Structure of the Alanine Dipeptide in Condensed Phases Determined by ^{13}C NMR

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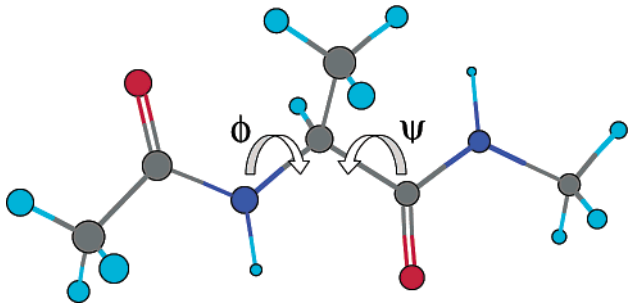
We report solution-state and solid-state ^{13}C NMR data for the alanine dipeptide (*N*-acetyl-L-alanine-*N*′-methylamide, AcAlaNHMe) in polycrystalline, lyophilized, and solvated states. Changes in the ^{13}C chemical shifts of individual carbonyl carbons between the solvated and lyophilized states for the alanine dipeptide dissolved in H_2O and $CHCl_3$ suggest preferential solvation at different carbonyl carbons. This effect depends on the solvent, and it suggests an alteration in secondary structure on removal of water. We employ a novel external referencing scheme (neat tetramethylsilane under magic-angle spinning), which allows for a direct comparison of solution- and solid-state chemical shifts. The structure of the alanine dipeptide in the crystalline state and after lyophilization out of H_2O and $CHCl_3$ solutions was determined using double-quantum dipolar recoupling solid-state NMR at high magnetic field strength (600 MHz). The compound adopts the same polyproline-II-like secondary structure when lyophilized from hydrogen-bonding (H_2O) and non-hydrogen-bonding ($CHCl_3$) solvents.

Solvent interactions are key to understanding protein folding. The capped alanine dipeptide (*N*-acetyl-L-alanine-*N*′-methylamide, AcAlaNHMe, shown below) has received sustained interest over the last two decades as a model system for the prediction of folded secondary structures in solvated environments.[1−7] Small molecules, such as dipeptides of amino acids, are able to adopt a greater variety of low-energy conformations than larger peptides and thus provide an added challenge for computational structure prediction. Recent computational analyses using either a molecular mechanics or a quantum mechanical/molecular mechanics force field indicate that in water AcAlaNHMe adopts either a β-sheet conformation or an α-helical conformation.[7] The ratio of populations for these two conformers and the energy barrier for interconversion depend on the force field employed. These predictions are in contrast to the computed results for AcAlaNHMe in vacuo, which predict that the C_{7eq} conformation should be favored.[3] A recent study has provided the first conclusive spectroscopic evidence for the C_{7eq} structure of the alanine dipeptide ($\phi \approx -80°$, $\psi \approx +71°$) in the gas phase.[8] Recent work on AcAlaNHMe in water and in a liquid−crystalline solvent medium has presented evidence that the structures in aqueous solution and the crystalline state are very similar, poly(proline)-II-like (P_{II}), with $\phi \approx -85°$, $\psi \approx +160°$.[9,10] However, these studies were inconclusive in ruling out the coexistence of an α_R conformation in water and assumed equal solvation of the two carbonyl groups. Additionally, controversy and disagreement exist in the literature about the stable structures this compound adopts in other solvated environments, and reports with hard experimental data are scant. Here we present solution- and solid-state ^{13}C NMR data at high magnetic field strength that provide evidence for preferential solvation of AcAlaNHMe in water and chloroform. The data indicate that the alanine dipeptide adopts a P_{II}-like structure in a non-hydrogen-bonding solvent environment, but a mixture of P_{II} and α_R conformers is more likely in water. Finally, removal of either solvent results in a P_{II}-like structure.



We first report the results of ^{13}C chemical shift measurements for AcAlaNHMe in the polycrystalline, lyophilized, and solvated states. For the lyophilized samples, AcAlaNHMe was dissolved in H_2O and $CHCl_3$ at low concentration (10 mM) then injected into *n*-pentane at −110 °C using a 27-gauge needle syringe. The resulting "snow" was centrifuged cold, and the *n*-pentane was decanted and dried under high vacuum for several days. Samples were held at −78 °C during the drying using dry ice.[11] The lyophilized states exhibited some diffraction in the X-ray powder patterns (data and discussion presented in the supporting section). When comparing chemical shifts between solid and liquid states, as we do here, proper referencing becomes critical for two reasons: first, a common external reference is necessary for liquids and solids and, second, isotropic bulk magnetic susceptibility must be taken into account. To address both concerns, we employed a novel referencing scheme suggested recently by Morcombe and Zilm, using neat tetramethylsilane (TMS) as the external reference for all spectra and applying magic-angle spinning (MAS).[12] This approach removes the isotropic bulk magnetic susceptibility contribution to the chemical shift, which affords a direct comparison of chemical shifts

