Hydrophobic analogues of the winter flounder 'antifreeze' protein

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Abstract The synthesis, solution conformation and ice-growth inhibition properties of four new analogues of the type I 37-residue winter flounder 'antifreeze' protein are reported. All four analogues contain two extra salt bridges to facilitate comparison of results with previously published data. In two analogues, all four threonine residues in the native polypeptide were mutated to 2-amino butyric acid (an unnatural amino acid) and isoleucine, respectively. The butyric acid analogue was ~85% helical at 3°C, modified the shape of ice growth, and exhibited reduced hysteresis compared to the native protein (9% at 4 mM). These results show that the γ-methyl group of threonine, which is present in the sidechain of 2-amino butyric acid, is not sufficient for activity. The isoleucine analogue, in which the threonine hydroxyl group is replaced by an ethyl group, was 100% helical at 3°C, showed no hysteresis but was able to modify the shape of ice crystal growth. In the third and fourth analogues, mutations of the aspartic acids 1 and 5 to alanine, and asparagines 16 and 27 to leucine in the threonine- and valine-substituted analogues did not affect the helicity of the polypeptides, but removed the ability to inhibit ice growth.

Key words: Antifreeze; α-Helical peptide; Ice-growth inhibition; Hydrophobicity; Ice/water interface; Hysteresis

1. Introduction

The type I 'antifreeze' proteins, found in the body fluids of fish inhabiting polar oceans, are alanine rich α-helical proteins that are able to inhibit the growth of ice (reviewed in [1,2]). The 37-residue winter flounder (*Pseudopleuronectes americanus*) protein 'HPLC6' (TTTT) [3], which contains three 11-amino acid repeats of the sequence $ThrX_2AsxX_7$ [4,5], modifies both the rate and shape (or 'habit') of ice crystal growth [6], displays hysteresis [7,8], and accumulates specifically at the $\{2\ 0\ \bar{2}\ 1\}$ ice plane [9]. In this paper we report studies on four new hydrophobic analogues of the winter flounder protein TTTT.

Structure–activity studies [10–13], and hemisphere etching [6] on TTTT have resulted in a number of models to explain specific accumulation at the $\{2\ 0\ \bar{2}\ 1\}$ ice surface by invoking hydrogen bonding involving the four threonine residues at positions 2, 13, 25 and 37. While molecular dynamics and computer simulations [14–18] supported these models, detailed simulations of the diffuse ice/water interface [19,20] do not support hydrogen bonding dominated mechanisms. Recent studies in which mutations of the threonine residues to hydrophobic residues (alanine, valine) [21,22], as well as mutations that retain hydrogen bonding characteristics (serine, *allo*-threonine) [21–24], have shown clearly for the first time that hydrophobic interactions play a significant, perhaps dominant, role in the mechanism of ice-growth inhibition. These recent experimental results [21–24] have prompted a re-evaluation of the putative ice-binding surface of the protein [1,25], and ice/vacuum simulations that explicitly incorporate hydrophobic interactions have been reported [26]. Residues that project onto the same face of the helix as the threonines, including the asparagine and aspartic acid (Asx) residues have also been proposed to be important [4,13,14,27,28] and it has recently been proposed that asparagine may be more important for increasing protein solubility than for its potential hydrogen bonding capabilities [29]. Very recent studies of synthetic alanine–lysine antifreezes, motivated by studies of sculpin antifreezes, by Wierzbicki et al. [30] are consistent with these findings.

In this paper we report studies on four new hydrophobic analogues of the winter flounder protein TTTT (Table 1). All four polypeptides studied retain two extra salt bridges to facilitate comparison with our earlier data [21,22]. The threonine residues were replaced by 2-amino butyric acid (B) and isoleucine (I), respectively, in order to provide further experimental data on the significance of hydrophobicity at positions 2, 11, 24 and 35 of the native protein TTTT. In addition we have studied analogues TTTTAL2KE and VVVVAL2KE (Table 1) in which hydrophobic mutations at the Asx positions have been introduced.

