Investigation of the Ice-Binding-Site of an Insect Antifreeze Protein using Sum-Frequency Generation Spectroscopy

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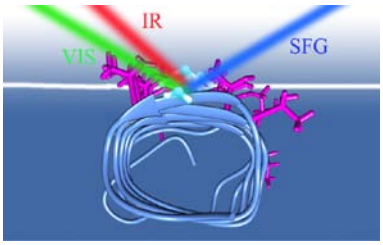
Abstract: We study the ice-binding site (IBS) of a hyperactive antifreeze protein from the beetle *Dendroides canadensis* (DAFP-1) using vibrational sum-frequency generation spectroscopy. We find that DAFP-1 accumulates at the air-water interface due to the hydrophobic character of its threonine-rich ice-binding site, while retaining its highly regular β-helical fold. We observe a narrow band at 3485 cm^{-1} that we assign to the O-H stretch vibration of threonine hydroxyl groups of the ice-binding site. The narrow character of the 3485 cm^{-1} band suggests that the hydrogen bonds between the threonine residues at the IBS and adjacent water molecules are quite similar in strength, indicating that the IBS of DAFP-1 is extremely well ordered, with the threonine side chains showing identical rotameric confirmations. The hydrogen-bonded water molecules do not form an ordered ice-like layer, as was recently observed for the moderate antifreeze protein type III (AFP-III). It thus appears that the antifreeze action of DAFP-1 does not require the presence of ordered water, but likely results from the direct binding of its highly ordered array of threonine residues to the ice surface.



KEYWORDS Antifreeze Proteins, Chiral Sum-Frequency Generation Spectroscopy, Phase-Sensitive Sum-Frequency Generation, Air-Water Interface, Protein-Water Interface

Antifreeze proteins (AFPs) have been isolated and structurally characterized from a wide range of organisms, including bacteria, plants, fungi, insects and polar fish[1,2]. Despite their structural heterogeneity and differences in activity, all AFPs are believed to function by an adsorption-inhibition mechanism in which the proteins recognize and irreversibly bind to the surface of small ice crystallites thereby preventing further growth of the crystallites at the adsorbed positions[3]. As a result, ice growth is limited to regions between adsorbed AFPs, and thus the ice is forced to grow in energetically unfavorable curved fronts. This effect eventually leads to the termination of crystal growth, and lowers the freezing point, a phenomenon known as the Kelvin effect. Ice binding involves a particular region of the antifreeze protein known as the ice-binding-site (IBS). Ice-binding-sites are usually relatively flat, hydrophobic and contain almost no charged residues. Molecular dynamics simulations provided evidence on the existence of preordered ice–like water molecules and/or decelerated water dynamics around the IBS of several AFPs, pointing towards an active involvement of water in their mode of action[4-6]. However, given the fact that AFPs bind ice nuclei in an environment that is largely liquid water makes it experimentally challenging to determine the structure and dynamics of the protein hydration shell. Consequently, up to now the mechanism of recognition and initial binding has barely been studied experimentally. We recently studied the properties of water hydrating the ice-binding site of AFP-III with vibrational sum-frequency generation (VSFG)[7]. This technique is ideally suited for this study as it is highly selective for molecules residing at interfaces.

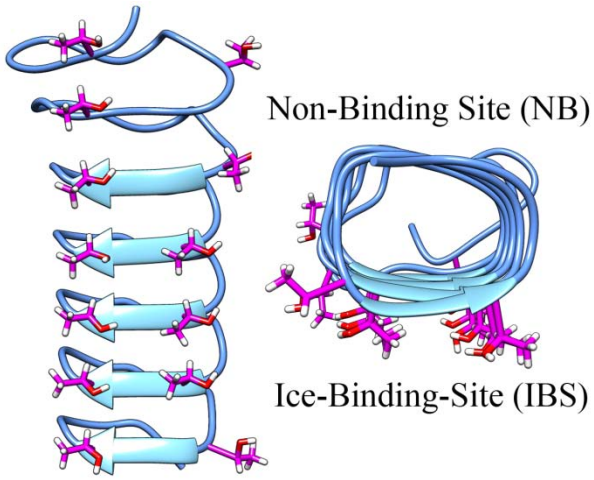


Figure 1. Model of the 3D-structure of the insect antifreeze protein DAFP-1. DAFP-1 consists of seven identical repeats that form an exceptional regular right-handed β- helix. The ice-binding-site consists of a flat, relatively hydrophobic and threonine-rich surface.

