# Winter Flounder "Antifreeze" Proteins: Synthesis and Ice Growth Inhibition of Analogues that Probe the Relative Importance of Hydrophobic and Hydrogen-Bonding Interactions

**A. D. J. Haymet,*,† Leanne G. Ward,‡ and Margaret M. Harding*,‡**

*Contribution from the Department of Chemistry, University of Houston, Houston, Texas 77204, and School of Chemistry, University of Sydney, NSW 2006 Australia*

*Received January 13, 1998. Revised Manuscript Received August 7, 1998*

**Abstract:** Two series of mutant polypeptides of the type I, 37-residue winter flounder "antifreeze" protein have been synthesized and analyzed by nanoliter osmometry, the "ice hemisphere" test, measurement of ice growth hysteresis and circular dichroism (CD) spectroscopy. In series 1 peptides the central two threonines and all four threonines of the native protein were mutated to serine. In series 2 peptides two additional salt bridges (K7, E11 and K29, E33) were incorporated, and all four threonine residues in this sequence were mutated simultaneously to each of serine, valine, alanine, and glycine, respectively. The CD studies showed that all mutants are 100% helical in structure at low temperature, except for the glycine derivative which was estimated to be 70% α-helical. Dilute solutions of serine-substituted series 1 peptides showed no detectable, nonbasal faceting, or hysteresis behavior, indicating either no or extremely weak interaction with ice. The analogous serine-substituted mutant in series 2, as well as the glycine derivative, displayed unfaceted growth and showed no hysteresis. Hysteresis values, ice growth patterns, and the helicity measurements showed that the additional salt bridges present in series 2 peptides do not alter significantly the properties of the protein. The valine-substituted mutant gave a distinct etching pattern in which polypeptide accumulates on the $\{2\ 0\ \bar{2}\ 1\}$plane of ice 1h, and exhibited thermal hysteresis comparable to that of the native protein. In the case of the alanine-substituted mutant, reduced hysteresis behavior was measured, together with a distinct etch pattern in the ice hemisphere test. These combined results show that existing hypotheses for the action of native winter flounder peptide are incorrect; these hypotheses include models in which the -OH groups on four threonine side chains, equally spaced 11 residues apart on the 37-residue native polypeptide, are responsible for "binding" of the molecule to the ice/water interface. The antifreeze activity of the valine- and alanine-substituted mutants indicate a significant contribution to the mechanism of ice growth inhibition by type I antifreeze proteins from the hydrophobic methyl group in threonine and valine. Arguments against the importance of the role of hydrogen-bonding are summarized, and alternate ice growth inhibition mechanisms that include hydrophobic interactions are discussed.

## Introduction

In contrast to temperate and tropical fish, which freeze at approximately −0.7 °C in the presence of ice and die, almost all Antarctic notothenoid and arctic gadoid (cod) fish contain biological antifreeze compounds in the blood and in many other body fluids.[1−4] These molecules prevent ice crystal growth down to −1.9 °C or below, thus allowing the fish to survive in arctic and Antarctic waters. In this paper we test the mechanism proposed to explain the action of one class of such molecules.

Several major classes of natural ice growth inhibition ("antifreeze")[5] molecules have been identified and characterized structurally.[3,4] The antifreeze glycoproteins are carbohydrate-rich 2.6−34 kDa proteins containing an $(Ala\text{-}Ala\text{-}Thr)_n$ repeat with a disaccharide attached to threonine.[6] At least four classes of structurally independent antifreeze proteins (AFP) have been identified: type I, alanine-rich 3.3−4.5 kDa proteins found in winter flounder and sculpin;[7−9] type II, cysteine-rich globular proteins that contain five disulfide bonds;[10,11] type III, approximately 6 kDa globular proteins,[12,13] and very recently type IV, glutamate- and glutamine-rich proteins that contain α-helices but appear to be unrelated to other AFPs.[14] While detailed

* To whom correspondence should be addressed: Professor A. Haymet, e-mail: haymet@uh.edu. Associate Professor Margaret Harding, e-mail: harding@chem.usyd.edu.au.

† University of Houston.

‡ University of Sydney.

(1) DeVries, A. L. *Annu. Rev. Physiol.* **1983**, *45*, 245−60.

(2) Duman, J. G.; DeVries, A. L. *Cryobiology* **1972**, *9*, 469−472.

(3) Yeh, Y.; Feeney, R. E. *Chem. Rev.* **1996**, *96*, 601−617 and references therein.

(4) Hew, C. L.; Yang, D. S. C. *Eur. J. Biochem.* **1992**, *203*, 33−42 and references therein.

(5) The term antifreeze is colloquially used to describe these proteins. They are ice growth inhibition molecules which kinetically depress the freezing point, on a molal basis, 300−500 times more than equilibrium colligative effects predict.

(6) Shier, W. T.; Lin, Y.; DeVries, A. L. *Biochim. Biophys. Acta* **1972**, *263*, 406−413.

(7) Duman, J. G.; DeVries, A. L. *Comp. Biochem. Physiol.* **1976**, *54B*, 375−380.

(8) Sicheri, F.; Yang, D. S. C. *Nature* **1995**, *375*, 427−431.

(9) Hew, C. L.; Joshi, S.; Wang, N.-C.; Kao, M.-H.; Ananthanarayanan, V. S. *Eur. J. Biochem.* **1985**, *151*, 167−172.

(10) Ng, N. F.; Trinh, K.-Y.; Hew, C. L. *J. Biol. Chem.* **1986**, *261*, 15690−15695.

(11) Slaughter, D.; Fletcher, G. L.; Ananthanarayanan, V. S.; Hew, C. L. *J. Biol. Chem.* **1981**, *256*, 2022−2026.

(12) DeLuca, C. I.; Davies, P. L.; Ye, Q.; Jia, Z. *J. Mol. Biol.* **1998**, *275*, 515−525.

(13) Sönnichsen, F. D.; Sykes, B. D.; Chao, H.; Davies, P. L. *Science* **1993**, *259*, 1154−1157.

(14) Deng, G.; Andrews, D. W.; Laursen, R. A. *FEBS Lett.* **1997**, *402*, 17−20.

