High-Resolution Magic Angle Spinning NMR Study of Resin-Bound Polyalanine Peptides

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Abstract: Polyalanine sequences of varying length and tethered to a solid support are studied in atomic detail by high-resolution magic angle spinning (HR MAS) NMR. At high densities, it is shown that aggregation of the sequences is at the origin of synthetic difficulties. Decreasing the peptide density by discharging the resin allows study of longer sequences without being hampered by aggregation, and α helix formation has been observed for the $(Ala)_{12}$ sequence. This demonstrates that the combined use of solid phase synthesis and high resolution magic angle spinning NMR spectroscopy is a new tool that can be used advantageously in the study of aggregating structures

The process of peptide conformational changes leading to irreversible aggregation is important both for chemistry and biology. In the field of solid-phase peptide chemistry, the difficulties encountered during the synthesis of certain sequences have been ascribed to interchain aggregation of the resin-bound peptides, leading to a higher effective degree of cross-linking, with subsequent problems of swelling and of accessibility of deprotection and/or coupling agents.[1] Similar processes leading to the interconversion of cellular-allowed peptide conformations (random coil and/or α-helix) to a cell-deleterious β-pleated-sheet conformation are thought to be at the origin of a number of amyloid diseases such as Alzheimer's disease (AD) and the prion diseases.[2] In the latter case, there clearly is a kinetic competition between productive folding and aggregation,[3] and understanding the factors that dominate this competition will be of considerable interest to intervene in the aggregation process.

Polyalanine has been studied extensively in both fields as an example of an aggregating system. It has been used as a model system to investigate the different factors that influence the coupling yield in solid-phase peptide chemistry, because of the notorious difficulties that arise when one wants to couple more than five subsequent Ala residues on a polystyrene-divinylbenzene resin (PS-DVB). The commonly accepted hypothesis is that short sequences do not provide enough hydrogen bonding to produce stable structures, but for $(Ala)_5$, $(Ala)_6$, and $(Ala)_7$, appreciable amounts of β-conformations are formed, leading to stable intermolecular sheets with nearby peptide chains.[4] The ease of deprotection and couplings after $(Ala)_7$, on the other hand, suggest that these structures are reversible at each synthetic cycle, with α-helical structures predominating at residues 8, 9, and 10, without formation of cross-links. For the problematic synthetic steps, many solutions have been devised, but only the explicit incorporation of β-sheet breakers such as D-Ala residues has truly resolved the problem.[5]

Polyalanine peptides have equally been used as solution models for the self-association process leading to β-sheet complexes.[6] Temperature studies on a soluble form of poly-Ala (Ac-KYA_{13}K-NH_2) indicated that the stabilization of complexes is resulting from hydrophobic interactions. A scan with different residues at position 10, most critical for the self-assembly of this peptide, showed that a Pro residue is indeed very effective at preventing the formation of β-sheet structures, whereas a Gly residue leads to essentially the same aggregation as the all-Ala peptide. This is in agreement with the results on the Syrian hamster prion protein peptide 113−120 (AGAAAAGA), which was found to be the most highly amyloidogenic peptide in a series of prion peptides predicted in α-helical conformation.[7]

Recently, we used high-resolution magic angle spinning NMR (HR-MAS) to study in atomic detail the resin-bound aggrecan sequence[8] and were able to correlate synthetic difficulties with a loss of mobility on the resin. Here, we extend our observations to the synthesis of poly-Ala and use the possibility of dilution that offers the resin to look beyond the fatal fifth alanine residue. The anchoring to the resin indeed allows the physical separation of the peptides, tipping therefore the balance of the aforementioned kinetic competition between aggregation and folding toward the latter without changing the peptide sequence of the aggregation model. This principle of immobilizing in order to promote the folding over aggregation, recently demonstrated for an expressed fusion protein linked to a polyanionic solid support,[9] can easily be implemented in solid-phase peptide

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chemistry and will probably be very useful to look for aggregation-prone-folding intermediates of small peptides and/or proteins.