2. Materials and methods

2.1. Polypeptides

Crude polypeptides were synthesised as the C-terminal amides by AusPep Pty Ltd, Melbourne, Australia and were purified by reverse-phase high-performance liquid chromatography (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. Electrospray ionisation mass spectrometry gave the expected molecular ion peaks: 3409.9 (BBBB2KE), 58% peptide content; 3522.4 (IIII2KE), 61% peptide content; 3384.4 (TTTTAL2KE), 59% peptide content and 3376.4 (VVVVAL2KE), 66% peptide content.

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

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and 0.5 mg ml^{-1} 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 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. Video microscopy

Ice crystals were observed through a microscope and time evolution recorded by a video camera linked to video recorder. Still images were obtained from the videotape record at regular intervals over a period of approximately 1 min. The absolute length scale in the video images was determined by taking an image of a 50 μm standard grid through the microscope at identical magnification.

3. Results

3.1. Polypeptide design

Two additional salt bridges (indicated by suffix 2KE) were incorporated into all polypeptides to allow direct comparison of results with our previous polypeptides [21]. Polypeptides BBBB2KE and IIII2KE contained mutations of all four threonines to 2-amino butyric acid (B) and isoleucine, respectively. Both amino butyric acid (B) and isoleucine contain a γ-methyl group analogous to the γ-methyl group present in threonine, but differ in the size and shape of the surrounding groups; IIII2KE contains an ethyl group in place of the hydroxyl group present in Thr, whereas BBBB2KE contains a hydrogen atom in place of the Thr hydroxyl group. These polypeptides were designed to complement our previous analogues in which the threonine residues were mutated to valine, alanine and glycine, respectively [21]. Since leucine is also a hydrophobic amino acid, a leucine analogue was considered. However, leucine lacks the methyl group present on the β-carbon, and the length of the sidechain protrudes significantly from the surface of the helix which creates a different class of interaction with the diffuse ice/water interface [20].

Polypeptides TTTTAL2KE and VVVVAL2KE were designed to assess the importance of hydrogen bonding of the Asx residues (Asp1, Asp5, Asn16 and Asn27) in the mechanism of inhibition of ice growth by TTTT. The Asp residues were replaced by Ala and the Asn residues by Leu, being hydrophobic residues that lack the ability to participate in hydrogen bonding interactions. As there are no naturally occurring amino acids with hydrophobic sidechains that mimic closely the approximate sidechain size of Asp and Asn, Ala and Leu were chosen as the sidechains most similar in size to Asp and Asn that did not introduce potential steric interactions through branching. TTTTAL2KE retains the key threonine residues and replaces only the other polar Asx residues with hydrophobic residues. VVVVAL2KE involved replacement of the threonine residues with valine residues, as well as the Asx residues with Ala/Leu. Hence, in this polypeptide, hydrophobic residues have replaced all potential hydrogen bonding residues that have been implicated in proposed ice-binding mechanisms. For enhancement in solubility and to allow comparison with related polypeptides [21], the additional salt bridges were also incorporated into the sequences.

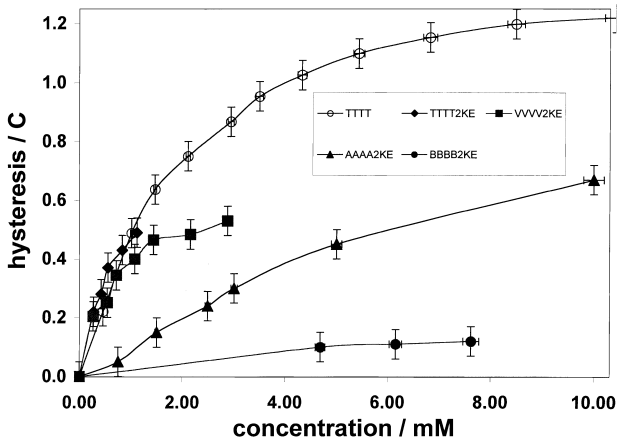

Fig. 1. Thermal hysteresis as a function of concentration for unbuffered solution of BBBB2KE (filled circles). For comparison, published hysteresis date for the native protein TTTT (open circles) [7] TTTT2KE (diamonds) and hydrophobic analogues VVVV2KE (squares) and AAAA2KE (triangles) [21] are also included.

