Physical Basis of Functioning of Antifreeze Protein

B. S. Melnik* and A. V. Finkelstein*

* Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia
b Biology Department, Moscow State University, Moscow, 119192 Russia
* e-mail: bmelnik@phys.protres.ru

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Abstract—Antifreeze proteins, expressed in cold-blooded organisms, prevent ice formation in their bodies, and thus help them to survive in extremely cold winter temperatures. However, the mechanism of action of these proteins is still not clear. In any case, it is not simply a decrease in the temperature of normal ice formation. In this work, investigating the ice-binding protein (a mutant form of the antifreeze protein cfAFP from the spruce budworm Choristoneura fumiferana, which overwinters in needles), we showed that this antifreeze protein does not at all lower the freezing point of water and, paradoxically, increases the melting point of ice. On the other hand, calculations based on the theory of crystallization show that at temperatures of 0° to −30°C ice can only appear on surfaces that contact water, but not in the body of water. These facts suggest a new perspective on the role of antifreeze proteins: their task is not (as it is commonly believed) to bind with nascent ice crystals already formed in the organism and stop their growth, but to bind to those surfaces, on which ice nuclei can appear, and thus completely inhibit the ice formation in supercooled water or biological fluid.

Keywords: ice-binding protein, antifreeze protein, ice melting, ice crystallization, ice crystallization temperature, ice nuclei, ice initiating surfaces, inhibition of ice formation

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Ice binding proteins (IBPs), also called antifreeze proteins (AFPs), are synthesized by various organisms in order to survive in freezing temperatures. These proteins were first found in the blood of fish living in Arctic and Antarctic waters, where the temperature drops to −2°C [1–3]. The mechanism of action of these proteins is still unclear [4, 5]. It is generally believed that IBP lowers the freezing point of body fluids by several degrees [6]. A number of studies have suggested that IBPs bind ice crystals and prevent their growth or affect the shape of ice crystals during repeated freeze–thaw cycles [7–9]. Analyzing the literature, one can also draw attention to the fact that the molar concentration of IBP required to prevent cell death at low negative temperatures is hundreds of times lower than the molar concentration of salt or alcohol required for the same decrease in the freezing point [10, 11]. This fact alone suggests that the mechanism of action of AFP is different from the mechanism of action of “ordinary” antifreezes such as low molecular weight alcohols and salts, which are known to lower the chemical potential of water in proportion to their molar concentration, so, these water-soluble molecules lower the chemical potential of water in solution more strongly than in ice [12], which leads to a decrease in the temperature of the point of thermodynamic equilibrium of ice water.

In this work, we show experimentally that IBP, taken by itself, does not work as an antifreeze, i.e. it does not lower either the freezing point of water nor the melting point of ice; and a theoretical study of the freezing process suggests that at 0°…−30°C ice, for kinetic reasons, cannot form in the water column, but can on ice-binding walls in contact with water, and the blockade of these walls is the most likely function of AFP.

EXPERIMENTAL

In experiments with IBP, we used a mutant form of AFP, cfAFP, which we named mIBP83 [13]. cfAFP from spruce budworm Choristoneura fumiferana that overwinter in needles was studied earlier in [14–16]. mIBP83 does not lower the freezing point of water and even slightly increases the melting point of ice [13] (such an increase was previously noted for AFP of fish [17]). This forced us to reconsider the generally accepted point of view [6, 18], according to which IBPs lower the freezing point of water and biological fluids by binding to ice.

Isolation and purification of mIBP83 and mIBP83-GFP. Previously, we designed a mutant form of the cfAFP protein from a spruce budworm Choristoneura fumiferana (hereinafter referred to as mIBP83), in
which some of the cysteines have been replaced. In addition, for experiments on binding to the ice surface, a chimeric protein was designed in which mIBP83 and the green fluorescent protein GFP are linked by a small linker [13]. mIBP83 and mIBP83-GFP are well isolated in monomeric form by standard methods at high concentrations without the addition of reducing substances [13].

For the expression of the IBP genes, the producer strain Escherichia coli BL21 (DE3) was used. Transformation of competent E. coli cells was carried out using the plasmid pET-22b encoding the desired protein (mIBP83 or mIBP83-GFP). The biomass was cultivated in LB liquid medium (Amresco, United States) with the following composition (per 1 L): tryptone, yeast extract, NaCl—10, 5, and 10 g, respectively, NaOH (10 M)—600 μL. As a substrate-specific marker, kanamycin was added to the medium, the resistance gene to which is encoded in the target vector plasmid, in the ratio of growth medium: antibiotic—1 : 100. To increase the yield of the target product, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 100 mM was added to the cells. A high pressure homogenizer was used to disrupt the cells. All solutions were prepared with distilled and dionized water.

