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1 **Purification, crystal structure determination and functional characterization of type III**
2 **antifreeze proteins from the European eelpout *Zoarces viviparus***

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16 **Abstract**

17 Antifreeze proteins (AFPs) are essential components of many organisms adaptation to cold
18 temperatures. Fish type III AFPs are divided into two groups, SP isoforms being much less
19 active than QAE1 isoforms. Two type III AFPs from *Zoarces viviparus*, a QAE1 (ZvAFP13)
20 and an SP (ZvAFP6) isoform, are here characterized and their crystal structures determined.
21 We conclude that the higher activity of the QAE1 isoforms cannot be attributed to single

22 residues, but rather a combination of structural effects. Furthermore both ZvAFP6 and
23 ZvAFP13 crystal structures have water molecules around T18 equivalent to the tetrahedral-
24 like waters previously identified in a neutron crystal structure. Interestingly, ZvAFP6 forms
25 dimers in the crystal, with a significant dimer interface. The presence of ZvAFP6 dimers was
26 confirmed in solution by native electrophoresis and gel filtration. To our knowledge this is
27 the first report of dimerization of AFP type III proteins.

28 **Keywords:** Antifreeze protein, *Zoarcetes viviparus*, crystal structure, dimerization, protein
29 purification

30 **Abbreviations:** TH (thermal hysteresis), T_{hf} (hysteresis freezing point), T_m (melting point),
31 T_f (freezing point), AFP (antifreeze protein), IBS (ice binding site)

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37 **1. Introduction**

38 Ectothermic animals that are frequently exposed to temperatures below the melting point
39 (T_m) of their body fluids must either avoid freezing of their body fluids and survive the low
40 temperatures or be able to tolerate ice formation in their tissues [22]. The body fluids of fish
41 living in ice laden waters have a temperature similar to that of the surrounding water. The T_m
42 of the body fluids is higher than that of the sea water, hence the fish are supercooled, and
43 should in principle freeze when they ingest or touch an ice crystal [9].

44 Antifreeze proteins (AFPs) are an essential component of the adaptations many of these
45 animals have evolved to survive low temperatures [7,9]. AFPs inhibit the growth of ice
46 crystals to a certain extent. The inhibition of the ice growth depresses the temperature at
47 which already present ice crystals grow, the hysteresis freezing point (T_{hf}), without changing
48 the melting point (T_m) that is predicted by Raoult's law (the colligative freezing point
49 depression). This separation of the T_{hf} and the T_m is termed thermal hysteresis (TH) or
50 antifreeze activity. It is still debated how AFPs inhibit ice crystal growth; however, the
51 general consensus is that the AFPs recognize and bind to various ice surface planes. Ice
52 growth is restricted to the regions between the adsorbed AFPs causing an increase in local
53 curvature which makes it less favorable for water molecules to join the ice crystal eventually
54 leading to an arrest in its growth [24,30]. The morphology of the ice crystals usually changes
55 to bipyramidal when fish AFPs are absorbed at the ice crystal's surface [9].

56 Type III AFPs have so far been found in fish belonging to the Zoarcoidei suborder (two
57 Antarctic and five Northern hemisphere species). Type III AFPs are divided into two groups
58 designated SP and QAE after their ability to bind to the ion-exchange matrices SP- and QAE-
59 sepharose, respectively. The two groups share ~50% sequence identity, while within groups
60 the sequence identity is ~90% for the SP group and ~75% for the QAE group. An alignment

61 of representative type III AFP sequences is shown in Figure 1. Several reports have shown
62 that the QAE isoforms are more active in terms of TH compared to SPs, which are inactive in
63 this respect. A QAE subgroup (QAE2) is also impaired in terms of antifreeze activity.
64 However, SPs, as QAEs, induce the characteristic bipyramidal ice crystal morphology [2,6].
65 The role of the SPs is still unknown, but *in vitro* the QAE1s and SPs from *Zoarces elongatus*
66 have been shown to co-operate and thereby increase the TH activity to the levels of TH found
67 *in vivo* [21].
68 The AFPs investigated in this study originate from *Zoarces viviparus* caught in Roskilde
69 fjord. Previous studies have shown by sequence analysis that the AFPs from *Z. viviparus*
70 belong to type III [1,27]. NMR studies of one of them, ZvAFP13, have shown secondary
71 structure elements similar to other type III AFPs [1].
72 Here we report the expression, purification and X-ray crystal structures for two type III AFPs
73 from *Z. viviparus*, ZvAFP13 representing the QAE1 and ZvAFP6 representing the SP
74 isoform classes, respectively.

