# Modeling Studies of Binding of Sea Raven Type II Antifreeze Protein to Ice

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Certain plants, insects, and fish living in cold environments prevent tissue damage due to freezing by producing antifreeze proteins or antifreeze glycoproteins that inhibit ice growth below the normal equilibrium freezing point of water in a noncolligative fashion. In polar fish these macromolecules, taking into account their structural characteristics, are grouped into three broad classes, namely Type I, Type II, and Type III. In this paper we report the results of our studies on the stereospecific binding of sea raven, a Type II antifreeze protein (AFP) to (111) hexagonal bipyramidal faces of ice. Earlier studies of Type I and Type III AFPs have shown that stereospecific binding of these proteins, recognizing specific planes of ice, is essential for their noncolligative antifreeze point depression. Moreover, as it has been shown for the AFT of Type I, this binding also occurs along specific vectors on these planes and also is enantioselective, distinguishing between the mirror related directions. In this study we will show, by using molecular modeling, that the fold of Type II AFP could facilitate a stereospecific mode of interaction with (111) planes of ice. Similar to Type I AFP, preferential directionality of binding was also observed in the simulations.

## INTRODUCTION

Some plants, insects, and fish living in cold environments are able to survive temperatures below the freezing point of water by producing antifreeze proteins (AFP) or antifreeze glycoproteins (AFGP).[1−4] These macromolecules prevent potential damage from freezing in a noncolligative fashion by binding to specific planes of seed ice crystals. The adsorption results in the cessation of ice growth from melt and a nonequilibrium depression of the freezing point. This freezing point depression depends upon AFP/AFGP concentration[5] and is believed to operate by the Kelvin effect.[6,7]

Fish antifreeze proteins are categorized into helical Type I AFPs, cysteine rich globular Type II AFPs, and small globular Type III AFPs.[4] The most extensively studied are the Type I AFPs which are generally alanine-rich, α-helical peptides of 36−44 residues, in some cases exhibiting 11 residue long repetitive segments. Helical structure has been also attributed to antifreeze glycoproteins, which have disaccharides attached to the threonine side chains of their ALA ALA THR repeat units. Type II and Type III AFPs, on the other hand, are nonhelical molecules without repetitive amino acid sequences. Type II AFPs are large (120+ residues), cysteine-rich proteins composed of $\beta$-sheets, reverse turns, and helices. Type III AFPs are of intermediate size, containing about 65 residues, and lack a predominant amino acid type. NMR studies of this protein from ocean pout (*Macrozoarces americanus*) have shown that it contains $\beta$-sheets, two anti-parallel triple $\beta$-strands, and one anti-parallel double $\beta$-strand. Ice etching[7] and molecular modeling studies[8] of Type III globular APF were not able to identify the preferred directionality of binding of these proteins on their respective adsorption planes. Mutation studies[9] and structural X-ray analysis[10] revealed that ice binding motif of these proteins was expressed on the protein surface that was characterized by unusual flatness, affording formation of hydrogen bonding network between the protein and flat ice surface.[8]

Whereas a wealth of structural information derived from X-ray and NRM spectroscopy,[10−12] is available for Types I and III AFPs, very little is known about the three-dimensional structure of the largest, Type II AFP. A three-dimensional structure of Type II antifreeze protein was recently proposed using comparative modeling.[13] Type II AFP shows significant sequence identify to C-type lectins, which are widely distributed Ca-regulated saccharide binding proteins with identical fold. The starting coordinates for the homology modeling were obtained from the X-ray crystallographic structure of rat-mannose binding protein-A. The modeling procedure focused on retaining the excellent protein-structural properties of these coordinates and resulted in a model that is indistinguishable from high-quality experimental structures in terms of backbone angles and side chain properties. Thus, in absence of an experimental structure of Type II AFP, the present model presents a high-quality starting point for the study of the ice-binding site presented here. This structure was derived using a sequence similarity to the carbohydrate recognition domain (CDR) of $Ca^{2+}$ dependent lecitin.[14,15] This more than 110 amino acid containing domain is a part of a superfamily of proteins that bind sugars specifically through contact with calcium ions.[16] In this proposed structure sea raven Type II AFP folds into one compact globular domain consisting of two helices and eight $\beta$-strands forming two antiparallel $\beta$-sheets. Similarly as in the Type III AFP the hydrophilic surface of the sea raven AFP protein that has been identified as being involved in binding to ice[13] is characterized by unusual coplanarity of threonine, serine, and lysine residues.

