The Ice-Binding Site of Atlantic Herring Antifreeze Protein Corresponds to the Carbohydrate-Binding Site of C-Type Lectins†

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ABSTRACT: The type II antifreeze proteins (AFPs) of smelt and Atlantic herring are homologous to the carbohydrate-recognition domains (CRDs) of Ca^{2+}-dependent (C-type) animal lectins and, like these lectins, acquire a stable and active structure upon binding Ca^{2+} ions. In the C-type lectin CRD, the carbohydrate-binding site is located at a Ca^{2+}-binding site. Site-directed mutagenesis was used to test the hypothesis that the ice-binding site of the type II AFP corresponds to the carbohydrate-binding site of the lectins. To disrupt this site in the herring AFP without perturbing the Ca^{2+}-dependent protein fold, a double mutant was constructed that changed the Ca^{2+}- and carbohydrate-binding motif from the galactose-type of wild-type AFP containing the sequence Gln-Pro-Asp to a mannose-type that has the sequence Glu-Pro-Asn and is also known to bind Ca^{2+}. The mutant AFP exhibited proper Ca^{2+} binding, folding, and stability as demonstrated by ruthenium red staining, proteolysis protection assays, and CD spectroscopy. However, it showed no antifreeze activity (thermal hysteresis) and did not alter ice crystal morphology to form bipyramidal crystals as does the active wild-type AFP. These results demonstrate that the ice-binding site of the herring type II AFP corresponds to the carbohydrate-binding site of the C-type lectin CRDs and further suggest that this ice-binding function evolved from the carbohydrate-binding site of a preexisting C-type lectin.

Antifreeze glycoproteins (AFGPs) and antifreeze proteins (AFPs) are found in many cold water marine teleosts. These proteins, despite their distinct chemical structures, share an ability to bind to ice crystals in solution which alters the pattern of ice crystal growth. This binding results in faceted ice crystal morphologies, inhibition of ice recrystallization during solution rewarming, and noncolligative lowering of the freezing points of solutions via a noncolligative mechanism (*1*−*3*).

Four types of AFGP/AFP have been identified and characterized, and significant work has been directed toward understanding their mechanism of ice binding. The glycoproteins of cods and nototheniids are polymers of Ala-Ala-Thr with a disaccharide attached to each Thr residue (*4*). Chemical modification of the glycoprotein revealed that the repeated carbohydrate hydroxyl groups interact with ice (*4*). The type I AFPs of the sculpins and righteye flounders are single amphiphilic α helices, and most contain defined 11-residue repeated elements. The repeating structures of the glycoproteins and the type I AFPs suggested a pattern of binding motifs that correspond to the lattice structure of ice crystals (*2*). Similarly, analysis of the protein crystal structure of a type I AFP has led to a hypothesis that recurring two- and three-residue ice-binding motifs specifically recognize the ice lattice (*5*). The type III AFPs of eel pouts are folded proteins, and one of these proteins has been shown to be globular with one flat surface (*6*). A cluster of residues on and near this surface mediates binding to ice crystals (*6*, *7*). More recently, a new type of AFP from the longhorn sculpin with a helix bundle structure has been reported (*8*). Its ice-binding surface is, at present, undefined. These studies demonstrate the widespread occurrence and varied modes of action of the fish AFPs.

In contrast to some of the fish AFGP/AFPs, the basis for molecular interaction of the type II AFPs with ice remains unknown. These proteins belong to the C-type (Ca^{2+}-dependent) lectin superfamily (*9*−*11*). Modeled structures of type II AFPs corresponding to the carbohydrate-recognition domains of the C-type lectins have been reported (*12*, *13*). In particular, the AFP of Atlantic herring (*Clupea harengus harengus*) has one Ca^{2+}-binding site, and its activity and conformation are Ca^{2+}-dependent (*13*).

