J|A|C|S
COMMUNICATIONS

Published on Web 12/17/2002

Reversible Binding of the HPLC6 Isoform of Type I Antifreeze Proteins to Ice Surfaces and the Antifreeze Mechanism Studied by Multiple Quantum Filtering−Spin Exchange NMR Experiment

Yong Ba,*,† Jeff Wongskhaluang,† and Jiabo Li‡

Department of Chemistry and Biochemistry, California State University Los Angeles, 5151 State University Drive, Los Angeles, California 90032, and Accelrys Inc., 9685 Scranton Road, San Diego, California 92121-3752

Received July 3, 2002; E-mail: yba@calstatela.edu

Polar and northern fishes are able to survive in cold seawater down to a freezing point of −1.9 °C by synthesizing antifreeze proteins (AFPs).[1] These proteins depress the freezing points of their aqueous solutions, but do not affect the corresponding melting points (thermal hysteresis).[2] This property was attributed to the binding of AFPs to specific ice surfaces. The HPLC6 isoform of type I AFPs purified from the serum of winter flounder[3] contains three 11-amino acid repeat units (TA_2NA_7) and additional N- and C-terminal capping sequences.[4] Its crystal structure shows a complete α-helical conformation with only an exception for the last peptide unit.[5] The equivalent groups within adjacent repeat units are separated by a distance of 16.5±0.5 Å. An ice-etching experiment[6] and a crystal morphology study[7] showed a strong structural match between the AFP's repeat units and the periodicity in ice oxygen repeat distance in the ice-binding planes[5,8,9] although the nature of the affinity between the ice surfaces and the AFP molecules has not been completely understood.[6,10] It was thought that the binding of AFPs to ice surfaces causes the growing ice fronts to advance in spaces between the AFP molecules leading to local surface curvatures. This physical change makes it energetically less favorable for water molecules to join the ice lattice, resulting in a local freezing-point depression. This mechanism was referred to as the Kelvin effect.[11,12] As was hypothesized,[6] the Kelvin-effect interpretation actually implies that AFPs essentially bind to ice surfaces in an irreversible manner, since desorption of AFPs would allow supercooled water to join the ice lattice instantly. However, thus far, no direct experimental evidence has been found to demonstrate whether the binding of AFPs to ice surfaces is reversible or irreversible. To elucidate this, we applied a newly developed MQ Filtering−spin exchange NMR experiment[13] to directly observe the molecular exchange of HPLC6 peptides between the ice surfaces and the liquid solution.

ND_4DCO_3 deuterated aqueous solutions of HPLC6 peptides were prepared for the NMR experiments.[14] A sample of these provided a two-phase system consisting of an ice phase in equilibrium with an electrolyte liquid phase containing the HPLC6 peptides at the experimental temperature of −1.0 °C. In the NMR experiment, proton NMR signals of the HPLC6 peptides adsorbed on deuterated ice surfaces were first labeled by MQ coherences due to the $^1H-^1H$ dipolar interactions. Then, with the elapse of molecular exchange time, desorption of the peptides from the ice surfaces to the aqueous solution was traced by observing the growth of the liquid-phase peptide signals with the MQ coherence label. Figure 1 shows the experimental result by using a double-quantum filtered spectrum for a sample of 1.0 mg/mL HPLC6 peptides, where the molecular exchange times are given along with the corresponding NMR spectra. A spectrum with 1 ms exchange time shows the signal, a typically broad line, of the HPLC6 peptides adsorbed on ice surfaces. With 50 ms exchange time, a narrower component consisting of the superposition of a number of proton peaks in the center of the broad spectrum appeared. This component came from the desorbed HPLC6 peptides from the ice surfaces into the aqueous solution. With longer molecular exchange times, the liquid-phase signals of the HPLC6 peptides increased monotonically, indicating increased amounts of desorbed peptides into the solution. The spectra show molecular exchange of the peptides between the ice surfaces and the aqueous solution because the amount of peptides desorbed into the solution is equal to that of peptides adsorbed on the ice surfaces after the adsorption/desorption process reached equilibrium observed by the unchanged 1D proton NMR spectrum with time. This exchange phenomenon was also observed for several other samples with different HPLC6 concentrations from 0.1 to 5.0 mg/mL although their exchange rates are different. The observed molecular exchanges clearly show that the binding of HPLC6 peptides to the ice surfaces is reversible.

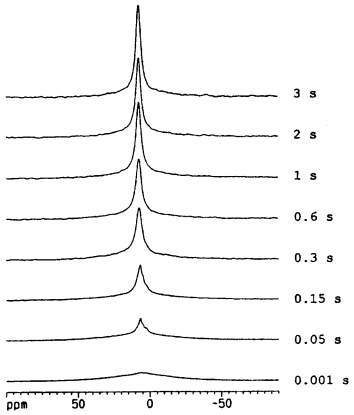

Figure 1. NMR spectra of HPLC6 peptide exchange between the ice surfaces and the aqueous solution.