10.1021/ja9801341 CCC: $18.00 
Published on Web 01/26/1999

**Table 1.** Summary of Sequences and Code Names of Series 1 and Series 2 Peptides Studied (Important Mutations Are Highlighted)

| | |
|---|---|
| **Series 1 :** | |
| TTTT | D TASDAAAAAAL TAANAKAAAEL TAANAAAAAAA TAR-CONH$_2$ |
| SSSS | D SASDAAAAAAL SAANAKAAAEL SAANAAAAAAA SAR-CONH$_2$ |
| TSST | D TASDAAAAAAL SAANAKAAAEL SAANAAAAAAA TAR-CONH$_2$ |
| **Series 2**: | |
| TTTT2KE | D TASDAKAAAEL TAANAKAAAEL TAANAKAAAEA TAR-CONH$_2$ |
| SSSS2KE | D SASDAKAAAEL SAANAKAAAEL SAANAKAAAEA SAR-CONH$_2$ |
| VVVV2KE | D VASDAKAAAEL VAANAKAAAEL VAANAKAAAEA VAR-CONH$_2$ |
| AAAA2KE | D AASDAKAAAEL AAANAKAAAEL AAANAKAAAEA AAR-CONH$_2$ |
| GGGG2KE | D GASDAKAAAEL GAANAKAAAEL GAANAKAAAEA GAR-CONH$_2$ |

structures of a number of these proteins are now available from NMR spectroscopy and X-ray crystallography,[8,13] the mechanism by which these proteins and glycoproteins inhibit ice growth is not understood.[3,4]

The type I AFPs, present in the blood of winter flounder, yellowtail flounder, Alaskan plaice and the shorthorn and grubby sculpin are the most widely studied class of fish AFPs.[3,4] These proteins contain 11 amino acid repeat units and adopt an α-helical conformation in solution. The most well-studied type I AFP is the winter flounder peptide (*Pseudopleuronectes americanus*) "HPLC6" (TTTT, see Table 1),[7,15−17] which adopts a highly helical conformation in both solution[18] and the solid state.[8] The relatively simple structure of this peptide has spawned a number of models of the interaction of the surface of the peptide with the $\{2\ 0\ \bar{2}\ 1\}$ice surface.[8,19−25] The match between the periodicity of the polar residues and the repeat distance that separates oxygens in the ice lattice along the *a*-axis of an ice crystal was first noted by DeVries and Lin.[20] Later, full analysis of the antifreeze behavior of several type I AFPs led to a model in which the threonine residues that are aligned along one face of the helix appear to match favorably with the 1.67 nm repeat spacing along the $\langle 0\ 1\ \bar{1}\ 2\rangle$ direction in ice 1h.[19] Subsequent models[8,21−27] have included alignment of the threonine hydroxyl groups to allow hydrogen bonding with the ice surface to occur. Several models have included hydrogen bonding involving other hydrophilic residues, (aspartic acid and asparagine) along with the threonine hydroxyls.[8,21,22,25,27] However, despite macroscopic evidence, there is little or no ***molecular*** evidence to support these models, and the hydrogen bonding dominated mechanism involving threonine residues is restricted to the type I AFPs which contain the 11-residue Thr-repeat structural motif.

In an effort to clarify the key residues required for activity, a number of structure−activity studies of TTTT have been reported, including the assessment of the effect of added bulky groups on activity,[28] the role of charged amino acids,[29] the role of polar neutral amino acids,[30] and determination of the minimum length of peptide required for activity.[31,32] However, due to the introduction of more than one mutation at a time and the enormous number of possible variations in a 37-residue peptide, definitive conclusions on the essential structural features required for antifreeze activity have not been possible. In addition, almost all analogues studied to date have retained the four threonine residues at positions 2, 13, 24, and 35. In one study, the polar residues, including threonine-13, were rearranged in TTTT, and it was proposed that the asparagine and aspartic acid residues play an important role in ice binding.[30] These conclusions were supported by the X-ray structure of TTTT[8] which showed that the threonine hydroxyls do not protrude sufficiently from the proposed ice-binding surface to clear sterically hindering groups, and a less stringent hydrogen-bonding criterion, including the asparagine/aspartic acid (Asx) residues, was discussed.[8] More recently, mutations of the central two threonines (T13 and T24) to valine did not show the expected loss of activity expected if hydrogen bonding was the dominant interaction in the ice-binding mechanism.[33] However, the presence of two threonine residues in the sequence (T2, T35), along with Asx residues, did not allow hydrogen bonding involving the threonine residues to be ruled out, and it was concluded that hydrogen bonds most probably contribute to the ice-binding mechanism, along with nonpolar interactions.

To further clarify the mechanism by which type I AFPs inhibit ice growth, this paper reports the design, synthesis, and ice growth inhibition studies of a series of analogues in which all four key threonine residues, which have been assumed to be essential for activity, have been systematically varied (Table 1). Preliminary data on several analogues have been reported[34,35] and led us to propose that the relative importance of hydrophobic[36] side chains in the mechanism of ice growth inhibition needs to be reassessed.[34] In this study, the relative size, hydrogen bonding, and hydrophobic properties of all four threonine side chains in two series of winter flounder AFPs (Table 1) were systematically varied by mutations to serine, valine, alanine, and glycine, respectively. These data show that the threonine hydroxyl groups do not play a crucial role in the accumulation of the native antifreeze protein at the ice/water interface and the inhibition of ice growth below the equilibrium melting temperature. The observed hysteresis and ice growth inhibition of both the valine-substituted and alanine-substituted AFPs suggest that interactions between these hydrophobic side chains

(15) Duman, J. G.; DeVries, A. L. *Nature* **1974**, *247*, 237−238.
(16) Hew C. L.; Yip, C. C. *Biochem. Biophys. Res. Commun.* **1976**, *71*, 845−850.
(17) Hew, C. L.; Joshi, S.; Wang, N.-C. *J. Chromatogr*. **1984**, *296*, 213−219.
(18) Gronwald, W.; Chao, H.; Reddy, D. V.; Davies, P. L.; Sykes, B. D.; Sönnichsen, F. D. *Biochemistry* **1996**, *35*, 16698−16704.
(19) Knight, C. A.; Cheng, C.-H. C.; DeVries, A. L. *Biophys. J.* **1991**, *59*, 409−418.
(20) DeVries, A. L.; Lin, Y. *Biochim. Biophys. Acta* **1977**, *495*, 388−392.
(21) Wen, D.; Laursen, R. *FEBS Lett.* **1993**, *317*, 31−34.
(22) McDonald, S. M.; White, A.; Clancy, P.; Brady, J. W. *AIChE Journal* **1995**, *41*, 959−973.
(23) Chou, K.-C. *J. Mol. Biol*. **1992**, *223*, 509−517.
(24) Lal, M.; Clark, A. H.; Ruddock, J. N.; White, D. N. J. *Faraday Discuss*. **1993**, *95*, 299−306.
(25) Cheng, A.; Merz, K. M., Jr. *Biophys. J.* **1997**, *73*, 2851−2873.
(26) Jorgensen, H.; Mori, M.; Matsui, H.; Kanaoka, M.; Yanagi, H.; Yabusaki, Y.; Kikuzono, Y. *Protein Eng.* **1993**, *6*, 19−27.
(27) Wen, D.; Laursen, R. A. *Biophys. J.* **1992**, *63*, 1659−1662.
(28) Wen, D.; Laursen, R. A. *J. Biol. Chem.* **1993**, *268*, 16401−16405.
(29) Wen, D.; Laursen, R. A. *J. Biol. Chem.* **1993**, *268*, 16396−16400.
(30) Wen, D.; Laursen, R. A. *J. Biol. Chem.* **1992**, *267*, 14102−14108.
(31) Chakrabartty, A.; Ananthanarayanan, V. S.; Hew, C. L. *J. Biol. Chem.* **1989**, *264*, 11307−11312.
(32) Chakrabartty, A.; Yang, D. S. C.; Hew, C. L. *J. Biol. Chem.* **1989**, *264*, 11313−11316.
(33) Chao, H.; Houston, M. E., Jr.; Hodges, R. S.; Kay, C. M.; Sykes, B. D.; Loewn, M. C.; Davies, P. L.; Sönnichsen, F. D. *Biochemistry* **1997**, *36*, 14652−14660.
(34) Haymet, A. D. J.; Ward, L. G.; Harding, M M.; Knight, C. A. *FEBS Lett.* **1998**, *430*, 301−306. A preliminary account of peptide VVVV2KE was presented at the Annual Meeting of the Society for Experimental Biology, York, England, March, 1998.
(35) Unpublished results on TSST were cited in footnotes of ref 33.
(36) Silverstein, K. A. T.; Haymet, A. D. J.; Dill, K. A. *J. Am. Chem. Soc*. **1998**, *120*, 3166−3175 and **1998**, submitted.

