A β-Helical Antifreeze Protein Isoform with Increased Activity

STRUCTURAL AND FUNCTIONAL INSIGHTS

The insect spruce budworm (Choristoneura fumiferana) produces a number of isoforms of its highly active antifreeze protein (CfAFP). Although most of the CfAFP isoforms are in the 9-kDa range, isoforms containing a 30- or 31-amino acid insertion have also been identified. Here we describe the functional and structural analysis of a selected long isoform, CfAFP-501. X-ray crystal structure determination reveals that the 31-amino acid insertion found in CfAFP-501 forms two additional loops within its highly regular β-helical structure. This effectively extends the area of the two-dimensional Thr array and ice-binding surface of the protein. The larger isoform has 3 times the thermal hysteresis activity of the 9-kDa CfAFP-337. As well, a deletion of the 31-amino acid insertion within CfAFP-501 to form CfAFP-501Δ-2-loop, results in a protein with reduced activity similar to the shorter CfAFP isoforms. Thus, the enhanced antifreeze activity of CfAFP-501 is directly correlated to the length of its β-helical structure and hence the size of its ice-binding face.

Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) are present in many organisms that must survive subzero temperatures. These proteins bind to seed ice crystals and inhibit their growth through an adsorption-inhibition mechanism. The structures of these proteins have proven to be remarkably varied, yet they perform the same freeze-protection function.

In all documented examples to date, an AFP-protected organism produces only one or two types of antifreeze, but they are typically comprised of multiple, independently active isoforms. The AFP-producing fishes of the Arctic and Antarctic regions are a good example of this. Among various isoforms of CfAFP, one 9-kDa isoform has been characterized in great detail (namely 337 or CfAFP-337) and has been the focus of recombinant expression and structure determination by NMR and x-ray crystallography. These investigations showed that CfAFP is a left-handed β-helix with a two-dimensional Thr array on one face of the protein that forms the ice-binding site. The antifreeze activity of another 9-kDa isoform (339) has also been assayed and found to be equivalent to that of isoform 337.

The long isoform 501 of CfAFP is only 66% identical to the characterized 337 isoform. Using CfAFP-501 as a representative of the longer isoforms, we were interested in determining whether the 31-amino acid insertion correlates with higher activity and, if so, what is the molecular and structural basis for the enhancement. To this end, we have expressed and measured the activity of the protein and determined its x-ray crystallographic structure. In addition, deletion of the 31-amino acid insertion (representing two turns of the β-helix) in a so-called “looptectomy” procedure has been carried out to further probe the activity-structure relationship.

EXPERIMENTAL PROCEDURES

CfAFP-501 and CfAFP-501Δ-2 Loop Expression and Purification—The cDNA of CfAFP-501 was cloned into the pET20(b) vector (Novagen) for recombinant expression in Escherichia coli. Refolded protein was purified using fast protein liquid chromatography and high pressure liquid chromatography column chromatography as reported for CfAFP-337 (14) with the exception that the majority of the CfAFP-501 protein eluted in a single, well folded peak from both the fast protein liquid chromatography and high pressure liquid chromatography columns without the splitting into incompletely folded and well folded peaks seen with CfAFP-337. To excise the 31-amino acid insertion found in the long isoform CfAFP-501 and to produce CfAFP-501Δ-2 loop, the pET20(b) vector
containing the cDNA for CfAFP-501 was cleaved with the restriction enzyme ClaI whose natural cleavage sites were found on either side of the cDNA encoding the 31-amino acid segment. Religation of the remaining vector backbone and cDNA produced an expression vector for the CfAFP-501-3-2 loop. Protein was reconstituted from the cDNA containing pET20(b) vector in BL21(DE3) E. coli cells. Again, the protein was present in inclusion bodies, and the refolding, expression, and purification were performed as in the case of CfAFP-337 (14) and CfAFP-501 described above.

CfAFP-501 Crystallization and Data Collection—Crystals of CfAFP-501 were grown at room temperature using the hanging drop vapor diffusion method. The protein was crystallized using a solution of 18% polyethylene glycol-4000 and 20% isopropanol in sodium citrate buffer (100 mM, pH 4.8–5.6) producing thin plate-like crystals. These crystallization conditions are different in both temperature and solution components used for CfAFP-337. Due to structural microheterogeneity found in recombinantly produced CfAFP-337, higher temperatures were required to reduce this heterogeneity and produce crystals of CfAFP-501. This conformational microheterogeneity is not seen in the preparation of CfAFP-501.

