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Unusual structural properties of water within the hydration shell of hyperactive antifreeze protein

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Many hypotheses can be encountered explaining the mechanism of action of antifreeze proteins. One widespread theory postulates that the similarity of structural properties of solvation water of antifreeze proteins to ice is crucial to the antifreeze activity of these agents. In order to investigate this problem, the structural properties of solvation water of the hyperactive antifreeze protein from *Choristoneura fumiferana* were analyzed and compared with the properties of solvation water present at the surface of ice. The most striking observations concerned the temperature dependence of changes in water structure. In the case of solvation water of the ice-binding plane, the difference between the overall structural ordering of solvation water and bulk water diminished with increasing temperature; in the case of solvation water of the rest of the protein, the trend was opposite. In this respect, the solvation water of the ice-binding plane roughly resembled the hydration layer of ice. Simultaneously, the whole solvation shell of the protein displayed some features that are typical for solvation shells of many other proteins and are not encountered in the solvation water of ice. In the first place, this is an increase in density of water around the protein. The opposite is true for the solvation water of ice – it is less dense than bulk water. Therefore, even though the structure of solvation water of ice-binding plane and the structure of solvation water of ice seem to share some similarities, densitywise they differ. © *2014 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4891810]

I. INTRODUCTION

Organisms exposed to temperatures below 0 °C face a lethal threat of freezing. But they have some ways and means to survive in the cold environment. To a certain extent, they can protect themselves against injuries caused by ice formation, using proteins capable of interaction with the surface of ice. These are so-called ice-binding proteins (IBPs) and ice-nucleating proteins (INPs).[1,2] They actively interfere with the process of freezing. Among the ice-binding proteins, two groups called antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) are distinguished.[1] Both groups help to keep the bodily fluids in a supercooled state (in a limited range of temperature, called thermal hysteresis). The antifreeze activity can be characterized as a length of hysteresis gap, which is a difference between melting temperature and freezing temperature when AFPs are present in the system.

A. The mechanism of action of AFPs

As we will briefly describe below, many theories can be found explaining the functioning of antifreeze agents[3–31] and sometimes they are mutually contradictive. Even a seemingly straightforward problem, i.e., whether the binding of AFPs with ice is a reversible or irreversible process, had not been unambiguously solved for a long time. Experimental results obtained recently[3,4] support the irreversibility hypothesis.

The AFPs are said to modify kinetics of freezing by inhibiting the formation[32] and growth[6] of ice seed crystals. They perform their function by adsorbing to the ice surface. As a result, they restrict the growth of the ice front to regions between the adsorbed protein molecules. Because of that any subsequent growth of an ice crystal occurs on a curved interface, and so it becomes less thermodynamically favorable[5–7] due to the so-called Gibbs-Thomson effect. Adsorbed AFPs can be eliminated from the ice surface by becoming incorporated into the growing ice crystal, as discussed by Sander and Tkachenko.[8] The AFP's "resistance to engulfment"[9] (meaning the ability not to be frozen into the newly accumulated layers of ice) is characterized by an engulfment angle, which is specific for each AFP.

Although the Gibbs-Thomson effect may appear to be sufficient to explain the AFPs functioning, it seems not to account for all of the subtleties of this phenomenon, as it will be briefly discussed in Subsection I B, devoted to the significance of solvation water. Moreover, it is even sometimes called into question.[10]

As mentioned by Sharp,[11] AFPs must be able to "notice" one phase of water, ice, in a great excess of another phase, liquid. They can do even more than that – various AFPs are able to distinguish different crystallographic planes of ice and to adsorb to only one or two of them.[26,33–35] The ability to bind to two ice planes, and especially to the basal one, has been proposed as a key prerequisite for an AFP to be hyperactive.[26] In the process of binding of AFPs to ice both hydrophobic and hydrophilic interactions appear to play a significant role.[12,13]

This immediately suggests the involvement of solvation water in the process. There are strong indications that the properties of solvation water are indeed important for the functioning of AFPs.[14–18]

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B. The significance of solvation water

There is a long-standing controversy regarding forces that bring AFPs and ice together. It was demonstrated that the number of hydrogen bonds between the active protein surface and surrounding water is roughly the same as the number of hydrogen bonds with the ice surface.[19] It seems that if there is practically no enthalpic contribution to the free energy of binding then there should be an entropic contribution. This entropic contribution could be attributed to characteristic features of hydrophobic solvation.[20] It seems that desolvation of hydrophobic groups upon the association may be an important stabilizing factor (they place themselves at the grooves at the ice surface).

In a model proposed by Kristiansen and Zachariasen[21] water plays a more direct role in the mechanism of AFP binding to ice. According to this model, when the appropriate fragment of AFP is close enough to the ice surface, the immediate crystallization of water present between the protein and ice occurs. The formation of ice from water entrapped between two surfaces of favorable geometry is known and has been described for other systems.[36,37] Herein, topography of both surfaces (ice and ice-matching AFP[7,22–26] surface) appears to be appropriate to promote the process.

This mechanism can be supported further by analyzing the structure of solvation water of a single AFP molecule, and specifically of its binding plane. Yang and Sharp[14,15] reported that water in the solvation layer of the active surface of AFP shows structural resemblance to ice. Smolin and Daggett,[16] Cui *et al.*,[17] and Nutt and Smith[18] also drew similar conclusions. It was proposed[14–18] that this modification of water structure facilitates binding with the ice surface. The most vivid confirmation of this supposition was given by Garnham *et al.*[26] who described the structure of water layer adjacent to AFP at a temperature equal to 200 K. The reported results suggested a possible binding mechanism, namely, that the AFP molecule carries its own "ice" ("*anchored clathrate water*") which is similar enough to bind to the ice nuclei, but also may be different enough to slow down the growth of crystals after attachment.[11] Bearing all that in mind, it is hardly possible to discuss the mechanism of AFP binding to ice as a simple protein-ligand recognition, especially taking into account the fact that solvation layer of ice is quite thick, reaching about 1–2 nm.[38–40]

In the light of the remarks above, it seems that the protein has to be properly oriented relatively to the ice surface in order to adsorb. But sometimes, as Nada and Furukawa have demonstrated,[41] more than one way of attachment can cause an antifreeze effect. According to them, *Cf*AFP may bind to the prism ice surface at least in two different ways and decrease the ice growth rate in both cases. They hypothesize that this may be an ability that underlies the hyperactivity.

It cannot be also ruled out that AFPs may accommodate in a very close proximity to the ice surface[12] rather than bind to the surface itself. Under this scenario, the protein would mainly interact with the solvation layer of ice. Zepeda *et al.*,[10] who investigated an AFGP (antifreeze glycoprotein), argue that ice/water interface is not sharp and the interactions within the transient region involving water molecules solvating the proteins and water molecules interacting tightly with the ice surface have to be taken into account. Therefore, a Gibbs-Thomson effect may not offer a sufficient or even appropriate explanation of antifreeze activity.