75

76 **2. Materials and methods**

77 Unless otherwise stated, general laboratory chemicals were from Sigma-Aldrich, vectors and
78 strains from Novagen, and enzymes for molecular biology from Fermentas.

79 *Genes, cloning and expression:* cDNA had been reverse transcribed from mRNA encoding
80 for ZvAFP6 and ZvAFP13 that was isolated from *Z. viviparus*, ligated into pGEM-T Easy
81 vector (Promega) and transformed into JM109 *Escherichia coli* cells (Promega). JM109 *E.*
82 *coli* cells (Promega) with pGEM-T Easy vector (Promega) carrying the genes encoding for
83 ZvAFP6 (KC622345) and ZvAFP13 (ABN42205) were amplified from the vector by PCR.
84 The mature genes were ligated with T4 DNA ligase into the pET-26b vector after digestion
85 with *NdeI* and *XhoI*. After transformation into the *E. coli* BL21 (DE3), cells were grown at

86 28°C in LB medium supplemented with 50 µg/ml Kanamycin until cell growth reached
87 OD₆₀₀ 0.7-0.8. To induce expression, 0.5 mM isopropyl thio-β-d-galactoside was added to the
88 LB medium, and the cultures were grown at 28°C for further 16-18 hours. The cells were
89 then pelleted (5000g for 20 min at 4°C), resuspended in 1/10 volume of 50 mM sodium
90 acetate at pH 8 and lysed by sonication.

91 *Purification:* the pH of the supernatant obtained after sonication was adjusted to 4 using 99%
92 acetic acid in order to precipitate most of the non AFPs. The samples were centrifuged
93 (12000g for 20 min at 4°C) and the supernatant applied to a 5 ml HiTrap SP HP cation-
94 exchange column (GE Healthcare) at a flow rate of 0.5 ml/min with a linear NaCl gradient
95 (0–1 M) in 50 mM NaOAc buffer pH 4. The peak fractions were checked for TH and
96 bipyrimidal ice crystal formation. The fractions containing the AFP were pooled and stored at
97 -20°C. The purity was checked on 15% SDS/PAGE gels. The pooled fractions were
98 concentrated using Amicon Ultra 15 centrifugal filter devices with a molecular cut off at 3
99 kDa (Millipore). The concentration of the protein samples was measured by the BCA Protein
100 Assay Kit (Thermo Fischer Scientific).

101 *Activity:* TH was determined as described in Nishimiya et al. [21] and Ramløv [23] using a
102 Nanoliter Osmometer (Otago Osmometers). A cooling rate of 1°C pr. min was used and an
103 annealing time of 1 minute. The ice crystals were as small as possible while still being visible
104 under the microscope.

105 *Native MW estimation:* Gel filtration was carried out at 4°C in a 50 mM NaOAc buffer at pH
106 4.0 containing 0.3 M NaCl using a Superdex 75 column (10/300 GL) and the following
107 proteins as MW standards: aldolase (158 kDa), ovalbumin (43 kDa), carbonic anhydrase (29
108 kDa) and ribonuclease A (13.7 kDa). Detection of ZvAFP6 was carried out at A₂₁₄ due to the
109 low abundance of aromatics.

110 For native PAGE 12% gels were used. The gels were run at RT at 150 V for 1.5 h (XCell
111 SureLock[®] Mini-Cell system; Invitrogen) in 30 mM MES, 30mM histidine, pH 6.1 and with
112 reversed cathode and anode. Ribonuclease A was used as standard.

113 *Crystallization:* Initial screenings were setup at RT with an Oryx 8 crystallization robot
114 (Douglas Instruments) in MRC 2 sitting drop plates (Douglas Instruments). Initial conditions
115 were optimized in hanging drops in 24 well VDX plates (Hampton Research) with a drop
116 volume of 4 µl and a reservoir volume of 1 ml. ZvAFP13 crystals grew in 2.5 M (NH₄)₂SO₄,
117 0.1 M citric acid, pH 4.5 with a protein stock concentration of 2mg/ml. ZvAFP6 crystals
118 grew in similar conditions; however, the pH of the citric acid was 4.0 and the protein
119 concentration was 10 mg/ml.