DAFP-1 consists of several identical 13-aa repeats that form a right-handed β-helix fold as seen in figure 1. The helix is stabilized by disulfide bonds and an extensive H-bond network, thus creating probably one of the most regular protein structures known[8]. The ice-binding site of this protein has been identified as a relatively flat, hydrophobic β-sheet that consists of two rows of threonine residues adjacent to a narrow trough. Several regularly spaced water molecules, H-bonded to the hydroxyl groups of the threonines, were observed in the crystal structure of a *Tenebrior molitor* AFP (TmAFP), a structural homolog of DAFP-1[9]. Molecular dynamics (MD) simulations confirmed the existence of regularly arranged water molecules at the surface, and showed that these water molecules remain associated with the protein in solution, thus constituting part of the AFP[6]. Further MD-simulations and Terahertz spectroscopic studies identified several layers of slowed hydrogen-bond dynamics of water sets of periodically arranged ordered water molecules parallel to the ice-binding site of the protein[5,10]. Contrasting

with these findings is a NMR study which found no indication for ice-like water or the presence of an extended hydration shell[11].

In this work we study the properties of the ice-binding site of DAFP-1 and the adjacent water molecules using a combination of different sum-frequency generation techniques. In VSFG an infra-red light pulse and a visible pulse are combined at an interface to generate light at their sum-frequency. The generation is enhanced in case the infrared light is resonant with a molecular vibration. For most systems the generation of sum-frequency light is symmetry-forbidden in the bulk which makes VSFG highly surface specific. By interfering the VSFG signal from the sample with a reference sum-frequency field, we determine the phase of the generated sum-frequency light, and thereby the phase of the second-order optical susceptibility $\chi^{(2)}$. The real (Re) and imaginary (Im) parts of $\chi^{(2)}$ provide direct information on the orientation of the vibrational transition dipole moments at the surface[12]. This phase-sensitive VSFG thus provides information that is not obtained in conventional intensity VSFG as the signal measured with this technique is proportional to $|\chi^{(2)}|^2$. VSFG and phase-sensitive VSFG (PS-VSFG) have been successfully applied to identify the orientation of interfacial water molecules next to various biomolecules[13,14].

We determined the surface propensity of DAFP-1 by measuring the surface tension of aqueous solutions containing different concentrations of the protein. We observed surface tensions of 45-60 mN/m, depending on the DAFP-1 concentration. This surface tension is significantly lower than the surface tension of ~72 mN/m of pure liquid water from which we conclude that DAFP-1 is a surface active molecule, comparable to AFP-III[15]. The IBS of DAFP-1 is quite hydrophobic, while the other surfaces of the protein are dominantly hydrophilic, making DAFP-1 an

amphiphilic system (FigS1). As a result, DAFP-1 proteins at the water-air interface will likely be oriented with their IBS towards the air phase and their hydrophilic surfaces to the water face[16].

Figure 2a shows the VSFG spectrum of the interface of pure liquid water (H_2O) and of a 20 μM DAFP-1 solution (pH 7.5), both at room temperature (20^o C). The VSFG spectrum of the water-air interface (in black) shows a broad double-peaked band between ~3100 and ~3600 cm^{-1}. This broad band is associated with the O-H stretch vibrations of H_2O molecules forming hydrogen bonds to other H_2O molecules. The spectrum also shows a relatively narrow band at ~3730 cm^{-1} that can be assigned to the OH-stretch vibrations of non-hydrogen bonded OH groups that are sticking out of the surface[17]. All spectra shown are background subtracted and normalized with respect to a reference SFG spectrum.

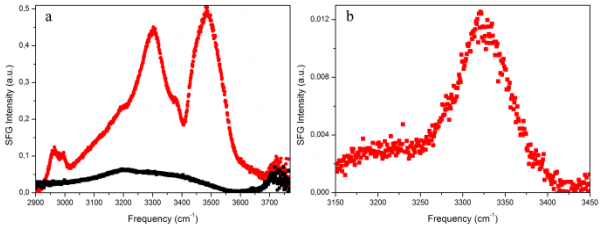


Figure 2. VSFG spectra of pure water (black) and a 20 μM DAFP-1 solution (pH 7.5) (red) at the air-water interface, measured in ssp and psp polarization configuration. (a) The VSFG spectrum of DAFP-1 (in red) in ssp shows several contributions belonging to CH-, NH- and OH-stretching vibrations. Among them are two sharp bands at ~3300 cm^{-1}and ~3485 cm^{-1}. (b) In the psp spectrum of DAFP-1 a single peak at ~3320 cm^{-1} is observed that we assign to the N-H stretch vibration of the chiral, β-helical fold of DAFP-1.

