NMR Analysis of Type III Antifreeze Protein Intramolecular Dimer

STRUCTURAL BASIS FOR ENHANCED ACTIVITY

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Kazunori Miura‡, Satoru Ohgiya‡, Tamotsu Hoshino‡, Nobuaki Nemoto†, Tetsuya Suetake‡, Ai Miura‡, Leo Spyračopoulos‡, Hidemasa Kondo‡, and Sakea Tsuda‡

‡Bioscience and Chemistry Division, Hokkaido National Industrial Research Institute, 2-17-2-1 Tsukisamu-Higashi, Toyohira, Sapporo 062-8517, Japan, §Varian Japan, Varian Japan Sumitomo Shibaura Building, 4-16-36 Shibaura, Minato-ku, Tokyo 108-0023, Japan, and ¶Department of Structural Bioinformatics, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

The structure of a new antifreeze protein (AFP) variant, RD3, from antarctic eel pout (Rigiphila dearborni) with enhanced activity has been determined for the first time by nuclear magnetic resonance spectroscopy. RD3 comprises a unique translational topology of two homologous type III AFP globular domains, each containing one flat, ice binding plane. The ice binding plane of the N domain is located ~3.5 Å “behind” that of the C domain. The two ice binding planes are located laterally with an angle of 32 ± 12° between the planes. These results suggest that the C domain plane of RD3 binds first to the ice (1010) prism plane in the (0001) direction, which induces successive ice binding of the N domain in the (001) direction. This manner of ice binding caused by the unique structural topology of RD3 is thought to be crucial for the significant enhancement of antifreeze activity, especially at low AFP concentrations.

Inhibition of ice crystal growth by antifreeze proteins (AFPs)1 is a unique biological function identified in various organisms such as fishes (1, 2), insects (3–5), and plants (6). Antifreeze function is macroscopically visible in aqueous solutions of some AFPs1 is a unique biological function identified in various organisms such as fishes (1, 2), insects (3–5), and plants (6). Antifreeze function is macroscopically visible in aqueous solutions of AFPs as the creation of an unusual bipyramidal shape which induces successive ice binding of the N domain in the (001) direction. This manner of ice binding caused by the unique structural topology of RD3 is thought to be crucial for the significant enhancement of antifreeze activity, especially at low AFP concentrations.

The atomic coordinates and structure factors (code 1C8A and 1C89) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

To whom correspondence should be addressed; Bioscience and Chemistry Division, Hokkaido National Industrial Research Institute, 2-17-2-1 Tsukisamu-Higashi, Toyohira, Sapporo 062-8517, Japan, Tel: 81-11-857-8912; Fax: 81-11-857-8893; E-mail: tsuda@hniiri.go.jp.

1 The abbreviations used are: AFP, antifreeze protein; Tf, freezing temperature; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; 3D, three-dimensional.

The coordination of the [6]-sheets and one [3]-helical turn. A remarkably flat and amphipathic surface plane that encompasses residues 9–21 and 41–44 is thought to be an essential ice binding site. The polar atoms of the putative ice binding residues Ghn14, Asn17, Thr15, Ala12, Thr18, and Gln44 are located to form hydrogen bonds with oxygen atoms of the ice lattice (1010) prism plane (11–14). RD3 is an activity-enhanced variant of type III AFP that is an intramolecular dimer (134 residues, Mγ 15,800; Ref. 15). In RD3 a 9-residue linker sequence (D(GTTSPGK)7) connects the two type III AFP domains in tandem and locates them in specific orientations, which determines in part the antifreeze activity of the intact molecule. Together with activity measurements, the present structural determination of RD3 will improve understanding of the ice binding mechanism and will provide insight into the potential for production of AFP with enhanced activity.