We have earlier postulated that the ice-binding site in the type II AFPs may have evolved at the Ca^{2+} site corresponding to site 2 of mannose-binding protein (MPB) and site 1 of E-selectin (*12*−*16*). This Ca^{2+}-binding site forms the carbohydrate-binding site in C-type lectins. To test this

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hypothesis, the herring AFP (hAFP) fused to a hexahistidine affinity tag was expressed in *E. coli* and refolded. Using this expression system, a mutant with Ca^{2+}-binding site residues identical to those in the mannose- and sialic acid-binding C-type lectins was prepared. The mutant protein folded properly and bound Ca^{2+} but lacked antifreeze activity. These studies have revealed that the ice-binding site is located at the Ca^{2+}-binding site of the herring AFP.

EXPERIMENTAL PROCEDURES

Materials. Ni^{2+} chromatography resin was purchased from Quiagen (Chatsworth, CA) and Invitrogen (San Diego, CA). Ruthenium red was from Fluka (Ronkonkoma, NY). Endoproteinase Glu-C was obtained from Promega (Madison, WI) and Boehringer Mannheim (Laval, Canada). All other chemicals were reagent grade.

Construction of the Expression Plasmid. Expression of the recombinant hAFP was performed using the pQE8 vector (Quiagen, Chatsworth, CA) in *E. coli* M15[pREP4]. A full-length hAFP cDNA was previously cloned in the *Bst*XI site of the plasmid pcDNAII (Invitrogen) (*11*). The cDNA was excised from the plasmid using *Xba*I and *Hin*dIII, gel-purified, and *Mae*I-digested to remove the sequences encoding the 5′ untranslated region and the predicted signal peptide of the hAFP. The remaining portion of the cDNA was purified, the ends were blunted using the *E. coli* DNA polymerase I Klenow fragment, and the cDNA was then ligated into a *Bam*HI-digested and blunt-ended pQE8 vector using T4 DNA ligase (Life Technologies Inc., Gaithersburg, MD). Positive clones (pQE8-HAFP) were identified by restriction digestion, and the clones expressing AFP were identified by screening expression cultures for hAFP by Western blotting using anti-herring AFP antiserum and a Protoblot detection system (Promega).

Site-Directed Mutagenesis. The mutant AFP was prepared from pQE8-HAFP using instructions supplied by Clontech (Palo Alto, CA). The selection primer (5′-AACAATTTCACACAGCATTCATTAAAGAGG-3′) was designed to remove an *Eco*RI site in the plasmid vector, and the mutation primer (5′-TGGTGCGCTGCAGAACCTAATACTACCTTAA-3′) was designed to change a Gln codon of the AFP cDNA into a Glu and an Asp codon into an Asn. The nucleotides that are changed in the primers are underlined. Following the preparation of mutant plasmids, freshly competent cells (*E. coli* BMH 71-18 *mutS*) were transformed and grown to amplify the plasmids. Plasmid DNA was isolated and the mutant plasmids were selected by repeated restriction digestion using *Eco*RI. Host cells used for protein expression (*E. coli* M15[pREP]) were transformed and screened by *Eco*RI digestion of plasmid DNA. Mutants were confirmed by dideoxy sequencing.

Expression and Purification of Recombinant and Mutant hAFP. For protein expression, 0.2−0.4 L of super medium containing 100 μg/mL ampicillin and 25 μg/mL kanamycin was inoculated with 10−15 mL of a stationary phase culture of M15[pREP4] containing pQE8-HAFP and grown until the OD_{600} was 0.6. IPTG was added to a final concentration of 3 mM, and cultures were grown for 5 h. Cells were harvested by centrifugation at 4000*g* for 20 min and frozen at −70 °C.

Protein purification was carried out under denaturing conditions following the Express System protein purification procedure (Invitrogen, San Diego, CA). Cells were suspended in guanidine lysis buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate, 0.5 mM NaCl, pH 7.8), rocked for 20 min, and sonicated for 30 s. The lysate was centrifuged at 2000*g* for 15 min, loaded on Ni^{2+} resin (3.5 mL of resin for every 100 mL of starting culture), and allowed to bind for 1 h at room temperature. The resin was then washed in phosphate buffer containing 8 M urea at pH 7.8, 6.0, and 5.3 following the protocol provided by Invitrogen. Elution was at pH 6.3 with the same washing buffer containing 250 mM imidazole.

