Rapid amyloid fibril formation by a winter flounder antifreeze protein requires specific interaction with ice

André Dubé1,*, Cindy Leggiadro2 and Kathryn Vanya Ewart1,3

1 Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Canada
2 Aquatic and Crop Resource Development, National Research Council, Halifax, Canada
3 Department of Biology, Dalhousie University, Halifax, Canada

Correspondence
K. V. Ewart, Department of Biochemistry and Molecular Biology, Dalhousie University, P.O. Box 15000, Halifax, NS B3H 4R2, Canada
Fax: 902 494 1355
Tel: 902 494 3149
E-mail: vewart@dal.ca

*Present address
College of Pharmacy, Dalhousie University, P.O. Box 15000, Halifax, NS B3H 4R2, Canada

(Received 26 February 2016, revised 31 March 2016, accepted 5 April 2016, available online 27 April 2016)

doi:10.1002/1873-3468.12175

Edited by Barry Halliwell

A typically α-helical antifreeze protein (wflAFP-6) from winter flounder, Pseudopleuronectes americanus, forms amyloid fibrils during freezing. In this study, the effects of distinct components of the freezing process were examined. Freezing of wflAFP-6 in the presence of template ice was shown to be necessary for rapid conversion to an amyloid conformation. Neither subfreezing temperature nor phase change was sufficient. Thus, specific interaction with the ice surface was essential. The ice-induced formation of amyloid appeared to be unique to this helical antifreeze, it required high concentrations of protein and it occurred over a range of pH values. These results define a method for rapid formation of amyloid by wflAFP-6 on demand under physiological conditions.

Keywords: amyloid; antifreeze protein; ice; Pseudopleuronectes americanus; winter flounder

A number of diseases have been associated with misfolded proteins [1] and several involve proteins that form amyloid fibrils. All amyloid fibrils share a similar conformation, β-sheets with hydrogen bond-associated strands, parallel or antiparallel, running normal to the axis of the fibril, which is referred to as ‘cross-β’ morphology [2]. Amyloid structures formed by different proteins or under distinct conditions can vary in the arrangement and interaction of their β-strands and in their higher order structures (reviewed by [3,4]). Nonetheless, amyloid fibrils have similar physical characteristics once assembled, including the formation of extended or branched ribbon-like structures, specific dye binding and protease resistance. Moreover, prefibrillar oligomeric forms of amyloid derived from distinct proteins are antigenically similar, consistent with shared conformational features [5].

It is notable that disease-associated amyloid-forming proteins, such as the beta amyloid (αβ) peptide in Alzheimer’s disease, α-synuclein in Parkinson’s disease, the polyglutamic portion of mutant huntingtin in Huntington’s disease and prion proteins share no sequence identity or native structural similarity. In fact, a large variety of proteins produce similar amyloid-like fibrils under particular conditions, suggesting that amyloid may be a universal conformation that proteins are able to adopt [6,7]. The amyloid conformation can be easily detected by dye-binding, scanning electron microscopy and related methods; however, study of the amyloid formation process is complicated by several factors,

Abbreviations
AFP, antifreeze protein; AFGP, antifreeze glycoprotein; BSA, bovine serum albumin; CAPS, N-cyclohexyl-3-aminopropanesulfonic acid; TEM, transmission electron microscopy; ThT, thioflavin T; wflAFP-6, winter flounder AFP isoform 6.
including the lag times normally required for nucleation of amyloid and the spurious ‘background’ amyloid oligomer or fibril formation in protein samples, which preclude reliable baselines [8,9]. Several methods have been developed to avoid these problems. Examples include a protein misfolding cyclic amplification assay originally devised for prions [9,10], a technique employing biotinylated αβ to detect aggregation [11], an oligomerization αβ assay for this peptide based upon a fluorescent fusion construct [8] and a universal amyloid-capture peptoid-based assay for multiple amyloid-forming proteins [12]. Nonetheless, an ideal alternative for studying amyloid formation would be a model protein that resists background amyloid formation, but that rapidly converts to an amyloid conformation on demand. This would allow focused studies on the natural pathways to amyloid formation. It would also provide a highly efficient screening platform for the identification and evaluation of compounds that modulate or inhibit amyloid formation.

