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A D-antifreeze polypeptide displays the same activity as its natural L-enantiomer

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The D- and L-forms of an α-helical antifreeze polypeptide (AFP) have been chemically synthesized. Circular dichroism spectra of the molecules show equal and opposite ellipticities. The D- and the L-enantiomers alone, and a 50:50 mixture of the two, all show identical antifreeze activity, but the enantiomeric forms are predicted to bind to the ice surface with different orientations. It is suggested that symmetry properties of certain ice surfaces permit the asymmetric binding of AFPs, and thus that AFPs are analogous to enzymes that act upon prochiral substrates.

D-Polypeptide; Antifreeze polypeptide; Ice; D-α-Helix; Circular dichroism

1. INTRODUCTION

Fishes inhabiting polar and other ice-laden seas are able survive at temperatures down to the freezing point of seawater, −1.8°C, by secreting antifreeze polypeptides (AFPs) which lower the plasma freezing point [1,2]. Of the several classes of AFPs, the best studied are the alanine-rich α-helical AFPs from the winter flounder, *Pseudopleuronectes americanus*. These AFPs exist as α-helical rods having regularly spaced polar amino acids (e.g. Thr and Asn), arranged along one side of the helix, which are believed [3–7] to bind to ice surfaces. In the presence of AFPs, ice crystals grow as hexagonal bipyramids, rather than the hexagonal plates seen in pure water. Knight et al. [4] have deduced that some AFPs bind to the hexagonal bipyramidal surfaces $\{20\bar{2}1\}$* with specific orientations $<\bar{1}102>$. Recently we have proposed a model that describes in detail how a specific AFP, HPLC-6 from the winter flounder [2] (referred to here as S00), binds to an ice surface by hydrogen bonding of the Thr, Asn and Asp residues [8]. We further suggest that complete inhibition of ice crystal growth is due primarily to cooperative, hydrophobic interactions between AFP molecules on the ice surface [8].

Recently, Milton et al. [9] have described the first synthesis of a D-enzyme, the D-form of HIV-1 protease. As expected, the D-protease is unable to hydrolyze a normal L-peptide substrate, but it does cleave its D-enantiomer. In contrast, we show with this communication that a D-AFP has the same activity as the natural L-AFP, and thus that both enantiomers bind equally well to the same ice surface.

2. MATERIALS AND METHODS

The D- and L-AFPs were synthesized on a MilliGen/Biosearch model 9050 peptide synthesizer, using Fmoc-L- (or D-) amino acid pentafluorophenyl esters and a PAL resin (Millipore), and purified by reverse-phase HPLC as described earlier [3]. Antifreeze activity, or thermal hysteresis (the difference between the freezing and melting temperatures), was measured using a Clifton Technical Physics nanoliter osmometer; circular dichroism measurements were made on an Aviv model 62DS CD spectrometer as previously described [3].

3. RESULTS

The D-analog of the 37-residue winter flounder AFP (S00), DTASDAAAAAALTAANAKAAAELTAA-NAAAAAAATAR, was synthesized and purified as described [3] for the L-enantiomer, except that D-Fmoc amino acid pentafluorophenyl esters were used. D-Threonine, and not D-allothreonine, was used in order to obtain the true enantiomeric D-AFP. The circular dichroism spectrum of D-S00 (Fig. 1) was the exact reflection of that of L-S00, indicating that the D-α-helix had been synthesized and that it was, like the L-AFP, essentially 100% helical [3]. The thermal denaturation curve

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*$\{20\bar{2}1\}$ designates the group of 12 equivalent surfaces of a hexagonal bipyramid, of which $(20\bar{2}1)$ is one specific surface; $<\bar{1}102>$ designates the group of 12 equivalent directions on these surfaces, of which $[\bar{1}102]$ is the direction appropriate to the specific surface $(20\bar{2}1)$. In the following discussion, for simplicity, we will refer to the specific directions (in square brackets) for the specific surface $(20\bar{2}1)$.

Abbreviations: AFP, antifreeze polypeptide; Fmoc, fluorenymethyloxycarbonyl-.

