Many marine fishes produce proteinaceous ‘antifreezes’ (DeVries, 1983; Davies and Hew, 1990; DeVries and Cheng, 1992; Duman et al. 1993). These proteins have been classified as antifreeze glycoprotein (AFGP) or antifreeze protein (AFP) types I, II or III depending on their very different chemical properties and tertiary structures (Davies and Hew, 1990). Type I AFP is an α-helical peptide with a relative molecular mass of 3300 (Sicheri and Yang, 1995). Type II AFP (Mr 14 000) is the largest of the three AFPs. It has a high cysteine content and shows homology to the carbohydrate-binding domain of C-type lectins (Ewart and Fletcher, 1990; Sönnichsen et al. 1995). Type III AFP (Mr 7000) has a unique β-sheet structure and shows no homology to proteins in the current database (Sönnichsen et al. 1993).

A fundamental property of these proteins is the ability to depress the freezing point of their solution (by at least 1 °C) without affecting the melting point (DeVries, 1983; Davies and Hew, 1990; DeVries and Cheng, 1992; Duman et al. 1993). This activity, which is also known as thermal hysteresis, is caused by the direct interaction of the AFP or AFGP with seed ice crystals (DeVries, 1983; Davies and Hew, 1990; Knight et al. 1991; DeVries and Cheng, 1992; Duman et al. 1993). In nature, the presence of AFPs allows fish from polar and north temperate regions to survive at temperatures below the freezing point of sea water (DeVries, 1983; Fletcher et al. 1986; Davies and Hew, 1990; DeVries and Cheng, 1992; Duman et al. 1993). Similarly, antifreeze activity is thought to aid in the survival of some overwintering insects (Duman et al. 1991, 1993). In these systems, exposure to temperatures that allow freezing results in the death of the organism (DeVries, 1983; Duman, 1984; Duman et al. 1991, 1993).

In contrast, certain species of insects, centipedes, intertidal molluscs and plants that produce AFPs can survive freeze–thawing (Knight and Duman, 1986; Duman et al. 1991, 1993; Urrutia et al. 1992; Duman and Olsen, 1993). Knight and Duman (1986) have proposed that the potent capacity of AFPs to inhibit recrystallization of ice could help protect these freeze-tolerant organisms during long-term exposure to subfreezing temperatures and recurrent bouts of freeze–thawing during winter. Recrystallization is a spontaneous process by which there is an increase in the average size of ice crystals and a decrease in the number of ice crystals. As explained by Knight and colleagues, these changes occur because they lead to a reduction in the overall interfacial surface area and free energy of the system (Knight and Duman, 1986; Knight et al. 1984, 1988, 1995). Although thermodynamically favorable, recrystallization can only occur as frozen samples are warmed to temperatures at which there is sufficient kinetic energy for the migration of water from one ice crystal to another. The process is, therefore, very rapid at high subzero temperatures, especially just below the melting point. As with thermal hysteresis, the interaction of AFPs with...
ice also appears to be necessary for the inhibition of recrystallization (Knight and Duman, 1986; Knight et al. 1984, 1988, 1991, 1995).

The purpose of the current study was to test further the hypothesis that inhibition of recrystallization could aid in survival of cells during freeze–thawing. In an earlier study, Carpenter and Hansen (1992) developed an in vitro model system, employing red blood cells (RBCs) and hydroxyethyl starch as a cryoprotectant, in which warming at suboptimal rates led to massive ice recrystallization and resultant destruction of the cells. With this system, they documented that type I AFP could enhance survival of RBCs by inhibiting ice recrystallization. This protection was maximum at a protein concentration of 62 µg ml⁻¹ and most evident at the lowest warming rate tested. In contrast, increasing the protein concentration to 1.54 mg ml⁻¹ fostered additional damage to the cells because of preferential, destructive growth of ice around the cells during warming. They suggested that the delicate balance of cell protection and damage hinges on the degree of this damaging ice growth and the beneficial effects of inhibition of ice recrystallization. They also proposed that similar effects on cell survival should be obtainable with other AFPs. To test this hypothesis, we have compared for the first time the effects of the three different AFP structural types (I, II and III) on RBC cryoprotection. Furthermore, we have investigated the effects of several type III ‘antifreeze activity’ mutants on RBC cryoprotection, inhibition of ice recrystallization and thermal hysteresis, and have obtained the first documentation of a direct correlation between these effects. Taken together, our results demonstrate that it is the capacity of a protein to influence ice crystal growth, and not the structural composition of that protein, that is dominant in determining RBC survival during freeze–thawing in hydroxyethyl starch.