120 *Data collection and processing:* A ZvAFP13 and a ZvAFP6 crystal were flash frozen in
121 liquid nitrogen and X-ray data were collected at beamline 911-2 at Maxlab, Lund, Sweden at
122 100 K with a maximum resolution of 1.45 Å for ZvAFP13 and 1.2 Å for ZvAFP6. Data were
123 processed using XDS [15]. For ZvAFP13 the space group was determined to be P2₁2₁2₁ with
124 1 molecule in the asymmetric unit. For ZvAFP6 the space group was determined to be C222₁
125 with 2 molecules in the asymmetric unit. Data collection statistics are shown in Table 1.

126 *Structure determination and refinement:* the structures of ZvAFP6 and ZvAFP13 were
127 determined by the molecular replacement method with PDB ID 1OPS and 4MSI respectively
128 as search models using Molrep [28]. Cycles of refinement using Refmac5 [20] were
129 alternated with cycles of manual model building in Coot [8]. In the last rounds of refinements
130 the structures were refined anisotropically. The final structures were evaluated with several
131 validation tools including Molprobity [3]. Refinement statistics are in Table 1.

132

Bioinformatics: For the sequence analysis the type III AFPs sequences deposited at the National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine (<http://www.ncbi.nlm.nih.gov/genbank/>) were retrieved and aligned using the program MAFFT [16]. The alignments were visualized using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Pymol was used to visualize 3D-structures (Schrödinger), while the PISA server was used to analyze the interfaces [18].

3. Results

Purification: The main purification step in the final procedure was a pH precipitation. The ion-exchange step originally carried out after the pH precipitation actually decreases the purity of ZvAFP13 with respect to higher MW contaminants; therefore this step was later omitted. Furthermore, difficulties were encountered in concentrating the fractions from the ion-exchange step, while the protein could be brought without difficulty to the concentrations necessary for crystallization by concentrating the supernatant from the pH precipitation step.

Activity: Both isoforms induced the previously observed bipyramidal ice crystal morphology. The QAE1 isoform ZvAFP13 had a TH of $0.38 \pm 0.03^\circ\text{C}$ at a concentration of 1 mg/ml and of $0.96 \pm 0.01^\circ\text{C}$ at a concentration of 5 mg/ml. This activity is comparable or higher than the activity reported for other QAE1 isoforms produced recombinantly, as for example in [21] or purified from fish [29]. The SP isoform ZvAFP6 was completely inactive on its own up to a concentration of 10 mg/ml. However, added to ZvAFP13 with both proteins at a concentration of 1 mg/ml nearly doubled the activity ($0.75 \pm 0.02^\circ\text{C}$).

Crystal structures: Well diffracting crystals were obtained for both ZvAFP6 and ZvAFP13. ZvAFP13 crystallizes isomorphously to many other reported type III AFPs [5,13,17], while ZvAFP6 crystallizes, as the first reported type III AFP, in the space group $C222_1$ with two molecules in the asymmetric unit forming a dimer in the crystal (see *Discussion*).

The crystal structure of ZvAFP13 was determined to a 1.45Å resolution. The Cα rmsd with the MR model (PDB ID 4MSI) was 0.384 Å over 52 residues aligned. The structure of the SP isoform, ZvAFP6, was determined to 1.2 Å resolution, representing the first high resolution structure of a SP isoform. The Cα rmsd with the MR model (PDB ID 1OPS) was 0.295 Å for the A chain of ZvAFP6 and 0.268 Å for the B chain, after omitting the two last C-terminal residues of the A chain which are clearly in a different conformation and are not modeled in the B chain. The fold of ZvAFP13 and ZvAFP6 (Figure 2a) is very similar to all other type III AFPs and comprises a compact, globular, single domain.

