. Type II AFP (M_r 14,000), the largest of the three AFPs, has a high cystine content and is homologous to the carbohydrate-binding domain of C-type lectins [3,4]. Type III AFP (M_r 7,000) has a unique β-sheet structure and shows no homology to proteins in the current database [5].

Unlike most solutes, which are rejected by the growing ice front, fish AFPs and AFGPs are preferentially adsorbed to the ice lattice via a network of hydrogen bonds and cause ice growth retardation by local surface curvature effects [6,7]. For the linear antifreezes (Type I AFP and AFGPs), specific adsorption planes have been deduced and a mechanism of action has been suggested based on a match of their repetitive polar groups to the ice oxygen atoms [7,8]. Specific matches to the ice lattice have not yet been made for the globular antifreezes (AFP Types II and III), but the adsorption planes they bind to are different from that recognized by Type I AFP [9].

It has been argued that individual AFP molecules might not have sufficient binding strength from their hydrogen bonds to adsorb tightly to the ice surface [8]. Knight et al. [8] have addressed this issue by suggesting that some binding groups on AFPs, such as -OH, can occupy positions in the ice lattice and thereby increase their number of H-bonds. Wen and Laursen [10] have proposed an alternative explanation. Based on regression analyses of early ice growth rate data, they argued that at low AFP concentrations, or during early time points in the freezing trial, Type I AFPs bind reversibly to ice [11]. They suggested that AFP binding becomes irreversible only when AFPs form patches on the surface of ice through hydrophobic interpeptide interactions. It is the co-operative interaction between neighbouring AFPs and the H-bonding between ice and protein patch that provide the total force needed to prevent ice-growth [11].

In an attempt to test this model, Wen and Laursen synthesized and mixed the D- and L-enantiomers of type I AFP [12]. They predicted that the D- and L-forms would bind to the same pyramidal plane but in mirror image orientations, with the potential to interfere with each other's binding. However, they found that a mixture of these enantiomers had the same thermal hysteresis activity (defined as the difference between melting and non-equilibrium freezing temperatures) as a sample of either form by itself. Although the non-interference of the two enantiomers could be explained if each AFP molecule acted independently, the authors interpreted the data as showing the formation of homogeneous D-AFP and L-AFP patches on the ice.

The requirement for protein–protein interaction can be put to a more stringent test by mixing AFP types which, due to their very different structures, are unlikely to bind to each other on the ice surface or even to the same pyramidal planes. If co-operative binding is necessary for antifreeze activity, the presence of multiple, non-interacting AFP forms should inhibit patch formation and thus reduce thermal hysteresis activity. On the other hand, if co-operative binding to a unique ice plane is not important, the activity should be unaffected. It is even conceivable that thermal hysteresis activity could increase, since a mixture of AFP types might provide more comprehensive ice coverage. To test these possibilities, we have examined the effect on thermal hysteresis and ice crystal morphology of various mixtures of the three AFP types.

2. Materials and methods

2.1. Preparation of AFP stock solutions and mixtures

Production and purification of Type I (winter flounder, HPLC-6) [13], Type II (sea raven, mature AFP) [2], and Type III (ocean pout, recombinant QAE m1.1) AFP [14] were done as previously described. Types I, II and III AFP stock solutions were prepared by adding 0.1 M NH_4HCO_3 (pH 7.9) to lyophilized AFP until the sample thermal hysteresis was 190 mOsmol. AFP combinations were prepared by mixing (v/v) stock solutions according to Table 1.

2.2. Antifreeze activity measurements and photomicroscopy

Thermal hysteresis was measured with a Nanolitre Osmometer as described by Chakrabartty et al. [15]. Ice crystal growth and morphology were observed using a Panasonic CCTV camera attached to a Leitz dialux 22 microscope and recorded by a JVC Super VHS video recorder. Still photographs of ice crystals were taken 1 min after the formation of the bipyramidal ice crystal under 0.1°C of supercooling.