Antifreeze proteins (AFPs) are ice-binding proteins that are widely distributed in nature, including all kingdoms [13], although they appear to be specific to species inhabiting low temperature environments where their ice-binding role is essential. Among temperate and polar teleost fish species, there are five structurally distinct types of AFP [13]. The winter flounder, Pseudopleuronectes americanus, produces a large family of type I alanine-rich α-helical AFPs that protect them from freezing in icy seawater [14]. The 37-residue winter flounder liver AFP-6 (wflAFP-6) isoform was shown to be monomeric in solution [15,16] but was found to form amyloid fibrils upon freezing and thawing [17,18]. The discovery of wflAFP-6 conversion into an amyloid conformation was first made in a repeatedly frozen and thawed preparation [17] and follow-up studies suggested that a single freezing event would be sufficient to trigger this conversion [18]. Although NMR analysis revealed no denaturation of the helical wflAFP-6 in ice, the spectra of the thawed product were consistent with β-sheet structure, amyloid was shown by thioflavin T (ThT) fluorescence and fibril morphology evident by transmission electron microscopy (TEM) [17]. Thus, wflAFP-6 showed potential as the basis for an inducible amyloid model system.

Before considering this system for model studies, there were intriguing questions to address. The parameters required for amyloid formation by wflAFP-6, including protein concentration, solution properties and other features were unknown. The specificity of this process for this peptide was also unclear. Most importantly, the aspect of the freezing process triggering amyloid formation was undefined. Therefore, this study was undertaken in order to determine the conditions under which this transition occurs. Components of the freezing process were evaluated independently for their effects on wflAFP-6 amyloid formation. Protein denaturation and concentration as well as solution properties were also examined in order to further define the parameters that affect amyloid formation. Finally, the specificity of the process to this protein was assessed. The results revealed ice binding to be essential for the transition of wflAFP-6 to an amyloid conformation and provided new insight into the mechanism of this conversion.

**Methods**

**Peptide synthesis and preparation**

Synthetic amidated wflAFP-6 (formerly named HPLC-6) was purchased from Biomatik (Cambridge, ON, Canada). Mass spectrometry and HPLC analyses were provided by the supplier, confirming the identity and purity of the peptides. Samples were stored dry at −20 °C until required for use. The wflAFP-6 peptide was warmed to room temperature before opening. Portions were suspended in water to 1 mM and protein was isolated a prepared Sep-Pak® C18 cartridge (Waters, Mississauga, ON, Canada) according to manufacturer’s instructions in order to remove trifluoroacetic acid and other possible contaminants. After methanol elution, the solvent was evaporated from the protein using a speed-vac (Vacufuge®, Eppendorf, Mississauga, ON, Canada) and the wflAFP-6 was stored dry, or diluted into buffers required for analysis. Protein solutions (15 mM) were normally prepared in 10 mM ammonium acetate, pH 6.0, or in other concentrations of other buffers for analysis. Antifreeze activity of the synthetic wflAFP-6 was confirmed by ice crystal morphology in solutions of 310 μM in 10 mM ammonium bicarbonate, pH 8.3, using a Clifton nanolitre osmometer and an Olympus microscope with a Moticam digital camera (Motic, Richmond, BC, Canada). The bipyrimalid crystal is typical of wflAFP-6 at this concentration in solution (Fig. 1).