Using the proposed 3D structure[13] we performed a molecular dynamics simulation of the sea raven AFP in a box of water. Structural, dynamical, and protein−water interactions during the simulation were investigated, and the dynamics structure was used to investigate possible protein−

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water binding interactions. The flat, exposed protein surface, containing ten hydrophilic residues, 90, 91, 92, 94, 103, 105, 109, 113, 120, and 122, indicated in ref 13 as responsible for interaction with the ice surface was used to model binding of sea raven AFP to the (111) ice face[17] and is discussed below in terms of protein−ice interactions.

## MATERIALS AND METHODS

The binding calculations were performed on a system consisting of a single 113-residue Type II AFP and a slab of ice composed of 1080 TIP3P water molecules, with dimensions of 70 × 70 × 8 Å$^3$. The following sea raven Type II AFP sequence was used for the purpose of modeling[13] (starting with amino acid residue 16):

16
DRCIY YETTAMTWAL AETNCMKLGG HLASIHSQEE HSFIQTLNAC VVWIGGSACL

QAGAWTWSDG TPMNFRSWCS TKRDDVLAAC CMQMTAAADQ CWDDLPCPAS

129
HKSVCAMTF

Polar hydrogens were added to the protein using QUANTA/CHARMm 4.0.[18]

The (111) ice plane was constructed from the fractional coordinates of ice using Cerius$^2$.[19] The space group used for ice, *Ih*, was no. 194 $P6_{3/mmc}$, unit cell constants were $a = b = 4.516$ Å, $c = 7.354$ Å, $\alpha = \beta = 90°$, and $\gamma = 120°$, and fractional coordinates used for ice were O(0.3333,0.6667,0.0629), $H_a$(0.3333,0.667,0.1989), and $H_b$-(0.4551,0.9102,0.0182). A slab of ice parallel to the (111) plane was cut along surface vectors [1 0−1] and [−1 1 0] of 8.63 and 7.82 Å dimensions, respectively, with an angle of 116.95 degrees between them. The size of this ice slab was approximately equal to 69 × 71 × 8 Å$^3$.

The binding calculations were done by minimizing the AFP/ice interactions using the molecular mechanics module of CHARMM 22g4.[20] Using the modeling tools of QUANTA 4.0 the protein was docked on ice surface by hand in its initial position to accomplish the best fit of the protein backbone to the surface corrugation, and the binding energy was minimized. The complex was subjected to 500 steps of steepest descent followed by 500 steps of ABNR.[20] During the energy minimization process all atoms were free to move except the oxygens of ice were held in their lattice positions. This process of manual docking and energy minimization was repeated many times to locate the "global" minimum for the AFP/ice complex. A nonbonded cutoff of 7.5 Å was used in the calculations, and the nonbonded list was updated every 100 steps. The electrostatic and van der Waals potential energy terms were gradually switched to zero over the range of 6.5−7.5 Å.

The binding energy, $\Delta E_{\text{binding}}$, was calculated using the following expression

$$\Delta E_{\text{binding}} = E_{\text{complex}} - (E_{\text{AFP}} + E_{\text{ice}})$$

where $E_{\text{complex}}$ is the minimized energy of the complex, $E_{\text{AFP}}$ is the energy of the Type II AFP, and $E_{\text{ice}}$ is the energy of the ice slab.

All calculations were performed using on an IBM RISC 6000 Model 350. Graphical analysis of the energy minimizations was conducted on IBM RISC 6000 Model 350 and Silicon Graphics Indigo$^2$ workstations using QUANTA 4.0.