EXPERIMENTAL PROCEDURES

Sample Preparation and Activity Measurement—Preparations of unlabeled, 13N-labeled, and 13C15N-labeled-RD3 were briefly described previously (10). Escherichia coli JM105 cells were transformed with a pKK223-3UC-based expression plasmid containing synthesized DNA encoding RD3 and were cultured in M10 minimal media. The media contained 13N-labeled NH4Cl for the expression of 13N-labeled RD3 and both 13N-labeled NH4Cl and 13C15N-labeled glucose for expression of 13C15N-labeled-RD3. The transformed cells were lysed by sonication, and the lysate was centrifuged at 21,000 × g for 30 min at 4 °C. The precipitate was suspended in TEN buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 300 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride). Inclusion bodies in the precipitate were purified by centrifugation in 40% (w/v) sucrose and washed with TEN buffer containing 1.0% Triton X-100. RD3 was extracted from the inclusion bodies with extraction buffer (acetonitrile:isopropanol:water, 46.7:23.3:30) containing 0.1%
trifluoroacetic acid at 22 °C. After centrifugation at 16,000 × g for 10 min at 4 °C, the supernatant was diluted with acetic acid buffer (pH 3.8) and loaded onto a fast protein liquid chromatography High-S column (Bio-Rad). Bound RD3 was eluted from the column with a linear gradient of aqueous ammonium sulfate. Fractions containing RD3 were further purified by reverse-phase liquid chromatography using a TSK-gel ODS-80Ts column (Tosoh, Tokyo, Japan). The RD3 sample was lyophilized after check of the purity by Tricine-SDS-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining. Approximately 50 mg of unlabeled RD3 and 15 mg of 15N- and 13C/15N-labeled RD3 were prepared from 2.4- and 6.0-liter cultures, respectively.

The thermal hysteresis measurements were performed using an osmometer (model OM 802, VOGEL GmbH) for lyophilized RD3 and type III AFP monomer (RD3-N1) samples. RD3 and RD3-N1 samples were dissolved in 0.1 M ammonium bicarbonate (pH 7.9) to give final concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.5, and 2.0 mM. Thermal hysteresis measurements were repeated three times using fresh solution for each point. The antifreeze activity of RD3 was further examined by observation of the ice crystal morphology using a Leica DMLB 100 photomicroscope equipped with a Linkam LK600 temperature controller. RD3 (2 μl) was dissolved in 0.1 M ammonium bicarbonate (pH 7.9, 0.1–1.0 mg/ml), momentarily frozen (approximately −22 °C), and warmed to 0 °C on the sample stage of the photomicroscope in order to create several ice crystal seeds in the solution. This solution was then cooled to approximately −1 to −5 °C, and growth of ice crystal seeds was monitored.

Structure Determination—A total of 1575 NMR-derived experimental restraints were obtained using a 500-MHz NMR spectrometer (Uni- Nova-500, Varian) for the structural determination. Intereproton distance restraints were obtained from the two-dimensional NOESY, 13C/15N-edited NOESY (17), and simultaneous 13C/15N-edited 3D NOE experiments (18) performed at 4 °C. The mixing time dependence of the transient NOE was determined from two-dimensional NOESY spectra to assess the effects of spin diffusion; subsequently, the mixing time was set to 50 ms for the NOESY experiments used to obtain experimental nuclear Overhauser enhancement (NOE) restraints. The intensities of two-dimensional and 3D NOESY data were calibrated on the basis of NOE peak intensity corresponding to a known distance such as Tyr H5–He (2.48 Å), and an error of 50% was assumed for the NOE peak intensities. The following distance constraints were used to calculate the 3D spectra:

\[
\begin{align*}
HN_1–HN_4 & = 2.70–3.05 \text{ Å} \quad \text{(for residues with negative } \psi \text{ value)}; \\
HN_2–HN_3 & = 1.7–3.6 \text{ Å} \quad \text{H–C–C–H} = 2.2–3.1 \text{ Å} \quad \text{H–C–CH} = 2.5–2.7 \text{ Å} \quad \text{H–C–H} = 1.7–1.8 \text{ Å.}
\end{align*}
\]

In cases in which direct calibration was not possible, the distance constraints were overestimated. For NOESYs with a mixing time of 120 ms, the upper bound was set to 6 Å. For all proton-proton restraints, the lower bound was set to 1.7 Å. Dihedral angle restraints for the φ angle were estimated from the 3D HNHA experiment, using a correction factor of 1.1. A 25% error on the peak intensities was assumed, and the minimum φ angle restraint range was set to ±10°. The initial set of restraints containing no dihedral angle restraints and a fraction of NOEs are listed in Table I. From this initial set of NOE restraints and starting from an extended structure, 100 structures were generated with the simulated annealing protocol in X-PLOR 3.851 (19) using 12,000 high-temperature steps (60 ps at 1,000 K) and 6,000 cooling steps (30 ps, final temperature of 100 K). Of the 100 calculated structures, 75 were converged and folded properly but had significantly high total energy, which is generally due to assignment errors and a lack of input structural constraints. Hence, the input constraints were refined by analysis of the inconsistency between the constraints and the coordinates of the calculated structures. The calculated structures were then used for a new round of peak picking of NOE spectra that enabled the assignment of larger numbers of NOEs. With refined distance constraints and the addition of dihedral φ angle constraints, the next round of structure calculation was performed starting with the 75 converged structures using the simulated annealing protocol with 6,000 high-temperature steps (30 ps) and 4,000 cooling steps (20 ps). Refinement of the input constraints with structure calculations was repeated over 30 times. The set of structures presented in this paper includes the 15 (see Fig. 2) and 40 (see Fig. 4b) lowest energy structures selected from the 50 structures obtained in the last round of refinement.

