Valine substituted winter flounder 'antifreeze': preservation of ice growth hysteresis

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Abstract Three mutant polypeptides of the type I 37-residue winter flounder 'antifreeze' protein have been synthesized. All four threonine residues in the native peptide were been mutated to serine, valine and glycine respectively and two additional salt bridges were incorporated into the sequences in order to improve aqueous solubility. The peptides were analyzed by nanoliter osmometry, the 'ice hemisphere' test, the 'crystal habit' test, measurement of ice growth hysteresis and CD spectroscopy. While the valine and serine mutants retain the α-helical structure, only the valine mutant retains 'antifreeze' activity similar to that of the native protein. 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.

Key words: Antifreeze; α-Helical peptide; Ice growth inhibition; Threonine residue; Hysteresis

1. Introduction

In both pure water and aqueous solutions, 'antifreeze' proteins (AFP) and glycoproteins prevent the growth of ice crystals at temperatures below the equilibrium melting point [1,2]. The type I AFPs found in the blood of the winter flounder *Pseudopleuronectes americanus*), Alaskan plaice (*Pleuronectes quadritaberulatus*) and the sculpin (*Myoxocephalus scorpius*) are alanine rich α-helical peptides that have been studied extensively (for review see [3–8]). A number of studies have been reported on type II and type III AFPs, which are larger globular proteins and structurally more complex than the type I AFPs [3]. The identification of an additional class of fish ice growth inhibitor proteins [9], and highly active natural antifreeze proteins in insect larvae [10,11], has increased the importance of establishing the exact molecular mechanism by which each class of AFPs is able to inhibit the growth of ice.

The best characterized AFP is the 37-residue, alanine rich α-helical type I polypeptide from the winter flounder. While the effect of the native protein (TTTT) on the growth of ice crystals has been known for some time [1,2], the exact molecular mechanism by which the peptide inhibits ice growth is unknown. A number of models have been proposed for the interaction of the polypeptide with the ice surface involving the threonine and asparagine/aspartic acid (Asx) residues in the protein [12–18]. These models rely on the formation of hydrogen bonds between hydroxyl groups of the threonine residues in the protein and certain crystallographic planes of ice with similarly placed hydrogen bonding sites. This picture has provided a useful model of the ice/protein interaction, since it keeps the molecules at the ice interface in approximately their bulk ice lattice positions.

While a large number of mutants of TTTT have been synthesized and their solution conformation and ice growth inhibition patterns studied [19–24], almost all analogues studied to date have retained the four threonine residues at positions 2, 13, 24 and 35, which have been assumed to be essential for activity. In one study, the polar residues were rearranged in TTTT and it was proposed that the asparagine and aspartic acid residues play an important role in ice binding [19]. During the last 5 years, at least two groups have replaced the central two threonine residues with either serine or valine ([25]; Haymet, A.D.J., Ward, L.G., Booth, M.J., Harding, M.M., Driggers, E. and Knight, C.A. (1995) unpublished results, reported in footnotes of [25]). While the serine analogue was virtually inactive in inhibiting ice growth, there was only a minor loss of activity in the valine analogue. Very recently, one group has proposed a diminished role for hydrogen bonds in antifreeze protein binding to ice [25].

In order to remove the ambiguity of having two threonine and two mutated residues, and to clarify the role of the threonine residues, this work describes systematic changes to all four threonines of TTTT to give the polypeptides VVVV2KE, SSSS2KE and GGGG2KE. Two additional salt bridges were incorporated into the peptide design in order to improve aqueous solubility, and since these additional residues had been incorporated previously into TTTT without affecting antifreeze activity [24].

