

# The effect of winter flounder antifreeze protein and its mutants on methane hydrate growth: A molecular dynamic simulation study

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**Abstract:** Natural gas hydrates (NGH) are widely found in seafloor sediments. In engineering, it is usually necessary to inject 60% of thermodynamic inhibitors, which makes hydrate extraction costly and polluting. Currently, kinetic inhibitors have attracted much attention due to their low injection dose and environmental friendliness, but the research is costly and time-consuming. In this study, we investigated the interaction between winter flounder antifreeze proteins (AFPs) and methane hydrate growth using molecular dynamics simulations. AFPs adsorbed on the hydrate surface and hindered the mass transfer of methane molecules. At the same time, the water molecules around the AFP adsorption surface are in a quasi-liquid state, a structure that facilitates the binding of AFPs to the hydrate surface. Analysis of the probability of amino acid adsorption showed that AFP was adsorbed to the hydrate surface through a combination of hydrophobic and hydrogen bonding interactions. Subsequent directional mutagenesis experiments showed that increasing the hydrophobicity of AFP rather weakens its adsorption capacity. This suggests that excessive hydrophobicity of AFP may be counterproductive to its adsorption on the hydrate surface. These findings deepen the understanding of the AFP mechanism and its potential for the development of novel hydrate inhalants.

## 1. Introduction

Gas hydrates are solid crystalline structures in which gas molecules are encapsulated within water cages formed through hydrogen bonding<sup>[1]</sup>. This unique configuration positions gas hydrates as a potential energy resource with significant global reserves, garnering widespread attention. However, the formation of hydrates in oil and gas production and transportation pipelines presents a considerable challenge, often leading to blockages and resulting in substantial economic losses<sup>[2-3]</sup>. This dichotomy underscores the dual nature of gas hydrates as both a resource and a challenge, highlighting the imperative for an in-depth understanding of their behavior and properties.

To address the issue of hydrate blockages, various remedial strategies have been proposed<sup>[4]</sup>. Among these, the method of inhibitor injection has gained widespread acceptance in the oil industry. There are primarily two categories of hydrate inhibitors: thermodynamic hydrate inhibitors (THIs) and low dose hydrate inhibitors (LDHIs)<sup>[5-6]</sup>. THIs are capable of effectively dissolving hydrate plugs by altering the phase equilibrium conditions of hydrates. However, their required injection concentration is notably high, around 60wt%<sup>[7]</sup>, which significantly escalates industrial costs<sup>[8]</sup>. In contrast, LDHIs are administered at substantially lower concentrations, typically less than 3wt%, compared to the concentration of THIs<sup>[9]</sup>. LDHIs function differently; for

instance, kinetic inhibitors impede the growth of crystal nuclei, while anti-agglomerants prevent the accumulation of hydrate particles<sup>[10-11]</sup>. Nevertheless, a major drawback of most LDHIs is their limited biodegradability, prompting a growing interest in the development of environmentally friendly hydrate inhibitors<sup>[12]</sup>.

Antifreeze proteins (AFPs), also known as ice structuring proteins, play a crucial role in enabling certain organisms to survive in sub-zero temperature environments<sup>[13]</sup>. Devries et al.<sup>[14]</sup> employed scanning electron microscopy to elucidate the impact of AFPs on ice growth, discovering that these proteins inhibit ice surface growth by adsorbing onto it. In subsequent research, Devries et al.<sup>[15]</sup> proposed that antifreeze glycopeptides likely adhere to the surface of ice crystals. This sparked extensive research into the adsorption mechanisms of AFPs, primarily focusing on hydrogen bonding<sup>[16-18]</sup> and hydrophobic interactions<sup>[19-21]</sup>. Molinero et al.<sup>[22]</sup> further explored the contributions of hydrophilic and hydrophobic groups in the TmAFP to the ice-binding affinity of these proteins through molecular dynamic simulations. Their findings indicated that hydrogen bonding and hydrophobic groups contribute equally to AFPs' affinity for ice.