(and/or the overall surface of the protein) and the growing ice crystals is more significant than previously proposed models. In addition, the results provide experimental data on which the design of potentially more active and readily available AFPs may be based.

## Experimental Section

**Peptides.** TTTT, VVVV2KE, SSSS2KE, and GGGG2KE were obtained as previously reported.[34,37] TTTT2KE, AAAA2KE, and SSSS were supplied as the *C*-terminal amides by AusPep Pty. Ltd., Melbourne, Australia. AAAA2KE was supplied HPLC purified >95% and was used without further purification. TTTT2KE was supplied ~80% pure and was purified by reverse phase HPLC.[34] TSST was synthesized by Macromolecular Resources, Department of Biochemistry, University of Colorado, and was supplied HPLC purified >95%. All peptides were >95% purity by analytical HPLC and were analyzed by electrospray mass spectrometry (see Supporting Information).

**Circular Dichroism Spectroscopy.** CD measurements were carried out on a Jasco J-710 spectropolarimeter as previously described.[34] Sample concentrations of all CD samples were determined by amino acid analysis which was carried out by AusPep Pty. Ltd., Melbourne, Australia. Molar ellipticity values were converted to mean residue ellipticity by $[\theta] = [\theta]_m/n$, where $[\theta]$ = mean residue ellipticity, $[\theta]_m$ = molar ellipticity and $n$ = number of residues. The percent helicity was calculated at 222 nm from the formula used by Greenfield et al.[38] where $[\theta]^{coil}$ represents 0% helix formation (assumed random coil) obtained from a 6 M guanidinium hydrochloride solution.

**Hysteresis Experiments and Video Microscopy.** An important fingerprint of antifreeze activity is thermal hysteresis, the difference between (i) the equilibrium melting temperature (by definition also the equilibrium freezing temperature) and (ii) the ice growth temperature, the temperature at which seed ice crystals will grow in the solution. In pure water, this difference is zero: any ice crystal seed inserted into the solution at any temperature below zero °C will grow. (The rate of growth is determined by the temperature.) The thermal hysteresis values for our peptides were measured in a Clifton nanoliter osmometer, using a method similar to ref 31. In addition, the behavior of ice growth in solutions of peptide was observed using microscopy and recorded on videotape. The temperatures at which a seed crystal in a single nanodroplet of solution shrinks and grows are defined to be the (i) melting temperature and (ii) ice growth temperature, respectively.

Measurements were made in unbuffered aqueous solutions on the original sample and at dilutions 1 in 2 and 1 in 4, with concentrations of each stock solution determined by amino acid analysis. Ice crystals were observed through a microscope, and time evolution was recorded by a JVC camera linked to a VHS video recorder. Still images (such as those shown below) were obtained from the videotape record at regular intervals over a period of 1 min. The absolute length scale in the video images was determined by taking images through the microscope at identical magnification of a 50-$\mu$m standard grid.

**Ice Hemisphere Test.** The ice hemisphere test was carried out according to a modification of the procedure of Knight et al. The first step is the same as that described by Knight et al.[19] In the second and final step,[39] this single crystal is placed in a hemispherical container holding 18 mL of the polypeptide solution, at an initial temperature of +4 °C. The crystal is grown for an additional 20−25 min. Approximately 14 mL of solution remains in the container after the crystal is removed. This yields the characteristic oriented single crystals shown below.

## Results

Two distinct families of peptides have been studied, series 1 and series 2 (Table 1). First, mutations of the four threonines in the native protein TTTT were carried out to yield SSSS and TSST (series 1). Due to the limited solubility and poor overall yield of the solid-phase synthesis of these peptides, the peptides in series 2 were designed and synthesized. To improve aqueous solubility, these peptides incorporated two additional salt bridges (K7, E11 and K29, E33: indicated by the suffix 2KE) compared to the native protein TTTT (which contains only K18, E22), on the face opposite to that containing the four key threonine residues. This modification was based on the previously reported study of TTTT2KE, which was prepared to increase the helicity of the native protein.[40]

The synthesis of peptides VVVV2KE, SSSS2KE, and GGGG2KE has been reported previously.[34] New peptides TTTT2KE and AAAA2KE were prepared to give the series 2 family of peptides. These peptides were designed to probe the importance of the threonine hydroxyl groups at positions 2, 13, 24, and 35 in the mechanism of antifreeze activity of the native protein TTTT, and the properties of the mutants were compared initially with the activity of the parent sequence TTTT2KE in this family. Compared to SSSS and TSST, the incorporation of the two additional salt bridges into the series 2 peptides greatly improved both the overall yield of the peptide synthesis and the purification of the peptides, with no problems experienced due to protein aggregation.

**Circular Dichroism.** Variable temperature CD spectra of all peptides were recorded to establish the effect of the mutations on the α-helical conformation of the peptides. We have previously reported that VVVV2KE and SSSS2KE are 100% helical at 3 °C, whereas GGGG2KE is only 70% helical under the same conditions.[34,41] Similar variable temperature spectra on peptides TSST, SSSS, TTTT2KE, and AAAA2KE showed an increase in the relative intensities of the 208/220 nm peaks as the temperature was lowered, and at 3 °C, these peptides were estimated to be 95−100% helical.[41] Heating the peptides to 50 °C destabilized the helical conformation, and CD spectra showing significantly reduced helical content and spectra approaching random coil were observed (data not shown).