Isolation of protein from the cell lysate of the producer strain E. coli was carried out sequentially using gel filtration, ion exchange, and hydrophobic chromatography. The purity of the isolated protein preparations was analyzed using 12% polyacrylamide gel electrophoresis. The concentration of mIBP83 was determined at a wavelength of 280 nm, taking into account its extinction coefficient (0.683). The concentration of GFP and mIBP83-GFP proteins was determined from the absorption at a wavelength of 395 nm, corresponding to its extinction coefficient (0.683). The concentration of mIBP83 was determined using 12% polyacrylamide gel electrophoresis. The purity of the isolated protein preparation was determined using 12% polyacrylamide gel electrophoresis.

In all experiments, we used the GFP variant, Cycle3-GFP [19]. Cycle3-GFP (for brevity, henceforth: GFP) folds well at 37°C (when expressed in E. coli), therefore, in many studies, this protein is used as a wild-type GFP protein. In addition, for this work, it is important that this protein does not dimerize under native conditions at low concentrations (up to 2 mg/mL) [20]. This has been verified by sedimentation and analytical gel chromatography. The chimeric protein mIBP83-GFP was also tested using gel chromatography. The protein came out in one narrow peak, as did GFP, which indicates that it does not dimerize.

**Devices and materials used in the work.** Experiments on ice freezing and melting were carried out in a setup assembled from a computer controlled thermostat [13]. The temperature was measured with an electronic thermometer, the relative accuracy (repeatability) of which is 0.01°C, and the absolute accuracy after adjustment in the region of 0°C was 0.1°C. The solutions were prepared with distilled water and filtered through 0.25 μm Corning Inc. filters (Germany), they were investigated in test tubes with a volume of 250 μL, Eppendorf (Germany). Experiments on melting and freezing protein solutions were carried out at a protein concentration of 0.6 mg/mL (which is close to the native concentration of cfAFP in Choristoneura fumiferana [11]), since at the same concentrations cfAFP was studied earlier in [18].

In experiments on the interaction of the GFP-mIBP83 protein with the ice surface, a VILBER ecx20.m transilluminator (France) was used to excite fluorescence.

### RESULTS

**Testing the Ability of mIBP83 to Bind to the Ice Surface**

To test the binding of mIBP83 (a mutant form of AFP—cfAFP) to the ice surface, we set up the following experiment. Two identical tubes with a buffer solution (1.0 mL, 20 mM sodium phosphate buffer, pH7) were frozen at —20°C and then kept at room temperature until the ice began to melt. Thus, each test tube contained a piece of ice surrounded by water. The mIBP83-GFP solution was added to one tube, and GFP to the other (in both cases, 0.20 ml at a concentration of 2 mg/mL). The tubes were illuminated on a transilluminator. If mIBP83 in the mIBP83-GFP chimeric protein had not lost its ability to bind to the ice surface, then it would cover the ice floating in the water, and the ice block should fluoresce more intensely than the solution. GFP does not bind to ice, so it must color the solution evenly. Figure 1 shows photographs of such an experiment. It can be seen that in the test tube with mIBP83-GFP it is mainly the piece of ice that glows. Its outline is visible. In the test tube with GFP, the ice contours are not visible—the solution around the piece of ice glows. Thus, it can be concluded that mIBP83 binds to the ice surface.

**Testing the Ability of mIBP83 to Influence the Freezing Process of Water**

To test the ability of mIBP83 to influence the freezing point of water, we conducted the following experiment. 50, 100, 150, or 200 μL of solution was poured into the test tube, the probe of an electronic thermometer was placed in the center of the test tube, and the test tube was closed and placed in a metal holder through which the cooling liquid of the thermostat circulated [13]. The thermostat cooled the test tube by changing the temperature linearly at a rate of 0.24 K/min. If no heat absorption or heat release occurs in the sample, then the temperature in the sample changes linearly with time at the same rate as in the thermostat. When the water freezes, heat is generated and the sample temperature rises, this jump in temperature at the moment of freezing of water was recorded by a thermometer.
Figure 2 shows the curves of temperature change in the samples depending on the cooling time. The curves, for convenience of comparison, are aligned according to the freezing time (marked with an arrow in Fig. 2).