Materials and methods

Purification and mutagenesis of antifreeze proteins

Type I AFP (recombinant, but identical to Type I AFP from winter flounder, Pseudopleuronectes americanus) was a generous gift from J. E. Villafranca (Agouron Pharmaceuticals, La Jolla, CA, USA). Type II AFP was isolated from the sea raven Hemitripterus americanus. A sample of the fish serum was fractionated twice on a Sephadex G-75 gel permeation column (Ng et al. 1986). Final purification was performed on a Vydac C18 reversed-phase HPLC column using a linear gradient of acetonitrile (from 30 % to 50 %) in 0.1 % trifluoroacetic acid. Type III AFPs were produced in Escherichia coli. Construction of the synthetic gene, and expression and isolation of the proteins have been described previously (Chao et al. 1993).

Type III AFP point mutations were produced according to the method of Kunkel et al. (1987). Mutants P29A and P33A differed from the wild-type AFP (M1.1) by the replacement of a proline by an alanine residue in positions 29 and 33, respectively (Chao et al. 1993). The point mutation Y63W was produced using the mutagenic oligonucleotide 5'-pGGTTAAGGTTGGCTGTTAAG-3', which replaced the tyrosine at position 63 with a tryptophan (Chao et al. 1993).

All proteins were pure as judged by homogeneity on HPLC, confirmed in some cases by N-terminal sequencing and mass spectrometry. Protein was quantified by weighing and spectrophotometric determination of absorbance at 280 nm.

Antifreeze activity measurements

Thermal hysteresis is defined as the difference between the melting and freezing point of the solution. The temperature difference was measured using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) as described by Chakrabartty and Hew (1991).

Preparation, cryopreservation and cryomicroscopy of red blood cells

Human blood was collected from healthy volunteers into heparinized tubes. The blood was processed to obtain red cells, and the red cells were cryopreserved in hydroxyethyl starch, as described by Carpenter and Hansen (1992). Samples were thawed in air at room temperature, which leads to very slow warming and, in the absence of AFP, the opportunity for extensive ice recrystallization. The cryomicroscopy protocol was that described by Carpenter and Hansen (1992) and mimicked the conditions (i.e. cooling and warming rates) arising during the red cell cryopreservation experiments. Cell suspensions were cooled at 600 °C min⁻¹ to −133 °C. The warming rates were 40 °C min⁻¹ between −133 and −50 °C, 25 °C min⁻¹ between −50 and −10 °C and 2.5 °C min⁻¹ during the remainder of the thawing cycle.

Results and discussion

Influence of AFP types I, II and III on red cell cryopreservation

Initially, we compared the capacity of AFP types I, II and III to alter the survival of red blood cells that were cryopreserved in hydroxyethyl starch solutions (Fig. 1). The cell samples were warmed slowly to foster ice recrystallization and resultant damage to the cells (Carpenter and Hansen, 1992). In the absence of AFP, the degree of hemolysis was about 40 %. At relatively low concentrations of type I AFP, hemolysis is maximally reduced to about 10 %. This protective effect was attenuated at higher AFP concentrations, and at the highest concentration tested (1.54 mg ml⁻¹) damage was greater than in the absence of AFP. These results are very similar to those obtained previously by Carpenter and Hansen (1992).

With type II AFP, the degree of hemolysis was also reduced to about 10 % (Fig. 1). However, across the concentration range tested, there was only a small increase in hemolysis at higher concentrations of the protein. Hemolysis in the presence of type III AFP at concentrations lower than 0.5 mg ml⁻¹ was also reduced to about 10 %. At the highest concentration tested (1.54 mg ml⁻¹), the results are intermediate between those for the other two proteins. There is an increase in damage relative to that obtained with lower concentrations of the protein, but the damage does not exceed the level noted in control samples.

Although the AFP types appear to have different efficiencies
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(I>III>II) in reducing and promoting hemolysis on a mg ml\(^{-1}\) concentration scale, on a molar basis they are very similar. By interpolation, the hemolysis minima for AFP types I (\(M_r\) 3300), II (\(M_r\) 14 050) and III (\(M_r\) 7031) occur at 32.1 mg ml\(^{-1}\), 27.4 mg ml\(^{-1}\) and 28.8 mg ml\(^{-1}\), respectively. At equivalent molar concentrations, it is quite likely that AFP types II and III could promote hemolysis to the same extent as the 1.54 mg ml\(^{-1}\) type I solution. Fig. 1 indicates that trend, but unfortunately these two AFPs could not be assayed at high enough concentrations because of their limited solubility in hydroxyethyl starch.

