The antifreeze proteins (AFPs) are structurally diverse molecules that share an ability to bind to ice crystals and inhibit their growth. The type II fish AFPS of Atlantic herring and smelt are unique among known AFPS in their requirement of a cofactor for antifreeze activity. These AFPS are homologous with the carbohydrate-recognition domains of Ca$^{2+}$-dependent (C-type) lectins and require Ca$^{2+}$ for their activity. To investigate the role of metal ions in the structure and function of type II AFPS, the binding of Ca$^{2+}$ and other divalent cations to herring AFP was investigated. Binding studies using $[^{99m}Tc]$-Ca$^{2+}$ demonstrated that the AFP has a single Ca$^{2+}$-binding site with a $K_d$ of 9 $\mu$m. Proteolysis protection studies and measurement of antifreeze activity revealed a conformational change from a protease-sensitive and inactive apoAFP to a protease-resistant active AFP upon Ca$^{2+}$ binding. Other divalent metal ions including Mn$^{2+}$, Ba$^{2+}$, and Zn$^{2+}$ bind at the Ca$^{2+}$-binding site and induce a similar change. A saturatable increase in tryptophan emission intensity at 340 nm also occurred upon Ca$^{2+}$ addition. Whereas antifreeze activity appeared normal when Ca$^{2+}$ or Mn$^{2+}$ were bound, it was much lower in the presence of other metal ions. When Ba$^{2+}$ was bound to the AFP, ice crystals showed a distinct difference in morphology. These studies demonstrate that herring AFP specifically binds Ca$^{2+}$ and, consequently, adopts a conformation that is essential for its ice-binding activity.

Many marine teleost fishes are protected from freezing in icy sea water by antifreeze proteins (AFPs) or glycoproteins (AFGPs). These proteins lower the freezing points of solutions in a noncolligative manner by inhibiting the growth of ice crystals (Conformational change; Davies and Hew, 1990). Four distinct types of antifreezes have been found in fishes, and each has a narrow phyletic distribution. The AFGs are found in the cods and in the nothotheids (see Davies and Hew (1990) and references therein), and they consist of a series of repeated tripeptide units (Ala-Ala-Thr) with an O-linked disaccharide linked to each Thr residue. The type I AFPs, found in scallops and in righteye flounders, are Alpha-rich amphiatic $\alpha$-helices (Davies and Hew, 1990). The type II AFPs are larger proteins with a folded structure and are found in smelt (Osmerus mordax), herring (Clupea harengus harengus), and sea raven (Hemitrépterus americanus) (Ewart et al., 1992; Ng and Hew, 1992; Ewart and Fletcher, 1993). Type III AFP is a protein with a $\beta$ sandwich structure and appears limited to eel pouts (Sönàichsen et al., 1993).

The type II AFPs from sea raven, smelt, and herring share a high protein sequence identity. All three proteins are homologous to the carbohydrate recognition domains (CRDs) of Ca$^{2+}$-dependent (C-type) lectins and correspond to the group VII C-type lectin family (Drickamer and Taylor, 1993). Multiple sequence alignment of the sea raven AFP with C-type CRDs and modeling of the AFP using coordinates from the CRD of rat mannose-binding protein (Weis et al., 1991a) has confirmed the structural similarity of the type II AFP to the C-type CRD fold (Sönàichsen et al., 1995). However, in contrast to the lectins, the AFPs do not bind to carbohydrates (Ewart, 1993).

Two Ca$^{2+}$-binding sites were identified in the crystal structure of a C-type CRD from rat mannose-binding protein (MBP), and a single site was identified in the CRD of E-selectin (Weis et al., 1991a; Graves et al., 1994). The Ca$^{2+}$-binding site that is shared by the lectins and the MBP was shown to be the carbohydrate-binding surface in a crystal structure of an oligosaccharide-MBP complex (Weis et al., 1992). Like the C-type lectins, the herring and smelt AFPs require Ca$^{2+}$ for their activity (Ewart et al., 1992; Ewart and Fletcher, 1993). In order to understand the role of Ca$^{2+}$ in the structural integrity and activity of these AFPS and the structural alterations underlying the evolution from carbohydrate to ice binding, we examined the role of Ca$^{2+}$ in the conformation and the antifreeze activity of the herring AFP (hAFP). Our findings show that hAFP contains a single Ca$^{2+}$-binding site. The protein binds Ca$^{2+}$ and several other ions which modulate the protein conformation as detected by proteolysis protection assays and by Ca$^{2+}$-induced fluorescence change. However, fully active AFP is only detected in the presence of Ca$^{2+}$ and Mn$^{2+}$.