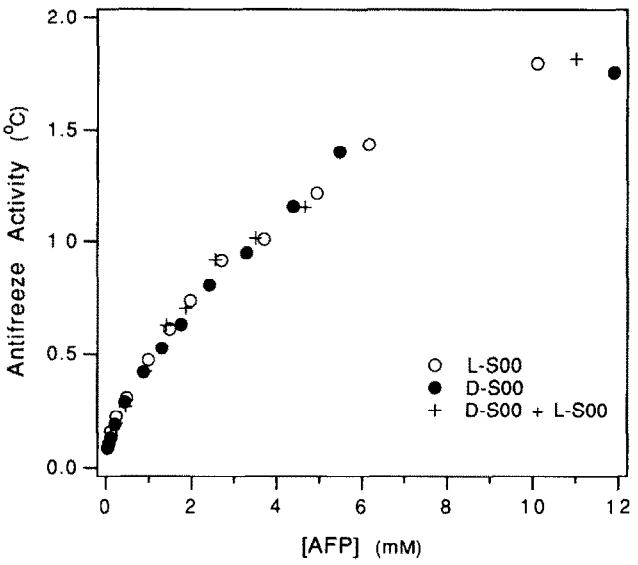

Fig. 1. Circular dichroism spectra of D- and L-AFPs (D-S00 and L-S00). Spectra were run on approximately 0.1 mM solutions at 1.0°C in 0.1 M ammonium bicarbonate (pH 8.5) in a 1-mm cell.

(not shown) also mirrored that of L-S00, with a T_m (temperature at which the fraction of α-helix is 0.5) value of 28°C for both enantiomers.

Plots of antifreeze activity (thermal hysteresis) of D-S00, L-S00 and a 50:50 mixture of the two were, within experimental error, superimposable (Fig. 2), as were plots (cf. [3]) of the rates of ice crystal growth for D- and L-S00 in dilute solution (not shown).

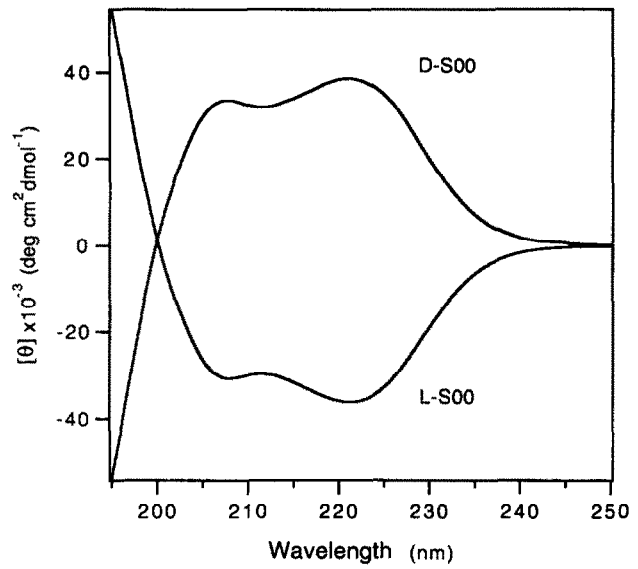

Fig. 2. Dependence of antifreeze activity on AFP concentration. Assays were run in 0.1 M ammonium bicarbonate buffer (pH 8.5). Activity is defined as the difference between the equilibrium melting point of ice and the freezing point.

4. DISCUSSION

We have proposed [8] that the α-helical AFPs, such as S00, inhibit ice crystal growth by a two-step mechanism. In the first step, AFP molecules bind to the 12 equivalent $(20\bar{2}1)$ surfaces of hexagonal bipyramidal ice along the direction $[\bar{1}102]$, as described by Knight et al. [4]. At low concentrations, binding, by hydrogen bonding of Thr hydroxyls and Asn amide side chains to the ice lattice, is reversible, and ice crystals grow slowly as hexagonal bipyramids. In the second step, when the AFP concentration becomes sufficiently high, properly aligned AFP molecules pack together, making efficient van der Waals contact between their hydrophobic sides, leading to much tighter (cooperative) binding to the ice lattice and complete inhibition of ice crystal growth.

In our original binding model [8], we suggested that AFP molecules might either completely cover the ice surface (Fig. 3B), which would require perfect alignment of all AFP molecules, or, more likely, might bind in patches of aligned molecules (Fig. 3C). To try to distinguish between these two possibilities, we synthesized and measured the activity of the D-enantiomer of a well-studied α-helical AFP, S00. We reasoned that D-S00 should bind equally well to the $(20\bar{2}1)$ ice surfaces, which is achiral with mirror symmetry, but in the direction $[0\bar{1}12]$, the reflection of $[\bar{1}102]$ (Fig. 3A). As seen in Fig. 2, D-S00 does have activity indistinguishable from the natural L-S00.

