THE MYSTERIES OF MEMORY EFFECT AND ITS ELIMINATION WITH ANTIFREEZE PROTEINS

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ABSTRACT

Crystallization of water or water-encaged gas molecules occurs when nuclei reach a critical size. Certain antifreeze proteins (AFPs) can inhibit the growth of both of these, with most representations conceiving of an embryonic crystal with AFPs adsorbing to a preferred face, resulting in a higher kinetic barrier for molecule addition. We have examined AFP-mediated inhibition of ice and clathrate hydrate crystallization, and these observations can be both explained and modeled using this mechanism for AFP action. However, the remarkable ability of AFPs to eliminate ‘memory effect’ (ME) or the faster reformation of clathrate hydrates after melting, prompted us to examine heterogeneous nucleation. The ubiquitous impurity, silica, served as a model nucleator hydrophilic surface. Quartz crystal microbalance-dissipation (QCM-D) experiments indicated that an active AFP was tightly adsorbed to the silica surface. In contrast, polyvinylpyrrolidone (PVP) and polyvinylcaprolactam (PVCap), two commercial hydrate kinetic inhibitors that do not eliminate ME, were not so tightly adsorbed. Significantly, a mutant AFP (with no activity toward ice) inhibited THF hydrate growth, but not ME. QCM-D analysis showed that adsorption of the mutant AFP was more similar to PVCap than the active AFP. Thus, although there is no evidence for ‘memory’ in ice reformation, and the structures of ice and clathrate hydrate are distinct, the crystallization of ice and hydrates, and the elimination of the more rapid recrystallization of hydrates, can be mediated by the same proteins.

Keywords: gas hydrates, kinetic inhibitors, antifreeze proteins, memory effect, polyvinylpyrrolidone, polyvinylcaprolactam, quartz crystal microbalance

NOMENCLATURE

AFP antifreeze protein
KI kinetic inhibitor
LDHI low dosage hydrate inhibitor
ME memory effect
PVP polyvinylpyrrolidone
PVCap polyvinylcaprolactam
TH thermal hysteresis
QCM-D quartz crystal microbalance with dissipation

INTRODUCTION

Easily accessible traditional hydrocarbon supplies will be supplemented in the coming decades with new stores situated in deeper off shore waters, in the permafrost, or sheathed in crystalline water as gas hydrates. Prospecting, recovery and transport of this energy will be not without its challenges. When pressure and temperature conditions are favorable, hydrocarbon gases can form hydrate plugs that can lead to shutdown and financial

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losses. As well, there is the potential for environmental damage due to unexpected hydrate formation. For example, methane hydrate accidents in the Sea of Azov, Ukraine, resulted in mass fish mortality even at sites located distant to the accident [1].

Traditionally, methanol is used to inhibit hydrates, but it is expensive due to the large amounts required (10 to 50% of the water phase), and there are also environmental concerns associated with its use in the Arctic and some European sectors [2]. Both methanol and ethylene glycol, which has been used in the Gulf of Mexico, act by lowering the crystallization point of the hydrate. Thus cost considerations as well as environmental concerns have motivated the search for more inexpensive, low dosage polymers [2,3]. One such alternative to these traditional polyol inhibitors are newer low dosage hydrate inhibitors (LDHIs), which can be classified as kinetic inhibitors (KIs) and anti-agglomerates. At relatively low concentrations (<1% by weight), LDHIs appear to function by slowing down hydrate growth or by reducing the likelihood of hydrate particle agglomeration so that there is a reduced probability of blockage. Results have been encouraging; when a KI was introduced into a flow line in the West Pembina (Alberta) field, “downtimes” were reduced [4]. Thus the search for such new inhibitors is to be encouraged.

The rapid addition of water molecules to embryonic crystals is an analogous problem faced by certain organisms that live at temperatures lower than their theoretical freezing points. Antifreeze proteins (AFPs) inhibit freezing in a non-colligative manner by adsorbing to ice [5]. Since melting is effected in a colligative manner, the association between AFPs and the ice surface results in a separation of the freezing point and the melting point, a phenomenon termed thermal hysteresis (TH). The inhibition is thought to derive from local ice surface curvature effects due to the presence of AFPs at the ice/solution interface (the adsorption-inhibition hypothesis [6]). Because of this action, small quantities of AFPs can have large effects on ice crystal growth.