## RESULTS AND DISCUSSION

In order to elucidate the structure function relationship of AFPs one has to address the importance of a great structural variability of three major classes of AFPs. The best studied Type I of AFPs seem to provide several clues about sufficient, although not necessary, conditions for a functional AFP macromolecule. This AFP macromolecule should have a reasonable rigid framework, supporting several hydrophilic residues organized in a flat array that is able to recognize and hydrogen bond to one of several low Miller index planes of ice. This hydrophilic surface of the macromolecule should be embedded into a generally hydrophobic superstructure that would protect from overgrowth with ice this part of the molecule that is not directly involved in binding to ice. Also the hydrophilic/hydrophobic compositions of the residue make-up of the protein has to be appropriately balanced to provide for sufficient water solubility of the AFPs macromolecules in the concentrations that are necessary to fulfill their biological function. All three types of AFPs seem to follow this pattern, although until recently the reason for such a great structural diversity among the macromolecules fulfilling the same biological function in different biological species was not established. Recent studies[14,15] point out that the AFP proteins have significant sequence similarity to proteins performing entirely different biological functions (CRDs, mannose binding proteins, pancreatic stone proteins). In some cases the genes that code these AFP/AFGPs can be traced to genes coding different macromolecules, like trypsinogen.[21−23] One may speculate that the diversity of the AFP/AFPG molecules occurred as a result of some form of evolutionary adaptation.

In the study of structure function relationship in short horn sculpin antifreeze protein[24] we have shown that binding to ice was facilitated via a general structural match between the protein and the ice surface when the protein is oriented along a specific vector in this plane. This complementarity was found to derive from the right-handed helical character of the AFP, which allowed the repetitive low-pitch spirals of side chains formed by every fourth residue to fit within the ice topography and align certain hydrophilic groups for hydrogen bonding. Further complementarity was seen in the projection of side chain Cα−C$\beta$ bonds from the AFP backbone, which directed side chains into accommodating ice surface cages. Specific interaction of the protein with the ice surface were based on the spacing of K9 and K31 (33.8 Å) as well as R12 and K23 (16.9 Å). Two binding models were proposed; the first requiring accommodation of binding surface residues within ice cages, and the second involving the inclusion of lysine side chain tetrahedral groups into the ice lattice. The structure and binding of shorthorn sculpin AFP when compared to that of the winter flounder AFP revealed the importance of the contoured fit of the protein into ice surface topography.

In this study of binding of sea raven Type II AFP protein to ice we used the approach of matching the hydrophilic surface of the protein with (111) hexagonal bipyramidal surface of ice, similar to the strategy that was used for the study of binding of shorthorn sculpin AFP to ice[24] and Type