**EXPERIMENTAL PROCEDURES**

Materials—Bio-Gel DEAE-agarose and Chelex 100 resin were purchased from Bio-Rad. Supported nitrocellulose was from Micron Separations (Westborough, MA), and Immobion polyvinylidene difluoride (PVDF) was from Millipore (Bedford, MA). Ruthenium red (99.3% pure) was obtained from Fluks (Ronkonkoma, NY). Proteases were from Promega (Madison, WI) and Boehringer-Mannheim (Laval, Canada). $[^{45}CaCl]_2$ (10–40 mCi/mg) was obtained from ICN Biomedicals (Costa Mesa, CA). All other chemicals were reagent grade.
The purification of hAFP—Serum proteins from Atlantic herring were fractionated on a Sephadex G-150 column as described previously (Ewart and Fletcher, 1990). The G150-fractionated protein was redissolved in 25 mM NH4HCO3 (pH 8.0) and applied to a Bio-Gel DEAE-agarose column (1 x 30 cm) equilibrated in the same buffer and eluted using a 25-250 mM buffer gradient. Fractions containing hAFP were lyophilized, further desalted on a metal-free Sephadex G-25 column and re-lyophilized. All buffers used for experiments with the "apoAFP" were made metal-free by passage through a Chelex column and stored in EDTA-treated glassware.

Binding and Ruthenium Red Staining of the hAFP—Ruthenium red dye binding was evaluated as described by Charuk et al. (1990). APO (4 μg/lane) was run on SDS-PAGE under nonreducing conditions and then electrophoretically transferred to 0.2-μm nitrocellulose as recommended by the supplier (Mini Protein II transfer unit Bio-Rad). Four identical blots were incubated in 20 mM Heps, pH 7.8, containing 0.1 mg/ml ruthenium red for 15 min at 4°C and photographed. Each blot was then washed at 4°C in the same buffer without the dye but containing 50 mM CaCl2, MgCl2 or NaCl, or buffer alone and rephotographed.

For 45Ca2+ binding study, hAFP (2 μg) was run on reducing Tricine-SDS-PAGE and blotted onto a 0.45-μm PVDF membrane. The blot was incubated with 2.5 μCi of 45CaCl2 in 0.1 mM CaCl2, 40 mM Tris-HCl, pH 7.5, for 30 min followed by washes in the same buffer without Ca2+ and autoradiography.

Equilibrium Ca2+ Binding—Binding studies were performed by ultrafiltration in microcentrifuge filter cartridges (Millipore Ultracel-MC) with a 5-kDa molecular mass cutoff as described by Martin et al. (1989). For each experiment, 250 μl containing 40 μg hAFP, with 7.5, 8.2, 10, and 20 μM hAFP, and 2-300 μM CaCl2, containing 45CaCl2, were incubated at 4°C for 15 min and partially filtered by centrifugation at 10-11,000 rpm. Following centrifugation, duplicate aliquots (40 μl) were taken from both the filtrate and the remaining unfiltered protein solution and placed in 10 ml of ACS scintillation fluid (Amer sham Corp.). The filters were blotted dry, placed in scintillation vials, and 350 μl of a solution of 10% SDS in 4 mM Tris-HCl, pH 7.5, were added to each filter, followed by 10 ml of scintillation fluid. Radioactivity was measured in a LKB Wallac 1219 Rackbeta scintillation counter. Significant nonspecific binding by performing identical experiments with no hAFP was detected at CaCl2 concentrations greater than 1.0 mM. Only values obtained at lower concentrations were used to determine Ca2+ binding to hAFP and for each measurement, nonspecific binding was subtracted.