We hypothesized that gas hydrates, with their regular crystal lattice might act as an alternative substrate for AFPs. Our experiments both with model gas hydrates (tetrahydrofuran clathrate; THF) and methane and propane hydrates have shown that certain AFPs inhibit gas hydrate crystallization as well as eliminate faster recrystallization [7-9]. Despite these promising results, we know little of the mechanism of this inhibition. The faster reformation or ‘memory effect’ (ME) is problematic in the field when downtimes are expensive [2,10-13]. It should be noted, however, that others have reported that ME is an experimental artifact [e.g., 14]. Nevertheless, we have explored the use of a quartz crystal microbalance to predict hydrate inhibition as a first step to explore the observed differences in hydrate recrystallization and eventually toward a more sophisticated understanding of how these unique proteins interact with hydrates.

**METHODS**

**Preparation and characterization of potential inhibitors**

Polyvinylpyrrolidone (PVP K30, ~40,000 da; kindly provided by Dr. E.D. Sloan) as well as wild type Type I AFP from winter flounder (kindly provided by Dr. G. Fletcher) solutions were at a final concentration of 2.5, 12.5 and 25 μM. Polyvinylcaprolactam (PVCap; ~110,000 da; kindly provided by Dr. L. Talley) solutions were at 2 and 10 μM. All solutions were prepared with Milli-Q® ultrapure water (Millipore, Bellerica, USA). A mutant Type I AFP with a Leu for Ala substitution at position 17 (A17L) was chemically synthesized using solid-phase peptide synthesis at the Queen’s University Protein Function Discovery Facility. Peptide concentrations were routinely determined by amino acid analysis and A17L was used in solutions at the final concentrations of 2.5, 12.5 and 25 μM.

TH was assessed using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) as previously described [15] and the ice crystal morphology was noted. Inhibition of ice recrystallization was assayed by rapidly freezing samples in microcapillary tubes (10 μl) and allowing them to incubate at 267 K overnight [16]. After viewing through crossed polarizing filters those samples with no recrystallization inhibition activity were recognized by the presence of large ice crystals. Ice nucleation assays were done as reported [17,16]. Briefly, small samples (10 μl) were loaded onto a polarizing filter and placed in a chamber where the temperature was lowered from 272 K to 258 K. Digital photographs were captured every 60 sec though a crossed polarizing filter and automatically analyzed and transferred to a spreadsheet. The temperature at which 90% of the samples froze was taken as the ice nucleation
Quartz crystal microbalance assessments
Surface adsorption was determined using a quartz crystal microbalance (QCM) equipped to determine the energy loss or dissipation factor (D). This QCM-D (Q-Sense D300, Q-Sense AB, Gothenburg, Sweden) with 5-MHz AT-cut crystals had a sensor crystal coated with SiO₂ (QSX-303) on one side of the gold electrode. This was cleaned and placed in a 250 μl measurement chamber with ultrapure water equilibrated at 299 K. Once a stable baseline was achieved, aliquots (0.5 ml) of the solutions (1.5 ml) to be tested were introduced into the measurement chamber, replacing the water. Frequency shift (f) and D were sampled at a rate of ~1 Hz with a sensitivity of <0.5 Hz and 1×10⁻⁷, respectively. After the initial adsorption measurements, the sensor was rinsed three times with 0.5 ml ultrapure water at 299 K. Assessments of f and D were obtained after each rinse. Control experiments were done with a hydrophobic substrate, a polystyrene surface (QSX-305), on the sensor crystal.

RESULTS
Characterization of ice-associating properties
Type I AFP showed TH activity consistent with previously published values. At a concentration of 1 mg/ml it showed a TH of approximately 0.3ºK. The A17L mutation showed no TH activity at this or higher concentrations. Ice crystals grown in the presence of Type I AFP showed the diamond-like morphology typical of this AFP (Fig. 1a). In contrast, the mutant A17L protein resulted in no change to the flat circular crystals seen in the absence of AFPs. Since ice recrystallization inhibition is a property that can be assessed at very low concentrations of AFPs, this assay was also used to assess the activity of the two AFPs. Type I AFP showed complete inhibition of ice recrystallization even at the lowest concentrations used, whereas the mutant A17L showed large crystals and the complete absence of ice recrystallization at every concentration tested (Fig. 1b; not shown). Neither AFP nor the mutant A17L showed any ice nucleation activity (Fig. 1c; not shown). PVP showed no ice association activity using ice recrystallization inhibition activity or ice nucleation assays (Fig. 1).