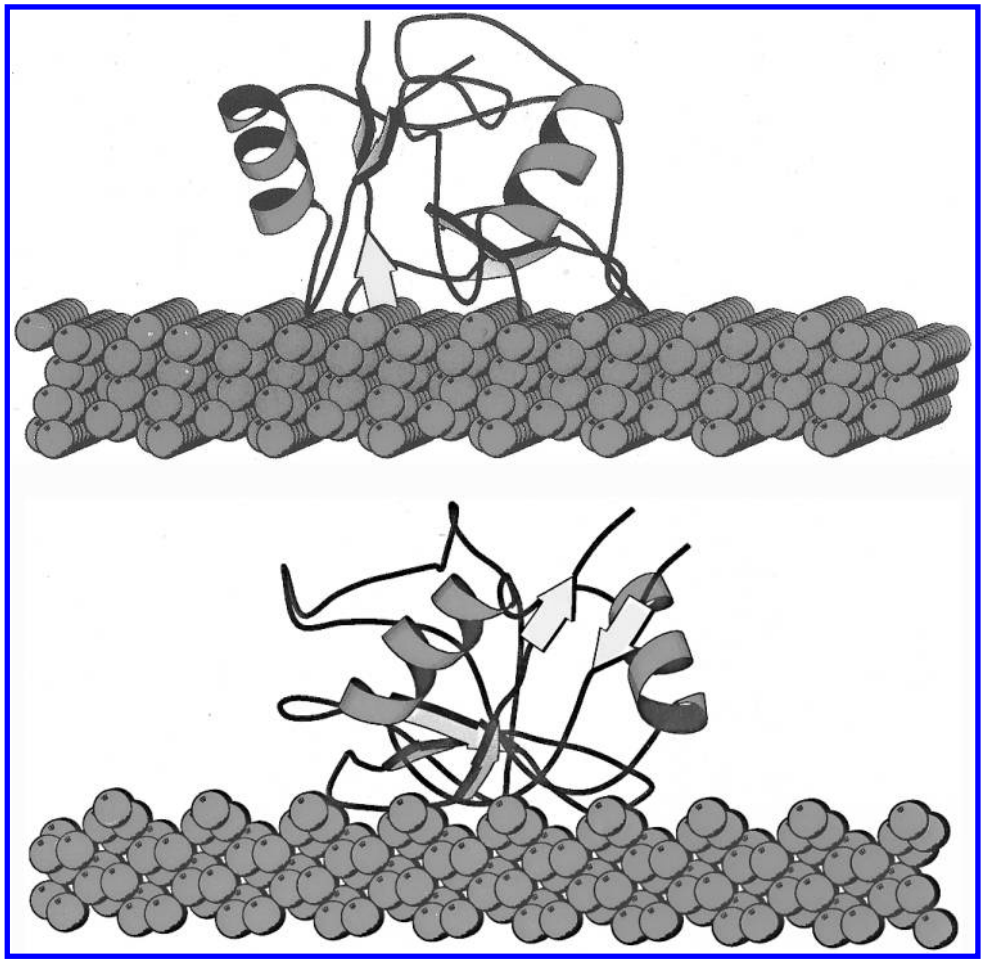

**Figure 1.** MOLSCRIPT representation of sea raven AFP binding to (111) planes of ice corresponding to a binding energy equal to −302 kcal/mol. (A). (top) The sea raven AFP protein backbone comfortably fits with its three loops (blue) and a part of the $\beta$ strand/random coil (yellow/gray) into the (111) ice surface corrugation with a 8.63 Å periodicity of (111) ice surface oxygen atoms along [1 0 −1] surface vector. B. (bottom) Three loops (blue) and a part of $\beta$ strand/random coil (yellow/gray) match another, 7.82 Å periodicity, along [−1 1 0] direction.

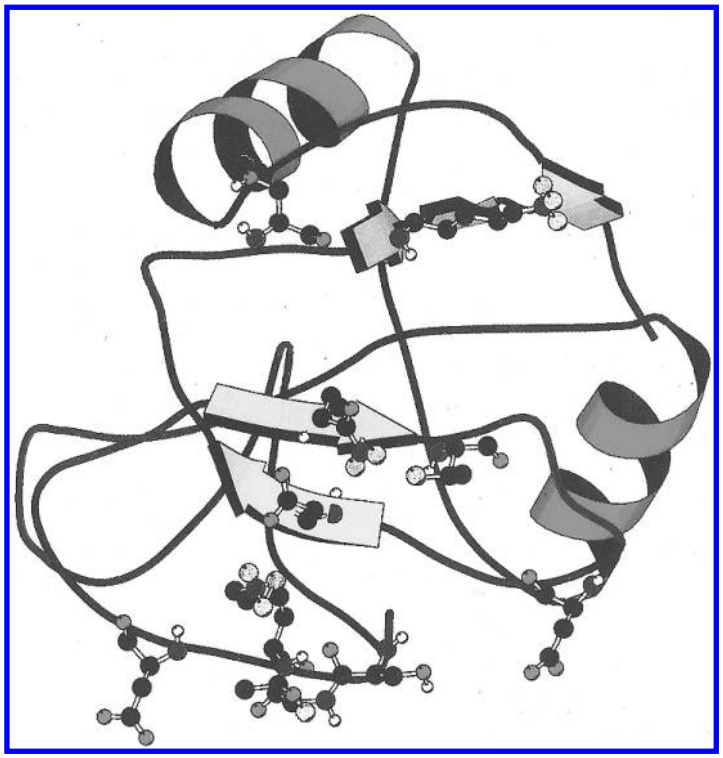

**Figure 2.** MOLSCRIPT rendering of sea raven AFP, showing the hydrophilic, ice binding surface of the protein.

III AFP.[8] Due to the low accuracy in determination of residue chains positioning in the proposed 3D structure of this protein[13] we first attempted to fit the backbone of the protein to overall corrugation of the surfaces allowing for the optimization of the side chains into the natural binding position on the ice surface. Our earlier attempts of "automated" matching of the AFPs to the ice surface by discrete, incremental rotation of the protein on the surface revealed almost continuous distribution of binding energy, not revealing any indication for a presence of a global minimum.