To the great importance of solvation water also pointed Ebbinghaus and his co-workers.[27–30] These authors used increasingly popular terahertz spectroscopy to detect a long-ranged retardation of water dynamics in the solvation shells of AFGPs.[28] They considered this phenomenon to be a possible factor disfavoring the freezing of water. The same authors also investigated an antifreeze peptide and its mutants[30] and found that the most active peptides had extended dynamical hydration shells. Such shell may be particularly important for enhancing the antifreeze activity of proteins at their low concentrations. However, there is no consensus regarding the mobility of solvation water of antifreeze agents and its significance. For example, these results are in contradiction with the conclusions reached by Modig *et al.*[31] They conducted the experimental study of the dynamics of solvation water of *Tm*AFP and concluded that it is qualitatively comparable to typical dynamics of solvation water of proteins which do not interact with the ice. The changes in water mobility were neither particularly far-ranged nor the dynamics was particularly retarded, with the exception of several ordered water molecules on the ice-binding plane.

Moreover, the aforementioned results are not consistent with the conclusions reached by Nutt and Smith,[18] who investigated the *Cf*AFP solvation water by computer simulations. These authors observed increased mobility of the solvent at some distance from two protein surfaces which do not interact with ice. It was proposed that this phenomenon helps keep the adjacent water in a liquid state.

C. The significance of our results

Although numerous uncertainties and controversies exist when it comes to defining the role of solvation water for biological functionality of AFP, the considerations above indicate that it is important. At the same time, it seems that the specific manner in which the properties of water within solvation shell are modified as well as how such modifications influence the mechanism of action of AFPs is still unclear. Because it seems that the modification of structural properties of solvation water may be crucial for biological functionality of AFPs, our attention has focused on this particular issue. Hyperactive *Cf*AFP has been chosen as the subject of the research based on the supposition that its high activity should facilitate observation of the solvation effects. We also took into account the recently expressed suggestion[2] that structural motif, characteristic for hyperactive AFPs (flat matrix with regularly arranged threonine residues), is probably also responsible for biological functionality of ice-nucleating proteins (INPs). If so, an obvious question arises: why AFPs do not act as INPs? The most likely explanation is that AFPs are relatively small molecules (from ∼3 to ∼30 kDa), and therefore they are able to arrange only a relatively small number of neighboring water molecules, i.e., several dozen to a few hundred. The critical number of water molecules which can effectively create an ice nucleus is from hundreds to thousands[42]

even at supercooling as high as 10 K or more. Indeed, INPs are large proteins, with a mass exceeding 100 kDa, and they usually act as multimers.[2] It seems, however, that this explanation of ice-nucleating inactivity is only partially true for two reasons. First, high stability of the ordered structure of solvation water near the active region on the protein surface (*anchored clathrate water*[26]) should compensate, at least partially, the tendency for decomposition (melting) of the ice nuclei caused by the Gibbs-Thomson effect. Second, we should remember that the natural environment of action of AFPs is very distant from the model systems containing only water, used by Pereyra *et al.*[42] to estimate the critical size of ice nuclei. Moreover, it was reported that above certain AFP concentration the protein itself, after having aggregated, can work as an ice nucleator.[32,43] It was also stated that the structural similarity of any surface to the ice is not sufficient to effectively promote ice growth[44] on itself. The main aim of our paper is to investigate the structural properties of water adjacent to the active region of AFP, and to demonstrate both the differences and similarities in comparison to the solvation water of the rest of the protein, other proteins and hydration layer of ice. Naturally, the surface of ice has got ice-forming capabilities, therefore the comparison of its solvation water with the solvation water of the AFP should provide us with some information on structural prerequisites necessary for liquid to be changed into solid. This is why we are going to analyze the solvation of two ice surfaces, to which the ice-binding plane of *Cf*AFP is fitted:[7] basal and primary prism. We also take into account the temperature dependence of the investigated structural parameters, in hope to gain a deeper insight into the properties of solvation water.

II. METHODS

A. Simulation procedure

The results were obtained using computer simulations. They were carried out with the help of molecular dynamics package Amber10[45] and ff03 force field suitable for proteins.[46] *NPT* conditions were applied, and the temperature was kept constant by the weak coupling to an external bath ($\tau_T = 1.0$ ps).

We used two water models: SPC/E and TIP5P. This choice is discussed and justified below. Our investigations covered the temperature range from 240 to 300 K for SPC/E water model, and from 260 to 300 K for TIP5P water model, with a 10° interval between isotherms. Pressure (1 bar) was kept constant by the weak coupling method ($\tau_p = 1.0$ ps). The particle-mesh Ewald method was used for electrostatic interactions, and the lengths of chemical bonds involving hydrogen atoms were fixed using SHAKE. A cutoff of 1.2 nm for nonbonding interactions was used. Trajectories were saved every 8 fs to examine structural properties and every 1 fs to calculate diffusion coefficients discussed in the supplementary material.[89]

B. Selection of water models

Nutt and Smith[18] chose TIP5P[47] water model to solvate *Cf*AFP molecule because the model's melting point is close to the real one (274 K[48]). However, this model is not extensively used for simulations of proteins. In general, simpler models such as, TIP3P, SPC, SPC/E, and TIP4P are favored. Using several common water models (TIP3P, SPC, SPC/E, TIP4P, and TIP4P-Ew) and a few popular force fields (Amber, Gromos, and OPLS), Hess and van der Vegt[49] reached a conclusion that the choice of water model significantly affects thermodynamics of the system and that SPC/E model generally works best with the force fields in question. This model is also known to reproduce a number of properties of liquid water reasonably well.[50,51] On the other hand, the disadvantage of SPC/E model with regard to the simulation of AFPs is the very low melting temperature (215 K[48]). Despite this drawback, the model was successfully used in the studies of supercooled water at the temperatures ranging from 210 K to 260 K.[52–54] Therefore, it appears that an inaccurate melting temperature is not a disqualifying factor when it comes to examining properties of water at lower temperatures.

To achieve our goal, we also had to construct a small ice crystal (ice Ih). Ice Ih is not a thermodynamically stable phase for SPC/E and TIP5P water models.[48] However, in our opinion, it does not make these models impossible to use. We were not interested in the process of the formation of the ice crystal and in the exact structure of ice crystals spontaneously formed by these particular models of water. As it is described below, we used crystallographic data and ensured that we got the crystal of well-defined structure and with the exact crystallographic planes we wanted to investigate. Moreover, for comparison purposes, we wanted to investigate a broad temperature range (up to 300 K). Naturally, in these conditions water models do not form a stable solid phase. At high temperature atoms in the ice crystal had to be, of course, restrained by some external force in order to suppress the melting.

In our study, we used both models, i.e., SPC/E and TIP5P what allowed us to compare the performance of the models as well as to extend the temperature range in which water occurs in its liquid form. This choice has proven to be very useful since we have encountered some difficulties during the analysis of systems with TIP5P water at temperatures near 273 K (discussed below). In those troublesome cases, only data for systems with SPC/E water was used instead. We believe that as long as we are primarily interested in differences between values rather than in the absolute values, the choice of water model should not have a big impact on the quality of the results and on overall conclusions.

C. Systems setup

Three different types of systems had to be constructed, as follows:

(1) systems consisting of *Cf*AFP molecule immersed in water (SPC/E or TIP5P),
(2) systems consisting of ice cuboid immersed in liquid water (SPC/E or TIP5P),
(3) systems consisting solely of liquid water.