	1 2	13	24	35
TTTT	D TASDAAAAAAL	TAANAKAAAEL	TAANAAAAAAA	TAR-CONH$_2$
VVVV2KE	D VASDAKAAAEL	VAANAKAAAEL	VAANAKAAAEA	VAR-CONH$_2$
SSSS2KE	D SASDAKAAAEL	SAANAKAAAEL	SAANAKAAAEA	SAR-CONH$_2$
GGGG2KE	D GASDAKAAAEL	GAANAKAAAEL	GAANAKAAAEA	GAR-CONH$_2$

2. Materials and methods

2.1. Peptides

The native antifreeze peptide from the winter flounder *P. americanus* is the same as the material used by Knight et al. [12] in earlier experiments. It was purified from fish serum by Sephadex G75 gel filtration and reverse-phase HPLC, and sequenced and characterized by the methods of Cheng and DeVries [26]. Crude mutant peptides were synthesized as the C-terminal amides by Macromolecular Resources, Fort Collins, CO, USA or AusPep Pty. Ltd., Melbourne, Australia and were purified by reverse-phase HPLC (Vydac 218TP1022 column, AB gradient of 10–45% B over 90 min; solvent A 0.05% trifluoroacetic acid/water, solvent B 0.05% TFA/acetonitrile).

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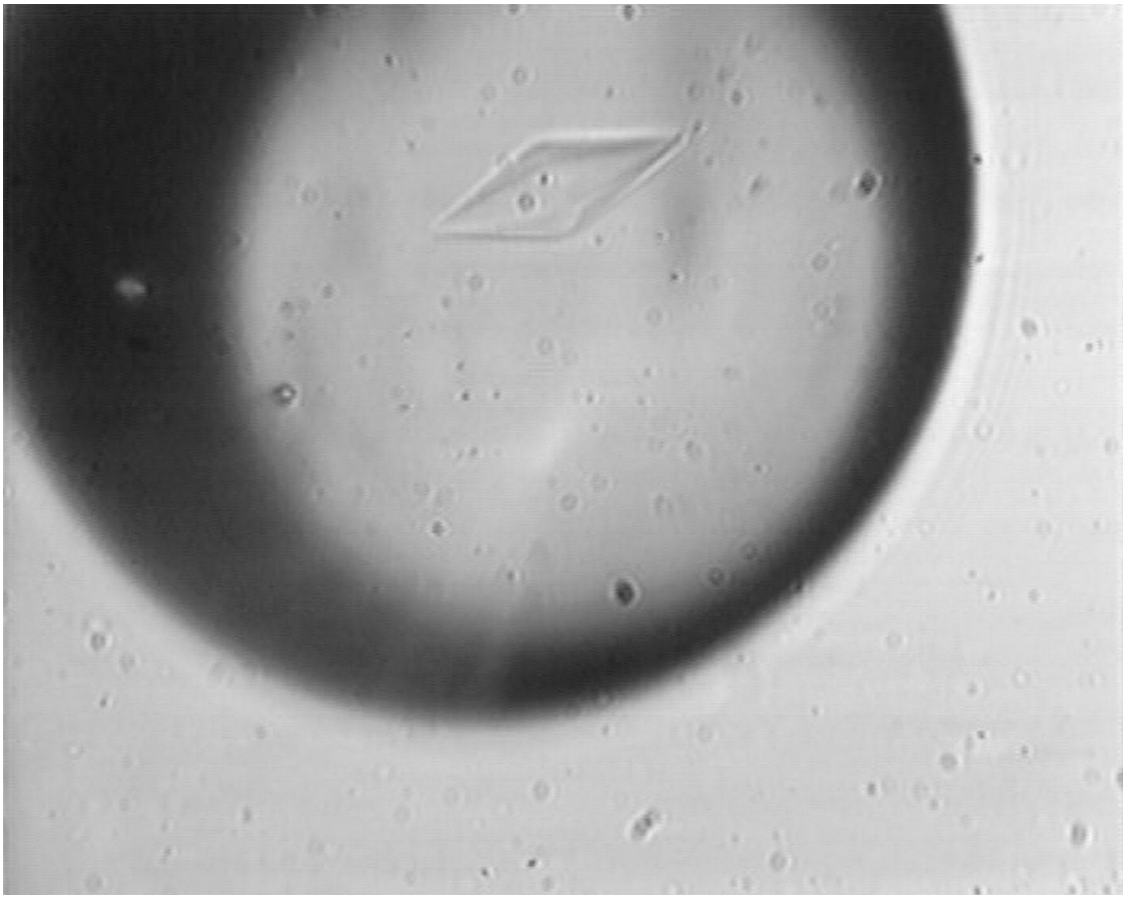