Influence of type III mutants on hemolysis of cryopreserved red cells

In the course of earlier studies analyzing the structure of type III AFP, three single amino acid substitution mutants (Y63W, P33A and P29A) were made. When these were assayed for thermal hysteresis activity, Y63W was not significantly less active than the starting recombinant type III AFP, which is designated M1.1 (Fig. 2). However, P33A and P29A were approximately 80% and 50% as active as M1.1, respectively, over the concentration range tested.

Since the effects of AFPs on red cell survival appear to be due to modulation of ice crystal growth by the protein, we hypothesized that the relative effects of the mutants on red cell survival should parallel their relative thermal hysteresis activities. The data in Fig. 3 support this hypothesis. The recombinant type III AFP (M1.1) and the mutant Y63W had almost identical effects on reducing damage to cryopreserved red cells. The curves for hemolysis versus protein concentration were very similar, and the maximum protection was the same. Also, there was a parallel increase in damage with both proteins as the concentration was increased above 0.34 mg ml\(^{-1}\).

The mutant P33A was slightly less effective at reducing hemolysis at concentrations lower than 0.34 mg ml\(^{-1}\), but provided essentially equivalent protection at 0.34 mg ml\(^{-1}\) concentration. However, this protein caused only a slight increase in damage to RBCs at a concentration of 1.54 mg ml\(^{-1}\). The different effects of P33A versus M1.1 on red cells survival were qualitatively similar to those of type III versus type II (Fig. 1), suggesting that an increased molar concentration of P33A could compensate for its reduced activity.

Finally, the mutant P29A, which had the least thermal hysteresis activity, provided the smallest degree of protection to the red cells. Even at concentrations above 1 mg ml\(^{-1}\), greater than 20% hemolysis occurred and an asymptote in the curve was not reached, i.e. maximum reduction of hemolysis was not realized. The solubility limits of the protein in the hydroxyethyl starch solution prohibited testing at higher concentrations.

Influence of type III protein and type III mutants on ice recrystallization

We have previously shown that the effects of type I AFP on...
The Student’s Data for the three mutants were compared with that for M1.1 using standard deviations shown by the vertical bars. Each point is the mean for three separate samples (Fig. 4). In contrast, the presence of 62\(\mu\)g ml\(^{-1}\) type III AFP (or any of the mutant forms), visible ice crystals often exceeded that of the cells, suggesting that both extracellular and intracellular water contributed to ice crystal growth. These results are also consistent with the earlier observations made with type I AFP (Carpenter and Hansen, 1992).

Cryomicroscopic analysis of the samples containing the type III mutants provided similar results (data not shown). First, with Y63W the effects of the protein on ice crystal growth during warming at concentrations of 62\(\mu\)g ml\(^{-1}\) and 1.54\(\mu\)g ml\(^{-1}\) were virtually identical to those shown for the natural type III (M1.1) protein in Fig. 4. This observation correlated well with the almost identical effects of the two proteins on the survival of red cells during freeze–thawing (Fig. 3).

With P33A at 62\(\mu\)g ml\(^{-1}\), the inhibition of recrystallization was slightly less than that noted for type III (M1.1) and Y63W (data not shown), which correlated well with the relative capacities of the proteins to enhance red cell survival at 62\(\mu\)g ml\(^{-1}\) (Fig. 3). The ice crystals observed with P33A at \(-2^\circ\)C were about two times larger than those seen with type III (M1.1) and Y63W. Cryomicroscopic results were similar for 1.54\(\mu\)g ml\(^{-1}\) P33A, in that there were numerous cells around which there was preferential growth of ice (data not shown). However, in this case, the ice growth did not seem to correlate with increased cellular damage, because 1.54\(\mu\)g ml\(^{-1}\) P33A was more effective than 62\(\mu\)g ml\(^{-1}\) protein at reducing hemolysis of frozen–thawed red cells (Fig. 3). The cause of this observation is not known.

With the P29A mutant at 62\(\mu\)g ml\(^{-1}\), the degree of recrystallization inhibition was much less than that noted with Y63W and type III (M1.1). At \(-2^\circ\)C, the ice crystals appeared to be about 4–8 times larger in diameter than those observed at this temperature for the other two proteins. Increasing the P29A concentration to 1.1 \(\mu\)g ml\(^{-1}\) (the solubility limit under the experimental conditions) led to more effective inhibition of recrystallization, such that ice crystals noted at \(-2^\circ\)C were about 2–3 times larger than those noted with the other proteins. Preferential ice growth around the cells did not occur at this concentration of P29A. These findings correlated well with the observation that a concentration of 1.1 \(\mu\)g ml\(^{-1}\) P29A provided the maximum protection to red cells during freeze–thawing (Fig. 3).

