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Hydration Dynamics of the Collagen Triple Helix by NMR

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The hydration of the collagen-like Ac-(Gly-Pro-Hyp)$_6$-NH$_2$ triple-helical peptide in solution was investigated using an integrated set of high-resolution NMR hydration experiments, including different recently developed exchange-network editing methods. This approach was designed to explore the hydration dynamics in the proximity of labile groups, such as the hydroxyproline hydroxyl group, and revealed that the first shell of hydration in collagen-like triple helices is kinetically labile with upper limits for water molecule residence times in the nanosecond to sub-nanosecond range. This result is consistent with a ''hopping'' hydration model in which solvent molecules are exchanged in and out of solvation sites at a rate that is not directly correlated to the degree of site localization. The hopping model thus reconciles the dynamic view of hydration revealed by NMR with the previously suggested partially ordered semi-clathrate-like cylinder of hydration. In addition, the nanosecond to sub-nanosecond upper limits for water molecule residence times imply that hydration-dehydration events are not likely to be the rate-limiting step for triple helix self-recognition, complementing previous investigations on water dynamics in collagen fibers. This study has also revealed labile proton features expected to facilitate the characterization of the structure and folding of triple helices in collagen peptides.

Keywords: chemical exchange; collagen; hydration; NOE; NMR

Collagen is the most abundant protein in mammals (Nimni, 1988) and represents the major structural protein in the extracellular matrix (Kadler, 1994). The basic structural motif of collagen is the triple helix (Figure 1), which is also found in some host defense and membrane proteins (Kodama *et al.*, 1990; Hoppe & Reid, 1994; Elomaa & Tryggvason, 1995) and results from the right-handed super-coiling of three left-handed poly-proline-II helices around a common axis (Rich & Crick, 1961; Fraser *et al.*, 1979; Bella *et al.*, 1994). The three chains are staggered by one residue, allowing for inter-chain backbone-to-backbone hydrogen bonds. In addition, the three poly-proline-II helices are closely packed around the super-coiling axis, requiring that a glycine residue be present at every third position. This leads to repeating primary sequences of the -(Gly-Xxx-Yyy)$_n$- type. In collagen, the most common triplet is Gly-Pro-Hyp, where Hyp denotes 4-hydroxyproline resulting from the post-translational enzymatic modification of proline. As cyclic imino acid, Pro and Hyp are both sterically favorable to the formation of extended poly-proline-II helices. Furthermore, the presence of the hydroxyl group of Hyp significantly contributes to the thermodynamic stability of collagen triple helices (Berg & Prockop, 1973; Kielty *et al.*, 1993).

The stabilizing role of the Hyp OH moiety cannot be explained by direct hydrogen bonds donated by the Hyp OH hydroxyl group because the available acceptors in collagen triple helices are not sterically accessible. Several alternative explanations have therefore been pro-

Abbreviations used: CW, continuous wave irradiation; Hyp, hydroxyl-proline; MIN, abbreviation of MINSY, an NOE experiment with CW irradiation of a labile proton; NOE, nuclear Overhauser effect; ORR, off-resonance ROE; PHOGSY, protein hydration observed through gradient spectroscopy; ROE, rotating frame nuclear Overhauser effect; WG, watergate block.

