The Role of N and C Termini in the Antifreeze Activity of Winter Flounder (Pleuronectes americanus) Antifreeze Proteins

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Antifreeze proteins (AFPs) are found in many marine fish and have been classified into five biochemical classes: AFP types I–IV and the antifreeze glycoproteins. Type I AFPs are α-helical, partially amphipathic, Ala-rich polypeptides. The winter flounder (Pleuronectes americanus) produces two type I AFP subclasses, the liver-type AFPs (wflAFPs) and the skin-type AFPs (wfsAFPs), that are encoded by distinct gene families with different tissue-specific expression. wfsAFPs and wflAFPs share a high level of identity even though the wfsAFPs have approximately half the activity of the wflAFPs. Synthetic polypeptides based on two representative wflAFPs and wfsAFPs were generated to examine the role of the termini in antifreeze activity. Through systematic exchange of N and C termini between wflAFP-6 and wfsAFP-2, the termini were determined to be the major causative agents for the variation in activity levels between the two AFPs. Furthermore, the termini of wflAFP-6 possessed greater helix-stabilizing ability compared with their wfsAFP-2 counterparts. The observed 50% difference in activity between wflAFP-6 and wfsAFP-2 can be divided into ~20% for differences at each terminus and ~10% for differences in the core. Furthermore, the N terminus was determined to be the most critical component for antifreeze activity.

Fish antifreeze proteins are diverse in structure and have been grouped into five biochemical classes based on their structural characteristics: antifreeze proteins (AFPs)\(^1\) types I–IV and antifreeze glycoproteins (AFGPs)\(^1\) (1–3). Although diverse in structure, all AF(G)Ps act by what is known as the adsorption inhibition mechanism (2, 4–6), where the AF(G)Ps lower the observed freezing point in a non-colligative manner creating a hysteresis between the equilibrium melting point and the observed freezing point. The degree of thermal hysteresis is used as a measure of AF(G)P activity.

Type I AFPs are Ala-rich, partially amphipathic single α-helical polypeptides found in several sculpins and righteye flounders (2, 7, 8). The winter flounder (Pleuronectes americanus) produces two subclasses of type I AFPs, the liver-type (wflAFPs) and the skin-type AFPs (wfsAFPs), which are encoded by distinct gene families (9). wflAFP-6 (formerly known as HPLC-6) is the major winter flounder plasma AFP and is produced in the liver as a prepro-precursor, and then secreted into circulation, where it is then processed into the mature polypeptide of 37 residues (10, 11). Conversely, the wfsAFPs have a wider tissue distribution and are produced as mature intracellular polypeptides (9).

The wflAFPs and wfsAFPs share a high level of structural identity even though the wfsAFPs have approximately half the activity of the wflAFPs (9). Both wflAFPs and wfsAFPs possess two 11-residue motifs of the structure XaaXAXAXAXX (where the first Thr is always conserved, uppercase A is a conserved Ala, lower case a is almost always Ala, and X can be one of several amino acids), with wflAFP-6 having a third full motif at the N terminus (see Fig. 1). Furthermore, the wflAFPs possess complete ice-binding motifs (IBMs), LTAAN (8), whereas the wfsAFPs have incomplete IBMs of the form ATAAA, and this difference in IBMs has been proposed to be the cause of the lower thermal hysteresis found in wfsAFPs (12). However, the introduction of two proposed IBMs (KT-D, and DT-K) into wfsAFP-2 did not produce any improvement in activity (12). Furthermore, the introduction of the LTAAN IBM into wfsAFP-2 only increased the activity by ~15%, yet lowered the helical content from 80 to 60\(^\%\).\(^2\)

The IBM hypothesis is based on earlier beliefs that wflAFP-6 requires regularly spaced polar groups to match the arrangement of water molecules on the ice surface for the formation of hydrogen bonds (5, 13–16). However, recent study of wflAFP-6 has suggested that the major ice-binding surface resides on the hydrophobic face of the polypeptide, with major contribution from conserved Ala residues. Furthermore, the driving forces behind ice binding are van der Waals interactions and the hydrophobic effect (17–21). The major sequence variations between wflAFP-6 and the wfsAFPs are in the N and C termini (see Fig. 1). Most notably, wflAFP-6 has the N-terminal sequence DTASDA, whereas all wfsAFPs begin with MDAP (9).

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N and C Termini of Winter Flounder Antifreeze Proteins

This study examines the contributions of the N- and C-terminal sequences to the antifreeze activity of the liver- and skin-type AFPs. Polypeptides were synthesized where the N- and C-terminal sequences of two representative AFPs, wflAFP-6 and wfsAFP-2, were systematically exchanged, then assayed for antifreeze activity and α-helical content. The results demonstrated that the difference in activity was caused primarily by sequence variations at the N and C termini. Furthermore, the N and C termini of wflAFP-6 were able to confer higher overall helical content and greater thermal stability on their skin-type AFP counterparts.