However, if total coverage of the ice surface by AFP molecules (Fig. 3B) is required for full antifreeze activity, a mixture of D- and L-S00 should have lower activity, since it would be impossible to achieve perfect coverage of the surface if the molecules have to align in two directions. Fig. 2 shows that a 50:50 mixture of D- and L-S00 is as active as either D-S00 or L-S00 alone, thus making the total coverage model (Fig. 3B) untenable. We therefore propose that D- and L-S00 bind as outlined in Fig. 4: D- and L-S00 first bind randomly and reversibly to $(20\bar{2}1)$, but specifically along $[0\bar{1}12]$ and $[\bar{1}102]$, respectively; at higher concentrations a network of aligned patches leads to maximal antifreeze activity. Individual AFP molecules act as nuclei for the ensuing patches. The size and density of these putative patches is unknown.

These results demonstrate for the first time that a biologically active L-polypeptide and its D-enantiomer can act on (bind to) the same substrate, in this case, ice. By contrast the recently synthesized D-HIV-1 protease acts only on a D-peptide substrate [9]. Ice is achiral, and thus the AFP is analogous to certain enzymes which act upon achiral substrates, for example, aconitase, which dehydrates citric acid [10]. Although citric acid is not chiral, it is prochiral, having two methylene groups that become non-equivalent on binding to the enzyme. Likewise, the $(20\bar{2}1)$ surface of ice is, in a sense, prochiral, since the constellation of ice lattice water molecules that