RESULTS AND DISCUSSION

Recombinant RD3 protein generated by bacterial expression gives rise to thermal hysteresis and a bipyrimalid shape for ice crystals as typically observed for all AFP variants. The noncollicative increase in antifreeze activity of RD3 is compared with that of type III AFP monomer (RD3-N1; Ref. 20) in the range of 0–2.0 mM protein (Fig. 1a). RD3 was initially reported to show 1.9-fold higher activity than type III AFP monomer (11), which is verified in the present study when the concentrations of RD3 and monomer are 0.5 mM (Fig. 1a, i and b). For AFP concentrations of 0–0.5 mM, a significant 5.9-fold enhancement of antifreeze activity is observed for RD3 compared with monomer (RD3-N1; Fig. 1b). Although RD3 consists of two type III AFP monomers, the increased antifreeze activity of RD3 does not correspond to simple additive activity of two monomers under nonsaturating conditions. It has been suggested that AFPs bind irreversibly to ice crystal nuclei according to the adsorption-inhibition mechanism at the ice-water interface (8), on which convex ice surfaces are created between the bound proteins. The height and curvature of the convex ice surface increases because of the Kelvin effect at subzero temperatures (21). The convex ice surface is energetically unfavorable for water to join the ice lattice and results in depression of the freezing point (ΔTf; Ref. 22). The freezing point depression was assumed to follow the relationship ΔTf = constant × hDp, where h is the height of the convex ice surface, and D is the average distance between the ice-bound AFPs on the ice surface (21). Assuming that the average separation distance (D) for RD3 is less than the distance between RD3-N1 monomers because of the unique interdomain topology of RD3, this equation predicts an increased ΔTf for RD3 compared with type III AFP monomer. It should be noted that this equation was proposed under the assumption that type I AFP is cylindrically shaped. Thus, modifications or additional hypotheses, or both, may be necessary to describe the ice binding properties of RD3. In any case, more coverage of the ice surface area or more effective ice binding of RD3 compared with monomer (RD3-N1), or both, is thought to alter efficiently the convex ice surface between ice-bound protein molecules especially at low protein concentrations (0.1–0.5 mM AFP). At higher AFP concentrations (0.5–2 mM), saturating amounts of AFP are bound to the ice crystal surface; thus no new convex ice surfaces can be formed, leading to an upper limit for ΔTf (Fig. 1a). Assuming that the average distance between RD3 is shorter than that between RD3-N1 molecules, a smaller amount of RD3 compared with RD3-N1 is enough to saturate the ice surface. Hence, the
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Table I
Summary of restraints and structural statistics

| Restraints                  | Value |
|-----------------------------|-------|
| Total                       | 1655  |
| Short                       | 672   |
| Medium (2 ≤ | i − j | ≤ 4) | 532   |
| Long (| i − j | ≥ 5) | 291   |
| H bond                      | 80    |
| Dihedral (ϕ)                | 80    |
| Restraint violations        |       |
| Distance (> 0.2 Å)          | 0     |
| Dihedral (> 2°)             | 0     |
| Precision (Å²)              |       |
| Intact molecule (residues 4–134) | 1.38 ± 0.38 |
| N domain (residues 4–64)    | 0.49 ± 0.07 |
| C domain (residues 74–131)  | 0.43 ± 0.06 |
| Linker (residues 65–73)     | 0.66 ± 0.12 |
| Deviations from experimental restraints |       |
| NOE distance restraints (Å) | 0.009 ± 0.002 |
| Dihedral angle restraints (deg) | 0.309 ± 0.070 |
| Deviations from ideal covalent geometry |       |
| Bonds (Å)                   | 0.002 ± 0.000 |
| Angles (°)                  | 0.517 ± 0.005 |
| Improper (°)                | 0.380 ± 0.004 |
| X-PLOR Energies (kcal/mol)  |       |
| Bond                        | 199.69 ± 6.15 |
| Angle                       | 55.22 ± 4.45 |
| Improper                    | 155.68 ± 2.91 |
| VDW (E_Eq,U)                | 21.62 ± 0.44 |
| NOE                         | 10.02 ± 1.80 |
| NOE distance restraints (Å) | 6.52 ± 2.16 |
| Dihedral (°)                | 6.64 ± 0.31 |
| Procheck (%; aa/aa)         | 81/19  |