Experimental Procedures

Peptide Synthesis. All starting materials were obtained from commercial suppliers and used without further purification. Peptide synthesis was performed on an ABI 431A (Applied Biosystems, Foster City, CA) using standard Fmoc/tBu strategy on acid-labile Wang resin or on a ABI 420 A using standard Boc/Bzl strategy on HF labile PAM resin. In both cases, HBTU was used as the coupling reagent. For a uniform dilution of peptide chains on the resin the following manually performed protocol (dilution to 2% of original charge is taken as an example) is used: PS/DVB-PAM-Val-Boc resin (0.754 mmol/g; 450 mg, 0,339 mmol; from ABI) is treated with TFA in order to cleave the temporary Boc-protecting group (2 × 5 mL, 15 min each). The resin is washed with DCM (3 × 5 mL) and NMP (3 × 5 mL). Fmoc-Gly-OH (1.993 mmol, 592.5 mg) and Boc-Ala-OH (0.041 mmol, 7.7 mg) are mixed and suspended in NMP (0.5 M). Preactivation of the amino acids by the addition of HBTU (2.034 mmol, 771.6 mg), HOBt (2.034 mmol, 311.4 mg), and DIEA (4.1 mmol, 680 μL) results after a few minutes in a clear solution. Three consecutive couplings were performed with 1/6, 1/6, and 4/6 of the activated amino acid solution. For each coupling, an additional 3 mL of NMP was added in order to obtain homogeneous resin−solvent suspensions. The reaction mixtures were agitated each time for 2 h. Between couplings, the supernatants were discarded by filtration. Complete reaction was confirmed by negative ninhydrin test for amines. The resin was washed with NMP (3 × 5 mL) and treated with 20% piperidine/NMP (2 × 7 mL, each 15 min) to cleave the Fmoc-protecting group. The resin was washed thoroughly with NMP (7 × 7 mL) and DCM (3 × 7 mL). The liberated N-termini were acetylated by the addition of pyridine (3.4 mmol, 274 μL), acetic anhydride (3,4 mmol, 321,4 μL), and DCM (7 mL).

Cleavage of (2%) H-Ala$_{12}$-Val-PAM-PS/DVB-PAM-Val-Gly-Ac (98%) and MS Analysis. Polyalanine-containing resin (350 mg) was cooled by the means of an ice bath. Anhydrous HF (5 mL) was added, and the resin−solvent suspension was agitated for 1 h. All solvents were evaporated under reduced pressure at 295 K. The resin was suspended in 50% ACN/TFA (50 mL), filtered, and washed once with TFA (20 mL). Collected filtrates were evaporated under reduced pressure at 35 °C. The addition of 10 mL of ACN, 10 mL of 50% ACN in water, and 10 mL of TFA formed a colorless precipitate that was separated from the solvent by centrifugation. For MS analysis, a small portion of the obtained colorless solid was suspended in 50% ACN in water and added directly on aluminum foil: (TOF-PDMS) $[M + 1]^+ = 974.3$ amu (expected 974.1 amu); $[M + Na]^+ = 996.1$ amu (expected 995.1 amu). For ESI-MS analysis, 50 μL of the polyalanine−ACN/H_2O (1/1) suspension was treated with 10 μL of acetic acid. The supernatant was separated by decantation and injected into the MS: $[M + Na] = 996.1$ amu, expected 996.1 amu.

Cleavage of (10%) H-Ala$_8$-Val-Wang-PS/DVB-Wang-Val-Ala-Boc (90%). Fifty milligrams of dry resin was treated with 3 mL of TFA/H_2O = (95/5) for 1 h. The resin was separated from the TFA solution by filtration and washed once with 1 mL of TFA. The combined solutions were evaporated under reduced pressure at 40 °C. The remaining precipitation was lyophilized in 3 mL of tBuOH/H_2O (4:1 v/v). A 0.3 mg portion of the obtained colorless solid was dissolved in ACN/H_2O (1:1 v/v) and subjected to MS analysis: TOF-PDMS $[M + 1]^+$ TOF-PDMS $[M + 1]^+ = 687$ amu (expected 686.8 amu) ESI-MS $[M + Na]^+ = 708.5$ amu (expected 708.8 amu).

Cleavage of (10%) H-Ala$_n$-Val-Wang-PS/DVB-Wang-Val-Ala-Boc (90%) (n = 3−8) in the HR MAS NMR Rotor. A 3.1−3.6 mg portion of resin was loaded into the rotor: (i) 50 μL of d^2-DCM or (ii) 40 μL of d^2-DCM and 10 μL of d^1-TFA or (iii) 50 μL of d^1-TFA were added. All spectra were recorded in an interval of 5 min.

NMR Spectroscopy. All spectra were recorded on a Bruker DMX600 spectrometer equipped with a 4 mm $^1H/^{13}C$ double resonance HR MAS probehead. Resin containing rotors were rotated at 6000 Hz. Chemical shifts for ^1H MAS NMR are reported relative to TMS as internal standard. 2D TOCSY[10] and NOESY[11,12] spectra were recorded with mixing times of 80 ms, respectively, 100 ms, where a MLEV-16 sequence[13] of 8.3 kHz was applied during the TOCSY mixing time. The final data set comprised 1K × 128 complex points and was transformed to a 2K × 1K matrix after application of a shifted sine bell in both dimensions.

