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Biophysical Chemistry 110 (2004) 223–230

Biophysical Chemistry

www.elsevier.com/locate/bpc

Translational dynamics of antifreeze glycoprotein in supercooled water

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Received 17 January 2004; received in revised form 23 February 2004; accepted 23 February 2004
Available online 27 April 2004

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 243 to 303 K using nuclear magnetic resonance (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.

Keywords: Supercooled water; Protein; Self-diffusion; Antifreeze glycoprotein (AFGP)

1. 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 pro-

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doi:10.1016/j.bpc.2004.02.005

teins 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 their function. This was addressed by measuring the self-diffusion coefficient of AFGP8, the smallest AFGP, as a function of temperature in the presence of pulsed field gradient (PFG) using nuclear magnetic resonance (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 (AFPs) 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 AFGPs, and non-glycosylated proteins, known as AFPs [5]. Extensive 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 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.

There are eight known fractions of AFGP that range in molecular mass from 33.7 to 2.6 kDa [7], and each consists of a number of repeating units of alanine–alanine–threonine*, with minor sequence variations (*represents the glycosylated threonines at the Cβ position with the disaccharide β-D-galactopyranosyl-(1,3)-2-acetamido-2-deoxy-α-D-galactopyranose). AFGP8 is the shortest, with four repeating units, and AFGP1 the longest, with 50 repeating units. 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 [8]. 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.

Biophysical characterizations of AFGP8 have clearly shown that it inhibits ice growth [5]. Solution state 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 PFG are a well-established method for sampling translational motion [9]. Self-diffusion coefficient measurements have been widely used to study protein oligomerization and hydration, as well as to follow changes in complex formation [10,11]. Here, we present the experimental self-diffusion coefficient of AFGP8 as a function of temperature, through the supercooled regime (25 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.

2. Materials and methods

2.1. Protein samples

AFGP was prepared from the arctic fish, *B. saida*, using the methods described previously, with no additional purification [12,13]. AFGP8 corresponds to the fractions of molecular weight, 2.7 kDa. Lysozyme, ribonuclease 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, Yonezawa, Japan) and a 0.05-μm polycarbonate membrane (Coster Scientific, MA). A total of 60-μl protein sample dissolved in

water was taken in a 3-mm (o.d., outer diameter) thin-walled NMR tube (Wilmad). This tube was inserted in a 5-mm (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).

2.2. Self-diffusion coefficient measurements

NMR experiments were performed in a Varian INOVA 600 MHz spectrometer equipped with a 5-mm inverse probe with triple-axes shielded magnetic field gradients. Self-diffusion coefficient measurements as a function of temperature were obtained using a *b*ipolar-gradient *p*ulse *p*air *s*elective *e*cho *d*ephasing (BPP-SED) sequence [14]. The basis of this experiment is a combination of the *b*ipolar-gradient *p*ulse *p*air *l*ongitudinal-*e*ddy-current *d*elay (BPP-LED) experiment [15] 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, 3 s; and water-selective pulse, 4 ms. Gradients were varied from 1 to 32 G cm^{-1} in units of 1.0 G cm^{-1}, while the other gradients were applied at a strength of 30 G cm^{-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 [14]. 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 [15]:

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

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^2 s^{-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^2 s^{-1} at 298 K [16]. The chemical shift difference between the methyl and hydroxyl groups in methanol was used for temperature calibration [17]. The cooling rate between the temperatures was approximately 0.1 K/min. More than 15 min was allowed for temperature stabilization between the experiments. Independent calibrations performed on a water sample under the same conditions show that a 15-min equilibrium period between the experiments for temperature stabilization is sufficient. All the experiments were repeated at least twice to obtain experimental error bars.

2.3. Hydrodynamic calculations

Translational diffusion tensor values were calculated based on the beads model approximation of García de la Torre and Bloomfield [18]. This method has been used successfully by several groups to calculate translational as well as rotational diffusion tensors of proteins [10,19,20]. 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 $3N\times3N$ matrix, where N is the number of atoms determined from the structure of the protein. The program DIFFC, based on the beads theory [19], was used in the present work. All atoms were con-

Table 1
Structural parameters of the proteins

Protein	PDB[a]	Molecular weight (kDa)	R_g^b (Å)	SASA[c] (Å^2)
AFGP8	PC[d]	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).

sidered as beads of equal size for three different values (σ=3.1, 5.1 and 6.6 Å) [21] for purposes of calculating the diffusion tensor as a function of temperature. Experimental values of the viscosity (in N sm^{-2}) of pure water in the supercooled regime [1,22] 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 to 313 K were calculated. For AFGP8, the average of the diffusion tensor calculated over the 10 NMR determined solution structures [23] 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).