Overall the electron density is well defined for both proteins, except for the termini. In ZvAFP13, the first three N-terminal residues (M0, N1 and Q2) and the last C-terminal residue modeled (P65) have poor electron density. Alternate conformations are noted in Table 1. All other side chains had excellent electron density showing a single conformation. In ZvAFP6 the N-terminal M could not be modeled, either due to disorder or protease activity found in *E. coli* [19]. Disorder was also observed at the C-terminus, where P65 and Y63 are the last modeled residues for chains A and B respectively, with poor density for the two last residues modeled. In chain B only the side chain density is less defined for the stretch K25-S30. E36 (both chains) and M56A show poor side chain density. Alternate conformations are given in Table 1. P29 is a *cis*-proline in both structures. Additional refinement and geometrical quality information are in Table 1. Final R-factors/R-frees were 13.4%/17.6% for ZvAFP13 and 17.9%/19.5% for ZvAFP6 respectively after anisotropic refinement.

The rmsd for Cα atoms between ZvAFP6 (chain A) and ZvAFP13 was 0.63 Å. Between the A and B chain of ZvAFP6 it was 0.49 Å for all atoms and 0.20 for Cα atoms only.

Native oligomeric state of ZvAFP6: in order to see if the dimeric state of ZvAFP6 in the crystals is also present in solution, gel filtration was carried out. Comparison with standards

shows a MW of around 11 kDa most consistent with a dimer for the most prominent peak (Figure 3A). Native PAGE cannot be run under standard conditions for ZvAFP6 because of its high pI, however by reversing the current it can be electrophoresed at pH 6.1. ZvAFP6 runs close to a ribonuclease A standard, which has approximately the same pI (9.5 vs 9.3 as calculated for ZvAFP6) and double the MW (13.7 kDa) of a ZvAFP6 monomer (Figure 3B). Similar experiments carried out for ZvAFP13 were not conclusive and the MW in solution could not be clearly established.

4. Discussion

Expression and purification: A modification of the protocol by Nishimiya *et al.* [21] for expression of soluble type III AFPs in *E. coli* was followed for the *Z. viviparus* AFPs. Since type III AFPs are still active at low pH [4], the pH of the lysate could be lowered to pH 4, where most *E. coli* proteins precipitated, and an almost pure AFP preparation was obtained, which was suitable for structural studies without further purification.

Activity: Nishimiya *et al.* [21] previously showed that SP and QAE1 type III AFPs from *Z. elongatus* act in a cooperative manner. *Z. viviparus* produces both QAE1s and SPs AFPs, and when combined they also act in a cooperative manner.

Structural determinants for QAEs and SPs differences in activities: The hydration layer of type III AFPs has been subject of many investigations [14,25,26] since the original studies showing that at the ice binding site (IBS) of HPLC12 from *M. americanus* the water structure is ice-like [12,31]. Howard *et al.* [14] found a cluster of four water molecules, one with weaker density that was close to a tetrahedral geometry in the vicinity of T18, which was used to build a model for the ice face. Equivalent of these waters could be identified in both ZvAFP13 and ZvAFP6, though they are not present in the previously published SP structure

204 (PDB ID 1OPS) [32], so no obvious difference can be seen in the water structure organization
205 near the IBS for QAE1 and SP variants according to the structures presented here.

206 The involvement of residues Q9, L10, I13, N14, T15, A16, T18, L19, V20, M21, V41 and
207 Q44 at the IBS has been verified by mutational analysis of the QAE1 isoform HPLC12 from
208 *M. americanus* [2,6,13]. Most of these residues are very well conserved in both QAE and SP
209 proteins, with some variation at Q9 (V in QAE2), I13 (sometimes M in SPs), L19 (V in
210 QAE2s and mostly P in SPs), V20 (G in QAE2s and mostly A in SPs), M21 (one exception),
211 V41 (sometimes an I in QAEs). Recently an inactive QAE2 protein (nfeAFP11) was
212 conferred active similar to an active QAE1 variant through triple or quadruple mutations
213 (V9Q/V19L/G20V and V9Q/V19L/G20V/I41V), underlying the importance of these residues
214 [10].