EXPERIMENTAL PROCEDURES

Polypeptide Synthesis and Purification—Polypeptides were synthesized by continuous flow Fmoc chemistry on NovaSyn KA 100 resin (25) using a Biolynx 4170 automated peptide synthesizer (Amersham Biosciences, Montreal, Quebec, Canada) by the Advanced Protein Technology Centre, Hospital for Sick Children (Toronto, Ontario, Canada). A 20% piperidine solution in dimethyl formamide was used for removal of the Fmoc protection group. For each gram of resin (0.1 mmol substitution), a 4× excess of Fmoc amino acid activated with O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphosphate and diisopropylethylamine (1:1:2, mol/mol/mol) (26) was used for the coupling reaction, with a reaction time of 1 h. Peptide-resin conjugates were washed with dimethyl formamide, diethyl ether, and dried under reduced pressure. Dry peptide-resin conjugates were cleaved with 20 ml of trifluoroacetic acid containing 4 ml of thioanisole, 0.4 ml of trifluoroacetic anhydride, and 0.4 ml of n-cresol, 2 ml of 1.2-ethanedithiol, 3 ml of ethylmethylsulphone, and 4 ml of bromotrimethylsilylane at 0°C for 1 h. Peptides were extracted, dissolved in 0.1% trifluoroacetic acid, and desalted on a Sephadex G10 column. Crude polypeptide preparations were further purified by reverse-phase HPLC using a Jupiter 10-μm C8 300 Å (250 mm × 21.20 mm) column (Phenomenex, Torrance, CA) using an acetonitrile gradient in 0.1% trifluoroacetic acid. Homogeneity of peptides was analyzed by C18 reverse-phase HPLC, amino acid analysis, and electrospray ionization mass spectrometry. All polypeptides were synthesized with free N termini and amidated C termini except where indicated.

Measurement of Antifreeze Activity—Synthetic polypeptides were assayed for antifreeze activity using a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NY) as previously described (27). Briefly, purified and lyophilized polypeptides were re-dissolved in 0.1 M ammonium bicarbonate (pH 7.9), and assayed for antifreeze activity using a Clifton Nanolitre Osmometer mini and amidated C termini except where indicated.

RESULTS

A comparison of wflAFP-6 and wfsAFP-2 sequences demonstrated that the region of highest identity between the two polypeptides occurs in the central region (wflAFP-6 core; L-core) and Table I (column 1) details the difference in activity when [S]-Nterm was replaced by [L]-Nterm (A1 value) and [S]-Nterm was defined as DTASDA, and the C-terminal sequence, [L]-Cterm, was defined as AR. Correspondingly, in wfsAFP-2, the N-terminal sequence, [S]-Nterm, was defined as MDAP, and C-terminal sequence, [S]-Cterm, defined as KAGGAAR. The synthetic polypeptides were designed to examine the effects of N-terminal exchange, C-terminal exchange, and simultaneous exchange of both termini. In the N-terminal exchange series, polypeptides consisting of wild-type sequences for wfsAFP-2 and wflAFP-6, Skin(wt) and Liver(wt), were also assayed. The Skin(wt) activity was ~50% of Liver(wt) (Fig. 2D), in agreement with previous results (9). For ease of comparison, all activity levels were expressed as a percentage of Liver(wt) activity. Skin(wt) was used as an internal control to allow comparison of results between the three groups of polypeptides.

The Exchange of N- or C-Terminal Sequences—Lack of C-terminal amidation in the wfsAFP-2 C terminus ([L]Skin-OOH) did not significantly affect activity (Fig. 2A). The conversion of the wflAFP-6 N or C terminus to the corresponding wfsAFP-2 sequences resulted in a loss of ~27% of activity (Fig. 2D; Table I, column 1, values A1 and C1). In wfsAFP-2, replacement of the N or C terminus with those found in wflAFP-6 resulted in a gain of ~15% in activity (Fig. 2D; Table I, column 1, values A3 and C3).
When [S]-Nterm was replaced by [L]-Nterm, the magnitude of the increased activity was ~14% greater when the remaining polypeptide contained L-core and [L]-Cterm (Table II, column 1, A1–A3 value). When the remaining polypeptide was composed of a mixture of components, the increase in activity associated with the same exchange was ~4% higher in the presence of S-core and [L]-Cterm as opposed to L-core and [S]-Cterm (Table II, column 1, A4–A2 value). Similarly, for the exchange of [S]-Cterm with [L]-Cterm, the increased activity was ~11% higher when wflAFP-6 components were present (Table II, column 1, C1–C3 value). Furthermore, the increased activity associated with this exchange was ~7% higher when [L]-Nterm and S-core were present as opposed to the opposite composition (Table II, column 1, C4–C2 value).