**Hysteresis Experiments.** For all peptides, the thermal hysteresis behavior, namely the separation of the ice growth temperature and the melting temperature, was measured using a Clifton nanoliter osmometer. Figure 1 summarizes the hysteresis values obtained with series 2 peptides along with the published data for the native protein TTTT.[15,42] The concentration range for the mutants TTTT2KE and VVVV2KE was limited by solubility and gelling of samples.[43] No hysteresis

(37) Cheng, C.-H. C.; DeVries, A. L. *Biochim. Biophys. Acta* **1989**, *997*, 55−64.

(38) Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108−4116.

(39) The hemispherical container was suggested originally by Dr. Mike Kuiper (Swinburn University, Australia).

(40) Chakrabartty, A.; Hew, C. L. *Eur. J. Biochem.* **1991**, *202*, 1057−1063.

(41) Ward, L. G., Ph.D. Thesis, University of Sydney, 1999.

(42) Data for TTTT taken from ref 15. We have verified this curve in approximate concentration range 0−10 mg mL$^{-1}$ using partially purified fish protein. This curve has been measured independently in the concentration range 0−8 mg mL$^{-1}$ and reported in ref 33.

(43) While the aqueous solubility of the series 2 peptides was not quantified, some qualitative comments on the solubility can be made. Somewhat surprisingly, of the three analogues shown in Figure 1, AAAA2KE was the most soluble as is indicated by the concentration range achieved in Figure 1a. At higher concentrations, gelling behavior of both TTTT2KE and VVVV2KE was observed, and accurate dilutions could not be made. Analytical ultracentrifuge experiments have shown that all peptides are monomeric at ~1 mM (Mackay, J. P.; Ward, L. G.; Harding, M. M.; Haymet, A. D. J. H. unpublished results). It was expected that the four hydroxyl groups in TTTT2KE would improve aqueous solubility compared with VVVV2KE; however, in our hands this was not the case, and similar behavior was displayed by both peptides. Our original aim in incorporating the additional salt bridges was to improve aqueous solubility. Our results show that the major improvement in incorporation of these bridges was in the purification of the peptides and the overall yield of the solid-phase synthesis, which allowed amounts of 100−200 mg of material to be readily synthesized. For comparison, significantly reduced yields of SSSS were obtained on a comparable scale.

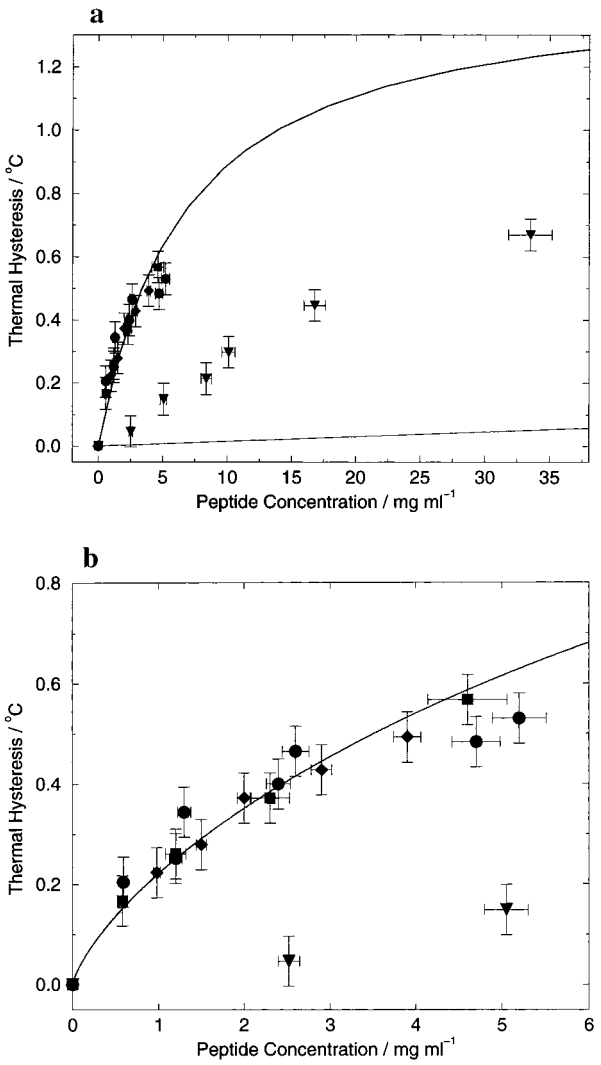


**Figure 1.** Measured thermal hysteresis as a function of concentration (a) 0 to 33 mg mL$^{-1}$; (b) 0 to 6 mg mL$^{-1}$ for solutions of TTTT (squares), TTTT2KE (diamonds), VVVV2KE (disks), and AAAA2KE (triangles). TTTT data summarized from Duman and DeVries[15] is denoted by the solid line. The thin line near zero temperature is the (thermodynamic) colligative depression of the equilibrium freezing point at these concentrations.

was observed with any of the serine-substituted analogues (TSST, SSSS, SSSS2KE) or the glycine-substituted derivative GGGG2KE.

**Video Microscopy.** The polypeptides studied here inhibit ice growth by accumulating at specific interfaces of ice, summarized in Figure 2. Images of growing ice crystals from the nanoliter osmometer are shown in Figure 3. Dilute solutions of serine-substituted polypeptides SSSS and TSST exhibit unfaceted growth. Both of the analogous mutants in series 2, SSSS2KE and GGGG2KE (Figure 3e), also display unfaceted growth. In contrast, distinct crystalline hexagonal bipyramids are observed for TTTT2KE (Figure 3f) and VVVV2KE (Figure 3g). At high concentrations, a similar but not identical ice growth pattern is observed over time with AAAA2KE (data not shown; for a single image see Figure 3d).

Figure 3a−d shows a comparison of the hexagonal bipyramids obtained with the native protein TTTT,[44] the two new

(44) Crystals in Figure 3a were grown from partially purified TTTT isolated from fish serum. The ice hemisphere was identical to that reported with pure protein and is included as a reference to compare the shape of the crystals obtained with mutants.

