Magic Angle Spinning Nuclear Magnetic Resonance in Solid-Phase Peptide Synthesis

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Abstract: Solid-phase peptide synthesis of certain sequences (commonly called "difficult sequences") suffers from the occurrence of incomplete coupling reactions and/or partial unmaskings of N^{α}-protection. The underlying reasons for these problems are thought to be a structuration and/or a poor solvation of the growing peptide chains. Few methods are available to study the structural aspects of the peptide chains when still anchored to the solid support. In most cases, they rely on the incorporation of a specific label and examine therefore a modified peptide analog. We describe the complete characterization by homonuclear and heteronuclear magic angle spinning nuclear magnetic resonance (MAS NMR) of the solid-phase synthesis of a 10-residue peptide. A detailed secondary structure determination of the growing peptide on the resin beads, based on the NOE analysis and the ^{1}H and ^{13}C chemical shift deviations, indicated an extended structure on the whole length of the sequence. At critical synthesis steps, a correlation between the coupling difficulties and the aggregation of the peptide chains was established by chemical measurements and MAS NMR. Upon titration with the hydrogen bond-accepting solvent DMSO, the mobility of the peptide chains on the resin beads increased, resulting in a significant line narrowing of the MAS NMR spectra. This increased mobility is linked to an enhanced peptidyl-resin solvation as reflected by the better coupling efficiency at the critical synthesis steps.

Introduction

Since the introduction of stepwise solid-phase peptide synthesis by Merrifield in 1963,[1] this method has developed into a powerful tool for the preparation of a great number of important biological polypeptides.[2,3] However it has been frequently reported that side products occur during solid-phase peptide synthesis.[4] The N^{α}-deprotection and, more frequently, the acylation stages, for example, can be highly inhibited or even totally prevented at some stage of the synthesis, leading to deleted or truncated sequences. Peptide sequences which display this behavior are commonly called "difficult sequences".[5,6] These difficulties in the peptide elongation have often been attributed to a sequence dependent tendancy of the growing peptide chains to aggregate through the formation of secondary structures.[7] The driving force for these inter- or intrachain associations is generally admitted to be hydrogen-bonding[8,9] and/or apolar interactions between side-chain protecting groups.[10] As this phenomenon clearly influences the yields of peptide syntheses,[11] insights at the molecular level of the peptide during its elongation while still linked to the solid support might suggest ways to overcome the synthesis difficulties.

Whereas numerous methods have been developed to quantify the final efficiency of the coupling and deprotection steps, very few methods allow direct access to the structural features of the growing peptide chain on the resin. Among them, the near-infrared Fourier transform (IR-FT) spectroscopy allows to evaluate the efficiency of deprotection reactions as the peptide chain is being elongated.[12] However efficient identification of specific groups requires the incorporation of a deuterium source into the protecting group. In a recent report, electron paramagnetic resonance (EPR) spectroscopy was used to study aggregation problems of peptides on resin beads, but a major problem is the need to include a paramagnetic amino acid in the sequence.[13] The ^{13}C gel-phase NMR spectroscopy on resin-bound material is a reliable technique to determine the success or failure of chemical transformations.[14] A drawback of the method is the low sensitivity inherently linked to 1% natural abundance of ^{13}C and to the small amount of compound attached to the resin. Consequently it can take several hours to acquire a spectrum with a suitable signal to noise ratio. The applicability of NMR spectroscopy to resin-supported material was recently extended through the combination of efficient swelling conditions and magic angle spinning (MAS)[15] techniques.[16−18]

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(1) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149−2154.

(2) Merrifield, R. B. *Science* **1986**, *232*, 341−347.

(3) (a) Gutte, B.; Merrifield, R. B. *J. Biol. Chem.* **1971**, *246*, 1922−1941. (b) Clark-Lewis, I.; Aebersold, R.; Ziltener, H.; Schrader, J. W.; Hood, L. E.; Kent, S. B. H. *Science* **1986**, *231*, 134−139. (c) Milton, R. C. L.; Milton, S. C. F.; Kent, S. B. H. *Science* **1992**, *256*, 1445−1448.

(4) Kent, S. B. H. *Annu. Rev. Biochem.* **1988**, *57*, 957−989.

(5) Milton, R. C. L.; Milton, S. C. F.; Adams, P. A. *J. Am. Chem. Soc.* **1990**, *112*, 6039−6046.