Metal ion competition studies were performed by combining 1.7 mM apoAFP and 0.1 mM CaCl2, containing 45CaCl2 alone or in the presence of metal ions (0.5 mM MgCl2, MnCl2, BaCl2, ZnCl2, or ruthenium red) in 40 mM Tris-HCl, pH 7.5, followed by centrifugation and analysis as described above.

Protection of Protein—Protection was carried out in aliquots of hAFP (0.2 mg/ml) in 10 mM Heps, pH 7.8, with different concentrations of endoproteinase Glu-C. The presence or absence of 20 μM CaCl2 for 2 h at 20-22°C. The reactions were stopped by the addition of an equal volume of SDS-sample buffer, resolved by nonreducing 14% acrylamide SDS-PAGE, and stained with Coomassie Blue.

Sequences of the proteolytic fragments were obtained by automated Edman degradation on PVDF according to LeGendre and Matsuda (1986), with 100 μg of protein loaded in each sample. The spectra for apo AFP. The spectra for apo AFP were obtained and recordings were repeated following the addition of aliquots of stock solutions of MgCl2, MnCl2, and EDTA in the same buffer.

Assay of Antifreeze Activity—Antifreeze activity was quantitated as thermal hysteresis, defined as the difference between the melting and freezing temperatures of the test solution. Melting and freezing temperatures were obtained by observing ice crystal behavior using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY) as described by Chakrabarty et al. (1989). Measurements for each sample were done in triplicate from three different sample wells. Ice crystal morphology was monitored by video recording.

To measure the effect of Ca2+ concentration on antifreeze activity, aliquots of apoAFP in Tris-HCl, pH 7.5, were combined with water or Ca2+ ion solutions to give a final protein concentration of 4 mg/ml (0.27 mM ion) in 40 mM Tris-HCl, pH 7.5. For thermal hysteresis measurements and crystal recordings in the presence of different metal ions, solutions of apoAFP in Tris-HCl, pH 7.5, were combined with water or metal ion solutions to give final metal ion concentrations of 10 mM, except for ruthenium red which was 3 mM.

Molecular Modeling—The program HOMOLOGY was used to construct a model of the hAFP using structural information from the human E-selectin and the rat MBP. In addition to sequence comparison, the statistics of structural alignment between the two crystal structures were used as a guide in assigning coordinates to the AFP sequence. Root mean squares errors in the superposition of fragments of the two structures were used as indicators of structural similarity. The model of the hAFP was constructed by splicing the two crystal structures while retaining all conserved regions and with minimal introduction of unknown coordinates. Arbitrary coordinates were assigned to a loop of eight residues that have no correspondence in the two crystal structures. The loop was then subject to 100 ps of molecular dynamic simulation using the program DISCOVER to arrive at the final conformation.

RESULTS

Binding of 45Ca and Ruthenium Red to hAFP—For activity and Ca2+ binding experiments, hAFP was purified using DEAE chromatography. The purified AFP showed a single band on nonreducing SDS-PAGE (not shown). The Ca2+-free apoAFP showed no thermal hysteresis. In addition, ice crystals in apoAFP solutions were found instead of the bipyramidal crystals seen normally in solutions of active AFP. Several techniques were used to characterize the nature and extent of Ca2+ binding by the hAFP. The result of protein blotting and detection with 45Ca was shown in Fig. 1A. The possibility of using an inorganic dye, ruthenium red, to further examine Ca2+-binding by the AFP was evaluated. Ruthenium red specifically stains Ca2+-binding proteins (Charuk et al., 1990), and it has been used in studies of several Ca2+-binding proteins. On nitrocellulose blots, two of the molecular mass markers, ovalbumin (43 kDa) and β-lactoglobulin (18 kDa), were stained with ruthenium red (Fig. 1B). Both of these proteins are known to bind Ca2+ ion. Incubation of the blots with ruthenium red resulted in strong staining of AFP (Fig. 1B). Washing the blots in 50 mM CaCl2 or MgCl2 displaced most if not all of the bound dye (Fig. 1B). In contrast, 50 mM NaCl or the buffer alone was ineffective (Fig. 1B). These results show that the hAFP is indeed a Ca2+-binding protein.