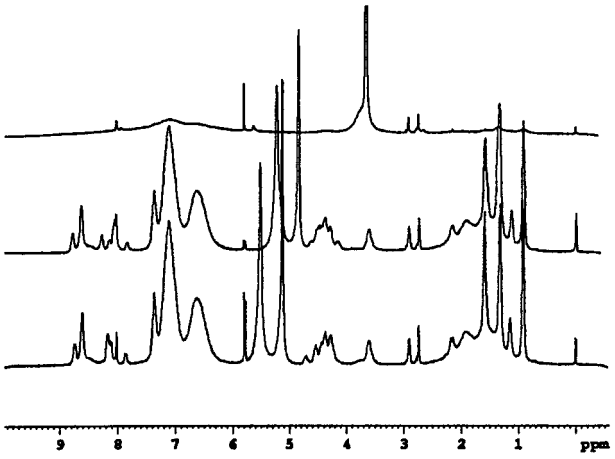

Figure 1. HR MAS RMN 1D spectra at 600 MHz of Val-(Ala)$_4$-NH_3^+ (bottom), Val-(Ala)$_5$-NH_3^+ (middle), and Val-(Ala)$_6$-NH_3^+ (top). Spectra were recorded at a spinning speed of 6 kHz in a 4 mm rotor with 5 mg resin charged at 0.754 mmol/g and swollen in DMF-d.[7]

Results

Assignment of (Ala)$_4$-(Ala)$_5$ Peptides. Initial 1D spectra of the shorter polyalanine peptides (PS/DVB-PAM-Val-(Ala)$_n$-NH_3^+, $n \leq 5$; 0.754 mmol/g) showed signals of the resin (large resonances at 6.2 and 7.5 ppm and in the range of 1.2−2.3 ppm), signals attributed to the residual solvent and reasonably sharp amide resonances, with an even better chemical shift dispersion than expected (Figure 1). Their assignment by 2D TOCSY spectra was facilitated by introducing isotopically labeled Ala residues at specific positions in the sequence (Figure 2). This allowed us to unambiguously assign both the (Ala)$_4$ and (Ala)$_5$ peptides on the resin. A consistent pattern was detected with the amide proton of the last but one residue resonating at the most downfield postion, followed by the amide proton resonance of the last residue, and then a monotonic series of chemical shift values upon approaching the anchoring position.

The physical state of the N-terminus of the peptide proved to be very important, however, as the (Ala)$_5$-Fmoc peptide gave very large signals, and no more signals were observed for the neutralized (Ala)$_5$-NH_2 peptide.

(Ala)$_6$ Peptide: Synthetic Difficulties Correlate with Poor HR MAS Spectra. In agreement with the poor synthetic results obtained for the sixth alanine residue, we obtained no workable NMR spectrum for the (Ala)$_6$ peptide anchored to the resin at a density of 0.754 mmol/g (Figure 1). The only signals remaining are resonances of residual DMF and water, both with much higher intensities than in the former spectra for $n = 4$ and 5. We attributed these solvent peaks to residual moisture and protonated solvent, captured in the badly swollen resin even after extensive drying, and uncapable to exchange with the

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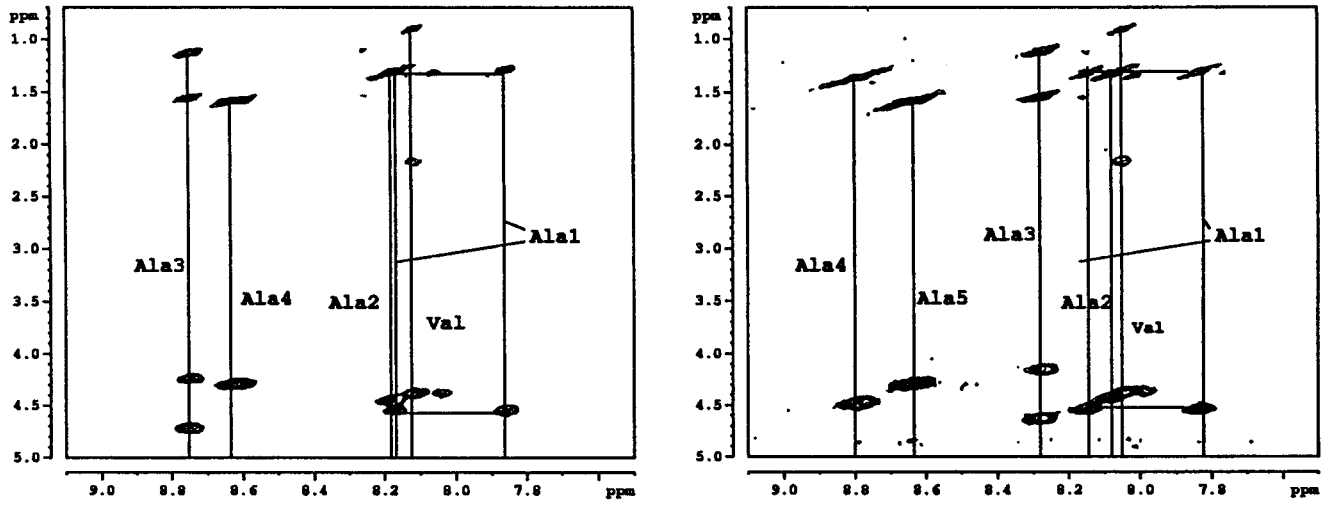

Figure 2. TOCSY spectra of Val-$(Ala)_4$-NH_3^+ (left) and Val-$(Ala)_5$-NH_3^+ (right), with Ala_1 ^{15}N labeled at its amide position and Ala_3 uniformly ^{13}C labeled. The amide resonance of Ala_1 appears as a doublet in the ω_2 direction, whereas both the Hα and methyl group of Ala_3 are doublets in the ω_1 direction.

supernatant due to hindered solvent exchange. The same phenomenon was observed for the $n = 7-10$ sequences.