215 The most consistent differences between QAE1s and SPs are L19, which in SPs is mostly P,
216 and V20, which in SPs is mostly an A. These two residues were shown to be important for
217 the differences between QAE1 and SP in an investigation by Granham et al. [11] who
218 produced the P19L/A20V variant of a SP isoform (nfeAFP6) from *Z. elongatus* improving its
219 ability to slow down the ice crystal growth 30-fold, however without conferring the ability to
220 arrest ice growth completely. Clearly these residues, while important, do not present the full
221 story, as for example the QAE1 isoform AB1 from *Austrolycithys brachycephalus* is active
222 (1.27 °C at 2.9mM) and contains P19/A20 [4] like the SP isoforms generally do. Another
223 QAE1, AM1 from *Anarhichas minor*, has P19/I20 at this position. P19/A20 is also not fully
224 conserved in SPs: the Uniprot sequence associated with PDB 1OPS has P19/V20, HPLC1
225 from *M. americanus* has P19/V20, nfeAFP1, 3 and 4 have L19/A20. All QAE2s have
226 V19/G20.

227 We note that the nature of the residue at position 37 (I in most QAEs and M in many SPs) is
228 correlated with position 19. AB1, the fully active QAE1 protein mentioned above, which
229 unusually has a P at position 19 also has a M at position 37. Am1 that contains P19 has I37
230 though, but the activity of Am1 is unknown. As exemplified by the superposition of the
231 ZvAFP13 and ZvAFP6 structures (Figure 2D), L19 and I37 are within contact distance ($3.8 \text{ \AA}$
232 from L CD2 to I CG2) and it maybe that I at this position is necessary to stabilize the V
233 position. Furthermore if residue 37 were a M it could be expected to destabilize V19, as it
234 would result in contacts as short as $2.5 \text{ \AA}$, if it were in the same position as in the ZvAFP6
235 structure. Thus it could be that an I at position 37 is necessary to fully reap the benefits of L
236 at position 19.

237 K61 is also a residue that has been discussed in the literature. Mutation of K61 to I affects ice
238 growth inhibition activity and the residue is thought to position N14 correctly, while R47 and
239 D58 form a salt bridge stabilizing the loop on which K61 resides [13]. Since SP isoforms
240 usually lack R47 and/or D58 these residues could play a role in the difference from QAEs as
241 suggested in [11]. However we observe that in both molecules of ZvAFP6 and in PDB ID
242 1OPS, K61 and N14 are positioned as in ZvAFP13 and other QAEs even though the SPs lack
243 the salt bridge forming residues.

244 *Dimerization of ZvAFP6:* The most interesting feature in the ZvAFP6 structure is that the
245 protein forms non-covalent dimers in the crystal as detected by analysis with the PISA server
246 [18]. The interface in the dimers covers an area of approximately $500 \text{ \AA}^2$ solvent accessible
247 surface per monomer (compared to about $3500 \text{ \AA}^2$ of total accessible surface for each
248 monomer). The dimer is formed by protein molecules related crystallographically by a two
249 fold axis. The observation of dimers formed by two separate polypeptide chains is to our
250 knowledge unprecedented for type III AFPs, and according to the PISA server prediction the
251 interaction observed in the crystal is strong enough to be of biological relevance in solution.

252 Gel filtration and native PAGE analysis further supported the formation of dimers in solution
253 for ZvAFP6. Since these techniques are affected by the molecular shape, the shape of the
254 ZvAFP6 dimer observed in the crystal was compared to the crystal structure of bovine
255 ribonuclease A (PDB code 5RSA). Both molecules had slightly elongated shapes.
256 Ribonuclease A has dimensions of about 38 Å in the longest dimension and 20-25 Å in the
257 shortest dimensions (excluding a small 2 residues N-terminal protrusion). The dimer of
258 ZvAFP6 formed in the crystal has also a longer dimension of about 38-39 Å and shorter
259 dimensions around 23-25 Å. Thus it seems reasonable to assume that the results of gel
260 filtration and the native PAGE are reliable.

261 It is interesting to note that ZvAFP13 is crystallized under similar high sulphate conditions,
262 but does not show dimerization in the crystals; unfortunately, determination of the MW in
263 solution was not conclusive. Two of the residues discussed up to now and which tend to be
264 different in SPs and QAEs, P19 and M37, are very important in dimerization (Figure 2C).
265 F34, fully conserved among the SPs and not found in any of the QAEs, seems also to be
266 essential in forming the interface (Figure 2C).

267 In the only other available SP crystal structure (PDB ID 1OPS), dimers are not formed. This
268 is hard to rationalize in terms of the few residues that are different between ZvAFP6 and
269 1OPS at the interface, but may be due to differences in the crystallization conditions for
270 1OPS, perhaps destabilizing dimerization.