Simultaneous Exchange of N- and C-terminal Sequences—The [L]Skin[L] polypeptide was similar to a previously studied wflAFP-6 mutation, ATAAA, which was reported to have ~85% of the activity of a wild-type analogue as determined by the activity curves presented by Loewen et al. (29). The ATAAA analogue differed from [L]Skin[L] at positions 8 and 30, where ATAAA possessed Ala instead of Lys. Loewen et al. (29) observed lowered solubility for ATAAA, which affected their analysis of the analogue; however, no solubility issues were observed with [L]Skin[L] for the concentrations used here. If the experimental error of ~5% between assays is considered, then the combined effects of N- and C-terminal exchange on thermal hysteresis levels appeared to be somewhat additive. For [L]Skin and Skin[L], activity was increased in each case to ~64%, and ~67% of Liver(wt) (Fig. 2D). In combination, the exchange of both N- and C-terminal sequences, [L]Skin[L], resulted in activity of ~89%. Similarly, the 54% level of activity for [S]Liver[S] was close to the level of activity expected from the combined reductions for each individual exchange.

The simultaneous exchange of both termini was also evaluated as a single exchange in the core region. Thus, replacement of L-core in wflAFP-6 with S-core (essentially a replacement of complete IBM with incomplete IBM) resulted in a loss of ~11% of activity (Table I, column 1, value B1). The corresponding exchange in wfsAFP-2 resulted in a gain of ~3% in activity (Table I, column 1, value B3). The magnitude of the effect of the S-core to L-core exchange was greater by ~7% in the context of the wflAFP-6 termini than for the wfsAFP-2 termini (Table II, column 1, B1–B3 value). In the mixed-termini case, the magnitude of the exchange was more prominent by ~3% when the termini were composed of [L]-Nterm and [S]-Cterm then when composed of [S]-Nterm and [L]-Cterm (Table II, column 1, B4–B2 value).

**Pairwise Comparison of the Effects of the N Terminus, Core Region, and C Terminus on Antifreeze Activity**—A pairwise comparison of activity levels between polypeptides that differed at a single region was performed to address the experimental error involved in each individual assay. Each region of wflAFP-6 demonstrated a higher level of activity than the corresponding region of wfsAFP-2. The [L]-Nterm provided an average improvement of ~20% (~6%), L-core an improvement of ~7% (~4%), and [L]-Cterm an improvement of ~22% (~5%) in activity over the corresponding wfsAFP-2 components (Table I, column 1). Only in the evaluation of the core region did the error significantly impact the average value determined; nevertheless, the general trend was toward higher activity when the L-core was present as opposed to the S-core. However, as demonstrated in Table II, the magnitude of the increased activity associated with a wflAFP-6 region over that of the corresponding wfsAFP-2 region was dependent on the identities of the remaining regions.

**The Magnitude of the Increase in Activity Associated with a wflAFP-6 Component Is Dependent on the Identity of the Remaining Regions**—In Table II (columns 3 and 5), a comparison of the values in Table I (column 1) was performed to determine the impact of the identities of the other regions that were present during the three exchanges. The presence of [L]-Cterm during exchange at the N terminus produced a ~9% higher improvement of activity compared with when the [S]-Cterm was present (Table II, column 3, A), and a ~5% higher improvement of activity was observed in the presence of L-core compared with S-core (Table II, column 5, A). Thus, a 4% higher improvement of activity (A1-A2 value) was observed when the wflAFP-6 component was present at the C terminus compared with when it was present within the core. Similarly, the improved activity of L-core over S-core demonstrated a 5% higher
increase in the presence of [L]-Nterm over [S]-Nterm (Table II, column 3, B). For this same exchange, a slightly higher increase in activity was noted in the presence of [L]-Cterm when [S]-Nterm was present. However, the observed increase in activity when [L]-Nterm was present was not statistically significant (Table II, column 6, B). In this exchange, the improvement of activity associated with L-core was higher when wflAFP-6 components were present at the N terminus as compared with when they were present at the C terminus (Bx1–Bx2 value). The improvement of activity with [L]-Cterm in place of [S]-Cterm demonstrated a 9% higher enhancement in the presence of [L]-Nterm (Table II, column 5, C). For this exchange, the increased difference in activity levels when L-core was present was not statistically significant when [L]-Nterm was also present; however, the result was significant in the presence of [S]-Nterm (Table II, column 6, C). For this exchange, the improvement in activity was higher when wflAFP-6 components were present at the N terminus compared with when they were present in the core (Cx1–Cx2 value). All trends indicated that the improvement of activity associated with each wflAFP-6 component was always greater when other regions of wflAFP-6 were present. Furthermore, the location of the other wflAFP-6 components demonstrated differing levels of influence on the increased activity associated with each exchange, with the order of dependence for other regions being N terminus > C terminus > core. This order of dependence was also apparent in the values determined in column 1 (Table II). For example, in the A4–A2 value (Table II, column 1, A), the degree of activity enhancement was higher for the S-core and [L]-Cterm combination than for L-core and [S]-Cterm because the effect of the C terminus took precedence. This dependence appeared to have a “long range” effect, as the increased activity associated with [L]-Nterm over [S]-Nterm was more dependent on the identity of the opposite terminus, not the core region that was adjacent to it. The dependence of activity levels on the identity of the opposite terminus was also observed with the C terminus exchange.

