Observation of Immobilized Water Molecules around Hydrophobic Groups

Y. L. A. Rezus* and H. J. Bakker

FOM–Institute for Atomic and Molecular Physics, Kruislaan 407, 1098 SJ Amsterdam, The Netherlands

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We have used femtosecond midinfrared spectroscopy to study the orientational mobility of water molecules in the hydration shells of hydrophobic groups. Our results show that hydrophobic groups are surrounded by a number of water molecules that display much slower orientational dynamics than the bulk liquid and that are therefore effectively immobilized. It turns out that each methyl group is surrounded by four immobilized water OH groups.

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Hydrophobic interactions play an important role in many biochemical processes [1–7]. The folding of globular proteins, the self-assembly of lipid membranes, and the binding of drugs to proteins are examples of processes driven by these interactions. In essence one can describe the hydrophobic effect as the tendency of apolar groups to associate in aqueous solution, thereby minimizing the total hydrophobic surface that is exposed to water.

The hydrophobic effect is intricately linked to the particular manner in which apolar compounds are solvated by water. It is well known that the dissolution of these compounds in water is accompanied by an anomalously large increase in the heat capacity of the solution. In the 1940s Frank and Evans introduced a model to account for this observation: they proposed that the water molecules around hydrophobic groups form rigid, icelike structures, which they coined icebergs [8]. According to this model the freeing of entropy associated with the transfer of water molecules from the solvation shell to the bulk forms the origin of the hydrophobic effect.

The iceberg model of hydrophobic hydration is founded on thermodynamic measurements, and, as such, the evidence for the molecular picture that it presents remains indirect. During the past decades many researchers have attempted to confirm the iceberg model using more direct, structural methods. Among these techniques are neutron diffraction, dielectric relaxation, and nuclear magnetic resonance (NMR). Neutron diffraction experiments can provide direct structural information about a solution by measuring the water-water radial distribution function (RDF). In the presence of hydrophobic solutes this RDF shows little change, from which it is concluded that the structure of the water around hydrophobic groups is identical to that of the bulk liquid [9,10]. NMR and dielectric relaxation, however, come to another conclusion [11–14]. These methods take a different approach at probing the water structure: the orientational dynamics of water molecules are used as an indicator of the rigidity of the hydrogen-bond network. Both methods show that the *average* mobility of water molecules in solutions containing hydrophobic solutes is decreased. However, as these methods measure a response that is averaged over all water molecules, the techniques cannot distinguish between water molecules in the bulk liquid and in the apolar solvation shell. As a consequence no information exists on the difference in behavior of the water molecules: is there an iceberg consisting of a single, well-defined layer of water molecules, or are many molecules slightly affected in their dynamical behavior? Summarizing, the experiments do not provide a consistent picture of the effect of hydrophobic groups on the structural dynamics of water.

Here we report on the use of polarization-resolved midinfrared pump-probe spectroscopy to study the rotational motion of water molecules in the solvation shells of apolar molecules. An essential advantage of this method is that it probes the dynamics of water molecules on a subpicosecond time scale, which is shorter than the exchange time of water molecules in the bulk liquid and the solvation shell. As a result the method allows the separation of the response of the aqueous solvation shell from that of the bulk.

In our experiments we use aqueous solutions of hydrophobic solutes of varying concentrations. A small amount of heavy water (D_2O) is added to the water, such that a solution of HDO (8%) in H_2O is formed. The OD-stretching vibration of the HDO molecules leads to a strong absorption around 2500 cm^{-1}, and the orientational dynamics of these molecules can be conveniently monitored using pump-probe spectroscopy. In the experiment an intense femtosecond pump pulse, tuned to resonance with the OD vibration, is used to excite a significant fraction of the HDO molecules. The pump-induced absorption changes are monitored by delayed probe pulses that are polarized parallel and perpendicular to the pump-pulse polarization. This leads to the transient absorptions $\Delta\alpha_{\parallel}(t)$ and $\Delta\alpha_{\perp}(t)$, respectively. These two signals are initially different because of the preferential excitation of HDO molecules that have their OD groups aligned parallel to the pump polarization. As the delay between the pump and probe pulses is increased, molecular reorientation causes the molecules to loose memory of their initial orientation, and the difference between the two signals vanishes. The *normalized* differ-

ence between the parallel and perpendicular absorption changes is called the anisotropy,

$$R(t) = \frac{\Delta\alpha_{\parallel}(t) - \Delta\alpha_{\perp}(t)}{\Delta\alpha_{\parallel}(t) + 2\Delta\alpha_{\perp}(t)}, \quad (1)$$

and the decay of this quantity reflects the molecular reorientation. The isotropic signal,

$$\Delta\alpha_{\mathrm{iso}}(t) = \tfrac{1}{3}[\Delta\alpha_{\parallel}(t) + 2\Delta\alpha_{\perp}(t)], \quad (2)$$

is constructed in such a way that it is free of reorientational processes, and it reflects the decay of the excitation by vibrational relaxation.

