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Abstract

Structure and dynamics of biomolecules in supercooled water assume a particular and distinct importance in the case of antifreeze glycoproteins (AFGPs), which function at sub-zero temperatures. To investigate whether any large-scale structural digressions in the supercooled state are correlated to the function of AFGPs, self-diffusion behavior of the AFGP8, the smallest AFGP is monitored as a function of temperature from 243K to 303K using NMR spectroscopy. The experimental results are compared with the hydrodynamic calculations using the viscosity of water at the same temperature range. In order to evaluate results on AFGP8, the smallest AFGP, constituting approximately two-thirds of the total AFGP fraction in fish blood serum, similar experimental and computational calculations were also performed on a set of globular proteins. These results show that even though, the general trend of translational dynamics of AFGP is similar to that of the other globular proteins, AFGP8 appears to be more hydrated (approximately 30% increase in the bead radius) than the others over the temperature range studied. These results also suggest that local conformational changes such as segmental librations or hydrogen bond dynamics that are closer to the protein surface are more likely the determining dynamic factors for the function of AFGPs rather than any large-scale structural rearrangements.

Key Words

Supercooled water, protein, self-diffusion, antifreeze glycoprotein (AFGP).

Introduction

Structure and dynamics of water in the supercooled regime have been an important field in physical chemistry for many years [1]. Despite a long history of experimental and theoretical investigations, however, the nature of supercooled water is still not well understood [2]. Characterization of biomolecules dissolved in supercooled water has been implicated only recently as a means to obtain improved structural and dynamic properties of proteins and nucleic acids, and to gain insights into protein hydration and cold denaturation [3, 4]. Though it is interesting and important to understand the physical chemistry of proteins and protein-water interaction in supercooled water, most of the proteins are not required to function at these low temperatures. However, protein-water interactions in the supercooled state take a particular and distinct interest in a special class of proteins called the antifreeze glycoproteins (AFGPs). In this manuscript we investigate whether AFGPs undergo any global structural rearrangements in the supercooled regime that may be responsible for its function. This was addressed by measuring the self-diffusion coefficient of AFGP8, the smallest AFGP, as function of temperature in the presence of pulsed-field-gradient using NMR spectroscopy. To quantitatively evaluate the results on AFGP8, similar experimental procedures on a set of globular proteins and hydrodynamic calculations were also performed. To our knowledge, this is the first study on the translational dynamics of several proteins, including antifreeze glycoproteins, in the supercooled state of water.

Antifreeze proteins are present in fishes found in polar regions, allowing these species to survive even at sub-zero temperatures. There are two major classes of proteins that are responsible for the antifreeze function: glycosylated proteins, known as antifreeze glycoproteins (AFGPs), and non-glycosylated proteins, known as antifreeze proteins (AFPs) [5]. Extensive

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structural and dynamic characterization of AFPs [6]has been done, while such details are more limited in the case of AFGPs. In both cases, either the mechanism of antifreeze action is still under debate, or it has not been possible to identify a unique mechanism based on the podium of structure and dynamics. In this study, we focus our attention on the smallest of the AFGPs, AFGP8, which constitutes approximately two-thirds of the total functional AFGP in fish blood serum.

Biophysical characterizations of AFGP have clearly shown that it inhibits ice growth [5]. Solution state nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for obtaining both structural and dynamical information of biomolecules in the liquid state. Self-diffusion coefficient measurements in the presence of pulsed field gradients (PFG) are a well-established method for sampling translational motion [7]. Self-diffusion coefficient measurements have been widely used to study protein oligomerization and hydration, as well as to follow changes in complex formation [8, 9]. Here we present the experimental self-diffusion coefficient of AFGP8 as a function of temperature, through the supercooled regime (25° C to –16° C), and show that it does not undergo any major structural reorganization in order to function. The results are compared with similar experiments on other commonly available globular proteins. Hydrodynamic calculations as a function of temperature are also presented to monitor any changes from the non-ideal behavior of these proteins.

Materials and Methods

Protein Samples

AFGP was prepared from the arctic fish, *Boreogadus saida*, using the methods described previously, with no additional purification [10, 11]. AFGP8 corresponds to the fractions of molecular weight, 2.7 kD. Lysozyme, Ribonculease A and Ovomucoid (Turkey-III domain) were purchased commercially (Sigma) and used with no further purification.