(6) (a) Krchnak, V.; Flegelova, Z.; Vagner, J. *Int. J. Pept. Protein Res.* **1993**, *42*, 450−454. (b) Van Woerkom, W. J.; Van Nispen, J. W. *Int. J. Pept. Protein Res.* **1991**, *38*, 103−113.

(7) Stewart, J. M.; Klis, W. A. In *Innovation and Perspectives in Solid Phase Synthesis. Peptides, Polypeptides, and Oligonucleotides*; Epton, R., Ed.; SPCC Ltd.: Birmingham, U.K., 1990; pp 1−9.

(8) (a) Deber, C. M.; Lutek, M. K.; Heimer, E. P.; Felix, A. M. *Pept. Res.* **1989**, *2*, 184−188. (b) Ludwick, A. G.; Jelinski, L. W.; Live, D.; Kintanar, A.; Dumais, J. J. *J. Am. Chem. Soc.* **1986**, *108*, 6493−6496.

(9) Tam, J. P.; Lu, Y. A. *J. Am. Chem. Soc.* **1995**, *117*, 12058−12063.

(10) Fields, G. B.; Fields, C. G. *J. Am. Chem. Soc.* **1991**, *113*, 4202−4207.

(11) Kent, S. B.; Alewood, D.; Alewood, P.; Baca, H.; Jones, A.; Schnölzen, M. In *Innovation and Perspectives in Solid Phase Synthesis*; Epton, R., Ed.; Intercept Ltd.: Andover, U.K., 1992; pp 1−22.

(12) (a) Larsen, B. D.; Christensen, D. H.; Holm, A.; Zillmer, R.; Nielsen, O. F. *J. Am. Chem. Soc.* **1993**, *115*, 6247−6253. (b) Russell, K.; Cole, D. C.; McLaren, F. M.; Pivonka, D. E. *J. Am. Chem. Soc.* **1996**, *118*, 7941−7945.

(13) Cilli, E. M.; Marchetto, R.; Schreider, S.; Nakaie, C. R. *Tetrahedron Lett.* **1997**, *38*, 517−520.

(14) (a) Giralt, E.; Rico, J.; Pedroso, E. *Tetrahedron* **1984**, *40*, 4141−4152. (b) Look, G. C.; Holmes, C. P.; Chinn, J. P.; Gallop, M. A. *J. Org. Chem.* **1994**, *59*, 7588−7590.

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Whereas the former confer a high degree of mobility to the attached molecules, the MAS efficiency averages out the inherent magnetic susceptibility differences over the heterogeneous sample making a detection of the proton signals feasible.[19,20] This alleviates the sensitivity problems of ^{13}C NMR and makes the classical techniques of 2D NMR readily available. The MAS NMR technique is therefore a sensitive and nondestructive analytical method to completely characterize molecules covalently anchored to the solid support.

We report here the MAS NMR characterization of the solid-phase peptide synthesis of a well-established difficult sequence formed by the 10-residue peptide NH_2-Thr-Glu-Gly-Glu-Ala-Arg-Gly-Ser-Val-Ile-OH.[21] First we report the NMR assignment of our model peptide during its elongation on resin beads. We complete the assignment by a detailed secondary structure determination based on the NOE analysis and the ^1H and ^{13}C chemical shift deviations. We also examine at critical synthesis steps the influence of the swelling solvent on the success of the synthesis, by both structural and chemical characterization of the peptide elongation in various aprotic polar solvent mixtures. Finally these results allow us to identify the limiting factors for the synthesis of the difficult sequence.