**α-Helical Content of Polypeptides at 0 °C**—Each polypeptide was assayed for its α-helical content utilizing circular dichroism (CD) spectroscopy. Spectra (data not shown) were recorded at 0 and 40 °C in 0.1 M ammonium bicarbonate (pH 7.9) at two different concentrations. At 0 °C, all polypeptides displayed spectra indicative of high α-helical content with typical negative minima at 222 and 208 nm, and positive maximums at approximately 192 nm. At 40 °C, the spectra were indicative of helix-random coil mixed structures and were identical at two different concentrations. Helical content determinations indicated Liver(wt) to be nearly entirely α-helical (~95%), which was in good agreement with previous structural studies of wflAFP-6 (8, 30, 31), and Skin(wt) was found to have a helical content of ~82% (Fig. 3).

An initial inspection of the results (Fig. 3A) for exchanges to wflAFP-6 demonstrated that the effects on helicity for each exchange appeared to be additive. The decreases in helical
content between Liver(wt) and [S]Liver (10.3%), and between
Liver(wt) and Liver[S] (8.9%), gave a combined decrease of
19.2% that would predict a helical content of 75.3% for [S]Liv-
er[S], which was close to the observed value of 77.9%. However,
for wfsAFP-2, replacement of the C terminus alone (Skin[L])
produced 100% helical content (Fig. 3A), whereas [L]Skin
demonstrated 90% helical content.

Pairwise Comparison of the Effects of the N Terminus, Core
Region, and C Terminus on α-Helical Content—The differences
in helical content between polypeptides that differed at a single
region are summarized in Table I, column 3. Several of the
differences were found not to be statistically significant in this
comparison; however, in three of these instances, the p values
were close to the α value of 0.05 used in the analysis (Table I,
column 4, a2, b1, and c2). Overall, the results of the pairwise
comparison of the effects of exchanges on helical content were
not as consistent as for the activity analysis, because of the
aberrant values found for Skin[L] (Table I, column 5). However,
the general trend observed in the pairwise comparison indicated that the [L]-Nterm induced a higher helical content than the [S]-Nterm, whereas the S-core induced a
higher helical content than the L-core.

For the C-terminal exchange, the [L]-Cterm always produced
higher helical content than [S]-Cterm. Only in the presence of
[S]-Nterm and L-core was the increased helical content (Table
I, column 3, c2 value) associated with [L]-Cterm found to be
statistically insignificant. However, a dependence on the na-
ture of the core sequence for the increased helicity associated
with [L]-Cterm was observed, as helical content was ~10% higher when the S-core was present (Table I, column 6). When
the L-core was present, the [L]-Nterm demonstrated a greater
effect on enhanced helicity in the presence of the [L]-Cterm
(8.9% - 6.3% = 2.6%). Conversely, when the S-core was present,
the [S]-Nterm provided higher enhancement of helicity in the
presence of the [L]-Cterm (16.5% - 20.3% = -3.8%). In both
instances, the difference values were based on a single sce-
nario, unlike earlier comparisons where trends could be dis-
cerned because of multiple values from independent compari-
sions. However, these results may indicate that the dependence
on the nature of the core region is further enhanced when the

| Table I: Pairwise comparison of the activity and helical contents of polypeptides differing at a single region |
|----------------------------------------------------------|
| The resulting values of column 1 were assigned labels (A1 to A4, B1 to B4, and C1 to C4) for use in the analysis presented in Table II. The * indicates data in column 4 containing Skin[L] data. For the statistical analysis: H0 = no difference between means, α = 0.05, and failure to reject the null hypothesis (H0) is indicated by gray highlights. For column 5 in A and B, averages are derived from values in column 3 without inclusion of data containing Skin[L]. For column 6 in C, averages are from the indicated values of column 3. |