The results on $(Ala)_5$ and $(Ala)_6$ are in good correlation with the observations during the chemical synthesis of the peptides. In the Fmoc/tBu peptide chemistry, it was observed in our and other groups that the cleavage of the N-terminal Fmoc-protecting group from the Ala_5 can be difficult.[5] Accordingly, we observed already a severely broadened spectrum before the deprotection step, and complete aggregation upon removal of the Fmoc group, where the chain ends with an amine function. The positively charged $(Ala)_5$-NH_3^+ resin, on the contrary, yields a workable NMR spectrum, indicating a significant degree of motional freedom of the chains and hence the absence of aggregation. This most probably is the reason why in the Boc/Bzl strategy, in situ neutralization gives better results upon coupling of the sixth alanine residue.[14] In the charged resin, all reagents including the incoming amino acid can penetrate before the addition of base neutralizes the amino function, provoking peptide aggregation and hence hindered solvent diffusion in and into the resin.

Adding a sixth alanine residue with its Boc protection group leads immediately to aggregation (Figure 1). As the D-Ala amino acid is known to facilitate the synthesis of polyalanine, due to its β-sheet breaking properties,[5] we added a D-Ala residue to the $(Ala)_5$ sequence. As expected, the spectral quality was not degraded as for $(Ala)_6$, and full assignment could be obtained.

Dilution of Longer Polyalanine Chains on the Resin. Starting from the observation that $(Ala)_6$ does aggregate, whereas the $(Ala)_5$ peptides are still too short to form aggregates on the resin, we explored how much $(Ala)_6$ can be added before the system aggregates. In a preliminary assay, 0.1 equiv of Boc-Ala-OH was added to the $(Ala)_5$ resin (PS/DVB-PAM-Val $(Ala)_5$-NH_2, 0.75 mmol/g). To circumvent assignment problems for this heterogeneous system containing simultaneously $(Ala)_5$ and $(Ala)_6$ chains, we used specifically labeled alanines: Ala_4 with ^{13}C-enrichment on the methyl carbon was incorporated in the orginal $(Ala)_5$ resin. For the shorter $(Ala)_5$ chains, that should represent a majority, Ala_4 is the second last residue, and according to the previously established pattern, its amide proton resonates at the most downfield value of the amide proton chemical shifts. Indeed, two doublets for the methyl group of Ala_4 with a ^1J(CH) coupling constant of 130 Hz appeared in the TOCSY spectrum, one corresponding to the more abundant $(Ala)_5$ sequence with its amide at 8.66 ppm and a line of lower intensity at 8.33 ppm corresponding to the longer $(Ala)_6$ chains where Ala_4 is the third last residue (data not shown).

Table 1. Aggregation State of the Different $(Ala)_n$ Peptides on the PS/DVB-PAM-Val Resin, As Detected by HR MAS NMR[a]

	n							
	5	6	7	8	9	12	18	21
100% $(Ala)_n$-NH_3^+	√	x	x	x	x	–	–	–
10% $(Ala)_n$-NH_3^+	√	√	√	√	x	–	–	–
2% $(Ala)_n$-NH_3^+	√	√	√	√	√	√	–	–
1% $(Ala)_n$-NH_3^+	√	√	√	√	√	√	√	√

[a] Density is given as percentage of the initial 0.754 mmol/g density. √ = no aggregation, x = aggregation, – = not determined.

Because the condensation of the sixth alanine by HBTU/HOBt coupling is a fast process, we did not expect a uniform distribution of the $(Ala)_6$ sequences on the resin. Reaction sites on the resin that come first into contact with activated Boc-Ala-OH (e.g., the exterior of resin beads) will have a higher concentration of $(Ala)_6$ sequences than more buried sites. To circumvent this problem, we adopted a different strategy to dilute the polyalanine sequences from the beginning on, and under better controlled conditions. Commercially available resin with a first amino acid already immobilized was treated by a mixture of Fmoc- and Boc-amino acids. For a 10% dilution of a PS/DVB-PAM-Val-NH_2, the resin was treated by a 9:1 mixture of Fmoc-AA-OH/Boc-AA-OH, where the ratio of the mixture represents the desired dilution rate. Even if the used amino acids have different coupling efficiencies, the three consecutive coupling reactions using 1, 1, and 4 equiv, respectively, of activated amino acids should lead to reasonably good equidistribution of the two species on the resin. After coupling of the mixture of amino acids on the PAM resin, the Fmoc protecting group was cleaved by the addition of piperidine and the freed amino termini was acetylated with acidic anhydride. The synthesis was continued on the Boc-Ala-Val-Pam-PS/DVB strand, using standard Boc/Bzl peptide chemistry. Using this methodology, resins with dilutions to 10%, 2%, and 1% of their original charge where synthesized. Table 1 summarizes the aggregation state of the polyalanine sequences on the original and diluted resins, as determined by the presence or absence of signals in the HR MAS NMR spectra.