These results with type III (M1.1), Y63W and P29A proteins are all fully consistent with our previous results with type I antifreeze protein (Carpenter and Hansen, 1992) and suggest that inhibition of ice recrystallization is sufficient to explain the protection by antifreeze proteins in our experimental protocol. And for these systems, preferential growth of ice around the cells appears to foster cellular damage, even when...
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Recrystallization of extracellular ice is greatly inhibited. The results with the P33A mutant are fully consistent with the former conclusion, but not the latter. With 1.54 mg ml\(^{-1}\) P33A, preferential ice growth around the cells is observed with cryomicroscopy, yet there is no increase in hemolysis in cells during cryopreservation and thawing (Fig. 3). Clearly the situation is more complicated than a simple direct relationship between ice crystal behavior observed by cryomicroscopy and recovery of red cells frozen and thawed in test tubes. However, we can conclude that, in the absence of preferential ice growth around cells, the inhibition of recrystallization during warming does increase cell recovery relative to that noted in control samples that do not contain antifreeze protein.

In summary, it appears that the relative thermal hysteresis activity of the type III protein and the mutants (Fig. 2) correlates directly with their capacity to inhibit ice recrystallization and to confer protection to red cells during freeze–thawing with hydroxyethyl starch (Fig. 3). Knight et al. (1995) have also noted a direct correlation between thermal hysteresis activity and ice recrystallization inhibition in a series of type I antifreeze peptide analogs. Since both activities depend on the same mechanism – namely interaction of the protein with ice crystals – the correlation is not surprising.

In conclusion, our results support the hypothesis set forth by Knight and Duman (1986) that inhibition of ice recrystallization by antifreeze proteins can help cells survive the rigors of freeze–thawing. It is important to stress that our experimental system was purposely chosen because it is known to allow a high degree of ice recrystallization and resultant cellular damage. Clearly, the cryoprotectant type (hydroxyethyl starch) and cooling rates (i.e. hundreds of degrees per minute) needed for these effects to be operative in our system would not be found in nature. However, even at modest cooling rates (e.g. 1–10 °C min\(^{-1}\)), small thermodynamically unfavorable ice crystals could be formed. Similarly, freezing after a high degree of supercooling, which is often noted in terrestrial invertebrates (Duman et al. 1991), can result in the formation of very small ice crystals. In both cases, there would be the propensity for recrystallization during subsequent warming or sustained exposure to high subzero temperatures; albeit the instability of

Fig. 4. Effects of type III AFP on ice recrystallization in the presence of human red blood cells. AFP was tested at concentrations of 0 (top row), 0.062 mg ml\(^{-1}\) (middle row) and 1.54 mg ml\(^{-1}\) (bottom row) after warming to \(-11 °C\) (left column) or \(-2 °C\) (right column). Scale bar, 50 μm.
the ice crystals would probably not be as extreme as in our model system. Whether, in general, this transition would be damaging to an organism and could be attenuated by an antifreeze protein needs to be established.

A recent study by Tursman and Duman (1995) has provided the first documentation of such effects under conditions relevant to nature. They clearly demonstrated that damage to cells of the freeze-tolerant centipede (Lithobius forficatus) during freeze–thawing could, at least in part, be attributed to ice recrystallization. They also found that the presence of AFP (from the beetle Dendroides canadensis) attenuated ice recrystallization and cellular damage. This activity was optimal at relatively low concentrations of AFP (i.e. 20–200 μg ml\(^{-1}\)), which corresponds to levels noted in freeze-tolerant organisms (see Tursman and Duman, 1995) and to the optimum protective levels in our red cell cryopreservation model. Additionally, at a relatively high concentration (i.e. 2.0 mg ml\(^{-1}\)) the protein fostered additional damage to the centipede cells during freeze–thawing.

Finally, Knight et al. (1995) have speculated that the inhibition of ice crystal growth by antifreeze proteins could also be important for preventing damage in systems exposed to temperature gradients. Liquid water inclusions between ice crystals would migrate slowly towards the warm end of a gradient, leading to melting at that end and freezing at the cold side. The resulting mechanical tissue damage should be preventable by antifreeze proteins which, in a process analogous to the inhibition of ice recrystallization, should inhibit ice migration. Because the capacity of antifreeze proteins to inhibit recrystallization appears to be a ubiquitous property, it would not be surprising if future research rigorously established protective roles for this behavior in models simulating exactly conditions encountered in nature.

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