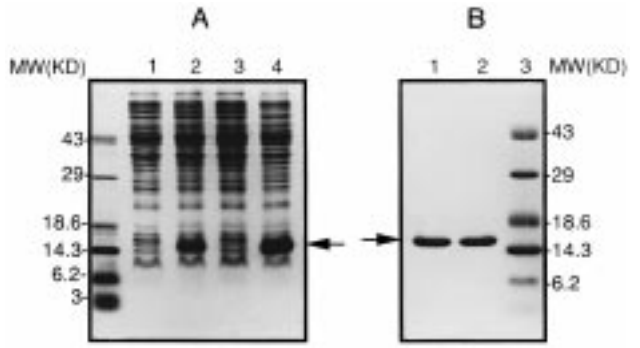

FIGURE 1: Expression of recombinant and mutant herring AFP in *E. coli.* (A) Expression studies. The bacterial extract was analyzed by SDS−PAGE. Recombinant AFP expression before (lane 1) and after IPTG induction (lane 2), and mutant AFP expression before (lane 3) and after IPTG induction (lane 4). (B) SDS−PAGE analysis of purified hAFP. Lane 1, rAFP purified by Ni^{2+}-NTA column; lane 2, mAFP purified by Ni^{2+}-NTA column; lane 3, protein low molecular mass markers. Arrows indicate the position of the hAFP.

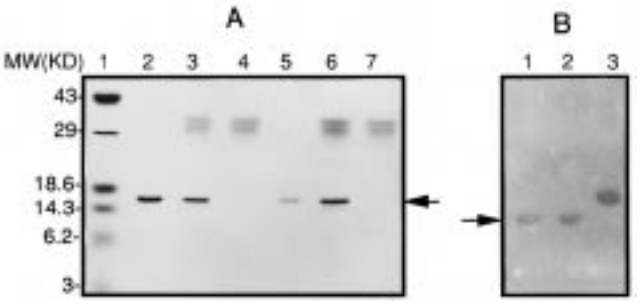

FIGURE 2: Ca^{2+} dependence of recombinant and mutant herring AFPs. (A) Proteolysis of recombinant hAFP in the presence and absence of added metals. Glu-C was added to rAFP (2 μL) as described under Experimental Procedures in the presence and absence of 1 mM Ca^{2+}. Lane 1, molecular mass markers; lane 2, rAFP alone; lane 3, with Ca^{2+} and Glu-C; lane 4, with Glu-C; lane 5, mAFP alone; lane 6, with Ca^{2+} and Glu-C; lane 7, with Glu-C. (B) Staining of herring AFPs with ruthenium red. Lane 1, rAFP; lane 2, mAFP; lane 3, β-lactoglobulin.

Refolding and Purification. The recombinant hAFP was refolded following the procedure of Klima et al. (*17*) with modifications. The protein eluted in urea-containing buffer was diluted gradually with washing buffer, pH 7.8, containing 0.2 mM β-mercaptoethanol until the final protein concentration was about 10 μg/mL. The solution was dialyzed against buffer A (100 mM NaCl, 1 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0) for 48 h and against 0.1 M NH_4HCO_3 for 72 h at 8 °C. The protein was freeze-dried.

Ruthenium Red Staining of the Recombinant hAFP. Ruthenium red dye binding was evaluated following the method of Charuk et al. (*18*) with minor modifications. AFP (4 μg per lane) was run on SDS−PAGE under nonreducing