We performed our experiments using four compounds that contain a varying number of hydrophobic groups (Fig. 1): tetramethylurea (TMU), trimethylamine-*N*-oxide (TMAO), the amino acid proline, and *N*-methylacetamide (NMA). These solutes all have an extremely high solubility in water (> 10 m) despite their considerable hydrophobic character.

Figure 2(a) shows a delay scan at the center of the OD absorption band (2500 cm^{-1}) for a 4-m solution of TMAO. At this frequency we observe a negative absorption difference, which is caused by the bleaching of the fundamental transition of the OD-stretching vibration. The signal decays with a time constant of ~ 2 ps, which is typical for the vibrational energy relaxation of HDO in H_2O [15,16]. At other solute concentrations a similar decay time is observed, which shows that the relaxation of the OD vibration is not affected by the solute. It can be seen that the signal does not fully decay to zero. This can be explained by the temperature increase in the sample that results from the thermalization of the energy of the pump-pulse. A temperature increase causes the OD-stretching band to shift to the blue, which results in a decreased absorption when observing the center of the band [15].

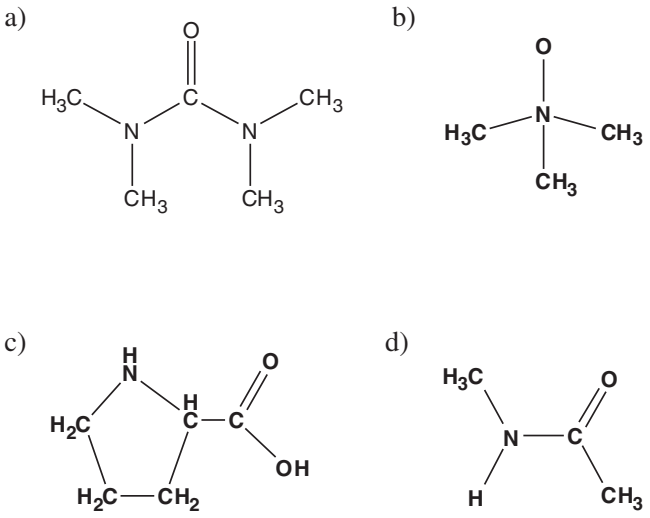

FIG. 1. Molecular structure of the solutes used in the experiment. (a) Tetramethylurea (TMU), (b) trimethylamine-*N*-oxide (TMAO), (c) proline, and (d) *N*-methylacetamide (NMA).

Previous studies have demonstrated that, in order to obtain the correct anisotropy decay, the isotropic signal [i.e., the denominator in Eq. (1)] must be corrected for the increasing temperature in the sample [15,16]. The heating contribution to the isotropic signal can be obtained by fitting the transient spectra to a two-step relaxation model. Such a model has been shown to provide an accurate description of the relaxation of the OD vibration [15]. The solid line in Fig. 2(a) represents this fit. From the fit we extract both the heating contribution to the signal (dashed line) and the bleaching of the excitation (dotted line). The latter contribution allows us to calculate the anisotropy of the excitation.

Using Eq. (1) in combination with the corrected isotropic signal we have calculated the anisotropy of our data. In Fig. 2(b) anisotropy decays are shown for TMAO solutions at four different concentrations. In all cases we observe a biexponential decay composed of a fast component (τ_{rot}) with a time constant ≤ 2.5 ps and a slow component with a time constant >10 ps. The fast component of ~ 2.5 ps has also been observed in the reorientation of pure water, indicating that this component is to be associated with the reorientation of the bulk water molecules in the solution [15]. To determine the origin of the slow component we have varied the TMAO concentration. For each concentration we have fit the anisotropy to a monoexponential decay with an offset [$R(t) = Ae^{-t/\tau_{\mathrm{rot}}} + B$]; the offset represents the slow component, the time constant of which falls outside our experimentally accessible time range. In Fig. 3(a) the amplitude of the slow component is plotted as a function of the solute concentration. The amplitude is directly proportional to the fraction of immobilized OH groups, the maximum value of 0.4 representing 100% immobilization. We observe a linear dependency that flattens at very high concentrations. The linear relation indicates that the slow component is associated with the water molecules that are part of the solvation shell of the TMAO molecule. The long time constant ($\tau_{\mathrm{rot}} > 10$ ps)

