Studies of a putative ice-binding motif in winter flounder skin-type anti-freeze polypeptide

Qingsong Lin[a], K. Vanya Ewart[a,1], Daniel S.C. Yang[b], Choy L. Hew[a,*]

[a] *Division of Structural Biology and Biochemistry, Hospital for Sick Children and the Departments of Laboratory Medicine and Pathobiology and Biochemistry, University of Toronto, 100 College Street, Room 351, Toronto, Ont. M5G 1L5, Canada*
[b] *Department of Biochemistry, McMaster University, Hamilton, Canada*

Received 20 April 1999; received in revised form 18 May 1999

Abstract Winter flounder contains two distinct anti-freeze protein isoforms, which are the liver-type extracellular anti-freeze proteins and the skin-type intracellular anti-freeze protein. The skin-type anti-freeze proteins exhibit lower anti-freeze activities than the liver-type isoforms and this might be due to their lacking complete ice-binding motifs. One of the skin-type anti-freeze proteins, skin-type anti-freeze protein-3, does contain putative overlapping ice-binding motifs with the sequences '-K—DT-' and '-DT–K-'. Synthetic anti-freezes containing 0–3 repeats of the '-DT–K-' motif were tested for stability and activity. Loss of the single '-DT–K-' of skin-type anti-freeze protein-3 increases the anti-freeze activity and increasing the number of motifs to two or three lowers the activity. The decrease in activity with an increasing frequency of the motif correlates with a decrease in the helical content of these peptides at 0°C.

Key words: Ice-binding motif; Winter flounder; Skin-type anti-freeze polypeptide; Synthetic peptide; Hysteresis

1. Introduction

The winter flounder *Pleuronectus americanus* contains the type I, alanine-rich, α-helical anti-freeze polypeptides (AFPs) to protect them from freezing in icy seawaters [1]. Recent studies from our laboratories have shown that the fish contains two distinct AFP isoforms that are encoded from different subsets of genes, namely the liver-type extracellular AFPs (wflAFPs) and the skin-type intracellular AFPs (wfsAFPs) [2]. Although these AFPs are structurally similar, the wfsAFPs, however, exhibit a much lower anti-freeze activity as compared to the liver-type isoforms. The presence of two distinct AFP isoforms in the same fish species with a different cellular localization and biosynthetic pathways implied that there may be differences between these proteins in conferring freezing protection, their structural/functional relationships as well as the transcriptional mechanisms controlling their tissue-specific expressions [3].

*Corresponding author. Fax: (1) (416) 978 8802.
E-mail: choy.hew@utoronto.ca

[1] Present address: Institute for Marine Biosciences, National Research Council, Halifax, N.S., Canada.

Abbreviations: AFP, anti-freeze protein and polypeptide; wflAFP, winter flounder liver-type anti-freeze polypeptide; wfsAFP, winter flounder skin-type anti-freeze polypeptide; IBM, ice-binding motif

The structure of the most abundant wflAFP (wflAFP-6, previously known as HPLC-6) has been extensively studied which included X-ray crystallography [4], NMR [5] and peptide synthesis [6,7]. It is a single rigid amphipathic helix with a regular 11 amino acid repeat of $ThrX_2Asn/AspX_7$, where X is mainly alanine. The distance of the Thr repeats in this AFP (16.5 Å) closely matches the ice lattice [8]. Based on the 1.5 Å X-ray crystal structure, Sicheri and Yang postulated the presence of ice-binding motifs (IBMs) which act as anchors or contact surfaces to strengthen the affinity of its ice-binding [4]. Because of the occurrence of IBMs with the sequence '-LT–N-' in wflAFP-6, putative IBMs were suggested to occur in other type I AFPs. These consisted of the sequences '-K—DT-' and '-KT–D-' [4]. In comparison with the wflAFPs, the skin AFPs are generally deficient in IBMs and this might account for the lower anti-freeze activity of the skin AFPs [2].

Based on the wflAFP-6 IBM and the putative ones suggested in other AFP sequences, two overlapping putative IBMs can be identified in a skin-type AFP, wfsAFP-3. One motif, '-K—DT-', corresponds precisely to one of those postulated previously in other AFPs [4]. However, it has been shown to have no role in the activity of wfsAFP [9]. The other compositionally equivalent motif '-DT–K-' remained to be tested. We have therefore investigated the role of this motif in wfsAFP-3 by measuring the stability and activity of synthetic peptides containing 0–3 repeats of this motif.

2. Materials and methods

2.1. Reagents and chemicals

Fmoc amino acid derivatives and Fmoc-Arg(Mtr)-Nava Syn KA 100 resin were purchased from Calbiochem-Novabiochem Group (San Diego, CA, USA).