There are two things to note here:

1. The freezing point of all samples is much lower than 0°C; which is quite consistent with the well-known fact that water can exist for a long time in a supercooled state.

2. “Instability of experiments,” i.e. a large spread of freezing temperatures even for the same solution (see Figs. 2a, 2b) in different (but identical) test tubes; Repetition of the same experiments with the same test tube with the same sample of the solution gives practically coinciding curves (the results are not shown).

The freezing point of a supercooled liquid depends not only on its temperature, but also on micro- and even nanoscopic defects of the tube surface. (see section “Theoretical estimation of the characteristic time of formation of an ice nucleus on various surfaces”). The presence of such microscopic defects is difficult to control, therefore, in freezing experiments it is impossible to achieve perfect repeatability of results obtained in different test tubes with the same liquids. However, comparing Figs. 2a and 2b, we can conclude that the presence of mIBP83 in solution has little effect (within the range shown in Fig. 2a, experimental errors ±1°C) on the freezing point of water, and at least does not lower it. In Fig. 2c for comparison, four curves are shown, reflecting the freezing processes of mIBP83 solution, carbonic anhydrase solution (an example of a protein for which antifreeze activity has not been detected), sodium phosphate buffer, and distilled water. If we take into account the error of exper-

Fig. 1. Binding of the mIBP83-GFP chimeric protein to the ice surface. Each tube contains a small piece of ice. On the left in the photo is a test tube to which the mIBP83-GFP solution was added, on the right is the GFP solution. The tubes were illuminated from below with ultraviolet light. In a test tube with mIBP83-GFP, it is mainly a piece of ice stained with adhered protein that glows; in a test tube with GFP, the piece of ice is not noticeable, since it does not glow.

Fig. 2. Dependence of the temperature of the samples on the cooling time. (a) Six identical freezing experiments with the same amount of the same solution (150 μL, 20 mM sodium phosphate buffer, pH 7) in identical tubes. (b) Two pairs of identical experiments on freezing a solution of mIBP83 (with a concentration of 0.6 mg/mL) in 20 mM sodium phosphate buffer, pH 7. Solid lines—two experiments with 150 μL of solution, dashed lines—two experiments with 200 μL of solution. (c) Cooling curves of a solution of mIBP83 (with a concentration of 0.6 mg/mL) in 20 mM sodium phosphate buffer, pH 7 (solid line), a solution of carbonic anhydrase (with a concentration of 0.6 mg/mL) in 20 mM sodium phosphate buffer (dashed line), the same sodium phosphate buffer (dotted line), and distilled water (dots). The curves are aligned with the freezing time of the solutions. The freezing point is indicated by an arrow. The temperature at which water begins to freeze in each of the experiments is labeled on the graph.
iments on freezing (see Figs. 2a, 2b), then we can conclude that the freezing of all the liquids shown in Fig. 2c occurs at the same low temperature.

Testing the Ability of mIBP83 to Affect Ice Melting

Figure 3 shows the dependences of the temperature of the samples on the heating time, i.e. the melting curves of ice with and without mIBP83. The technical features of the experiment are described in [13]. The “stability” of ice melting experiments is incomparably higher than the stability of ice formation experiments [13]; the error is ±0.1°C. Figure 3a is a diagram showing all stages of temperature change in the sample. At low temperatures, ice heats up at the same rate as liquid in a thermostat, but it does not melt (I in Fig. 3a). Approaching the ice melting temperature, line II in Fig. 3a begins to deviate from this speed, since when the tube is heated, the ice begins to melt at its wall, where the melting point is reached earlier, while the temperature at the center of the tube, where the measuring thermocouple is located, is still negative. If we measure a sample of large volume, then the curve, approaching the melting point, will be almost horizontal for some time (III in Fig. 3a); the larger the sample volume, the longer the curve will remain horizontal, since the mixture of water and ice maintains its melting point. After the ice has melted, the water quickly heats up to the temperature of the thermostat (IV in Fig. 3a). The last part of the curve (V in Fig. 3a) should reflect the heating process of the liquid in the test tube, going at the same rate as the liquid in the thermostat.

