Kinetics of antifreeze protein-induced ice growth inhibition

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Abstract Antifreeze proteins (AFPs) depress the freezing temperature of a solution in a non-colligative manner, by arresting the growth of ice crystals. The kinetics of this effect, studied here for the first time using a new technique called temperature gradient thermometry, are consistent with an adsorption-mediated inhibitory mechanism. The results obtained by this approach provide a new experimental basis for understanding AFP interaction with ice.

Key words: Antifreeze proteins; Protein adsorption; Temperature gradient thermometry; Ice

1. Introduction

Antifreeze proteins (AFPs) and glycoproteins (AFGPs) are synthesized by a broad range of cold-adapted organisms, including fish, invertebrates, plants, fungi, and bacteria [1]. In fish, three classes of AFPs, designated Types I, II, and III, have been identified [2]. Type I AFP, the focus of the present study, occurs as a single, amphipathic α-helix with a molecular weight of 3.3 kDa [3,4]. AFPs inhibit freezing in a non-colligative manner, by arresting the growth of ice crystals over a concentration-dependent temperature range. Melting, by contrast, is affected in the predicted colligative manner. AFPs thus engender a separation, termed thermal hysteresis, between the temperatures at which ice crystals grow (the non-equilibrium freezing temperature) and melt (the melting point). The inhibitory influence of AFPs on ice crystal growth is thought to derive from local ice surface curvature effects induced by the adsorption of these proteins at the ice/solution interface. This is referred to as the adsorption-inhibition hypothesis [5]. It has been proposed that adsorption of AFPs to ice is stabilized by stereospecific hydrogen bonding between the protein and the crystal lattice [6,7].

While it is widely agreed that AFP-induced ice growth inhibition results from the adsorption of these proteins at the ice/solution interface, the kinetic implications of such a mechanism have not been studied. Thus whereas the freezing temperature in solutions of AFPs has been the subject of extensive investigation (e.g. [5,8]), only its concentration dependence has been considered; no attempts to measure its time dependence have been reported. If ice growth inhibition is a consequence of AFP adsorption, however, the freezing temperature must of necessity be governed by the kinetics of the adsorption process. The goal of this study was to determine whether ice growth inhibition in solutions of AFP exhibits kinetics commensurable with those of protein adsorption. To this end we developed a new experimental technique for studying the time dependence of the freezing temperature in solutions of AFP. Applying this technique to solutions of Type I AFP, we found that the kinetics of ice growth inhibition are consistent with those of protein adsorption. A molecular interpretation of these results is proposed.

2. Materials and methods

2.1. Temperature gradient thermometry and the directional solidification stage

The kinetics of ice crystal growth inhibition were studied by means of a new technique based on the principles of temperature gradient thermometry [9] and implemented using a directional solidification microscope stage [10]. Temperature gradient thermometry is a method for measuring the temperature on an ice/solution interface from a space-temperature correlation. The correlation is established by subjecting reference solutions with known freezing points to a linear temperature gradient. The freezing interfaces in these solutions define the space-temperature correlation by demarcating isotherms of known temperature. The position of the freezing interface in a sample solution subjected to the same temperature gradient then specifies the temperature on this interface.

Fig. 1 illustrates the temperature gradient thermometry technique, as implemented using the directional solidification stage. The directional solidification stage is an apparatus for producing a linear, one-dimensional temperature gradient within the field of view of a microscope [10]. A detailed description of this apparatus can be found in Rubinsky and Ikeda [10]. Briefly, the stage consists of two temperature-controlled bases separated by a small (3 mm) gap lying in the optical path of the microscope (Fig. 1A). When a glass microscope slide (26×76×1 mm) is placed on the stage, it experiences a linear, one-dimensional temperature gradient within the gap region [10]. Glass microcapillaries (20 μm×200 μm×10 cm) (Vitro Dynamics, Rockaway NJ) containing reference and sample solutions are laid on top of, and in good thermal contact with, the glass microslide. The temperature of one base is maintained above (T_{hot}), and the other below (T_{cold}), the freezing points of these solutions. As a result, when freezing is initiated at the cold end of the capillaries, freezing interfaces come to lie within the gap, where they are imaged and recorded by means of a video camera attached to the microscope (Fig. 1B). The positions of the freezing interfaces are measured from a monitor.

