Polypeptide Hydration in Mixed Solvents at Low Temperatures

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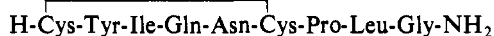
Abstract: The hydration of the nonapeptide oxytocin dissolved in a mixed solvent of 60% H_2O and 40% [$^2H_6$]acetone was investigated by nuclear magnetic resonance (NMR) spectroscopy. In the temperature range between 0 and −15 °C the nuclear Overhauser effects (NOEs) between the individual proton resonances of the peptide and the water signal were found to change their sign. At the temperature of the sign change the residence time of the hydration water molecules on the peptide surface is about 500 ps. The temperature-dependent transition from positive to negative NOEs provides a sensitive probe for assessing differences in the residence times of the hydration water molecules bound to different atom groups of a polypeptide chain.

The characterization of three-dimensional protein structures in solution at atomic resolution by nuclear magnetic resonance (NMR)[1] spectroscopy[2] has recently been extended to include a description of surface hydration.[3] A view at individual hydration sites in solution is afforded by the observation of nuclear Overhauser effects (NOEs) between the individual protons of the polypeptide and nearby water molecules.[4] Quite generally, hydration sites on polypeptide and protein surfaces are characterized by hydration lifetimes shorter than about 500 ps, as indicated by the positive sign of the cross-relaxation rate, σ^{NOE}, measured in NOESY cross peaks between polypeptide protons and water protons.[3] Water molecules bound with significantly longer residence times were so far observed only in interior cavities of globular proteins; their NOEs with the protein protons are characterized by large negative σ^{NOE} rates indicating residence times much longer than 500 ps for these waters.

In this paper we further investigate the residence times of water molecules near different atom groups of a polypeptide chain using the nonapeptide oxytocin



as a model system. Oxytocin is flexibly disordered in aqueous solution[5] and is devoid of interior cavities that could contain bound water molecules. Correspondingly, all NOEs between oxytocin protons and water protons were found to have positive σ^{NOE} rates at 8 °C, which is characteristic of hydration lifetimes shorter than 500 ps.[3] In the following we show that as the temperature is lowered in the range between 0 and −15 °C the sign of the σ^{NOE} rates becomes negative for all oxytocin–water NOEs. The temperature-dependent transitions from positive to negative values of σ^{NOE} enable a distinction of different hydration sites by their hydration lifetimes.

Results and Discussion

To investigate the water proton–polypeptide proton NOEs at temperatures below 0 °C, oxytocin was dissolved in a mixed solvent of 60% H_2O and 40% [$^2H_6$]acetone. This solution has a freezing point of approximately −28 °C. Two-dimensional NOE spectra in the laboratory frame (NOESY) and the rotating frame (ROESY) were recorded with the experimental schemes of Figure 1, as well as of Figure 1B of ref 8. Figures 2 and 3 show respectively cross sections through the NOESY and ROESY spectra taken at the ω_1 chemical shift of the water signal. Positive cross peaks in the ROESY spectrum indicate chemical exchange with the water, whereas all NOE cross peaks are negative (Figure 3).[9] Over the entire temperature range from 10 to −25 °C chemical exchange peaks are observed for the labile protons of the N-terminal α-amino group and the –OH group of Tyr 2. For these protons the exchange with the water is sufficiently rapid to result in efficient bleaching of the cross-peak intensities at 10 °C. At all four temperatures studied, the intrapeptide NOEs with these two resonances are therefore transferred to the water resonance, so that the intensities of the cross peaks with αCH and βCH_2 of Cys 1 and ϵCH of Tyr 2 (Figures 2 and 3) are partly or entirely due to such transfer effects rather than to direct NOE cross peaks with the water line. The only other exchange peak in Figure 3 is with the amide proton of Tyr 2 at +10 °C. Since this proton exchanges much more slowly than the aforementioned examples, the NOE magnetization transfer from the water is more effective than chemical exchange at temperatures below 0 °C (Figure 3). All other peaks are negative throughout and represent direct NOE interactions with hydration water at all temperatures studied. With only a few exceptions due to overlap with intrapeptide cross peaks (circles and crosses in Figures 2 and 3), the NOE cross peaks with the water line could readily be assigned to individual protons of oxytocin.