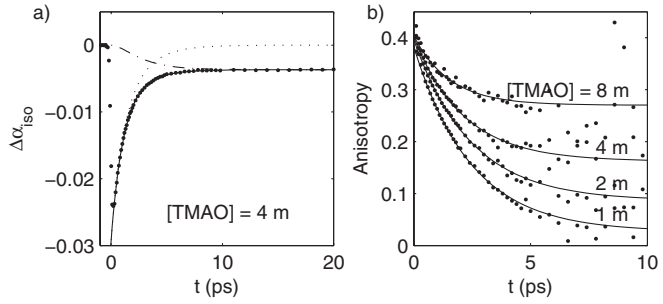

FIG. 2. Time-resolved data for a 4-m solution of TMAO in isotopically diluted water. (a) Delay scan taken at the center of the OD absorption band (2500 cm^{-1}). The solid, dashed, and dotted lines represent the fit to the relaxation model, the heating contribution, and the true pump-probe contribution, respectively. (b) Anisotropy decays of the OD vibration of HDO in H_2O at different TMAO concentrations. The solid lines represent fits to monoexponential decays with an offset.

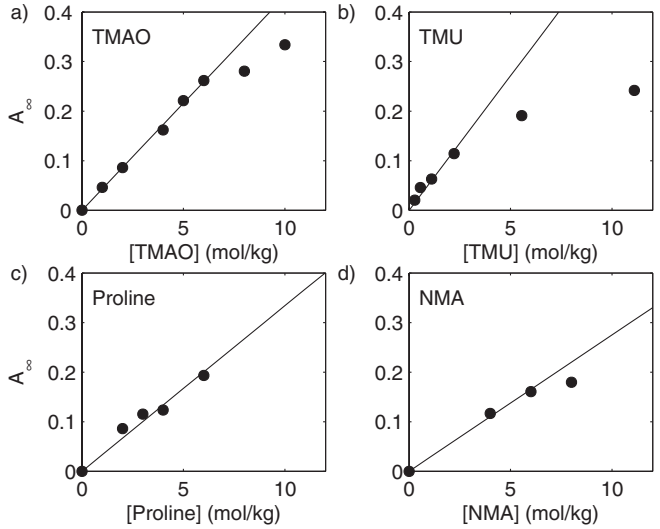

FIG. 3. Long-time anisotropy of the OD vibration of HDO as a function of the concentration of the four different solutes: (a) TMAO, (b) TMU, (c) proline, and (d) NMA. The uncertainty in the data points is 0.02.

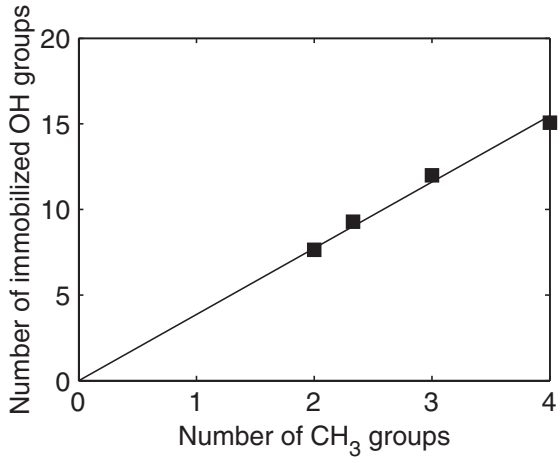

FIG. 4. Number of immobilized water OH groups as a function of the equivalent number of CH_3 groups in the solute molecule.

shows that these water molecules are strongly immobilized by TMAO. From the slope of the linear part of Fig. 3(a) we can calculate that the solvation shell of a TMAO molecule contains approximately 12 strongly immobilized OH groups.

As the TMAO molecule is amphiphilic we are faced with the question as to which part of the molecule is the cause of the immobilized water molecules: the hydrophilic NO group or the hydrophobic methyl groups? To investigate this issue we have varied the nature of the solute and repeated our measurements. We observe a similar pattern for each of the remaining solutes in Fig. 1. The anisotropy decays biexponentially with a fast component (~ 2.5 ps) and a slow component (> 10 ps). At low concentrations the amplitude of the slow component scales linearly with the solute concentration [Figs. 3(b)–3(d)]. For each of the solutes in Fig. 1 we have determined the number of OH groups immobilized per solute molecule. We have summarized these results in Fig. 4, where the number of immobilized water molecules is plotted versus the equivalent number of CH_3 groups in the solute molecule. The observed linear relation unambiguously shows that the immobilized water molecules are part of the hydration shell around the *hydrophobic* methyl groups of the solutes. Apparently the *hydrophilic* groups of the solutes do not lead to the immobilization of water molecules. The slope of the graph in Fig. 4 has a value of 3.9, indicating that every methyl group is responsible for the immobilization of approximately 4 water OH groups. At high solute concentrations the curves in Fig. 3 flatten, which shows that for these concentrations fewer than 4 OH groups are immobilized per methyl group. This is explained by the fact that in these solutions part of the immobilized OH groups are shared by different solute molecules.