271 Dimers as observed in the ZvAFP6 crystals could affect ice binding (either favourably or
272 unfavourably), since when the IBS in one of the monomers is bound, the N- and C-terminal
273 tail of the other monomer in the dimer as well as loop 26-35 would protrude towards the ice
274 face. While the biological relevance of dimerization is at this moment in time highly
275 speculative, the demonstration of dimers both in the crystal and in solution for the SP type

276 ZvAFP6 opens a new dimension for research in the structure-function relationships of AFP
277 type III.

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282 **6. References**

- 283 [1] C.N. Albers, M. Bjorn-Mortensen, P.F. Hansen, H. Ramløv, T.F. Sorensen, Purification
284 and structural analysis of a type III antifreeze protein from the European eelpout *Zoarces*
285 *viviparus*, *CryoLett.* 28 (2007) 51-60.
- 286 [2] J. Baardsnes, P.L. Davies, Contribution of hydrophobic residues to ice binding by fish
287 type III antifreeze protein. *Biochim. Biophys. Acta* 1601 (2002) 49-54.
- 288 [3] V.B. Chen, B. Arendall III, J.J. Headd, D.A. Keedy, R.M. Immormino, G.J. Kapral, L.W.
289 Murray, J.S. Richardson, D.C. Richardson, MolProbity: all-atom structure validation for
290 macromolecular crystallography, *Acta Cryst. D* 66 (2010) 12-21.
- 291 [4] C.-H.C. Cheng, , A.L. DeVries, Structures of antifreeze peptides from the Antarctic eel
292 pout, *Austrolycithys brachycephalus*, *Biochim. Biophys. Acta* 997 (1989) 55-64.
- 293 [5] C.I. DeLuca, P.L. Davies, Q. Ye, Z. Jia, The effects of steric mutations on the structure of
294 type III antifreeze protein and its interaction with ice, *J. Mol. Biol.* 275 (1998) 515-525.
- 295 [6] C.I. DeLuca, H. Chao, F.D. Sönnichsen, B.D. Sykes, P.L. Davies, Effect of type III
296 antifreeze protein dilution and mutation on the growth inhibition of ice, *Biophys. J.* 71 (1996)
297 2346-2355.
- 298 [7] J.G. Duman, Antifreeze and ice nucleator proteins in terrestrial arthropods, *Annu. Rev.*
299 *Physiol.* 63 (2001) 327-357.

300 [8] P. Emsley, B. Lohkamp, W.G. Scott, K. Cowtan, Features and development of Coot,
301 Acta Cryst. D53 (2010) 486-501.

302 [9] G.L. Fletcher, C.L. Hew, P.L. Davies, Antifreeze proteins of teleost fishes, Annu. Rev.
303 Physiol. 63 (2001) 359-390.

304 [10] C.P. Garnham, Y. Nishimiya, S. Tsuda, P.L. Davies, Engineering a naturally inactive
305 isoform of type III antifreeze protein into one that stop the growth of ice, FEBS Lett. 586
306 (2012) 3876-3881.

307 [11] C.P. Garnham, A. Natarajan, A.J. Middleton, M.J. Kuiper, I. Braslavsky, P.L. Davies,
308 Compound ice-binding site of an antifreeze protein revealed by mutagenesis and fluorescent
309 tagging, Biochemistry 49 (2010) 9063-9071.

310 [12] K.R. Gallagher, K.A. Sharp, Analysis of thermal hysteresis protein hydration using the
311 random network model, Biophys. Chem. 105 (2003) 195-209.

312 [13] S.P. Graether, C.I. DeLuca, J. Baardsnes, G.A. Hill, P.L. Davies, Z. Jia, Quantitative and
313 qualitative analysis of type III antifreeze protein structure and function, J. Biol. Chem. 274
314 (1999) 11842-11847.

315 [14] E.I. Howard, M.P. Blakeley, M. Haertlein, I. Petit-Haertlein, A. Mitschler, S.J. Fischer,
316 A. Cousido-Siah, A.G. Salvay, A. Popov, C. Muller-Dieckmann, T. Petrova, A. Podjarny,
317 Neutron structure of type-III antifreeze protein allows the reconstruction of AFP-ice
318 interface, J. Mol. Recognit. 24 (2011) 724-732.