Water used to dissolve the protein was passed through a MilliQ reverse osmosis system (Milli-RX12, Nihon Millipore Ltd., Yonezawa, Japan) and a 0.05 μm polycarbonate membrane (Coster Scientific Corp., MA). A total of 60 μL protein sample dissolved in water was taken in a 3mm (o.d., outer diameter) thin-walled NMR tube (Wilmad Inc.,). This tube was inserted in a 5mm (o.d.) thin-walled NMR tube, which was filled with $CDCl_3$ and used as the external lock for the NMR experiment. The concentration of AFGP8 was 10 mg/ml, while the other protein concentrations were: Lysozyme (12.6 mg/ml), Ribonuclease A (4.6 mg/ml) and Ovomucoid (6.5 mg/ml).

Self-Diffusion Coefficient Measurements

NMR experiments were performed in a Varian INOVA 600 MHz spectrometer equipped with a 5mm inverse probe with triple-axes shielded magnetic field gradients. Self-diffusion coefficient measurements as a function of temperature were obtained using a BPP-SED (bipolar-gradient pulse pair selective echo dephasing) sequence [12]. The basis of this experiment is a combination of the BPP-LED (bipolar-gradient pulse pair longitudinal-eddy-current delay) experiment [13] with improved water suppression. The experimental parameters were as follows: acquisition time, 0.328 s; spectral width, 12,500 Hz; signal averaging and 128 scans; recycling delay, 3s and water-selective pulse, 4 ms. Gradients were varied from 1 Gcm^{-1} to 32 Gcm^{-1} in units of 1.0 Gcm^{-1}, while the other gradients were applied at a strength of 30 Gcm^{-1} for 1 ms each, yielding a total echo time ($\tau_1+\tau_2$) of 14.026 ms. Phase cycling was used to advantageously utilize the radiation damping effects for water suppression as previously reported [12]. Time domain self-diffusion coefficient data were zero filled once, and a cosine bell apodization applied prior to complex Fourier transformation. The area under each spectrum from 5 to –1 ppm was integrated, and a non-linear least squares fit to Eq. [1] was used to estimate the diffusion coefficient [13]:

$$S(q) = S(0)\exp(-D_s q^2(\Delta-\delta/3-\tau/2)). \quad [1]$$

Here, S(q) is the measured integral value as a function of q, S(0) is the value at q=0, and q is the effective area of the gradient pulse, given by ($\gamma g_z \delta$), where γ is the proton gyromagnetic ratio (2.6752×10^8 s^{-1} T^{-1}) and g_z and δ are the amplitude and duration of the gradient pulse, respectively. In Eq. [1], D_s is the translational diffusion coefficient, represented in units of m^2s^{-1}, while Δ and τ are delays employed in the pulse sequence, represented in seconds.

The gradient strength was calibrated using the known diffusion constant of water, 2.30×10^{-9} m^2s^{-1} at 298 K [14]. The chemical shift difference between the methyl and hydroxyl groups in methanol was used for temperature calibration [15]. The cooling rate between the temperatures was approximately 0.1K/minute. More than 15 minutes was allowed for temperature stabilization between the experiments. Independent calibrations performed on a water sample under the same conditions show that a 15-minute equilibrium period between the experiments for temperature stabilization is sufficient. All the experiments were repeated at least twice to obtain experimental error bars.

Hydrodynamic Calculations

Translational diffusion tensor values were calculated based on the beads model approximation of García de la Torre and Bloomfield [16]. This method has been used successfully by several groups to calculate translational as well as rotational diffusion tensors of proteins [8, 17, 18]. In this method, the protein is modeled as a collection of point sources of friction (denoted as beads) with hydrodynamic tensor interactions between them. The rotational diffusion tensor is calculated from a set of linear equations solved by integrating a $3N \times 3N$ matrix, where N is the number of atoms determined from the structure of the protein. The program DIFFC, based on the beads theory [17], was used in the present work. All atoms were

considered as beads of equal size for three different values (σ = 3.1, 5.1 and 6.6 Å) [19] for purposes of calculating the diffusion tensor as a function of temperature. Experimental values of the viscosity (in Nsm^{-2}) of pure water in the supercooled regime [1, 20] were used. The overall isotropic translational self-diffusion coefficient was calculated by taking the average of the principal values of the diffusion tensor. A total 15 temperature values ranging from 243 K to 313 K were calculated. For AFGP8, the average of the diffusion tensor calculated over the 10 NMR determined solution structures [21] was used. The standard deviation of the isotropic diffusion coefficient is less than 5%. Three-dimensional structural coordinates for all the other proteins were obtained from the protein data bank (http://www.rcsb.org/pdb/) (Table 1).