*The average rms deviation for the coordinate set was calculated by superimposing each of the 40 structures onto the minimized average coordinate set. The superposition was over backbone, N, O, and C of the indicated regions.

*The force constant for the van der Waals energy calculation was 4.0 kcal mol⁻¹ Å⁻¹.

*Force constants for the calculation of NOE and dihedral energies were 50 kcal mol⁻¹ Å⁻² and 200 kcal mol⁻¹ rad⁻², respectively.

*Φ and ϕ dihedral angles in the most favored (mf) and additionally allowed (aa) regions of the Ramachandran plot were determined using the program PROCHECK (33).

ΔT curve becomes a plateau even at the low concentration of RD3. The validity of this idea requires more experimental evidence and consideration, and detailed information about the structural difference between RD3 and the type III AFP monomer would have a significant contribution in this regard.

The NMR solution structure of intact RD3 (Fig. 2) has been determined on the basis of complete assignments of the ¹H, ¹³C, and ¹⁵N resonances of RD3 at pH 6.8 and 4 °C, which is approximately the freezing point of the solvent (BioMagResBank accession number 4449; Ref. 16). The determined structures have high stereochemical quality as well as sufficiently small root mean square deviation values (Table I). The structural quality of each domain is due to the β-strand contribution (48%). The elements of secondary structure for the N domain consist of eight short β-strands (β1–β8; residues 3–7, 9–13, 15–18, 22–26, 31–33, 43–45, 47–49, and 53–55; Fig. 3a) followed by a type III turn. The eight β-strands are connected through a type II turn (residues 40–43), a type III turn (residues 18–21), and an α-helix (β1; residues 36–39). The eight β-strands are also identified in the corresponding regions of the C domain (β9–β16; residues 73–77, 79–83, 85–88, 92–96, 101–103, 113–115, 117–119, and 122–125). Similar to the N domain, the other C domain secondary structural elements are an α-helix (α2; residues 106–109), a type II turn (residues 110–113), and two type III turns (residues 88–91 and 126–129). For the type III AFP monomer (QAE isoform), there are coordinates available for the NMR solution structure (Protein Data Bank code 1KDF; Refs. 11, 12) and the x-ray structure (Protein Data Bank code 1MSI; Ref. 13). There are slight inconsistencies in the secondary structure assignment between the NMR and x-ray structural coordinates. For example, one β-strand (residues 2–13) identified in the x-ray structure (1MSI) was assigned as two strands (residues 3–7 and 9–13) in the NMR structure (1KDF). Our determination of the lengths and locations of the α-helices and β-strands for each globular domain of RD3 are similar to those identified in the NMR structure (1KDF; Ref. 12). The structures of the type II turns at residues 40–43 and 110–113 were not identified in the QAE isoform and are due, in part, to the presence of residues Gly⁴² and Gly¹¹² of RD3, which favor turns. The root mean square deviation value between the globular domains of RD3, 1KDF, and 1MSI is 1.17 ± 0.23 Å for the backbone atoms, indicating that the overall structural motif of

Fig. 2. Solution structure of RD3 determined at 4 °C. a, stereo view showing 15 of the lowest energy structures of RD3 superimposed on residues 4–64 (N domain). b, stereo view showing 15 of the lowest energy structures superimposed on residues 74–131 (C domain). When the calculated structures of one domain are superimposed (black), the structures for the counter-domain are dispersed (gray). This implies that the flexible linker region (residues 65–73) causes divergence in the calculated structures of one domain are superimposed (black), the structural quality of the counter-domain are dispersed (gray).
the N- and C-terminal globular domains of RD3 is typical of type III AFP monomer.