The VSFG spectrum of the aqueous DAFP-1 solution (in red) also shows a broad response in the spectral region of the O-H stretch vibrations and bands that can be assigned to methylene and

methyl vibrations (2880 – 3000 cm^{-1}). At frequencies >3100 cm^{-1} the VSFG spectrum of the DAFP-1 solution shows two strong, relatively narrow bands at ~3300 cm^{-1} (FMHW ~65 cm^{-1}) and ~3485 cm^{-1} (FMHW ~95 cm^{-1}). We assign these bands to protein specific signals. This frequency region also shows weaker bands at 3200 cm^{-1}, 3380 cm^{-1} and 3730 cm^{-1} that we assign to water background signals. Previous VSFG studies of non-AF protein solutions also showed the presence of a narrow peak at 3300 cm^{-1}. This peak was assigned to either the amide A mode related to the protein backbone or to side-chain related amine resonances[18-20]. Both assignments are possible for DAFP-1. The VSFG spectra of Fig. 2 are measured in ssp polarization configuration (s-polarized SFG, s-polarized visible and p-polarized infrared), and this polarization configuration does not allow for a distinction between the two possible assignments.

In order to determine the molecular origin of the signal at 3300 cm^{-1} we performed chiral VSFG-measurements using a psp polarization configuration (p-polarized SFG, s-polarized visible and p-polarized infrared). Chiral VSFG has been used before to determine the secondary structures of proteins at interfaces as it is highly selective for signals from macromolecules with a chiral architecture[21]. In Figure 2b we show the VSFG spectrum of DAFP-1 in psp polarization. This spectrum shows a narrow band at 3320 cm^{-1} that can be unambiguously assigned to the N-H vibrations of the helical protein backbone of DAFP-1[19,20]. The observation of a chiral VSFG signal demonstrates that DAFP-1 remains in its regular β-helical fold, since unfolding of the helix at the interface would eliminate the chirality and consequently the psp VSFG signal. In view of the clear presence of the N-H vibrations of the helical protein backbone in the psp VSFG spectrum, the band at 3300 cm^{-1} in the ssp spectrum likely has the same origin.

The other narrow band observed in the ssp spectrum of Fig. 2 at ~3485 cm^{-1} has not been observed before in VSFG spectra of proteins. We exclude further contributions from N-H

vibrations since these vibrations are expected to have lower frequencies. Hence, the narrow band at 3485 cm^{-1} is likely associated with the O-H vibration of particular amino acid residues or water molecules adsorbed at the protein surface.

In previous infrared spectroscopic studies of hydrogen-bonded alcohol clusters, a vibrational signal at ~3500 cm^{-1} has been as-signed to the O-H stretch vibration of an alcohol molecule that is donating but not accepting a hydrogen-bond[22,23]. Previous VSFG studies have assigned signals at ~3500 cm^{-1} to alcohol dimers and carbohydrate-water systems[24,25], which also constitute systems in which an O-H group of an organic molecule is only donating and not accepting a hydrogen bond. Threonine possesses an alcoholic O-H group, and thus is an amino-acid that complies with these characteristics. Moreover, threonine residues are abundantly present in DAFP-1, especially at the IBS. DAFP-1 consists of nearly 20% of threonine and within the IBS more than 60% of the residues are threonine[8]. Furthermore, 7 of the 11 threonine residues in the IBS have been proposed to form a strong hydrogen-bond with a fixed water molecule[9]. We therefore assign the band at 3485 cm^{-1} to the O-H stretch vibrations of the hydroxyl groups of threonine that donate hydrogen bonds to water molecules. The frequency of 3485 cm^{-1} is somewhat lower than the frequency of 3500-3520 cm^{-1} of the O-H stretch vibration of the last hydroxyl group donating a hydrogen bond in a hydrogen-bonded cluster of alcohol molecules[21,22], indicating that the hydrogen bond donated by the hydroxyl group of threonine is relatively strong. However, the frequency of 3485 cm^{-1} is high compared to the center of the absorption band of the O-H stretch vibrations of liquid HDO:D_2O, indicating that the hydrogen bond donated by the hydroxyl group of threonine is weaker than the average hydrogen bond in water. In bulk water the hydrogen bonds constitute extended conjugated hydrogen-bond networks, and this conjugation makes the hydrogen bonds stronger. The width of ~95 cm^{-1} of the

threonine OH peak is much narrower than the spectral width of ~300 cm^{-1} of the O-H stretch absorption bands of alcohol clusters and of HDO:D_2O. The large bandwidth of the latter systems results from the large variation in structures and hydrogen-bond strengths. The much smaller width of the threonine OH peak shows that the hydrogen bonds donated by the threonine residues have a quite well-defined strength. This finding implies that the IBS of DAFP-1 would be well ordered, with the threonine side chains showing motional rigidity and identical rotameric conformations[26,27].