3.2. Nanoliter osmometry

The thermal hysteresis values for all polypeptides were measured by nanoliter osmometry using similar methods to those previously reported [21]. The behaviour of ice growth in polypeptide solutions was observed from the thermal hysteresis experiments using microscopy and recorded on videotape. Measurements were made in unbuffered aqueous solutions on 4, 2 and 1 mM polypeptide samples, with concentrations of each stock solution determined by amino acid analysis. The initial solution concentration was as high as possible without producing gelling of the sample (i.e. approximately 10 mM). Of the four polypeptides, only BBBB2KE showed thermal hysteresis. Fig. 1 shows the thermal hysteresis values along with the published data for the native protein TTTT [7] and our previously published hydrophobic analogues [21].

Fig. 2 summarises the patterns of ice crystals grown from solutions of BBBB2KE, IIII2KE, TTTTAL2KE and VVVVAL2KE. BBBB2KE displayed a pronounced needle shaped hexagonal bipyramid (Fig. 2a). In the case of IIII2KE and TTTTAL2KE (Fig. 2b,c), distinct faceting was observed. However, the characteristic hexagonal bipyramid formed in solutions of ice-growth inhibitors, such as TTTT2KE or VVVV2KE [21], was not seen. In solutions of IIII2KE and TTTTAL2KE, the seed crystal faceted to a hexagonal shaped crystal that then continued to grow steadily until the entire solution was encompassed and no rapid growth was evident. VVVVAL2KE did not show any faceting (Fig. 2d).

Table 1
Polypeptide sequences highlighting mutations of Thr (bold), mutations of Asx residues (underlined) and additional salt bridges (italicised)

	1	2	13	24	35
TTTT	D	**T**ASDAAAAAAL	**T**AANAKAAAEL	**T**AANAAAAAAA	**T**ARCONH$_2$
IIII2KE	D	**I**ASDA*K*AAA*E*L	**I**AANAKAAAEL	**I**AANA*K*AAA*E*A	**I**ARCONH$_2$
BBBB2KE	D	**S**ASDA*K*AAA*E*L	**S**AANAKAAAEL	**S**AANA*K*AAA*E*A	**S**ARCONH$_2$
TTTTAL2KE	A	**T**AS<u>A</u>A*K*AAA*E*L	**T**AA<u>L</u>AKAAAEL	**T**AA<u>L</u>A*K*AAA*E*A	**T**ARCONH$_2$
VVVVAL2KE	A	**V**AS<u>A</u>A*K*AAA*E*L	**V**AA<u>L</u>AKAAAEL	**V**AA<u>L</u>A*K*AAA*E*A	**V**ARCONH$_2$

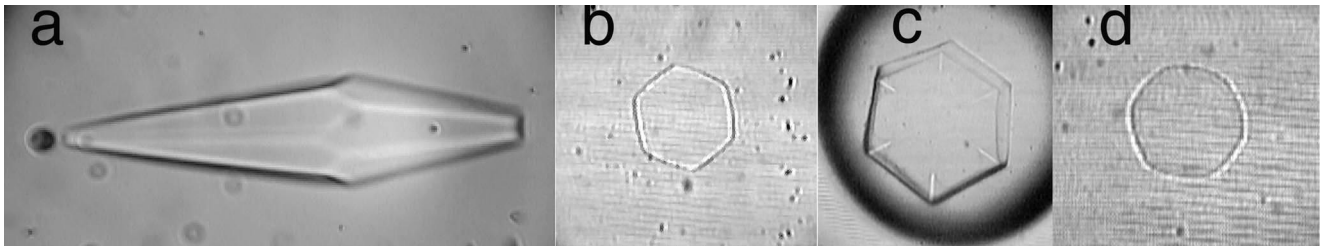

Fig. 2. Video microscopy images of ice crystal growth in the presence of (a) BBBB2KE, (b) IIII2KE, (c) TTTTAL2KE and (d) VVVVAL2KE. All concentrations are in the range 6–7 mM.