The side chain oxygen atoms of Gln9, Asn14, Thr15, Thr18, and Gln44 and main chain carbonyl oxygen atoms of Asn14 and Ala16 are clustered together and form a flat surface plane on the outer face of the two-fold symmetry motif of the N domain (Fig. 4a). These atoms were identified as putative ice binding residues for type III AFP monomer. Mutation of these residues reduces protein thermal hysteresis activity and causes changes in morphology of the ice crystal (13). The corresponding ice binding residues of the C domain of RD3 are Gln79, Asn84 (O'), Thr85, Ala86, Thr88, and Gln114 (Fig. 4a). It appears that the nonpolar side chain groups of Pro12, Thr15, Ala16, Thr18, Ile20, Met21, and Gly42 fill the gaps between the ice binding residues of the N domain. The corresponding residues of the C domain are Pro62, Thr65, Ala66, Thr68, Ile69, Met71, and Gly112. The location of these nonpolar residues in each domain is thought to stabilize the spaced polar groups and the planarity of the ice crystal (13).
Fig. 4. a, stereo view of the minimized average structure of RD3. Shown on the ice binding planes are all polar side chain atoms assumed to be involved in complementary hydrogen bonds with water oxygen atoms of the ice lattice [1010] prism plane. The ice binding O and N atoms are represented in Corey-Pauling-Koltun (red and blue, respectively). b, stereo view of the 40 calculated structures of RD3 superimposed on the C domain (residues 74–131). The various solid lines for the N domain indicate the range of the movement of this domain allowed by the flexible linker. The region from Val65 to Glu64 is not drawn to avoid congestion. The oxygen atoms of the C domain essential for ice binding (a) are shown in the Corey-Pauling-Koltun representation. The backbone of the minimized average structure is shown as a thick black rod. The ice binding plane of the structure with a backbone represented by the thick blue rod is nearly aligned with that of the C domain, whereas the structure represented by the red rod is located farthest behind the ice binding plane of the C domain. The average values of the backbone N–NH bond vector order parameter ($S^2$) are 0.84 ± 0.08 and 0.87 ± 0.08 for the N and C domains (residues 4–64 and 74–131), respectively. The overall correlation times ($\tau_m$) were estimated separately for each domain (11.38 ± 1.09 ns for the N domain and 11.29 ± 1.03 ns for the C domain; Refs. 31, 32). The $S^2$ profile of the linker (residues 65–73) shows a V shape as found in RD3-Nl (20). The average $S^2$ value for the linker (0.60 ± 0.15) indicates that the backbone atoms of the linker are more flexible compared with the globular domains.
binding planes to achieve appropriate ice binding function (12, 13). The side chain groups of the key ice binding residues Asn\textsuperscript{14} in the N domain, and the corresponding residue Asn\textsuperscript{44} in the C domain are located slightly behind the ice binding plane (Fig. 4a). For the type III AFP monomer, Asn\textsuperscript{14} plays a pivotal role in initiation of binding to transition points between prism and basal ice planes (13). The 9-residue linker of RD3 (residues 65–73) forms a bent structure, as found in RD3-Nl (20), that places the N and C domains in close proximity but does not allow for direct association. This short linker locates the two ice binding planes of each domain laterally with an angle of 32 ± 12° between the planes (Fig. 4, a and b). To our knowledge, this interdomain topology is structurally unique among smaller proteins (<30 kDa) that comprise two similarly sized domains. One well-known, small intramolecular dimer is calmodulin (148 residues), a dumbbell-shaped calcium regulatory protein. Calmodulin is composed of two homologous N- and C-terminal globular domains connected through a 28-residue linker that forms a long α-helix (23). Disparate from RD3, the two homologous domains of calmodulin have no preferred orientation with respect to each other (24). The domains have the propensity to come together and “grip” the target protein, much like two hands capturing a rope (25). The N and C termini of RD3 (both type III AFP monomers) point to almost opposite directions in the ice-free state and presumably in the ice-bound state as well. On the other hand, the N and C termini of calmodulin point to nearly the same direction when bound to the target protein; i.e. the functional difference between RD3 and calmodulin may be ascribed to the difference in the interdomain topologies.