| N-terminal exchange (N-terminus) DITASDA vs. MDAP |
|--------------------------------------------------|
| [L] Liver(wt) [L] - [S] Liver [L] |
| [L] Liver [S] - [S] Liver [S] |
| [L] Skin [S] - [S] Skin(wt) [S] |
| [L] Skin [S] - [S] Skin [L] |

| P-value | P-value |
|---------|---------|
| a1 = 10.3 | 0.0008 |
| a2 = 7.7 | 0.0505 |
| a3 = 8.2 | 0.0090 |
| a4 = 3.8 | 0.1549 |

| Average | 20.3 |
|---------|------|
| Std. Dev. (σ) | 5.9 |

| Core exchange (Liver/Core) vs. Skin (Core) |
|------------------------------------------|
| [L] Liver(wt) [L] - [L] Skin [L] |
| [S] Liver [S] - [S] Skin [S] |
| [S] Liver [S] - [S] Skin(wt) [S] |
| [L] Liver [L] - [L] Skin [L] |

| P-value | P-value |
|---------|---------|
| b1 = -4.3 | 0.0501 |
| b2 = -18.4 | 0.0066 |
| b3 = -4.9 | 0.1960 |
| b4 = -4.4 | 0.0451 |

| Average | 6.6 |
|---------|----|
| Std. Dev. (σ) | 3.7 |

| C-terminal exchange (C-terminus) TAR vs. TKAGAAR |
|-----------------------------------------------|
| [L] Liver(wt) [L] - [L] Liver [S] |
| [S] Liver [S] - [S] Liver [S] |
| [S] Skin [S] - [S] Skin(wt) [S] |
| [L] Skin [L] - [L] Skin [L] |

| P-value | P-value |
|---------|---------|
| c1 = 8.9 | 0.0080 |
| c2 = 6.3 | 0.0597 |
| c3 = 20.3 | 0.0090 |
| c4 = 16.5 | 0.0331 |

| Average | 22.0 |
|---------|-----|
| Std. Dev. (σ) | 5.3 |

| Column number | 1 | 2 | 3 | 4 | 5 | 6 |
core region is associated with its native N terminus. An analysis of the helicity values obtained for the N-terminal exchange demonstrated that the relationship existed for the N terminus. The effect of higher helical content associated with (L)-Nterm was greater in the presence of L-core, but when either core was present, the increased helical content was higher when each core was associated with its own C terminus (Table I, column 3, A).

**Correlation of Activity Levels and Helical Content**—When comparing all analogues studied, activity levels increased as helical content increased (Fig. 4A). However, for all analogues, a rigorous direct correlation between helical content and activity was not observed (Fig. 4A, overall linear regression line). If the L-core and S-core analogues are analyzed separately, the analogues containing L-core demonstrated a very good direct correlation between activity and helical content ($R^2 = 0.990$), whereas the S-core containing analogues still demonstrated a low level of direct correlation. Examination of the residuals for the linear regression for all analogues demonstrated that a reasonably good correlation between activity and helical content existed until the polypeptides achieved a helical content of $90\%$, beyond which the correlation became very poor.

**Comparison of Observed $\alpha$-Helical Content with Predicted Values Based on a Simple Two-state Helix Prediction Method**—

### Table II

Pairwise analysis of the increased activity of a wflAFP-6 component and the dependence on the characteristics of the remainder of the polypeptide