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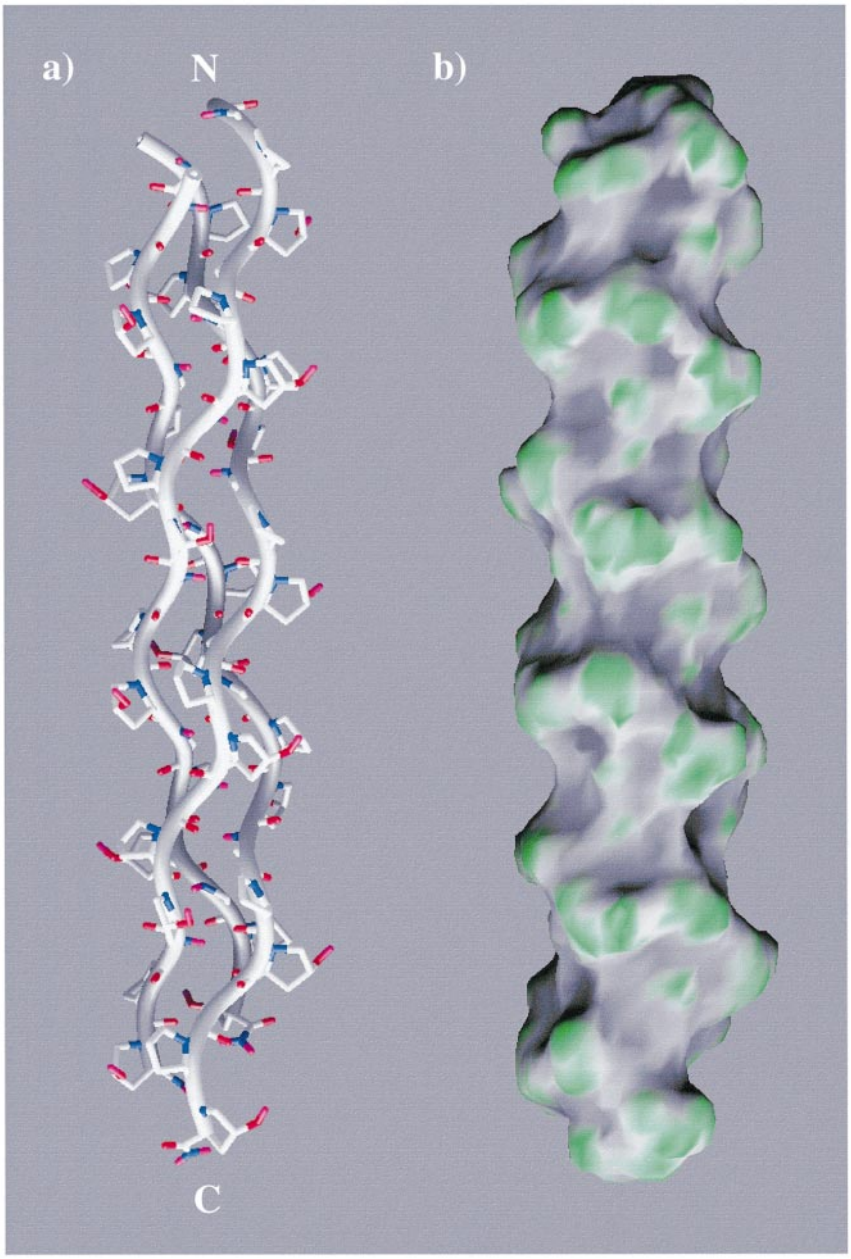

Figure 1. Structure (a) and molecular surface (b) of the collagen-like peptide [Ac-(Gly-Pro-Hyp)$_6$-NH$_2$]$_3$ triple helix. (a) Minimized model assembled starting from the triple-helical parts of the 1cgd structure (Bella *et al.*, 1995). Carbon atoms, white; oxygen atoms, red; nitrogen atoms, blue; polar hydrogen ions, magenta. (b) The molecular surface is colored accordingly to the surface curvature from convex (green) to concave (gray.) This Figure was generated with GRASP (Nicholls *et al.*, 1991).

posed, including inductive effects of the Hyp OH on the hydroxylprolyl peptide bond *cis-trans* isomerism (Holmgren *et al.*, 1998) and the involvement of the Hyp hydroxyl group in water-mediated hydrogen bonded bridges (Ramachandran *et al.*, 1973; Suzuki *et al.*, 1980). The latter hypothesis is also consistent with thermodynamic evidence showing that the enthalpy and entropy of collagen denaturation are significantly affected by bound water molecules (Fraser & MacRae, 1973; Privalov, 1982). In addition, the structure of water in collagen fibrils has been shown to differ significantly from that of water as bulk solvent (Berendsen & Migchelsen, 1965; Nomura *et al.*, 1977; Hoeve & Tata, 1978; Grigera & Berendsen, 1979; Cusack & Lees, 1984; Peto *et al.*, 1990).