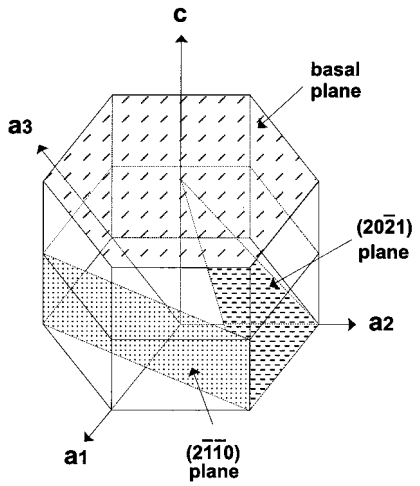


**Figure 2.** The ice crystal planes at which winter flounder antifreeze and mutants accumulate: {2 0 $\bar{2}$ 1} native compound TTTT, control compound TTTT2KE, and valine mutant VVVV2KE; {2 $\bar{1}$ $\bar{1}$ 0} alanine mutant AAAA2KE, which is the sculpin plane. The basal plane is also indicated.

active mutants in series 2, TTTT2KE and AAAA2KE, and for direct comparison previously reported VVVV2KE[34] (scales not directly comparable). For TTTT, TTTT2KE, and VVVV2KE (Figure 3a−c) the shapes of the bipyramids are essentially identical, consistent with the hemisphere results below, indicating interaction with the same interface of ice. Most interestingly, AAAA2KE (Figure 3d) displays a different, more needle-shaped hexagonal bipyramid, again consistent with evidence below that this molecule accumulates at a ***different*** interface of ice.

**Ice Hemisphere Test.** The ice hemisphere test has been described fully by Knight et al.[19] It is a simple test to determine which crystal planes (if any) are recognized by the AFP. A single ice crystal in a dilute antifreeze solution is grown into a large hemispherical single crystal, such that all interfacial orientations are present during growth. The solution is dilute so as to allow essentially unretarded ice growth. The crystallographic orientation of the antifreeze molecules is subsequently determined from measurement of the interface orientations at which antifreeze is incorporated into the growing crystal. This is done by evaporation etching of the grown crystal.

Knight et al.[19] have shown that the native peptide TTTT accumulates on the 12 equivalent {2 0 $\bar{2}$ 1}bipyramidal planes of the ice 1h crystal. The original etching experiments on TTTT were consistent with interaction with the growing ice surface. In contrast, etching of a hemisphere grown identically from the dilute solution of SSSS shows no adsorption or incorporation of the polypeptide into the ice crystal. Careful visual inspection, in a cold room at −8 °C, of a hemisphere grown from a solution of polypeptide SSSS at double the concentration used in ref 19 showed a faint etch pattern in the same location as the pattern found with the native peptide, but in our hands this very faint pattern could not be captured photographically. Polypeptide TSST showed no etch pattern (data not shown) and yielded an ice hemisphere visually identical to that grown from deionized water.

In the case of series 2 peptides, the etching pattern of the parent sequence TTTT2KE (Figure 4b) is identical to the original TTTT etch pattern (Figure 4a)[19] and yields similar patterns to those reported for VVVV2KE (Figure 4c).[34] In the case of AAAA2KE (Figure 4d), the pattern is not the same pattern observed in Figure 4a−c, characteristic of native winter flounder peptide, but instead is identical to that observed with

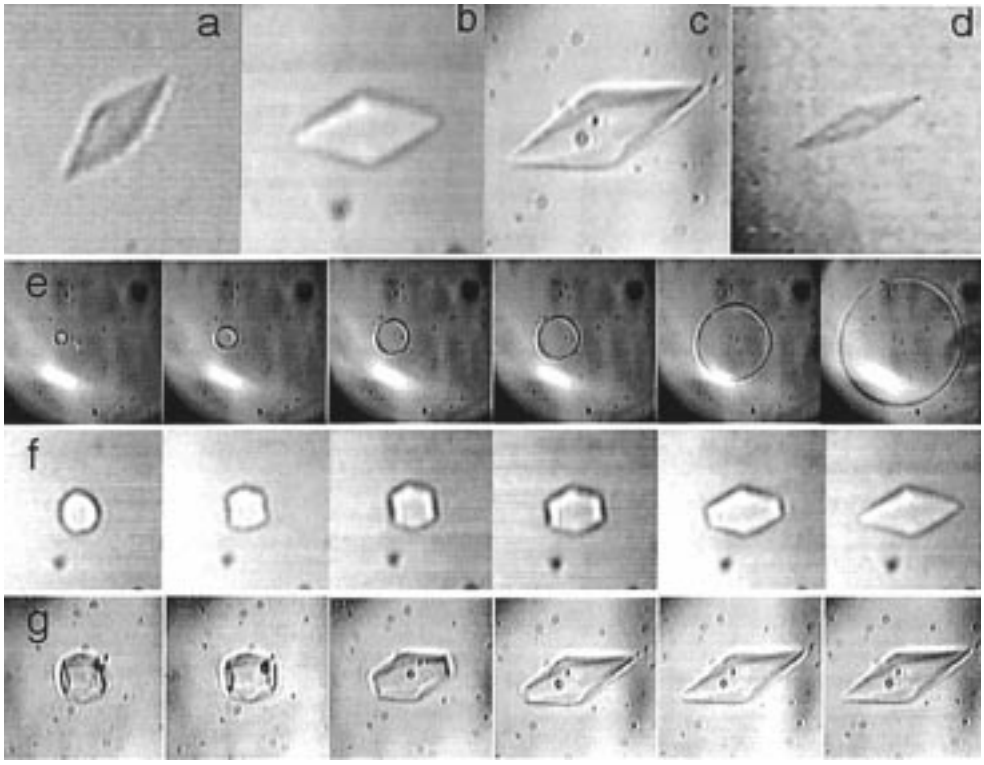


**Figure 3.** Still images of ice crystals grown from solutions of (a) TTTT (~5 mg $mL^{-1}$), (b) TTTT2KE (4 mg $mL^{-1}$), (c) VVVV2KE[34] (10 mg $mL^{-1}$), and (d) AAAA2KE (5 mg $mL^{-1}$). Video microscopy of ice crystals in the absence and presence of antifreeze proteins: (e) GGGG2KE (10 mg $mL^{-1}$), (f) TTTT2KE (4 mg $mL^{-1}$), (g) VVVV2KE (10 mg $mL^{-1}$). Still images are taken at regular intervals over a period of 1 min.

shorthorn sculpin AFP.[9,45] For comparison, Figure 4e shows the results of the ice hemisphere test with the inactive mutant GGGG2KE.

Parts f and g of Figure 4 show the side views of the VVVV2KE and AAAA2KE patterns, respectively, and the modification of ice growth caused by the peptides. Instead of the hemispherical shape of ice grown from pure water and inactive compounds under these conditions, Figure 4f shows that flattening of surfaces is caused by VVVV2KE. Data consistent with these findings were also obtained with the crystal habit test.[41,46] Figure 2 shows a diagram of the two totally different ice faces at which the ice hemisphere test proves the molecules VVVV2KE and AAAA2KE accumulate, despite the fact that only four residues differ by one -$CH_3$ group.

