Detection of Localized Water Clusters in a Charged Peptidyl Resin

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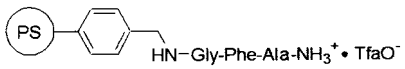
The understanding of local solvent structure and especially that of water in the proximity of ions represents a challenging area of research due to its importance in many chemical and biological processes[1]. Very recently, the isolated dynamics of water molecules in the solvation shell of an ion in bulk aqueous solution was obtained using femto-second mid-infrared nonlinear spectroscopy,[2] but the distinction of the water molecules in the solvation shells from those in the bulk solvent, because of the unfavorable population ratio and the rapid exchange between both components, remains a enormeous challenge. Therefore, most of the information regarding the physicochemical properties of water-solvated species originates from gas-phase experiments,[3,4] where microclusters composed of an ion or a neutral species surrounded by a few water molecules are studied, or from theoretical calculations[5,6] on similar clusters. The extension of those clusters to macroscopic systems, however, including possible interactions with higher solvation shells, remains an open question. Here, we report the presence of a distinct water component around the ion pair formed by the terminal ammonium group of a resin-anchored peptide and its counterion and study its localization and dynamic behavior in atomic detail by high-resolution magic angle spinning (HR MAS) NMR.

Our experimental system, a model peptide (Ala-Phe-Gly) in the charged form synthesized on a *p*-aminomethyl polystyrene (PS) support swollen in deuterated dimethylformamide (DMF), corresponds to the status of the peptidyl resin after the last Boc deprotection step with trifluoroacetic acid concentrate (TFA). After extensive washing of the resin with dichloromethane and drying under vacuum conditions, all excess acid was removed, leaving the peptide on the resin as an ammonium salt with trifluoroacetate as counterion.



1-TFA

HR MAS NMR, which is emerging as a powerful analytical tool for the study of heterogeneous systems,[7] was used to characterize this supramolecular system. After total ^{1}H NMR assignments of the proton resonances corresponding to the peptide at 10 °C using TOCSY and NOESY spectra, and confirmation of the full assignment of the peptide by the carbon resonance assignment from a ^{1}H−^{13}C HSQC experiment, two well-separated resonances remained unassigned in the ^{1}H NMR spectra (Figure 1A). The signal at 3.60 ppm has the same chemical shift of water in DMF at 10 °C, and displayed a narrow line width (12 Hz at half-maximum). Both arguments identify it as residual water in the resin, that might have come with the DMF solution or just captured from air moisture. We will further denote it as H_2O_{bulk}. The other resonance at 3.77 ppm was characterized by a larger line width of 46 Hz, even larger than that of the peptide resonances (36 and 39 Hz for the Phe amide or H_α protons).

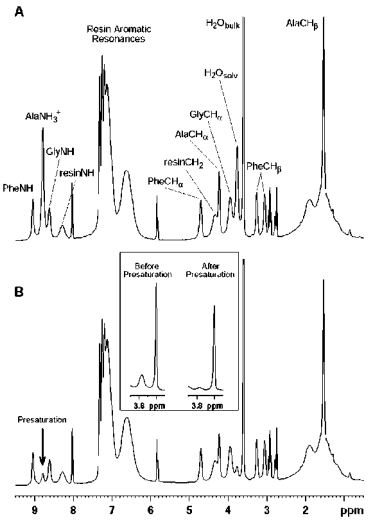


Figure 1. HR MAS ^{1}H NMR 1D spectra at 600 MHz of **1-TFA**. Spectra were recorded at 283 K, spinning rate 6 kHz, in a 4 mm rotor with 10 mg of resin swollen in 100 μL of DMF-d_7: (A) single pulse sequence; (B) 1D proton spectra with presaturation on the $AlaNH_3^+$ resonance. The boxed insert shows the water resonances before and after presaturation of the ammonium protons.

Saturation NMR experiments gave a first indication of the chemical nature of the resonance at 3.77 ppm. The presaturation of H_2O_{bulk} during 1 s led to small decrease in intensity of both this resonance and the NH_3^+ ammonium signal, whereas the rest of the peptide resonances were basically unaffected. On the other hand, presaturation of the peak at 3.77 ppm not only induced the expected symmetrical small intensity decrease of the H_2O_{bulk} resonance but also generated an almost complete loss of the intensity of the NH_3^+ resonance, but not of the other amide protons. This contrasts to earlier TOCSY studies where the water stripe showed correlations to many exchangeable protons on a peptide.[8] More importantly, the presaturation of the ammonium protons' resonance induced a considerable intensity loss for the resonance at 3.77 ppm and a small loss of the H_2O_{bulk} line, whereas all of the other resonances were unaffected (Figure 1B). The good chemical shift correlation and complete coupling by chemical exchange of the ammonium protons and those corresponding to the signal at 3.77 ppm not only identified the latter as water molecules (that we will further indicate as H_2O_{solv}) but also allowed us to localize them in the immediate vicinity of the N terminus of the charged peptide moiety.