319 [15] W. Kabsch, Automatic processing of rotation diffraction data from crystals of initially
320 unknown symmetry and cell constants, J. Appl. Cryst. 26 (1993) 795-800.

321 [16] K. Katoh, M.C. Frith, Adding unaligned sequences into an existing alignment using
322 MAFFT and LAST, Bioinformatics 28 (2012) 3144-3146.

323 [17] T.-P. Ko, H. Robinson, Y.-G. Gao, C.-H.G. Cheng, A.L. DeVries, A.H.-J. Wang, The
324 refined crystal structure of an eel pout type III antifreeze protein RD1 at 0.62-Å resolution

325 reveals structural microheterogeneity of protein and salvation, *Biophys. J.* 84 (2003) 1228-
 326 1237.

327 [18] E. Krissinel, K. Henrick, Inference of macromolecular assemblies from crystalline state,
 328 *J. Mol. Biol.* 372 (2007) 774-797.

329 [19] J.-Y. Li, Y.-M. Cui, L.-L. Chen, M. Gu, J. Li, F.-J. Nan, Q.-Z. Ye, Mutations at the S1
 330 sites of methionine aminopeptidases from *Escherichia coli* and *Homo sapiens* reveal the
 331 residues critical for substrate specificity, *J. Biol. Chem.* 279 (2004) 21128-21134.

332 [20] G.N. Murshudov, A.A. Vagin, E.J. Dodson, Refinement of macromolecular structures by
 333 the maximum-likelihood method, *Acta Cryst. D* 53 (1997) 240-255.

334 [21] Y. Nishimiya, R. Sato, M. Takamichi, A. Miura, S. Tsuda, Co-operative effect of the
 335 isoforms of type III antifreeze protein expressed in Notched-fin eelpout, *Zoarces elongatus*
 336 Kner, *FEBS J.* 272 (2005) 482-492.

337 [22] H. Ramlov, Aspects of natural cold tolerance in ectothermic animals, *Human*
 338 *reproduction* 15 (2000) 26-46.

339 [23] H. Ramløv, Measuring antifreeze activity. In Graether, S.P (Ed.), *Biochemistry and*
 340 *Function of Antifreeze Proteins*, Nova Science Publishers Inc., New York, 2010, pp. 7-42.

341 [24] J.A. Raymond, A.L. DeVries, Adsorption inhibition as a mechanism of freezing
 342 resistance in polar fishes, *Proc. Natl. Acad. Sci. U. S. A.* 74 (1977) 2589-2593.

343 [25] A.B. Siemer, K.-Y. Huang, A.E. McDermott, Protein-ice interaction of and antifreeze
 344 protein observed with solid-state NMR, *PNAS* 107 (2010) 17580-17585.

345 [26] N. Smolin, V. Daggett, Formation of ice-like water structure on the surface of an
 346 antifreeze protein, *J. Phys. Chem. B* 112 (2008) 6193-6202.

347 [27] T.F. Sorensen, C.H. Cheng, H. Ramlov, Isolation and some characterization of antifreeze
 348 protein from European eelpout *Zoarces viviparus*, *CryoLett.* 27 (2006) 387-399.

349 [28] A. Vagin, A. Teplyakov, MOLREP: an automated program for molecular replacement, J.
350 Appl. Cryst. 30 (1997) 1022-1025.

351 [29] X. Wang, A.L. DeVries, C.-H.C. Cheng, Antifreeze peptide heterogeneity in an
352 Antarctic eel pout includes an unusually large major variant comprised of two 7 kDa type III
353 AFPs linked in tandem, Biochim. Biophys. Acta 1247, 163-172.

354 [30] P.W. Wilson, Explaining thermal hysteresis by the Kelvin effect, CryoLett. 14 (1993)
355 31-36

356 [31] C. Yang, K.A. Sharp, The mechanism of the type III antifreeze protein action: a
357 computational study, Biophys. Chem. 109 (2004) 137-148.

358 [32] D.S. Yang, W.C. Hon, S. Bubanko, Y. Xue, J. Seetharaman, C.L. Hew, F. Sicheri,
359 Identification of the ice-binding surface on a type III antifreeze protein with a "flatness
360 function" algorithm, Biophys. J. 74 (1998) 2142-2151.