**Subjecting samples to components of the freeze/thaw process**

Solutions of wflAFP-6 were subject to different components of the freeze/thaw process. For each wflAFP-6 sample undergoing ice interaction, 5 μL of distilled water were first frozen in a 500-μL centrifuge tube on dry ice with no protein solution present in order to act as an ice seed. Then, 5 μL of liquid wflAFP-6 sample, equilibrated for approximately 1 min at −2 °C in a refrigeration unit (RTE-III; Neslab, Montreal, QC, Canada), was immediately added to each tube containing frozen water held at −2 °C in the same unit. For samples undergoing rapid phase change,
5 μL of 15-mM wflAFP-6 sample was snap-frozen in 500-μL centrifuge tubes in a dry ice–ethanol bath. The phase change samples were monitored visually for full freezing and absence of liquid in order to ensure that they froze completely during this incubation, and then transferred directly to the −2 °C refrigeration unit. For samples subject to low temperature without phase change, 5 μL of 15 mM wflAFP-6 sample was placed in 500-μL centrifuge tubes and then placed in the same refrigeration unit at −2 °C.

All samples were then incubated for 1.5 h at −2 °C. The low temperature sample tubes were monitored visually for changes in opacity or growing ice during the incubation, in order to ensure that these samples did not freeze during incubation. All sample tubes were removed from the refrigeration unit and placed at 4 °C for 30 min to allow the frozen samples to thaw. A portion of each sample was then diluted to 0.1 μg·μL⁻¹ with 10 mM ammonium acetate, pH 6.0, for use in ThT assays. A solution of 15 mM wflAFP-6, kept at 4 °C for the same total time interval (2 h), was also diluted to 0.1 μg·μL⁻¹, using 10 mM ammonium acetate, pH 6.0, as buffer, to serve as a negative control. A diagram outlining the different sample treatments is shown in Fig. 2. All experiments were performed in triplicate.

Thioflavin T dye binding and measurement

Thioflavin T dye-binding assays were carried out largely as described previously [17,19,20]. Work using ThT was performed in low light conditions. The 50-mM stock solutions of ThT dye were prepared in distilled water, filtered through a 0.45-μm syringe filter and stored in the dark at 4 °C for up to 1 month. The stock was diluted into 50 mM glycine–NaOH, pH 8.5 at 1 μg·mL⁻¹ to prepare assay dye solution. In a black 96-well plate, 1 μg of protein sample, or 10 μL of the 0.1 μg·μL⁻¹ wflAFP-6 samples prepared during the various experiments, was added to the wells along with 250 μL of assay dye solution. Samples were read on a SpectraMax Gemini XS dual-scanning microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) with excitation set to 450 nm and emission set to 482 nm.

Analysis of wflAFP-6 by transmission electron microscopy

Samples of 15 mM wflAFP-6 that were subjected to the ice interaction and 4 °C controls were diluted to 2.5 mM in water and dispensed as drops into wells of 16-well plates lined with parafilm. A carbon-coated copper TEM grid (Canemco, Canton de Gore, QC, Canada) was then floated on each sample drop for 5 min, removed and then allowed to dry for approximately 10 min. The sample grids were then floated on drops of 45-μm syringe-filtered 2% uranyl acetate, pH 4.0, for 5 min and washed by floating on a series of four drops of 45-μm syringe-filtered distilled water for approximately 1 min each. The grids were then dried by briefly touching their sides to Whatman filter paper and then allowed to sit for 10 min. They were viewed using a Hitachi S300N electron microscope.