Given the unique effects and mechanisms of antifreeze proteins (AFPs) on ice, their potential role as inhibitors of hydrate formation is an intriguing area of exploration. Zeng et al.<sup>[23]</sup> delved into this possibility by examining the influence of AFPs from winter flounders and insects on the formation of tetrahydrofuran hydrates. Their findings

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revealed a significant impact of AFPs on hydrate morphology, shifting the growth from three-dimensional to two-dimensional patterns, and notably extending the induction time for hydrate nucleation. Additionally, these AFPs demonstrated greater inhibitory activity compared to the commonly used kinetic inhibitor polyvinylpyrrolidone (PVP). Further investigations by Zeng et al.<sup>[24-25]</sup> proposed that AFPs could mitigate the 'memory effect' in the reformation of methane and tetrahydrofuran hydrates. Complementing these findings, Mu et al.<sup>[26]</sup> observed that 2250 ppm of mSA-RmAFP1 exhibits a similar effect on methane hydrate nucleation as PVP at the same concentration. Conversely, research by Servio et al.<sup>[27]</sup> on the temperature and pressure dependencies of AFP's inhibitory action revealed that under conditions of higher hydrate formation driving force (lower temperatures and higher pressures), AFPs might actually promote hydrate growth. This observation was echoed by Solms et al.<sup>[28]</sup>, who suggested that RmAFP1 can facilitate hydrate growth in fresh solutions.

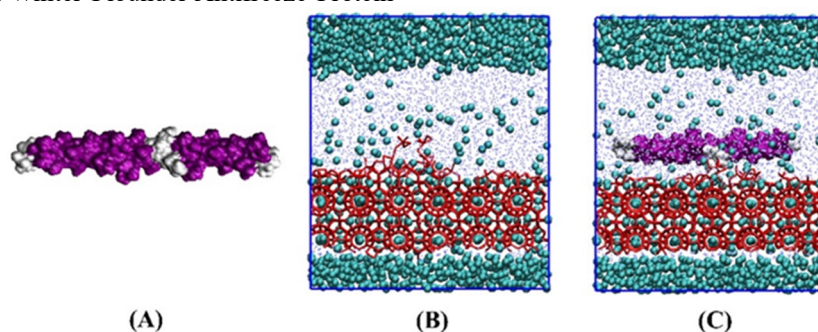
The existing body of research indicates that AFPs can either inhibit or promote hydrate formation under varying conditions, highlighting the intricate nature of AFPs' action mechanisms in hydrate formation. This complexity suggests that insights into the anti-hydrate mechanism may be gleaned from understanding the anti-ice mechanism of AFPs. Englezos et al.<sup>[29]</sup> pioneered the use of molecular dynamics simulation to assess the effect of AFPI on methane hydrate growth. Their study revealed that the hydrophobic groups in AFP-I play a crucial role in influencing hydrate growth. Complementing this, Peyvandi et al.<sup>[30]</sup> demonstrated that hydrogen bonding in AFP-III contributes minimally to its hydrate growth inhibition. Furthermore, they posited that the geometry and surface structure of AFPs are critical factors in determining the interaction dynamics between hydrates and AFPs.

In this study, we conduct a comprehensive examination of the hydrogen bonding and hydrophobic interactions between Winter Flounder Antifreeze Protein

(WFAFP) and the hydrate surface, specifically investigating the impact of various WFAFP mutants on its inhibitory activity. Our findings reveal that WFAFP predominantly adheres to the surface of hydrate crystal nuclei through a synergistic combination of hydrophobic interactions and hydrogen bonds. Intriguingly, we observed that enhancing the hydrophobicity of the antifreeze protein via localized mutation adversely affects its adsorption onto the hydrate surface. This research not only sheds light on the complex interplay between antifreeze proteins and hydrates but also offers valuable insights for the development of novel inhibitor designs, thereby contributing significantly to the advancement of hydrate research.