1. CfAFP in water

The initial coordinates of *Cf*AFP molecule were taken from Protein Data Bank (PDB ID: 1L0S). The original file contained one iodated tyrosine (not present in natural protein), which was changed into tyrosine. Moreover, two counterions (chloride anions, Cl^-), and all missing residues and atoms were added in LEaP program, which is a part of Amber suite. Amino acids with charged side chains were Arg, Asp, Glu and Lys. Finally, the protein was placed inside a truncated octahedral box and solvated with SPC/E or TIP5P water, with a minimal distance between the protein and the box walls equal to 2.5 nm. As a result, each system contained approximately 15 000 water molecules. To carefully remove possible bad contacts, restraints were applied to protein atoms at the initial steps of preparation of the systems. Later, no restraints for the positions of atoms were applied. A preliminary period of equilibration lasted about 1.6 ns. Total simulation time at each temperature was equal to 15 and 30 ns for TIP5P and SPC/E models, respectively.

2. Ice cuboid in liquid water

We were interested in obtaining an ice crystal of well-defined structure and with the exact basal {0001} and primary prism $\{10\bar{1}0\}$ crystallographic planes (*Cf*AFP is said to be able to bind to both of them[7]). The structure of hexagonal ice (Ih) was found in Inorganic Crystal Structure Database,[55] Fachinformationszentrum Karlsruhe (number 27837). The original file defined the arrangement of the oxygen atoms as well as the hydrogen atoms.

We built a cubic crystal of ice that consisted of 2090 SPC/E water molecules. The crystal was solvated with water up to 2.5 nm from its surface and placed in rectangular box. In the starting configuration of ice, the orientation of bonds between oxygen and hydrogen atoms was ordered. In the real ice Ih these bonds are distributed randomly. To facilitate and speed up the process of reorientation of water molecules, the system was heated up to 1000 K under *NVT* conditions. To avoid the crystal's destruction, its oxygen atoms were restrained using a harmonic potential (force constant 100 kcal $mol^{-1}A^{-2}$). Subsequently, one configuration with fairly low overall dipole moment of ice was chosen. It was a starting point to further equilibration under *NPT* conditions at a temperature range 240–300 K and using restraining potential for oxygen atoms of ice (a force constant was equal to 10 kcal $mol^{-1}A^{-2}$). Next, the proper production run began. Total simulation time was 8 ns. Two opposite faces of the ice cuboid were basal planes, while the two other were primary prism planes. Therefore, the values of parameters calculated for the solvation water of basal and primary prism planes presented here are averages computed from two sets of results.

3. Liquid water

For the purpose of calculation of pure water properties and the values of parameters for fictitious solvation layers filled with bulk water (see below), the cubic box containing approximately 13 000 water molecules (SPC/E or TIP5P, as required) has been constructed.

D. Definition of solvation layers

In our paper we aimed at analyzing the properties of water contained within a thick solvation shell that covers about three water layers. Moreover, the properties of water present only within the first solvation layer of AFP were also investigated. To achieve the aforementioned goals, we adopted the following two definitions of solvation layer.

1. Definition of the thick solvation shell

The investigated *Cf*AFP molecule has a specific prismatic shape. Its function is closely connected to its structure. Three well-defined planes that constitute three walls of the prism can be distinguished within the protein. One of them (herein called "plane 1") interacts with the surface of ice, while the other two remain in contact with the liquid. Because of this well-defined structure it seems to be rational to select a definition of solvation shell which reflects the regular, prismatic shape of the investigated AFP. To this aim, we chose the backbone atoms of the protein as a reference. We are aware that this definition is non-standard, but we believe that it is applicable in this particular case. The definition should be rational especially when the water layer is relatively thick. Plane 2 and plane 3 are equipped with protruding and relatively mobile side chains, but with increasing distance from the protein (and elongating time of simulation), its local geometrical irregularities become less distinct. Thus, the primary criterion for defining the extent of solvation layer for each plane was the distance separating water molecules from the surface created by β-strands running through every prism wall, as illustrated in Figure 1. The range of solvation layer was determined based on the density distribution functions (described in the supplementary material[89] and shown in Figure S1). It is

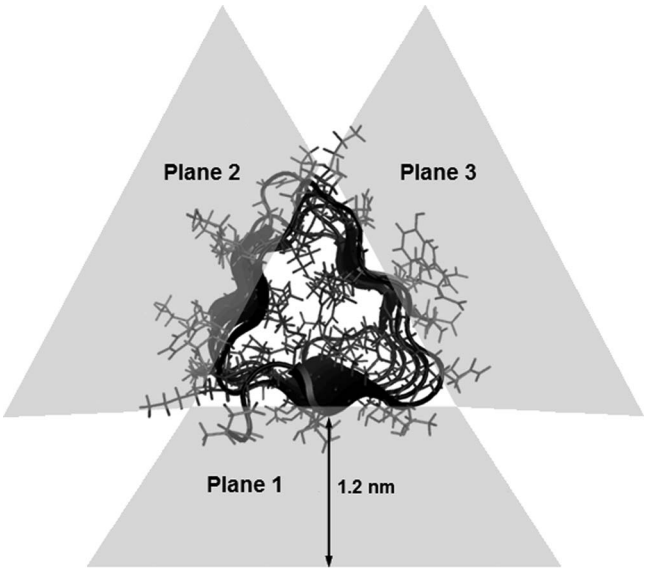

FIG. 1. Definition of the overall solvation layer around the investigated *Cf*AFP molecule.

apparent that the distance of 1.2 nm is the upper limit of the observed density changes. For this reason, we used this value as the range of solvation layer.

2. Definition of the first solvation shell of the protein

This shell has been defined in Refs. 56–58 as the nearest neighbor layer of water around the protein surface. It is comprised of water molecules whose distance from the protein surface does not exceed 0.4 nm.

3. The "reference state" for structural parameters describing the properties of water within solvation shell

In order to detect small changes in the structure of solvation water (compared to bulk water), two series of simulations were performed. The first series was the standard simulation of AFP in water. The second one was the simulation of water alone under identical pressure and temperature conditions. The next step was to read the (time-dependent) coordinates of AFP from the first simulation and insert them into the box with bulk water. After the insertion, water molecules from the region occupied by the protein molecule were removed. As expected, we encountered the problem of choosing the proper cut-off value, r_{cut}, which is a minimum acceptable distance of any water molecule from the protein. This problem has been examined previously (see the supplementary material attached to our previous papers, Refs. 56 and 57). We concluded that the r_{cut} value does not influence the outcome of analysis as long as it is within a reasonable range (we tested the r_{cut} values in the range (0.15–0.19) nm). Thus, in this work we used $r_{cut} = 0.17$ nm, which is the same value as the one used in our previous publications.

Following the aforementioned procedure, we obtained the AFP molecule surrounded by water displaying properties that correspond (by definition) to the properties of bulk water. Further treatment was the same as in the case of standard system, i.e., after selection of water molecules belonging to fictitious solvation shells, the appropriate structural parameters of this solvation water were determined. The new parameters described the properties of water with bulk-like structure therefore we obtained the "reference state" for further interpretation of properties of water enclosed within the real solvation shells. This procedure has a significant advantage because it allows us to circumvent the problem of the space volume inaccessible to water molecules due to the presence of the protein (excluded volume). As a result, we obtain values $\Delta(X) = (X)_{solv} - (X)_{bulk}$, where X is a calculated parameter.

