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Contributions from hydration of carboxylate groups to the spectrum of water–polypeptide proton–proton Overhauser effects in aqueous solution

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SUMMARY

Nuclear Overhauser effects (NOE) were measured between water protons and protons of the glutamic acid side chain of the bicyclic decapeptide c-[Arg-Arg-Cys-Asn-Ala-Glu-Ala-Cys-Lys-Gly] in aqueous solution. Positive NOEs were observed between the γCH_2 group of Glu and the water resonance, with similar NOE intensities at pH 2.0 and pH 6.3 in both the laboratory frame and the rotating frame of reference. These results indicate that the residence times of the hydration water molecules near the side-chain methylene protons are shorter than 500 ps for both the charged form and the uncharged form of Glu, and hence comparable to the water residence times near uncharged amino acid side chains. Furthermore, this study shows that the acidic proton in protonated carboxylic acid groups is not likely to interfere with the observation of polypeptide–hydration water NOEs, which is in contrast to the hydroxyl protons of the side chains of serine, threonine and tyrosine.

The characterization of the molecular surface of biological macromolecules in aqueous solution has recently been extended to include surface hydration, making use of the fact that the residence times of hydration water molecules near individual protons of a polypeptide chain can be estimated from the signs and intensities of the NOE cross peaks with the water signal observed in NOESY and ROESY experiments performed in H_2O solution (Otting et al., 1991c). The

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Abbreviations: NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy in the laboratory frame, ROESY, NOE spectroscopy in the rotating frame; 1D, one-dimensional; 2D, two-dimensional; HPLC, high-pressure liquid chromatography.

general picture of protein hydration that emerges from these experiments is that NOEs with the water resonance can be observed for all polypeptide protons exposed to solvent and that the residence times of individual surface hydration waters in all, or nearly all, hydration sites are shorter than 500 ps (Otting et al., 1991c). In DNA duplexes it was found that water molecules belonging to the spine of hydration in the minor groove of A=T base pairs (Kopka et al., 1983; Westhoff, 1988) are bound with residence times longer than 10 ns, although their solvent accessibility is comparable to that of other hydration waters in the minor and major grooves, which have residence times shorter than 500 ps (Kubinec and Wemmer, 1992; Liepinsh et al., 1992b). On the basis of this information, it is of special interest to investigate whether solvent-accessible hydration sites with outstandingly long residence times are also present in proteins, and to study the functional significance of this specific surface hydration.

Special care has to be taken when interpreting NOE experiments with water as the solvent when a polypeptide contains labile protons that exchange so rapidly that intramolecular NOEs with these protons appear also at the water chemical shift. This situation is, for example, encountered with the side-chain hydroxyl protons of Thr and Ser, where the same NOEs are observed with the resolved hydroxyl proton resonances at pH 6.3 and with the water resonance at pH 3.5, where rapid exchange (Wüthrich, 1986) causes these protons to appear at the chemical shift of the water signal (Liepinsh et al., 1992a). These cross peaks with hydroxyl protons at the water chemical shift must be identified properly because, if they were interpreted as direct NOEs with hydration water molecules, they would suggest the presence of long-lived surface hydration sites. The present study was undertaken to investigate whether a similar interference is likely to arise from the acid proton in protonated carboxylic acid groups.

For this study on carboxylate groups we designed the bicyclic decapeptide c-[Arg-Arg-Cys-Asn-Ala-Glu-Ala-Cys-Lys-Gly] (referred to hereafter as the decapeptide). The disulfide bridge was introduced to make the peptide more rigid, and to separate the Glu residue from the rapidly exchanging labile side chain protons of Arg and Lys. Arg and Lys were inserted to enhance the solubility of the peptide and prevent self-aggregation, since previous experiments showed that highly concentrated solutions were needed to obtain the sensitivity of the NMR experiments that is required for observation of NOEs with surface hydration water (Otting et al., 1991c). For the solid-phase synthesis of the linear sequence H-Arg-Arg-Cys-Asn-Ala-Glu-Ala-Cys-Arg-Gly-OH, 9-fluorenylmethoxycarbonyl (Fmoc) was used as a temporary α-amino-protecting group, and the following acid-labile protecting groups were used for the side chains: Arg, 2,2,5,7, 8-pentamethyl-chromane-6-sulfonyl; Cys and Asn, trityl (Sieber and Riniker, 1991); Glu, tert. butylester; Lys, tert. butyloxycarbonyl. The coupling reaction and the optimized Fmoc-cleavage were as described (Rink and Ernst, 1991). Mild cleavage from the trialkoxy-benzhydrylester resin with acetic acid (Rink, 1987) yielded the peptide with fully protected side chains. Subsequent cyclization with dicyclohexylcarbodiimide/hydroxybenzotriazole, removal of the protecting groups with trifluoroacetic acid/scavengers, and disulfide formation through air-oxidation at pH 8.0 resulted in the crude bicyclic peptide, which was then purified by reverse-phase HPLC. The purity and molecular weight of the intermediates and the product (purity >> 95%) were checked by HPLC, thin-layer chromatography, and mass spectrometry with plasma desorption and fast atom bombardment.