Fig. 1. Bipyramidal ice crystal grown from an unfaceted seed crystal in a 9.8 mg/ml solution of VVVV2KE in water.

Electrospray ionization mass spectrometry gave the expected +2 and +3 molecular ion peaks: 1733.3 and 1155.3 (VVVV2KE), 1709.3 and 1139.8 (SSSS2KE) and 1649.5 and 1099.7 (GGGG2KE).

2.2. Circular dichroism (CD)

CD measurements were made using a Jasco J-710 spectropolarimeter equipped with a water-jacketed cell of 0.1 cm connected to a NESLAB RTE-111 water bath. Peptide samples were between 0.1 and 0.5 mg/ml in water or 100 mM NH_4HCO_3 buffered solutions, at pH 8.5. The sample pH was adjusted using 0.1 M NaOH and HCl solutions as required. Variable temperature measurements were made at regular intervals between 3°C and 50°C. Sample concentrations for CD and ice binding studies were determined by amino acid analysis which was carried out by AusPep Pty. Ltd., Melbourne, Australia.

2.3. Crystal habit test [27]

To observe ice growth, 30 μl of a dilute solution of peptide, concentration <2 mM (<1 mg/ml), was placed into a flat, glass capillary, of internal dimensions 0.3 mm × 3 mm × 5 cm. This was mounted on a temperature-gradient microscope stage inside a cold room at −8°C. One end of the solution is frozen, and the gradient adjusted so that the water/ice interface is near the middle of the capillary. Then the capillary is moved so that the ice grows into the solution. The solution and the capillary are cooled by slow, even thermal conduction through the glass plates, and hence the ice crystal grows evenly, quickly at first, then more slowly, as the steady-state thermal gradient is re-established. At magnifications of ×50–100, different solutions display characteristic facets on the growing ice crystal.

2.4. Ice hemisphere test

The ice hemisphere test was carried out according to the procedure of Knight et al. [12]. Ice hemispheres of peptides were grown at a concentration of 0.06 mg/ml, for direct comparison with existing data from ice hemisphere grown in the same concentration of native peptide TTTT [12]. In addition, a hemisphere was grown from water as a reference. In all cases, single crystals of ice 1h were grown from a single crystal seed.

3. Results

3.1. Peptide design and synthesis

Peptides VVVV2KE, SSSS2KE and GGGG2KE 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 contained mutations of all four threonines to serine, valine and glycine respectively. Two additional salt bridges (indicated by the suffix 2KE) were incorporated into the native protein on the opposite face to that it contained the four key threonine residues as a means of improving aqueous solubility. This modification was based on the previously reported study of the native protein TTTT containing two additional salt bridges as a means of increasing the helicity of the native protein [24]. All peptides were purified to $>95\%$ by reverse-phase HPLC and were characterized by electrospray mass spectrometry. The incorporation of the additional salt bridges greatly improved both the overall yield of the peptide synthesis and the purification of the peptides with no problems experienced due to protein aggregation.

3.2. Nanoliter osmometry

Fig. 1 shows the result of growing an unfaceted seed crystal of ice in a solution of VVVV2KE in water at a concentration of 9.8 mg/ml (2.8 mM), using a Clifton Nanoliter Osmometer.