III. RESULTS

The most obvious measure of structural ordering of water is its entropy, which measures the overall ordering of water. However, its value is not suited for describing specific structural properties of water. The degrees of freedom of solvation water near the protein surface may be limited due to the protein's presence (the space volume it occupies is inaccessible to water) or as a result of internal changes in water structure. We define the *internal structural changes*[56] as modifications of intermolecular distances and mutual orientations of water molecules compared to the properties of bulk water. Unfortunately, determining the properties of solvation water presents many difficulties due to heterogeneity of the system, and specifically the excluded volume. To overcome this obstacle, we postulated in our previous paper[56] to construct fictitious solvation shells of the same volume and shape as the real ones but filled with bulk water instead (the procedure is described in Sec. II of this paper). In this way, we were able to eliminate the misleading impact of the protein's local geometry on the results. The method allowed us to capture even subtle changes in the structure of solvation water compared to the properties of bulk water.[56]

As a result of our investigations of the solvation layer of *Cf*AFP described herein, we found significant differences between the structural properties of solvation water of ice-binding plane (plane 1) and the solvation water of the two remaining planes, oriented towards liquid water (planes 2 and 3). Most strikingly, we have also observed an opposite direction of changes with temperature. However, the three solvation layers also share some similarities. Around the whole protein, increased water density is observed. The value of the density increase is comparable to that reported for the solvation water of the majority of proteins.

Several characteristic features observed in the structure of solvation water of plane 1 (along with its temperature changes) resemble the structure of solvation water of ice. The major difference is that the solvation water of ice is less dense than bulk water. Following our previously published considerations,[57,58] we speculate that the density increase near the protein can be caused by the destructive influence of the protein surface on low-energy, high-volume, ordered structures stabilized by hydrogen bonds.

Our method is based on the analysis of several functions characterizing the correlations of mutual orientations and distances between water molecules. We start from the less complex ones and proceed to the more complex ones. To make the presentation of the results clear, we have divided it into two parts.

- In Part (a) we discuss the results obtained with the use of parameters describing radial and angular distributions of water molecules. We observed some differences between solvation water of various planes, and we have reached a conclusion that the density of solvation water of the whole protein increased, while the density of solvation water of ice decreased.
- Part (b) is devoted to the analysis of the two-particle correlation function, which depends on both the distance between two water molecules and their mutual orientation. The two-particle correlation function provides most of the information on the water structure, as it will be discussed later. This analysis highlights significant differences between the solvation water of plane 1 and two remaining planes (in the structure of water and its dependence on temperature). It also illustrates some similarities between solvation of plane 1 and solvation of ice.

A. Radial correlations and short-ranged angular ordering of water molecules

To gain some information on the distribution of the distances between particles, we used a radial distribution function, $g_{OO}(r)$ and a parameter called local structure index (LSI).[59,60] LSI is a measure of heterogeneity of radial distribution of water molecules within short distances (up to about 0.37 nm). In order to analyze the angular distribution of water molecules, we used the previously adapted[57] probability distribution of angle α between vectors connecting the central water molecule with water molecules from its first solvation layer. This parameter measures the heterogeneity of spatial distribution. It also takes into consideration the nearest neighborhood only (molecules present within the radius equal to 0.33 nm).

Liquid water, as opposed to many other popular solvents, possesses a lot of unusual properties, such as a density maximum at 4 °C. In order to explain these anomalies, Tanaka put forward an interesting model.[61–63] The model is based on the assumption that there are two types of structures in liquid water, namely, (1) low-energy, low-density ordered structures occupying high volume and stabilized by hydrogen bonds and (2) high-energy, high-density disordered structures. Recently, Sciortino *et al.*[53,54] analyzed structural changes in supercooled water using LSI and interpreted the results with the help of Tanaka model. By employing some of their conclusions and expanding the analysis with the use of the distribution of angle α, we have demonstrated[57] that the local density changes in the solvation water of proteins may be explained by changes in the relative amounts of the two types of structures in question. These changes result from the destabilizing influence of protein surface on the ordered structures of high volume, low energy, and low density. Our model also explains different behavior of water in the process of hydrophobic solvation. Therefore it seems to be well-suited to describe the solvation of *Cf*AFP.

The probability distributions obtained for the parameters describing radial and angular ordering are depicted in Figure 2. As previously explained, the values obtained for water in the solvation layer were compared to the values obtained for bulk water filling the fictitious solvation layer of the same shape and volume as those of the real solvation layer. Therefore in Figure 2 the differential probability distributions are depicted (the distributions before subtraction are added for comparison). Differential histograms in Figure 2 illustrate that the structure of solvation water of plane 1 is different from the structure of solvation water of two remaining planes. This statement applies to the radial parameters as well as to the angular parameter.

The characteristic features of the probability distribution of angle α are three extrema localized at angles α equal to about 58°, 71°, and 109°. Neighboring water molecules arrange themselves around the central molecule roughly in a tetrahedral manner[64] (angles α would be then equal to about 109°). However, when the first hydration shell becomes more crowded, excess water molecules place themselves between the corners of a tetrahedron or somewhere above the middle of the faces, which disturbs the geometry of the original structure. These situations can be detected by examining the differential distribution of angle α (increase in the values of probability distribution of angle α around 58° and 71°, respectively[57,65]). Inversely, less extensive crowding of the solvation shell should be connected to a decrease in these values.

First of all, it should be noted that the results obtained for solvation water of ice-binding plane (1) somehow resemble the results observed by us for purely hydrophobic hydration[57] (minima at 58° and 71°, and a maximum at 109°). It is understandable because ice-binding plane has partly hydrophobic character due to multiple threonine residues (with methyl groups). On the other hand, the results for water adjacent to two remaining planes (plane 2 and 3) seem to resemble distributions that are typical for other proteins that we previously investigated, such as kinesin and tubulin[57,58] (maxima around 58° and 71°, a minimum at 109°). Previously, we have argued that this shape of the distribution is an indicator of increased density of water. The supposition of increased density of solvation water of plane 2 and 3 finds another support in the radial distribution function, since it can be used as an estimate of local density of water[66] too. A density increase is suggested by positive or near-zero values of differential radial distribution function $\Delta g_{OO}(r)$ within the range 0.0–0.4 nm. The extent of local density increase can be quantified by integrating function $\Delta g_{OO}(r)$ in a given radius R_C (according to the formula in the supplementary material[89]). As a result, the average difference between the number of water molecules surrounding the central molecule in solvation water and in bulk water, enclosed by a sphere of a radius R_C was obtained. The difference was positive (see Table S1) not only for solvation water of plane 2 and 3, but for solvation water of plane 1 as well. When it comes to plane 1, we have to recall that the values of the differential distribution of angle α in the range 45°–85° were negative. Normally, we would expect that to indicate a density decrease. This way, we encounter an apparent contradiction. We are going to explain it below.