Size exclusion chromatography of wflAFP-6

A 16/60 HiPrep Sephacryl S-200 HR column was employed using an Akta-FPLC system (both GE Life Sciences, Mississauga, ON, Canada). The column was washed with several volumes of water, and then equilibrated with 10 mM ammonium acetate, pH 6.0. Molecular mass standards used to calibrate the column included γ-globulin, bovine serum albumin (BSA), ovalbumin, trypsinogen and cytochrome c, in duplicate runs. Standards contained a protein mass of approximately 2–10 mg, and were dissolved in 2 mL of 10 mM ammonium acetate, 6.0. The flow rate was 0.5 mL·min⁻¹ and two column volumes were used to ensure complete removal of one standard before addition of the next.
Several 15-mM wflAFP-6 samples, subjected to the ice interaction freezing protocol, were combined in order to obtain a minimum of 1 mg of protein. The sample volume was then adjusted to 2 mL with 10 mM ammonium acetate, pH 6.0, for use with the column. After the wflAFP-6 was applied to the column, protein was monitored by absorbance at 214 nm, to detect the peptide bond, as wflAFP-6 does not contain any aromatic amino acids that would absorb light at 280 nm. Each chromatography run was performed in duplicate. Several control samples of 15-mM wflAFP-6, which had been held at 4 °C and not exposed to ice, were combined in order to obtain over 1 mg of protein and analysed in the same way.

Denaturation and evaluation of wflAFP-6

To test the effect of denaturation, 5 μL of 15-mM wflAFP-6 sample were placed in a 500-μL centrifuge tube, incubated in a boiling water bath for 5 min and then chilled to 4 °C for approximately 30 min. A portion was diluted to 0.1 μg·μL⁻¹ with 10 mM ammonium acetate, pH 6.0, for use in ThT-binding assays. Identical samples of the 15-mM wflAFP-6, kept at 4 °C, were also diluted to 0.1 μg·μL⁻¹ with 10 mM ammonium acetate, pH 6.0, as controls.

Preparation and evaluation of wflAFP-6 over a range of pH values

wflAFP-6 samples were dissolved to 15 mM in solutions containing 10 mM sodium acetate, 10 mM ammonium acetate, 10 mM Tris-HCl and 10 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) adjusted to pH 4.0, 6.0, 8.0 and 10.0. Prepared samples were treated as described above, and prepared for ThT assay, ensuring triplicates. Samples in 10 mM ammonium acetate, pH 6.0, maintained at 4 °C were used as controls.

Preparation of wflAFP-6 over a range of concentrations

Solutions of wflAFP were prepared at concentrations of 1 mM, 5 mM, 10 mM and 15 mM wflAFP-6 in 10 mM ammonium acetate, pH 6.0, and then frozen using the ice interaction procedure above, for ice interaction and prepared for ThT assay. Portions of each wflAFP-6 sample concentration, held at 4 °C, were also diluted to 0.1 μg·μL⁻¹ in 10 mM ammonium acetate, pH 6.0, to serve as negative controls.

Preparation and evaluation of wflAFP-6 and suitable control proteins following ice interaction

Soybean (Glycine max) trypsin inhibitor and a preparation of antifreeze glycoproteins (AFGP) from Atlantic cod (Gadus morhua), were used in order to test the specificity of freeze-induced amyloid formation. Solutions of 3.1-mM solution of soybean trypsin inhibitor, 3.5 mM AFGP and 15 mM wfl AFP were prepared in 10 mM ammonium acetate, pH 6.0. These concentrations were chosen in order to approximate the same molar concentration of total amino acid residues in each solution. The solutions were frozen using the procedure indicated for ice interaction and prepared for ThT assay, ensuring triplicates. Portions of the original unfrozen stock solutions were held at 4 °C as negative controls and the wflAFP-6 samples subject to ice interaction were included as positive controls. Samples were then prepared for ThT assays.

Results and discussion

Requirement of ice interaction for amyloid formation

Any solute, and particularly an AFP that binds to ice, may undergo changes during a freeze/thaw cycle. Therefore, the freezing process was dissected out into its component aspects in order to more precisely determine the trigger for amyloid formation by wflAFP-6. Three events can be considered during the cooling of an AFP: (a) a decline in temperature, (b) a phase change (freezing and thawing), and (c) interaction with an ice surface. Experiments were designed to examine these events separately. Equilibrium freezing of 15 mM wflAFP-6 in the presence of an ice seed resulted in strong ThT fluorescence (Fig. 3), while spontaneously nucleated rapid phase change in the absence of an ice seed and maintenance of a low temperature without