|                  | P-value | C-terminus dependence | Core dependence |
|------------------|---------|-----------------------|-----------------|
|                  |         | XLL-XLS=               | XLL-XSS=        |
| A                |         | A1-A2= 8.7             | A4-A3= 9.2      |
| N-terminus       |         | 0.0004                | 0.0000          |
| exchange         |         | 0.0219                | 0.0014          |
|                  | Avg.=A_x= 9.0 |                     |
|                  | A_{x1}-A_{x2}= 4.0 |                 |
|                  |         | XSL-XLS=               | XLS-XSS=        |
|                  |         | A4-A2= 4.0             | A2-A3= 5.2      |
|                  |         | 0.0000                | 0.0000          |
|                  | Avg.=A_x= 5.0 |                     |
|                  |         | A_{x1}-A_{x2}= 4.0 |                |
|                  |         | Bx_{1}-Bx_{2}= 2.7 |                |
|                  |         | 0.0024                | 0.0000          |
|                  | Avg.=B_{x1}= 5.0 |                     |
|                  |         | Bx_{1}-Bx_{2}= 2.7 |                |
|                  |         | 0.2321                | 0.0000          |
| B                |         | LXL-SX=               | SXL-SX=        |
| C-terminus       |         | B1-B3= 7.2             | B2-B3= 5.2      |
| exchange         |         | 0.0001                | 0.0012          |
|                  | Avg.=C_{x1}= 9.0 |                     |
|                  |         | C_{x1}-C_{x2}= 6.7 |                |
|                  |         | 0.0000                | 0.0000          |
|                  | Avg.=C_{x1}= 2.3 |                     |
|                  |         | C_{x1}-C_{x2}= 6.7 |                |
|                  |         | 0.2016                | 0.0366          |
| C                |         | LLX-SS=               | SLX-SS=        |
| C-terminus       |         | C1-C3= 11.2            | C2-C3= 9.2      |
| exchange         |         | 0.0000                | 0.0000          |
|                  | Avg.=C_{x1}= 9.0 |                     |
|                  |         | C_{x1}-C_{x2}= 6.7 |                |
|                  |         | 0.0000                | 0.0000          |
|                  | Avg.=C_{x1}= 2.3 |                     |
|                  |         | C_{x1}-C_{x2}= 6.7 |                |
|                  |         | 0.0000                | 0.0000          |

*All values (A1 to A4, B1 to B4, and C1 to C4) are derived in Table I. In the three letter notation, X is the site of exchange, and core regions are abbreviated to single letters. The values obtained in on the right of each column are derived from the calculations shown on the left. For statistical analysis: $H_0 = $ no difference between means, $\alpha = 0.05$, and failure to reject the null hypothesis is indicated by gray highlighting.*
In Table III, the predicted helical contents were derived by using a simple two-state helix prediction method where each polypeptide component was isolated and assigned a total number of helical residues likely to be found in each region. For wflAFP-6, helix predictions were based on the assumption that each region ([L]-Nterm, L-core, and [L]-Cterm) was 100% helical based on structural data (8, 31). For wfsAFP-2, the assignments were based on current theories of helix capping (see “Discussion” for rationale behind assignment of helical and non-helical residues). In the [S]-Nterm, 3 of 4 residues were assumed to be non-helical (all residues preceding Pro-4), the S-core was assumed to be 100% helical, and in [S]-Cterm, 4 of 6 residues were assumed to be non-helical (all residues following and including Gly-36). The predicted helical contents for each polypeptide was then simply arrived at by determining the total number of residues in a helical conformation under the assumption that each region was independent of effects caused by the identity of the other regions. Examination of all polypeptides that contained the S-core demonstrated a remarkably high correlation of the predicted values with the observed values, except for Skin[L], which was underestimated by ~11%. In polypeptides that contained L-core, including Liver(wt), the helical content was consistently overestimated by ~5%.

**Thermal Denaturation of Polypeptides**—The polypeptides were subjected to thermal denaturation from 0 °C to 60, 64, or 65 °C, and their helical contents were assayed by CD spectroscopy at 222 nm (data not shown). The thermal denaturation of each polypeptide was fully reversible, but demonstrated very broad transitions over the temperature ranges used. Because of the broad nature of the unfolding process, standard practices of curve fitting for thermal denaturation data (32, 33) could not be performed on data collected here without producing significantly large values of error (data not shown). Thus, only a qualitative assessment of unfolding is presented by evaluating the helical fraction \( f_\text{H} \) of each polypeptide during thermal denaturation.

The two wild-type polypeptides displayed partial cooperativity in helix unfolding as evidenced by “S-shaped” denaturation profiles and a slight degree of thermal stability as temperatures increased (Fig. 3B). At temperatures below ~25 °C, Liver(wt) demonstrated enhanced helical content over Skin(wt). All polypeptides demonstrated some degree of cooperative unfolding except for [S]Liver and [S]Liver[S], which lacked S-shaped unfolding profiles and any indications of thermal stability (Fig. 3C). For [L]Skin, there was only a slight increase in helical content at 0 °C compared with Skin(wt), but an increase...
Thus, the improved activity of the [L]Skin mutation was because of the introduction of a more efficient ice-binding surface centered around the γ-methyl group of Thr-2, and not because of the increased helical content of the entire polypeptide afforded by the exchange of [S]-Nterm by [L]-Nterm.