Figure 1 shows a MOLSCRIPT[25] representation of sea raven AFP binding to (111) planes of ice corresponding to a binding energy equal to −302 kcal/mol. Figure 1A shows a 8.63 Å periodicity of (111) ice surface oxygen atoms along [1 0 −1] surface vector. The sea raven AFP protein backbone confortably fits with its three loops (blue) and a part of the $\beta$ strand/random coil (yellow/gray) into the (111) ice surface corrugation. The uniqueness of this fit can be also seen in Figure 1B where three loops (blue) and a part of $\beta$ strand/random coil (yellow/gray) match another, 7.82 Å periodicity, along [−1 1 0] direction. This contour fit of the protein backbone into the ice surface minimizes the repulsion between the protein backbone and ice surface and allows the hydrophilic residues of the protein surface to approach the ice surface and form a network of hydrogen bonds with the surface.

The arrangement of hydrophilic, potentially ice binding residues, in the sea raven protein is unusually planar, allowing formation of strong hydrogen bonding with the surface. Ten hydrophilic residues of the sea raven AFP that have been identified in[13] as equivalent to $Ca^{2+}$ binding site 2 in MBP-A were also recognized as potentially important for ice binding. These ten residues are listed below along with the number in the parentheses indicating the number of hydrogen bonds formed with the (111) ice surface:

S90(1), T91(1), K92(2), D94(0), Q103(0), T105(3), D109(0), D113(1), S120(1), and K122(3).

Additionally we have found that T23, that has not been identified in ref 13 as a part of sea raven AFP equivalent to $Ca^{2+}$ binding site 2 in MBP-A, was participating in formation of one hydrogen bond. The next residue in the sequence, T(24), was not involved in binding, pointing away from the surface; although possibly due to some uncertainty in the side chain position determination, this threonine could also participate in binding to the surface. Figure 2 shows MOLSCRIPT rendering of sea raven AFP, with the emphasize put on the ice binding surface of the protein.

To summarize the findings on the hydrogen bonding of sea raven AFP to the (111) surface of ice we can state that the bonding to the ice surface occurs predominantly via threonine/serine and lysine residues. This has been established previously for the winter flounder AFP[7,26] where threonine residues were determined essential for ice recognition/binding to the (201) faces of ice, and for shorthorn sculpin AFP, where lysine, theronine/serine, and arginine[24] residues were the key residues for recognizing/binding to the (2−10) faces. In sea raven AFP, very much like the shorthorn sculpin case, thereonine/serine residues, having short side chains, are approaching the ice surface from the top, whereas lysines residues, with long side chains, are entering the tunnels in the ice surface. S90 and T91 residues of sea raven AFP are bound to the tops of the steps along [1 0 −1] direction, while K90 and K122 are entering large hollow openings in the (111) ice surface, parallel to the *c* axis of ice crystal. Interestingly the asparitic acid residues listed above, with one exception, do not participate in bonding to the surface of ice. We may speculate that they are essential to the overall solubility of the protein, and also since this surface of the protein was identified as equivalent to $Ca^{2+}$ binding site 2 in MBP-A, they were probably involved in calcium binding for the evolutionary analog/predecessor of sea raven AFP, since the importance of aspartic acid for calcium binding has been shown before.[27,28]

## CONCLUSIONS

In this study molecular modeling has been used to investigate the interactions between sea raven AFP and the (111) faces of ice. Our analysis revealed strong, face specific binding of this Type II AFP to this face of ice, that involved threonine/serine and lysine residues recognized earlier as the key residues, is stereospecific binding of winter flounder and shorthorn sculpin AFPs to ice. Contoured fit of the protein backbone to the corrugation of the (111) surface and coplanarity of hydrophilic, ice binding residues was essential for the protein−ice recognition. Although a single "global" minimum was found, there were several closely spaced, on the energy scale, local minima, differing by slight variations in the amino acid side chain position. Although no clear evidence of directionality of bonding was found for this globular Type II AFP protein, uniqueness of binding determined by the contoured backbone fit to the (111) surface of ice seem to suggest that the preferred angle of binding of the sea raven AFP may follow the alignment of K91-K122 vector along the bisector of the angle determined by [1 0 −1] and [1 −1 0] surface vectors.

We have found that the structure−function relationship in the antifreeze proteins, determined earlier for the α-helical Type I antifreeze proteins like winter flounder or shorthorn sculpin, extends to another class of structurally entirely different Type II AFP proteins from sea raven. The key features of this universality of structure−function relationship, with respect to protein−ice recognition and binding, rely upon the contoured fit of the protein backbone to the corrugation of ice surface and utilization of threonine/serine and lysine residues for creating a hydrogen bond network between the surface and the protein.

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