Fig. 3. Fluorescence of thioflavin T (ThT) dye in the presence of wflAFP-6 incubated under different freezing and nonfreezing conditions in 10 mM ammonium acetate, pH 6.0. Fluorescence levels in wflAFP-6 samples that were maintained at 4 °C (controls), cooled to −2 °C (low temperature), snap frozen and then maintained at −2 °C (phase change) or frozen at −2 °C with seed ice (ice interaction) prior to dye addition are shown. RFU indicates relative fluorescence units. Values shown are means ± SEM of three samples.
freezing each produced far weaker signals similar to those of control samples held at 4 °C. Thus, it is the specific binding of wflAFP-6 to the ice surface that favours its transition to an amyloid structure, rather than temperature or phase changes that occur during the freezing process.

When examined by TEM, the samples that had undergone ice interaction (ice-seeded freezing) revealed fibrils (Fig. 4), whereas none were present in the control samples that were held unfrozen at 4 °C. The protein size profile analysed by size exclusion chromatography also shifted in ice interaction samples compared with unfrozen (4 °C) controls (Fig. 5). Early-eluting protein with relative masses > 67 kDa (the largest marker) increased sharply (peaks 1 and 2), whereas the major peak (4) corresponding to a mass of 10 kDa, which is the predicted mass of monomeric wflAFP-6 in size exclusion chromatography due to its extended structure [21,22], was diminished. These results suggest extensive protein association. A 32-kDa peak (3) was also present in both samples, which may represent modest oligomerization under both conditions. The size exclusion and TEM results are consistent with the ThT fluorescence indicating amyloid formation.

The mechanism by which ice interaction favours the amyloid transition in wflAFP-6 is unclear. NMR analyses have revealed the AFP to be α-helical both in supercooled solution and when frozen in ice [15,23], consistent with the absence of an amyloid transition in the low temperature and phase change samples. The requirement shown here for ice binding suggests that either the alignment of wflAFP-6 molecules on the ice surface or a physical characteristic of the intermediate quasi-liquid layer between liquid water and frozen ice allows the amyloid transition to take place. Surfaces such as those generated by phospholipid membranes, the air–water interface or oil–water interfaces have been implicated in protein misfolding and amyloid formation (reviewed by [24–26]). Thus, alignment of AFP specifically bound to the ice surface could be considered to favour interaction leading to unfolding and aggregation. However, studies on other AFPs have suggested the binding of individual molecules to ice [27]. Furthermore, scanning tunnelling microscopy study of the wflAFP-6 revealed ice surface grooves corresponding in size to individual adsorbed monomers approximately 50 Å apart, which would preclude protein–protein interaction on the ice surface [28]. Therefore, it is unlikely that the ice serves as a template inducing interaction between AFPs. It is possible that a denaturation event upon thawing leads to the amyloid conversion, as suggested by Graether and Sykes [18], but this denaturation would be a consequence of specific ice binding rather than straightforward freeze-concentration of the protein.

The effects of protein and solution parameters on amyloid formation by wflAFP-6

When a series of concentrations of wflAFP-6 were subject to ice interaction, the 1 mM solution showed no appreciable ThT fluorescence, whereas fluorescence became detectable at 5 mM of protein and levels in solutions of 10 and 15 mM appeared equivalent,
suggesting a maximal level of amyloid for this system (Fig. 6). This dependence on a highly elevated protein concentration suggests that freeze-concentration of the AFP does not have a role in amyloid formation, as the solution concentration would not be expected to be limiting at 1 mM.