3. Results

Fig. 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 [24]. 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 [25], 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. Fig. 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. Fig. 1c–e

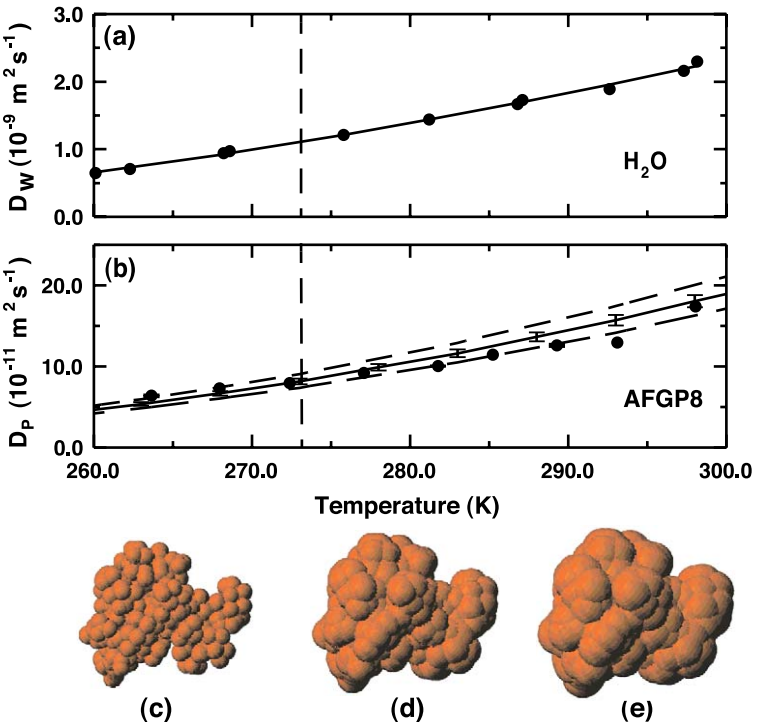

Fig. 1. Plot of the self-diffusion coefficient of supercooled water (a) and supercooled solution of AFGP8 in water (b). Curves in (a) and (b) correspond to VTF relationship and hydrodynamic calculations, respectively. Hydrodynamic calculations were performed using beads model approach with three different sizes for the beads: σ=3.1 Å (small dashed), 5.1 Å (continuous) and 6.5 Å (long dashed). The respective molecular representation of the size of the beads are depicted in (c), (d) and (e). The error bars in the continuous curve of (a) corresponds the standard deviation of the calculated diffusion constants across the ensemble 10 NMR determined structures. Experimental points in (a) were obtained from Price et al. [24]. 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).

shows the representation of the three-dimensional structure of AFGP8 at these bead sizes. The error bars in the continuous lines (σ=5.1 Å) in Fig. 1b corresponds to the standard deviation of the diffusion coefficient over the 10 different structures of AFGP. The error bars for the other σ values are not shown. Typically, the diffusion coefficients across the structures varies between 4.5% and 6%. 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. Fig. 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.

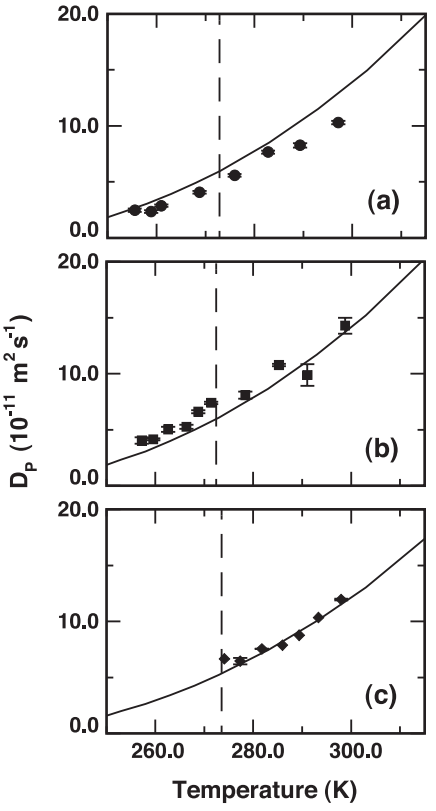

Fig. 2. Plots of the temperature dependence of self-diffusion coefficients. (a) Ovomucoid, a glycoprotein (b) ribonuclease A and (c) lysozyme. Continuous curves are the respective hydrodynamic calculations using beads model (uniform radius of 5.1 Å for all atoms). No attempt is made to fit the experimental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

Fig. 2 shows the plots of the experimental self-diffusion coefficients as a function of temperature for three other proteins, and their respective hydrodynamic calculations. Fig. 2a shows results of ovomucoid, a globular glycoprotein, while Fig. 2b and c corresponds 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 σ=5.1 Å. Larger deviations were observed in the room temperature regime for ovomucoid (Fig. 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 Figs. 1 and 2, the experimental and theoretical values are plotted as they are and no attempt has been made to fit the data.