3.5. CD

Variable temperature CD spectra of all analogues were recorded in order to establish the effect of the mutations on the α-helical conformation of the polypeptides (Fig. 3). At 3°C, polypeptides IIII2KE, TTTTAL2KE and VVVVAL2KE were estimated to be 100% α-helical, as expected from helix propensities of the amino acids [31]. Heating the polypeptides to 50°C destabilised the helical conformation, but even at this temperature TTTTAL2KE and VVVVAL2KE were still 75–85% helical. In the case of BBBB2KE, the helicity at 3°C was estimated to be 85%. Since 2-amino butyric acid is an unnatural amino acid, there is no known data available in regards to its helical propensity.

4. Discussion

Compared to the native protein TTTT, analogues BBBB2KE and IIII2KE contain mutations of the four threonine sidechains to hydrophobic sidechains that are complementary to our published analogues VVVV2KE and AAAA2KE [21]. Both 2-amino butyric acid and isoleucine retain the γ-methyl group of threonine, but replace the potential hydrogen bonding hydroxyl group with hydrogen and an ethyl group, respectively (Fig. 4). Thus, the local environment of the threonine γ-methyl group has been systemically varied.

BBBB2KE displayed the ability to modify the shape of a seed crystal to form a hexagonal bipyramid, and exhibited thermal hysteresis (0.1°C at 5 mM), but at a significantly reduced level compared to the native protein, and lower hysteresis than either VVVV2KE or AAAA2KE (Fig. 1). The helicity of BBBB2KE is estimated at ~85% which may account for a minor reduction in hysteresis. However, the results show that the presence of the γ-methyl group of threonine alone is not sufficient to confer ice-growth inhibition properties; the presence of the OH as well as the γ-methyl in TTTT or a second methyl group (present in VVVV2KE) confers significantly higher thermal hysteresis.

In the case of IIII2KE, the hexagonal bipyramidal crystal characteristic of ice-growth inhibitors was not observed and the polypeptide exhibited no thermal hysteresis. However, the polypeptide was able to modify the crystal shape forming a hexagonal shaped crystal, i.e. the polypeptide is an ice-growth modifier [1]. As this polypeptide is fully helical at low temperatures, the presence of an ethyl group in place of the OH in TTTT, or in place of one of the two methyl groups in VVVV2KE (see Fig. 4), removes the ability to inhibit ice growth. This result is consistent with the ethyl group preventing close contact with the ice-binding face of the polypeptide and the ice surface, and is consistent with previous studies in which close contacts between the Thr sidechains and the ice surface are necessary for activity [4,14,29].

Comparison of the results obtained with BBBB2KE and IIII2KE with our previously published hydrophobic analogues VVVV2KE and AAAA2KE included in Fig. 1 also allows comparison of the effect of the sequential addition of methyl groups at positions 2, 13, 24 and 35 of the polypeptide on thermal hysteresis. Thus, addition of a single methyl group to the Ala residues of AAAA2KE (to give BBBB2KE) reduces hysteresis. In contrast, addition of two methyl groups to the Ala residues (to give VVVV2KE) gives maximum hysteresis. Further addition of another methyl group to give IIII2KE gives no thermal hysteresis.