Experimental Procedures

Solid-Phase Peptide Synthesis. The sequence selected as a difficult sequence model was the Aggrecan (a human cartilage large aggregating proteoglycan) residues 370−379 (NH_2-Thr-Glu-Gly-Glu-Ala-Arg-Gly-Ser-Val-Ile-OH), which were initially required as a model substrate for aggrecanase that cleaves Aggrecan at the Glu^{373}−Ala^{374} peptide bond.[21] This sequence was manually synthesized according to the solid-phase method using the Fmoc/*t*-Bu strategy, starting with 1.37 g (1 mmol) of Fmoc-Ile-Wang-resin (0.730 mmol/g). Side chain protecting groups were as follows: Ser(O*t*-Bu), Arg(Pmc), Glu(O*t*-Bu), and Thr-(O*t*-Bu). The Fmoc deprotection steps were performed twice with 20% (v/v) piperidine in *N*,*N*-dimethylformamide (DMF) for 15 min each. Double couplings were systematically performed with 4-fold excess of activated amino acids (4 mmol) using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium (HBTU) and 1-hydroxybenzotriazole (HOBt) in the presence of *N*,*N*-diisopropylethylamine (DIEA) (4 mmol:4 mmol:8 mmol) in DMF for 45 min each. All these compounds were previously incubated together for 2 min before transfer to the reaction vessel. Coupling and deprotection steps were monitored by ninhydrin tests.[22] A third coupling was carried out for the couplings of Ala^{374} on Arg^{375}, Glu^{373} on Ala^{374}, and Thr^{370} on Glu^{371}. At the end of each cycle, a capping step of unreacted free amino groups was performed with 10% (v/v) acetic anhydride, 5% (v/v) DIEA in CH_2Cl_2 for 10 min. One aliquot of 100 mg of peptidyl-resin was removed at the end of each synthesis step and submitted twice to 20% (v/v) piperidine in DMF for 15 min each to deprotect the amino terminal function. In this manner, the first sample contained one amino acid on the resin, the second contained two, ..., up to the tenth sample which contained the complete anchored peptide, protected on its side chains. Two additional samples were also analyzed: the 5-residue sample with its N-terminal extremity protected with a Fmoc group and the same sample acetylated on its N-terminal extremity, after cleavage of the Fmoc group.

MAS NMR Experiments. At each synthesis step and after N-terminal Fmoc deprotection, 10 mg of resin-supported peptide was taken for MAS NMR analyses. As throughout the synthesis DMF was used as the solvent for deprotection and coupling steps, the NMR analyses were equally carried out with DMF. The peptidyl-resins were swollen with a minimal volume of deuterated DMF (DMF-d_7) before intoducing them into a full 4 mm MAS rotor. The solvent mixture titration was performed using deuterated dimethyl sulfoxide (DMSO-d_6). Tetramethylsilane (TMS) was added as internal reference to the solvent before the resin swelling. Theoretical and experimental aspects of the MAS NMR technique applied for the characterization of resin-supported molecules have been previously described in detail.[20] All NMR experiments were performed at room temperature on a Bruker DRX 300 MHz spectrometer equipped with a 4 mm high-resolution solid-state MAS probe. A spinning of 4 kHz was sufficient to obtain line narrowing and to avoid spinning side bands in the ^1H NMR spectra. TOCSY[23] spectra were acquired with 70 ms 10 kHz MLEV-17 spin−lock field strength. NOESY[24] spectra were acquired with a 150 ms mixing period. Both spectra consisted of 2048 complex points in t_2 and 256 complex t_1 increments with 16 scans/increment utilizing the States method[25] to achieve phase discrimination in ω_1. The ^1H−^{13}C HMQC[26] spectra were obtained using the same procedure with 32 scans/increment.

Bead Diameter Measurement. To study the effect of the solvent composition on the bead size of the 9- and 10-residue samples, the bead mean diameters have been measured with the peptidyl amino terminal function in deprotected form under a light microscope (ocular ×10; objective ×50) on a population of 250 beads/solvation condition. Beads, preswollen overnight in DMF or in different mixtures of DMF/DMSO, were spread over a slide with a coverglass to minimize solvent evaporation. A graduated ocular was used for the measurements and was calibrated with the help of a calibrated micrometric ruler printed on a slide.

Coupling Yield Evaluation. The coupling yields under two conditions were measured for the 9-residue sample (Fmoc-Thr(O*t*-Bu) coupled to the peptidyl-resin) and the 10-residue sample (Fmoc-Ile coupled to the peptidyl-resin), according to two different conditions in DMF and DMF/DMSO solvent mixtures. First, 1 equiv of activated amino acids (in comparison to the theoretical loading) was used with HBTU (1 equiv) and HOBt (1 equiv) in the presence of DIEA (2 equiv) in each solvent for 20 min. Peptidyl-resin was pre-equilibrated with the solvent mixture for 1 h before the coupling. The yield of the reactions was measured by the colorimetric picric acid test.[27] Second, we used 4 equiv of activated amino acids (in comparison to the theoretical loading) with HBTU (4 equiv) and HOBt (4 equiv) in the presence of DIEA (8 equiv) in each solvent for 45 min. Peptidyl-resin was pre-equilibrated with the solvent mixture for 1 h before the coupling. Here, the yield of the reaction was measured by the colorimetric quantitative ninhydrin reaction.[22] The theoretical loadings of the 9- and 10-residue samples before coupling were respectively 0.386 and 0.364 mmol/g.