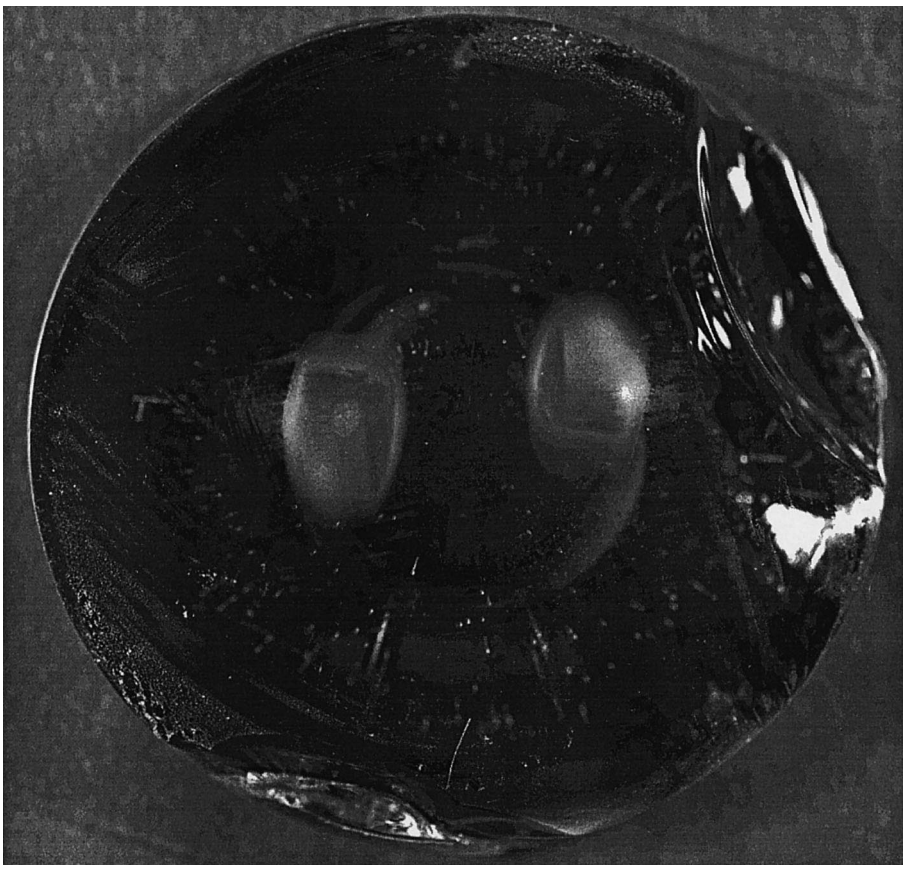

Fig. 2. Top view of oriented, single crystal ice hemisphere grown from a 0.10 mg/ml solution of VVVV2KE in water. The etch patterns are oriented on the $\{2\ 0\ \bar{2}\ 1\}$ plane of ice 1h.

As the temperature is lowered below the melting temperature, the ice seed immediately facets and does not grow further. At approximately 0.1°C below the melting temperature, the seed crystal grows out to the regular hexagonal bipyramidal crystal (approximate length 70 μm) shown in Fig. 1, captured from a video tape of the entire experiment. Upon further supercooling to 0.5°C below the melting temperature, no change is observed in this bipyramid, even after 2 h. As the temperature is lowered still further, the bipyramid grows slowly until essentially the entire solution phase has been incorporated into the bipyramidal ice crystal. This process was cycled repeatedly with no change to the result. This hysteresis behavior for