Neither of the analogues TTTTAL2KE or VVVVAL2KE exhibited thermal hysteresis. As the parent sequences TTTT2KE and VVVV2KE show similar ice-growth inhibition properties at low concentrations (<1 mM, Fig. 1), it was expected that similar thermal hysteresis and ice-growth patterns would be observed for TTTTAL2KE and VVVVAL2KE, if the Asx residues play an insignificant role in the inhibition of ice growth. As a result of the replacement of Asp at positions 1 and 5 with Ala, the extensive capping network present in TTTT [4] is lost, which could have a detrimental effect on the stability of the helix. However, CD and molecular modeling were consistent with a fully helical polypeptide at low temperatures and hence the removal of the capping network was not considered to have had a significant

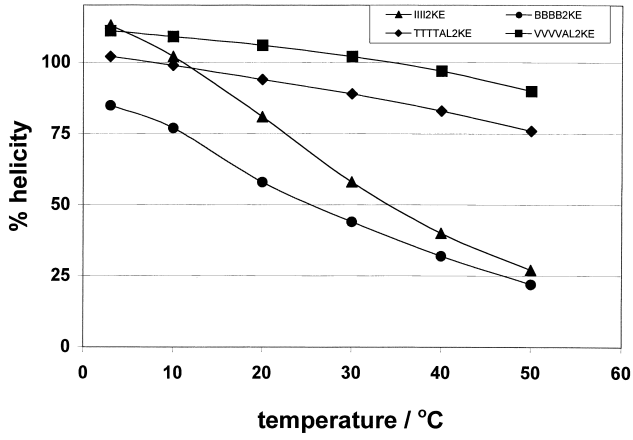

Fig. 3. Variation of % helicity (±10%) with temperature of polypeptides in water, pH 8.5, BBBB2KE (circles), IIII2KE (triangles), TTTTAL2KE (diamonds) and VVVVAL2KE (squares). Values >100% are due to errors in sample concentrations determined by amino acid analysis and in the estimation of the theoretical value for the polypeptide in a random coil conformation.

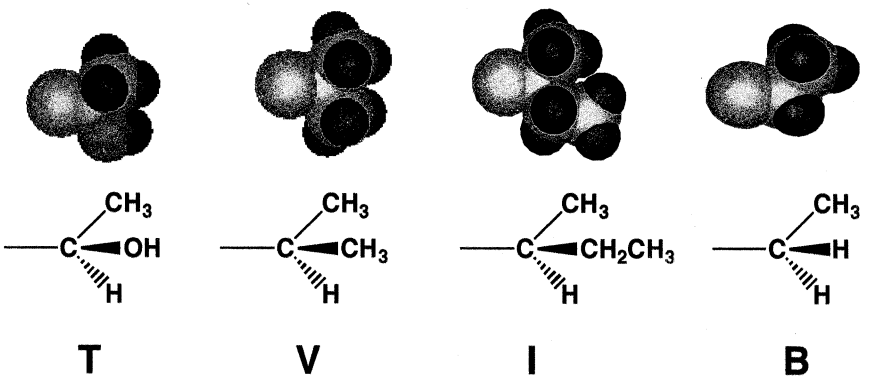

Fig. 4. Comparison of relative size and hydrophobicity of sidechain sizes present in hydrophobic analogues.

impact on the helicity of the polypeptide. TTTTAL2KE showed a distinct ice-growth pattern, forming hexagonal crystals, but in our hands yielded no measurable thermal hysteresis (< 0.05°C). Thus, the mutations introduced confirm the involvement of the Asx residues in the mechanism of ice-growth inhibition.

In a recent study [29], replacement of the asparagine residues with amino acids of shorter sidechains (alanine or threonine) resulted in little change in ice-growth inhibition properties, while substitution with larger sidechains (glutamine) abolished thermal hysteresis. In addition, replacement of leucine residues with alanine gave more active, but less soluble analogues. Thus it was proposed that asparagine may be more important for increasing protein solubility than for its potential hydrogen bonding capabilities, with a primary role for leucine in preventing aggregation [29]. Our results are consistent with this hypothesis, as leucine is larger than asparagine, and would introduce steric interactions similar to glutamine.