The synergy between the Hyp hydroxyl groups and hydration is supported by the crystal structure of a triple-helical peptide containing mainly Gly-Pro-Hyp triplets (Bella *et al.*, 1994, 1995). This crystallographic structure revealed an ordered cylinder of hydration surrounding collagen-like triple helices and characterized by semi-clathrate-like repetitive patterns that involve the Hyp OH moiety, the carbonyl groups of Gly and Hyp and an extensive network of hydrogen-bonded water molecules. The water-mediated bridges are either inter or intra- triple-helices and the latter type includes links both within and between polypeptide chains. Hence, the hydration model obtained by X-ray analysis represents a good basis for interpreting experimental data on triple-helix stability and assembly. However, this model does not provide

any information on the kinetics of hydration. The focus of this investigation is therefore to characterize the hydration dynamics of collagen-like triple helices in solution. One of the most powerful tools to obtain local information on the residence times of hydration water molecules is high-resolution NMR (Otting, 1997). This method relies on the sign determination for the pure laboratory-frame dipolar cross-relaxation rate between water proton magnetization and ''probe'' protons belonging to the macromolecular solute (Otting *et al.*, 1991a,b, 1992), that is, here, the collagen-like Ac-(Gly-Pro-Hyp)$_6$-NH$_2$ peptide (Feng *et al.*, 1996; Melacini *et al.*; 1996).

Triple helix characterization

The 1D-WG (Piotto *et al.*, 1992; Sklenar *et al.*, 1993) spectrum of Ac-(Gly-Pro-Hyp)$_6$-NH$_2$ (Figure 2(a)) shows the characteristic resonances of collagen-like triple-helices, including the intense peak of Gly NH at 7.94 ppm, the Pro $C^{\delta}H_{l,h}$ signals at 3.52 and 3.20 ppm and the Hyp $C^{\beta}H_l$ resonance at 2.18 ppm (Brodsky *et al.*, 1992; Li *et al.*, 1993; Melacini *et al.*, 1996). The observation of these peaks confirms that under the experimental conditions used for this investigation, the peptide is well assembled into the typical collagen-like triple helices. In addition, the 1D-WG spectrum (Figure 2(a)) reveals two peaks that were not previously detected: a minor peak at 6.35 ppm and a major peak shifted upfield to 6.08 ppm. This ppm region is typical of hydroxyl protons in slow chemical exchange with water (Liepinsh *et al.*, 1992; Liepinsh & Otting, 1996) and the observed resonances are clearly assigned to the Hyp OH protons. The minor set of Hyp OH resonances can arise either from the well-known end effects of collagen-like triple helices (Li *et al.*, 1993; Melacini *et al.*, 1996) and/or from residual unassembled polypeptide chains. These interpretations are also supported by the observation of a resonance close to 6.3 ppm in the 1D-WG spectrum of a short unassembled peptide (Ac-(Gly-Pro-Hyp)$_3$-NH$_2$) under experimental condition similar to those used for the present investigation (G.M., unpublished results).