2.2. Technique for measuring the kinetics of ice crystal growth inhibition

We used temperature gradient thermometry to study the kinetics of ice crystal growth inhibition in solutions of Type I AFP from the winter flounder, *Pseudopleuronectes americanus* (A/F Protein, Waltham, MA). The experimental procedure was as follows. Capillaries containing AFP solutions (prepared in distilled water) and reference solutions (water and saline) were placed on the directional solidification stage and dabbed with a liquid nitrogen-cooled swab at their cold end to initiate freezing. Once the freezing interfaces had stabilized, a gradual, controlled reduction in the base temperatures was initiated. A slow migration of the reference interfaces ensued as the interfaces adjusted their positions to the changing temperature distribution. The freezing interfaces in the AFP-containing capillaries, by contrast, did not move as long as the interfacial temperature remained above the freezing temperature, T_f. At the freezing temperature, ice growth recommenced in a sudden burst and continued down the capillary until coming to rest at a new interfacial temperature. Growth was observed to stop not at the equilibrium melting point of the solution, T_m, but at a lower halt temperature, T_h. Once the interface had come to rest, the base temperatures were held constant for a prescribed period before

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being again reduced to initiate a new round of growth. The elapsed time between the halt of ice growth and its subsequent resumption defined the exposure time, t_{exp}, of the ice surface. The variables T_f, T_h and t_{exp} describe the kinetics of ice growth inhibition in the presence of AFPs.

We measured T_f and T_h as a function of t_{exp} in solutions of Type I AFP, for three different protein concentrations (1.0, 0.5, and 0.25 mg/ml, in water). These concentrations were chosen to produce smooth interface morphologies, for the sake of facilitating precise temperature determination. (Higher concentrations produce spicular ice crystals which do not permit precise determination of the site from which ice crystal growth begins.)

3. Results

Fig. 2 shows ΔT_f and ΔT_h plotted as a function of t_{exp}, for the three protein concentrations studied. ΔT_f is the non-equilibrium freezing point depression, defined as $T_m - T_f$. Similarly, ΔT_h is defined as $T_m - T_h$. Since for the sub-millimolar concentrations studied here, $T_m < 0.001$ °C, $\Delta T_f \approx -T_f$ and $\Delta T_h \approx -T_h$. All experiments were performed using a temperature gradient of 1 °C/mm. For a given experimental condition, each pair of points in the figure represents a single growth/halt cycle performed in a separate capillary.

The time dependence of ΔT_f is immediately apparent from Fig. 2. Equal at $t_{exp} = 0$ to ΔT_h, ΔT_f first increases rapidly and then tends towards a plateau where its value is approximately five times that of ΔT_h, e.g. ~0.2 °C vs. ~0.04 °C in the 0.25 mg/ml solution. The time dependence of ΔT_f is suggestive of a Langmuir adsorption process. (Indeed, it is interesting to note that the characteristic time of increase of ΔT_f (~30 min) is comparable to the time constant observed for the adsorption of AFGP to ice, measured at a similar protein concentration by means of ellipsometry [11].) In contrast to ΔT_f, ΔT_h is seen to be nearly constant for a given protein concentration. This fact suggests that ΔT_h, which cannot be measured using standard, non-gradient techniques, possesses some sort of physical significance.

4. Discussion

Both the halt temperature and the time-dependent freezing temperature can be explained in terms of the kinetics of AFP adsorption at the ice/solution interface. According to the adsorption-inhibition hypothesis [5], ice crystal growth ceases when the surface concentration of adsorbed AFP molecules exceeds a critical concentration, the value of which is a decreasing function of temperature. When ice crystal growth occurs in a positive temperature gradient, the advancing interface experiences progressively higher temperatures. As a result, the critical concentration of adsorbed AFP molecules needed to arrest the advance of this interface becomes smaller