Quartz crystal microbalance assessments
When AFP, A17L, PVP and PVCap were analyzed using QCM-D all showed an initial decrease in f before leveling out at a value consistent with the mass of the adsorbent on the surface (Fig. 2; not shown). This adsorption was also concentration dependent; more molecules were adsorbed to the SiO₂ surface as the concentration of the potential inhibitors was increased (Table 1 and not shown). Adsorption masses, as assessed by the final values of f, were higher for the PVP solutions than for...
PVCap and these masses were less for the two proteins than those of the two polymers. For example, at 10-12 μM, the value of $f$ for PVP was -19.1 Hz, -0.59 Hz for PVCap, -2.1 Hz for AFP and -3.7 for A17L.

QCM-D analysis not only reveals the adsorption mass, but the dissipation factor represents the viscoelastic properties of the adsorbed molecules [18,19]. The $D$ value for PVP, PVCap and A17L increased throughout the assay period, but that for AFP remained almost constant (Fig. 3; not shown). The slope ($R$), of these curves was calculated as $R = \Delta D/\Delta m$. Thus $R$ indicated the status of the adlayer. PVP, PVCap and A17L showed two distinct steps of adsorption, $R_1$ and $R_2$, and for A17L at high concentrations ($\geq$ 12.5 μM), there was some evidence of a third step (Table 1). The final status of the adlayer is reflected in the last $R$ values. PVP showed the highest final $|R|$ values ($R_2$) of the four molecules tested, whereas the lowest final $|R|$ values (0 Hz) were seen with the wild type AFP (Table 1).

When adsorption on the hydrophobic polystyrene surface was tested with the potential

![Representative graphs showing (a) the adsorption of AFP (25 μM, solid line) and PVP (25 μM, dotted line) as well as (b) AFP (2.5 μM, solid line) and A17L (2.5 μM, dotted line) on the SiO$_2$ surface at 299 K. Frequency shift ($f$) vs. time was assessed using QCM-D.](image)

![Table 1. Kinetic parameters for adsorption of antifreeze proteins (wild type AFP and A17L mutant) as well as kinetic inhibitors on SiO$_2$ and polystyrene, a control hydrophobic surface, as assessed by QCM-D.](table)

<table>
<thead>
<tr>
<th>Sample</th>
<th>AFP</th>
<th>A17L</th>
<th>PVP</th>
<th>PVCap</th>
<th>AFP</th>
<th>A17L</th>
<th>PVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>~ Conc. (μM)</td>
<td>25</td>
<td>12</td>
<td>25</td>
<td>12</td>
<td>25</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>$R1$ (x 10$^{-6}$ Hz$^{-1}$)</td>
<td>-0.04</td>
<td>-0.03</td>
<td>-0.04</td>
<td>-0.06</td>
<td>-0.14</td>
<td>-0.12</td>
<td>-0.36</td>
</tr>
<tr>
<td>$R2$ (x 10$^{-6}$ Hz$^{-1}$)</td>
<td>0.00</td>
<td>0.00</td>
<td>-0.01</td>
<td>-0.22</td>
<td>-0.19</td>
<td>-0.18</td>
<td>-0.06</td>
</tr>
<tr>
<td>$R3$ (x 10$^{-6}$ Hz$^{-1}$)</td>
<td>-</td>
<td>-</td>
<td>-0.07</td>
<td>-0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$f_{total}$ (Hz)</td>
<td>-11.1</td>
<td>-7.6</td>
<td>-19.9</td>
<td>-18.03</td>
<td>-29.5</td>
<td>-27.33</td>
<td>-1.6</td>
</tr>
</tbody>
</table>
inhibitors (PVP, AFP and A17L; PVCap was not assayed) the kinetic parameters for adsorption showed final $|R|$ values that were the same for all the tested molecules (-0.12 to -0.13 x $10^6$ Hz$^{-1}$; Table 1).