(15) (a) Andrew, E. R.; Bradbury, A.; Eades, R. G. *Nature* **1959**, *183*, 1802−1803. (b) Lowe, I. J. *Phys. Rev. Lett.* **1959**, *2*, 285−287.

(16) (a) Fitch, W. L.; Detre, G.; Holmes, C. P.; Shooreley, J. N.; Keifer, P. A. *J. Org. Chem.* **1994**, *59*, 7955−7956. (b) Anderson, R. C.; Stokes, J. P.; Shapiro, M. J. *Tetrahedron Lett.* **1995**, *36*, 5311−5314. (c) Anderson, R. C.; Jarema, M. A.; Shapiro, M. J.; Stokes, J. P.; Ziliox, M. *J. Org. Chem.* **1995**, *60*, 2650−2651. (d) Garigipati, R. S.; Adams, B.; Adams, J. L.; Sarkar, S. K. *J. Org. Chem.* **1996**, *61*, 2911−2914. (e) Maas, W. E.; Laukien, F. H.; Cory, D. G. *J. Am. Chem. Soc.* **1996**, *118*, 13085−13086. (f) Sarkar, S. K.; Garigipati, R. S.; Adams, J. L.; Keifer, P. A. *J. Am. Chem. Soc.* **1996**, *118*, 2305−2306. (g) Keifer, P. A.; Baltusis, L.; Rice, D. M.; Tymiak, A. A.; Shoolery, J. N. *J. Magn. Reson. A* **1996**, *119*, 65−75. (h) Wehler, T.; Westman, J. *Tetrahedron Lett.* **1996**, *37*, 4771−4774. (i) Shapiro, M. J.; Chin, J.; Marti, R. E.; Jarosinski, M. A. *Tetrahedron Lett.* **1997**, *38*, 1333−1336. (j) Jelinek, R.; Valente, A. P.; Valentine, K. G.; Opella, S. J. *J. Magn. Reson.* **1997**, *125*, 185−187.

(17) Pop, I.; Dhalluin, C.; Déprez, B.; Melnyk, P.; Lippens, G.; Tartar, A. *Tetrahedron* **1996**, *52*, 12209−12222.

(18) Keifer, P. A. *J. Org. Chem.* **1996**, *61*, 1558−1599.

(19) Stoll, M. E.; Majors, T. J. *Phys. Rev. B* **1981**, *24*, 2859−2862.

(20) Dhalluin, C.; Pop, I.; Déprez, B.; Melnyk, P.; Lippens, G.; Tartar, A. In *Conference Proceedings Series Molecular Diversity and Combinatorial Chemistry: Libraries and Drug Discovery*; Chaiken, I. M., Janda, K. D., Eds.; American Chemical Society: Washington, DC, 1996; pp 255−272.

(21) (a) Simmonds, R. G. *Int. J. Pept. Protein Res.* **1996**, *47*, 36−41. (b) Lohmander, S.; Neame, P. J.; Sandy, J. D. *Arthritis Rheum.* **1993**, *36*, 1214−1222.

(22) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147−157.

(23) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521−528.

(24) Kumar, A.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1−6.

(25) States, D. J.; Haberkorn, R. A.; Ruben, D. J. *J. Magn. Reson.* **1982**, *48*, 286−292.

(26) Bax, A.; Griffey, R. H.; Hawkins, B. L. *J. Magn. Reson.* **1983**, *55*, 301−315.

(27) Arad, O.; Houghten, R. A. *Pept. Res.* **1990**, *3*, 42−50.