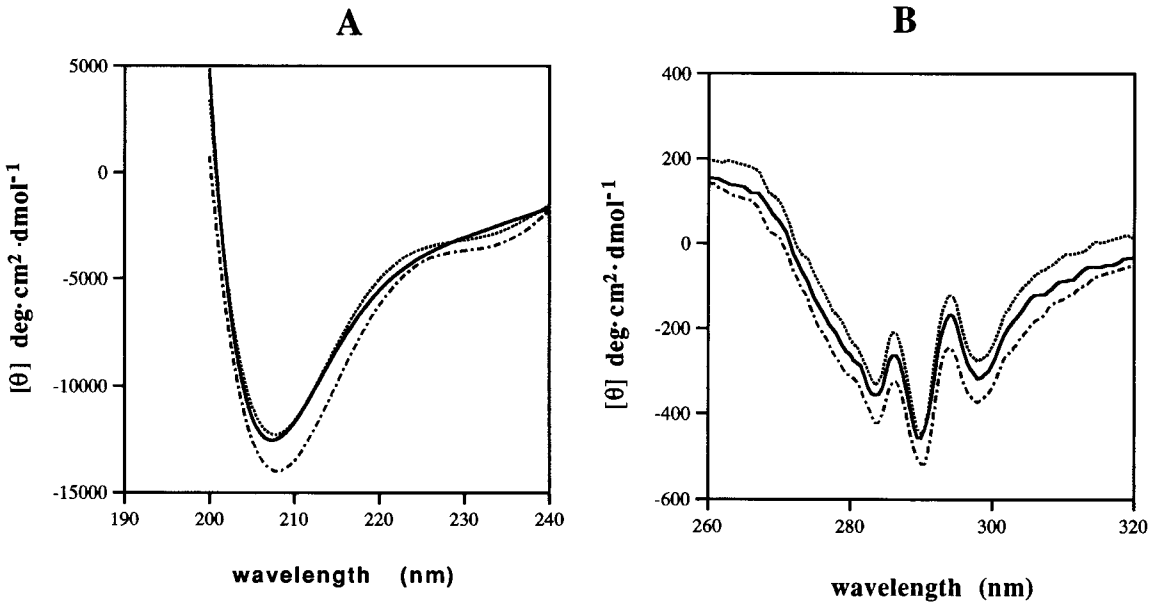

FIGURE 3: Circular dichroism studies. The native (—·—), recombinant (—), and mutant herring AFPs (···) are studied as described under Experimental Procedures. (A) Far-UV region. (B) Near-UV region.

conditions and then electrophoretically transferred to 0.2 μm nitrocellulose and stained with ruthenium red (*13*).

Proteolysis Protection Assay. Proteolysis was carried out on aliquots of AFP (0.2 mg/mL) in 10 mM Hepes, pH 7.8, with and without Ca^{2+}. The reactions contained 0.2 mg/mL endoproteinase Glu-C in the presence and absence of 10 mM $CaCl_2$ for 3 h at 20−22 °C. The reaction mixtures were resolved on nonreducing 16% acrylamide SDS−PAGE gels and stained with Coomassie Blue (*13*).

Circular Dichroism (CD) Studies. Protein samples for CD measurements were dialyzed for 12−16 h in 1 mM sodium phosphate, pH 7.8, and centrifuged before use. CD spectra were determined with an AVIV (Model 62 DS) CD spectrometer at 0 °C. Far-UV CD spectra (200−240 nm) were measured using a 0.1 cm path length cuvette, and near-UV CD spectra (260−320 nm) were in a 0.5 cm path length cuvette at a scanning speed of 0.5 nm/s and a bandwidth of 1.00 nm. The protein concentrations of the native recombinant and mutant AFPs in the far-UV spectra were 18.1, 24.7, and 19.5 μg/mL, respectively. The protein concentrations of the native recombinant and mutant AFPs in the near-UV spectra were 1.134, 1.120, and 1.120 mg/mL. The ellipticities were determined assuming a mean residue weight of 110 Da.

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 using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY) as described by Chakrabartty et al. (*19*). Each measurement value was the average of triplicate readings obtained from three different sample wells. Video recording was used to monitor the ice crystal morphology.