Results

Figure 1 shows the temperature dependence of the self-diffusion coefficient of AFGP8 (Fig. 1b) in comparison with the self-diffusion coefficient of pure water (Fig. 1a), obtained from the literature [22]. In this temperature range, the diffusional behavior of water is well represented by the Vogel-Tamman-Fulcher (VTF) type relationship derived for glass-forming liquids [23], as evidenced by the continuous curve in Fig.1a. In order to understand the temperature dependence of AFGP8, however, a complete set of hydrodynamic calculations was performed. Figure 1b shows the plots of calculations for three different bead sizes, σ = 3.1, 5.1 and 6.6 Å, as small dashed, continuous and long dashed lines, respectively. Figures 1c, d and e show the representation of the three dimensional structure of AFGP8 at these bead sizes. Molecular properties obtained from the three dimensional structure (pdb codes, molecular weight, radius of hydration and solvent accessible surface area) are given in table 1. Figure 1b shows that the experimental temperature dependence of AFGP8's self-diffusion coefficient closely follows the

hydrodynamic calculations. This suggests that there are no major changes in the three-dimensional structure of AFGP8 as a function of temperature, including the supercooled regime.

Figure 2 shows the plots of the experimental self-diffusion coefficients as a function of temperature for three other proteins, and their respective hydrodynamic calculations. Figure 2a shows results of Ovomucoid, a globular glycoprotein, while Figures 2b and 2c correspond to the results for Ribonuclease A and Lysozyme, respectively. All these proteins qualitatively tend to follow the behavior predicted by the hydrodynamic calculations using a bead size of $\sigma = 5.1$ Å. Larger deviations were observed in the room temperature regime for Ovomucoid (Figure 2a). In the case of Lysozyme, though it was not possible to perform measurements in the supercooled state (because the sample was frozen), the data from zero to room temperature provide a good qualitative fit to hydrodynamic calculations. Table 1 also lists the relevant structural parameters for these proteins. In figures 1 and 2, the experimental and theoretical values are plotted as they are, and no attempt has been made to fit the data.

Discussion

Temperature dependence of the self-diffusion of proteins in solution can, in general, be represented by the generalized Stokes-Einstein relationship [24],

$$D_s = k_B T/(6\pi\eta R_H), \qquad [2]$$

where k_B is Boltzmann's constant (1.3806×10^{-23} $m^2kgs^{-2}K^{-1}$), T is the temperature in K, η is solvent (water) viscosity (Nsm^{-2}) and R_H is the effective hydrodynamic radius of the protein. R_H can also be expressed in terms of molecular weight (MW) as

$$R_H = F(3\ MW\ V_p/4\pi N_A)^{1/3}. \qquad [3]$$

Here, F is Perrin's shape-factor [25], V_p is the partial specific volume (in m^3kg^{-1}) and N_A is Avogadro's number (6.02217×10^{23} mol^{-1}). In the case of nearly spherical proteins (shape-factor,

$F \approx 1$), it is possible to estimate either R_H (Eq. [2]) or the shape-factor (Eq. [3]) to account for the hydrodynamic radius from these measurements. However, this is particularly difficult for proteins of arbitrary shape, as no independent estimation of F is available. Even though calculation of the hydrodynamic properties using the beads model accounts for the exact shape of the protein, these calculations do not account for protein hydration directly. Qualitatively, uniform hydration effects can be included indirectly by assuming a radius for the beads larger than that of the atoms. In our calculations for AFGP8, beads of size 6.6 Å tend to provide a good agreement with the experiment, while a bead size of 5.1 Å seems appropriate for all the other proteins. This nearly 30% increase in bead size suggests that AFGP8 is considerably more hydrated than the other globular proteins, including ovomucoid another glycoprotein. Garcia de la Torre et al. [26] have shown that a bead size of approximately 5 Å fits the majority of the experimental rotational correlation times. The need for a larger bead size in the case of AFGP8 probably suggests that it is much more hydrated than regular proteins (nearly a factor of two on the basis of volume ratios), and this concept is in agreement with the structural studies by quasi-elastic light scattering experiments [11] and NMR [21].