Ca2+-binding Sites and Binding Affinity of hAFP—The interaction of Ca2+ with the AFP was evaluated by equilibrium

![Fig. 1. Ca2+ and ruthenium red binding to immobilized hAFP.](image-url)
**Fig. 2. Binding of metal ions to hAFP.** A, Ca²⁺ binding. Equilibrium bindings were carried out as described under "Experimental Procedures." The data shown are from a representative experiment. The curve was generated by best-fit to a single site ligand binding model. The inset shows a Scatchard plot of the data obtained by linear regression. The x intercept of this regression line is 1.09. B, competition studies. Ca²⁺ binding was determined in the presence of 0.1 mM Ca²⁺ alone or with 0.5 mM added competitor ion. The average level of Ca²⁺ binding in the absence of competitor was normalized to 100%. Each bar represents the mean ± S.E. of three separate filtration experiments.

binding using ⁴⁵CaCl₂. A Scatchard plot revealed the presence of a single Ca²⁺-binding site with a Kₐ of 9 μM (Fig. 2A). This value is similar to the Kₐ values for Ca²⁺ binding (2.5–3.5 μM) by the high affinity site of E-selectin (Anostario and Huang, 1995).

The possibility that other ions may bind at the Ca²⁺-binding site was evaluated through competition experiments (Fig. 2B). Mg²⁺ did not compete with Ca²⁺ binding in this assay. However, the presence of other ions resulted in markedly reduced Ca²⁺ binding. Thus, Mn²⁺, Ba²⁺, Zn²⁺, and ruthenium red can compete with Ca²⁺ for binding at the Ca²⁺ site.

Metal Ions Protect AFP from Proteolysis—The AFP was resistant to digestion by both trypsin and endoproteinase Glu-C in the presence of added Ca²⁺, whereas without Ca²⁺, the apoAFP was completely digested (results for trypsin not shown). Glu-C was employed for further studies because the hAFP contains 14 potentially susceptible acidic residues outside the Ca²⁺-binding site. Thus, any cleavage of these peptide bonds by Glu-C should provide an indication of the conformation of the AFP as modulated by the metal ions. In the presence of 20 mM Ca²⁺, the AFP remained fully resistant to Glu-C digestion at all protease concentrations (Fig. 3A). However, in the absence of added Ca²⁺, complete digestion of AFP was evident (Fig. 3A). Major proteolytic products of the AFP were resolved in the Tricine-SDS-PAGE system. The N-terminal sequences of these fragments were LHWAD and STFVK which indicated cleavage sites between residues Gln-22 and Leu-23 and between residues Glu-47 and Ser-48 as numbered in the mature hAFP (Ewart and Fletcher, 1993). Both sites are far from the predicted Ca²⁺-binding residues. They correspond by sequence alignment to a position between α helix 1 and β sheet 1 and to the loop joining β sheet 2 and loop 1 in the homologous MBP CRD structure (Weis et al., 1991a). Thus, the resistance to proteolysis conferred by Ca²⁺ appears to involve a modulation of the overall protein conformation.

The effect of other divalent cations (20 mM each) on the conformation of AFP was similarly evaluated. Digestion of AFP in the presence of Ba²⁺, Mn²⁺, and Zn²⁺ was markedly reduced compared to that in the absence of added metal (Fig. 3B). In contrast, Mg²⁺ ions and ruthenium red did not protect the AFP from digestion by Glu-C. A similar assay using trypsin also showed a lack of protection by Mg²⁺ ions (results not shown).

Fluorescence Emission—The modulation of hAFP conformation by divalent cations was investigated using fluorescence spectroscopy. The addition of 1 mM Mg²⁺ did not affect the emission spectrum of the apoAFP but the subsequent addition of 3 mM Ca²⁺ elicted an increase in emission intensity (Fig. 4). No shift in emission wavelength was detected. Addition of 1 mM Ca²⁺ followed by 3 mM Mg²⁺ resulted in an initial increase in emission intensity upon Ca²⁺-binding and no further change with Mg²⁺ (results not shown). The Ca²⁺-induced spectral changes were observed at 5 and 15 °C. At 15 °C, the spectral changes were completely reversible with excess EDTA. These findings further document the conformational change that occurs in the AFP upon Ca²⁺ binding.