Figure 3b shows the experimental curves of melting ice with and without mIBP83. It can be seen that the shape of the experimental curves is similar to the schematic curve shown in Fig. 3a. The only difference is that stage III of the experimental curves is very small, since the volumes of the studied samples are only 50, 100, and 150 μL. At the same time, it is noticeable that with an increase in the sample volume, stage III also increases. Figure 3b also shows that at this stage the curves measured for samples without mIBP83 approach the 0°C line, that is, to the melting point of “pure” ice, while the curves for samples containing mIBP83 cross the 0°C line and approach +1°C. This means that the melting point of ice in the presence of mIBP83 increases. On average, the presence of mIBP83 at a concentration of 0.6 mg/mL increases the melting point of ice by one degree. This can be explained by the fact that mIBP83 binds to the ice surface (see Fig. 1), which under normal conditions is like supercooled water [21], and “strengthens” it. The phenomenon of overheating of crystals is known, although it is observed extremely rarely [22–24].

Theoretical Estimate of the Characteristic Time of Formation of an Ice Nucleus in the Water Column at Different Temperatures

Theoretical estimates show that, for kinetic reasons, ice nucleation in a pure water column is possible only at very low temperatures, below −30°C, and ice formation at near-zero temperatures is possible only on ice-binding surfaces in contact with water [25].

To prove this, we first consider the formation of a three-dimensional ice nucleus in a fluid column. The increment of free energy upon the formation of a compact fragment of a new three-dimensional phase, consisting of n particles can be estimated approximately [12, 26] as follows:

\[ G(n) = n\mu + \alpha_s n B, \]  

(1)
where \( \mu < 0 \) is the chemical potential of the molecule in the “new” phase (for example, in ice) minus its potential in the “old” phase (for example, in water), \( B > 0 \) is the additional free energy of the molecule on the surface of the “new” phase, \( \alpha, n^2 \) is the number of molecules on the surface of a compact piece of a new phase, consisting of \( n \) particles (for our estimates, where only the order of magnitude is important, we can take \( \alpha, n \approx 5 \), since for a sphere \( \alpha = \sqrt{3}/4 \) and for a cube \( \alpha = 6 \)). The free energy of less compact (with higher \( \alpha, n \) values) nuclei of nucleation of the new phase are higher than those of compact ones; therefore, one can ignore the “uncompetitive,” slow paths passing through the non-compact nuclei of the new phase [27].

In a biological object, the appearance of ice is expected at relatively low negative temperatures, when, as we will see below, ice nucleation nuclei are quite large. This justifies our disregard for the details of the structure of ice nucleation nuclei.

At slightly below the point of thermodynamic equilibrium of the phases (\( 0^\circ \text{C}, \text{i.e., } T_0 = 273 \text{ K} \)), ice is more stable than the liquid state of water (\( \mu = 0 \) at \( T_0 = 273 \text{ K} \)), and \( 0 < -\mu \) at \( T < T_0 \), but only a little (\( -\mu \ll k_B T_0 \), where \( k_B \) = Boltzmann’s constant, and \( -\mu \ll B \)). In this case \( G(n) \) first grows with increasing \( n \) (see equation 1), passes through the maximum \( G^\text{u} \) in the “transitional” state (determining the critical size of the nucleation nucleus), and then decreases. Maximum \( G^\text{u} \) functions \( G(n) \) is achieved at \( \frac{dG}{dn}_{n=n_0} = \mu + \frac{2}{3} \alpha, n^2 \frac{1}{B} = 0 \), that is, when the nucleation nucleus of a piece of ice contains \( n_0 = \left( \frac{2}{3} \frac{\alpha, B}{-\mu} \right)^3 \) H\(_2\)O molecules. Then the “germ,” i.e. minimum stable block of ice (with \( G(n) = 0 \) at \( n > 0 \)), contains

\[
n_{\text{need}} = \left( \frac{\alpha, B}{-\mu} \right)^3.
\]

H\(_2\)O molecules, and the free energy of the transition state is:

\[
G^\text{u} = G(n_0) = \frac{\alpha, B}{3} \left( \frac{2}{3} \frac{\alpha, B}{-\mu} \right)^2.
\]