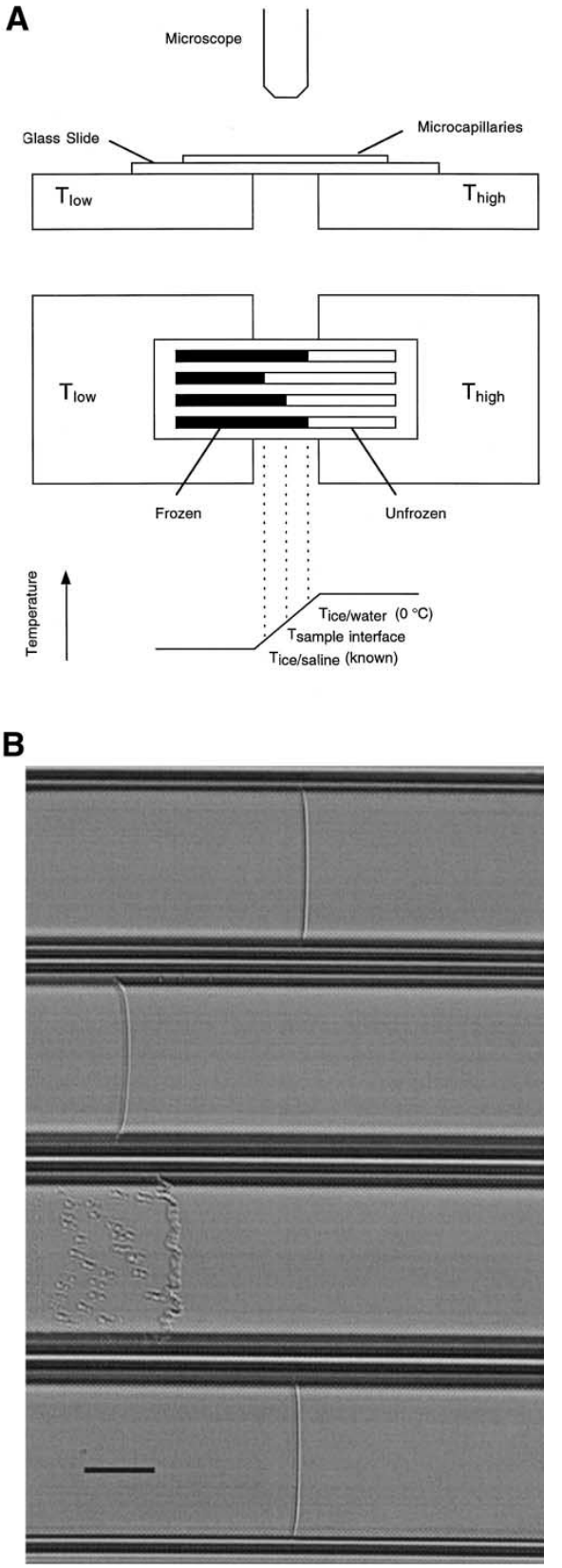

Fig. 1. (A) Schematic of the directional solidification stage illustrating the principle of temperature gradient thermometry. (B) View through the microscope, showing four glass capillaries lying side by side (horizontal lines). From top to bottom, the capillaries contain: water, saline with a freezing point of −0.2°C, AFP solution, and water. The solutions in the capillaries, which experience a temperature gradient, are partially frozen. The freezing interfaces are the vertical lines in each capillary separating the frozen region (left) from the unfrozen region (right). The two ice/water interfaces, which are observed to lie along the same vertical line, define the 0°C isotherm. The freezing interface in the saline capillary defines a second isotherm, parallel to the first. Assuming a linear temperature gradient along the horizontal axis of the capillaries, the temperature on the ice/AFP solution interface can be determined by interpolation between the two known isotherms. Scale bar = 100 μm.