## Discussion

The peptides in series 1 and 2 were designed to probe the importance of the threonine hydroxyl groups at positions 2, 13, 24, and 35 in the mechanism of antifreeze activity of TTTT. In both the solid state[8] and solution,[18] TTTT adopts a highly α-helical conformation in which the four threonine residues project from one face of the helix (Figure 5). In solution, NMR studies have shown that these polar side chains are mobile and dynamic prior to interaction with the ice surface.[18] To date, models proposed[8,19,20−27] for the molecular level picture of how these proteins interact with the surface include alignment of the threonine hydroxyl groups, which are spaced at 11-residue intervals along the chain, on the ice surface in a conformation that allows hydrogen bonding with the ice surface to occur. These models assume that the threonine side chains orient themselves such that the $\gamma$-methyl groups point back toward the peptide, exposing the -OH groups for possible hydrogen bonding. Note, however, that the threonine side chains may also orient themselves such that the $\gamma$-methyl groups project onto the surface of the protein (see CPK models in Figure 5) and hence provide a highly hydrophobic surface on the protein. In fact this latter orientation is seen to be preferred in computer simulations of TTTT in water[47] and also in the crystal structure of TTTT.[8] From the mutants and truncated analogues of TTTT that have been studied to date,[27−32,40] significant ice growth inhibition has always been accompanied by a highly helical conformation, estimated by circular dichroism to be >80%.

The peptides reported in this paper and our previous study,[34] are the first examples of winter flounder analogues that have incorporated systematic changes to all four threonine side chains to test the hypothesis that the mechanism depends on hydrogen bonding by threonine residues. Our studies show unambiguously that the threonine -OH groups are ***not*** required for antifreeze activity. There are six major differences between the side chains incorporated into the mutated sequences that may have an influence on the antifreeze mechanism: (i) hydrogen bonding, (ii) hydrophobicity, (iii) relative size of side chain, (iv) helix propensity of mutations, (v) dipole moment, and (vi) chirality of threonine side chain.

There is a simple general argument against the role of hydrogen bonding. In the past authors have cited[46] a favorable enthalpy of interaction between the solute and the ice surface formed by four or more threonine residues (and, in addition, possibly other residues). We shall denote that quantity $\Delta H_{\text{solute}}^{\text{ice}}$. The traditional argument implies simply that this number is negative and hence provides a mechanism for interaction between the solute and ice surface. However, this argument neglects the interactions which these same residues would have

(45) Wierzbicki, A.; Taylor, M. S.; Knight, C. A.; Madura, J. D.; Harrington, J. D.; Sikes, C. S. *Biophys. J.* **1996**, *71*, 8−18.

(46) Knight, C. A.; DeVries, A. L.; Oolman, L. D. *Nature* **1984**, *308*, 295−296.

(47) Haymet, A. D. J.; Kay, P. A. Molecular Dynamics Simulation of a Fish "Antifreeze" polypeptide (AFP), *Society for Cryobiology 29th Annual Meeting (CRYO '92)*, Ithaca, New York, June 15, 1992; page 17.

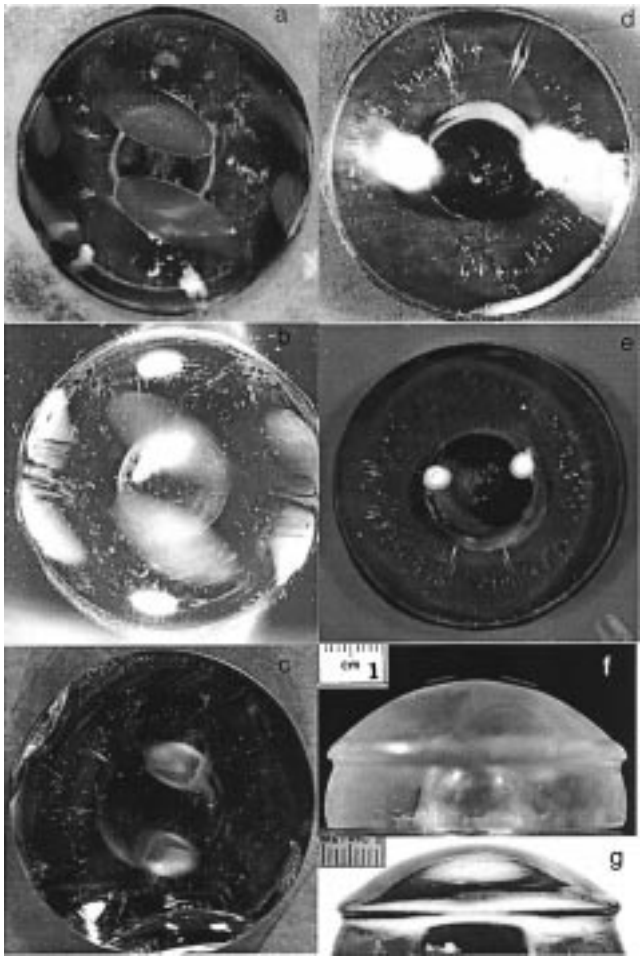


**Figure 4.** Top view of oriented single-crystal ice hemispheres grown from 0.03 mg $mL^{-1}$ solutions of (a) TTTT,[19] (b) TTTT2KE, and (c) VVVV2KE[34] for comparison (d) AAAA2KE and (e) GGGG2KE. The ice 1h *c*-axis points vertically up the page. Lighting is provided by two lamps above the hemispheres, seen clearly in (e) the etch-free pattern. Side views of (f) the VVVV2KE hemisphere in panel (c), where straight white lines have been added above the crystal to highlight the distortion of the hemisphere into planes due to the presence of peptide, and (g) the AAAA2KE hemisphere in panel (d).

with water, which we denote $\Delta H_{\text{solute}}^{\text{water}}$. The crucial quantity is of course the **difference** $\Delta H_{\text{solute}}^{\text{ice}} - \Delta H_{\text{solute}}^{\text{water}}$. Although the sign of this difference is unknown from basic physical chemistry, and awaits further experiments and/or theory, its magnitude can be confidently estimated to be small, at most a few tenths of a kcal/mole; hence, it likely plays little or no role in the mechanism of action of these polypeptides. General considerations of the ease of hydrogen bonding between the solute and molecules in the water phase, which can adjust their position to make a more optimal hydrogen bond as opposed to molecules in the ice phase constrained to oscillate about lattice positions, lead to an argument that the difference $\Delta H_{\text{solute}}^{\text{ice}} - \Delta H_{\text{solute}}^{\text{water}}$ is in fact positive and *further* argues against hydrogen bonding as a possible mechanism. Note that the earlier discussions of this mechanism assign (implicitly or otherwise) the quantity $\Delta H_{\text{solute}}^{\text{water}}$ the erroneous value zero. Further elaboration of this argument requires calculation or estimation of relative free energies, a task underway via molecular simulation.[48]

Specifically, we discuss the results of series 1 peptides, TSST and SSSS. Incorporation of serine residues into these sequences

(48) Hayward, J. A.; Haymet, A. D. J. *J. Chem. Phys*., manuscript in preparation.