Homonuclear 2D NOESY and 2D ROESY spectra were recorded with a 50 mM solution of the decapeptide in a mixed solvent of 90% H_2O and 10% D_2O at 4°C, as described in the legend to

Fig. 1. The figure compares the 1 D cross sections taken along ω_2 at the ω_1 frequency of the water resonance through the NOESY spectra recorded at pH 6.3 (Fig. 1A) and at pH 2.0 (Fig. 1B) with a conventional 1D NMR spectrum recorded at pH 2.0 (Fig. 1C). Only the spectral region between 1.2 and 4.1 ppm is shown, because at lower field the spectrum was obscured by the residual water resonance, and near pH 6.3 by the chemical exchange peaks of the amide protons. The results obtained coincide largely with those obtained previously with a peptide of similar size, oxytocin (Otting et al., 1991c; 1992). Thus, all intramolecular NOESY cross peaks between different protons of the decapeptide were positive, which shows that the overall rotational tumbling rate is in the slow motional regime at 4°C, with a rotational correlation time greater than about 1.5 ns. (Note that positive NOESY cross peaks correspond to negative NOEs, and vice versa.) Furthermore, intermolecular NOESY cross peaks with water were observed for all proton resonance lines of the decapeptide. The fact that all these cross peaks were found to be negative (Figs. 1A and 1B) indicates that the residence times of the individual hydration water molecules near to any

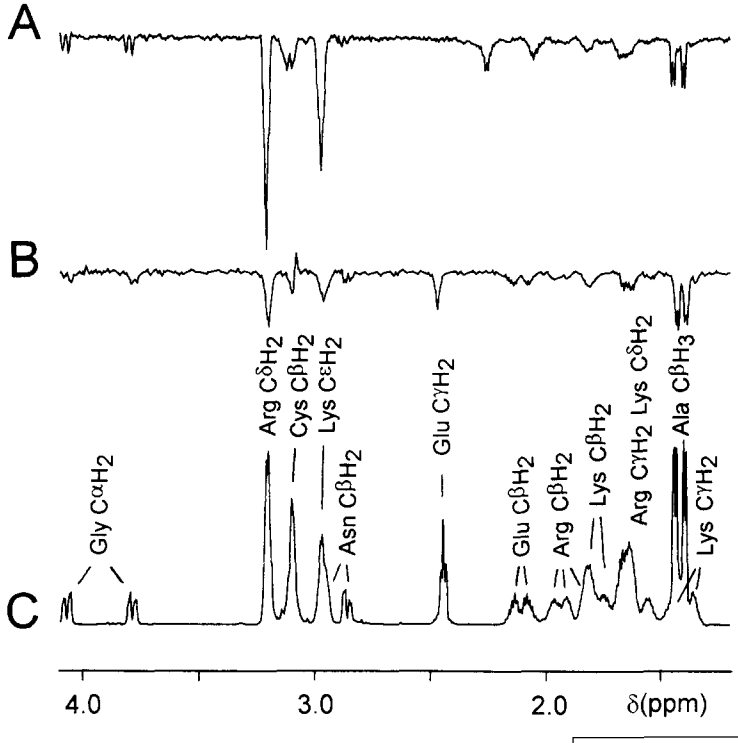

Fig. 1. ^{1}H NMR spectra of a 50 mM solution of the bicyclic decapeptide c-[Arg-Arg-Cys-Asn-Ala-Glu-Ala-Cys-Lys-Gly] in 90% H_2O/10% D_2O at 4°C recorded on a Bruker AM 600 spectrometer. (A) One-dimensional cross section taken along ω_2 through the diagonal peak of the water resonance in a 2D NOESY spectrum recorded at pH 6.3. The experimental scheme of Otting et al. (1991a) was used, with t_{1max} = 80 ms, t_{2max} = 205 ms, recorded data size 800 × 2048 points, mixing time 50 ms, spin-lock purge pulses of 0.5 and 2.0 ms duration, and a total recording time of about 10 h per spectrum. A delay of 167 μs between the two spin-lock pulses gave the spectral excitation profile sin(3.15–0.63δ), where δ is the chemical shift in ppm; the maxima of the excitation profile were thus near 2.5 and 7.5 ppm. (B) Same as (A), except that the pH was 2.0. (C) One-dimensional ^{1}H NMR spectrum at pH 2.0. The individual resonance lines are identified with the three-letter amino acid symbols and the designation of the group of hydrogen atoms.