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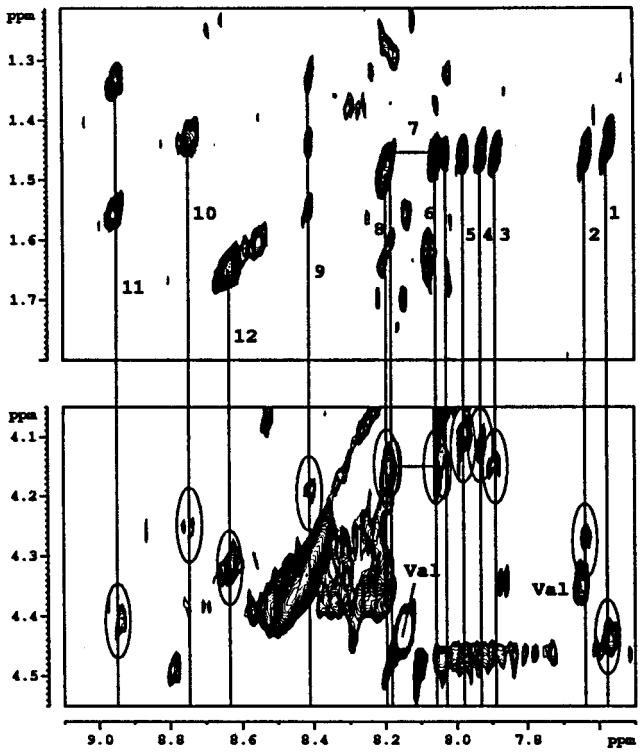

Figure 3. TOCSY spectrum of the PS/DVB-PAM-Val-$(Ala)_{12}$-NH_3^+ resin (0.015 mmol/g with the methyl (top) and fingerprint region (bottom) and full assignment based on differential labeling schemes.

Mass Spectroscopy of the Longer Polyalanine Peptides Indicates Unique Sequences. To verify the synthesized sequences, Val-$(Ala)_8$-NH_3^+ from a 10% charged resin and Val-$(Ala)_{12}$-NH_3^+ from a 2% charged resin were cleaved from the solid support. The mass spectroscopy spectra of the obtained products showed the expected mass values. More importantly, however, was the absence of peaks that might correspond to truncated sequences, as are usually observed in crude products of polyalanine synthesis.[5]

2D NMR Analysis of PS/DVB-Pam-Val-$(Ala)_{12}$-NH_3^+. For PS/DVB-Pam-Val-$(Ala)_{12}$-NH_3^+ (0.015 mmol/ g), complete characterization was achieved by HR MAS NMR TOCSY spectra (Figure 3). Peak attribution of the 12 alanine residues was done by synthesizing several PS/DVB-Pam-Val-$(Ala)_{12}$-NH_3^+ batches, with specific labeling at different positions composed of (i) Ala ($^{13}CH_3$), yielding a doublet for the methyl protons; (ii) a 1:2 mixture of unlabeled Ala and Ala label at the methyl position ($^{13}CH_3$), leading to a triplet for the methyl signal; or (iii) Ala (^{15}NH), giving a doublet for the amide proton. Full asignments are given in the Supporting Information.

Structural information on resin-bound peptides can be extracted from NOE spectra, as even under MAS conditions the dipolar effect remains effective as a relaxation mechanism.[8] We therefore recorded a NOESY spectrum on the PS/DVB-Pam-Val-$(Ala)_{12}$-NH_3^+ resin but obtained only very limited information about eventual secondary structure. A first reason was the low sensitivity of the experiment, due to the extreme dilution of the peptide on the resin (2%). Second, the pattern of chemical shift values of poly-Ala is such that amide protons on consecutive residues resonate very closely (Figure 3). This, together with the decreased resolution of the HR MAS spectra compared to liquid-state spectroscopy, resulted in the absence of interpretable NOE connectivities, with the exception of a weak NOE between the amide protons of Ala_2 and Ala_3 (data not shown).