**DISCUSSION**

Evolutionary pressures have designed AFPs to adsorb to the surface of seed ice crystals thereby increasing the energy barrier for ice growth due to the Kelvin effect [20]. This inhibition mechanism occurs subsequent to heterogeneous nucleation. AFPs show a remarkable variation in structure: some fish AFPs, including the Type I AFP used here are α-helices, but with other fish and insect AFPs showing β-helices, β-rolls or more globular structures. Therefore, it appears that the structure per se does not dictate ice association properties, but a more general complementarity with particular residues exerting van der Waals attractions and possibly a combination of hydrogen bonding and other hydrophobic interactions [21] facilitating the snug, irreversible fit to ice. Indeed, the 17th residue in Type I AFP is so important for this interaction that a Leu substitution at this site results in a loss of all ice association activity. We report here that there is no affinity for ice by the mutant as assayed by ice crystal morphology and ice recrystallization inhibition activity (Fig. 1). The complete loss of TH activity in A17L was reported previously and was used to support a new proposed “ice binding” face of the Type I AFP α-helix [22]. PVP also did not show ice association as revealed by assays to detect inhibition of ice recrystallization or ice nucleation (Fig. 1).

AFPs have been shown to inhibit the growth of model and gas hydrates [7-9], but the mechanism for this inhibition is unknown. Significantly, the A17L mutant showed similar inhibition of THF hydrate growth as the active AFP (Zeng et al., unpublished), demonstrating that adsorption to hydrate and ice crystals is not mediated by identical residues. Curiously, however, although the active AFP eliminated the more rapid recrystallization of hydrate after a brief melt [8], the A17L mutant did not eliminate ME. Significantly, ME was also not eliminated by either of the tested KIs, PVP or PVCap (Zeng et al., in revision). We reasoned that active AFP may be unique in its ability to eliminate ME due to inhibition of heterogeneous nucleation. However, since A17L was inactive against ice, it was a formal possibility that ice crystals could act as heterogeneous hydrate nucleators, which grew to a critical size only in the absence of AFP activity. By ensuring that the temperature of THF hydrate was kept above 272 K, this risk was eliminated (Zeng et al., unpublished).

A second possibility was that active AFP could inhibit hydrate recrystallization by inhibiting heterogeneous nucleation on the surface of silica, a ubiquitous stable ice nucleator [23]. QCM-D analysis showed that the four tested molecules could be grouped into three different classes based on their interaction with silica. PVP loosely associated with silica; the adsorption mass was high, and this KI had the highest final $|R|$ values of any of the tested molecules, consistent with a porous adlayer with tapped water molecules. PVCap and A17L formed a somewhat more compact film, with final $|R|$ values that were 3-4 fold less than the PVP values, indicating that less water was trapped on the surface. Remarkably however, the lowest final $|R|$ values of the tested molecules were found for wild type AFP, consistent with a highly dense, compact adlayer, with very little water. These differences in silica adsorption reflect the different effect the proteins and polymers had on ME, and further suggest that...
such film differences could have a distinct influence on heterogeneous nucleation.

We propose that hydroxylated silicon or hydrated silica nano or micro particles present during the initial hydrate crystallization become ‘imprinted’ and thus become more effective nucleators for ME recrystallization. This work shows that the hydroscopic polymers PVP and PVCap as well as A17L can form films on SiO2 but since the aggregation is either relatively loose or can easily wash off (not shown), there is no elimination of ME. In contrast, active AFP adsorbed to silica and retained a tight compact surface with little trapped liquid. As a consequence, water and guest molecules cannot so easily reach the nucleating sites making heterogeneous nucleation on such a surface much less probable (Fig. 4). As well, it appears that the ice binding site and the surface that tightly binds silica coincide, given the differences observed with QCM-D in the adlayers for the active AFP compared to the A17L mutant. The ice adsorption face of Type I AFP is relatively hydrophobic [22, 24], and therefore the more hydrophilic side of the active AFP would consistently face away from the silica surface.

In conclusion, these studies demonstrate that the properties of adsorbed layers can be monitored effectively by QCM-D. The results have provided useful information about the inhibition mechanism of heterogeneous nucleation of clathrate hydrate. This technique offers opportunities to screen potential LDHIs, and to examine residues in AFPS that are involved in silica adsorption, and by extension the inhibition of heterogeneous nucleation. However, the most important practical ramification may be that these studies offer a procedure to further investigate the mysterious memory effect, a phenomenon that has been hotly debated by lab scientists who have denied its existence and those in the field who see its destructive capability first hand.

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