The lateral orientation of the N- and C-terminal domains of RD3 directs the faces of two ice binding planes in nearly the same direction with a slight difference in their alignments. The ice binding plane of the N domain is located ~3.5 Å behind that of the C domain in the minimized average structure (Fig. 4a). This orientation of the two domains suggests that the C domain ice binding face of RD3 attaches to the ice crystal first, which follows the second attachment of the ice binding face of the N-domain in a cooperative manner. Presumably, the ice-bound form of RD3 would have the ice binding faces of each domain oriented nearly identically with respect to the ice surface. Indeed, an “aligned” form of RD3 was identified in one of the present solution structures (Fig. 4b, thick blue rod). If a structural change occurs between ice-free and ice-bound states of RD3, it is likely that the conformation of the 9-residue linker changes between the two states. Consistent with a view of conformational flexibility between ice-free and ice-bound forms of RD3, greater flexibility for the backbone in the linker region was identified by \textsuperscript{15}N NMR relaxation measurements, and fewer experimental NMR constraints were found compared with the structured regions (Fig. 4b).

Manual docking of RD3 to ice indicated that interatomic distances between key atoms of ice binding residues of the two domains of RD3 form a close match to thespacing of water oxygen atoms in the ice (1010) prism plane of hexagonal ice crystal \(\left(\text{I}_6\right); \text{c-axis} = 7.361\text{Å}; \text{a-axis} = 4.507\text{Å}; \text{Ref. 26}.\) For example, the distance between the oxygen atoms of Thr\textsuperscript{88} and Ala\textsuperscript{86} (CO) is 4.5 Å, and that of Ala\textsuperscript{86} and Gln\textsuperscript{114} is 7.4 Å. The corresponding residues in the N domain are Thr\textsuperscript{18}, Ala\textsuperscript{16} and Gln\textsuperscript{44}. These atoms are located in line in each domain and match the positions of three water oxygen atoms lined up in the (0001) direction. The placement of the C domain in this direction leads to the positioning of Asn\textsuperscript{44} (Fig. 4a) at the intersection between the prism and basal planes of the ice crystal. These results are in good agreement with the ice-docking model proposed for type III AFP monomer (12, 13). Assuming that the C domain binds ice first in the (0001) direction, one possible target ice surface for the N domain might be the same prism plane in the (0101) direction. Respective ice binding in the (0001) and (0101) directions would be in good agreement with the 32° difference in alignment of the two ice binding planes. In such an ice-bound model for RD3, close proximity is still identified between the oxygen atoms of the N domain and the water oxygen atoms in the prism plane. The positioning of the nonplanar residue Asn\textsuperscript{44} to the intersection between prism and basal planes is unexpected in this model because of steric hindrance by two globular domains. Note that the two ice binding planes can be manually aligned in the (0001) direction to achieve better interoxygen space matching of the two domains by adjustment of backbone φ/ψ angles of the linker sequence (residues 67–69).

It is likely that the ice binding of the C domain induces the specific ice binding of the N domain to the prism surface. In addition, recent proposals implicate flatness of the ice binding plane and hydrophobicity as important determinants of ice binding (12–14). These determinants are also expected to contribute to the specificity of ice binding of the N domain. DeLuca et al. (27) reported that genetically expressed type III AFP connected through the N terminus to thioredoxin (12 kDa) or maltose-binding protein (42 kDa) possess ~2–3-fold higher antifreeze activity than type III AFP monomer on a molar basis. Thioredoxin and maltose-binding protein do not possess antifreeze activity, and the higher activities of the fusion proteins were ascribed to ~3–8-fold wider coverage (without ice binding) of the ice surface. For RD3 we can assume just a 2-fold increase in coverage of the surface area compared with the monomer. Hence, the observed 5.9-fold increased activity of RD3 compared with the monomer cannot be explained on the basis of increased ice surface coverage of RD3 but can be explained on the basis of increased ice binding strength of the molecule. It is likely that the on rate for ice binding of the type III AFP monomer is a diffusion-controlled process. The same process is likely to dominate ice binding of the C domain of RD3, whereas the on rate of ice binding of the N domain is influenced by its close proximity to the ice surface because of initial ice binding of the C domain. Hence, the affinity of intact RD3 for ice would not be a simple sum of the affinity of two type III AFP monomers but, rather, would be greater than the sum of the monomer affinities, leading to the 6-fold activity enhancement of RD3. We conclude that specific ice binding of the two domains, guided by the unique structural topology of RD3, is an important factor in determination of the high antifreeze activity of RD3. The 9-residue linker presumably plays a key role in connecting the N- and C-terminal AFP domains laterally and potentially affords simultaneous ice binding of the two domains. An artificial AFP consisting of multiple type III AFP monomers connected through 9-residue linker sequences may possess highly enhanced antifreeze function compared with unconnected monomers of type III AFP.