A distinct peak observed at ∼0.27 nm for function $\Delta g_{OO}(r)$ points to an increase in the number of the nearest neighbors of water molecules in the solvation shell of the whole *Cf*AFP (on average). We suppose that these neighboring molecules are placed at the tetrahedron's corners. Moving further away from the central water molecule, to the distance values ranging from 0.30 nm to 0.40 nm, we encounter water molecules in close contact with the first hydration shell. Probably, these molecules attempt to squeeze into the tetrahedron and are responsible for the peaks at 45°–85° in the distribution $\Delta P(\alpha)$ of angle α, as discussed previously. For solvation water of planes 2 and 3, the values of function $\Delta g_{OO}(r)$ are positive for the distance values between 0.30 and 0.40 nm, which suggests increased density in this region. However, for plane 1, they are near-zero. This scenario is accompanied by more distinct increase in the value of the first peak at ∼0.27 nm, as well as the second peak at ∼0.45 nm. These features can be indicators of increased ordering of water. When the first solvation shell is not exposed to immediate attack of water molecules from the outside and has more free space to arrange itself comfortably, it adopts a more tetrahedral geometry.

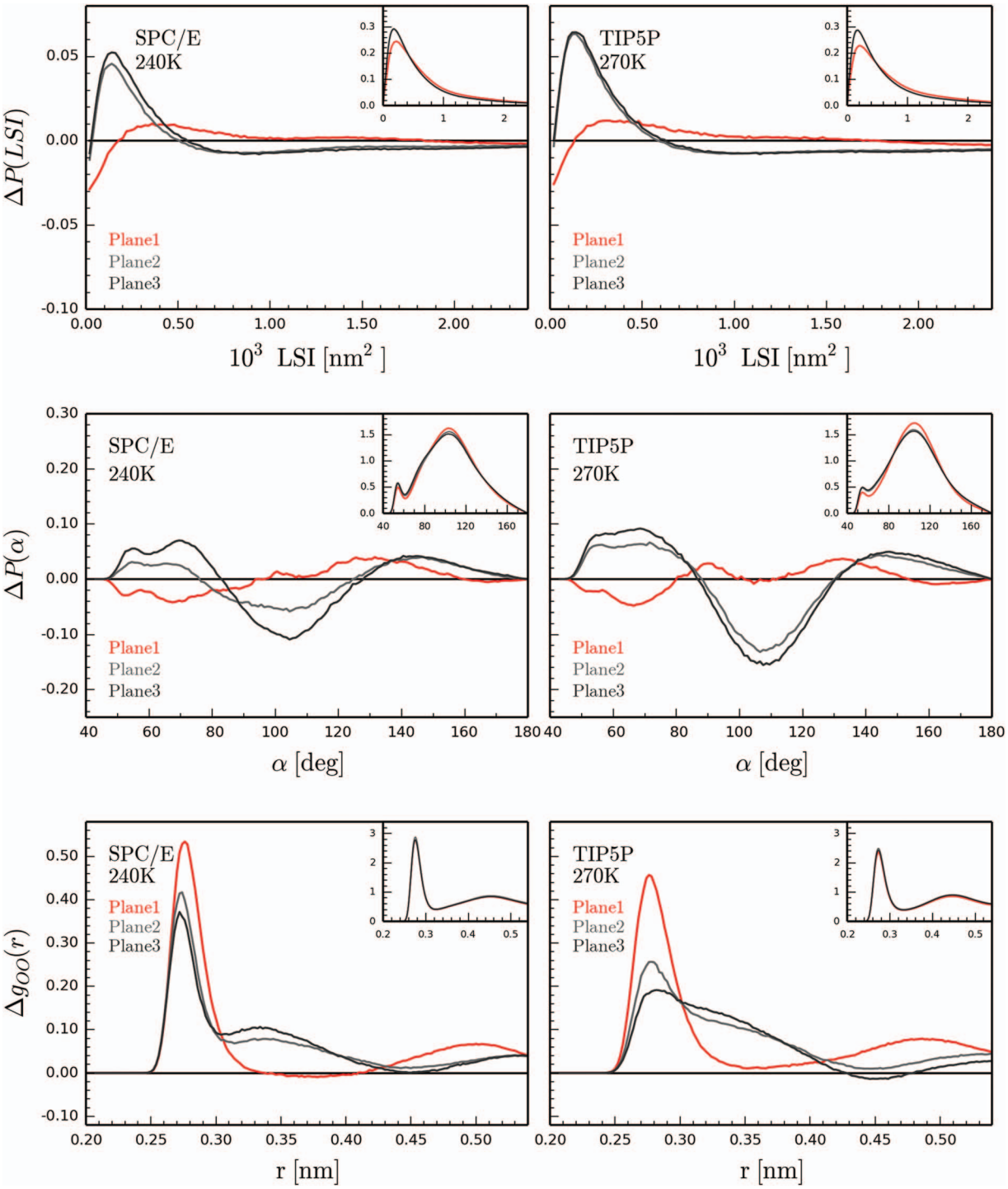

FIG. 2. The differential probability distributions ΔP(LSI), $\Delta P(\alpha)$, and $\Delta g_{OO}(r)$ of the LSI, angle α, and radial distribution function (see text) for water in the first solvation shells (at the distance 0–0.4 nm from the surface atoms of the protein) of the three planes of *Cf*AFP, determined at 240 K for SPC/E and at 270 K for TIP5P water model. The insets depict original distributions P(LSI), $P(\alpha)$, and $g_{OO}(r)$.

Similar reasoning was conducted by Sciortino *et al.*[53,54] with the help of LSI. The value of this parameter is a measure of the heterogeneity of radial distribution of surrounding water molecules around some arbitrary chosen central one. It was defined by Shiratani and Sasai[60] as follows. For each water molecule i one orders the rest of the water molecules depending on the radial distance r_j between the oxygen atom of the molecule i and the oxygen atom of molecule j: $r_1 < r_2 < \ldots < r_j < \ldots < r_n < r_{n+1}$, while n is chosen so that $r_n < 0.37$ nm $< r_{n+1}$. Then, LSI is defined as[60]

$$\mathrm{LSI} = \frac{1}{n}\sum_{j=1}^{n}[\Delta(j) - \overline{\Delta}]^2, \tag{1}$$

where $\Delta(j) = r_{j+1} - r_j$ and $\overline{\Delta}$ is the average value of $\Delta(j)$ (over all molecules).

Let us at first discuss in more details why LSI can be helpful in characterizing water structure. If we moved from the center of some random water molecule, at first we would encounter, ideally, four hydrogen-bonded and tetrahedrally arranged water molecules. The second layer of water molecules would appear after a gap. Therefore, up to the limit of the first solvation shell, we would find water molecules at very similar distances from the central one. But the distance to the first molecule in the second solvation shell would be significantly different. This would have an impact on LSI, since the mean value of distances between aligned molecules would be quite different from each distance on its own. On the other hand, if the molecules were arranged in such a way that they would be more uniformly distributed and there would not be any gap after the first solvation shell, then we would not have one value significantly different from the others and the mean value would be similar to every one of the distances. To sum up, small value of LSI is an indicator of a more uniform radial distribution of surrounding molecules and more crowding at the border between the first and the second solvation shell of a molecule of water. Large value of LSI is an indicator of a non-uniform radial distribution and a gap between the first solvation shell and the second solvation shell.

Differential distributions of LSI values for solvation water of plane 1 and two remaining ones are not alike. For planes 2 and 3, we observe positive values at the left side of the histogram (small LSI). Moving to the right side, the values become slightly negative (large LSI). As mentioned, this can be interpreted as an indicator of more crowded and disturbed structure. For plane 1, we find smaller peak and negative values on the left. Such features were previously observed by us in case of hydrophobic solvation[57] and can be interpreted as indicators of less crowding and less disturbed structure than for solvation water of planes 2 and 3. It agrees with the values of the integral from the function $\Delta g_{OO}(r)$ for a radius 0.33 nm and 0.40 nm given in the supplementary material.[89] While for 0.33 nm these values for plane 1 are bigger than for plane 2 and 3, for 0.40 nm they become smaller.