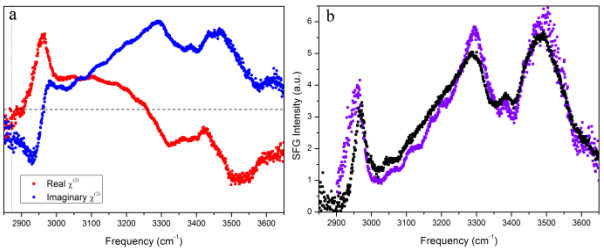


Figure 3. (a) Real and Imaginary χ(2) of a 40 μM DAFP-1 (pH 7.5) solution at the air-water interface measured with ssp polarization. (b) (Im $\chi^{(2)}$)^2 + (Re $\chi^{(2)}$)^2 (purple) and conventional VSFG spectrum of 40 μM DAFP-1.

To further elucidate the absolute orientation of DAFP-1, threonine and its surrounding interfacial water, we performed PS-VSFG measurements. Figure 3a shows the PS-VSFG spectrum of a 40 μM DAFP-1 solution (pH 7.5) at room temperature (20ºC). In Figure 3b we compare | $\chi^{(2)}$|^2 constructed from the PS-VSFG data with the conventional VSFG spectrum. The two spectra are in excellent agreement, thus validating the PS-VSFG spectrum. The PS-VSFG spectrum shows two negative bands at ~2880 cm^{-1} and ~2940 cm^{-1} that can be assigned to the methyl symmetric stretching ($CH_{3,SS}$) and methyl Fermi resonance ($CH_{3,FR}$), respectively[13]. A positive band with a maximum at~2975 cm^{-1} can be assigned to the methyl antisymmetric

stretching vibration ($CH_{3,AS}$). The negative signs of the $CH_{3,SS}$ and $CH_{3,FR}$ bands and the positive sign of $CH_{3,AS}$ indicate the presence of terminal methyl CHs oriented towards the air[14]. DAFP-I's ice-binding site is rather flat and consists almost entirely of threonine residues, as seen in figure 1. As a result, the upward facing methyl groups are likely associated with the side chains of threonine residues. The $Im\chi^{(2)}$ spectrum of DAFP-I shows two distinct positive peaks at ~3300 cm^{-1} and ~3500 cm^{-1}. The positive sign of the latter peak implies that the threonine O-H groups are also pointing towards the air. The PS-VSFG spectrum thus shows that the threonine side chains are pointing up, in agreement with the conformation of a TmAFP dimer that was found in low-temperature X-ray diffraction studies[9]. Here we observe this conformation for a single DAFP-1 in the aqueous phase at room temperature.

In Figure 4 we compare VSFG spectra of DAFP-1 measured at 20^o C and 0^o C. The similarity of the two VSFG spectra of Figure 4 shows that no significant structural change occurs upon lowering the temperature. Decreasing the temperature appears to lead only to a small decrease of the intensity of the VSFG spectrum and a small red shift of the threonine O-H band. The red shift indicates that the hydrogen bond donated by the threonine hydroxyl group to water becomes slightly stronger when the temperature is decreased. This is a quite common observation for hydrogen-bonded systems. We conclude that lowering the temperature enhances the hydrogen bonding but has little effect on the secondary structure of DAFP-1, as suggested before by circular dichroism and linear infrared spectroscopy[28].

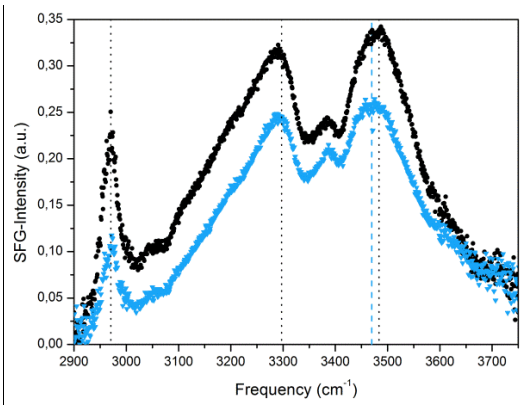


Figure 4. VSFG spectra of a 40 µM DAFP-1 solution (pH 7.5) at 20° C (black) and 0° C (blue). Lowering the temperature enhances hydrogen bonding and leads to a red shift of the threonine OH signal and a decrease of the amplitude. The dashed vertical lines indicate the central frequency positions of the threonine OH bands at the two different temperatures.