## 2. Methods

In this investigation, molecular dynamics simulations were employed to study the influence of Winter Flounder Antifreeze Protein (WFAFP) on the growth of methane hydrate. We chose WFAFP for its relatively simple structure, as depicted in Figure 1A. The initial configuration of WFAFP was derived from X-ray crystallography data<sup>[31]</sup>. The simulation setup involved placing a  $6 \times 3 \times 2$  sI hydrate crystal nucleus within the initial system, accompanied by a gas layer serving as the methane source. The gap between the lower surface of WFAFP and the upper surface of the hydrate crystal nucleus was approximately 9 Å, as illustrated in Figures 1B and 1C. The simulation model comprised 2900 water molecules, 74 methane molecules in the liquid phase, and 900 methane molecules in the gas phase, both with and without the presence of WFAFP. The system underwent an initial 20 ns pre-equilibrium phase at 300 K and 1 bar, during which the methane molecules within the hydrate and the WFAFP were immobilized. Subsequently, the simulation conditions were adjusted to 250 K and 650 bar, and a 350 ns dynamic simulation was conducted.



**Figure 1.** Depiction of the Molecular Dynamics Simulation Setup. (A) The molecular structure of Winter Flatfish Antifreeze Protein (WFAFP). (B) The simulation system post-pre-equilibrium phase without the presence of antifreeze protein, and (C) with the inclusion of antifreeze protein. In these visualizations, methane molecules are represented by cyan spheres, oxygen atoms of water molecules are denoted by blue dots, and the red lines illustrate the hydrogen bond network within the hydrate structure.

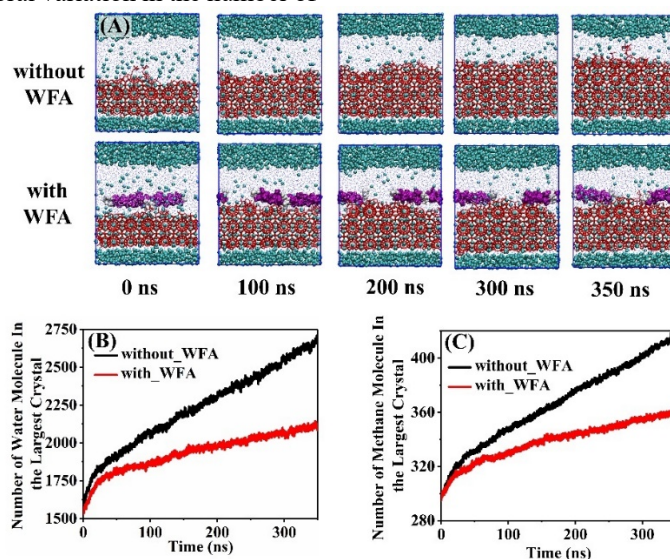
To optimize computational efficiency, we omitted the provision of an external gas source for hydrate growth in our simulations. Instead, we allowed the antifreeze proteins to adsorb freely onto the surface of the initial hydrate, focusing on identifying the adsorption groups within the proteins. The distance between the lower

surface of the antifreeze proteins and the upper surface of the hydrate was maintained at approximately 5 Å. The simulations were conducted for a duration of 100 ns, under controlled conditions of 250 K temperature and 650 bar pressure.

### 3. Results and discussion

In this study, we explored the impact of antifreeze proteins (AFPs) on the growth of hydrates using a gas-liquid biphasic growth model. To negate the structural effects of the AFPs themselves, we conducted ten separate simulation runs for both systems—with and without AFPs—ensuring varied initial orientations of the AFPs towards the hydrate in each iteration. As illustrated in Figure 2(A), in the system without AFPs, the hydrates grew upwards, reaching approximately four layers by 350 ns. In contrast, in the presence of AFPs, hydrate growth appeared to halt at about three layers within the same timeframe. Further analysis, as depicted in Figures 2(B) and 2(C), revealed the temporal variation in the number of