The major set of Hyp OH resonances arises from the Hyp residues that are in the core triple-helical environment. This assignment is supported by several NOEs observed between the major Hyp OH signal and the other resonances typical of collagen-like triple-helical structures (Figure 3(c)). As expected, the most intense NOESY cross-peaks involve protons belonging to the Hyp rings (i.e. cross-peak at 2.18 ppm in the strip of Figure 3(c)), while weaker cross-peaks are observed with selected protons of Pro residues (i.e. boxed cross-peaks in Figure 3(c)). The cross-peaks between the assembled Hyp OH resonance and the assembled Pro $C^{\delta}H_{l,h}$ (3.52 and 3.20 ppm) and Pro $C^{\beta}H_l$ (2.32 ppm) are particularly interesting because they can arise uniquely from inter-chain interactions based

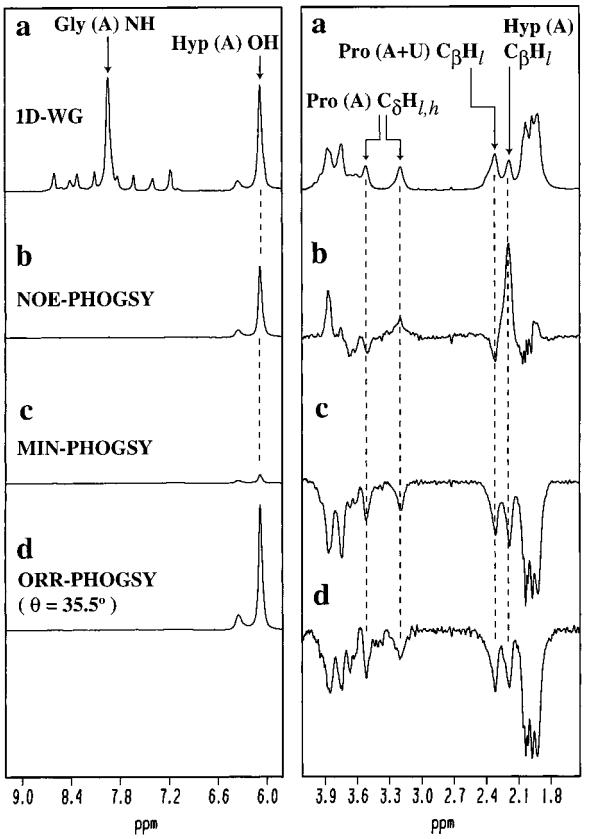

Figure 2. (Left) Low-field region of the (a) 1D-WG, (b) NOE-PHOGSY, (c) MIN-PHOGSY and (d) ORR-PHOGSY with $\theta = 35.5^\circ$ spectra of Ac-(Gly-Pro-Hyp)$_6$-NH$_2$ in 94.7 % H_2O, 5.3 % ^2H_2O (v/v) at 5 °C and pH 6.5. The concentration of single polypeptide chains (MW 1663) is 5.5 mM. All data were acquired at 500.13 MHz. The total mixing time is 85.3 ms for the NOE-PHOGSY and MIN-PHOGSY experiments, while it is only 40.3 ms for the ORR-PHOGSY spectrum in order to remain in the linear build up regime. The vertical scale of spectrum (d) is corrected proportionally to its reduced mixing time in order to facilitate comparisons in relative intensities with spectra (b-c). Labels indicate representative resonances and (A) denotes signals resulting from the assembled triple-helical structure. In spectrum (c) the Hyp (A) OH resonance is irradiated using a trapezoidal 224 Hz CW pulse. The residual Hyp (A) OH signal in (c) is approximately 12 % of that observed in (b). (Right) (a-d) show the high-field region of spectra (a-d), respectively. Spectrum (a) is vertically scaled to fit the Figure window, while spectra (b-d) are vertically scaled by a factor 75 as compared to the corresponding low-field regions in the left panels. Representative assignments are shown. The small peak close to 2.7 ppm arises from an impurity. All PHOGSY experiments were preceded by a weak gradient spin-echo with a hard 180° pulse and total length of 62 ms which avoids any ''leak-through'' from the $C^{\alpha}H$ protons of proline residues (Mori *et al.*, 1996; Melacini *et al.*, 1999b). Water magnetization was then selectively inverted by a 50 ms Gaussian pulse. Gradients were used for coherence selection in order to avoid biases arising from radiation damping effects.