The underlying process of chemical exchange between the Ala NH_3^+ and the water protons of H_2O_{solv} was subsequently confirmed by the presence of a strong exchange cross-peak between both species with the same sign of the diagonal peak in a ROESY[9] 2D experiment. Two-dimensional EXSY experiments[10]

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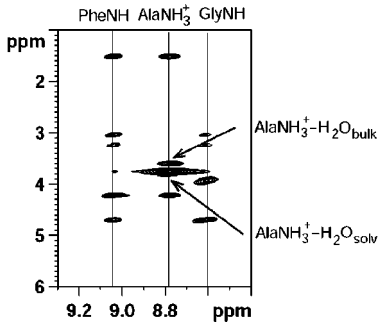

Figure 2. EXSY spectrum at 600 MHz of **1-TFA** showing the exchange between the ammonium protons and the two water components.

with increasing mixing times yielded an exchange rate $k_{ex} = (48\ \text{ms})^{-1}$ for the ammonium protons and H_2O_{solv}. In this spectrum, we equally noted an exchange peak between H_2O_{bulk} and the protons of H_2O_{solv} (and thus indirectly with the ammonium protons), but the reduced intensity of their exchange peak indicated a slower time scale for this exchange process with a $k_{ex} = (1183\ \text{ms})^{-1}$ (Figure 2). The exchange process between H_2O_{bulk} and the $AlaNH_3^+$ protons was on a similar time scale as the latter process, with a value for $k_{ex} = (967\ \text{ms})^{-1}$.

Whereas the chemical exchange between the protons of the ammonium moiety and the H_2O_{solv} signal probably contributes to the line width of both, the time scale is too slow compared to their chemical shift separation to explain the important line width of the latter. A valid alternative is the quasi-permanent attachment of these water molecules to the N-terminal moiety of the anchored peptide chains, leading to similar processes of line broadening as for the tethered peptide chains. Because this should lead to a loss in translational mobility, we used the LED diffusion pulse sequence[11] to probe the degree of anchoring of both water species to the resin. The diffusion experiments confirmed the absence of diffusion for the peptide resonances but equally showed that H_2O_{solv} corresponds to a very slowly diffusing species, with a diffusion coefficient $D_c = 2.2 \times 10^{-10}$ m^2/s, hence 55 times lower compared to that of water in DMF ($D_c = 1.2 \times 10^{-8}$ m^2/s at 10 °C). The other water component of the system (H_2O_{bulk}) diffused only twice more slowly, with a value for $D_c = 6.8 \times 10^{-9}$ m^2/s.

Both observations of fast exchange with the ammonium protons and very slow diffusion suggest that the protons corresponding to the H_2O_{solv} resonance are associated with water molecules that reside with an important lifetime in direct contact with the N-terminal ion pair. Interestingly, integration of the signal yielded a value of two to three water molecules in the first hydration shell of every N-terminal ion pair, which should be compared with the five water molecules that were found in the first solvation shell of the free ammonium ion.[12] The second water signal corresponds to twice this amount of molecules and might be putatively assigned to a second hydration shell. Significantly, even at the longest mixing times, no exchange peak was seen with the Gly or Phe amide protons, confirming the localized character of even this more mobile water component. Whereas the use of Cl^- as a counterion equally led to the observation of two distinct water components, the absolute necessity of a stable ion pair became clear when we used a weaker acid such as acetic acid. This led to the disappearance of not only the distinct water signals, but also of the proton signals of the ammonium moiety, probably due to rapid exchange with the unique water signal at 3.58 ppm. A similar behavior was previously noticed upon neutralization of the peptide amino group by washing with a base, where the signal of the NH_2 terminal disappeared from the spectrum[13] and only one water signal could be observed in the HR MAS ^{1}H NMR spectra. Presaturation of this unique water resonance resulted in an intensity decrease of the Phe amide proton,[14] indicating a more diffuse character for the water molecules in the absence of a stable ion pair at its N terminus.

At 10 °C, the two water resonances were best resolved, which was the primary reason for the temperature choice of our experiments. However, varying the temperature yielded some interesting results as well. For temperatures below 50 °C, both resonances could be distinguished individually, and a linear relationship with temperature coefficient of 7 ppb/K was observed for both line positions. Upon raising the temperature from 10 to 20 °C, the exchange rate between H_2O_{solv} and the NH_3^+ protons increased from $(48\ \text{ms})^{-1}$ to $(15\ \text{ms})^{-1}$, whereas the exchange processes implying protons of the other water component did not show any change in dynamics. Above 50 °C, both the NH_3^+ and H_2O_{solv} signals became severely broadened, probably because the exchange rate approaches their chemical shift separation.

To study the dynamical behavior of both water components, we carried out T_1 relaxation experiments at 10 °C. We cannot exclude completely that the measurement is hampered by radiation damping,[15] but if so, the effect should be more pronounced for the most intense H_2O_{bulk} water component. The experimental results, however, were quite the opposite: the T_1 value of H_2O_{bulk} was found to be 956 ms, nearly double the T_1 value of H_2O_{solv} at 565 ms. Whereas molecular dynamics simulations probably will be necessary to separate the intra- from intermolecular contributions to those relaxation rates,[16] the results point to a reduced rotational freedom for the water molecules that are in the direct vicinity of the anchored ammonium moiety.

Similar to the case of an aggregating peptide,[17] the solid-phase resin offers the possibility of dilution of the peptide chains on the resin, to further isolate the water clusters. We thus believe that the present demonstration of two distinct water components in commonly used peptidyl resins opens up prospects for new theoretical and experimental work related to the study of solvation and applicable to solid-phase chemistry.

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Supporting Information Available: HR-MAS diffusion experiment on **1-TFA** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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