Antifreeze Activity—The dose response of hAFP antifreeze activity to Ca²⁺ was evaluated by measuring the antifreeze activity at a series of Ca²⁺ ion concentrations (Fig. 5A). However, this response curve cannot be used as a true measure of
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![Graph](image)

**Fig. 4.** Ca\(^{2+}\)- and Mg\(^{2+}\)-induced changes in the intrinsic fluorescence of hAFP. Emission spectra of hAFP (0.6 \(\mu\)M) were recorded as described under “Experimental Procedures” in the absence of divalent metal ions and following the addition of 1 mM Ca\(^{2+}\) and then 2.5 mM Mg\(^{2+}\).

Ca\(^{2+}\) binding. Activity measurement requires very high concentrations of AFP. In addition, the activity of fish AFPs increases in a nonlinear fashion with protein concentration forming a hyperbolic curve (Kao et al., 1986). This study does, however, provide a clear illustration of the metal ion requirements of the AFP when present at physiological concentrations in herring blood.

The activity of AFP was also assayed in the presence of an excess (10 mM) of other metal ions. Activity was detected in the presence of each of the added metals but the AFP appeared fully active only in the presence of Ca\(^{2+}\) and Mn\(^{2+}\) (Fig. 5B). In the presence of Mn\(^{2+}\), activity was 80% that of the Ca\(^{2+}\)-activated AFP. In contrast, activity was less than 25% of that obtained for the Ca\(^{2+}\)-activated AFP in the presence of the other metal ions. Ice crystals in AFP solutions that contained Ca\(^{2+}\) and Mn\(^{2+}\) formed bipyramids typical of those seen in fully active AFP at high concentrations. Crystals in AFP solutions containing the other metals formed cylinders with hexagonal cross sections consistent with those seen in the presence of minimal concentrations of herring AFP (Fig. 6). However, in the presence of Ba\(^{2+}\), depressions formed on the basal planes of the ice crystals suggesting a different pattern of AFP interaction with ice when this metal is bound.

Control experiments demonstrated that the effects of the metal ions on hAFP activity resulted from their specific interaction with this protein. Neither the ice crystal morphology nor the activity of an unrelated type III AFP from the ocean pout was affected by the removal of metal ions or by the addition of specific metal ions.

**Fig. 6.** Antifreeze activity of hAFP in the presence of metals. Ice crystals were grown in solutions of 4 mg/ml AFP. Crystals are shown with the \(a\) and \(c\) axes indicated. An exception is the crystal grown in the absence of added ion that has the \(a\) axis parallel to the page and the \(c\) axis normal to the page. Added metal ions are indicated. Crystals were videotaped at temperatures below the melting point. Individual frames were then transferred into image files.

Control experiments demonstrated that the effects of the metal ions on hAFP activity resulted from their specific interaction with this protein. Neither the ice crystal morphology nor the activity of an unrelated type III AFP from the ocean pout was affected by the removal of metal ions or by the addition of specific metal ions.

Modeling Structure of the hAFP—A model of the hAFP was constructed based upon alignment with crystal structures of C-type CRDs from MBP and E-selectin. Sequence alignments of hAFP based on structural alignment between the two CRD crystal structures revealed five structurally invariant regions (Fig. 7A). The five Ca\(^{2+}\) ligands predicted by alignment among MBP and various other C-type lectins (Weis et al., 1991a; Drickamer, 1993) are shown in a ribbon diagram of the hAFP (Fig. 7B). Only four of the five residues corresponding to those indicated are Ca\(^{2+}\) ligands in the E-selectin. The Glu-88 residue in E-selectin that corresponds by alignment to Glu-99 in hAFP is in a position and orientation that precludes a role in Ca\(^{2+}\)-binding (Graves et al., 1994). Similarly, in the model of hAFP, this residue does not appear to be associated with the Ca\(^{2+}\) ion (Fig. 7B).