We now consider the physical mechanism that underlies the immobilization of water molecules in the vicinity of hydrophobic groups. One could presume that these immobilized molecules are connected by very strong hydrogen bonds, resembling the hydrogen bonds encountered in ice. However, as appealing as this notion may be, it cannot be correct. The fact that the addition of the hydrophobic solutes does not shift the OD-stretch vibration to the red indicates that the hydrogen bonds in the investigated solutions are of a strength comparable to those in pure water.

A number of molecular dynamics studies on liquid water have appeared in the past years. Sciortino *et al.* have shown that the relatively high orientational mobility of pure water is related to the presence of defects (i.e., five-coordinated water molecules) in the tetrahedral hydrogen-bond network of liquid water [17]. They have suggested that the slowing down of water dynamics around hydrophobic groups is the consequence of a steric effect, which prevents the creation of five-coordinated water molecules around these groups. Recently, Laage and Hynes proposed a detailed mechanism for water reorientation involving five-coordinated water molecules [18]. In this mechanism the pathway for reorientation involves a rotating water molecule that concertedly breaks a hydrogen bond with an overcoordinated first-shell neighbor and reforms one with an undercoordinated second-shell neighbor. In another molecular dynamics study by Sharp *et al.* the effect of hydrophobic solutes on the structure of water was investigated [19]. These researchers observed that hydrophobic solutes tend to preferentially displace water molecules that overcoordinate a second water molecule, providing a rationale for why hydrophobic solutes lower the amount of network defects. These studies together with our results form compelling evidence for the notion that the immobilization of water molecules around a hydrophobic solute arises from a steric effect, in which the hydrophobic group prevents a fifth water molecule from approaching a tetrahedrally coordinated water molecule, and as such prevents the molecule to reorient.

It has been suggested by several researchers that two regimes can be distinguished as far as hydrophobic effects are concerned: the regime of a small hydrophobic solute and that of a large hydrophobic particle or a plane surface [2,20]. Small hydrophobic solutes can be accommodated by the hydrogen-bond network of water without breaking hydrogen bonds, whereas an extended hydrophobic surface can only be solvated if hydrogen bonds are sacrificed. The notion that the hydrogen-bond network of water does not have to be perturbed in order to solvate small hydrophobic particles corroborates the interpretation of our results.

In a number of molecular dynamics studies it has been suggested that the hindered reorientation of water molecules, as it occurs in the proximity of hydrophobic groups and protein surfaces, may follow stretched-exponential dynamics [21,22]. Whereas the fast component in our measurements follows monoexponential dynamics, the possibility exists that the slow component exhibits such stretched-exponential dynamics. Therefore it would be interesting to investigate the decay of the slow component experimentally. Unfortunately, the dynamics of the slow component lie outside the time range that is accessible with our technique.

It is interesting to compare the number of immobilized water molecules surrounding a methyl group with the size of its hydration sphere, as it follows from neutron diffraction. Neutron diffraction data on methanol solutions in water were reported by Soper and Finney [23]. By integrating the first peak in the carbon-oxygen radial distribution function, these authors find that the first solvation shell of the methyl group contains about 10 water molecules (20 OH groups). Let us assume that the solvation structure of the methyl group of methanol is representative of that of a methyl group in general. Our experiments show that only 4 OH groups are immobilized per methyl group, which means that not all OH groups in the solvation shell of a methyl group have the same configuration. Apparently, approximately 80% of the OH groups in the hydration shell are in ''open'' configurations that can be approached by new hydrogen-bonding partners and therefore show bulklike dynamics. Only 20% of the OH groups in the hydration shell represent immobilized OH groups; these OH groups are in such close proximity to the methyl group that there is no space for the creation of network defects.

We conclude by returning to the iceberg model of Frank and Evans. Our results provide a molecular picture of these icebergs: they consist of four strongly immobilized water OH groups for every methyl group in solution. They are the consequence of a decrease in the configurational space available to water molecules around hydrophobic solutes. This notion also explains Frank and Evans' original observation of a decreased entropy upon the dissolution of hydrophobic compounds in water. The structure of the iceberg, however, is not the ordered structure observed in ice, but it rather resembles the disordered hydrogen-bond network of bulk water: the icebergs are icelike from a dynamical perspective but waterlike as far as structure is concerned. This provides an explanation for why hydrophobic icebergs were not previously observed using structural methods.

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*rezus@amolf.nl

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