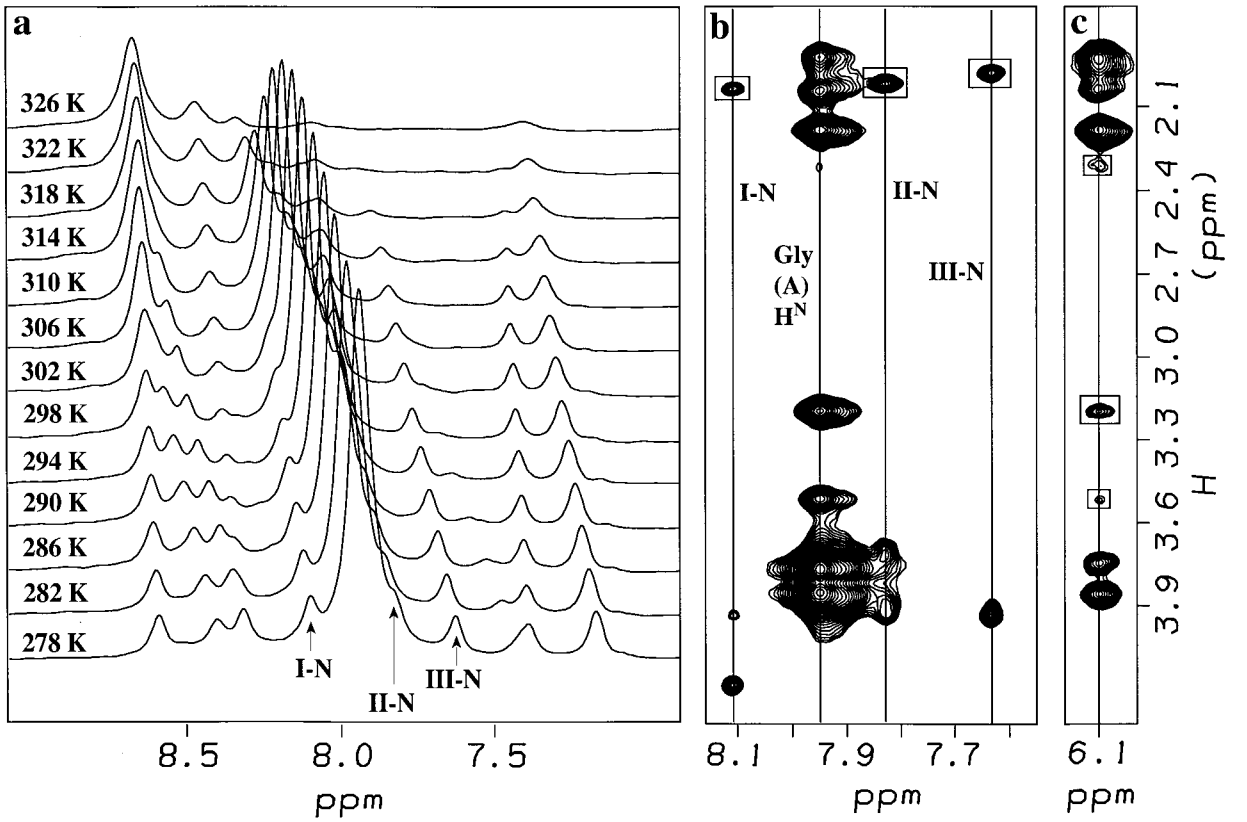

Figure 3. (a) Expanded amide region of 1D-WG spectra of Ac-(Gly-Pro-Hyp)$_6$-NH$_2$ in 94.7% H_2O, 5.3% ^2H_2O (v/v), pH 6.5 and acquired at different temperatures. The ppm scale refers to the spectrum at 278 K, while the spectra at high temperatures are shifted to the left by a constant offset to obtain a clearer representation. The labels I-N to III-N indicate the resonances at 8.11, 7.83, and 7.63 ppm, respectively. These peaks arise from the N-terminal Gly residues of the three collagen chains and are split by the collagen triple-helix tail effects. The intense peak at 7.94 ppm arises from the amide protons of Gly residues in the core of the triple-helical structure (assembled (A) set). (b) Expanded region of the 2D NOESY-WG spectrum (Kumar *et al.*, 1980) ($\tau_{mix} = 58$ ms) of Ac-(Gly-Pro-Hyp)$_6$-NH$_2$ in the same experimental conditions as in Figure 2. The labels have the same meaning as in (a). Boxed cross-peaks correspond to sequential NOEs between the acetyl methyl protons and the N-terminal Gly H^N. (c) Expanded region of the same NOESY-WG spectrum as in (b) showing the cross-peaks involving the major Hyp OH resonance. The boxed cross-peaks here indicate inter-chain NOEs beween Hyp OH and Pro side-chain protons. Quadrature detection was obtained using the States-TPPI scheme (Marion *et al.*, 1989). All data were processed using NMRPipe (Delaglio *et al.*, 1995).

on the collagen zones of the X-ray structure of a similar -(Gly-Pro-Hyp)$_n$- peptide (Bella *et al.*, 1994, 1995). However, the NOEs involving the Hyp OH group lead to exchange-relayed artifacts in the measurements (*vide infra*) aimed at characterizing the direct transfer of magnetization from water (van de Ven *et al.*, 1988; Otting *et al.*, 1991a; Otting, 1997).