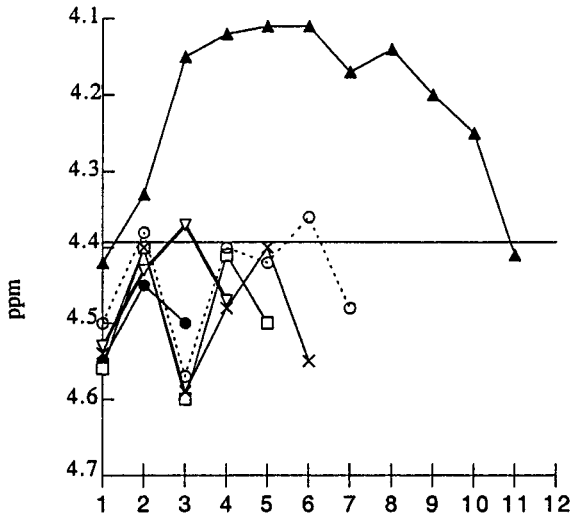

Figure 4. Hα chemical shift values for the different poly$(Ala)_n$ peptides as a function of residue number. Symbols used are as follows: ●, $(Ala)_4$; ▽, $(Ala)_5$; □, $(Ala)_6$; ×, for $(Ala)_7$; ○, $(Ala)_8$; ▲, $(Ala)_{12}$. The random coil value is at 4.41 ppm.

More information about secondary structure came from the chemical shift values of the different protons. We first observed that the length of the sequence correlated with the spread in amide proton chemical shift values. Whereas for $n = 4$, we observed a Δppm value of 0.63 ppm, this value increased monotoneously as follows: $n = 5$ (Δppm = 0.79), $n = 6$ (Δppm = 0.81), $n = 7$ (Δppm = 0.89), $n = 8$ (Δppm = 0.96), $n = 12$ (Δppm = 1.38), to reach a value greater than 1.5 ppm for the $n = 18$ sequence, where the second last residue (based on the previous results, although no attempt to assign this peptide was performed) resonates beyond 9 ppm. Second, the chemical shift pattern observed for Val-$(Ala)_n$-NH_3^+ ($n = 4, 5, 6, 7, 8$) was altered for the 12 residue peptide. Not only the second last alanine, but also the third last alanine residue has a more downfield chemical shift value than the last amino acid. Direct information on the formation of secondary structure came from the observed pattern for the H_α chemical shift values. H_α protons corresponding to residues in the middle of the sequence were found to be shifted to the high field, whereas protons belonging to residues close to one of the two termini approach the chemical shift values observed for Val-$(Ala)_n$-NH_3^+ ($n = 4, 5$), or for the alanine H_α proton in a random coil sequence GG***A***AGG synthesized on the resin (4.4 ppm; Fruchart, J.-S.; Boutillon, C.; Lippens, G., submitted). In Figure 4, we show graphically the H_α chemical shift values of Val-$(Ala)_n$-NH_3^+ ($n = 4, 5, 6, 7, 8, 12$) for comparison. The N terminal H_α of the sequence (not shown in the diagram) always has a higher chemical shift value, probably due to the presence of the ammonium ion complexed with a TFA molecule at the N-terminal. Whereas some general pattern can be observed for the shorter sequences, with the third to last H_α proton resonating at 0.15 ppm downfield the second last one, all other values remain reasonably close to the random coil value. The situation is radically different for the longer chain, where the third H_α proton signal is shifted upfield by 0.18 ppm, and where the pattern resembles a bell shaped curve. As we have shown for other peptides that for resin bound peptide chains swollen in DMF the Chemical Shift Index method,[15,16] developped to deduce secondary structure

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information from peptide and/or protein chemical shift values, remains valid (J.-S. Fruchart, C. B. and G. L., submitted), this bell shaped curve is a clear indication of helix formation in the center of the $(Ala)_{12}$ chain.

Cleavage of a peptide in the rotor. To prove that the physical separation of polyalanine sequences on the resin rather then the presence of a large excess of polystyrene prevents the structures from aggregation, the peptides of PS/DVB−Wang-Val-$(Ala)_n$-NH_3^+ ($n = 3-8$; $n = 3$ at 0.73 mmol/g, $n = 4-8$ at 0.073 mmol/g) were cleaved with TFA and TFA/DCM in the HR MAS NMR rotor. Since TFA and DCM are unusual solvents for NMR analysis of resin bound structures, especially for polyalanine sequences, control spectra were taken with (i) PS/DVB-PAM-Val-$(Ala)_7$-NH_3^+ in TFA and TFA/DCM, and (ii) PS/DVB-Wang-Val-$(Ala)_7$-NH_3^+ in DCM. Because the PAM linker resists treatment with TFA, the polyalanine sequences remain immobilized on these latter resins. The spectral quality decreased in DCM and in TFA upon comparison with the same spectra in DMF, but despite their broader appearance, the H_α protons could be clearly distinguished. As the Wang linker does not resist to TFA treatment, we used it to monitor the cleavage in the rotor. The cleavage of Val-$(Ala)_3$-NH_3^+ resulted in the nonaggregated soluble tripeptides, and TOCSY spectra taken under the conditions of the cleavage (in TFA/DCM (4:1 v/v)) led in a straightforward manner to a complete peak assignment. By treating a PS/DVB-Wang-Val-$(Ala)_7$-NH_3^+ (0.073 mmol/g) with TFA/DCM (4:1 v/v) in the rotor under MAS conditions, the H_α signals of the $(Ala)_7$ disappeared, leading us to the conclusion that the solubilized sequences do aggregate. The only signals remaining at 4.2−4.6 ppm were attributed to the dipeptide used to dilute the polyalanine chains. This Val-Ala-Ac dipeptide is also cleaved from the resin, but does not aggregate, and we could detect the sharp NMR resonances corresponding to its side chains.