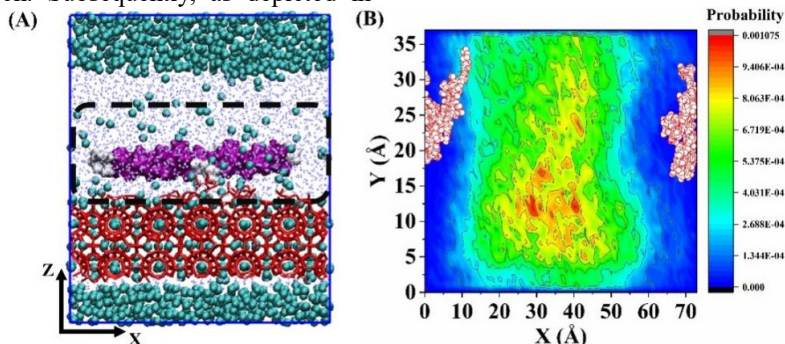
water and methane molecules within the hydrate nuclei. During the initial 25 ns, the growth rates of hydrates were similar in both systems, as the AFPs had not yet adsorbed onto the hydrate nuclei. Post 25 ns, as the liquid-phase methane was depleted and the diffusion of gas-phase methane to the liquid phase slowed down, the growth rate decreased in both systems. This slowdown was more pronounced in the presence of AFPs, which, upon adsorption on the hydrate nuclei, further hindered the arrival of methane to the hydrate surface. Additionally, the presence of AFPs altered the growth pattern of hydrates from planar to curved. Thus, our findings indicate that AFPs can effectively inhibit the growth process of hydrates.



**Figure 2.** (A) The hydrate growth morphology of the system without and containing antifreeze protein changes with the simulation time. The green ball represents the methane molecule, the blue dot represents the oxygen atom of the water molecule, and the red line segment represents the internal hydrogen bond network structure of the hydrate. Change of the number of (B) water molecules and (C) methane molecules in the maximum hydrate crystal nucleus with time

We utilized the Visual Molecular Dynamics (VMD) tool to observe the adsorption process of antifreeze proteins (AFPs), noting that the structure of AFPs underwent certain degrees of vibration. Such structural fluctuations could potentially influence the adsorption efficacy of AFPs. Accordingly, we identified three amino acids from the AFP structure to serve as reference points for measuring angular changes, as shown in Figure 3(A). These amino acids, numbered 1, 17, and 37, were selected based on prior research. Subsequently, as depicted in

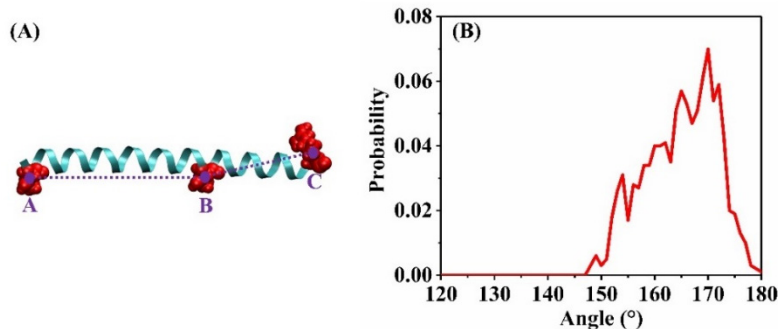
Figure 3(B), we analyzed the angle  $\angle ABC$  formed by these amino acids. Our observations indicated that prior to adsorption, the structure of AFPs changes, with the angle fluctuating between approximately  $150^\circ$  to  $180^\circ$ , and most frequently around  $170^\circ$ . These structural variations in AFPs could lead to alterations in their adsorption state, potentially impacting the adsorption strength and stability, and thereby influencing the effectiveness of AFPs as hydrate growth inhibitors.