At a temperature \( T_0 \), \( \mu = 0 \), so when \( T_0 - \Delta T \) (where \( \Delta T \ll T_0 \)), \( \mu = -\Delta S(H) (\Delta T) = -\Delta H(H) \left( \frac{-\Delta T}{T_0} \right) \) according to the classical equations of thermodynamics (here, \( \Delta S(H) \) and \( \Delta H(H) \) — entropy and enthalpy of freezing, respectively, per H\(_2\)O molecule). According to experimental data (see [28]), for water \( \Delta H(H) \approx -6.0 \text{ kJ/mol} \approx -2.6 k_B T_0 \), so

\[
\mu = \frac{-\Delta T}{100^\circ \text{C}} k_B T_0.
\]

The magnitude \( V = 0.85 k_B T_0 \) near \( T_0 = 273 \text{ K} \) follows from the experimental estimate of the free energy of the ice/water interface [29], \( \approx 32 \text{ erg/cm}^2 \) and the fact that the molecule H\(_2\)O takes \( \approx 10 \text{ Å}^2 \) on this surface. Therefore, for water \( \frac{B}{-\mu} = \frac{85^\circ \text{C}}{\Delta T} \), and, as a result,

\[
\frac{G^u}{k_B T_0} = 12 \left( \frac{100^\circ \text{C}^2}{\Delta T} \right).
\]

Then, according to the classical theory of nucleation of phase transitions [27, 30] based on the theory of transition states [31, 32], the characteristic time of the appearance of an ice nucleus around one given water molecule can be estimated as

\[
t_1 \sim \tau \exp \left( \frac{G^u}{k_B T} \right),
\]

where \( \tau \) is the characteristic time of adding one more water molecule to the growing ice, not less than \( \tau \approx 10^{11} \text{ s} \), which is the flip time of the molecule H\(_2\)O at 273 K [33]. A more detailed estimate shows that \( \tau \) is several orders of magnitude greater than \( \tau_0 \) [25], but this is not important for us, since we only need to estimate the lower limit of the ice formation time.

If in the considered volume (in a cell, in a test tube) there is \( N_v \) water molecules, and ice can form around any of them, then the characteristic time of the appearance of the first (and only) ice formation nucleus in such a volume can be estimated (see equations (5), (6)) as

\[
\text{TIME}_{3D} \sim \tau \frac{N_v}{N_v} \Delta \exp \left( 12 \left( \frac{100^\circ \text{C}^2}{\Delta T} \right) \right) \text{ s}.
\]

Because \( \tau > 10^{11} \text{ s} \), then the nucleus of an ice floe may appear in the water column in a test tube (containing \( N_v \approx 10^{23} \) molecules H\(_2\)O in \( \sim 1 \text{ cm}^3 \)) at \( >10^{482} \text{ s} = 10^{973} \text{ years} \) at \(-10^\circ \text{C} \), and after \( >10^{14} \text{ years} \) at \(-30^\circ \text{C} \) (i.e., from a biological point of view, an ice nucleus cannot appear in water at rest).

Theoretically, it can appear in the water column at rest, at \(-38^\circ \text{C} \) (experiment: at \(-35^\circ \text{C} \) [34]), and with sharp shaking at \(-15^\circ \text{C} \) [35]. An ice nucleus in the water column (see equation (2) and Fig. 4a) is quite large: it contains \( \sim 10^3 \) H\(_2\)O molecules at \(-35^\circ \text{C} \) (and its diameter is \( \sim 4 \text{ nm} \)), and at \(-10^\circ \text{C} \) its diameter is \( \sim 15 \text{ nm} \).
Theoretical Estimation of the Characteristic Time of Formation of an Ice Nucleus on Various Surfaces

While an ice nucleus can not appear in a water column at near-zero temperatures, the appearance of ice on ice-binding surfaces looks different. When such a surface can bind ice (according to Faraday, this property is possessed, for example, by flannel, but not gold [36]), an ice core appears on it, not as a three-dimensional object (Fig. 4a), but as a 2-dimensional object (Figs. 4b, 4c). We are not interested in the first monomolecular layer of ice: if ice is strongly bound by the surface, such a layer can appear (but cannot grow further) even at positive temperatures. We are interested in the beginning of further growth of the ice layer on the ice, which occurs only at subzero temperatures. And although the formation of a 2-dimensional layer, strictly speaking, is not a first-order phase transition [37], this layer is still quite similar to a crystal [37], and we can estimate its nucleation using equations similar to those given above.