There are eight known fractions of AFGP that range in molecular mass from 33.7 to 2.6 kD [27], and each consists of a number of repeating units of alanine-alanine-threonine, with minor sequence variations. AFGP8 is the shortest, with 4 repeating units, and AFGP1 the longest, with 50 repeating units. The threonines are glycosylated at the Cβ position with the disaccharide β-D-galactopyranosyl- (1,3) –2-acetamido-2- deoxy-α-D-galactopyranose. The longer glycopeptides, typified by AFGP2-5, are as much as 20 times more active on a molar basis in lowering the freezing temperature of solution than the shorter ones, here represented as AFGP8 [28]. Structurally, AFGP8 has proline following some threonine, whereas AFGP2-5 exhibits a regular

alanine-alanine-threonine sequence. In this work, glycopeptides from the Greenland cod, *Boreogadus saida,* were studied. Even though AFGP8 is only about 25% as active as the larger AFGPs on a weight basis, its role is significant in function. For example, though the serum of the fish contains all AFGP fractions, it is interesting to note that the tissues contain only AFGP8. Further, it has been noted that the function of AFGP8 is potentiated in the presence of higher molecular weight fractions of AFGPs.

In contrast to the structural and dynamic information on AFPs [6], including the sampling of rotational correlation time in the supercooled state [29], structural and dynamic information on AFGPs are scarce. Unfortunately, no single molecular mechanism has emerged, to date, to explain AFGP ice binding affinity and specificity. AFPs show considerable variation in secondary structure, from single α or β helices, to mixed α/β, fold. The only common characteristic between AFPs and AFGPs is that they are all stable at or near 0°C.

The NMR based methods used here can sample the translational motion of AFGP8. One of the major problems in performing these experiments in the supercooled regime is the relatively bulky nature of the sample itself. Even when a given sample is cooled slowly, the lowest possible temperature obtained varies, and it is sometimes not possible to reach the supercooled state due to spontaneous nucleation due to small disturbances. The purity of the protein and water, as well as the cleanliness of the NMR tubes, also tend to play critical roles. In the case of pure water, Price et al. [22] have been able to attain supercooled temperatures as low as 238 K by using a small volume (0.5 μL) in a fine capillary (0.13-mm.13 mm inner diameter). In order to achieve the sensitivity necessary for the NMR signal, we required a much larger amount of protein and hence larger solution volume). Temperature dependence of self-diffusion coefficients (not in the supercooled regime) has been used to study translational dynamics of peptides [30]

and DNA oligomers [31]. In these studies, instead of monitoring the changes in D vs. T directly, $D\eta/T$ (where η is the solution viscosity) vs. T is monitored. $D\eta/T$ is sometimes referred to as the shape-factor (not Perrin's shape factor), and is inversely related to effective hydrodynamic radius (Eq. [2]). However, in the supercooled regime, the rate of change of the solution viscosity as a function of temperature is much more rapid than at room temperature [1, 20].

Conclusions

Although there has been a great interest in understanding the mechanism of antifreeze action in the last decade [5, 6, 32, 33], there is no consensus description. The problem is in general approached from two different points of view: methods that attempt to understand the ice inhibition process, and methods that correlate structure (dynamics) to function. These can be roughly considered to be macroscopic and microscopic (molecular level) methods, seldom with cross-talk between them. On the macroscopic level, the mechanism is regarded as an adsorption-inhibition process, in which AFGP binds to the surface of a growing ice crystal. At the microscopic level the question of how exactly these proteins bind to the surface of ice remains a source for intense debate and investigation [5, 6, 33]. The only known family of AFGP structures is that of fraction 8 [21]. However, these structures were determined above the ice-point temperature, and lack long-range order.