**DISCUSSION**

Taking into account the results of individual exchanges, the pairwise comparison, and earlier studies, we conclude that the ~50% difference in antifreeze activity between wflAFP-6 and wfsAFP-2 is derived from (i) average contributions of ~20% by both the N (DTASDA versus MDAP) and C termini (AR versus KAGAAR), and (ii) a 10% contribution by the core region (complete IBM (LTAAN) versus incomplete IBM (ATAAA)). Furthermore, the magnitude of change in antifreeze activity generated by the exchange of a wfsAFP-2 component with a wflAFP-6 component was dependent on the characteristics of the other polypeptide regions. The region that imparted the greatest influence on activity between wfsAFP-2 and wflAFP-6 was the N terminus, because of its own ability to modulate activity levels and its influence on the activity of other regions of the polypeptide. The termini of wflAFP-6 demonstrated a greater ability to impart both higher helical character and thermal stability as compared with the wfsAFP-2 termini. However, the wflAFP-6 core region exhibited lower helix propensity than the wfsAFP-2 core region. Thus, the activity level of each region is likely related to a complex interplay between its own inherent ice-binding ability, helix propensity, and the helix propensity imparted upon the entire polypeptide by each region.

The thermal hysteresis levels associated with type I AFPs have been shown to be positively correlated with helical content (34), which is likely the result of the ability of ice-binding residues to adopt the proper configuration for ice recognition. This positive correlation of activity with helical content was confirmed with the analogues studied here (Fig. 4). However, the results clearly demonstrated that other factors as well as helical content affect activity levels, especially after relatively high helical content (>90%) is achieved, an example of which was demonstrated by the [L(T2S)]Skin mutation. Furthermore, a more direct correlation was observed for analogues containing L-core than for S-core, indicating that the activity of analogues containing L-core were more sensitive to the helical content.

From current theories of helix capping, it can be predicted that Gly-36 of wfsAFP-2 occupies the C’ position in a C-cap, or at least acts as a helix terminator, and Pro-4 is constrained to occupy the N1 position of the N-cap under the conventional notation for residues at helix termini (35). If all intervening residues adopt α-helical (ϕ, ψ) angles, the predicted helical content of Skin(wt) would be ~80%, which is very close to the observed value of ~82% (see Fig. 3A). Thus, from this analysis, the helix propensity of the S-core is equivalent to, or more likely, greater than (see below) the helix propensity of the L-core (at 0 °C), even though the CD measurements consistently demonstrated a lower overall helical content at 0 °C for wfsAFP-2. The nearly identical helical content of [S]Liver[S] to Skin(wt) at 0 °C (~78% versus ~82%) could be interpreted in the same manner as for Skin(wt). However, the lack of thermal stability of [S]Liver[S] compared with Skin(wt) (see Fig. 3C) would suggest that the L-core possesses a lower helical propensity than the S-core, which would be expected because of the higher Ala content in the S-core (36). Furthermore, the L-core consistently demonstrated a lower helical content than S-core (Table I, column 2, B). The assessment of a slightly lower than 100% helical propensity for L-core led to an overestimation of the helical content for polypeptides containing L-core in the

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**Fig. 4. A comparison of the observed activity levels and the helical content for each analogue.** A, a plot of the correlation between helical content and the activity level, as a percentage of Liver(wt), for each analogue. Error bars in the x and y axes directions are ±1 standard deviation. For Liver(wt), there is no y axis error bar as all activity values were normalized to Liver(wt). The solid black line (regression) is a plot of predicted activities from a linear regression analysis for all analogues. L-core (solid grey line) is a plot of predicted activities from a linear regression analysis for analogues containing L-core, and S-core (broken line) is a plot of predicted activities from analysis of analogues containing S-core. B, plot of the residual values from the linear regression analysis. L-core points are the residuals from analysis of only the L-core-containing analogues, and S-core points are the residuals of the analysis of analogues containing S-core.

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**N and C Termini of Winter Flounder Antifreeze Proteins**

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simple two-state helix prediction model (see Table III). The analysis presented in Table III was not an attempt to fully describe the structural characteristics of type I AFPs, but was used merely to illustrate that, in direct comparison, L-core was in fact not 100% helical whereas S-core maintains high helical content under several differing contexts. Thus, the S-core contributed more overall helical character than L-core when present in type I AFPs. Furthermore, the lower helix propensity of L-core compared with S-core manifested itself in the correlation between helical content and activity (Fig. 4), where the activity levels were more directly dependent on helical content for analogues containing L-core than for those containing S-core. Thus, inherent in a higher propensity for helix cap formation is a greater ability to impart helical character to the entire polypeptide. The ability of [L]-Nterm to impart higher thermal stability and its consistent demonstration of the ability to impart higher helical content to the entire polypeptide at 0 °C may indicate a greater N-capping propensity for [L]-Nterm compared with [S]-Nterm. In native winter flounder AFPs, the higher helix-inducing ability of the [L]-termini as compared with the corresponding [S]-termini may be necessitated by the lower helix propensity of the L-core caused by the presence of the more active complete IBMs. Thus, when L-core replaced S-core in [S]Liver[S], only a ~3% increase in activity was noted (see Table I) because the [S]-termini were not able to promote a high enough helical content within the core region to produce the full antifreeze activity of L-core. Conversely, nearly the full magnitude of the effect on activity for the core exchange was