**Figure 3.** (A) The model of the system containing antifreeze protein. The cyan sphere represents the methane molecule, the blue dot represents the oxygen atom of the water molecule, and the red line segment represents the hydrogen bond network structure of the hydrate. (B) The distribution relationship between antifreeze protein core and hydrate crystal nucleus. White dots represent the distribution of antifreeze protein core, and different shades of color represent the distribution probability of water molecules in hydrate

Following structural adjustments, certain amino acids of the antifreeze protein (AFP) adsorb onto the hydrate surface, achieving a dynamically stable adsorption structure. As shown in Figure 4(A), we monitored the variation in the number of atoms from AFP adsorbed on the hydrate surface over time. An AFP atom was considered adsorbed on the hydrate surface if, within a 7 Å radius, it encountered over 12 water molecules from the hydrate (as shown in Fig. 4(A)). Our analysis indicated that, starting from around 200 ns, the quantity of adsorbed AFP atoms progressively increased, signifying a gradual

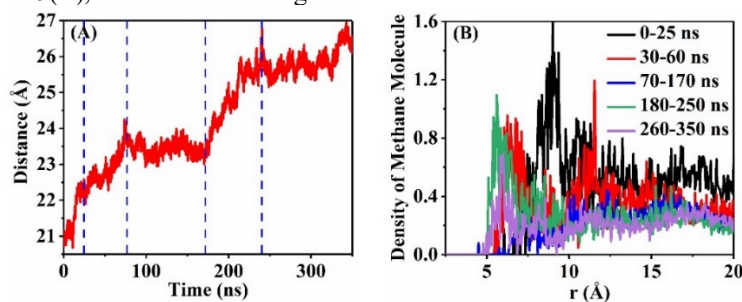
adsorption of AFPs onto the hydrate surface and the establishment of a stable adsorption state. The surface of hydrates is characterized by an abundance of semi-cage structures, some containing methane molecules (termed non-empty hydrate cages) and others devoid of methane (termed empty hydrate cages). As shown in Figure 4(B), our findings revealed that the probability of AFP amino acids being located in empty hydrate cages was 87.11%, compared to 12.89% in non-empty cages. This suggests a preferential binding of AFPs to the empty cage structures of hydrates.



**Figure 4.** (A) The selection of antifreeze protein angle, where A, B and C respectively represent arginine 37, alanine 17 and aspartic acid 1. (B) Probability distribution of selected three amino acid composition angles

The growth of hydrates necessitates the timely arrival of methane molecules at the hydrate surface. The binding of antifreeze proteins (AFPs) to this surface may impede the mass transfer process of methane, thereby inhibiting further hydrate growth. As shown in Figure 5(A), we calculated the distance between the centroid of the AFP and the initial surface of the hydrate. It was observed that AFP adsorption is not static but exhibits a step-like pattern. After a period of stable adsorption, the AFP moves with the growing hydrate, eventually achieving a new stable adsorption state. This indicates that AFPs engage in dynamic adsorption on the hydrate surface. Further analysis, shown in Figure 5(B), involved assessing the

methane molecule density around the AFP centroid during different stages. During the initial 0-25 ns period, the density of methane molecules around the AFP was relatively high, corresponding to a faster hydrate growth rate (as shown in Figures 2(B) and 2(C)). Subsequently, we noted a decrease in the density of methane molecules around the AFP over time, with a tendency for the methane molecules to approach the AFP. This suggests that AFPs form a natural 'barrier' on the hydrate surface, obstructing the mass transfer process of methane to the hydrate surface. Consequently, the presence of AFPs inhibits hydrate growth.

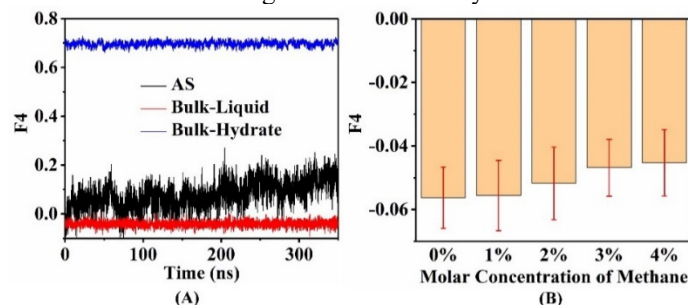


**Figure 5.** (A) The distance between the antifreeze protein core and the initial upper surface of the hydrate changes with the simulation time, and the dotted line represents different stages. (B) Changes of methane molecular density around the heart of antifreeze protein at different time periods