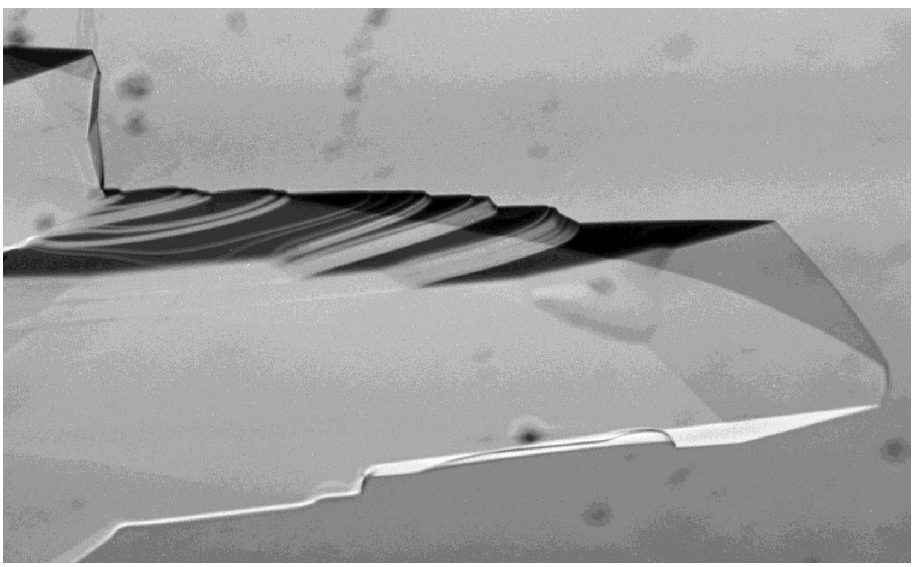

Fig. 3. Ice crystals grown in a linear temperature gradient from a 0.15 mg/ml solution of VVVV2KE in water.

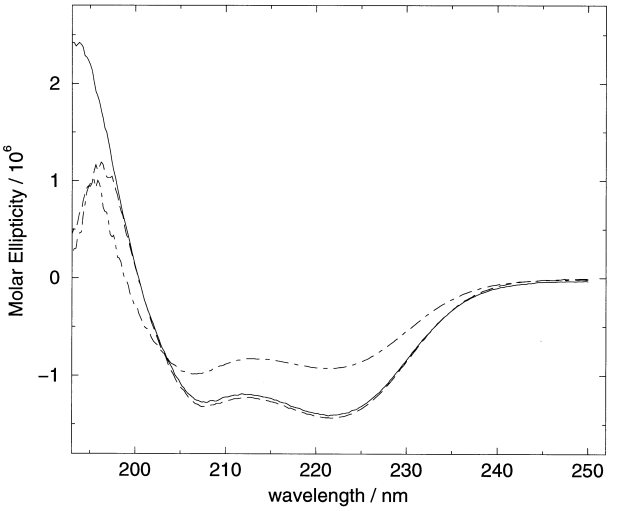

Fig. 4. CD spectra of VVVV2KE (solid line), SSSS2KE (dashed line) and GGGG2KE (dot dashed) in water at 3°C and pH 8.5.

VVVV2KE (the separation of the ice growth temperature and the melting temperature) is concentration dependent, and compared with the native protein TTTT is about half as active at a concentration of 10 mg/ml [28]. Similar experiments were carried out with SSSS2KE and GGGG2KE, but neither peptide exhibited any hysteresis behavior.

3.3. Ice hemisphere test

The ice hemisphere test has been described fully by Knight et al. [12]. 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 so dilute 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.

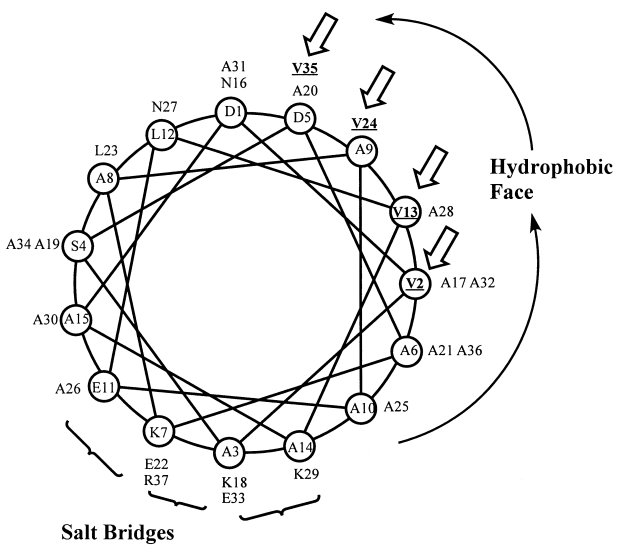

Fig. 5. Helical wheel representation of VVVV2KE, looking down the helix axis, derived from the crystal structure coordinates of TTTT [16]. Valine mutations indicated by arrows.