**DISCUSSION**

Antifreeze proteins depress the freezing temperatures of solutions by binding specific planes of ice crystals and thereby inhibiting crystal growth. The ice-binding mechanism of the type II fish AFPs is not understood, and the surface that forms the ice-binding site has not been identified. In this context, the evolutionary relationship and structural similarity between the type II AFPs and the C-type lectin CRDs may provide a rational approach to the study of AFP function. The AFPs of...
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Fig. 7. Predicted Ca$^{2+}$-binding site in type II AFPs. A, sequence alignment of MBP (Drickamer et al., 1986), E-selectin (SEL) (Bevilacqua et al., 1989), and hAFP (H) sequences (Ewart and Fletcher, 1993). Conserved residues corresponding to the Ca$^{2+}$-binding site residues in MBP are in bold letters. The bottom row indicates the source of the herring coordinates (E from SEL, M from MBP, and L indicating a loop region). The structurally conserved regions (SCR) are also indicated. B, a ribbon diagram of the herring AFP structure modeled as described under "Experimental Procedures" by sequence alignment with MBP and E-selectin. Residues that were identified as potential Ca$^{2+}$ ligands by alignment with MBP and E-selectin are shown. The Ca$^{2+}$ atom is represented by a sphere.

herring and smelt are unique among all AFPs in their absolute requirement of a cofactor (Ca$^{2+}$) for activity. Thus, the Ca$^{2+}$-dependent AFPs provide an interesting model for the study of the specific role of metal ions in ice-binding activity. Their Ca$^{2+}$-dependent interaction with ice suggests that they represent an intermediate between two different forms of the CRD, the typical C-type lectin CRD with Ca$^{2+}$-dependent carbohydrate-binding activity and the distinct CRD of the sea raven AFP which is a Ca$^{2+}$-independent ice-binding protein.

Blotting experiments using $^{45}$CaCl$_2$ and ruthenium red dye showed that Ca$^{2+}$ is indeed bound directly by the hAFP. In our experiments, excess Mg$^{2+}$ appeared to displace the dye from the AFP and the positive controls as effectively as did Ca$^{2+}$ (Charuk et al., 1990). However, under the conditions used in $^{45}$Ca$^{2+}$ binding studies, Mg$^{2+}$ did not compete with $^{45}$Ca$^{2+}$ binding to the AFP.

Binding measurements using $^{45}$Ca$^{2+}$ revealed the presence of a single Ca$^{2+}$-binding site with a $K_d$ of 9 μM on the hAFP. Sequence analysis and prediction with known (C-type CRDs) crystal structures has also shown that the ACPs contain those conserved residues that form the Ca$^{2+}$-binding site that corresponds to Ca$^{2+}$-binding site 2 of the galactose-binding C-type lectins (Fig. 7A), whereas CRD Ca$^{2+}$-binding site 1 does not appear to be conserved in the herring and smelt AFPs (Weis et al., 1991a, 1992; Ewart et al., 1992; Ewart and Fletcher, 1993). Together, these studies argue strongly for the presence of a unique Ca$^{2+}$-binding site on the hAFP that corresponds to Ca$^{2+}$-binding site 2 in the structure of rat MBP (Weis et al., 1991a). The presence of site 2 in a C-type CRD without site 1 has also been described in the E-selectin (Graves et al., 1994). Ca$^{2+}$-binding site 2 forms the carbohydrate-binding site on the C-type CRD (Weis et al., 1992) and may therefore be more conserved among lectins than site 1.

Previous studies on C-type lectin CRDs have shown that other metal ions can substitute for Ca$^{2+}$. Using various assay methods, E-selectin and chicken hepatic lectin have been shown to bind Sr$^{2+}$ and Ba$^{2+}$ (Loeb and Drickamer, 1988; Anostario and Huang, 1995). Neither of these proteins appeared to interact with Mg$^{2+}$ ions. In the case of hAFP, several divalent metal cations, with the exception of Mg$^{2+}$, displaced Ca$^{2+}$ from the ACP. Ca$^{2+}$ and Mg$^{2+}$ are the most abundant divalent cations in fish blood (McDonald and Milligan, 1992). Our results show that in herring blood plasma, Ca$^{2+}$ is the ion that is bound at site 2 of the ACP.