After antifreeze proteins (AFPs) bind to the hydrate surface, the side of the AFP that is in contact with the hydrate is referred to as the binding face. As shown in Figure 6(A), we calculated the F4 order parameter of water molecules within a 5 Å radius of the AFP binding face and compared these to the F4 order parameters of water molecules in the liquid and hydrate phases. The F4 parameter value of 0.7 corresponds to water molecules in hydrates, -0.04 to those in the liquid phase, and -0.4 to those in the ice phase. We observed that the effective parameter values for water molecules around the AFP

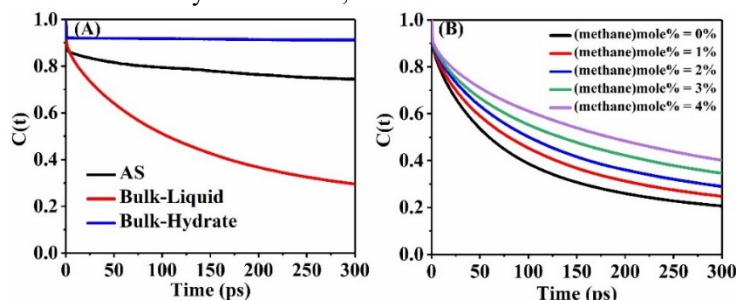
binding face were higher than -0.04, indicating these molecules do not belong to a pure liquid phase. Prior to 200 ns, when the AFP had not yet bound to the hydrate surface, the non-pure liquid state of the surrounding water molecules could be attributed to methane adsorbed onto the AFP binding face. To substantiate this interpretation, we established a methane-water mixed system, as depicted in Figure 6(B). By adjusting the molar concentration of methane, we noted that the F4 order parameter value of water molecules increased with the rising methane molar concentration in the liquid phase. In the pure water system

without methane, the F4 parameter value was lower than -0.04 due to the system's low temperature, resulting in a supercooled state of the water molecules. Post 200 ns, as the AFP progressively bound to the hydrate surface, the F4 order parameter values gradually increased. This rise is attributed to the amino acids of the AFP binding face



**Figure 6.** (A) The F4 order parameters of water molecules in the surrounding (black), liquid (red) and hydrate (blue) phases of the interface change with the simulation time, and the interface is AS. (B) F4 ordering parameters of water molecules in methane-water system with different molar concentrations of methane

To identify the specific amino acids of the antifreeze protein (AFP) involved in adsorption, we removed the gas source necessary for hydrate growth, and maintained the temperature and pressure at 250 K and 650 bar, respectively, ensuring stable hydrate existence. This allowed the AFP to freely adsorb onto the hydrate surface,

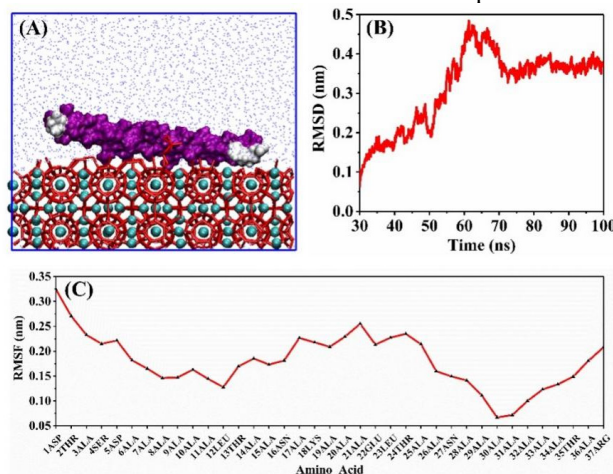


**Figure 7.** (A) The hydrogen bond autocorrelation function of water molecules around the binding surface (black), in the liquid phase (red) and in the hydrate phase (blue) changes with the simulation time. The adsorption surface is AS. (B) Hydrogen bond autocorrelation function of water molecules in systems with different molar concentrations of methane