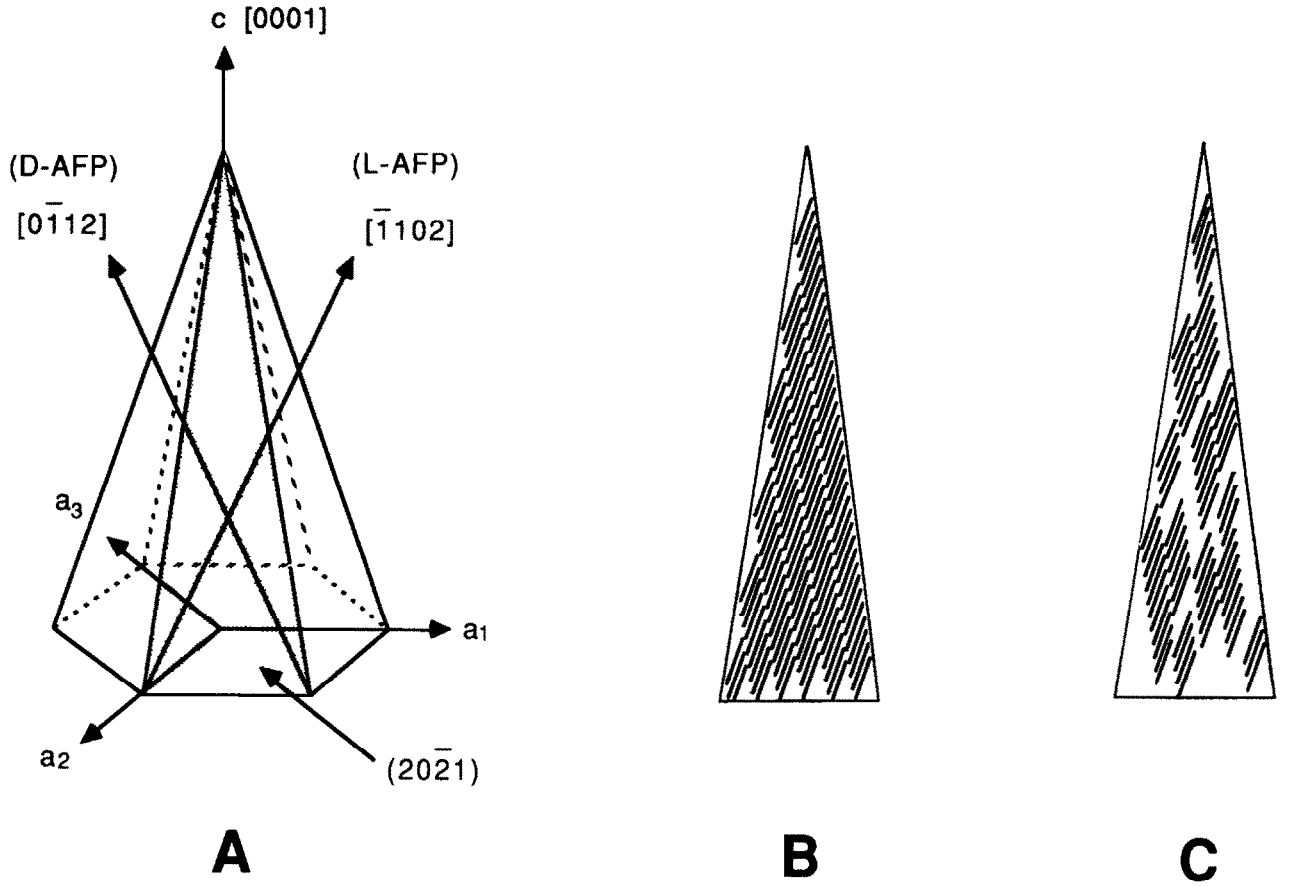

Fig. 3. Mode of binding of AFPs to an ice surface. (A) Diagram of one half of a hexagonal bipyramidal crystal of ice, showing the proposed directions [0$\bar{1}$12] and [$\bar{1}$102] of binding of D-S00 and L-S00, respectively, to the specific surface (20$\bar{2}$1) (shaded area). (B) Hypothetical packing pattern of AFPs assuming that all molecules are in perfect register. (C) Hypothetical 'patchy' packing pattern assuming that patches grow from nucleating AFP molecules which are not in perfect register (cf. Fig. 4).

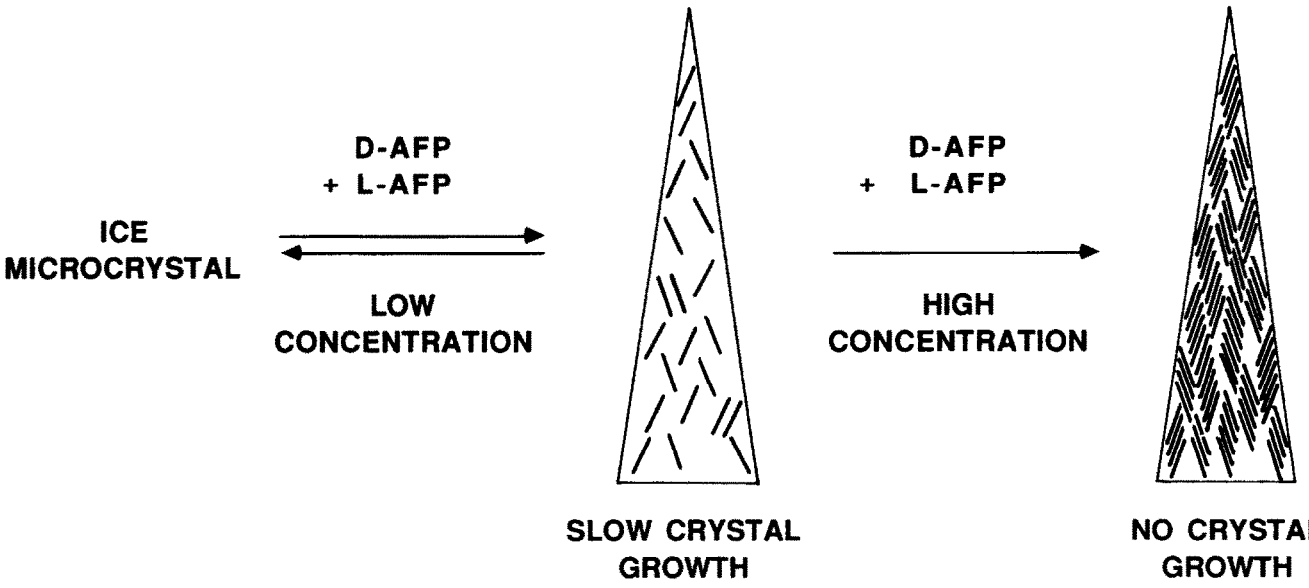

Fig. 4. Proposed mode of binding and packing of a mixture of D- and L-AFPs to the (20$\bar{2}$1) surface of ice.

constitutes a site for binding an L-AFP in the direction [$\bar{1}$102] has a mirror image along [0$\bar{1}$12] for binding to a D-AFP.

We further suggest that AFPs will bind effectively to an ice surface only if (i) the polar side chains of the AFP hydrogen bond efficiently with the ice lattice, and (ii) the number of orientations possible on the surface is highly restricted, so as to maximize the opportunity for orderly interpeptide interactions, or packing. For the (20$\bar{2}$1) surface, there is only one possible orientation (according to our model), but for the basal plane (0001), there are six equivalent orientations. The inability of AFPs to bind to the basal plane in an orderly way may explain why ice crystals prefer to grow along the c-axis [0001], i.e. normal to the basal plane.

Implicit in our model is the assumption that D-S00 binds in the direction [0$\bar{1}$12]. This has yet to be proved, but it should be possible to demonstrate it directly using the ice crystal etching technique described by Knight et al. [4]. Such experiments are planned.

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