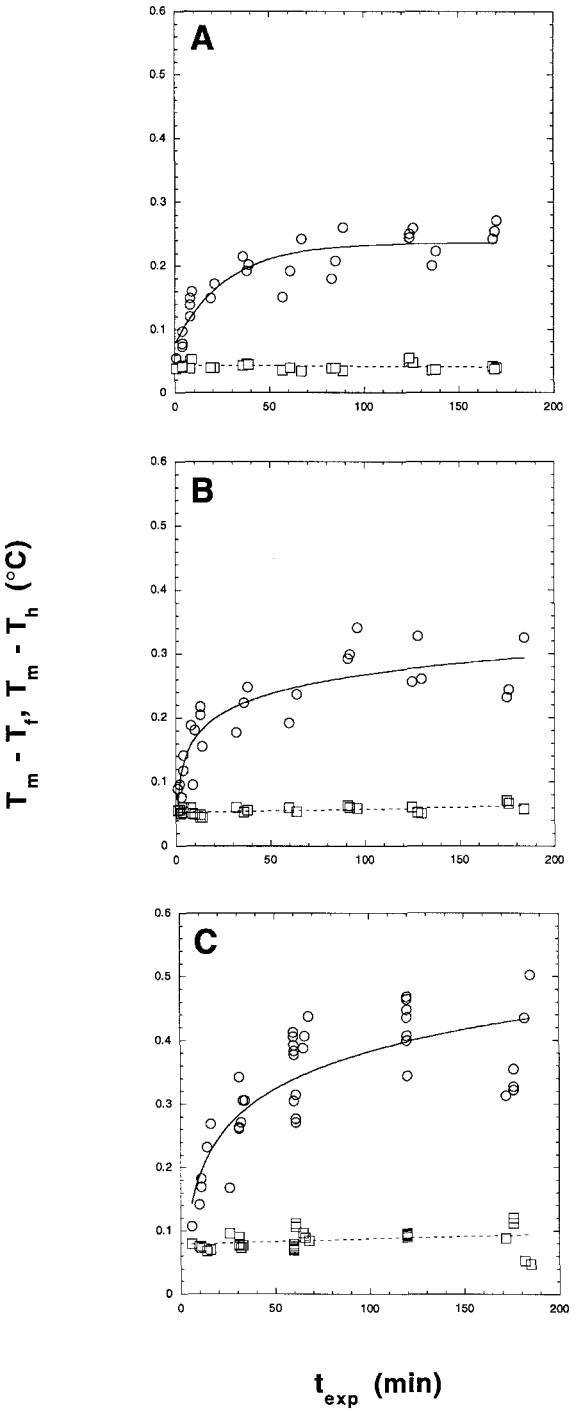

Fig. 2. The kinetics of ice growth inhibition in solutions of Type I AFP. ΔT_f (open circles) increases with the exposure time, t_{exp}, of the ice surface to solution, whereas ΔT_h (open squares) is nearly constant for a given protein concentration. (A) 0.25 mg/ml, (B) 0.5 mg/ml, (C) 1.0 mg/ml.

and smaller. At the halt temperature, this concentration becomes equal to the concentration of AFP molecules adsorbing at the moving interface 'instantaneously', i.e. on the time scale at which the interface advances over molecular distances, and growth comes to a stop. The time dependence of the freezing temperature can be accounted for in a similar way, by considering AFP adsorption at the stationary ice/solution interface. At the moment the interface comes to rest, only a fraction of AFP binding sites on the crystal surface will be occupied. Subsequent exposure of the ice surface to solution leads to progressive filling of these sites and a concomitant decrease in the freezing temperature. The precise time dependence of the freezing temperature will be determined by the details of the adsorption process.

To interpret the kinetics of inhibition at the stationary interface, we must first ask whether the kinetics of AFP adsorption are transport or surface-reaction-rate limited. For the concentration regime studied here (10^{-4} M), a simple diffusion analysis shows that if every molecule striking the ice surface were to adhere (the Smulochowski model), coverage of the surface would occur very rapidly, with a time constant of a second or so. Transport from the bulk can therefore be ruled out as the rate-determining step in the adsorption kinetics. This leaves the surface reaction steps involved in protein adsorption, which include [12]: (1) attachment to the interface, (2) rearrangement of adsorbed molecules by diffusion, reorientation, and/or conformational changes, and (3) detachment from the interface. The decrease in the freezing temperature over time could therefore reflect progressive AFP adsorption, rearrangement of adsorbed AFPs, or some combination of the two. Although our data do not allow us to distinguish between these possibilities, the stereospecific ice-binding property upon which the inhibitory activity of AFP is thought to depend [6,7,13] suggests a possible role for surface rearrangement of AFP molecules in the kinetics of ice growth inhibition. If we suppose that to inhibit ice growth AFP molecules must be bound on the ice surface in a specific orientation, and if we further suppose that AFP molecules can and do adsorb in other, non-optimal orientations as well (as appears likely given that Type I AFP has been shown to adsorb to the basal plane of ice [14], wherein the lattice spacing identified with the stereospecific adsorption of this protein does not occur [7]), then ordering of adsorbed AFP molecules into their preferred binding orientation through surface diffusion and/or reorientation would be a necessary step for achieving maximal inhibition [see 7 for an alternative hypothesis in this regard]. If this hypothesis is correct, the kinetics of the rearrangement process might well govern those of the freezing temperature.

To provide context to the foregoing discussion, we briefly mention two related findings, one bearing on the kinetics of crystal growth inhibition, the other on ordering in adsorbed protein layers. First, we note that our observation of a time-dependent threshold for crystal growth is new only insofar as the inhibitory effects of AFPs on ice are concerned. Analogous effects have been reported for solution growth of several types of inorganic crystals, including ammonium dihydrogen phosphate (ADP) [15], potassium dihydrogen phosphate (KDP) [16] and potassium bichromate (KBC) [17]. These crystals are susceptible to growth blockage by various atomic or low-molecular-weight inhibitors. The critical supersaturation at which growth resumes on an inhibited crystal face is found to depend on the length of time the face has been exposed to inhibitor-containing solution. This effect has been ascribed to the kinetics of inhibitor adsorption [15–17]. Second, we note that a study of diffusion in proteins adsorbed at a solid/liquid interface has found evidence suggesting that rearrangement of adsorbed molecules can lead to ordering of the adsorbed protein layer [18]. In this case, ordering presumably resulted from interactions between the adsorbed protein molecules them-

selves rather than from a preferred orientation of the proteins on the surface.

In summary, the kinetics of AFP-induced ice growth inhibition, studied here for the first time using a new experimental technique, are consistent with an adsorption-mediated inhibitory mechanism. By opening a window on the kinetics of AFP/ice interaction, this technique provides a new experimental basis for understanding the mechanism of AFP action.

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