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Their length (c-axis) and width (a-axis) were measured directly from the monitor screen where 1.0 mm is equivalent to 0.93 μm in actual size.

3. Results

When stock solutions of different AFP types that had been prepared to give an identical thermal hysteresis activity of 190 mOsmol (equivalent to 0.35°C) were combined in v/v ratios of 1:3, 1:1 or 3:1, the resulting mixtures had thermal hysteresis activities that were not significantly different from that of the stock solutions (Table 1). This same result was obtained when all three AFP types were mixed in a v/v ratio of 1:1:1 (sample 13, Table 1).

Stock solutions of each AFP type produced hexagonal bipyramidal ice crystals with distinct dimensions that were diagnostic for its type (samples 1, 5 and 9; Fig. 1). The ice crystal formed in the presence of Type I AFP was the largest of the three, with the longest c- and a-axial lengths, and the highest c/a ratio (Table 2) The Type II AFP ice crystal had the shortest c-axial length and the lowest c/a axial ratio. It also showed a characteristic rounding of the crystal faces at the pyramidal junction. The Type III AFP ice crystal had the shortest a-axial length and an intermediate c/a axial ratio.

AFP mixtures produced hexagonal bipyramidal ice crystals with dimensions that varied depending on the type and proportion of AFPs in the mixture. The most noticeable changes occurred when Type I AFP was mixed with either of the globular AFPs (Fig. 1). As little as 25% of Type II AFP reduced the c/a axial ratio from 2.2 to 1.5 (Table 2). When the proportion of Type II AFP increased to 75%, the c/a axial length ratio (1.4) resembled that of a Type II ice crystal (1.3). Additional evidence for the participation of Type II AFP in shaping the ice crystal, even in the 25% mixture, was the pronounced curvature of the pyramidal junction that is characteristic of ice crystals formed in the presence of this antifreeze (Fig. 1).

Ice crystals formed in the presence of Type I and Type III AFP mixtures also had a hybrid morphology. The c/a axial length ratio in the Type I/Type III 75/25% and 25/75% mixtures was 2.0, intermediate between that of the Type I AFP crystal (2.2) and the Type III AFP crystal (1.9) (Table 2). The pyramidal junction was angular, with no evidence of the curvature seen in the presence of Type II AFP (Fig. 1)

Table 1
Effect of mixing types I, II and III AFP on the antifreeze activity

Sample	Type I (% v/v)	Type II (% v/v)	Type III (% v/v)	Thermal[a] hysteresis (°C)
1	100	0	0	0.35 ± 0.01
2	75	25	0	0.35 ± 0.02
3	50	50	0	0.34 ± 0.02
4	25	75	0	0.35 ± 0.01
5	0	100	0	0.35 ± 0.01
6	0	75	25	0.35 ± 0.02
7	0	50	50	0.34 ± 0.02
8	0	25	75	0.35 ± 0.02
9	0	0	100	0.35 ± 0.01
10	25	0	75	0.35 ± 0.01
11	50	0	50	0.35 ± 0.02
12	75	0	25	0.35 ± 0.02
13	33.3	33.3	33.3	0.34 ± 0.01

[a] Three independent measurements were made for each mixture. The mean and standard deviations are shown.

Table 2
The dimensions of the ice crystals produced by a mixture of AFPs

Sample[a] AFP type:%	c-axial length (μm)	a-axial length (μm)	Ratio[b] (c/a)
I: 100	23.3 ± 4.5	10.7 ± 1.5	2.2
II: 100	11.7 ± 1.2	8.8 ± 0.8	1.3
III:100	14.8 ± 1.6	7.8 ± 1.1	1.9
Type I and Type II			
I:II 75:25	14.1 ± 1.5	9.3 ± 1.6	1.5
I:II 50:50	13.9 ± 0.8	9.5 ± 1.3	1.5
I:II 25:75	12.7 ± 1.7	9.3 ± 0.8	1.4
Type I and Type III			
I:III 75:25	19.2 ± 2.3	9.8 ± 1.1	2.0
I:III 50:50	15.4 ± 1.3	10.2 ± 1.5	1.5
I:III 25:75	17.3 ± 3.3	8.5 ± 1.1	2.0
Type II and Type III			
II:III 75:25	9.7 ± 0.4	7.6 ± 0.84	1.3
II:III 50:50	12.4 ± 0.7	11.7 ± 1.2	1.1
II:III 25:75	15.6 ± 3.3	9.4 ± 1.7	1.7
Type I, Type II and Type III			
I:II:III	11.5 ± 2.6	10.5 ± 2.2	1.1