In Figure 8(C), we analyzed the fluctuations of individual amino acids after AFP adsorption. Lower RMS fluctuation values suggest a higher likelihood of amino acid adsorption. Notably, amino acids numbered 30 and 31

integrating into the cages on the hydrate surface, causing an increase in the F4 order parameter values of the surrounding water molecules. This indicates that the water molecules around the AFP binding face are in a quasi-liquid state, which may facilitate the adsorption of AFPs on the hydrate surface.

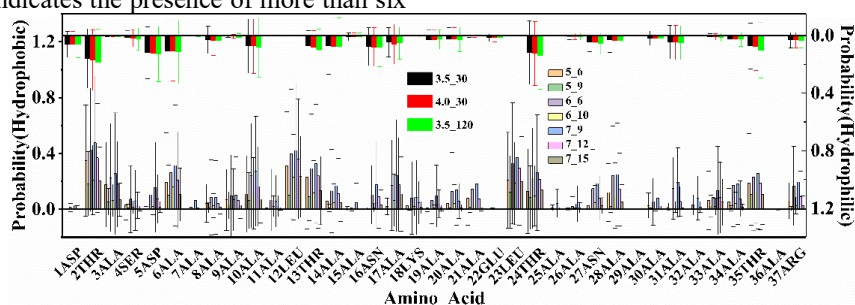
as shown in Figure 7(A). As depicted in Figure 7(B), we calculated the root-mean-square deviation (RMSD) during the AFP adsorption process. The RMSD value of the AFP approached stability around 70 ns, indicating that the protein had reached a stable state of adsorption.



**Figure 8.** (A) The morphology of antifreeze protein binding on the hydrate surface. The cyan sphere represents the methane molecule, the blue dot represents the oxygen atom of the water molecule in the liquid phase, and the red line segment represents the hydrogen bond network structure in the hydrate. (B) The root-mean-square deviation of antifreeze protein adsorption process changes with simulation time. (C) Root mean square fluctuation of antifreeze protein after adsorption

To mitigate the randomness of adsorption, we performed ten molecular dynamics simulation runs, varying the initial orientation of the antifreeze protein (AFP) relative to the hydrate surface in each simulation. Molinero et al. have indicated that both hydrophobic and hydrogen bonding interactions contribute to AFP binding on ice surfaces. Accordingly, we separately analyzed the hydrophobic and hydrogen bonding interactions between the AFP and the hydrate surface. As shown in Figure 9, to ensure the accuracy of our criteria for determining amino acid hydrogen bonding and hydrophobic interactions, we employed various standards. For hydrogen bonds, three different criteria were used, while seven were applied for hydrophobic interactions. For instance, '3.5\_30' refers to a hydrogen bond criterion of 3.5 Å in length and 30° in angle between AFP amino acid atoms and water molecules in the hydrate, and '5\_6' indicates the presence of more than six