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References

[1] Harding, M.M., Ward, L.G. and Haymet, A.D.J. (1999) Eur. J. Biochem. 264, 653–665.
[2] Yeh, Y. and Feeney, R.E. (1996) Chem. Rev. 96, 601–617.
[3] Fourney, R.M., Joshi, S.B., Kao, M.H. and Hew, C.L. (1984) Can. J. Zool. 62, 28–33.
[4] Sicheri, F. and Yang, D.S.C. (1995) Nature 375, 427–431.
[5] Gronwald, W., Chao, H., Reddy, D.V., Davies, P.L., Sykes, B.D. and Sönnichsen, F.D. (1996) Biochemistry 35, 16698–16704.
[6] Knight, C.A., DeVries, A.L. and Oolman, L.D. (1984) Nature 308, 295–296.
[7] Duman, J.G. and DeVries, A.L. (1974) Nature 247, 237–238.
[8] Duman, J.G. and DeVries, A.L. (1976) Comp. Biochem. Physiol. B 54, 375–380.
[9] Knight, C.A., Cheng, C.-H.C. and DeVries, A.L. (1991) Biophys. J. 59, 409–418.
[10] Wen, D. and Laursen, R.A. (1992) J. Biol. Chem. 267, 14102–14108.
[11] Wen, D. and Laursen, R.A. (1993) J. Biol. Chem. 268, 16401–16405.
[12] Wen, D. and Laursen, R.A. (1993) J. Biol. Chem. 268, 16396–16400.
[13] Wen, D. and Laursen, R.A. (1993) FEBS Lett. 317, 31–34.
[14] Cheng, A.L. and Merz, K.M. (1997) Biophys. J. 73, 2851–2873.
[15] McDonald, S.M., Brady, J.W. and Clancy, P. (1993) Biopolymers 33, 1481–1503.
[16] Jorgensen, H., Mori, M., Matdui, H., Kanaoka, M., Yanagi, H., Yabusaki, Y. and Kikuzono, Y. (1993) Protein Eng. 6, 19–27.
[17] Lal, M., Clark, A.H., Lips, A., Ruddock, J.N. and White, D.N.J. (1993) Faraday Discuss. 95, 299–306.
[18] Chou, K.C. (1992) J. Mol. Biol. 223, 509–517.
[19] Karim, O.A. and Haymet, A.D.J. (1988) J. Chem. Phys. 89, 6889–6896.
[20] Hayward, J.A. and Haymet, A.D.J. (2001) J. Chem. Phys. 114, in press.
[21] Haymet, A.D.J., Ward, L.G. and Harding, M.M. (1999) J. Am. Chem. Soc. 121, 941–948.
[22] Haymet, A.D.J., Ward, L.G., Harding, M.M. and Knight, C.A. (1998) FEBS Lett. 430, 301–306.
[23] Chao, H., Houston, M.E., Hodges, R.S., Kay, C.M., Sykes, B.D., Loewen, M.C., Davies, P.L. and Sönnichsen, F.D. (1997) Biochemistry 36, 14652–14660.
[24] Zhang, W. and Laursen, R.A. (1998) J. Biol. Chem. 273, 34806–34812.
[25] Baardsnes, J., Kondejewski, L.H., Hodges, R.S., Chao, H., Kay, C. and Davies, P.L. (1999) FEBS Lett. 463, 87–91.
[26] Dalal, P. and Sönnichsen, F. (2000) J. Chem. Inf. Comput. Sci. 40, 1276–1284.
[27] McDonald, S.M., White, A., Clancy, P. and Brady, J.W. (1995) AICHE J. 41, 959–973.
[28] Wen, D. and Laursen, R.A. (1992) Biophys. J. 63, 1659–1662.
[29] Loewen, M.C., Chao, H.M., Houston, M.E., Baardsnes, J., Hodges, R.S., Kay, C.M., Sykes, B.D., Sönnichsen, F.D. and Davies, P.L. (1999) Biochemistry 38, 4743–4749.
[30] Wierzbicki, A., Knight, C.A., Rutland, T.J., Miuccio, D.D., Pybus, B.S. and Sikes, C.S. (2000) Biomacromolecules 1, 268–274.
[31] Chakrabartty, A., Kortemme, T. and Baldwin, R.L. (1994) Protein Sci. 3, 843–852.