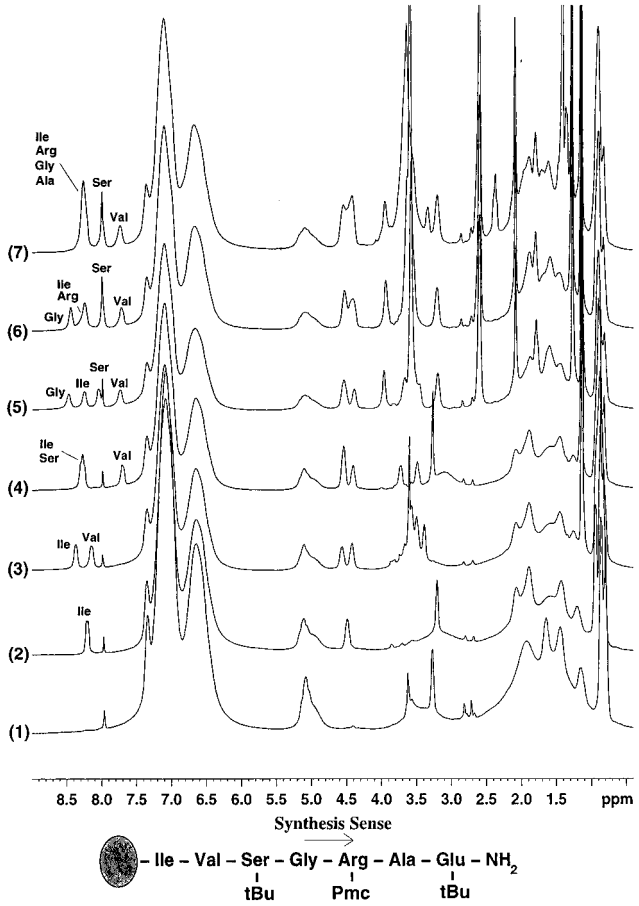

Figure 1. ^{1}H 1D MAS spectra for the first seven peptidyl-resins swollen in DMF-d_7 at room temperature and 4 kHz spinning rate. The assignment of the NH resonances at each synthesis step is shown.

Results

NMR Assignment. As previously shown, correct swelling of the resin beads is a prerequisite to obtain workable spectra.[17,18] In DMF, the peptidyl-resin swelling was satisfactory, and the MAS NMR technique allowed us to obtain high-resolution NMR data for resin-supported peptides (Figure 1). The ^{1}H sequential assignment of each peptide was performed according to the method described by Wüthrich[28] using the MAS TOCSY and MAS NOESY spectra. All residues displayed good TOCSY patterns, from both the backbone amide and Hα protons to the side-chains protons. By combining the information provided by the MAS TOCSY and MAS NOESY spectra in the fingerprint region, the ^{1}H sequential assignment for resin-supported peptides became straightforward. The ^{1}H spin system of the *t*-Bu and Pmc protecting groups could also be identified. The ^{1}H assignment is illustrated in Figure 2 with the MAS TOCSY spectrum of the 5-residue peptidyl-resin.

The full assignment of the peptides was completed with the attribution of the carbon resonances based on the ^{1}H−^{13}C MAS HMQC experiments (Figure 3). This analysis, analogous to the resonance strategy for a peptide in solution, readily shows the power of the MAS NMR. Whereas the results were also comparable to solution data, one highly unusual ^{1}H chemical shift was observed at each synthesis step: the Hα resonance of the last coupled residue presented systematically an important upfield shift to 3.30 ppm. At the following coupling step, the Hα resonance of this residue returned to its usual value for Hα resonance in peptides (4.50 ppm), whereas now the anomalous

(28) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley and Sons: New York, 1986.

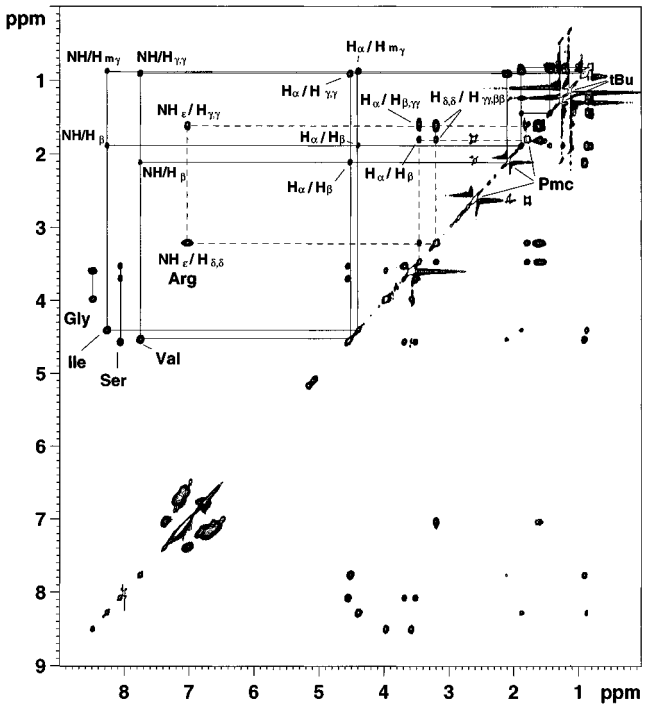

Figure 2. 2D MAS (4 kHz) TOCSY (70 ms) spectrum for the 5-residue peptidyl-resin (resin-Ile^{10}-Val^9-Ser^8(O*t*-Bu)-Gly^7-Arg^6(Pmc)-NH$_2$) swollen in DMF-d_7. The full assignment of the peptide and its side-chain protection is shown.

