Characterization of Waters of Hydrophobic Hydration by Microwave Dielectric Relaxation

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In the present paper, a temperature dependent dielectric relaxation near 5 GHz (at a frequency just lower than that of bulk water) is observed in aqueous solutions of hydrophobic elastic protein-based polymers, such as $(GVGVP)_{251}$ and $(GVGIP)_{260}$. On dilution at low temperatures of the solution, this relaxation becomes more intense approaching different hydrophobicity dependent limits as the hydrophobicity increases from Val (V) with the side chain $-CH(CH_3)_2$ to Ile (I) with the addition of a CH_2 moiety (i.e., $-CH(CH_3)CH_2CH_3$). The relaxation decreases in intensity to near 0 as the temperature of solutions of the elastic protein-based polymers are raised from below to above their respective inverse temperature transitions of hydrophobic folding and assembly. Furthermore, using the polymers $(GEGXP\ GVGVP\ GVGVP\ GVGVP\ GVGX-P)_n$ where the two X residues are either two V or two Phe (F) residues with the aromatic phenyl side chain of $-CH_2C_6H_5$, ionization of glutamic acid (E) side chains (i.e., the formation of COO^- from $COOH$) destroys the majority of the waters of hydrophobic hydration in a charge density dependent manner down to a limit suggestive of remaining pentagonally arranged waters previously observed in crystal structures[1,2] adjacent to hydrophobic moieties. This paper characterizes, for the first time, waters of hydrophobic hydration (N_{hh}) in terms of the variables of dilution, temperature and polymer charge density. In the absence of charge, N_{hh} appears to be more extensive than the first shell of pentagonally arranged waters. The significance of this characterization resides in the widely held view that the thermodynamics of waters of hydrophobic hydration is central to the hydrophobic folding and function of proteins and protein-based polymers.[3−7]

Previous dielectric relaxation studies extending into the microwave (supra gigahertz) range have been reported on proteins such as myoglobin,[8,9] lysozyme,[10] and collagen,[11] and the ca. 10 GHz relaxation was, indeed, recognized as arising from protein hydration. For several reasons, however, the previous protein studies were unable to correlate with hydrophobic hydration any part of the relaxations ascribed to protein hydration. First, only a very small part of the hydration could
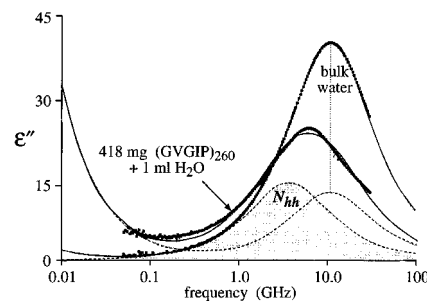

Figure 1. Imaginary part of the dielectric permittivity, ϵ'', in the 0.1− 26.5 GHz range for pure (bulk) water and for 1 mL of pure water plus 418 mg of $(GVGIP)_{260}$. The solution curve is resolved into the curve for bulk water and a more intense additional relaxation which represents the water interacting with the protein-based polymer.

be due to the presence of hydrophobic groups on the surface of these native proteins. Second, transitions to hydrophobically unfolded states (e.g., cold denaturation) would have to occur under conditions where a substantial part of the total water was hydrophobic hydration. Third, the transitions would have to be thermally accessible and well-characterized as dominantly hydrophobic. Finally, the variables that favor or disrupt hydrophobic hydration have not been identified in more complex proteins as they have for the elastic protein-based polymers.