Discussion

Association of peptide chains during the solid-phase peptide synthesis has been recognized early on as a potential source of problems, as the aggregation leads to additional effective cross-links that can possibly affect the desired reactivity. A number of analytical techniques have been used to monitor the interchain aggregation process, ranging from infrared spectroscopy,[17] electron spin resonance,[18] NMR spectroscopy based on natural abundance ^{13}C signals,[19] deuterium signals of ^2D labeled amino acids,[20] or, recently, proton NMR.[8] Whereas this last nucleus definitely has the most advantageous magnetic properties related to its omnipresence, 100% natural abundance and high sensitivity, its broad line width in the heterogeneous system that is the swollen resin with its attached peptide chains long hindered its application to the study of solid-phase peptide synthesis. The advent of high-resolution magic angle spinning NMR (HR MAS NMR), that uses the MAS to successfully average out most line broadening mechanisms from residual dipolar interactions or from magnetic heterogeneity of the sample has eliminated this factor, and has allowed to follow reactions on the solid phase, both in peptide and general organic chemistry.[8,21−26] The possibility of identifying peptides while bound on the resin, and simultaneously obtain structural information makes it an interesting technique to address long-standing problems in peptide synthesis.

Poly-alanine is probably one of the most studied homopolymers in peptide science, because it is thought to form a stable α helix when the number of monomers is sufficiently high. Fiber diffraction studies,[27] infrared (IR) spectroscopy,[28] FTIR on surface-immobilized polyalanine peptides,[29] solid-state NMR spectroscopy,[30,31] circular dichroism and NMR spectroscopy on the solubilized peptide,[32−34] and finally theoretical studies by Monte Carlo (MC) and Molecular Dynamics (MD) simulations[35−38] all have indicated the presence of an α helix in this polypeptide, but the critical length and the mechanisms of helix formation remain unclear, mainly due to problems of insolubility. To add to the complexity of the problem, it was shown that another polyalanine-based peptide, Ac-$KA_{14}K$-NH_2, can undergo conformational changes from monomeric random coil conformations into soluble, macromolecular *β*-pleated sheet complexes without any covalent modification.[6,39]

In the realm of peptide synthesis, polyalanine is thought to form *β* sheet structures, either intra- or interchain, at 5, 6, and 7 residues, correlating with important difficulties at those synthetic steps. As beyond those critical steps the synthesis again is easier, it is thought that the α helix dominates for the longer peptides. In this study, we have addressed both aspects: first, we have correlated the synthetic difficulties with intermolecular aggregation, through the presence or absence of well resolved lines in the HR MAS NMR spectra. Second, we have used the dilution on the resin and the resulting physical separation of the peptides to look into longer sequences and their secondary structure, without being hindered by aggregation.

For the shorter sequences up to five residues, good-quality NMR spectra could be obtained and fully assigned. A characteristic pattern of H_N chemical shift values was observed. Similar to a 21-residue polyalanine-based peptide in aqueous solution,[33] the second residue (counting from the N terminus) resonates at the most downfield value, followed immediately by the first one, and then monotoneously by the remaining amide protons in the sequence. However, even at this short length of five

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alanine residues, problems arose for the neutralized form, in agreement with FT-IR spectra of protonated resins vs neutralized ones, that led to the conclusion of more non-hydrogen-bonded NH functions in the former.[40] In peptide synthesis, this observation formed the basis for an improved strategy based on in situ neutralization simultaneous with the coupling, leading to improvements in yield for difficult sequences.[41,42]

At the sixth amino acid, the synthetic difficulties become severe, and we observe no more signals of the peptide in the HR MAS NMR spectrum. This absence of signals comes from the complete loss of mobility of the resin-bound structures on the nanosecond time scale and clearly points toward a totally aggregated system. Whereas in a former study we had tried to monitor the effect of DMSO as aprotic polar cosolvent on both the reaction efficiency and the spectral quality,[8] we decided here only to test the insertion of a D-Ala residue as a potential β sheet breaker.[5] Disrupting the aggregated structure by coupling of a sixth D-Ala residue indeed led to workable spectra.