The 1D-WG spectrum of Ac-(Gly-Pro-Hyp)$_6$-NH$_2$ also reveals an interesting dispersion of the signals in the amide region (Figure 2(a)): besides the intense peak at 7.94 ppm assigned to the Gly NH protons in the core of the triple-helical structure (Li *et al.*, 1993; Melacini *et al.*, 1996), several other less intense signals are observed between 7 and 9 ppm. The peak at the lowest field resonates at 8.60 ppm as also found for Gly NH residues belonging to the core of single unassembled Ac-(Gly-Pro-Hyp)$_n$-NH$_2$ polypeptides chains (Melacini *et al.*, 1996). In the vicinity of the core assembled Gly NH signal, three additional satellite peaks are detected at 8.11, 7.83 and 7.63 ppm. These peaks are assigned to N-terminal Gly NH residues based on the sequential acetyl-methyl-Gly NH NOEs observed in the 2D-NOESY-WG spectrum (boxed cross-peaks in Figure 3(b)) and they are denoted as I-N, II-N and III-N, respectively.

Similar to the Gly NH resonances in the core of the triple helix, the I, II and III-N peaks are upfield-shifted as compared to the N-terminal Gly NH resonance in unassembled single chains, which resonates at 8.34 ppm (Melacini *et al.*, 1996). This observation suggests that the I, II and III-N signals correspond to Gly NH protons in an environment at least partially structured as expected for Gly NH residues close to the triple-helix ends. In addition, the dispersion of the I, II and III-N resonances is fully consistent with the well-known triple-helix

end asymmetries (Li *et al.*, 1993; Melacini *et al.*, 1996). This interpretation is further supported by the intensity ratios for signals I, II and III-N, which is close to 1:1:1 (Figure 3(a)) as expected for the collagen trimer. Furthermore, melting experiments (Figure 3(a)) show that the chemical-shift dispersion observed at low temperatures is reduced when the triple helix is denatured. Consistent with these observations, renaturation experiments show that the I, II and III-N Gly amide signal dispersion reappears when the triple helix is formed again upon cooling. The temperature dependence of the spectra shown in Figure 3(a) also indicates the presence of other signals that are associated with the formation of triple-helical structures. While these additional peaks are consistent with the triple-helix C-terminal end-effects and the C-terminal primary amide group, no unambiguous assignment was possible for these signals.