Table III

A comparison of predicted α-helical content values based on a simple two-state model including helix capping with those observed by CD spectroscopy

| AFP analogue | No. of residues | No. of predicted non-helical residues | Predicted helix | Observed helix | Predicted helix minus observed helix |
|--------------|----------------|--------------------------------------|----------------|---------------|-------------------------------------|
| Skin(wt)     | 39             | 7                                    | 82.1           | 82.3          | -0.2                                |
| [L]Skin      | 41             | 4                                    | 90.2           | 90.5          | -0.3                                |
| Skin[L]      | 35             | 3                                    | 91.4           | 102.6         | 11.2                                |
| [L]Skin[L]   | 37             | 0                                    | 100.0          | 98.8          | 1.2                                 |
| Liver(wt)    | 37             | 0                                    | 100.0          | 94.5          | 5.5                                 |
| [S]Liver     | 35             | 3                                    | 91.4           | 84.2          | 7.2                                 |
| Liver[S]     | 41             | 4                                    | 90.2           | 85.6          | 4.6                                 |
| [S]Liver[S]  | 39             | 7                                    | 82.1           | 77.9          | 4.2                                 |

*Average for L-core polypeptides = 5.4 ± 1.4.

Fig. 5. Increased activity of [L]Skin polypeptide is dependent on presence of γ-methyl group of Thr-2. A, sequences of the synthetic polypeptides analyzed. All polypeptides were synthesized with free N termini and amidated C termini. The identical residues in the core region between all polypeptides are highlighted in gray. The Thr-2 → Ser mutation in [L(T2S)]Skin is highlighted in black. B, thermal hysteresis levels of the polypeptides. [L]Skin control is data collected in Fig. 2A and is included for comparison purposes. C, helical fraction of each polypeptide as determined by CD spectroscopy (222 nm) at 0 °C. Values are averages of three to four independent readings, and error bars are ±1 standard deviation.
observed in [L]Skin[L], because all components in this polypeptide were able to produce maximal helical content.

In wfAFP-2, Lys-6, which is normally found i + 4 to Asp-2, was not transferred along with [S]-Nterm in the various exchanges. The Asp-2/Lys-6 pair (see Fig. 1, D2-K6) may be necessary to form a salt bridge to stabilize the helix by forming an N-cap. Furthermore, the C terminus of wfAFP-2 may be involved in a complex C-cap structure, because C-capping residues usually reside outside the helix proper and often require a Gly residue at C’ to allow for positive (φ, ψ) angles (35). Although the lower capping ability of [S]-Nterm may suggest that the Asp-2/Lys-6 salt bridge is necessary in wfAFP-2, the improved thermal stability of [L(Skin over SkinL)] would suggest that [L]-Nterm still possessed a superior ability to impart higher helix propensity to the entire polypeptide. Furthermore, the inherent thermal stability of the S-core suggests that the helix-nucleating capabilities of the termini are not as critical for S-core. The putative Asp-2/Lys-6 salt bridge may also explain the seemingly aberrant results observed for SkinL in that some form of interaction between regions caused the three residues preceding Pro-4 to adopt a helical conformation. Further work is necessary to investigate the validity of the proposed Asp-2/Lys-6 salt bridge.

The “long range” dependence of the increased activity associated with each [L]-termini on the identities of the opposing termini was likely related to the relative helix propensity of the remainder of the polypeptide. Thus, the order of the activity dependence (N terminus > C terminus > core) was a reflection of the importance of each region on the overall helix propensity. The placement of the core last in the order does not imply that the helix propensity of the core is inconsequential to activity. The low level of influence was observed because both L-core and S-core were highly helical, and exchanges between the two likely did not significantly influence the character at the termini or significantly change the nature of the hydrophobic binding face. However, relatively distant termini likely affected the activity through their ability to induce helical content to the overall polypeptide through helix capping, thereby enhancing the ice-binding sites in other portions of the polypeptide.

In wfAFP-6, the first 11-residue motif occurs in the N terminus. A study of a minimized peptide consisting of the N and C termini of other type I AFPs (N terminus, core, and C terminus) and the level of antifreeze activity. Thus, the conclusions presented here should be used as a first step in more in-depth studies of the determinants of antifreeze activity that reside in the N and C termini of other type I AFPs.

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