Characterization of waters of hydrophobic hydration becomes possible with elastic protein-based polymers, because these model proteins exhibit phase transitional behavior.[7] When the temperature is raised, the elastic protein-based polymers hydrophobically fold and assemble to form a more dense phase, called a coacervate, which is of the order of 50% polymer and 50% water by weight. The temperature at which the transition occurs decreases on increasing the hydrophobicity of the amino acid side chains and increases on increasing the polar character of the amino acid side chains. Utilizing this property, a hydrophobicity scale for amino acid residues has been developed based on the temperature T_t for the onset of the hydrophobic folding and assembly transition.[12] Furthermore, a host of variables that change the expression of hydrophobicity (i.e., that raise or lower T_t) have been systematically studied.[7]

Previous dielectric relaxation studies ranging from 1 MHz to 1 GHz on elastic protein-based polymers had suggested a hydrophobicity dependent relaxation at frequencies greater than 1 GHz.[13,14] Additional studies on the inverse temperature transitional properties of these compositionally varied hydrophobic elastic protein-based polymers had been interpreted to indicate (1) that the waters of hydrophobic hydration should increase to different limits as dilution occurs in a manner which increases with increasing hydrophobicity, (2) that the quantity of water of hydrophobic hydration should decrease as the temperature is raised from below to above the temperature of the hydrophobic folding and assembly transition, (3) that waters of hydrophobic hydration should be destroyed as functional side chains such as carboxyls become ionized,[15,16] and (4) that this competition for hydration between hydrophobic and charged groups can result in large hydrophobic-induced pK_a shifts.[17−20]

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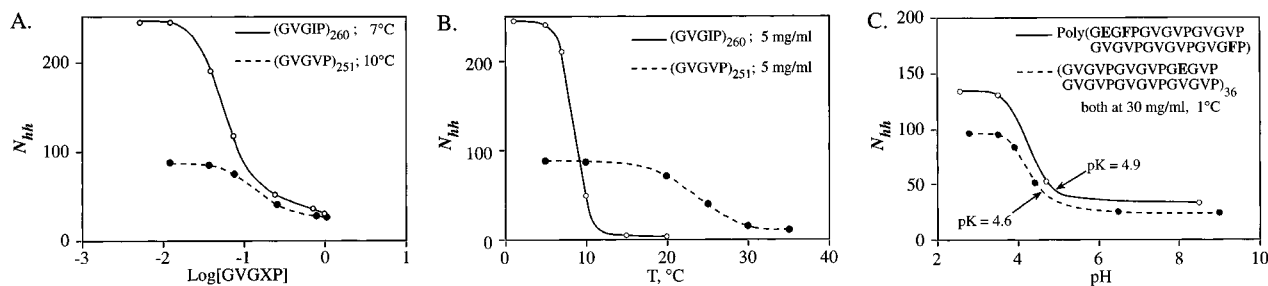(19) Urry, D. W.; Gowda, D. C.; Peng, S. Q.; Parker, T. M. *Chem. Phys. Lett.* **1995**, *239*, 67−74.

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Figure 2. Plots of the estimated numbers of waters of hydrophobic hydration, N_{hh}, for solutions of several protein-based polymers as a function of several variables. (A) Plots of N_{hh} as a function of the pentamer concentration as log[GVGXP] for (GVGIP)$_{260}$ at 7 °C and (GVGVP)$_{251}$ at 10 °C. Note, on dilution, that the values of N_{hh} increase to limits that are greater for the former which has one additional CH_2 moiety per pentamer. (B) Plots of N_{hh} as a function of temperature for 5 mg/mL each of (GVGIP)$_{260}$ and (GVGVP)$_{251}$. Note that the values of N_{hh} begin at the dilution limit and decrease to 0 or near 0 over the temperature ranges for the respective hydrophobic folding and assembly transitions. (C) Plots of N_{hh} for two glutamic acid residue-containing polytricosapeptides, namely, chemically synthesized poly(GEGFP GVGVP GVGVP GVGVP GVGVP GVGFP) and microbially prepared (GVGVP GVGVP GEGVP GVGVP GVGVP GVGVP)$_{36}$, as a function of pH at 1 °C and 30 mg/mL solutions. Note, when fully protonated, that the polymer with the more hydrophobic phenylalanine residue, Phe(F), has a higher N_{hh} and that the values of N_{hh} for both protein-based polymers decrease to slightly different limits as the number of charges increases, but not in a manner proportional to the degree of ionization.