Due to poor diffraction and high mosaicity of the thin crystals, diffraction quality of most crystals was unsatisfactory. After screening many CfAFP-501 crystals (between 50–75%) under cryogenic conditions, a data set was eventually collected to a 2.45-Å resolution using a copper Rigaku rotating anode x-ray generator and a Mar Research imaging plate. The diffraction images were processed using the HKL program suite (18), and data statistics are summarized in Table I.

CfAFP-501 Structure Determination and Refinement—The positions of four molecules in the asymmetric unit of the CfAFP-501 crystals were determined by molecular replacement using the program EPMR (version 3.1) (19). A theoretical search model was used for molecular replacement and was composed of the crystal structure of CfAFP-337 (15) (PDB code 1L0S) with two extra β-helical loops manually modeled into the structure using Sybyl (Tripos, St. Louis, MO) and Turbo-Frodo (20). These two loops were residues 29–59 of CfAFP-337 inserted between residues 28 and 29. Amino acids in the model were mutated in silico to those of CfAFP-501 without additional energy minimization. Structure refinement was performed using the CNS program package (21) with gradual reduction of non-crystallographic symmetry restraints. Ribbon diagrams in figures were generated using MolScript (22).

Antifreeze Activity Measurements—Measurement of AFP activity was performed according to a previously established routine process (23) in the presence of 0.1 mM ammonium bicarbonate employing a nanoliter osmometer (Clifton Technical Physics).

RESULTS AND DISCUSSION

Overall Structure of CfAFP-501—CfAFP-501 retains the extremely regular left-handed β-helical structure seen with CfAFP-337 (15) (Table I). The β-helix is formed by a series of 15-amino acid turns, which result in an elongated protein with a triangular cross-section (Fig. 1). The 31-amino acid insertion found in the CfAFP-501 sequence forms two extra loops (16 + 15 amino acids) within the central region of the β-helix (Fig. 1), extending the length of the β-helical structure from 31 Å (in CfAFP-337) to 39.6 Å. Although each turn of the CfAFP β-helix is usually 15 amino acids in length, one of the additional loops in CfAFP-501 contains 16 residues. An extra Ser within this repeat unit is accommodated within a corner of the triangular β-helix, not within the putative ice-binding site, and is directed away from the core structure of the protein.

Interior and exterior amino acid positions form stacks of similar residues that contribute to the rigidity of the β-helical structure. One side of the exterior of the protein features two stacks of Thr residues (Fig. 2A), while three interior stacks are composed of hydrophobic residues (Fig. 2B). Interior corner residues are mainly Ser, Thr, and Cys residues producing a hydrogen-bonding network to form the sharp 60° interior turns or corners required for the formation of the triangular cross-section of the protein (Fig. 2C).

By analogy with CfAFP-337, the side of CfAFP-501 containing the repetitive Thr-Xaa-Thr array forms the ice-binding face of the protein. On this face of CfAFP-501, Val residues replace Thr-5 and Thr-52, whereas Thr-37 is substituted with an Ile. These Val and Ile residues have side-chain χ1 torsion angles of 177° and −60°, respectively, whereas the Thr side-chains are at a χ1 torsion angle of −60°. In terms of the definition of χ1 torsion angles for Val, Ile, and Thr residues these values represent geometrically equivalent rotamers. The positioning of the side-chains places all the residues on this face of the protein in the same orientation arrangement enabling the continued formation of the regularly spaced ice-binding platform (Fig. 3).
As in CfAFP-337 (15), this face forms an ice-binding site with extensive surface complementarity to ice, and the addition of two extra loops within CfAFP-501 increases the size of the interfacial surface between the AFP and ice by 34%.

Molecular Replacement Using a Theoretical Model—A theoretical model of CfAFP-501 was used in the molecular replacement stage of CfAFP-501 x-ray structure determination. Because CfAFPs have such a repetitive protein backbone this procedure is a very rational approach to structure determination. Although there has been some success in using theoretical models for molecular replacement, in most cases the probing models have been built from the combination of a number of core structures from homologous proteins, and these models were usually not larger than the starting templates. However, in this case a larger model had to be constructed instead of employing the usual approach of trimming the original structure. The theoretical model was based on the structure of CfAFP-337 (15) with residues 29–59 repeated and incorporated between residues 28 and 29. The amino acid residues were changed to that of CfAFP-501 with no additional energy minimization. The actual resulting root mean square deviation value between the theoretical search model and the final structure of CfAFP-501 is 1.53 Å for all atoms and 0.63 Å for the backbone atoms, respectively. The latter value is also repre-
sentative of the overall backbone structural similarity between CfAFP-501 and 337, even though the amino acid identity between them is only 66%.