An increase in $\Delta P(\alpha)$ values for the α values close to 105° for plane 1 also suggests a more tetrahedral ordering. Molecules are more neatly arranged in the corners of tetrahedron, not disturbed by the molecules from the outside of the first solvation layer.

It seems that although there is a density increase for solvation water of plane 1 (as follows from the analysis of function $\Delta g_{OO}(r)$), it is accompanied (on average) by a better (not worse) tetrahedral arrangement of water molecules surrounding each molecule of solvation water. This way, this density increase would not be properly detected by the distribution of angle α.

Naturally, the next question should concern the cause of this density increase and the internal structural changes in solvation water that lead to it. Undoubtedly, methyl groups of threonine contribute to the arrangement of water molecules. These groups can promote the increased tetrahedral ordering as it has been previously observed in the vicinity of hydrophobic molecules. This effect has been extensively debated over many years, and recently, it was reported by Galamba.[67] But contrary to purely hydrophobic hydration, in the case of solvation water of plane 1, solvation shell is anchored to the surface by hydrogen bonds via hydroxyl groups of threonine. It resembles the *anchored clathrate model*, described by Garnham *et al.*[26] In a previous study concerning *Cf*AFP, Nutt and Smith[18] also have found greater ordering of water around plane 1 and disruption of the ordering of water around planes 2 and 3.

The results in Figure 2 describe water from the first solvation shell of three planes of *Cf*AFP. However, as we can see in Figure S2, changes similar in direction, but smaller in intensity, can also be observed when the thicker solvation shell of protein is considered. Therefore our conclusions apply to the averaged properties of the whole solvation layer, not only to water in immediate contact with the surface of the protein.

The solvation layer of *Cf*AFP is diverse, which can be connected to its function. It is known that plane 1 is structurally fitted to two crystallographic planes of ice, i.e., basal and primary prism planes.[7] It is also said that it arranges water molecules in such a way that they fit into the lattice of ice.[18] But in order to adsorb to ice, an AFP first has to move through its solvation layer. After that, it can freeze to the ice with the fraction of solvation water still present between the surfaces and solidified. Alternatively, it can move all the way through the solvation layer to the ice surface. In both cases, the properties of solvation water of ice may be important to the process. Because of that we decided to analyze the differences and similarities of structural properties of solvation water of *Cf*AFP and of the two just mentioned ice planes.

In Figure 3 the differential plots of radial distribution function $\Delta g_{OO}(r)$, probability distribution ΔP(LSI) of LSI values and probability distribution $\Delta P(\alpha)$ of angle α are presented. These plots depict the properties of water remaining in immediate contact with the surface of ice (in a layer of 0.4 nm thickness). Based on the comparison with the three planes of the protein, it can be said that structural changes in the solvation water of ice are most similar to structural changes in the solvation water of plane 1. The general direction of these changes seems to be the same, although the intensity is greater in the case of the solvation water of ice. The only difference is that the density of solvation water of ice is considerably smaller than the density of bulk water (as can be deduced from $\Delta g_{OO}(r)$ in Figure 3 and data in Table S1). However, a decrease in density does not apply to the first hydration shell of a water molecule (at radius 0.33 nm, Table S1). On the contrary, the first solvation shell of water is denser, but the second solvation shell is better separated from it (deep minima at 0.30–0.45 nm for $\Delta g_{OO}(r)$, deep minimum at the left side of the ΔP(LSI) histogram and significantly positive values at the right side of the ΔP(LSI) histogram) – hence the overall density decrease.

To sum up, solvation water of plane 1 is denser than bulk water, and the density increase seems to be roughly the same as that observed at planes 2 and 3. In spite of that, its structure seems to share some common features with the structure of solvation water of ice, but the latter is modified deeper compared to bulk water.

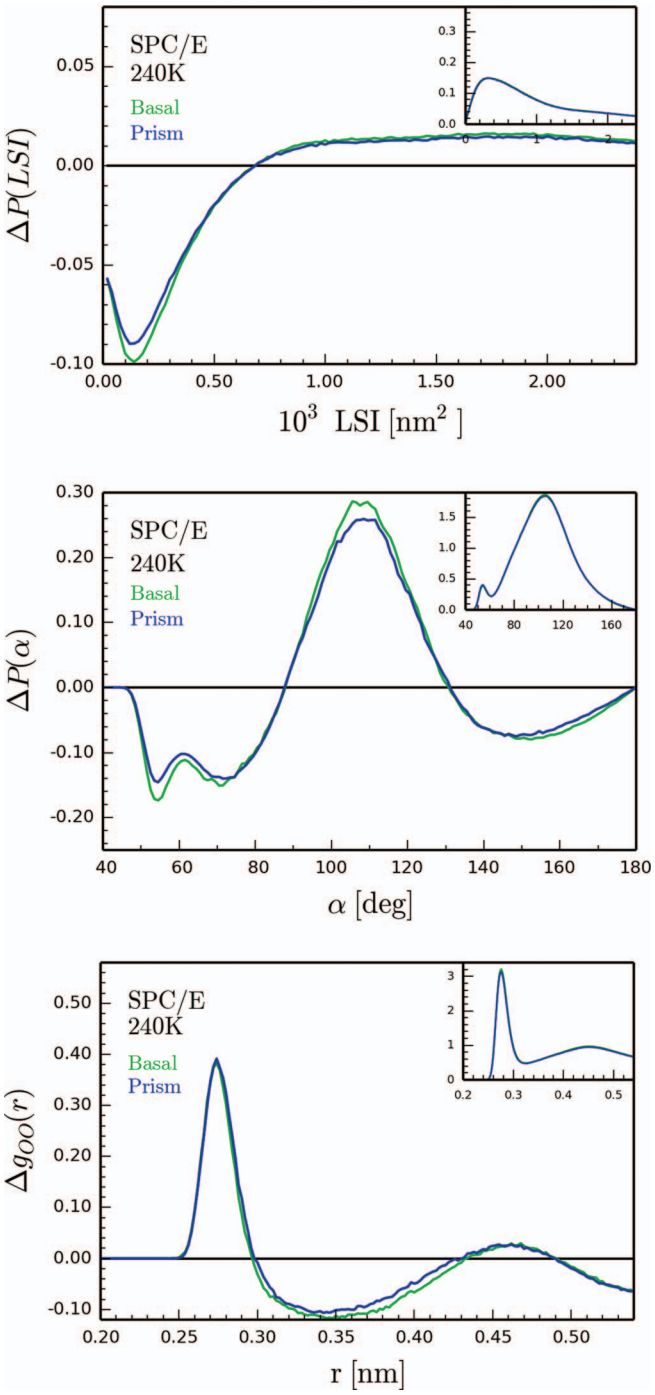

FIG. 3. The differential probability distributions $\Delta P(\text{LSI})$, $\Delta P(\alpha)$, and $\Delta g_{OO}(r)$ of the LSI, angle α, and radial distribution function (see text) for water in the first solvation shells of two crystallographic planes of ice (basal and primary prism), determined at 240 K (for SPC/E water model). The insets depict original distributions $P(\text{LSI})$, $P(\alpha)$, and $g_{OO}(r)$.