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REFERENCES

1. Davies, P. L., and Hew, C. L. (1990) \textit{FASEB J.} 4, 2460–2468

2. Sicheri, G., and Yang, D. S. C. (1995) \textit{Nature} 375, 427–431

3. Graham, L. A., Liou, L.-C., Walker, V. K., and Davies, P. L. (1997) \textit{Nature} 388, 727–728

4. Liou L.-C., Tocilj, A., Davies, P. L., and Jia, Z. (2000) \textit{Nature} \textit{406}, 322–324

5. Graether, S. P., Kuiper, M. J., Gagné, S. M., Walker, V. K., Jia, Z., Sykes, B. D., and Davies, P. L. (2000) \textit{Nature} \textit{406}, 325–328

6. Worrall, D., Elias, L., Ashford, D., Smallwood, M., Sidebottom, C., Lillford, P., Telford, J., Hold, C., and Bowles, D. (1998) \textit{Science} \textit{282}, 115–117
Structure of Type III AFP Intramolecular Dimer

and NMR, Yale University Press, New Haven

20. Miura, K., Ohgiya, S., Hoshino, T., Nemoto, N., Odaira, M., Nitta, K., and Tsuda, S. (1999) J. Biochem. 126, 387–394
21. Wilson, P. W. (1993) Cryo-Letters 14, 31–36
22. Wilson, P. W. (1994) Cryobiology 31, 406–412
23. Babu, Y. S., Sack, J. S., Greenough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) Nature 315, 37–40
24. Barbato, G., Ikura, M., Kay, L. E., Pastor, R. W., and Bax, A. (1992) Biochemistry 31, 5269–5278
25. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) Science 256, 632–638
26. Hobbs, P. V. (1974) Ice Physics, pp. 18–39, Oxford University Press, London
27. DeLuca, C. I., Comley, R., and Davies, P. L. (1998) Biophys. J. 74, 1502–1508
28. Wishart, D. S., Willard, L., Richards, F. M., and Sykes, B. D. (1994) VADAR: A Comprehensive Program for Protein Structural Evaluation, Version 1.2, University of Alberta, Edmonton
29. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
30. Merritt, E. A., and Bacon, D. J. (1997) Methods Enzymol. 277, 505–524
31. Lipari, G., and Szabo, A. (1982) J. Am. Chem. Soc. 104, 4546–4559
32. Lipari, G., and Szabo, A. (1982) J. Am. Chem. Soc. 104, 4559–4570
33. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291

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and NMR, Yale University Press, New Haven

20. Miura, K., Ohgiya, S., Hoshino, T., Nemoto, N., Odaira, M., Nitta, K., and Tsuda, S. (1999) J. Biochem. 126, 387–394
21. Wilson, P. W. (1993) Cryo-Letters 14, 31–36
22. Wilson, P. W. (1994) Cryobiology 31, 406–412
23. Babu, Y. S., Sack, J. S., Greenough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) Nature 315, 37–40
24. Barbato, G., Ikura, M., Kay, L. E., Pastor, R. W., and Bax, A. (1992) Biochemistry 31, 5269–5278
25. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) Science 256, 632–638
26. Hobbs, P. V. (1974) Ice Physics, pp. 18–39, Oxford University Press, London
27. DeLuca, C. I., Comley, R., and Davies, P. L. (1998) Biophys. J. 74, 1502–1508
28. Wishart, D. S., Willard, L., Richards, F. M., and Sykes, B. D. (1994) VADAR: A Comprehensive Program for Protein Structural Evaluation, Version 1.2, University of Alberta, Edmonton
29. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
30. Merritt, E. A., and Bacon, D. J. (1997) Methods Enzymol. 277, 505–524
31. Lipari, G., and Szabo, A. (1982) J. Am. Chem. Soc. 104, 4546–4559
32. Lipari, G., and Szabo, A. (1982) J. Am. Chem. Soc. 104, 4559–4570
33. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291

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and NMR, Yale University Press, New Haven
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