Denaturation by heating under specific buffer conditions has been employed to bring about an amyloid transition in globular proteins [29,30]. To determine whether denaturation had a role in the ice interaction-induced wflAFP-6 amyloid formation, 15 mM wflAFP-6 samples were boiled and then analysed. Boiling resulted in the formation of an amorphous gel, which made pipetting more challenging, although still possible. As noted previously [17,18], elevated concentrations of wflAFP-6 gradually formed an amorphous gel over several days of storage at 4 °C. The gel appeared to be distinct from amyloid, as it did not increase ThT fluorescence [17]. The gel formed by boiling appeared to be consistent with the nonamyloid amorphous gel observed in solutions of this protein at pH 8 [18]. Positive control (ice interaction-treated) wflAFP-6 samples showed increased ThT fluorescence, whereas the negative controls (4 °C) showed no increase and the boiled samples were indistinguishable from the controls (Fig. 7). Thus, as shown previously [18], the wflAFP-6

**Fig. 5.** Size distribution of wflAFP-6 samples with and without ice interaction. Size exclusion chromatography was performed in 10 mM ammonium acetate, pH 6.0, on wflAFP-6 subject to ice interaction as well as an unfrozen control. mAU indicates milliabsorbance units. The elution positions of the protein mass standards are indicated by arrows and notable peaks are numbered (1–4).

**Fig. 6.** Fluorescence of thioflavin T (ThT) dye in the presence of wflAFP-6 subject to ice interaction at different concentrations. Samples of wflAFP-6 were prepared at 1, 5, 10 and 15 mM in 10 mM ammonium acetate, pH 6.0, subjected to ice interaction and then assayed using ThT. Control samples of 15 mM wflAFP-6, held at 4 °C, were also measured. RFU indicates relative fluorescence units. Values shown are means ± SEM of three samples.

**Fig. 7.** Fluorescence of thioflavin T (ThT) dye in the presence of wflAFP-6 subject to denaturation by heating. Samples of 15 mM wflAFP-6 were prepared in 10 mM ammonium acetate pH 6.0, then subjected to either ice interaction or heating and then assayed using ThT. Control samples of 15 mM wflAFP-6, held at 4 °C, were also measured. RFU indicates relative fluorescence units. Values shown are means ± SEM of three samples.
can form nonamyloid aggregates under specific conditions.

The denatured, gelled aggregates of wflAFP-6 and the amyloid form appear to be the products of alternative processes rather than steps in a single pathway. In several proteins, the unfolding of α-helices does not appear to be a prerequisite for the conversion into an amyloid structure. Instead, the existence or formation of α-helices is proposed to be a step leading directly to amyloid formation [31]. In huntingtin, islet amyloid polypeptide and α-synuclein, the α-helical intermediates appear to correspond to the lag phase that typically precedes more rapid appearance of β-strands and ensuing amyloid structure [32–34]. In a model peptide bundle, 310-helix was shown to form during the transition from an α-helical to a β-strand conformation [35]. The crystal structure of wflAFP-6 revealed 310-helical conformation in the last peptide unit [36], which could be further investigated in light of this finding. It may provide a starting point for conversion of the wflAFP-6 helix into the β-strand required for amyloid.

The pH of a solution can affect interactions within and among proteins by shifting the ionization of charged groups. The helical content and antifreeze activity of wflAFP-6 are steady between pH 4 and 9 [37], which implies native structure and ice binding over this pH range. However, pH has been implicated in the behaviour of wflAFP-6, as it was shown to form amyloid at pH 4 and an amorphous gel at pH 8 [18]. Therefore, ice interaction studies were performed in solutions of wflAFP-6 over a range of pH values. ThT fluorescence was elevated at pH 4.0, 6.0 and 8.0, with the highest mean obtained at pH 6. In contrast, there was no fluorescence increase at pH 10.0 (Fig. 8). The loss of positive charge on the ε-amino group of Lys is the key change that would occur with this rise in pH and this would disrupt a salt-bridge that stabilizes the α-helix [38]. The absence of amyloid conversion at pH 10 would suggest either a requirement for a stable α-helix or a separate role for charged Lys involvement in the conversion of wflAFP-6 to the amyloid conformation. Alternatively, since the protein has been reported to form an amorphous gel at pH 8.0, it may be that gelation at high pH sequesters it, precluding ice association and the ensuing amyloid formation. However, no gel was observed in the high pH samples.

