学 位 論 文 内 容 の 要 旨

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学 位 論 文 題 名

Structural basis for the binding of antifreeze proteins from a snow mold fungus to ice
（担子菌由来不凍タンパク質の構造と機能に関する研究）

Approximately 80 % of the Earth's surface is permanently cold, that is, at temperatures below 5 ºC. These area include cold oceans, the polar regions and high mountains. Although these cold habitats seem to be extremely inhospitable to life, cold-adapted organisms have successfully evolved their survival strategies to inhabit ice-laden environments. Antifreeze proteins (AFPs), which are identified from some cold-adapted organisms, are known as an adaptation molecule for cold stress. AFPs specifically adsorb to the surface of ice crystals and inhibit their growth, which result in a non-colligative depression of the freezing temperature of a solution and a slight elevation of the melting temperature owing to the Gibbs-Thomson effect. The difference between these temperatures is termed thermal hysteresis (TH) and is used as a definitive evaluation of the antifreeze activity.

AFPs are categorized into two subgroups based on their antifreeze activities, moderately active and hyperactive AFPs. Moderately active AFPs from fishes and plants show THs of 0.5–1.0 ºC at millimolar concentrations. On the other hand, hyperactive AFPs from insect and microorganism exhibit potent TH activities, which are up to ~5 ºC at micromolar concentrations. To date, three-dimensional structures of various hyperactive AFPs have been determined to reveal characteristic β-helical folds composed of highly repetitive sequences. The ice-binding site (IBS) of hyperactive AFPs is often constructed of well-conserved sequences, which contain regularly arrayed motifs. It has been thought that IBS with repetitive structure leads to the attachment of AFP to the basal plane of ice crystals, which seems to be a key determinant of hyperactivity. The IBS also has been hypothesized to organize surface waters into an ice-like network that resembles the quasi-liquid layer of ice, thus merging the AFP with ice.

Typhula ishikariensis, a snow mold fungus that grows on the turf and grass under snow cover, secretes *Tis*AFPs as a mixture of seven isoforms of 223 residues, which share a high sequence identity with each other. We previously determined the crystal structure of an isoform, *Tis*AFP6, which exhibits moderate TH activity. *Tis*AFP6 folds into a right-handed β-helix with a triangular cross-section despite lacking any repetitive motifs. Within all the isoforms, *Tis*AFP8 shows the lowest sequence identity (83.4 %) with *Tis*AFP6. A preliminary study reported that *Tis*AFP8 exhibit a high antifreeze activity, yet the detailed antifreeze activities and structural difference between these two isoforms have not been well studied. Besides, homologous AFPs to *Tis*AFPs have been identified from various kinds of microorganisms including fungus, diatom, yeast and bacteria, which form a unique protein family. To date, crystal structures have been reported for four microbial AFPs, all of which fold into a right-handed β-helix. Interestingly, these microbial AFPs exhibit a wide variety of antifreeze activities both of moderately active and hyperactive despite their close structural similarity. It has been reported only for the microbial AFP family that hyperactive and moderately active AFPs are identified in the same family, suggesting that a non-repetitive IBS could contribute to hyperactive TH activity. Nevertheless, it has been unclear about a detailed mechanism that explains how hyperactive microbial AFPs interact with ice crystals in the absence of repetitive ice-binding motifs as seen for insect AFPs.

In order to answer the above questions, I constructed the recombinant expression system of *Tis*AFP8, observed the antifreeze activity, and determined its crystal structure. Based on the crystal structure role of the IBS residues in TH activity was examined by the mutational study. Furthermore, I constructed docking models with ice crystal to gain an insight into the ice-binding properties of *Tis*AFPs.

In the present study I successfully constructed *E. coli* expression system for *Tis*AFP8. *Tis*AFP8 exhibited TH activity of approximately 2 ºC at a concentration of 0.11 mM and induced rapid growth of ice crystals in the hexagonal directions. Fluorescence-based ice plane affinity analysis showed that *Tis*AFP8 binds to multiple ice planes, including the basal planes of ice crystals. These observations indicated that *Tis*AFP8 is a member of hyperactive AFPs. In contrast, *Tis*AFP6 showed TH activity of ~0.3 ºC at 0.11 mM and an elongation of the ice crystal in two directions, which is comparable with moderately active AFPs. X-ray crystal analysis of *Tis*AFP8 revealed that it folds as a right-handed β-helical domain and a long α-helix, showing a high similarity for the main chain structure with *Tis*AFP6. Furthermore, close comparison of the β-sheet of the putative IBS and the adjacent loop region of *Tis*AFP8 and *Tis*AFP6 revealed that *Tis*AFP8 bear more hydrophobic character in the ice-binding β-sheet and the adjacent loop region than *Tis*AFP6. A series of *Tis*AFP8 mutants were prepared for residues on the β-sheet and the adjacent loop region with the corresponding residues of *Tis*AFP6. All introduced mutations resulted in TH activity decrease. A double mutant A20T/A212S, which comprised a hydrophobic patch between the β-sheet and the loop region, caused the greatest depression of antifreeze activity of 75 %, when compared with that of *Tis*AFP8 wild type. This showed that the loop region is involved in ice binding and hydrophobic residues play crucial functional roles. In addition, bound waters around the β-sheet and the loop region were organized into an ice-like network and can be divided into two groups that appear to mediate *Tis*AFP and ice separately. The docking model of *Tis*AFP8 with the basal plane via its loop region reveals a better shape complementarity than that of *Tis*AFP6. In conclusion, I present new insights into the ice-binding mechanism of *Tis*AFP8 by showing that a higher hydrophobicity and better shape complementarity of its IBSs, especially the loop region, may render *Tis*AFP8 hyperactive to ice binding.