4. Discussion

Temperature dependence of the self-diffusion of proteins in solution can, in general, be represented by the generalized Stokes–Einstein relationship [26],

$$D_{\mathrm{p}} = k_{\mathrm{B}}T/(6\pi\eta R_{\mathrm{H}}), \tag{2}$$

where k_{B} is Boltzmann's constant (1.3806×10^{-23} m^2 kg s^{-2} K^{-1}), T is the temperature in K, η is solvent (water) viscosity (N sm^{-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

$$R_{\mathrm{H}} = F(3\mathrm{MW}V_{\mathrm{p}}/4\pi N_{\mathrm{A}})^{1/3}. \tag{3}$$

Here, F is Perrin's shape-factor [27], V_{p} is the partial specific volume (in m^3 kg^{-1}) and N_{A} is Avogadro's number (6.02217×10^{23} mol^{-1}). In the case of nearly spherical proteins (shape-factor, $F\approx1$), 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. [28] 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 (QELS) experiments [13] and NMR [23]. Though we have not tried to fit the experimental results to a particular model, the comparison of the temperature dependence of AFGP8's diffusion coefficient with hydrodynamic calculations inherently depends on the kind of boundary layer conditions (slip, stick or intermediate). The theoretical lower limit for the intrinsic viscosity of a spherical particle obeying stick hydrodynamic boundary conditions is the Einstein value. Though this limit is different for slip boundary conditions, there is a general consensus in that proteins in water are better described by stick rather than slip behavior [21]. In comparison with other hydrodynamic models, the current model tends to predict the hydrodynamic properties of proteins in general with a single variable parameter, σ.

Comparison of the diffusion behavior of the other proteins (Fig. 2) and that of AFGP8 (Fig. 1) though tend to suggest that it behaves like a globular protein in the solution state, other NMR-based dynamic measurements at room temperature by Lane et al. [29] show that these molecules do not assume a particular secondary structure but are segmentally flexible. These are further supported by the temperature dependent QELS [13] and FT-IR experiments [29,30].

As AFGPs are known to undergo segmental motion, on the translational diffusion time scale we assume that the various conformations exchange rapidly such that the measured diffusion coefficient represents the population weighted average of those inter converting conformations that have similar three-dimensional structures. The 10 different structures that we have used to predict the hydrodynamic behavior of AFGP8 are NMR determined structures. The experimentally measured diffusion coefficient reflects the population of AFGP8 molecules that dominates the NMR spectrum have not undergone any large-scale structural transition upon supercooling. If there were another measurable population of structures present upon supercooling the NMR data would reflect these effects by (a) changes in the spectral features due to chemical shift changes triggered by the structural changes and (b) failure to fit the experimental diffusion constants to single exponential (Eq. (1)). Neither of these were observed in our experimental data.

5. Conclusions

If AFGP assumes a distinct structural scaffold in order to function, this might involve intermolecular (with water) as well as intramolecular hydrogen bonds. NMR and molecular modeling studies of mucin type model glycopeptides suggest that intramolecular hydrogen bonding between the amide proton of *N*-acetylgalactosamine (GalNAc) and the carbonyl oxygen of the threonine to which it is covalently linked is probably a necessary interaction to stabilize the backbone structure with respect to the side chain sugars [31]. Molecular dynamics simulations [32] suggest a high degree of mobility for the side-chain sugar moieties relative to the protein backbone. It is normal to expect that the hydrogen bonding interactions, which are stronger in the supercooled regime, are significantly weaker at room temperature. Hence, the variation in the overall structure is considered to be a combination of destabilizing interactions (lack of hydrogen bonds) and increased mobility. Recently, in a comprehensive study using solid-state NMR and FT-IR experiments, Tsvetkova et al. [30] have shown that AFGPs (both AFGP8 and AFGP-2-5) undergo dynamical changes when frozen.

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. As the AFGP8 does not show deviation from ideal behavior upon supercooling, any anticipated structural changes in the frozen state may be predominantly due to protein-ice interaction and must arise from local conformational changes, such as hydrogen bond dynamics or internal rotations. Residue specific sampling of dynamics in the presence of water, supercooled water and in ice, are necessary to gain additional insight.

Acknowledgements

The authors thank the members of AFGP research group (Dr. N. Tsvetkova, D. Nguyen, S. Zepeda and S. Mielke) for many discussions. This work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory, under Contract No. W-7405-Eng-48.

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