[a] The type of AFP present in the sample is indicated by I for Type I, II for Type II, III for Type III. The relative proportion of the component AFP types is shown as a percentage of the final volume. Sample I:II:III contained equal volumes of all three AFP types.
[b] Three independent measurements were made for each mixture of AFPs. The mean and standard deviations are shown.

Mixtures of AFP Types II and III generated unique ice crystal shapes with a general shortening of the c/a axial length ratio. Thus the presence of 25% Type II AFP reduced the c/a ratio from 1.9 to 1.7, while the presence of 25% of Type III AFP failed to increase the ratio (1.3) above that due to Type II AFP alone. Somewhat surprisingly, the 50/50% mixture had a lower c/a axial length ratio than that produced by either AFP alone. (A similar result was observed for the Type I/III 50/50% mixture.) Again the presence of Type II AFP produced a less angular pyramidal junction. A mixture of all three AFP types (Fig. 1, sample 13) generated a short ice crystal with an a/c axial length ratio of 1.1, similar to that found in the Type II/Type III 50/50% mixture.

4. Discussion

Two lines of evidence indicate that different AFP types in a mixture each contribute to the inhibition of ice crystal growth. Firstly, the shape and dimensions of the ice crystals formed in an AFP mixture are intermediate between those of the individual constituent AFPs, and are influenced by the proportions of the AFPs in the mixture. Thus, instead of having two or more crystal forms in a mixture, each with only one AFP type on their surface, the crystals are hybrids. Since the dimensions of these ice crystals are different from those formed in the presence of the individual AFP types, the facets on these ice crystals should not be considered as crystallographic planes with rational indexes. Rather, each crystal 'face' is a collection of surfaces with different orientations. This would agree with Knight et al.'s interpretation that the bipyramid faces are surfaces with the greatest deviation from the prism direction that still support adsorption and inhibition [8].

Secondly, there is no decrease (or increase) in thermal hysteresis activity when iso-active solutions of different AFP types are mixed. If one or more AFP types were excluded from the