Reagents used in peptide synthesis were purchased separately as stated. *N,N*-dimethylformamide (DMF) (ACS grade, BDH Canada, Etobicoke, Ont., Canada), trifluoroacetic acid (TFA, Chem-Impex International, Wood Dale, IL, USA), thioanisole, *m*-cresol, 1,2-ethanedithiol, ethylmethylsulfide, diisopropylethylamine and bromotrimethylsilane (Aldrich Chemicals, Milwaukee, WI, USA), *o*-(7-azabenzotriazol-1-y1)-l,l,3,3-tetramethyluronium hexafluorophosphate (HATU, Perspective Biosystems, Burlington, Ont., Canada) and piperidine (ACS grade, Anachemia Canada, Mississauga, Ont., Canada).

2.2. Peptide synthesis

Peptides were synthesized at the Hospital for Sick Children Biotechnology Service Centre, Toronto, on the Pharmacia-LKB-Biolynx 4170 Automated Peptide Synthesizer (Pharmacia Biotech, Montreal, Que., Canada) using the continuous flow Fmoc chemistry on the NovaSyn KA 100 resin [10]. A solution of 20% piperidine in DMF was used for the removal of the Fmoc protection group. For each gram resin (0.1 nmol substitution), a four times excess of Fmoc amino acid activated with HATU and diisopropylethylamine (1:1:2, mol/mol/mol) [11] was used for the coupling reaction. The reaction time was 1 h.

PII: S0014-5793(99)00752-8

Table 1
Amino acid sequences of the synthetic AFPs

	1	11	22	33
0IBM	MDAPAKAAAA	TAAAAKAAAEA	TAAAAAKAAAA	TKAAAAR
1IBM	MDAPAKAAAA	TAAAAKAAAEA	TAAAAAKAAAD	TKAKAAR
2IBM	MDAPAKAAAA	TAAAAKAAAED	TAAKAAKAAAD	TKAKAAR
3IBM	MDAPAKAAAD	TAAKAKAAAED	TAAKAAKAAAD	TKAKAAR

The peptides were nominated based on the number of putative IBMs. 1IBM has the same sequence as wfsAFP-3 and contains one IBM '-DT–K-', the other three peptides contain zero, two and three IBMs, respectively. The amino acid residues involved in the IBMs are underlined.

After the synthesis, the side chain-protected peptide-resin conjugates were acetylated separately using acetic anhydride/diisopropylethylamine (2:1) at room temperature for 1 h. The acetylated peptide-resin conjugates were washed with DMF, diethyl ether and dried under reduced pressure. The dry peptide-resin conjugates were cleaved with 20 ml TFA containing 4 ml thioanisole, 0.4 ml *m*-cresol, 2 ml 1,2-ethanedithiol, 3 ml ethylmethylsulfide and 4 ml bromotrimethylsilane at 0°C for 1 h. The peptides were extracted, dissolved in 0.1% TFA and desalted on a Sephadex G10 column.

The homogenity of the peptides was analyzed by reverse phase HPLC on a Waters μBondapak C_{18} column, amino acid analysis and by atmospheric pressure ionization mass spectrometry.

2.3. Measurement of anti-freeze activity

Anti-freeze activity was measured as thermal hysteresis (difference between the melting and freezing temperatures) essentially following the procedure of Chakrabartty et al. [6]. Proteins were dissolved in 0.1 M NH_4HCO_3. Activity measurements were performed on a series of dilutions using a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NY, USA). For each dilution, measurements were made from three wells and the average was taken.

2.4. Measurement of circular dichroism (CD) spectra

CD spectra were measured using a Jasco J-600 spectropolarimeter. A water-jacketed (0.1 cm) quartz cell was used. The concentrations of peptides for CD measurement were between 0.1–0.2 mg/ml in 0.1 M NH_4HCO_3, pH 8.5. The mean residue ellipticity at a given wavelength, $[\theta]_\lambda$, was expressed in degree cm^2 dmol^{-1}. The fraction helix was calculated as described by Greenfield and Fasman [12] employing 222 nm instead of 208 nm:

$$\text{Fraction helix} = \frac{[\theta]_{222}^{\text{obs}} - [\theta]_{222}^{\text{coil}}}{[\theta]_{222}^{\text{helix}} - [\theta]_{222}^{\text{coil}}}$$

The ellipticity $[\theta]_{222}^{\text{coil}}$ of each peptide in 6.0 M guanidine hydrochloride at 25°C was assumed to represent 0% helix formation and the ellipticity $[\theta]_{222}^{\text{helix}}$ for 100% helix formation was calculated from Yang et al. [13]: $[\theta]^n = [\theta]^\infty(1-k/n)$, where n is the chain length and k is a wavelength-dependent factor (at 222 nm, $k = 2.5$ and $[\theta]^\infty = 37\,400$ degree cm^2 dmol^{-1}).