Figure 2 shows the fractions of the signal intensities of the HPLC6 peptides desorbed into the aqueous solution to the total HPLC6 signals versus molecular exchange time. A fraction was calculated by comparing the area under the line shape of the adsorbed peptides and the total area of a spectrum. Theoretical analysis of the Langmuir theory for monolayer adsorption[15] resulted in the concentration of the desorbed AFP into the liquid phase as a function of exchange time as:

$$[\mathrm{AFP}(t)] = [\mathrm{AFP}]_E(1 - \exp[-kt]) \qquad (1)$$

where $[AFP]_E$ denotes the equilibrium concentration of the peptides in the liquid phase, t represents the exchange time, and

† California State University Los Angeles.
‡ Accelrys Inc.

10.1021/ja027557u CCC: $25.00

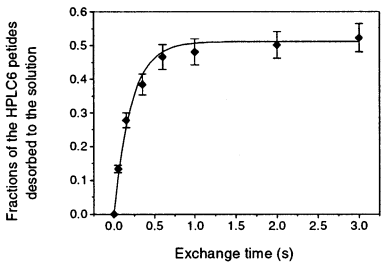

Figure 2. Experimental intensity fractions of the HPLC6 peptides desorbed from the ice surfaces to the solution versus molecular exchange time, and the theoretical fitting curve with the model of Langmuir adsorption.

$$k = \frac{k_- n_0/S}{n_E/V} = \frac{k_+ n_{A0}(1 - n_0/S)}{n_E}$$

where n_0 represents the amount of original MQ coherence labeled peptides on the ice surfaces, n_E represents the amount of equilibrium peptides labeled by the MQ coherence desorbed in the solution, n_{A0} denote the original amount of peptides in the liquid phase, V and S are the total volume of the liquid phase and the total available protein-binding sites on the ice surfaces, respectively, and k_+ and k_- represent the adsorption and desorption rate constants, respectively. Equation 1 was used to fit the experimental signal fractions. The best theoretical fitting curve is shown as the solid line in Figure 2, which gives the equilibrium fraction to be 0.50 ± 0.04 and $k = 4.70 \pm 0.04\ s^{-1}$. k_+ and k_- have not been determined in this communication since a proper method to measure S and V was not found. The fine agreement between the experimental data and the theoretical curve shows that the reversible binding of HPLC6 peptides to the ice surfaces obeys the model of monolayer adsorption. This indicates that direct interactions between HPLC6 peptides and the binding sites on the ice surfaces are the driving force for the binding of the AFP to ice surfaces, and, furthermore, the inter-peptide interactions on ice surfaces[6,16] should not exist.[15] The latter was also concluded from an earlier antifreeze activity study.[17] Our experimental observation supports earlier research where reversible Langmuir adsorption was used to explain the concentration dependence of the thermal hysteresis of antifreeze glycoproteins.[18]

The reversible ice-surface binding of the AFP suggests that the Kelvin effect is not suitable for explaining the antifreeze mechanism. It was speculated that the binding of AFPs to ice surfaces is perhaps reversible, but the consequence of the reversible ice-surface binding is to allow the Kelvin effect for freezing point depression to manifest itself.[19] We think that it is not likely for a reversible binding of AFPs to ice surfaces to maintain local surface curvatures, since desorption of AFPs would allow the supercooled water to join the ice lattice instantly. On the basis of the experimental facts that specific ice surfaces possess affinities to AFPs, and AFPs bind to ice surfaces in a reversible manner, we propose that a concentration gradient of AFP from an ice surface to the solution exists, which results in a dense solution layer of AFP to be in contact with the ice surface. This dense layer of AFP is able to depress the local freezing point through the colligative property[15] by reducing the chemical potential of the local liquid water. Consequently, a lower temperature has to be reached before equilibrium between the seed-ice crystals and the liquid layers is achieved. When the local equilibrium has been reached, the rest of the solution not in close contact with the ice surfaces is in a supercooled condition. We expect that an AFP that has a stronger attractive interaction with ice surfaces will be more efficient for local freezing point depression due to the induced higher concentration layer of AFP in contact with the specific ice surfaces.

The thermal hysteresis property of AFPs[2] and AFP molecules incorporated into ice crystals observed in the ice-etching experiment[6] was seen as indirect evidence to support the Kelvin-effect explanation. However, the antifreeze mechanism proposed here is capable of explaining these two phenomena as well. Once a seed-ice crystal appears in an AFP solution, AFP molecules begin to be adsorbed reversibly on its specific ice surfaces and to form concentration gradient layers between the ice surfaces and the supercooled water. Therefore, the growth of the seed-ice crystal on the unadsorbed surfaces generates a crystal with a characteristic shape determined by the adsorption surfaces of the AFP.[2] As a result, the freezing point of this crystal is not depressed by AFP. Because AFP molecules can capture seed-ice crystals in an AFP solution, supercooled solution is formed at a temperature lower than 0 °C at 1 atm. When a supercooled water of AFP solution is frozen at a lower temperature, according to the Boltzmann distribution, there is always a portion of AFP molecules adsorbed on the ice surfaces that have smaller desorption velocities than the advancing velocity of the growing ice crystal. Therefore, AFP molecules can be embedded into ice crystals on the specific planes.