Resistance to proteolysis has been employed as a useful indicator of the modulation of C-type CRD conformation (Loeb and Drickamer, 1988; Weis et al., 1991b; Anostario and Huang, 1995). Upon binding Ca$^{2+}$, CRDs typically undergo a conformational change from a functionally inactive conformation susceptible to proteolysis to one that is protease-resistant and contains a fully functional binding site for a specific carbohydrate or other ligand. The present investigation revealed that hAFP undergoes a similar alteration with the addition of Ca$^{2+}$. The enhancement of resistance to proteolysis is a good diagnostic indicator of a Ca$^{2+}$-induced conformational change in the ACP. Two protease cleavage sites on the ACP are found to be at locations separate from the Ca$^{2+}$-binding sites. This suggests that a conformational change involving a large region of the herring ACP occurs in response to Ca$^{2+}$. Metals that conferred protease resistance to the ACP included Mn$^{2+}$, Ba$^{2+}$,
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and Zn\(^{2+}\), but not Mg\(^{2+}\). The Ca\(^{2+}\)-induced change in conformation was also detected by fluorescence spectroscopy. This finding corroborates competition data suggesting that Mg\(^{2+}\) does not bind strongly at the Ca\(^{2+}\)-binding site.

To understand the structure-activity relationship in the hAFP, the response of the antifreeze activity to Ca\(^{2+}\) ions was evaluated. The approximately 1:1 stoichiometric molar ratio of Ca\(^{2+}\) to AFP at which maximal activity was attained is in agreement with Ca\(^{2+}\) binding data showing a single site on the AFP. The response of AFP activity to different metals revealed that Mn\(^{2+}\), which results in an ice crystal morphology similar to Ca\(^{2+}\), can be an effective substitute for Ca\(^{2+}\) in forming an active AFP. In contrast, the ice crystals formed in the presence of AFP with Ba\(^{2+}\) contained depressions on the basal planes (Fig. 6) that are not seen with the addition of Ca\(^{2+}\) or other metals. Similar differences among metal ions have been reported for E-selectin. Ba\(^{2+}\) and Sr\(^{2+}\) ions protected the selectin from proteolysis but in each case the resulting apparently stably folded selectin did not interact normally with its carbohydrate ligand (Anostario and Huang, 1995).

The ice-binding site in the type II AFPs is most likely to have evolved from the Ca\(^{2+}\) site corresponding to site 2 of MPB in a lectin CRD as suggested by Ewart (1993) and Sönnichsen et al. (1995). Binding of C-type lectins to carbohydrate and binding of AFPs to ice both require a motif that is formed exclusively in the Ca\(^{2+}\)-induced "active" CRD conformation. In addition, carbohydrate binding by C-type lectins is mediated primarily through a precise arrangement of hydrogen bond-forming groups which would be an ideal starting point for the evolution of an ice-binding site. The formation of an unusual ice crystal morphology when Ba\(^{2+}\) is added to the AFP further supports the notion that the Ca\(^{2+}\) site is directly involved in ice binding.

The residues that form the Ca\(^{2+}\)-binding site in the MBP are conserved in hAFP. However, in the AFP, residues 1 and 2 of the Ca\(^{2+}\)-binding site are Gin and Asp as in the galactose-binding lectins, and in contrast to the MBP and the E-selectin in which they are Glu and Asn (Fig. 7B). The architecture of the galactose and mannose-binding motifs are expected to be quite similar because simultaneous mutations changing Glu-Pro-Asn to Gin-Pro-Asp in MBP cause it to bind galactose (Drickamer, 1992). The role of Glu-99 in Ca\(^{2+}\) binding is not yet clear. In MPB, this residue is a Ca\(^{2+}\) ligand, whereas its location in the E-selectin is not compatible with a Ca\(^{2+}\)-binding role (Wes et al., 1991; Graves et al., 1994). A crystal structure of hAFP will allow a precise identification of the Ca\(^{2+}\) and ice-binding residues in the AFP. The ion-binding site identified here will allow us to generate protein crystals in the presence of heavy atoms suitable for structural determination. Site-directed mutagenesis of selected residues will provide another avenue that will be useful in mapping both the metal and ice-binding site residues.

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