When a layer of ice freezes onto a more or less flat ice surface (Figs. 4b, 4c), the additional free energy associated with the boundary of the new layer depends only on its perimeter, but not on its area (because in this case one ice surface only replaces another, see Figs. 4b, 4c). Therefore, the free energy of a 2-dimensional layer on a more or less flat surface (Figs. 4b, 4c) can be expressed in the same way as in Eq. (1):

$$\tilde{G} (n) = n\mu + \frac{1}{2} \alpha_2 n^2 \tilde{B},$$  \hspace{1cm} (8)

(where the degree $n$ is not $\frac{2}{3}$, but $\frac{1}{2}$, and $\alpha_2 \tilde{B}$ refers to the perimeter, not the entire surface, as $\alpha_2 \tilde{B}$). Now $\alpha_2 = 4$, because $\alpha_2 = \sqrt{4\pi}$ for a circle, and $\alpha_2 = 4$ for a square. The magnitude $\tilde{B}$ for the layer perimeter on a flat surface (Fig. 4b) is close to the value $\tilde{B}$ for the surface of a 3-dimensional body (Fig. 4a), but for a molecule of the perimeter of the corrugated layer (Fig. 4c) the value $\tilde{B}$ is close to zero due to the small contact of molecules inside the corrugated layer (see inset to Fig. 4c).

The magnitude $\tilde{G} (n)$ has a maximum $\tilde{G}^* (n)$ at $n = \bar{n}_0$,

where $\left.\frac{d\tilde{G}}{dn}\right|_{n=\bar{n}_0} = \mu + \frac{1}{2} \alpha_2 \bar{n}_0^2 \tilde{B} = 0$, i.e. the nucleation core of the ice layer contains $\bar{n}_0 = \left[ \frac{1}{2} \alpha_2 \left( \tilde{B} - \mu \right) \right]^2$ molecules H$_2$O, and the “nucleus” of the layer contains

$$\bar{n}_{\text{seed}} = 2^2 \bar{n}_0$$  \hspace{1cm} (9)

H$_2$O molecules. The free energy of the transition state during the formation of the layer is

$$\tilde{G}^* \equiv \tilde{G} (\bar{n}_0) = \frac{\alpha_2 \bar{B}}{2} \left( \frac{1}{2} \alpha_2 \frac{\tilde{B}}{-\mu} \right).$$  \hspace{1cm} (10)

If a layer of ice builds up on a flat surface,

$$\tilde{B} = B = 0.85 k_B T_0$$ [25], and $\bar{n}_{\text{seed}} = 2^2 \bar{n}_0 \approx \left( \frac{340^\circ C}{\Delta T} \right)^2$, but

$$\tilde{G}^* \approx \left( \frac{\alpha_2 \tilde{B}}{2} \right)^2 \frac{k_B T_0}{4 \mu k_B T_0} \approx 300^\circ C / \Delta T.$$  \hspace{1cm} (11)

If the layer is corrugated (as in Fig. 4c), then $\tilde{B}$ is close to zero, and so are $\bar{n}_0$, $\bar{n}_{\text{seed}}$, $\tilde{G}^*$.

The characteristic time for the appearance of a two-dimensional ice core on a flat surface capable of accommodating $N_k$ H$_2$O molecules, is estimated according to the classical theory of nucleation of phase transitions (see Eqs. (6), (7), (11)), as:

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where \( \tau > \tau_0 \approx 10^{-11} \text{s} \) (see above; according to a detailed calculation [25], more accurate estimate is \( \tau \approx 10^{-5} \text{s} \) at a temperature on the order of \(-1^\circ\text{C}\), but, due to the dominance of the exponential term, this is not so important for the estimates below). According to them, a 2-dimensional ice nucleus appears on a wall with an area of 1 cm\(^2\) (which contacts \( N_S \approx 10^{25} \text{H}_2\text{O molecules} \)) after years (!) at \(-4^\circ\text{C}\), and in seconds at \(-6^\circ\text{C}\). This is a colossal acceleration of the appearance of ice with a slight decrease in temperature! In seconds, an ice nucleus will appear on a wall with an area of \( \approx 100 \mu\text{m}^2 \) (which contacts \( N_S \approx 10^9 \text{H}_2\text{O molecules} \)) at \(-9^\circ\text{C}\) (compare with the characteristic temperatures in Fig. 2); such nuclei should have a diameter of \( \approx 10 \text{~nm} \) [25]. Note that such a high sensitivity of the ice formation time to the experimental conditions is directly related to the above “instability of experiments” on its formation.