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TABLE 1: ^{13}C Chemical Shifts (ppm) of AcAlaNHMe in Various Environments[a]

	experimental							calculated (in H_2O)				
	poly crystal CPMAS[b]	$CHCl_3$ soln ZAS[c]	$CHCl_3$ soln HRMAS[d]	lyoph $CHCl_3$ CPMAS	H_2O soln ZAS	H_2O soln HRMAS	lyoph H_2O CPMAS	coil	P_{II}[g] (−80,150)	α_R[g] (−80,−30)	$P_{II}+\alpha_R$[h]	C_{7eq}[g] (−80,71)
DSS					0							
TMS	2.00[e]	2.63	2.00[e]	2.00[e]		2.00[e]	2.00[e]					
C_β[f]	20.61	21.05	21.18	20.53	19.34	19.49	20.63	19.10	20.44	18.94	19.69	19.39
acetyl CH_3	25.22	25.65	25.72	25.06	24.37	24.51	25.08					
amide CH_3[f]	28.29	28.86	29.00	28.21	28.52	28.76	28.23					
C_α	51.66	51.53	51.70	51.58	52.59	52.77	51.60	52.50	51.01	54.72	52.86	51.37
acetyl C═O	173.44	173.13	173.40	173.52	176.86	176.94	173.54					
alanine C═O	178.13	175.93	176.12	178.21	178.41	178.51	178.23	177.80	176.25	179.17	177.71	176.21

[a] MAS spectra were externally referenced to neat TMS (2.0 ppm compared to DSS in H_2O under MAS Conditions).[12] Data are adjusted to DSS to allow direct comparison with calculated chemical shifts based on prediction programs using the empirically derived protein chemical-shift dependencies corresponding to particular (ϕ, ψ) torsion angles.[13] Data were also taken in water and chloroform using a standard high-resolution (zero-angle) probe with internal reference standards and are shown for comparison. All data were collected at 20 °C and 600.377 MHz (^{1}H), 150.987 MHz (^{13}C). MAS spectra were collected at a spinning speed of 4 kHz; MAS solution-state spectra were collected using a sealed rotor insert. All spectra were collected without a ^{2}H lock with 200 scans. The drift rate for the magnet was less than 0.188 Hz/h (^{13}C, 0.0012 ppm/h). [b] Cross polarization-magic angle spinning [c] Zero angle spinning (traditional liquids) [d] High-resolution magic angle spinning [e] Neat TMS external reference [f] Peaks show an uneven doublet splitting in solid samples [g] (ϕ,ψ) torsion angles given in parentheses [h] Equal populations of P_{II} and α_R

between solids and liquids with varying magnetic susceptibilities.

When the susceptibility portion of the chemical shift is removed under MAS, what remains are contributions due to chemical environment, solvent interactions, and secondary structure. Chemical shifts of the C_α, C═O, and methyl carbons for the crystalline, lyophilized, and solubilized compound are summarized in Table 1 along with predicted chemical shifts for particular secondary structures.[13] The C_α and C_β shifts in Table 1 for the crystalline, lyophilized, and dissolved in chloroform samples show agreement with the predicted chemical shifts for a P_{II}-like conformation. The C_α and C_β shifts for AcAlaNHMe dissolved in H_2O are in better agreement with predictions for an almost equal mixture of α_R and P_{II} rapidly interconverting. These data largely support earlier conclusions that AcAlaNHMe adopts a P_{II}-like conformation in a crystalline form and when dissolved in a non-hydrogen-bonding environment and a mixture of two conformers in water.[9,10] Since the chemical shifts for the C_α and C═O carbons do not change in a coordinated fashion upon lyophilization out of chloroform, conformational changes on freezing or removing solvent are unlikely,[13] a point further supported by double-quantum dipolar recoupling (DQDR) NMR experiments (vide infra). However, our data do show a marked change in chemical shifts for the acetyl carbonyl (−3.40 ppm), C_α (−1.17 ppm), and C_β (+1.14 ppm) upon lyophilization out of aqueous solution, suggesting an overall change in secondary structure on lyophilization. However, the data show only a slight change in chemical shift for the alanine carbonyl (−0.28 ppm). Furthermore, the changes in chemical shift of the carbonyl carbons on lyophilization out of chloroform or water are markedly different. On lyophilization from chloroform, a large change in chemical shift is seen for the alanine carbonyl carbon (+2.09 ppm) and only a slight change for the acetyl carbonyl carbon (0.12 ppm). Conversely, on lyophilization from water a large change in chemical shift is seen for the acetyl carbonyl carbon(−3.40 ppm) and only a slight change for the alanine carbonyl carbon (−0.28 ppm). These differences suggest a large, selective solvent effect for the individual carbonyls, which are solvent dependent. Our data do not corroborate the double-water-bridge model, in which a sizable chemical-shift effect should be exhibited by both carbonyl carbons, as they would both participate in a hydrogen bonding network.[4,9] In the same fashion, our data show a preferentially large interaction with a non-hydrogen-bonding solvent (chloroform) at the alanine carbonyl carbon. The chemical shift data of Table 1 echo the cautious, sobering conclusions of Bolhuis et al. that the solvent degrees of freedom are much more subtle and complex, in their structure as well as dynamics, than static electronic structure calculations might suggest.[14]