Triple-helix hydration

The NOE-PHOGSY (Dalvit, 1995; Dalvit & Hommel, 1995) spectrum (Figure 2(b)) indicates that the laboratory-frame dipolar cross-relaxation rate between water and the Pro $C^{\delta}H_l$ protons in the core of the collagen-like triple-helix is positive. The NOEs exchange-relayed by the Hyp OH spins do not allow us to draw any conclusion about the other assembled (A) peaks based on the NOE-PHOGSY spectrum (Figure 2(b), see Supplementary Material). However, these exchange-relay biases are significantly reduced in the MIN-PHOGSY experiment with saturation of the OH signal (Olejniczak *et al.*, 1986; Massefski & Redfield, 1988; Fejzo *et al.*, 1991; Macura *et al.*, 1994; Zolnai *et al.*, 1995; Zwahlen *et al.*, 1996; Dalvit, 1998; Phan *et al.*, 1999; Melacini *et al.*, 1999a) (Figure 2(c)). Figure 2(c) shows that the effective dipolar cross-relaxation rates between water and the Pro $C^{\delta}H_h$, Pro $C^{\beta}H_l$ and Hyp $C^{\beta}H_l$ protons are positive (see Supplementary Material). This conclusion is also consistent with the pattern of negative peaks independently observed for the ORR-PHOGSY experiment (Desvaux *et al.*, 1994, 1995; Desvaux & Goldman, 1996; Birlirakis *et al.*, 1996; Hwang *et al.*, 1997; Guennegues, 1999) shown in Figure 2(d) (see Supplementary Material).

Considering that the measured positive cross-relaxation rates probe all the previously characterized hydration sites (Bella *et al.*, 1995) (Table 1), an average picture emerges of a kinetically labile first hydration shell of collagen-like triple helices with residence time upper limits in the ns or sub-ns time-scales (Otting *et al.*, 1991a; Bruschweiler & Wright, 1994; Otting, 1997; Denisov *et al.*, 1997). Since proton exchange between bulk-phase water molecules occurs in longer times scales under the experimental conditions adopted (Meiboom, 1961), the ns or sub-ns residence time upper limits refer to the exchange of entire water molecules between the hydration sites and the bulk phase (Otting, 1997). This conclusion is further supported by solvent accessible-surface area computations (Figure 1(b) and Table 1). Table 1 shows that all the water molecules in the different hydration sites characterized by X-ray crystallography (Bella *et al.*, 1994, 1995) are significantly more exposed to the bulk solvent than long-lived water molecules previously detected in globular proteins. For these long-lived water molecules, the solvent accessible surface area is usually lower than 10 $Å^2$ (Denisov & Halle, 1996). Moreover, the ill-localized hydration sites that may escape detection through X-ray crystallography are also expected to be considerably solvent exposed, because large internal cavities are absent, as can be seen from the molecular surface of the collagen peptide in Figure 1(b).

The short residence times measured in solution by NMR cannot be employed to assess the extent of hydration site localization in single triple helices. Furthermore potential crystal packing biases may cause differences between the spatial definition of hydration patterns observed in single crystals (Bella *et al.*, 1994) and those occurring in solution.

Table 1. Solvent-accessible surface areas of collagen hydration sites and their distances to NMR probe protons

Hydration site[a]	Minimal SASA[b]	Triple-helix probe protons[c]			
		Pro $C^{\beta}H_l$	Pro $C^{\delta}H_l$	Pro $C^{\delta}H_h$	Hyp $C^{\beta}H_l$
Gly W_N	39.5	-[d]	2.5-4.0	3.0-4.0	-[d]
Hyp W_A	55.5	3.5-4.0	-[d]	-[d]	-[d]
Hyp W_N	43.5	3.5-4.0	-[d]	3.0-4.0	2.5-3.5
Hyp W_B	44.5	-[d]	-[d]	-[d]	2.5-4.0
Hyp W_D	26.0	-[d]	2.5-4.0	3.0-3.5	-[d]

The reported ranges refer to the water oxygen-collagen probe proton distances lower than the cutoff distance of 4.0 Å (Sunnerhagen *et al.*, 1998). All distances are in Å and are rounded to 0.5 Å.