Acknowledgment. We thank the Cal State LA MBRS RISE program and NIH MBRS-SCORE Grant 5S06 GM08101 for partial support of this research.

References

(1) Ewart, K. V.; Lin, Q.; Hew, C. L. Structure, function and evolution of antifreeze proteins. *Cell Mol. Life Sci.* **1999**, *55*, 271.
(2) Duman, J. G.; Wu, D. W.; Olsen, T. M.; Urrutia, M.; Turaman, D. Thermal-Hysteresis Proteins. *Adv. Low-Temp. Biol.* **1993**, *2*, 131.
(3) Duman, J. G.; DeVries, A. L. Isolation, characterization and physical properties of protein antifreeze from the winter flounder Pseudopleunectus americanus. *Comp. Biochem. Physiol.* **1976**, *B54*, 375.
(4) Harding, M. M.; Ward, L. G.; Haymet, A. D. Type I "antifreeze" proteins. Structure−activity studies and mechanisms of ice growth inhibition. *Eur. J. Biochem.* **1999**, *264*, 653.
(5) Sicheri, F.; Yang, D. S. Ice-binding structure and mechanism of an antifreeze protein from winter flounder. *Nature* **1995**, *375*, 427.
(6) Knight, C. A.; Cheng, C. C.; DeVries, A. L. Adsorption of alpha-helical antifreeze peptides on specific ice crystal surface planes. *Biophys. J.* **1991**, *59*, 409.
(7) Houston, M. E., Jr.; Chao, H.; Hodges, R. S.; Sykes, B. D.; Kay, C. M.; Sonnichsen, F. D.; Loewen, M. C.; Davies, P. L. Binding of an oligopeptide to a specific plane of ice. *J. Biol. Chem.* **1998**, *273*, 11714.
(8) Devries, A. L.; Lin, Y. Structure of a peptide antifreeze and mechanism of adsorption to ice. *Biochim. Biophys. Acta* **1977**, *495*, 388.
(9) Wen, D.; Laursen, R. A. Structure−function relationships in an antifreeze polypeptide. The role of neutral, polar amino acids. *J. Biol. Chem.* **1992**, *267*, 14102.
(10) Chao, H.; Houston, M. E., Jr.; Hodges, R. S.; Kay, C. M.; Sykes, B. D.; Loewen, M. C.; Davies, P. L.; Sönnichsen, F. D. A diminished role for hydrogen bonds in antifreeze protein binding to ice. *Biochemistry* **1997**, *36*, 14652.
(11) Wilson, P. W. Explanation Thermal Hysteresis by the Kelvin Effect. *Cryo-Lett.* **1993**, *14*, 31.
(12) Yeh, Y.; Feeney, R. E. Antifreeze proteins: Structures and Mechanisms of Function. *Chem. Rev.* **1996**, *96*, 601.
(13) Ba, Y.; Ripmeester, J. A. Multiple quantum filtering and spin exchange in solid-state nuclear magnetic resonance. *J. Chem. Phys.* **1998**, *108*, 8589.
(14) The HPLC6 peptide (90−99%) purified from the serum of winter flounder was purchased from A/F Protein Inc. The peptide was pretreated twice by being dissolved in deuterated water (99.996%, Isotec Inc.) and then being freeze-dried. By doing this, the amino, carboxylate, and hydroxyl hydrogen atoms were replaced by deuterium atoms, which can introduce fewer protons from the AFP molecules into the deuterated water. The pretreated HPLC6 peptides were then dissolved in a 0.1 M ND_4DCO_3 deuterated aqueous solution.
(15) Atkins, P.; de Paula, J. *Physical Chemistry*; W. H. Freeman and Company: New York, 2002.
(16) Wen, D.; Laursen, R. A. A model for binding of an antifreeze polypeptide to ice. *Biophys. J.* **1992**, *63*, 1659.
(17) DeLuca, C. I.; Comley, R.; Davies, P. L. Antifreeze proteins bind independently to ice. *Biophys. J.* **1998**, *74*, 1502.
(18) Burcham, T. S.; Osuga, D. T.; Yeh, Y.; Feeney, R. E. A kinetic description of antifreeze glycoprotein activity. *J. Biol. Chem.* **1986**, *261*, 6390.
(19) Hew, C. L.; Yang, D. S. Protein interaction with ice. *Eur. J. Biochem.* **1992**, *203*, 33.

JA027557U