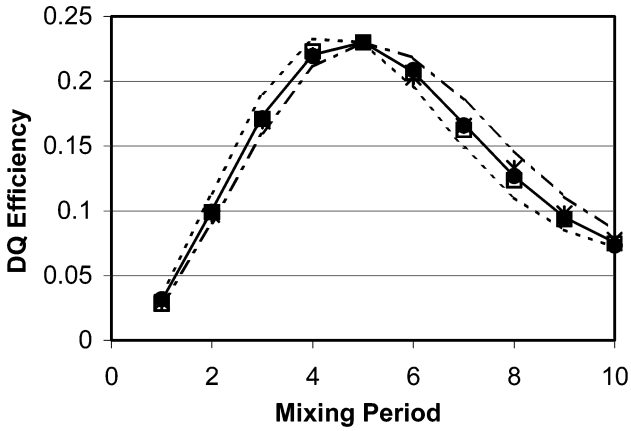

Figure 1. Double-quantum buildup curves for crystalline (●) and lyophilized states of AcAlaNHMe (water, □; chloroform, *). Simulated curves are shown for $\phi = -75°$ (dashed line), −85° (solid line), and −95° (dot-dashed line).

To investigate local structure in the crystalline and lyophilized states, we employed DQDR NMR experiments using dipolar recoupling with a windowless sequence (DRAWS) pulse sequence.[15] We synthesized AcAlaNHMe with ^{13}C labels at the two carbonyl carbons using existing procedures. In DQDR experiments, the DQ excitation efficiency is directly dependent on the mixing time. When used in conjunction with numerical simulations, the DQ buildup profile (the exact dependence on mixing time) can be used to determine the internuclear distance. In AcAlaNHMe, the distance between the two carbonyl carbons is a direct measure of the $\pm\phi$ angle. The $+\phi$ value is often ruled out for non-glycine residues based on steric considerations. Figure 1 shows the DQ buildup curves for the crystalline and two lyophilized states of AcAlaNHMe. The three sets of data lie virtually on top of each other, suggesting very similar ϕ angles. Numerical simulations bear this out, giving an internuclear distance of 3.24−3.26 Å consistent with $\phi \approx -85°$.

A 2D version of the DQDR experiment yields a spectrum in which the amplitudes of the spinning sidebands encode both ϕ

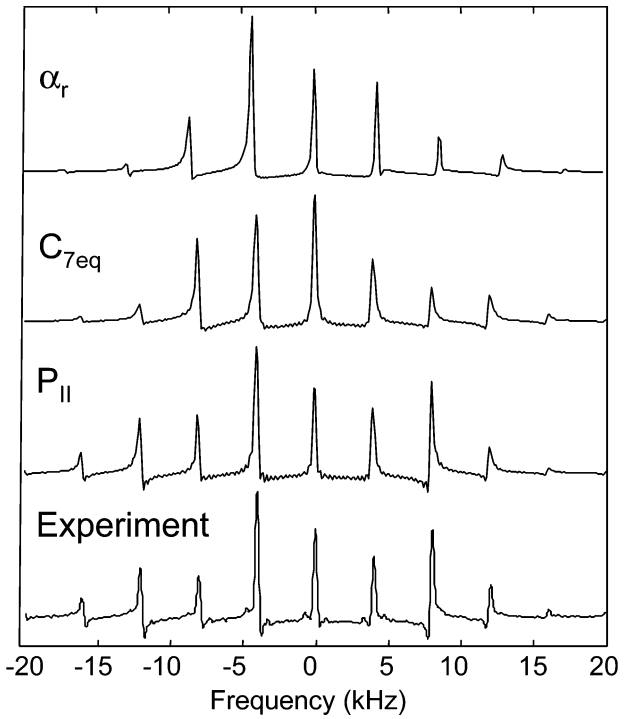

Figure 2. DQ projection of the 2D-DQ-DRAWS spectrum for crystalline AcAlaNHMe and simulated spectra for P_{II}, C_{7eq}, and α_R as indicated.