B. Analysis of the full form of two-particle correlation function: A local ordering parameter

So far, we have analyzed parameters that are able to measure only local structure of liquid (in immediate vicinity of a given water molecule), which are angle α and LSI. To calculate them, we take into account water molecules within short distances from a given water molecule (up to 0.33 nm[57] or up to about 0.37 nm, respectively). The radial distribution function is free from these restrictions, but the amount of information that it supplies is limited.

Much more information can be obtained from the analysis of the full water-water two-particle correlation function, which depends on both positions and relative orientations of two water molecules. In the past, Green[68] proposed to write the expression for entropy as a series of the n-particle correlation functions. Nettleton and Green,[69] followed much later by Raveché,[70] have derived a similar expansion for the entropy of open systems, and this original concept was used and broadened by many authors.[71–83] Although it allows for calculation of the absolute entropy of the system, it is difficult – or even impossible in practice – to determine the n-particle correlation functions for n higher than 2. Therefore, the higher terms are usually omitted; this is a so called two-particle approximation. However, the results obtained for Lennard-Jones fluid indicate[71,72] that the two-particle term, $s^{(2)}$, contributes about 85%–95% to the total excess entropy, which describes the difference between total entropy of the system and the one of an ideal gas. A very similar finding has also been reported[64] for liquid water. Thus, this leads to an important conclusion that two-particle correlation function describes, with sufficient accuracy, the overall structure of liquid phase. Therefore, the $s^{(2)}$ term can serve as a convenient and comparatively accurate measure of overall structural ordering of liquid.

Within the two-particle approximation, and using some simplifying assumptions, the expression for $s^{(2)}$ may be defined[75–78] as an integral from $g^{(2)}(r,\vec{\omega})\cdot\ln(g^{(2)}(r,\vec{\omega}))$, where $g^{(2)}(r,\vec{\omega})$ is a two-particle correlation function. The function depends on distance r and relative orientation $\vec{\omega}$ of two water molecules. This idea has been adopted to analyze the results of Monte Carlo and molecular dynamics simulations in order to derive thermodynamic properties of individual water molecules.[79,80] Moreover, as Lazaridis and Karplus[81] have demonstrated, it is possible to decompose the $s^{(2)}$ parameter into a sum of two components. The first component is radial; it is a translational ordering parameter s_{tra}, which depends only on distance between a pair of molecules. The second component is angular; it is an orientational ordering parameter s_{ort}, which depends on the relative orientation of a pair of molecules.

Basing on the ideas described above, we have introduced a local ordering parameter in order to describe structural properties of water around biomolecules.[82] It measures structural changes in hydration shell around a water molecule up to some cut-off distance, R_c. The local ordering parameter has been defined as the following integral of the two-particle correlation function, $g^{(2)}(r,\vec{\omega})$:

$$s^{(2)}(R_c) = -k_B\frac{\rho_W}{16\pi^2}\int_{r=0}^{r=R_c}\int_{\vec{\omega}}\{g^{(2)}(r,\vec{\omega})\ln[g^{(2)}(r,\vec{\omega})] - g^{(2)}(r,\vec{\omega})+1\}\,r^2\,dr\,d\vec{\omega}, \quad (2)$$

where r represents distance between two water molecules, while $\vec{\omega}$ represents five angles describing their relative orientation. Parameter ρ_W represents the number density of bulk water, k_B is the Boltzmann constant, and R_c is the cut-off distance corresponding to the limit of the second hydration shell

around a water molecule (equal to 0.58 nm[82,83]). Moreover, following the idea of Lazaridis and Karplus,[81] we also decomposed this quantity into three parts, called s_{tra}, s_{con}, and s_{orient}, where $s_{ort} = s_{con} + s_{orient}$, as described previously.[56,82]

Because these parameters depend on how the molecules are distributed and oriented around each other, even subtle structural changes in solvation water of proteins are expected to have an effect on their values.[56] Based on this assumption, we employed some concepts from the papers by Truskett *et al.*[84] and Esposito *et al.*[85] to construct ordering maps. Previously, we used two parameters, i.e., s_{tra} and s_{ort} to construct an ordering map that would depict structural changes occurring in water of simple polypeptides.[82] Herein, we would like to use the same idea to construct ordering maps in a slightly modified form.

The modification of local ordering parameters was put forward in Ref. 56. The modified values were calculated as differences between the parameter values for solvation water and bulk water filling space volume of real solvation shells (and of the same shape and size – just as it has been done to calculate the differential distributions of angle α and $g_{oo}(r)$). Moreover, we also argued that we can often replace the s_{ort} parameter, which is more difficult to calculate, with the much simpler s_{con} parameter. Parameter s_{con} usually converges quite well, but the value of parameter s_{ort} have to be obtained through extrapolation.[86] Parameter s_{con} uses only two angles (spherical coordinates) instead of five angles describing the orientation of a molecule from the surrounding area in relation to the central molecule. Therefore, the neighboring molecule is represented as a point (a center of mass).

In our modified ordering maps we use Δs_{tra}, Δs_{ort}, and Δs_{con} instead of s_{tra}, s_{ort}, and s_{con} to eliminate the influence of excluded volume, which is inaccessible to water molecules because of the protein's presence. Thanks to that the location of points on the modified map reflects changes in internal water structure comparing to properties of bulk water. It is a sensitive, convenient, and illustrative way to visually present changes in the properties of solvation water of the three surfaces of *Cf*AFP and the two crystallographic planes of ice. The relations $\Delta s_{tra} = f(\Delta s_{con})$ and $\Delta s_{tra} = f(\Delta s_{ort})$ are depicted in Figure 4.

In the case of parameter Δs_i (i = *tra, con*, or *ort*), the smaller (more negative) its values, the greater the local ordering of the molecules in solvation layer compared to ordering of bulk water. On the other hand, positive values of Δs_i indicate decreased ordering of water molecules relatively to bulk water.

The modified ordering maps were prepared for SPC/E water solvating *Cf*AFP. Unfortunately, we were unable to produce analogous maps for TIP5P water. As suggested by Kim and Yethiraj in their paper,[87] the reason for that might be the presence of chloride ions. Ions influence strongly dynamics and structure of TIP5P water at temperatures close to its melting temperature (274 K). Further discussion of this problem can be found in the supplementary material.[89]

The analysis of ordering maps brings up the differences between the structure of solvation water of plane 1 versus planes 2 and 3. The overall ordering of water once again appears to be greater near plane 1 than near the other two planes.