[a] Hydration sites are defined and named according to Bella *et al.* (1995). The distance ranges are based on the collagen zones of the Protein Data Bank structure 1cgd (Bella *et al.*, 1994, 1995) with added hydrogen atoms.

[b] Solvent-accessible surface areas (SASA) were computed using the program Naccess (Hubbard & Thornton, 1993) with a probe radius of 1.4 Å. Only the collagen zones of the 1cgd structure (Bella *et al.*, 1994, 1995) were considered in the analysis and for each hydration site the minimum SASA computed in the absence of water molecules belonging to other hydration sites is reported in $Å^2$.

[c] Probe protons were stereospecifically assigned as previously described (Li *et al.*, 1993; Melacini & Goodman, 1998).

[d] Distances >4.0 Å.

However, thermodynamic data show a significant effect of bound water molecules on the denaturation enthalpy and entropy of collagen (Fraser & McRae, 1973; Engel *et al*., 1977), suggesting that the triple-helical structure causes more ordering of the hydration water than the denatured single polypeptide chains (Berendsen & Migchelsen, 1965; Privalov, 1982). In addition, molecular dynamics simulations of triple-helical Ac-(Gly-Pro-Hyp)$_6$-NH_2 confirm the presence of localized hydration sites in solution (A.M.J.J.B *et al*., unpublished results). The resulting at least partially localized picture of triple-helix hydration and the short residence times measured in solution by NMR are fully reciprocally consistent if a ''hopping'' hydration model is considered (Teeter, 1991; Gu & Schoenborn, 1995; Sunnerhagen *et al*., 1998). According to this model, water molecules bounce in and out of localized hydration sites and the rate of in/out exchange is not directly correlated to the degree of localization of the site.

The cylinder of hydration surrounding collagen triple helices has also been proposed to play a role in triple helix to triple helix assembly, which leads to higher order structures such as micro-fibrils and fibrils found in biological tissues (Bella *et al*., 1995; Kramer *et al*., 1998). The NMR results on ns or sub-ns hydration dynamics suggest that hydration-dehydration steps are not likely to be rate limiting (Otting, 1997) in triple-helix/triple-helix recognition (self-recognition). However, the hydration of single triple helices is still expected to be relevant for self-recognition because it provides the basis for structural complementarity (Bella *et al*., 1995).

Once the triple-helical units are assembled in higher order structures, a fraction of water molecules is expected to become at least partially buried from the bulk solvent, and therefore can reside in the vicinity of collagen molecules for times longer than those determined here. This prediction is in agreement with previous results on collagen fibers that indicate the presence of water molecules with correlation times $\geqslant \sim 1$ ns (Grigera & Berendsen, 1979; Peto *et al*., 1990; Renou *et al*., 1994; Knauss *et al*., 1996). In addition, it was noticed that the remaining water molecules, constituting the majority of the total water present, formed a relatively more mobile multi-layer with quasi-liquid-like properties and rotational correlation times shorter than 100 ps (Grigera & Berendsen, 1979; Henkelman *et al*., 1994). The mobility of this major fraction of water molecules is also consistent with the rapid dynamics of hydration here reported for collagen-like model triple helices in solution.

In conclusion, this study reveals that the first hydration shell of the collagen-like [Ac-(Gly-Pro-Hyp)$_6$-NH_2]$_3$ triple-helix is kinetically labile, with upper limits for water molecule residence times in the ns to sub-ns range. This highly dynamic picture of collagen hydration can be reconciled with the previously suggested partially localized semi-clathrate-like cylinder of hydration by an hopping hydration model in which the rate of in/out exchange and the degree of localization are not directly correlated. The labile hydration shell has important implications for the kinetics of triple-helix self-recognition.

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