Macroscopic, oriented, single crystals of ice, grown at temperatures slightly below the ice growth inhibition temperature from a dilute solution (0.1 mg/ml) of VVVV2KE, showed the etching pattern shown in Fig. 2. Similar patterns were generated over a wide range of concentrations. Analysis of this hemisphere shows that the polypeptide accumulates on the $\{2\ 0\ \bar{2}\ 1\}$ plane of ice 1h, the same interface at which the native protein TTTT is known to accumulate [12]. Under identical conditions, no patterns were observed for either SSSS2KE or GGGG2KE.

3.4. Crystal habit test

The crystal habit [27] test relies upon the fact that the antifreezes alter ice growth habit, because their inhibition of crystal growth is orientation specific. Pure water exhibits only basal faces as facets, at any growth rate. For most solutes, ice growing from slightly supercooled, dilute solution forms rounded plates or dendrites normal to the *c*-axis. However, in the presence of fish antifreeze proteins and glycoproteins, the ice growth is qualitatively different. At high concentration the ice grows as 'spicules', and at low concentration the ice is bounded by facets [27,29]. The growth forms are observed easily at magnifications of 50–100×. Sharp faceting anywhere except on the basal plane is a diagnostic of orientation-specific surface interaction, which is the fundamental antifreeze property. This is an extremely sensitive test, which can detect antifreeze-active compounds above 10^{-7} weight% concentration of the fish antifreezes, including the native winter flounder protein. Measurement of the actual, non-equilibrium ice growth point is not nearly as sensitive.

Crystals of ice grown in a linear temperature gradient in a dilute solution (0.1 mg/ml) of VVVV2KE showed facets and equally remarkable stepped ledges in the ice, shown in Fig. 3. In previous studies by our group, no stepped ledges have been observed in solutions of the native compound TTTT. Pure water, and solutions of SSSS2KE and GGGG2KE, displayed unfaceted growth.

3.5. Circular dichroism

Variable temperature CD spectra of all mutants were recorded in order to establish the effect of the mutations on the α-helical conformation of the peptides. Fig. 4 shows the % helicity at various temperatures for the three analogues VVVV2KE, SSSS2KE and GGGG2KE. At 3°C, analysis of these spectra shows that both VVVV2KE and SSSS2KE are essentially 100% α-helical, while GGGG2KE shows only 70% helical structure. CD measurements of VVVV2KE in water showed no variation in the helicity of this peptide in the pH range 3–11.

4. Discussion

In both the solid state [16] and solution [30], the native winter flounder protein TTTT adopts a highly α-helical conformation in which the four threonine residues project from one face of the helix. In solution, NMR studies have shown that these polar sidechains are mobile and dynamic prior to interaction with the ice surface [30]. All proposed models 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 protein surface in a conformation that allows hydrogen bonding with the ice surface to occur [12–16]. However, the threonine sidechains may also orient themselves such that the γ-methyl groups project onto the surface of the protein, and hence provide a highly hydrophobic surface on the protein. Indeed, the crystal structure of TTTT reported in 1995 [16] showed 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 was proposed.

Variable temperature CD measurements showed that VVVV2KE and SSSS2KE adopt a similar, highly helical conformation in solution, comparable to that of the native protein TTTT. As expected, based on helical propensities of amino acids [31], the presence of four glycines in GGGG2KE reduced the helicity of this peptide to ~70%. The two additional salt bridges have been incorporated previously into the native protein as a mechanism of increasing the helicity of the peptide through formation of two stabilizing salt bridges [22]. The antifreeze activities of the native protein TTTT and the salt bridge analogue (TTTT2KE) were identical; while both peptides affected the growth rates of ice crystals, the TTTT2KE mutant was active at 7–8-fold lower concentrations than the native protein [24].