Since oxytocin is flexibly disordered at all temperatures studied,[5] only intraresidual and sequential NOEs[2] were observed. The NOESY cross peaks between different peptide resonances were positive throughout, indicating that the peptide is in the slow motional regime. In contrast, the sign of the NOESY cross peaks between polypeptide protons and the water changed with temperature (Figure 2), although the corresponding peaks were always negative in the ROESY spectra (Figure 3). This behavior is a consequence of the different dependence of the cross relaxation rates in NOESY and ROESY on the spectral density function, $J(\omega)$, where ω_0 is the angular Larmor frequency:

$$\sigma^{NOE} = 6J(2\omega_0) - J(0) \quad (1)$$

$$\sigma^{ROE} = 3J(\omega_0) + 2J(0) \quad (2)$$

The spectral density function $J(\omega)$ depends further on the effective correlation time for the vector connecting the water proton with the proton of the peptide. σ^{NOE} is respectively positive and negative for rapid and slow changes in orientation and length of this interproton vector, where positive σ^{NOE} values are manifested by negative NOESY cross peaks and vice versa. Quite independent of the motional model used to calculate $J(\omega)$,[10,11] the sign change

(1) Abbreviations and symbols used: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; σ^{NOE}, cross-relaxation rate in the laboratory frame; σ^{ROE}, cross-relaxation rate in the rotating frame; 2D, two-dimensional; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; ROESY, two-dimensional nuclear Overhauser enhancement spectroscopy in the rotating frame.

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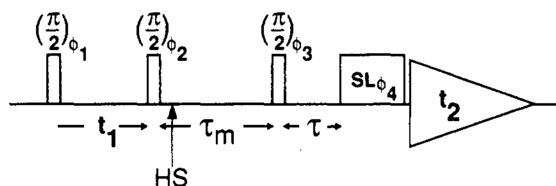

Figure 1. Experimental scheme used to record the NOESY spectra of Figure 2. One or several homospoil pulses, HS, at the beginning of the mixing time, τ_m, defocus transverse magnetization and thus prevent radiation damping.[6] The carrier is set at the water frequency and the water signal is suppressed using a spin lock pulse of 1–2 ms duration, $SL_{\phi 4}$, which is separated from the mixing time by a delay τ of about 1/SW, where SW is the spectral width in Hz. The excitation profile along the ω_2 frequency axis is given by sin ($\Omega\tau$), where Ω is the angular frequency relative to the carrier frequency. The phase cycle is $\phi_1 = 8(x)8(-x)$, $\phi_2 = 8(x,-x)$, $\phi_3 = 4(x,x,-x,-x)$, $\phi_4 = 2(4(x),4(-x))$, $\phi_5 = 2(x,-x,-x,x)2(-x,x,x,-x)$, which can be extended to 64 steps by CYCLOPS.[7] The experiment is also well suited for the measurement of water proton–peptide proton NOEs with mixing times shorter than 10 ms. (In a previously published NOESY pulse sequence (Figure 1A of ref 8) an additional spin lock pulse was applied immediately after the $\pi/2$ pulse following τ_m, instead of using a homospoil pulse during the mixing time. The advantage of the present experiment lies in the fact that the additional spin lock pulse used in ref 8 could contribute significant amounts of ROE mixing when used with short mixing times τ_m.)