To summarize: although ice cannot appear in the water column (through homogeneous nucleation) at temperatures above \(-30^\circ\text{C}\) (see above), it can occur at approximately \(-6^\circ\text{C}...-10^\circ\text{C}\) on the walls surrounding the water by heterogeneous nucleation (which is generally much more common than homogeneous nucleation [38]).

Until now, we have only talked about the time required for the nucleation of an ice block, but not for its subsequent growth, since the ice growth time can be neglected in comparison with the usually much longer time of its nucleation [25]. The sharp jump in temperature during freezing (Fig. 2) also shows that ice growth is a very fast process.

Also, we have so far only talked about the kinetics of freezing, but not about the kinetics of melting, because the initiation of ice melting is fast: it does not require noticeable preliminary overheating of water, since the ice surface is initially similar to supercooled water [21, 25].

**Possibility of “Ice Birth without Nucleation” on Exotic Surfaces**

The time of the above-considered nucleation of an ice layer on a flat surface (Fig. 4b) goes to infinity when the temperature approaches \(0^\circ\text{C}\) (see Eq. (9)). This effect arises from the loss of contacts at the periphery of the ice layer. However, this loss (resulting in an infinitely long crystallization time at \(0^\circ\text{C}\)) can be avoided if the ice-binding surface is corrugated (Fig. 4c).

Then ice may not form cooperatively, without a slow phase transition, but this requires an exotic (in this case, corrugated) surface that initiates ice formation.

The above estimates show that the existence, size, and nanoscopic structure of ice-binding surfaces are extremely important for the formation of ice nuclei; they determine the temperature at which ice occurs.

**DISCUSSION**

By analyzing the data, several conclusions can be drawn.

It is not so easy to freeze clean water or protein solutions, in a test tube until \(-8^\circ\text{C}\) solutions exist in a supercooled state. At the same time, the addition of IBP does not affect the freezing process, but it clearly increases the melting point of ice.

Based on the results of theoretical calculations, it can be concluded that a significant factor that increases the freezing point of supercooled water is the presence and structure of ice-binding surfaces. If we assume that the task of IBP is to inactivate such surfaces, then it becomes clear how such proteins can act as antifreezes.

When a cell or blood vessel has visible (larger than a few \(\mu\text{m}^2\) in size) surfaces on which ice nuclei can appear at negative temperatures, then some “antifreeze” molecules are needed to bind to these surfaces and block the growth of ice on them.

To date, we know almost nothing about ice-initiating surfaces (which could be targets for AFP). Identifying such surfaces and examining their properties will be the next step in investigating the operation of AFP.

Ice prevention is not the only property of IBP. Since these proteins bind to ice (Fig. 1), they can stabilize it (this is indeed the case—see Fig. 3). Thus, IBP can serve not only as an antifreeze, but also as a protein that initiates ice formation. However, as follows from the above calculations, the linear size of the surface suitable for ice nucleation should be at least \(\approx 10 \text{~nm} \). This is consistent with studies showing that large (\(\approx 200 \text{kDa}\), i.e., with a diameter of at least \(7 \text{~nm} \)) IBPs can not only prevent, but also, under certain conditions, initiate ice formation [39], while small IBPs work only as antifreeze, preventing the formation of ice.

In addition, adhesion of IBPs to cell surfaces should stabilize them and may protect cells from damage by hypothermia (preice), this is consistent with the hypothesis of Hirano et al. [5].

Thus, the supposed adhesion of IBP with cell surfaces can explain two phenomena observed experimentally during freezing of cells [9, 39]: (i) resistance of cells to cold shock under moderate cooling to \(0^\circ\text{C}\) (possibly due to stabilization of cell surfaces when ice-binding proteins adhere to them), and (ii) survival of cells under stronger cooling (apparently due to the fact that IBP blocks the surfaces that initiate the nucleation of ice, and thereby prevent the formation of ice crystals).

It is worth emphasizing that this work provides a fundamentally different view of the functioning of AFP. Their task is not to bind to ice crystals and stop
their growth, but to bind directly or through a thin layer of water molecules to those surfaces of cells or tissues where ice nuclei can form, thereby preventing the formation of ice there, in supercooled water. Blockage of ice nucleated surfaces is the only way to prevent freezing using small amounts of antifreeze molecules.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare they have no conflicts of interest.

This study does not contain any research involving humans or animals as research objects.

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ADDITIONAL INFORMATION

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