We study the properties of the hyperactive insect antifreeze protein DAFP-1 with conventional, chiral and phase-resolved vibrational surface sum-frequency generation. We observe that DAFP-1 accumulates at the air-water interface, probably as a result of the predominantly hydrophobic character of the threonine-rich IBS. Owing to its particular regular β-helical structure, contributions arising from N-H stretching vibrations of the chiral protein backbone could clearly be assigned, confirming that DAFP-1 does not undergo structural changes or unfolding upon adsorption to the interface. The VSFG spectrum of DAFP-1 shows a strong narrow band at 3485 cm^{-1} that we assign to the hydroxyl groups of threonine residues located at the ice-binding site that donate hydrogen bonds to water molecules. The narrow character of the 3485 cm^{-1} band indicates that the hydrogen bonds between the threonine residues at the IBS and the water molecules are quite similar in strength, indicating that the IBS of DAFP-1 is extremely well ordered, with the threonine side chains showing identical rotameric confirmations. Using PS-VSFG measurements we were able to determine the absolute

orientation of the threonine side chains of DAFP-1 with both the terminal methyl and hydroxyl group pointing up.

In a recent study of AFP-III we observed that the O-H stretch vibrational spectrum of water to the IBS has the form of a relatively narrow peak at ~3250 cm^{-1}, quite similar to the O-H stretch vibrational spectrum of water ice[7]. This observation supports an ice-binding mechanism of AFP-III in which the IBS has a high affinity for ice surfaces thanks to the ice-like character of its hydration shell. The VSFG spectrum measured for DAFP-1 does not show the presence of such highly structured ice-like water layers. The VSFG spectrum is in fact dominated by the O-H stretch vibration of threonine residues that donate hydrogen bonds to adsorbed water molecules. This difference in VSFG spectra between DAFP-1 and AFP-III indicates that the water adsorbed to the IBS of DAFP-1 is of quite different character than the water adsorbed to the IBS of AFP-III[7]. It was observed in a crystallographic study on the related MpAFP that the water molecules bound to the IBS are quite regularly positioned[29]. The regular positioning is likely due to the fact that the water molecules accept hydrogen bonds from the hydroxyl groups of a highly ordered array of threonine residues. It should be noted that the regular positioning of the water molecules does not imply that the water molecules themselves would be donating strong hydrogen bonds to other water molecules. In the crystallographic study on MpAFP the authors speculate that in solution water near the IBS is likely very mobile[29]. Here we find strong evidence that this is indeed the case, as the water molecules are observed not to form a well-ordered ice-like layer.

The IBS of DAFP-1 consists of a well-ordered array of threonine residues that already by itself provides a good surface similarity to ice. Consequently it seems that DAFP-1 does not require additional ordered water layers to obtain a high ice-surface affinity. In contrast to that, AFP-III consists of no repetitive sequence or structural match on its surface and would require stable

water layers to provide a structural match to the ice-plane. We speculate that different water layers for moderate and hyperactive AFPs might actually be a mandatory requirement in order to be able to distinguish and bind to different planes of ice, which themselves have different growth rates and orientations of water molecules. For instance, the more disordered water at the IBS of DAFP-1 may provide sufficient malleability for docking onto two distinct ice crystal planes. Moreover, if ice-binding requires solidification of hydration water at the IBS, this may also be faster for preformed ice-like water associated with AFP-III than for the more disordered surface water of DAFP-1. This could explain the more pronounced time-dependent activity for DAFP-1 compared to AFP-III, as was recently reported[30].

Summarizing, we used a combination of VSFG, chiral and phase-sensitive vibrational sum-frequency generation spectroscopy to investigate the ice-binding site of the hyperactive antifreeze protein DAFP-1.The VSFG spectra reveal that the IBS of DAFP-1 is extremely well ordered, with the threonine side chains showing identical rotameric confirmations. Using PS-VSFG measurements we were able to determine the absolute orientation of the threonine side chains of DAFP-1 and we found that both the terminal methyl and hydroxyl group are pointing up. Our findings reveal that moderate and hyperactive AFPs have structurally different IBS and hydration layers, which are likely optimized to their specific task.

ASSOCIATED CONTENT

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Notes

The authors declare no competing financial interests.

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SUPPORTING INFORMATION (SI)

Supporting Information Avaiable: Experimental Details, Figures S1-S3. This material is avaible free of charge via the internet http://pubs.acs.org.

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