The specificity of ice interaction-induced amyloid formation

The specificity of amyloid induction by ice interaction was examined by testing two control proteins in the same manner as the wflAFP-6. One was an AFP (the AFGP of Atlantic cod) that binds ice, but that is non-homologous and structurally distinct from the wflAFP-6 [39]. The other was soybean trypsin inhibitor, chosen because its pI is similar to that of wflAFP-
6. The ThT fluorescence was not elevated in samples of those proteins when subjected to ice interaction (Fig. 9), suggesting ice interaction-induced amyloid to be neither a general property of proteins that bind to ice nor of proteins with particular charge features. With the variety of closely related winter flounder AFPs, it is possible that some of them share the amyloid propensity of wflAFP-6. Investigation of this process in naturally occurring and engineered sequence variants would help in identifying the structural determinants of this conversion. Amyloid propensity was engineered into the β-solenoid structures of AFPs from spruce budworm (Choristoneura fumiferana) and ryegrass (Lolium perenne), but these were chosen based on their geometric arrangements of β-sheets in their native form and the transition did not involve ice interaction or low temperature [40]. Thus, no generalization can be made from these examples taken together in terms of antifreeze propensity for amyloid formation.

Conclusions

This study has shown that specific interaction with the ice surface is necessary for the conversion of the wflAFP-6 from its native α-helix to an amyloid conformation; subzero solution temperature and phase change do not otherwise bring about this conversion. Ice interaction-induced amyloid formation appears specific to this protein and it requires no nonphysiological solution components.

Numerous proteins contain amyloidogenic segments, but the majority of these sequences are buried in the native fold of the protein or they are twisted in a manner that precludes the extended β-strand formation and interdigitation that occur in amyloid [41]. Furthermore, sequences with high amyloid propensity often occur within so-called discordant α-helices in proteins; this appears to be a common mechanism by which spurious amyloid formation is avoided [42,43]. In such proteins, the structural constraints prevent formation of β-strand in the native protein. Thus, denaturation, proteolysis, additives or extreme solution conditions are normally required to unfold the protein and release the amyloid-forming segment. In contrast, the wflAFP-6 is not restrained; it is a lone helix devoid of tertiary structure. In this case, it is the presence of a trigger rather than the removal of a constraint that allows wflAFP-6 to convert to an amyloid structure.

As a model for amyloid formation, wflAFP-6 brings distinct advantages. There appears to be no spurious amyloid formation in untreated samples, whereas the conversion takes place in minutes with ice interaction. The diversity of naturally occurring flatfish AFPs and well-characterized mutant sequences along with the ease of peptide synthesis provide a plethora of options for exploring the roles of specific residues and structural elements in the amyloid propensity of wflAFP-6. Additionally, since the wflAFP-6 amyloid fibrils were found to be stable once formed and to continue growth at 20 °C [18], the possibility of cross-templating with other amyloid-forming proteins can be explored, making information gained from this protein more relevant to human amyloid diseases.

Further work will be required in order to determine whether the amyloid conversion of wflAFP-6 in response to ice interaction occurs in vivo in the winter flounder. In addition, the possibility of AFP interaction with cell surfaces, conferring possible hypothermic protection, has been the subject of several studies with mixed and conflicting results [44]. The soluble, gelled and amyloid forms of wflAFP-6 could be examined separately for differences in this regard, as these forms may offer an explanation for the divergence in those findings.

Acknowledgements

We thank Garth Fletcher (Memorial University of Newfoundland) for generously providing the antifreeze glycoprotein (AFGP) used as a control in this study. This work was supported by an NSERC Discovery Grant (KVE).

Author contributions

KVE conceived and designed the experiments. AD, KVE and CL performed the experiments. AD and KVE analyzed the data and wrote the manuscript.

References