The ice growth inhibition and interaction properties of polypeptides VVVV2KE, SSSS2KE and GGGG2KE were analyzed by (i) nanoliter osmometry, (ii) the 'ice hemisphere' test [12], (iii) the 'crystal habit' test [27], and (iv) measurement of ice growth hysteresis. Using these four tests, no evidence for the inhibition of ice growth by either SSSS2KE or GGGG2KE was observed. In contrast, in the case of VVVV2KE, the hysteresis and ice growth patterns (Figs. 1–3) show that the replacement of the hydroxyl groups on all four threonines by a methyl group (threonine to valine) does not change the fundamental ice growth inhibition and ice interface interaction properties of the native protein TTTT, and may indicate a weakened inhibition of ice crystal growth. Hence, these data do not support models that have proposed that the threonine -OH groups play a special role in the interaction with the ice surface.

The results obtained with VVVV2KE, SSSS2KE and GGGG2KE, which are the first examples of type I AFPs in which all four threonines have been mutated, show conclusively that hydrogen bonding through threonine residues to the ice surface is not necessary for strong antifreeze action. These results are consistent with our own studies which mutated the middle two threonines (Haymet, A.D.J., Ward, L.G., Booth, M.J., Harding, M.M., Driggers, E. and Knight, C.A. (1995) unpublished results, reported in footnotes of [25]), and by recent ice binding studies of TVVT and TSST which infer a reduced role for hydrogen bonding in ice binding [25]. However, since these mutants still contain two threonine residues, hydrogen bonding through these threonine residues cannot be ruled out. It remains possible that hydrogen bonding may still be involved in the mechanism, through the asparagines (N16/N27) and perhaps the aspartic acid residues (D1/D5); hydrogen bonding involving these residues has been included in several models of the AFPs interacting with the ice surface [15,16]. The reduced hysteresis and the appearance of steps in the faceted crystals of VVVV2KE may be attributed to the reduced number of hydrogen bonding groups. It is also possible that hydrogen bonding plays no role in the action of the antifreeze molecule and the overall shape of the molecule may be sufficient to inhibit ice growth.

An alternative hypothesis is that the molecular level action of all families of 'antifreeze' compounds is due to distinct hydrophobic and hydrophilic sides of the molecule [32]. In the case of type I α-helical polypeptides, candidate hydrophobic and hydrophilic sides are shown in Fig. 5, a helical diagram of VVVV2KE consistent with the crystal structure of the native protein TTTT [16]. This diagram shows (i) the four residues VVVV which have been mutated in this study (highlighted with arrows), (ii) an alanine-rich hydrophobic side (for TTTT this is achieved with threonine methyl groups pointing out), and (iii) a localized salt-bridge-rich region. In this alternative hypothesis, the proposed orientation of molecule is with the hydrophobic side facing the ice surface, and the other side interacting strongly with the surrounding liquid water molecules, consistent with the detailed molecular level picture of the ice/water interface [33,34]. This proposed orientation is essentially a 90° rotation of the arrangement in [8]. Perturbation of the hydrophobic face (encompassing A10/25 to A9/V24) in a number of synthetic analogues has *always* been accompanied by a decrease in antifreeze activity [19,21]. However, it should be noted that, in its current form, this alternative hypothesis does *not* explain the specific interaction of the native and mutant polypeptides with the $\{2\,0\,\bar{2}\,1\}$ plane of ice 1h.

The above hypotheses may be tested explicitly by further molecular level perturbations to the protein, and may lend themselves to generalization to the other three families of fish 'antifreezes', and insect 'antifreezes'. Further detailed studies on VVVV2KE are under way, as well as the design and synthesis of mutants to further clarify the mechanism of ice growth inhibition by type I AFPs.

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