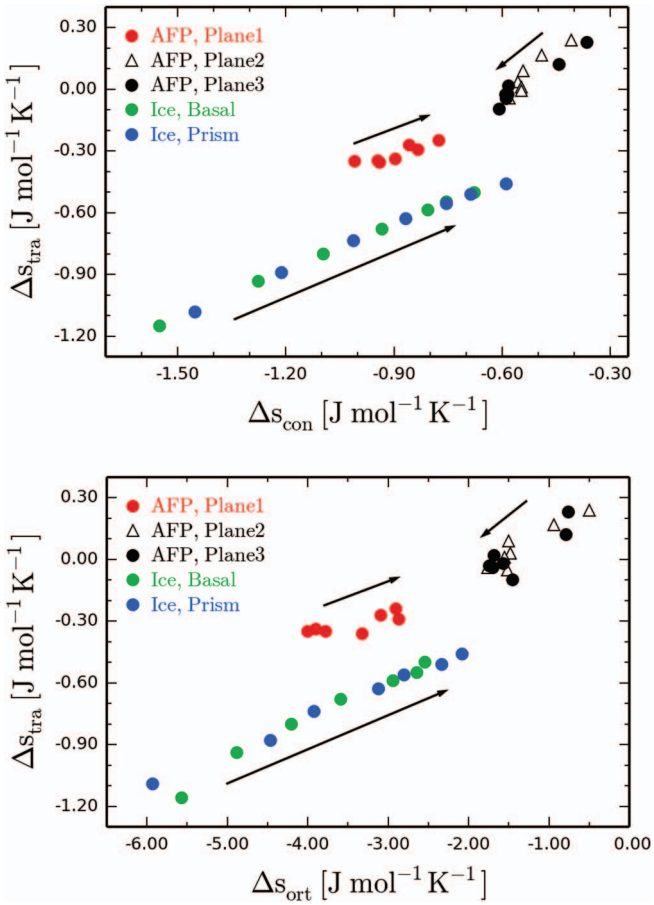

FIG. 4. Ordering maps – temperature dependences of the relation $\Delta s_{tra} = f(\Delta s_{con})$ and $\Delta s_{tra} = f(\Delta s_{ort})$, determined for solvation water (at the distance 0–1.2 nm) around three planes of *Cf*AFP for SPC/E water model and for solvation water (at the distance 0–1.0 nm) of two crystallographic planes of ice (basal and primary prism). The arrows indicate the direction of the increase of the temperature. The estimated statistical uncertainty is as follows: ±0.04 for Δs_{tra}, ±0.07 for Δs_{con}, and ±0.4 for Δs_{ort}.

Moreover, for plane 1, we observe a completely opposite temperature dependence of the values of these parameters compared to the rest of the protein. For solvation water of planes 2 and 3, a decrease in temperature is associated with increasing values of both translational and orientational local ordering parameters of solvation water (a decrease in local ordering compared to bulk water). For plane 1 the relationship is opposite, namely, the values of both parameters become more negative (an increase in local ordering compared to bulk water). Altogether, with decreasing temperature the overall (translational and orientational) ordering of solvation water of planes 2 and 3 becomes more similar to that of bulk water – the sum of parameters Δs_{tra} and Δs_{ort} is closer to zero. On the other hand, in the case of plane 1, this sum becomes more negative, therefore solvation water becomes less similar to bulk water.

As the discussed values suggest, these three solvation shells appear to be more similar to each other at higher temperatures and less similar at lower temperatures, what generally agrees also with findings of Nutt and Smith.[18] In one point, however, our conclusions differ. According to Nutt and Smith, the structural differences between solvation water of planes 2 and 3 and bulk water diminish at higher

temperatures. Our values of local ordering parameter indicate that the difference in structural ordering with bulk water increases with temperature for planes 2 and 3. The overall structural ordering of solvation water of the whole protein measured with local ordering parameter is greater than bulk water at all investigated temperatures. However, the greater ordering does not necessary have to mean greater tetrahedral ordering – it seems that we have in fact found less tetrahedral ordering around planes 2 and 3 at all temperatures, as described in Subsection III A.

It appears that at low temperatures the significance of directional interactions in solvation water of plane 1 is more profound, while planes 2 and 3 seem not to favor specific orientations to the same extent. On the other hand, the changes in values of the discussed parameters with temperature suggest that perhaps for a high enough temperature, we might be able to observe more orientationally ordered water near the planes 2 and 3 instead of plane 1. These remarks may bring to mind the unusual temperature changes in hydrophobic solvation entropy, which starts from negative values in cold water and ends with positive values in hot water. The observed relationship was explained by Xu and Dill[88] by directionality of hydrogen bonds. At low temperature, the drive to maximize hydrogen-bonding interactions is very strong. A hydrophobic solute restrains the orientational freedom of nearby molecules because it is incapable of forming hydrogens bonds. On the contrary, a hydrophilic solute offers alternative donors or acceptors of hydrogen bonds. In our case, plane 1 can be considered as partly hydrophobic, while planes 2 and 3 as hydrophilic. At high temperature, molecules move faster, many hydrogen bonds are broken, and a hydrophobic solute stimulates rearrangement of water molecules because it partly frees them from the restraining influence of their fellow water molecules.

The same temperature-dependent changes as for solvation water of plane 1 can be observed in the solvation water of basal and primary prism planes of ice. This finding supports the hypothesis that from the three planes of *Cf*AFP, the structural changes in solvation water of plane 1 are closest to changes in solvation water of ice. Yet again we can see that the structure of solvation water of ice is certainly different, that is, the respective points on the ordering map are quite far away from each other (but generally closer to plane 1 than to planes 2 and 3). Moreover, the sensitivity to temperature changes (within the investigated range 240 K–300 K) is significantly greater for solvation water of ice. It would certainly be difficult to call the surface of ice hydrophobic. Therefore we have to explain this phenomenon by simply assuming that highly ordered (and translationally immobile) water molecules at the ice surface force water molecules in the solvation layer to adopt only a limited range of orientations even more effectively than plane 1 does. This time, obviously, these restraints would originate not from avoiding the solute but from fitting into the ice structure to maximize the interactions.

IV. CONCLUDING REMARKS

The conducted analysis allows us to conclude that solvation water of *Cf*AFP is versatile. Water near the ice-binding plane (plane 1) is structurally different from water near the two remaining planes. The structural properties of solvation water at planes 2 and 3 seem to resemble typical structural properties of solvation water in "ordinary" proteins. However, water close to plane 1 behaves differently. Despite a similar density increase, the local tetrahedral ordering and general structural ordering, as measured with the aid of the two-particle correlation function, is greater. Surprisingly, this water also responds differently to temperature changes. As temperature grows, the structural changes of water at plane 1 measured with the local ordering parameters (Δs_i) increasingly resembles bulk water, just like solvation water of ice but opposite to solvation water of planes 2 and 3. We are inclined to explain it by the differences in chemical character and geometry of the protein surfaces.

Although there are some similarities between solvation water of plane 1 and the solvation water of ice, there are also significant differences, e.g., density variation. For solvation water of ice, density decreases. In the context of Tanaka model, we can suspect that the amount of low-energy, low-density structures in solvation water of plane 1 decreases, while it increases in the case of solvation water of ice. As we have previously hypothesized,[57,58] the proximity of a rough surface of a protein and its electric field both may contribute to destabilizing of high-volume structures of water and lead to their distortion and collapse. As a result, the density of nearby water will increase. These ordered structures can be considered as little seeds of ice in liquid water.[63] If there is a decrease in number of high-volume structures then it suggests that *Cf*AFP will probably not promote ice-like ordering and subsequent formation of some little ice nuclei in its vicinity, regardless of the size of the protein.

In the case of the solvation water of ice, the situation is different. Namely, water molecules in the neighborhood of ice surface accommodate to the crystal network. This most probably stimulates the creation of high-volume structures described in Tanaka model because water molecules simply recreate the pattern provided by the molecules on the ice surface. As a result, the density of solvation water decreases.

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