RESULTS

Expression and Purification of Recombinant and Mutant Herring AFP. A cDNA fragment encoding the predicted mature herring AFP (hAFP) was cloned in-frame into pQE8 with a hexahistidine-encoding sequence, and the fusion protein with a predicted molecular mass of 16 240 Da was expressed in *E. coli* (Figure 1A, lane 2). The yield of AFP was approximately 35 mg from 400 mL of culture. No cytosolic or periplasmic AFP was detected in bacterial lysates (results not shown). The recombinant herring AFP (rAFP) was therefore isolated from the inclusion bodies by Ni^{2+}-affinity chromatography under denaturing conditions, and the purity of the refolded rAFP is shown in Figure 1B. Only monomer was detected. To modify the presumptive ice binding site, a double mutant (mAFP) was generated by site directed mutagenesis. In this mutant, the sequence at positions 92−94 was changed from Gln-Pro-Asp (QPD) to Glu-Pro-Asn (EPN). The mutant AFP was isolated and purified using the same protocol (Figure 1A,B).

Both Recombinant and Mutant hAFPs Are Ca^{2+}-Dependent and Resistant to Proteolysis. An inorganic dye, ruthenium red, specifically stains Ca^{2+}-binding proteins (*18*), and the binding of ruthenium red to AFP is shown in Figure 2. These results show that the recombinant AFP and the mutant AFP both bind Ca^{2+} in a manner indistinguishable from the native hAFP (*13*). Resistance to proteolysis was used to detect conformational changes in AFP upon metal addition. The recombinant and mutant AFPs were resistant to proteolysis in the presence of Ca^{2+} ions, indicating that both were correctly folded (Figure 2).

Circular Dichroism Studies. CD spectra of the native, His-tagged recombinant and mutant AFPs coincide in both the near- and far-UV regions (Figure 3). These data indicate that these proteins fold in the same way and contain the same overall secondary structures, in agreement with the protease protection assay results.

The Mutant AFP Lacks Antifreeze Activity. The concentration dependence of native and recombinant AFP antifreeze activity was evaluated through thermal hysteresis measurements, and the activities of the proteins were found to be similar (Figure 4A). The ice crystal morphologies, which provide a qualitative indicator of antifreeze activity, were recorded in solutions used for thermal hysteresis measurements. Ice crystals in AFP solutions that contained rAFP formed bipyramids typical of those seen in the presence of