TABLE 2: Geometrical Parameters of AcAlaNHMe Determined Using DQDR NMR[a]

physical state	C═O···C═O distance (Å)	ϕ from DQ buildup	ψ from 2D-DQ-DRAWS
crystalline	3.25 ± 0.02	$-85° \pm 2°$	$146° \pm 19°$
lyophilized from H_2O	3.24 ± 0.02	$-84° \pm 2°$	$147° \pm 20°$
lyophilized from $CHCl_3$	3.26 ± 0.02	$-86° \pm 2°$	$145° \pm 22°$

[a] Reported values are from best-fit numerical simulations.

and ψ. Figure 2 shows the DQ Projections of 2D-DQ-DRAWS spectra of the crystalline state and the best-fit numerical simulation (P_{II}: $\phi = -85°$, $\psi = 146°$). Simulations for the α_R and C_{7eq} conformations are shown for comparison ($\phi = -80°$, $\psi = -30°$ and $\phi = -80°$, $\psi = 71°$, respectively). The sideband envelopes in the DQ spectra of the two lyophilized samples are very similar to the crystalline sample, indicating the same conformation. On the basis of these data, as well as the line widths and isotropic chemical shifts observed in the chemical shift spectra, we see no evidence for conformations other than P_{II} in any of the solid samples.

The DQ-DRAWS buildup curves and 2D-DQ-DRAWS data show that the crystalline and the two lyophilized states share nearly identical ϕ and ψ values, summarized in Table 2. Given the experimental evidence for similar structures in the three solid samples, the large changes in chemical shift only at select positions upon freeze drying from chloroform point to preferential solvent interactions rather than changes in secondary structure. However, for the sample lyophilized from water, significant changes in the chemical shifts of all the backbone carbons except one indicate changes in secondary structure on lyophilization as well as the presence of preferential solvent interactions.

Our results suggest that in water there exist two conformers that rapidly interconvert on the NMR time scale. Removal of the water on lyophilization destabilizes the second structure and favors the P_{II} conformer. Full conversion to the P_{II} state for AcAlaNHMe is more rapid than changes in secondary structure observed on freezing of larger peptides,[11] suggesting a relatively low activation barrier. In their thorough examination of AcAlaNHMe in a liquid−crystalline environment, Weisshaar and coworkers present data and simulations that support the presence of a single P_{II} conformer in water.[13] Nevertheless, their residual dipolar coupling data are insufficient to rule out the existence of a second conformer. Instead, they rely on the $J(H^N,H^\alpha)$ coupling constant to limit ϕ to a value between -75 and $-95°$. This type of constraint is well-established in the NMR community, but their assertion that it rules out any α_R conformation is not necessarily correct. Estimates of the torsion angles for the AcAlaNHMe α_R state are closer to $\phi = -80°$ and $\psi = -30°$ than $\phi = -60°$ and $\psi = -60°$ when water molecules are explicitly modeled.[14] Additionally, they argue that the interaction of AcAlaNHMe with bicelles does not affect its secondary structure. However, our data indicate differential solvation of the two carbonyls in water and chloroform, suggesting their arguments regarding the relative stabilities of the two conformers at the water/bicelle interface may be too simplistic. Unfortunately, the magnetic susceptibility effects of the liquid−crystal additive preclude direct comparison of the non-MAS chemical-shift data presented in their paper with data for AcAlaNHMe dissolved in water alone. Finally, vibrational spectra support a possible mixture of P_{II} and α_R conformers of in water.[4]

Data presented here pose a substantial challenge for modern, atomic-level structure-prediction models. This work, in agreement with previous NMR data,[13] supports a further constraint on the torsion angle ϕ in solvated environments (between -75 and $-95°$). Any conformational changes due to solvent effects will primarily affect the ψ torsion angle. Further experiments are underway in our laboratory to understand changes in chemical shift and dynamics for AcAlaNHMe in the course of hydration.

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Supporting Information Available: Details of the chemical synthesis, X-ray diffraction, NMR experiments, and numerical simulations are presented (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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