3. Results

3.1. Peptide design and synthesis

In order to test the role of the putative IBM '-DT–K-' in ice-binding in the wfsAFPs, a series of synthetic peptides based on the structure of wfsAFP-3 were synthesized (Table 1). One peptide (1IBM) was synthesized with the sequence of wfsAFP-3 and it contained a single complete IBM. In other peptide analogues, residues corresponding to the Asp and Lys of the complete IBM were substituted for Ala at specific residues in order to create new IBMs (Fig. 1). Thus, two peptides (2IBM and 3IBM) which contain two or three of this IBMs were synthesized. A peptide containing only incomplete IBM (0IBM) was synthesized by placing an Ala at the positions corresponding to the D and K of the IBM in the 1IBM peptide. These peptides were N-terminal acetylated to mimic their native analogue. All the synthetic peptides were purified by reverse phase HPLC and confirmed by amino acid analysis and mass spectrometry.

3.2. Anti-freeze activities

Fig. 2 shows the anti-freeze activities of the synthetic peptides as thermal hysteresis in a concentration-dependent manner. The 0IBM peptide, which lacks any complete IBM, has a slightly higher activity than the 1IBM peptide, suggesting that loss of this IBM has no effect on anti-freeze activity. Peptide

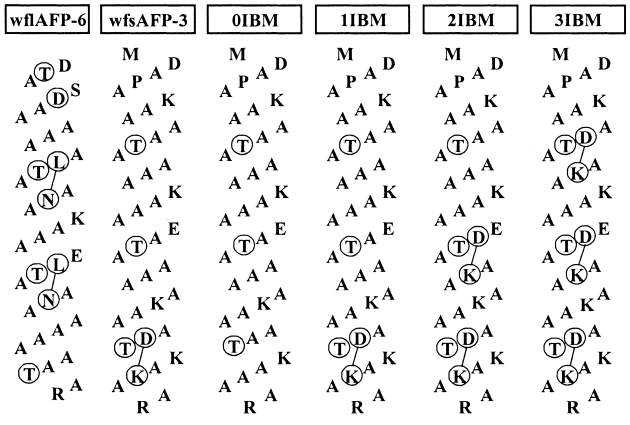

Fig. 1. Helical net representation of the wflAFP-6, wfsAFP-3 and its synthetic analogues. The presumptive complete IBMs are circled. Residues that interact or are predicted to interact in order to stabilize ice-binding conformations are joined. The incomplete IBMs, Thr residues are also circled.

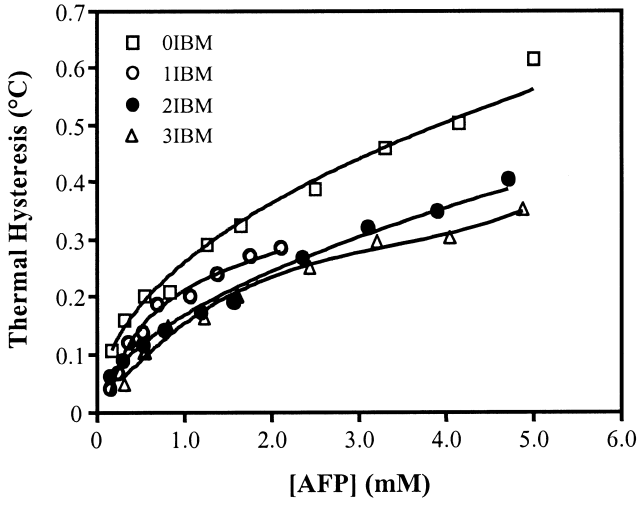

Fig. 2. The concentration-dependence of anti-freeze activities of the synthetic AFPs. Anti-freeze activities (thermal hysteresis) of solutions containing a series of AFP concentrations were measured as described under Section 2. The symbols representing different AFP analogues are indicated in the figure.

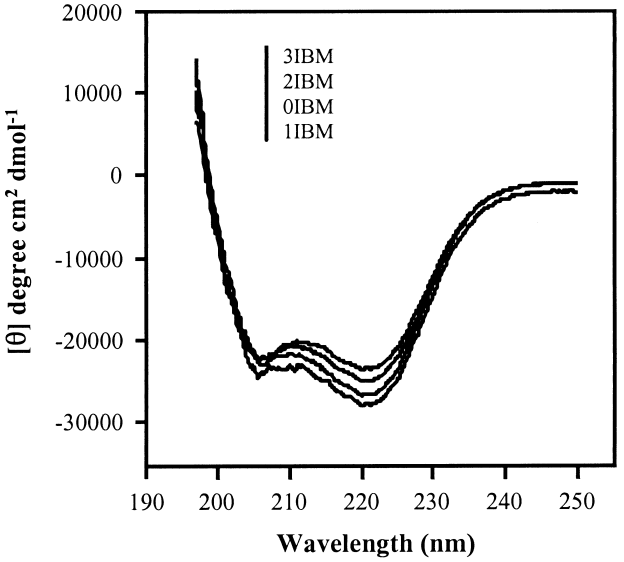

Fig. 3. CD spectra of synthetic AFPs. The CD spectra of the synthetic peptides were measured at 0°C using a Jasco J-600 spectropolarimeter, as described under Section 2. The four spectra from bottom to top are 1IBM, 0IBM, 2IBM and 3IBM, respectively.

analogues containing two and three IBMs were slightly less active than the 1IBM peptide.