The protein-based polymers were prepared by genetic engineering and microbial biosynthesis and purified utilizing the inverse temperature transition as previously described.[21]

The dielectric relaxation studies utilized the Hewlett Packard HP 8722C Network Analyzer, an HP Vectra PC with HP 85070 software, and an HP 85070B coaxial probe with frequency ranging from 50 MHz to 26.5 GHz. The calibration standards were air, a short circuit, and water. The water reference was obtained under exactly the same conditions of temperature and pH as the sample, and considerable care was taken to obtain a uniform, homogeneous, equilibrated sample completely filling the sampling region. Each measurement was repeated at least four times, using different prepared samples, and the data was well-fitted by two simple Debye functions, one corresponding to the water reference and the second due to the water interacting with the polymer.

For example, the imaginary part of the dielectric relaxation for water at 7 °C and for 1672 mg of (GVGIP)$_{260}$ dissolved in 4 mL of water at 7 °C are shown in Figure 1. At this temperature, below the temperature interval of the transition, the resolved curve for the water interacting with polymer is greater than that of bulk water. Since the sum of the two resolved curves represents essentially all of the water and the total amount of water is known, an estimate of the number of water molecules interacting with the polymer is possible. Furthermore, knowing the concentration of polymer allows estimation of the number of interacting water molecules per pentamer, N_{hh}. Now, N_{hh} can be estimated as a function of dilution, as the temperature is raised from below to above T_t and as a function of the ionization of the glutamic acid side chain. The reproducibility of the calculated value of N_{hh}, for example, resulting from seven determinations at 16 mg/mL and 7 °C was 101±1.3 water molecules. The accuracy of the values is poorest at the extremes of concentration. As can be seen in the fit to a simple Debye function to the high-concentration data of Figure 1, there is an error of ±6%. At the lowest concentration of 5 mg/mL, the error is believed to be better than ±20%, because the curves are so well-behaved as a function of concentration and temperature, as seen in Figures 2A and B and because it is possible to predict, within an accuracy of 12%, the value of N_{hh} for poly(GEGFP GVGVP GVGVP GVGVP GVGVP GVGFP) at 30 mg/mL in Figure 2C by using the data of Figure 2A and the T_t-based hydrophobicity scale.[7,12]

Plots of the putative waters of hydrophobic hydration, N_{hh}, versus log[GVGXP] are shown in Figure 2A at 10 and 7 °C for (GVGVP)$_{251}$ and (GVGIP)$_{260}$, respectively.

The critical test for identifying this N_{hh} as waters of hydrophobic hydration would be the decrease in N_{hh} as the polymer passes through the temperature interval for the hydrophobic folding and assembly transition during which waters of hydrophobic hydration become less-ordered bulk water and the polymer becomes more ordered. This behavior of the N_{hh} is seen on raising the temperatue of 5 mg/mL solutions in Figure 2B. Each polymer loses its water of hydrophobic hydration as it hydrophobically folds and self-assembles (i.e., as the temperature is raised through its value of T_t which is 10 °C for (GVGIP)$_{260}$ and 25 °C for (GVGVP)$_{251}$).

A further test (for the identification of N_{hh} and for previous interpretations of the physical characterizations of the dependence of T_t and the enthalpy[22] ΔH_t of the transition on the extent of ionization) would be the decrease in N_{hh} as the side chains of glutamic acid become charged. This is seen in Figure 2C for 30 mg/mL solutions at 1 °C. The results are as expected; even the greater N_{hh} for the polymer with two Phe (F) residues per repeating 30mer is as expected on the basis of the T_t-based hydrophobicity scale. Two aspects of the effect of charge were unexpected, however: (1) the effect of ionization does not follow the degree of ionization (note the pK_a values) but rather depends on the density of charge and (2) there is a residual N_{hh} suggesting that there exists a water of hydrophobic hydration that is less readily destroyed by charged groups as the charges strive to achieve their own hydration shells. Perhaps this residual N_{hh} represents the polymer itself shielding hydrophobic hydration on the far side of the polymer from the charged groups as previously depicted schematically.[23] From the larger values of N_{hh} obtained on dilution, we suggest, in accordance with a number of authors, that there occurs a more extended shell of thermodynamically significant water of hydrophobic hydration.[24]

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