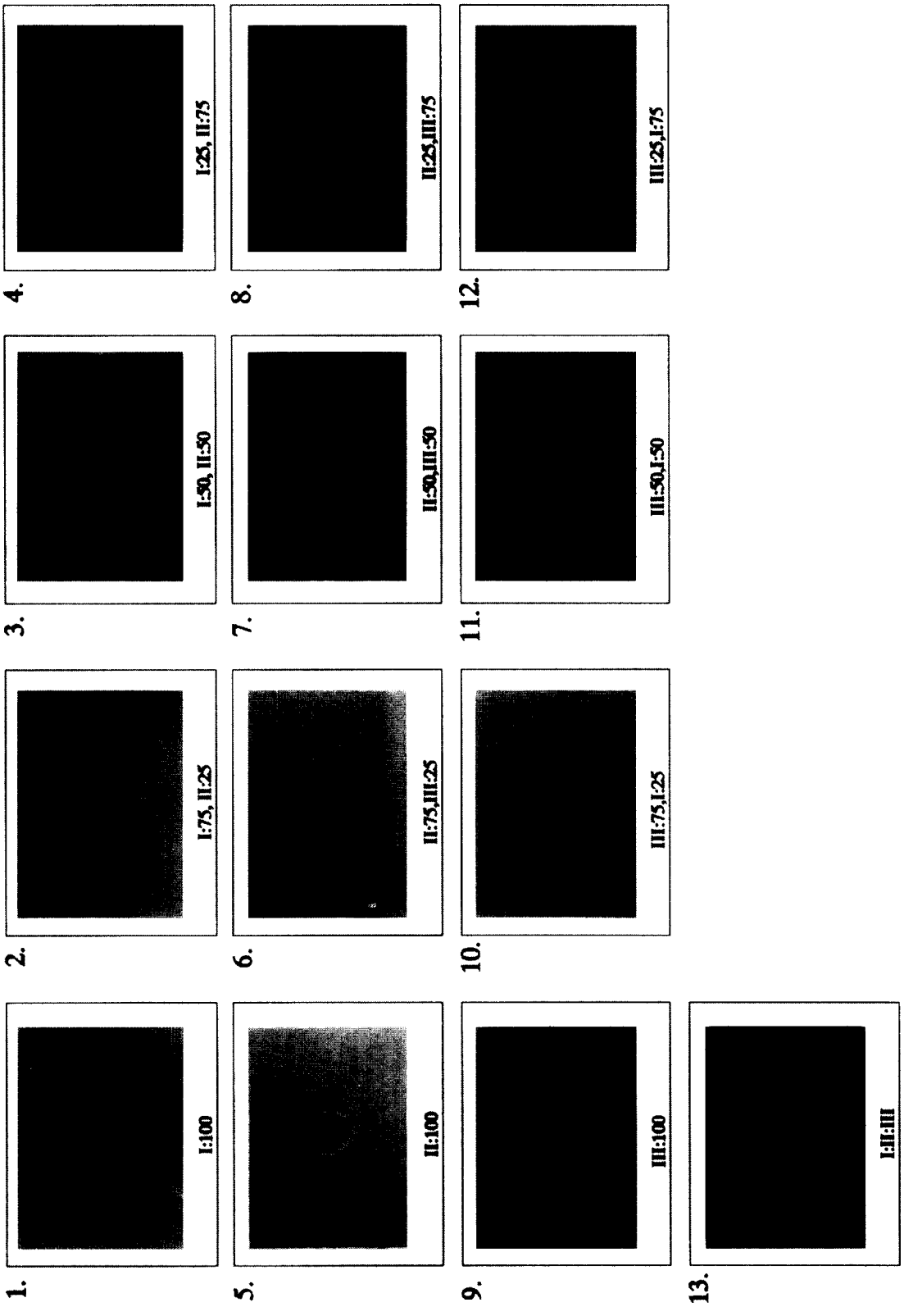

Fig. 1. Photographs of ice crystals formed in stock solutions and mixtures of AFP Types I, II and III. The AFP Type(s) present and their proportions (% v/v) are indicated below each crystal.

surface of an ice crystal by the dominant expression of a particular ice plane or the requirement for specific protein–protein interaction, the effective concentration of the controlling AFP in the mixture would be less than that in the stock solution, and should result in lowered thermal hysteresis values.

When AFP types are mixed, does each AFP molecule make a contribution to ice-binding that is independent of its neighbour on the ice surface, or do AFPs bind to form numerous homogeneous patches? While it is not possible to exclude the latter possibility with the available data, this model would require some sorting or rearrangement on the ice surface of up to three AFP types that have very diverse structures and different binding planes. The binding of competing AFP types to an ice lattice site adjacent to a formative homogeneous AFP patch would most likely retard patch formation and decrease thermal hysteresis activity. Even if different AFP types could freely diffuse on the ice surface and coalesce to form their respective patches, one would expect some interference with each other at high AFP concentrations.

The simplest explanation for these data are that each AFP molecule binds to the ice lattice independently of its neighbour, and that the activity of an AFP mixture is more a function of the number of ice-binding molecules than of their type. This conclusion is in agreement with elipsometry measurements that suggest only 30% of the ice surface is covered by AFPs when crystal growth is inhibited [16]. If AFPs formed protein-protein patches, coverage of the surface would be inadequate to retard ice crystal growth.

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