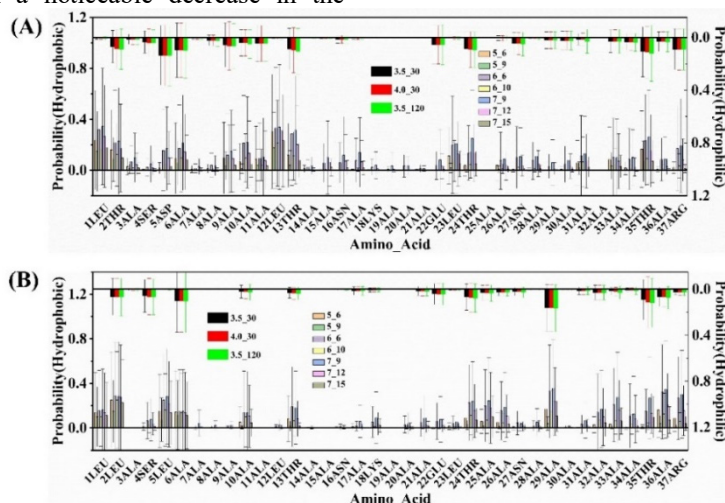
water molecules within a 5 Å radius of an amino acid atom. We observed minimal variation between these different criteria. The results highlighted a relatively higher adsorption probability for amino acids such as Alanine (ALA), Threonine (THR), Leucine (LEU), Aspartic Acid (ASP), and Asparagine (ASN), suggesting a propensity for these amino acids to adsorb onto the hydrate surface. Interestingly, Leucine exhibited only hydrophobic interactions without forming hydrogen bonds with the hydrate surface. In contrast, Alanine, Threonine, Asparagine, and Aspartic Acid engaged in both hydrophobic and hydrogen bonding interactions. This suggests that AFPs bind to the hydrate surface through a combination of hydrophobic and hydrogen bonding interactions, with varying contributions from different amino acids.



**Figure 9.** The probability of amino acids contained in antifreeze protein adsorbed on the surface of hydrate, the upper column represents the probability of hydrogen bond formation between amino acids and the surface of hydrate, the lower column represents the probability of binding of hydrophobic groups of amino acids on the surface of hydrate, and different colors represent different judgment criteria

From the analysis above, it is evident that Leucine primarily binds to the hydrate surface through hydrophobic interactions and exhibits a high probability of adsorption. Additionally, existing research has shown that hydrate surfaces are hydrophobic. Therefore, if an antifreeze protein (AFP) contains more of such amino acids, its adsorption stability could potentially improve. As illustrated in Figure 10(A), we mutated the first amino acid of the AFP to Leucine and observed that compared to the native AFP, the probability distribution of adsorbed amino acids in the mutant did not change significantly. Consequently, we further mutated the second and fifth amino acids to Leucine, as shown in Figure 10(B). This modification resulted in a noticeable decrease in the

adsorption probability of the AFP amino acids, with a reduction in the number of adsorbed amino acids. We also calculated the binding energies of the native and mutant AFPs. The binding energy of the native AFP was approximately -590.122 kJ/mol, while the mutant in Figure 10(A) had a binding energy of about -591.925 kJ/mol, and the mutant in Figure 10(B) had a binding energy of approximately -525.019 kJ/mol. These results indicate that an increase in the hydrophobicity of the AFP leads to less stable adsorption on the hydrate surface. Consequently, enhancing the hydrophobicity of an AFP may not be conducive to its adsorption on the hydrate surface, thus failing to effectively inhibit hydrate growth.



**Figure 10.** The probability of the amino acid contained in the antifreeze protein mutant adsorbed on the surface of the hydrate, and (A) amino acid 1 was replaced with leucine. (B) Amino acids 1, 2 and 5 were replaced with leucine

## 4. Conclusion

This study employed molecular dynamics simulations to investigate the effect of antifreeze proteins (AFPs) from winter flounder on hydrate growth. Our findings demonstrate that AFPs can inhibit the growth process of hydrates. By examining the microscopic processes, we observed that AFPs adsorb on the hydrate surface, impeding the mass transfer of methane molecules. Additionally, water molecules surrounding the AFP binding face were found to be in a quasi-liquid state, a structure that may facilitate the binding of AFPs to the hydrate surface. Further analysis of amino acid adsorption probabilities revealed that AFPs adsorb onto hydrate surfaces through a combination of hydrophobic and hydrogen bonding interactions. Subsequent targeted mutagenesis experiments, replacing certain amino acids with the more hydrophobically inclined Leucine, indicated that an increase in the hydrophobicity of AFPs paradoxically weakens their adsorption capability. This suggests that excessive hydrophobicity in AFPs may be counterproductive for their adsorption on hydrate surfaces, thereby diminishing their effectiveness in inhibiting hydrate growth. These insights are theoretically instructive for the design of novel hydrate inhibitors.

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