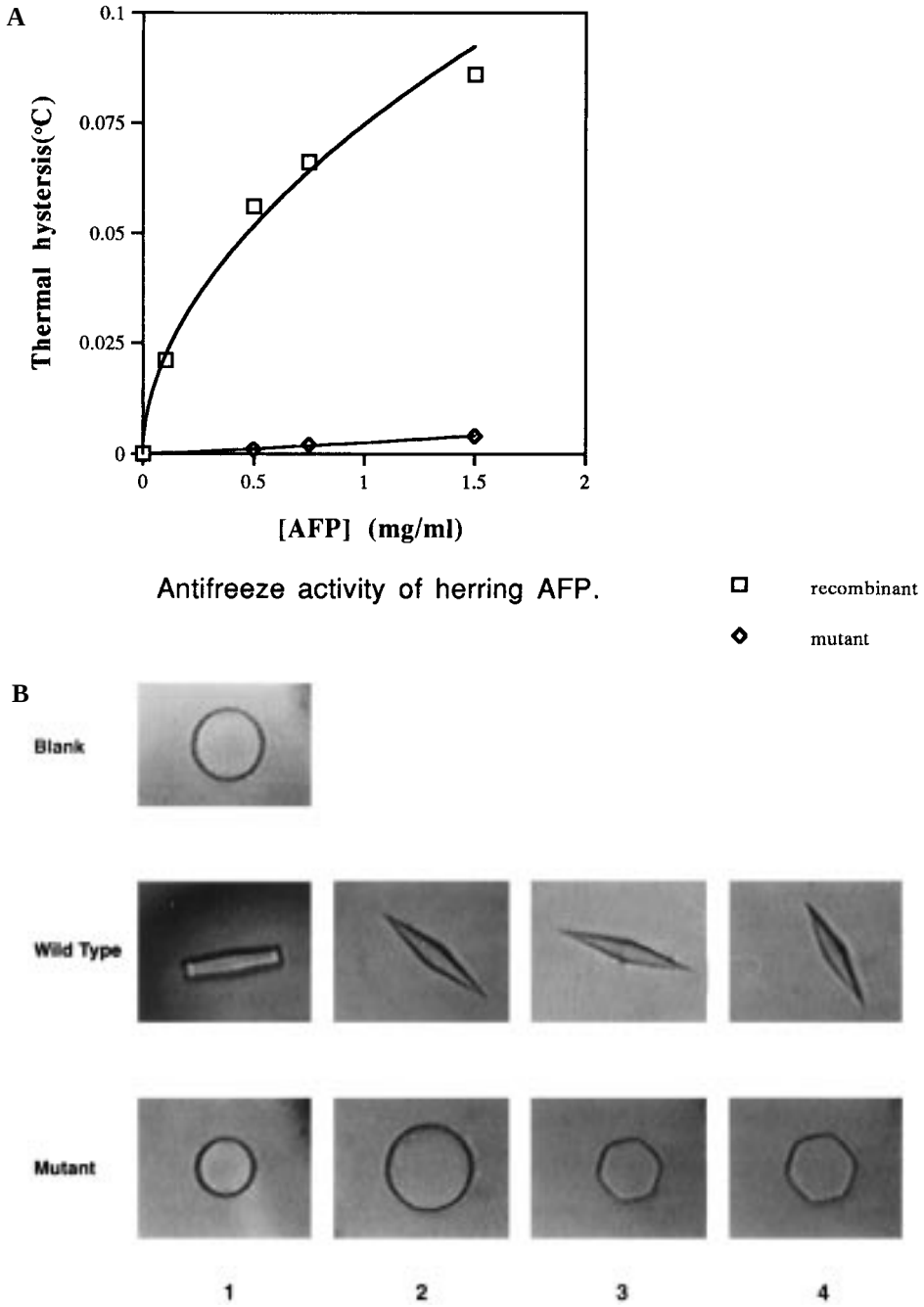

FIGURE 4: Antifreeze activity of recombinant and mutant herring AFP's as a function of added metals. (A) Antifreeze activity (thermal hysteresis) of solutions containing a series of AFP concentrations was measured as described under Experimental Procedures using a Clifton nanoliter osmometer. Values shown represent means ± SD of triplicate measurements done on single samples. The curve was fitted using the data pooled from the native and recombinant AFPs. (B) Ice crystal morphologies in the AFP solutions used to measure thermal hysteresis. Protein concentrations are 0.10, 0.5, 0.75, and 1.5 mg/mL in panels 1−4, respectively. A protein-free control is also shown. The magnification used was 400×.

native hAFP (Figure 4B). Thus, it appears that the histidine tag on the recombinant protein does not interfere with ice binding. The thermal hysteresis of the mutant AFP was also measured and was found to be minimal compared to the control (Figure 4A). This result indicates that the ice-binding site is located at the Ca^{2+}-binding site. Crystals in AFP solutions containing the mutant AFP formed mainly hexagonal prisms (6-sided cylinders) consistent with those seen in the presence of trace levels of native hAFP (Figure 4B). Ice crystal morphology is altered by AFPs at concentrations approximately 100-fold lower than those at which measurable hysteresis occurs.

DISCUSSION

The hAFP cDNA encoding the predicted mature AFP was cloned in-frame with a polyhistidine sequence in a bacterial expression vector and was successfully expressed in *E. coli.* The refolded rAFP binds Ca^{2+} and exhibits a Ca^{2+}-dependent protease resistance as the native AFP does. Similarly, the antifreeze activity of the recombinant AFP is indistinguishable from the native AFP (results not shown). The full activity of the recombinant AFP suggests that the ice-binding site is not situated close to the N- or C-termini of the protein. The termini are located together at the opposite end of the

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hafp        89   CAAQPDT------TLTECC
rsl         93   DKNQPDHY-----QNKEFC
cpgcp      249   RPNQPDNFF-----AGEDC
rhep1      235   RPGQPDDWYGHGLGGGEDC
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FIGURE 5: Alignment of amino acid sequences from three lectins and an AFP showing residues that may influence carbohydrate binding. The positions of aromatic residues that may stack with galactose are indicated by asterisks, and the Gly residues of the Gly-rich loop of the hepatic lectins are indicated by circumflexes. Sequences are hafpl, herring AFP (*11*); rsl, rattlesnake lectin (*26*); cpgcp, cartilage proteoglycan core protein (*29*); and rhep1, rat hepatic lectin (*30*).