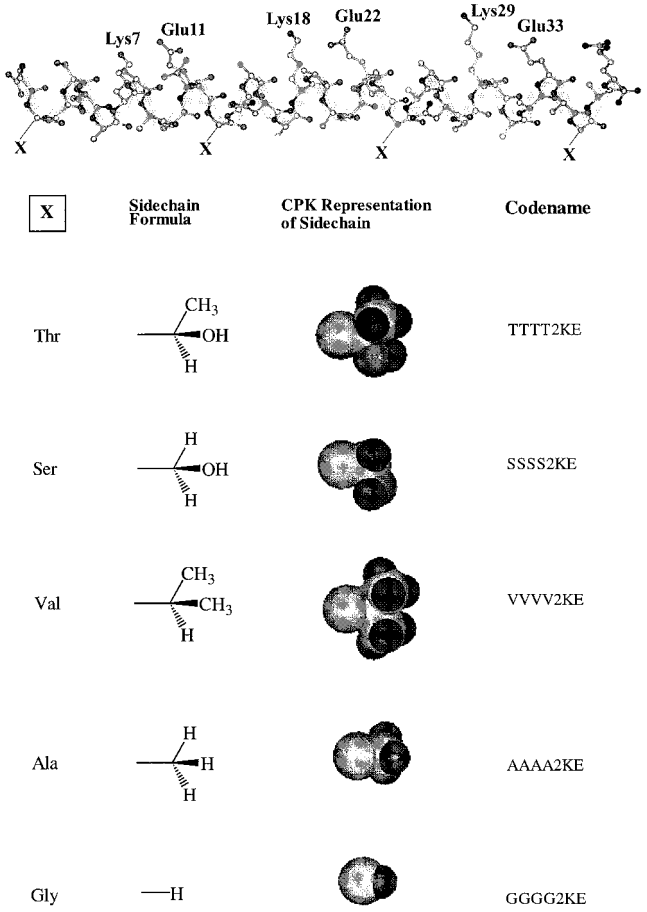


**Figure 5.** Computer-generated representation (MSI InsightII) of the α-helical conformation of series 2 peptides, highlighting the structural features of the side chains of the mutated residues. For clarity, the α-carbon is included in the CPK representation.

did not alter significantly the α-helical conformation of the peptides, which still retain the side chain mobility, and ability to hydrogen bond to the ice/water interface in a manner similar to that predicted by both solid-state[8] and solution structures[18] of TTTT. Peptide SSSS lacks the $\gamma$-methyl groups present in TTTT and hence can only provide the hydroxyl groups of the four serines on the surface of the protein in an α-helical conformation. Peptide TSST also has the potential to adopt a conformation in which all four hydroxyl groups are aligned on the surface. No interaction with the ice surface was detected for either SSSS or TSST by the ice hemisphere test or the crystal habit test, and no measurable hysteresis was detected. These results alone point to the importance of the $-CH_3$ group rather than -OH. These conclusions are supported by an independent study of TSST and TVVT:[33] the results for TVVT showed ice growth patterns similar to that of the native protein suggesting that hydrogen bonding involving the central two threonines is not crucial to antifreeze activity.[33]

Series 2 peptides confirmed our conclusions made from series 1 peptides. Due to the improved overall synthetic yield of series 2 peptides,[34,43] the additional mutations of alanine and threonine were incorporated in the 2KE family of peptides to further probe the hydrophobic contributions of the threonine methyl groups in the mechanism of antifreeze activity. The alanine analogue AAAA2KE contains a hydrophobic methyl group, but the side chain is significantly smaller than either threonine or valine, and thus provides complementary data to VVVV2KE, and SSSS2KE (which lack the hydroxyl or the methyl group

respectively) and GGGG2KE (which lacks both the methyl and hydroxyl group).[34]

The solution conformations of the series 2 peptides were studied using CD spectroscopy. The CD studies on TTTT2KE and AAAA2KE gave very similar results to VVVV2KE and SSSS2KE,[34] and all four peptides are 100% helical in structure at low temperature as expected on the basis of helix propensities of amino acids.[49]

Prior to performing antifreeze studies, experiments were carried out to show that the introduction of the additional two salt bridges does not alter the ice-binding properties of the native peptide. Limited ice growth inhibition properties of TTTT2KE have been reported previously.[40] Hysteresis values, ice growth patterns, and the helicity of TTTT2KE all gave results comparable to those obtained with the native protein TTTT, showing that the additional salt bridges do not alter significantly the properties of the protein.

Of the four mutations listed in series 2, only the valine and alanine analogues exhibited antifreeze activity. The valine analogue VVVV2KE showed the ability to inhibit ice growth in a manner similar to the parent sequence TTTT2KE. This analogue altered the growth habit of the seed crystal, forming a bipyramidal shaped crystal. Ice growth was stopped completely in a temperature range where growth should have occurred thermodynamically, and hysteresis was observed at a magnitude comparable to both TTTT and TTTT2KE. Since the substitution of threonine to valine does not alter the overall size of the side chain or the helical conformation of the peptide (but does remove the inherent chirality present in threonine), we conclude that the hydrophobic methyl group in the threonine (and valine) residues plays a dominant role in the way the protein interacts with the diffuse ice/water interface.[48,50,51]

The importance of hydrophobic interactions is supported by the results obtained with AAAA2KE. This analogue, while altering the growth habit of a seed crystal, appears to be about half as "active" as the native and valine-substituted proteins at any fixed concentration: the hysteresis curve (Figure 1) is reduced in comparison to the other active analogues. In the ice hemisphere test, AAAA2KE shows no winter flounder pattern, but rather a clear, sculpin pattern,[45] which corresponds to preferential accumulation of the peptide on the $\{2\ \bar{1}\ \bar{1}\ 0\}$ plane. When one considers the surface of the protein (see CPK representation of side chains shown in Figure 5), the smaller size of the side chain means that the alanine methyl groups do not protrude on the protein surface as far as the methyl groups in either TTTT2KE or VVVV2KE. Since this peptide is also 100% helical, the results may indicate that the weaker interaction of AAAA2KE with the ice surface is due to the fact that the hydrophobic alanine methyl groups are unable to interact as closely with the ice surface due to steric effects of the neighboring (more bulky) amino acid side chains. The sculpin pattern observed with AAAA2KE is unusual; it has been proposed that the charged residues in the shorthorn sculpins interact with specific water molecules in the ice surface.[45] Since the series 2 peptides contain additional charged residues, interaction of these residues in AAAA2KE with the ice surface in a manner analogous to the shorthorn sculpin cannot be ruled out. However, since these patterns have not been observed for any of the other active 2KE mutants and the spacing of the salt bridges differs from the spacings in the sculpin sequence, this mechanism seems unlikely.