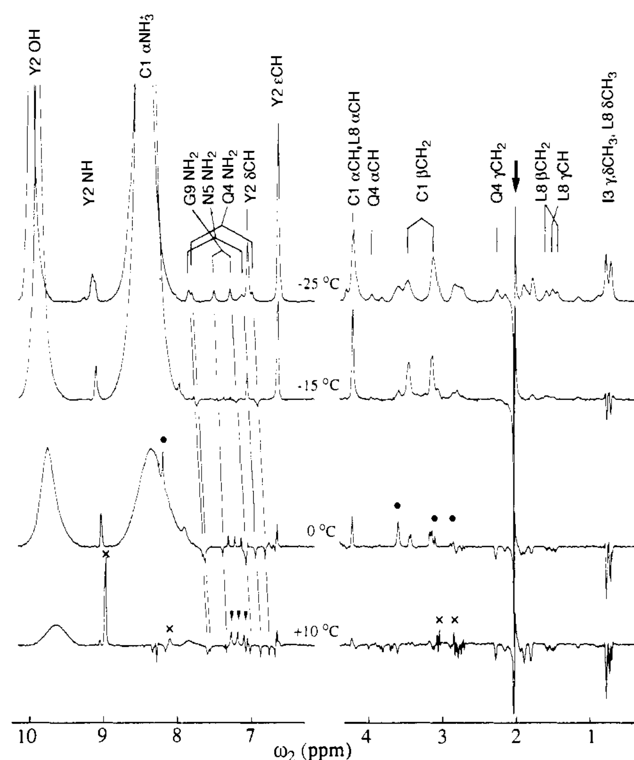

Figure 2. Cross sections along ω_2 taken in NOESY spectra of oxytocin at the ω_1 chemical shift of the water resonance; the peaks represent either NOEs or chemical exchange between oxytocin protons and water protons (oxytocin concentration 50 mM, solvent 60% H_2O/40% [$^2H_6$]acetone, $pH_{app} = 3.5$, ^1H frequency 600 MHz, time domain data size 450 × 2048 points; the experimental scheme of Figure 1 was used with $t_{1max} = 36$ ms, $t_{2max} = 156$ ms, 30 ms mixing time, $\tau = 135$ μs, and a 1 ms spin lock pulse). Since the excitation profile leads to sign inversion of all signals on both sides of the water line, the low-field region of each cross section was inverted in this figure for improved readability. The spectra were baseline corrected in both dimensions using polynomials. Selected peaks are labeled with the one-letter amino acid symbol and the sequence position. The temperature-dependent resonance positions of the amide NH_2 protons are identified by lines between the spectra. The cross peak of acetone at 2.05 ppm (arrow) was used as a chemical shift reference. Chemical exchange peaks from NH_4^+ ions present as an impurity are labeled with triangles, and intrapeptide cross peaks overlapping with the water line are identified with filled circles in the spectrum at 0 °C and with crosses at 10 °C.

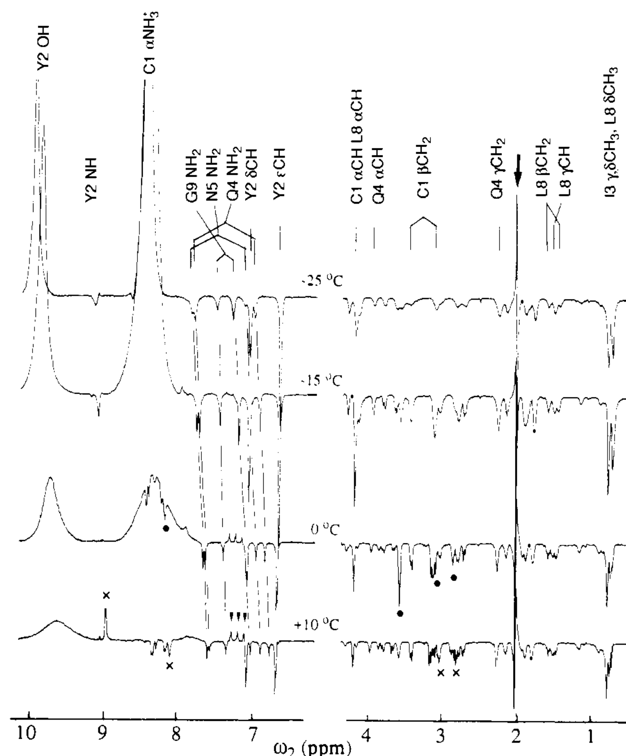

Figure 3. Cross sections along ω_2 taken in ROESY spectra of oxytocin at the ω_1 chemical shift of the water resonance. The spectra were recorded with an experimental scheme that corresponds to the NOESY scheme of Figure 1 and is described in detail in ref 8. See Figure 2 for sample conditions and presentation of the spectra.

in σ^{NOE} is expected to occur when the effective correlation time is of the order of the inverse of the angular Larmor frequency, i.e., about 500 ps at 600 MHz.[3] This time span is much shorter than the lifetime of a water proton with respect to hydrolysis,[12] so that these NOE observations must represent exchange of all the water molecules rather than proton exchange by hydrolysis. At 10 °C the negative NOESY cross peaks (Figure 2) are due to the translational diffusion of water molecules relative to the peptide protons, with diffusion coefficients that are only about four times smaller than the self-diffusion coefficient of pure water at the same temperature.[3] The positive cross peaks seen at −25 °C show that at this temperature the hydration water molecules bind to the peptide surface with residence times that are longer than 500 ps.

With regard to future applications of these principles for detailed studies of protein hydration, it is of special interest that individual polypeptide protons in oxytocin show different behavior in the experiment of Figure 2. Thus, although the NOE cross peaks with the amide protons (spectral range 8.1–8.5 ppm) and the α-protons (spectral range 3.7–5.0 ppm) vanish in the NOESY spectrum at 0 °C (Figure 2) (at the same temperature the corresponding cross peaks have nonvanishing intensity in the ROESY spectra of Figure 3), most NOESY cross peaks with side chain protons invert their sign at lower temperatures, i.e., between 0 and −15 °C, and negative NOESY cross peaks for the side chain methyl groups of Ile 3 and Leu 8 and the side chain amide group of Gln 4 are observed even at −15 °C. It remains to be seen to what extent the decreased effective correlation time for the water proton–polypeptide proton interactions is due to increased segmental mobility of the side chains or to shorter water residence times on the side chains than on the backbone, respectively.

Increased side chain mobility is indicated by the smaller $\sigma^{NOE}/\sigma^{ROE}$ ratio observed for the NOEs between backbone protons and side chain protons than between protons of the backbone. A quantitative evaluation of the polypeptide–water NOEs would

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therefore require the calculation of the spectral density function $J(\omega)$ in the presence of side chain motions. To date, no analytical formula has been derived for a model that would describe that situation. Assuming similar mobilities for all side chains, it is interesting to note that the sign inversion of σ^{NOE} occurs at a similar temperature for the side chain amide protons as for the methyl resonances of Ile 3 and Leu 8. This seems to indicate that the water does not bind with significantly increased residence times to the polar amide groups when compared to the apolar side chains.