One fundamental question that remains to be addressed is whether AFGPs undergo any large-scale conformational change upon binding to ice. The current experiments examine the possibility of an impending onset of changes that might allow for binding to ice. If any rearrangement of the AFGP structure is mandated, those must arise from local conformational changes, such as hydrogen bond dynamics or internal rotations as the self-diffusion coefficient measurements of AFGP8 through the supercooled regime do not show any large-scale structural

rearrangements. Residue specific sampling of dynamics in the presence of water, supercooled water and in ice, in addition to molecular dynamics might provide additional insight toward solving the puzzle of antifreeze function in AFGPs.

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Table 1: Structural parameters of the proteins.

Protein	[a]PDB	Molecular Weight (kD)	[b]R_g (Å)	[c]SASA (Å^2)
AFGP8	[d]PC	2.7	9.26	2012
Ovomucoid	2OVO	6.0	11.90	3239.3
Ribonuclease A	7RSA	13.7	15.66	7442.9
Lysozyme	1E8L	14.3	15.53	7580.2

[a] Three dimensional structural coordinates from protein data bank (http://www.rcsb.org/pdb/)

[b] R_g: Radius of gyration calculated from respective coordinates

[c] SASA: Solvent accessible surface area calculated with a probe radius of 1.4 Å using MOLMOL.

[d] Obtained through personal communication (Andrew Lane).

Figure Captions

Figure 1: Plot of the self-diffusion coefficient of supercooled water (a) and supercooled solution of AFGP8 in water (b). Curves in (a) and (b) correspond to Vogel-Tamman-Fulcher (VTF)-relationship and hydrodynamic calculations, respectively. Hydrodynamic calculations were performed using beads model approach with three different sizes for the beads; σ=3.1Å (small dashed), 5.1 Å (continuos) and 6.5 Å (long dashed). The respective molecular representation of the size of the beads are depicted in (c), (d) and (e). Experimental points in (a) were obtained from Price et al [22]. Standard deviation in the experimental values of AFGP (b) was obtained by duplicate measurements (size of the symbols larger than the error bars) and no attempt has been made to fit the experimental data to hydrodynamic calculations. Vertical dashed lines are drawn at 273 K (0 °C).

Figure 2: Plots of the temperature dependence of self-diffusion coefficients. (a) Ovomucoid, a glycoprotein (b) Ribonculease A and (c) Lysozyme. Continuous curves are the respective hydrodynamic calculations using beads model (uniform radius of 5.1 Å for all atoms). No attempt is made to fit the experimental data to hydrodynamic calculations. Experimental error bars are obtained with duplicate measurements and the vertical dashed lines represent 273 K (0 °C).

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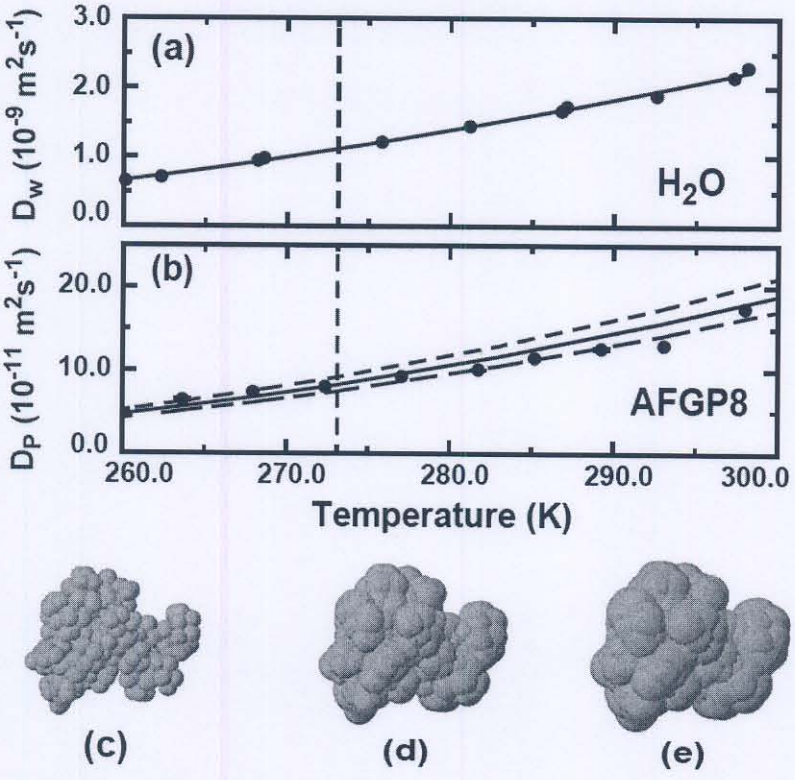
Fig 1