No evidence for the interaction of either SSSS2KE or GGGG2KE with any ice surface was detected. The results for SSSS2KE are consistent with that for the other series 1 serine-substituted analogues TSST and SSSS. The negative result for GGGG2KE may be attributed to the reduced helicity of the peptide,[34] the lack of a methyl group, or both. We believe it is most likely that the lack of a methyl group is the major reason for no activity.

It has been suggested that both the asparagine (N16, N27) and aspartic acid (D1, D5) residues in the native protein may be important in hydrogen bonding to the ice surface.[8,21,22,25,27] In the solid state[8] these side chains are in fact more accessible compared with the threonine hydroxyls. Note that our view of the ice/water interface,[48,50,51] derived from computer simulations and supported by ellipsometry,[52] is very different from the ice/vacuum model implicit in other work.[8,21,22,25] In this paper, we have addressed only the hydrogen-bonding contribution of the threonines to antifreeze activity, and since the Asx residues are present in all our sequences, hydrogen bonding involving these residues remains a possibility. However, we believe that the activity demonstrated by both VVVV2KE and AAAA2KE has identified an important hydrophobic contribution in the activity of the type I AFPs. While the complete molecular mechanism of ice growth inhibition is not fully understood, and may involve dipolar and other interactions, we have suggested in a previous paper[34] that the overall shape of the molecule may be sufficient to inhibit ice growth and that hydrophobic effects may dominate this interaction.

Series 2 peptides contain three distinct regions, the mutations introduced at positions 2, 13, 24, and 35 (Figure 5, residues labeled X) which project onto one face of the α-helix, the hydrophilic region containing the Asx residues (D1, D5, N16, N27), and the localized salt bridge region of the peptide. The fact that introduction of two additional salt bridges (K7, E11 and K29, E33) does not alter the ice growth inhibition properties of the peptide is consistent with the interaction of the opposite face of the protein with the ice/water interface. Analysis of the features of the active mutants (X = threonine, valine, and alanine) shows the presence of a common hydrophobic face on the protein. In the case of threonine, this is achieved with the methyl group on the surface of the protein. We propose that this hydrophobic side is facing the ice/water interface, with the opposite side interacting strongly with the surrounding liquid water molecules, consistent with the detailed molecular level picture of the ice/water interface.[48,50,51] Perturbation of the hydrophobic face (encompassing A10/25 to A9/X24) in a number of synthetic analogues[28,30] has always been accompanied by a decrease in antifreeze activity. The hydrophilic region encompassing L12/N27 to D5/X35 may also interact with the ice/water interface or a combination of the residues in the hydrophilic and hydrophobic regions. Systematic mutations of the hydrophilic residues will allow the importance of any hydrogen bonding involving these residues to be determined and will clarify the residues that are directly involved in contact with the ice surface. We note, however, that the detailed molecular mechanism which causes the native compound TTTT and TTTT2KE and VVVV2KE to accumulate on the $\{2\ 0\ \bar{2}\ 1\}$-face, but the very closely related AAAA2KE to accumulate on the $\{2\ \bar{1}\ \bar{1}\ 0\}$face, is not known, and this alternate hypothesis involving the hydrophobic face of the protein does not explain

(49) Chakrabartty, A.; Kortemme, T.; Baldwin, R. L. *Protein Sci*. **1994**, *3*, 843−852.

(50) Karim, O. A.; Haymet, A. D. J. *J. Chem. Phys*. **1988**, *89*, 6889−6896.

(51) Karim, O. A.; Kay, P. A.; Haymet, A. D. J. *J. Chem. Phys*. **1990**, *92*, 4634−4635.

(52) Beaglehole, D.; Wilson, P. W. *J. Phys. Chem.* **1993**, *97*, 11053−11058.

the specific interaction of the native and mutant polypeptides with specific faces.

Finally, since our VVVV2KE mutant appears to be as active as the native protein, one may ask why the natural protein TTTT (as well as other type I AFPs) have evolved with threonines instead of valines (or a mixture of both). Although solubility may be a contributing factor, our results to date suggest this effect is minimal.[48] However, solubility effects may manifest themselves in longer type I AFPs containing more than three repeat units. The chirality of this side chain may also be important and play a role in recognition of specific ice planes. Although the chirality of the amino acids has been studied and the all D-isomer synthesized and shown to interact with the mirror-related ice plane,[21] the chirality of the threonine side chain that determines the orientation of the methyl group has not been studied.

## Conclusions

The ice growth inhibition properties of analogues of the winter flounder antifreeze protein, in which the four threonine residues have been mutated systematically, do not support the current hypotheses for the mechanism of action of the native protein. Models discussed in the literature to date include the -OH groups on four threonine residues, equally spaced 11 residues apart on the 37-residue native polypeptide, binding the protein to the ice/water interface. By replacing these four -OH groups by $-CH_3$ (threonine to valine mutation) and showing that the ice growth inhibition properties are unchanged, we have disproved hypotheses that are dominated by hydrogen bonding involving these threonine -OH groups. Further, by mutating these four -OH groups to a smaller side chain (threonine to alanine mutation), we have found a peptide with closely related (but not identical) ice growth inhibition properties. Although the involvement of other hydrophilic residues in hydrogen bonding, notably asparagine and aspartic acid residues, cannot be ruled out, our results show that there is a significant contribution of the hydrophobic methyl groups in the threonine (and valine) side chains in antifreeze activity. The general argument against the importance of hydrogen bonding in this process is presented. Molecular simulations of the interaction of the hydrophobic face (see Figure 5) with the diffuse ice/water interface[50] may provide further insight into the exact mechanism of action. The synthesis and study of further mutants are underway to further test this new hypothesis.

**Acknowledgment.** Financial support in Sydney from the Australian Research Council (A.D.J.H. and M.M.H.) and in Houston from the ACS-PRF (A.D.J.H.) is acknowledged gratefully. A.D.J.H. thanks Dr. Charles Knight and Professor Art DeVries for many helpful discussions and Jennifer Hayward for assistance with Figure 2. A.D.J.H. and L.G.W. thank Yumi Jin (Illinois) for assistance with the Clifton nanoliter osmometer. L.G.W.'s travel to the University of Illinois was funded by a University of Sydney James Kentley Memorial Scholarship for which grateful acknowledgment is made. We thank Dr. Charles Knight for assistance with the ice hemisphere tests.

**Supporting Information Available:** HPLC chromatograms of crude and purified peptides, electrospray mass spectra of purified products, and temperature-dependent CD of peptides (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA9801341