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7. Figures and tables

Table1: Data collection and refinement statistics.

Figure 1: Sequence alignment of type III antifreeze proteins mentioned in the discussion. Asterisks indicate ice binding residues, residues with grey background are the mutations that are mentioned in text, and residues with black background are the ones that are important in dimerization of ZvAFP6.

Figure 2a-d: A) Superimposition of ZvAFP6 (orange), ZvAFP13 (green), RD1 from *Lycodichthys dearborni* (pink) (PDB ID: 1UCS), HPLC12 from *Zoarces americanus* (yellow) (PDB ID: 4MSI), and HPLC3 from *Zoarces americanus* (blue) (PDB ID: 1OPS). B) superimposition of presumed ice binding residues of ZvAFP6 (orange) and ZvAFP13 (green). C) The interface between ZvAFP chain a (orange) and chain b (purple). F34 and M37 are presented as sticks and the ice binding site residues are presented as spheres, with carbon atoms colored as the rest of the monomer, sulfur in yellow, oxygen in red and nitrogen in blue. D) Overlay of residues 19 and 37 from ZvAFP6 (orange) and ZvAFP13 (green).

Figure 3: Native oligomeric state of ZvAFP6 in solution. A) Gel filtration trace, with Ribonuclease A in black (13.7 KDa) and ZvAFP6 in grey. The major peak corresponds to a size of circa 11 kDa Inset: SDS-PAGE of loaded ZvAFP6 sample (S) and selected fractions (F1-F3, 1 mL fractions between 12-15 mL elution volume). M are the MW markers (97 to 14 kDa)) B: Native PAGE of ZvAFP6 and Ribonuclease A.

Table1: Data collection and refinement statistics.

Protein	ZvAFP13	ZvAFP6
PDB code	4UR4	4UR6
Space group	P2 ₁ 2 ₁ 2 ₁	C222 ₁
Unit cell dimensions (a, b, c in Å)	32.26; 39.69; 44.22	75.57; 108.17; 38.25
Resolution	1.45-29.54 (1.45-1.49)	1.20-30.0 (1.20-1.23)
Completeness (%)	98.0 (92.5)	98.5 (94.9)
Redundancy	4.4 (3.4)	6.2 (5.0)
R _{rim} (%)	4.0 (31.1)	3.1 (42.7)
No. of reflections	45146 (2448)	300182 (17280)
No. of unique reflection	10321 (715)	48601 (3431)
I/ σ _I	24.2 (4.7)	25.2 (5.2)
R _{work}	0.134	0.179
R _{free}	0.176	0.195
Rmsd bonds (Å)	0.015	0.018
Rmsd angles (°)	1.864	1.819
Modelled solvent and other components	90 water molecules	205 water molecules 2 sulphate ions
Residues in alternate conformation	Q2, Q9, L10, E25, T28, M30, P38, L40, S42, M43, L55 and Y63	L10(A), I13(B), S30(A/B), M37(A/B), Q39(A), L55(A/B), D58(A).
Ramachandran plot		

(Molprobit)		
Residues in favored regions (%)	98.4	100.0
Residues in allowed regions (%)	100.0	100.0
Outliers	None	None

Figure 1

	1	20	40	60
HPLC1 (SP) (P19608)	-SQSVVATQLIPMNTALTPVMMEGKVTNP			
1OPS (SP) (1OPS_A)	-SQSVVATQLIPMNTALTPAMMEGKVTNP			
nfeAFP6 (SP) (BAD95781)	-GESVVATQLIPINTALTTPAMMEGKVTNP			
ZvAFP6 (SP) (KC622345)	-GESVVATQLIPINTALTTPAMMAGKVTNP			
Ab1 (QAE1) (P12100)	-TKSVVASQLIPINTALTTPAMMKAKEVSP			
ZvAFP13 (QAE1) (ABN42205)	NQASVVANQLIPINTALTLMVMMRAEVVTP			
HPLC12 (QAE1) (P19614)	NQASVVANQLIPINTALTLMVMMRSEVVT			
nfeAFP11 (QAE2) (BAD95786)	NQESVVAQLIPINTALTVMGMMTRRVVSP			
Am1 (QAE1) (ABA41378)	HQAIVANQLIPINTALTPIMTKAQVVTP			
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Figure 2

Figure 3