molecule from the Ca^{2+}-binding site (*13*). The N-terminal histidine tag is likely to obstruct that end of the molecule, and it might therefore inhibit activity of the AFP if the ice-binding site was close to it. This finding reinforced the hypothesis that the Ca^{2+}-binding end of the molecule contains the ice-binding site. To determine whether the ice-binding site corresponds to the Ca^{2+}-binding site, a double mutation was designed in order that Ca^{2+} binding would be maintained to allow proper folding and an active protein confomation but the orientation of the ligands at the site would be altered. The AFP mutant showed normal Ca^{2+} binding and protease resistance, but it showed no antifreeze activity. This demonstrates that the mutant was properly folded but inactive, thus confirming that the ice-binding site of the AFP is located at the Ca^{2+}-binding site.

The Ca^{2+}-binding site of the C-type CRDs is formed by the Ca^{2+}-site residues that are shared by the AFPs and the lectins (*20*). Among the C-type lectins, there are CRDs with a variety of distinct carbohydrate specificities. There are two overall types of Ca^{2+}-binding site compositions among the C-type lectins: (1) within the lectins that bind galactose and its derivatives; and (2) within the lectins that bind mannose, fucose, sialic acid, or *N*-acetylglucosamine (*21*). The mutation in the AFP was designed to change the galactose-type site of the AFP into a mannose-type site. The opposite mutation has been carried out in mannose- and sialic acid-binding lectins and resulted in a new binding specificity for galactose (*22*−*25*).

The wild-typeAFP does not bind to carbohydrate despite its galactose-type (QPD) Ca^{2+}-binding site (results not shown). One contributing factor may be the absence of a key aromatic residue in type II AFPs that is present in galactose-binding lectins and stabilizes the bound sugar (Figure 5). High-affinity interaction of the human hepatic lectin with galactose was shown to require a Trp residue close to the Ca^{2+}/carbohydrate-binding site (*23*). Human proteoglycan core protein and rattlesnake C-type lectin contain Phe and Tyr, respectively, in similar positions (*23*, *26*). The human hepatic lectin also contains a Gly-rich loop that holds the Trp in position for packing with galactose and also excludes mannose from the binding site (*27*), and this loop is missing in the herring AFP (Figure 5). Likewise, the AFP mutant containing a mannose-type (EPN) motif does not bind to mannose (results not shown). This may also be explained by the absence of other key residues. In the macrophage mannose receptor, there are multiple CRDs containing the EPN motif, but only one of these binds to mannose. Mannose binding by this CRD relies on interactions with a unique Tyr residue (*28*). Binding to ice requires a relatively flat surface (*5*, *6*) that would appear to be incompatible with the presence of an aromatic residue or of a Gly-rich loop. The CRD of C-type lectins is a versatile domain that, with minor changes, can bind several different carbohydrates. This has evidently made it an ideal candidate for the evolution of ice-binding and antifreeze activity.

Modeling of the antifreezes and analysis of the Ca^{2+}-binding properties of the herring AFP confirmed the structural similarity between the AFPs and the C-type lectins, and analysis of the Ca^{2+}-binding properties of the AFP revealed ion binding and a conformational change that is typical of those in the C-type CRDs (*12*, *13*). The Ca^{2+}-site mutant has now revealed that the ice-binding site of the herring AFP is located at the Ca^{2+}-binding site. This corresponds to the carbohydrate-binding site in the C-type lectins. Thus, the ice-binding site of type II AFPs is likely to have evolved from the rearrangement of carbohydrate ligands on a preexisting C-type lectin.

Identification of the ice-binding site in the herring AFP provides several new avenues to determine the precise constellation forming the ice-binding motif. Further mutagenesis can be undertaken in order to define the ice ligands. In addition, the similarity between carbohydrate and ice recognition at this site can be evaluated by competition experiments. The availability of both refolded recombinant and mutant AFP should facilitate the structural determination of these proteins.

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