Figure 3 shows that the intensities of the ROESY cross peaks between the water signal and the polypeptide resonances increase with decreasing temperature between +10 and −15 °C and are again decreased at −25 °C. This behavior is a consequence of the fact that the cross-relaxation rates increase with decreasing molecular motions. With the mixing time of 30 ms used to record the spectra of Figure 2 and 3, only the spectra at 10 °C fulfill the initial rate condition, where the cross-peak intensities are directly proportional to σ^{ROE}. At lower temperatures the increased cross-relaxation rates lead to more pronounced relaxation and spin diffusion, and therefore to a decrease of the overall intensities in the ROESY spectrum at −25 °C. Additional NOESY and ROESY spectra recorded with mixing times as short as 3 ms confirmed that both σ^{NOE} and σ^{ROE} increase steadily with lower temperature (data not shown).

While the sign inversion of σ^{NOE} provides a sensitive tool to probe the residence times of the hydration waters around individual atom groups of a polypeptide chain, the *size* of σ^{ROE} is far less sensitive to the hydration lifetime in the temperature range studied, and increases only about 3-fold between 0 and −15 °C. Therefore, the size of σ^{ROE} is mainly indicative of the average spatial proximity of the water protons to the polypeptide proton and of the number of water protons involved in the interaction. Variations in the size of σ^{ROE} between different polypeptide protons are most easily assessed by comparison of the one-dimensional ^{1}H NMR spectrum of the solute with the one-dimensional cross section along ω_2 taken at the ω_1 chemical shift of the water resonance in a 2D ROESY spectrum recorded with a short mixing time. Interestingly, no significant variations in σ^{ROE} were observed for the oxytocin–water NOEs in aqueous solution at 8 °C (Figure 3, A and B, of ref 3). This observation supports the view that as a consequence of the motional and orientational disorder of polypeptide hydration the average number, orientation, and residence time of the hydration water molecules are similar for all types of protons in a polypeptide chain. Similar behavior was observed with the oxytocin solution in 60% H_2O and 40% [$^2H_6$]acetone at even lower temperatures.

To assess the role of acetone in the peptide environment, we recorded NOESY and ROESY spectra at 10 and −15 °C with a solution of oxytocin in 60% ^2H_2O/40% acetone. The same experimental schemes were used as for the spectra of Figures 2 and 3, and the carrier was set to the frequency of the acetone resonance for solvent signal suppression. At either temperature only a few very weak cross peaks with the acetone signal were observable, and all these cross peaks had positive σ^{NOE} values. This shows clearly that in this mixed solvent oxytocin is more stably solvated by water than by acetone.

Conclusions

Quite generally, the ratio $\sigma^{NOE}/\sigma^{ROE}$ may be used to characterize the hydration lifetimes of different hydration sites on a polypeptide or a protein surface. However, the quantitative determination of the cross-relaxation rates is difficult for weak NOEs, since it would require that NOESY and ROESY spectra are recorded with sufficiently short mixing times to fulfill the initial rate condition, where the cross peaks are weak. As a favorable alternative, the temperature-dependent transitions from positive to negative values of σ^{NOE} can be reliably observed even in experiments with longer mixing times, which enables an accurate distinction of different hydration sites by their residence times in a much simpler way than by quantitative measurements of $\sigma^{NOE}/\sigma^{ROE}$ ratios at a single temperature.

The experiments of Figures 2 and 3 present a clearcut corroboration of the previous findings[3] that the residence times for water molecules on peptide or protein surfaces are in the subnanosecond time range at temperatures above 5 °C. These measurements clearly have the potential to discriminate between different individual hydration sites on the basis of the different effective correlation times (Figure 2). If effects from segmental mobility can be assessed, it will be possible to obtain a quantitative description of possible variations of the hydration water residence times in different sites on the protein surface. This information is not otherwise available with present methods; in particular, it cannot be derived from observations on protein hydration made by X-ray diffraction in single crystals.[3] Experiments of the type shown in Figures 2 and 3 can thus contribute to a more detailed characterization of the hydration of polypeptide chains in solution, which is of fundamental interest for a better understanding of protein folding, protein stability, and intermolecular interactions with proteins. As is indicated by the aforementioned experiments with oxytocin in 60% ^2H_2O/40% acetone, similar experimental approaches can be employed for investigations on preferred solvation in solutions with mixed solvents. This could be particularly attractive for studies of the solvation of unfolded forms of proteins resulting from the addition of chemical denaturants.

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