3.3. CD spectra

The secondary structures of these synthetic peptides were determined by CD spectroscopy (Fig. 3). All these peptides showed typical double-minimum spectra of α-helices at 0°C. The calculated helical contents for 0IBM, 1IBM, 2IBM and 3IBM peptides at 0°C were 75.2, 78.5, 70.0 and 65.9%, respectively, indicating that introduction of more than one such IBM results in destabilization of the helical structure.

4. Discussion

Anti-freeze proteins have been postulated to retard ice crystal growth by an adsorption-inhibition process [14] in which a set of hydrophilic groups on the AFPs match to the oxygen atoms via hydrogen bonds on a particular plane of the ice lattice. Knight et al. [8] have hypothesized that some hydrogen-binding groups on the AFPs might become incorporated into the ice lattice so that they can each form three hydrogen bonds. More recently, X-ray crystallographic studies of wflAFP-6 [4] showed that the Thr side chains are sterically constrained ($\chi1 = -61 \pm 4°$) by the backbone helical structure and the side chains of Asp-5, Asn-16 and Asn-27 (Fig. 1) adopt a similar conformation ($\chi1 = -75 \pm 3°$, $\chi2 = 13 \pm 13°$). Two leucine side chains (Leu-12 and Leu-23) stabilize the asparagine conformation through close Van der Waals interaction. This led to the postulation of IBMs in which the composed residues are rigidly constrained to form an exceptionally flat ice-binding surface. In this model, the threonine hydroxyls do not protrude sufficiently into the ice lattice and become tetrahedrally linked and a less stringent hydrogen-binding criterion was proposed. The IBM hypothesis has been challenged by an NMR study [5] which showed that the proposed ice-binding side chains (threonines and asparagines) are not constrained but rather adopt many possible rotameric states in solution. However, this does not rule out the possibility that these side chains form a stable IBM upon binding to ice.

Our studies clearly showed that '-DT–K-' in wfsAFP-3 is not a 'functional IBM'. Moreover, the existence of more than one of this 'putative IBM' actually resulted in lowering of the anti-freeze activity and destabilization of helix. Thus it is implied that the IBM hypothesis based on the structure of wflAFP-6 may be restricted and may not be extended to other type I AFPs. A recent ice-binding model for a shorthorn sculpin AFP based on ice crystal etching and molecular dynamics simulation [15] did not support the existence of IBM '-KT–D-' as postulated by Sicheri and Yang [4]. Similarly, a four repeat AFP (AFP-9) isolated from flounder serum showed an enhanced anti-freeze activity compared to the four repeat yellow tail flounder AFP [16], yet it lacks any complete IBM as postulated by Sicheri and Yang [4].

The lower helical contents of peptide analogues containing two and three IBMs are likely due to the replacement of Ala with Lys and Asp which have lower helix propensities [17]. These may also correlate with the lower anti-freeze activities. This is consistent with our recent finding that the lower activities of some expressed mutants of wfsAFP-2 correlated with their lower helical content [9]. Although the relationship between helix stability and anti-freeze activity has not been studied systematically, there is evidence that indicates that helix stability does affect anti-freeze activity. Chakrabartty and Hew [7] introduced two additional salt bridges into wflAFP-6 and created a peptide analogue with an enhanced α-helicity. This highly helical analogue was able to affect the ice crystal growth rates at 7–8-fold lower concentrations than the native AFP. Another study [18] showed that introducing an internal lactam bridge reinforced the α-helicity of a minimized AFP analogue and thus conferred the anti-freeze activity.

This study clearly rules out the role of '-DT–K-' in wfsAFP-3 as a functional IBM. It also provides further evidence that helix stability affects anti-freeze activity.

Acknowledgements: We thank Linda Mark for the preparation of the manuscript. This work was financially supported by the Medical Research Council of Canada (to C.L.H.). K.V. Ewart was a recipient of a MRC Fellowship and Q.L. is a recipient of a U of T Open Fellowship, University of Toronto and a Research Training Fellowship, Hospital for Sick Children. We thank Dr V.S. Ananthanarayanan for helpful discussion and the use of the CD spectrometer.

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