of the NMR-observable protons must be shorter than about 500 ps (Otting et al., 1991c). The sign and intensity of the NOESY cross peaks between the water and the protons of the decapeptide did not vary significantly between pH 6.3 and pH 2.0. Exceptions were the βCH_2 signals of the Cys residues, which were affected by overlap with the intraresidual H^{α}–H^{β} cross peaks at pH 2.0, and the increased intensities of the cross peaks with εCH_2 of Lys and δCH_2 of Arg at pH 6.3 (Fig. 1A), attributed to the prevalence of a different magnetization transfer pathway for these protons. In a first step, magnetization from water to the εNH_3^+ of Lys or εNH of Arg is transferred by chemical exchange during the mixing time, and in a second step the magnetization is relayed from these labile side chain protons to the neighboring CH_2 groups via a TOCSY transfer during the 2-ms spin-lock before the detection period (Otting et al., 1991a). The increased cross-peak intensities at higher pH thus reflect the faster chemical exchange of the labile side-chain protons of Lys and Arg with the water (Wüthrich, 1986).

The chemical shift of the γCH_2 group of Glu 6 changed from 2.47 ppm at pH 2.0 to 2.25 ppm at pH 6.3, which corresponds to the protonated form at pH 2.0 and the deprotonated form at the higher pH (Bundi and Wüthrich, 1979). There was also a chemical shift change of the β-protons, which were non-degenerate at pH 2.0 and degenerate at the higher pH (Fig. 1). The most important observation in the present context is that the NOESY cross peak between the water resonance and the γCH_2 signal of Glu was negative throughout and its intensity did not change significantly with pH (Figs. 1A and 1B). This shows that the observed NOESY cross-peak intensity represents predominantly NOEs with highly mobile hydration water molecules, and that the additional peak intensity that might arise from dipole–dipole coupling between γCH_2 and the acid proton is negligible. For residence times of the acid proton between about 1 ns and 1 ms, any such NOE intensity would be expected to have a positive sign and to be at the chemical shift of the water resonance (Otting et al., 1991b; 1991c). The apparent low intensity of this NOE can be rationalized by a local conformation where the acid proton points predominantly away from the γCH_2 group (Almenningen et al., 1969; Grindley, 1982), so that the γCH_2–acid proton distance would be greater than 3.5 Å. Furthermore, in an aqueous environment at 25°C, the residence time of the acid proton is limited to values shorter than 100 ns by the self-dissociation rate of the carboxyl group, and significantly shorter times were found to arise from acid–base catalysis (Lankhorst et al., 1983). If the residence time of the acid proton were about 1 ns, the NOE would be partially quenched in the NOESY experiment by the translational dislocation of the acid proton. This quenching effect is expected to be less pronounced in ROESY than in NOESY experiments (Farmer et al., 1988). There is evidence that the cross peak between the γCH_2 of Glu and the water resonance in a ROESY experiment recorded at pH 2.0 under conditions identical to those of the NOESY experiment of Fig. 1B contained a small contribution from the NOE with the acid proton. Otherwise, the same sign of the NOEs and similar cross peak intensities were observed in the NOESY and ROESY experiments at both pH values. As described previously (Otting et al., 1991c), these results show that the water–peptide NOEs arise from dipole–dipole coupling modulated by translational diffusion of the hydration water molecules relative to the polypeptide surface, with residence times in the hydration sites shorter than 500 ps. Furthermore, the similar intensities of the NOESY cross peaks at high and low pH (Figs. 1A and 1B) show that these residence times are not affected noticeably by the state of protonation of the Glu side chain.

In conclusion, the present study with a model peptide shows that the carboxylic acid proton of Glu does not interfere with studies of hydration by observation of NOEs between the water line

and non-labile protons of the side chain of Glu. By analogy, this statement can be generalized to hold also for Asp side chains and for the C-terminal carboxylate in polypeptides. This behavior of carboxylate groups contrasts with the marked interference of the hydroxyl protons of Ser, Thr and Tyr (Liepinsh et al., 1992a). The charge on the carboxylate group did not have a measurable effect on the very short hydration lifetimes seen near the γCH_2 of Glu. In principle, the present data do not make it possible to exclude that more slowly exchanging hydration water molecules are bound to the carboxylate group at a position where they are too far away from γCH_2 to be observed by intermolecular NOEs. However, the results of a recent molecular dynamics simulation of a hydrated protein suggest that the residence times of hydration water molecules near charged carboxyl groups are shorter than 500 ps (Brunne et al., 1993), which would be in full agreement with the present experimental observations.

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