Antifreeze Activity of CfAFP-501—To assess the effect on antifreeze activity of the increased length of the β-helical structure and the greater overall area of the putative ice-binding face, thermal hysteresis measurements were taken. This assay evaluates the ability of the AFP to non-colligatively depress the freezing point of a solution by inhibiting the growth of a seed ice crystal. Activity was assayed over a range of concentrations from 0 to 1.2 mg/ml (0—0.1 mmol). CfAFP-501 was considerably more active than CfAFP-337 over all concentration ranges (Fig. 4) with thermal hysteresis values up to 3-fold higher. For example, at a concentration of 0.05 mOsm, CfAFP-337 has a thermal hysteresis value of 1.8 °C, whereas CfAFP-501 has an activity of 4.7 °C.

CfAFP-501—Lopectomy—To determine whether the increased antifreeze activity of CfAFP-501 was due to sequence differences between the two isoforms or due to the increased length of CfAFP-501, a shorter CfAFP-501 was recombinantly produced. This involved the deletion of a 31-amino acid segment from CfAFP-501 at the cDNA level to form the protein CfAFP-501-Δ2 loop in which two loops of the β-helix were removed from the protein.

Antifreeze activity measurements were performed on the shorter CfAFP-501-Δ2 loop resulting in decreased thermal hysteresis values that are similar to those of the 9-kDa 337 isoform (Fig. 4). This result convincingly demonstrates that rather than its sequence differences, the increased size of CfAFP-501 is responsible for its greater activity.

AFP-specific Surface Coverage versus Activity—The results of this study suggest that the size of the ice-binding face of CfAFP is an important determinant of its antifreeze activity. These findings parallel those where a longer isoform of the α-helical fish type I AFP, containing four ice-binding repeats as compared with the usual three, was found to be a more potent antifreeze protein (24). The four-repeat protein had a freezing point depression of 1.0 °C at 10 mg/ml, whereas the three-repeat protein had a thermal hysteresis activity of 0.68 °C at the same concentration. Although in the case of type I AFP there has been no structure determined for the longer isoform, given the regular α-helical structure observed in shorter isoforms (25) it would not be surprising if the longer isoform displayed the same repetitive α-helical folding. By the same token, removal of two ice-binding repeats from the three-repeat isoform left a 15-amino acid peptide that had lost all its thermal hysteresis activity but retained the ability to shape the ice crystal (26).

An additional correlation between AFP length and activity has also been described for the AFGPs. Evaluation of the thermal hysteresis activity of various molecular weight isoforms of AFGPs determined that lower molecular weight, not amino acid composition, was responsible for the reduced activity of smaller AFGP isoforms (27). Given the predicted linear nature of these AFGPs (28) the lower molecular weight isoforms are likely shorter, thereby linking AFGP length with activity.

Our work suggests that the higher activity of CfAFP-501, as compared with the shorter CfAFP-337, is due to the length of the β-helical platform and consequently the increased ice-binding area. It also indicates that as long as the basic β-helical folding is maintained, a certain degree of sequence variation in the Thr-Xaa-Thr motif can be tolerated. A question that naturally arises from this study is what are the limits to the effective length of the AFP β-helical platform. Although it can be envisioned that there is definitely a limit that can be experimentally determined, as described below, it is important to note that such a limit comes with some structural reasoning. The overall stability of a “free-standing” α-helix or β-helix has to be maintained by the helix itself, which has no additional stabilization interaction with other parts of the overall structure as usually seen in many other proteins. As the length of the helix increases, the flexibility will naturally increase. Given the regularity of ice lattice, too much flexibility on the part of AFP might have detrimental effects on AFP-ice interaction. Therefore, the effective length will be a compromise or delicate balance between the ice-binding area and structural flexibility.

To more directly map out the relationship between antifreeze activity and AFP length (ice-binding surface area) a series of AFGPs of varying sizes must be examined. This could be done with the addition or deletion of more loops within CfAFP or could be more directly accomplished using the beetle TmAFP, which contains three different lengths of its natural isoforms.

Acknowledgment—We thank Sherry Gauthier for technical assistance in the production of the shortened CfAFP-501.

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J. Biol. Chem. 2002, 277:33349-33352.
doi: 10.1074/jbc.M205575200 originally published online June 24, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205575200

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