(a)
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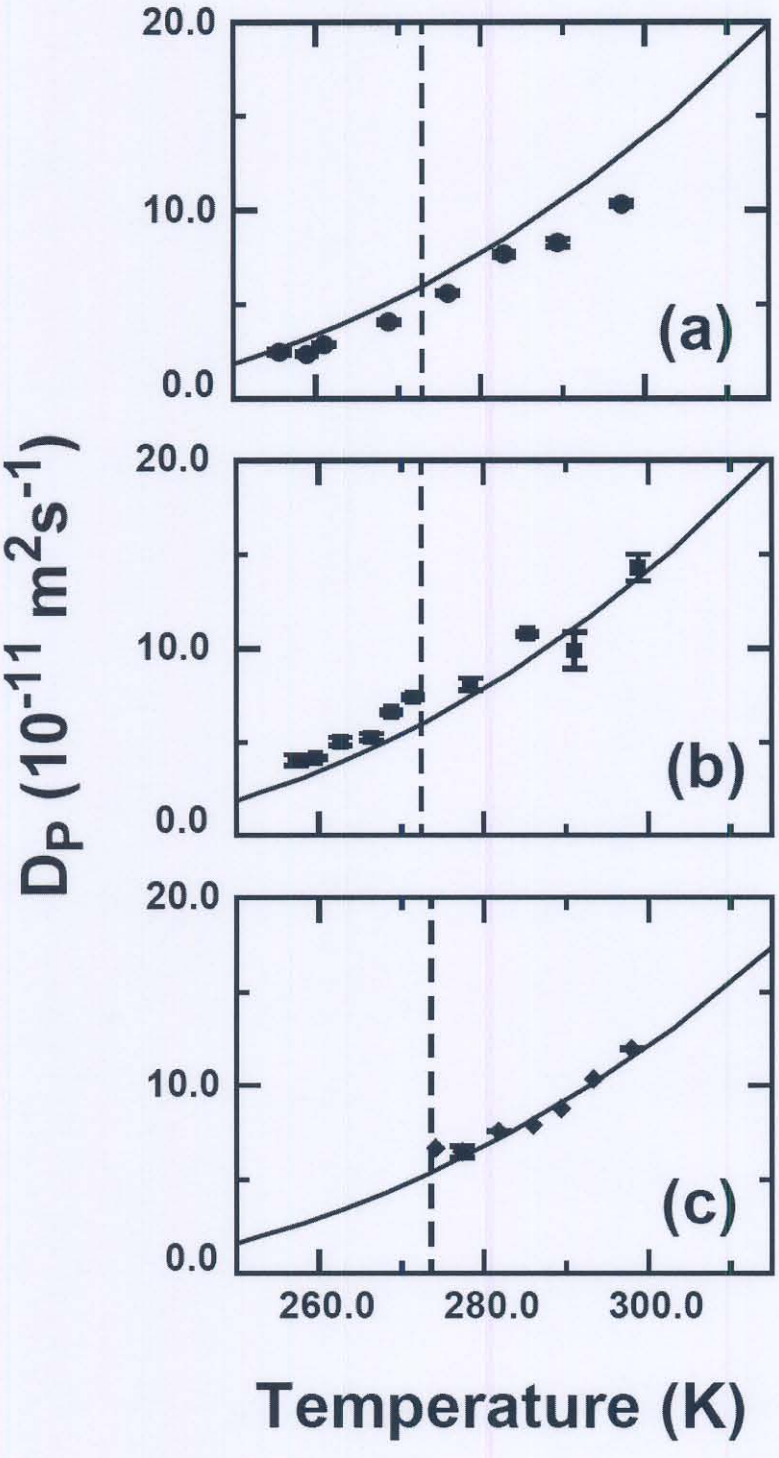
2.0
1.0
0.0
H_2O
(b)
D_P (10^{-11} m^2s^{-1})
20.0
10.0
0.0
AFGP8
260.0
270.0
280.0
290.0
300.0
Temperature (K)
(c)
(d)
(e)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Temperature (K)
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Figure 2