An alternative approach to prevent aggregation is to physically separate the objects, thereby preventing their possible encounter. Nature has used this approach in a very effective way through the chaperone proteins. They capture in their hydrophobic cavity the unfolded or partially folded protein chains that have not yet reached their native conformation and therefore risk aggregation through their partially exposed hydrophobic patches and give them the time to fold properly by shielding them from other proteins.[43] This principle of physical separation to prevent aggregation during the folding process has been elegantly applied to the folding step of recombinant proteins.[9] Here, we show its application in peptide synthesis and the possibility to obtain structural information in atomic detail on the separated chains through the use of HR MAS NMR.

For $(Ala)_6$, a dilution of a factor of 10 was obtained in two fashions. First, we coupled 0.1 equiv of Boc-Ala-OH to the $(Ala)_5$ resin (PS/DVB-PAM-Val-$(Ala)_5$-NH_2, 0.75 mmol/g) and observed both the $(Ala)_5$ and $(Ala)_6$ chains on the same supramolecular system. However, considering the relative speed of the condensation process and the diffusion of the incoming activated amino acid into the resin, we cannot expect a homogeneous distribution of pending chains throughout the volume of the bead. Therefore, the second approach exploited the orthogonal chemical strategies based on Fmoc and Boc protection and allowed a homogeneous dilution of the growing chains in the middle of a majority of acetylated dipeptides. Good-quality spectra were obtained for chains of up to eight Ala residues, but at the ninth synthesis cycle, aggregation set in again. Further dilution to 1% of the initial charge of 0.754 mmol/g led to workable NMR spectra for peptides as long as 18 Ala residues, although this is probably not yet the ultimate limit at this degree of dilution.

To rationalize this effect of dilution, both interchain separation and length can be estimated in the following way. Rotational isomeric state calculations for an atactic polystyrene chain charged at 0.5 mmole/g of resin led to an estimated separation of 28 Å between pendant sites.[20] A chain composed of one valine residue and five alanine residues in the extended conformation, on the contrary, has a length of 19 Å. Therefore, if we model two pending chains with their anchoring points separated by 25 Å (corresponding to the somewhat higher density of 0.754 mmol/g that we used) in an antiparallel β sheet conformation, two residues of each chain can contribute to the intermolecular sheet, bringing in two hydrogen bonds and one hydrophobic interaction with the methyl group per residue. If we decrease the density by a factor of 10, the average separation increases to 52 Å, too far for two 20 Å peptides to interact, but addition of four Ala residues leads to a Val-$(Ala)_9$ chain that is 12 Å longer compared to its Val-$(Ala)_5$ counterpart. Again, two residues can contribute in the same fashion as before and lead to an effective cross linking on the resin. It is important to note here that it is truly the physical attachment of the peptide on the resin that prevents the aggregation, and not just the presence of the polymer matrix, as cleavage of the longer peptides ($n > 4$) invariably leads to aggregated structures, that this time do not form an effective cross-linking anymore. At the 2% dilution, according to the same rule, we would expect that a Val-$(Ala)_n$ peptide with $n > 12$ would aggregate, if the peptides remain in the same secondary structure. However, our NMR results clearly indicate that between $(Ala)_8$ and $(Ala)_{12}$ a structural transition occurs, with a stable α helix for the longer peptide. This will immediately reduce the length of the peptide (Val-$(Ala)_{12}$ in the extended conformation measures 42 Å, whereas it extends no further than 20 Å in the α helix conformation) and hence further separate the peptides. Together with the presence of intrachain hydrogen bonds in the α helix, the reduced size of the helix prevents the formation of aggregated structures on the resin, and good spectra could be obtained for peptides containing up to 18 alanine residues. It was somewhat amazing that the helix formation did not start for the shorter peptide containing eight amino acids, as it is generally believed that the end of synthetic difficulties coincides with the domination of the helix structure. Molecular Dynamics simulations on α-methylalanine (MeAla) homopolymers in vacuo indicated moreover a possible onset of helical formation with a 3_{10} helix, where at an equilibrium length of 7.5 residues, the two helical states should be approximately isoenergetic.[44,45] However, our results on $(Ala)_8$ still indicate a random coil conformation at this length, and it therefore is not clear why the synthetic difficulties at those steps become less severe.

Whereas this study has no direct implications in the field of protein folding, as all NMR spectra were recorded on resins swollen in DMF rather than in aqueous solution, the principle of physical separation of peptides remains valid on resins that do swell correctly in aqueous solution (for a review, see ref 43). The long-chain poly(ethylene glycol) (PEG) chains used to enhance the physicochemical properties of the resin in aqueous solution[46,47] have been shown to be compatible with the native structure of proteins and peptides[48,49] and can be used to study polyalanine sequences in aqueous solution. Finally, it is clear that the combined use of solid-phase synthesis and high-resolution magic angle spinning NMR spectroscopy is a new tool that can be used advantageously in the study of aggregating structures such as β amyloid and other aggregating peptides that are at the origin of an ever growing number of molecular diseases.[2]

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