chemical shift is observed for the residue that is just coupled. This phenomenon was only observed for peptides with a free amino group and disappeared when the N-terminal extremity of the peptide on resin was acetylated or Fmoc protected (data not shown). In Figure 3, we demonstrate this observation for the 5- and 7-residue peptides: the Hα resonances of Arg^6 and Glu^4 in the 5- and 7-residue peptidyl-resins, respectively, are located at 3.30 ppm, whereas the Hα resonance of Arg^6 in the 7-residue peptidyl-resin has returned to its standard value of 4.45 ppm. At the ninth and tenth steps, the assignment of the resin-supported peptides became impossible because of excessively broad ^{1}H resonances (see below).

Secondary Structure. The secondary structure of the peptides on resin was determined by a qualitative analysis of the NOE peaks. The results are in agreement with an extended structure throughout the peptide chains, characterized by strong and medium sequential NH−Hα NOEs and by weak sequential NH−NH NOEs (Figure 4a−c). The absence of medium and long range ^{1}H−^{1}H distances does reinforce the model of an extended structure over the whole length of the peptide sequence.

Because spin diffusion effects can possibly distort the information of the observed NOE pattern, we completed the secondary structure analysis by the method of chemical shift indices (CSI) proposed by Wishart[29] for peptides and proteins in aqueous solution. This method, independent of the NOE information and therefore of spin diffusion, uses the deviation of the chemical shifts of the Hα, Cα, Cβ, and carbonyl nuclei from their random coil values as an indication of the secondary structure. The CSI method is a two-stage digital filtration process. In the first stage, a chemical shift index (a ternary index having values −1, 0, and 1) is assigned to all identifiable residues on the basis of their Hα, Cα, Cβ, and carbonyl chemical

(29) (a) Wishart, D. S.; Sykes, B. D.; Richards, F. M. *Biochemistry* **1992**, *31*, 1647−1651. (b) Wishart, D. S.; Sykes, B. D. *J. Biomol. NMR* **1994**, *4*, 171−180. (c) Wishart, D. S.; Bigam, C. G.; Holm, A.; Hodges, R. S.; Sykes, B. D. *J. Biomol. NMR* **1995**, *5*, 67−81.

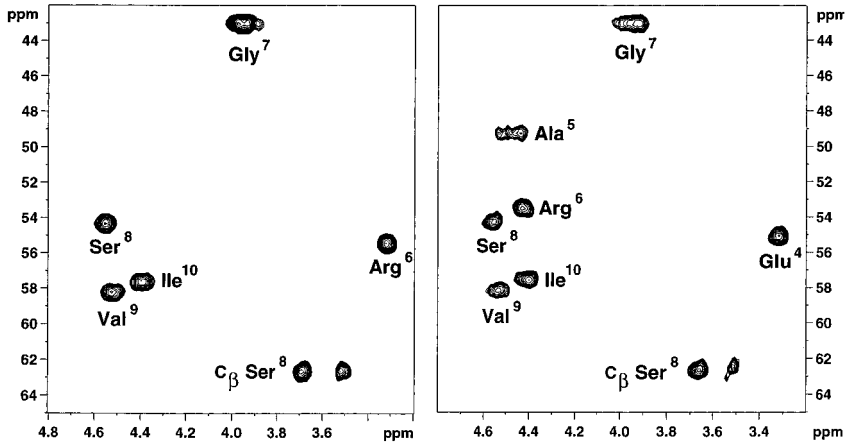

Figure 3. $^{13}C\alpha-^{1}H\alpha$ domain of the MAS (4 kHz) HMQC spectra for the 5-residue (left) and 7-residue (right) peptidyl-resins swollen in DMF-d_7. Notice that the Hα chemical shifts of the last coupled residues (Arg^6 and Glu^4) in the 5-residue (resin-Ile^{10}-Val^9-Ser^8(O*t*-Bu)-Gly^7-Arg^6(Pmc)-NH$_2$) and 7-residue (resin-Ile^{10}-Val^9-Ser^8(O*t*-Bu)-Gly^7-Arg^6(Pmc)-Ala^5-Glu^4(O*t*-Bu)-NH$_2$) peptidyl-resins, respectively, are located at 3.30 ppm.

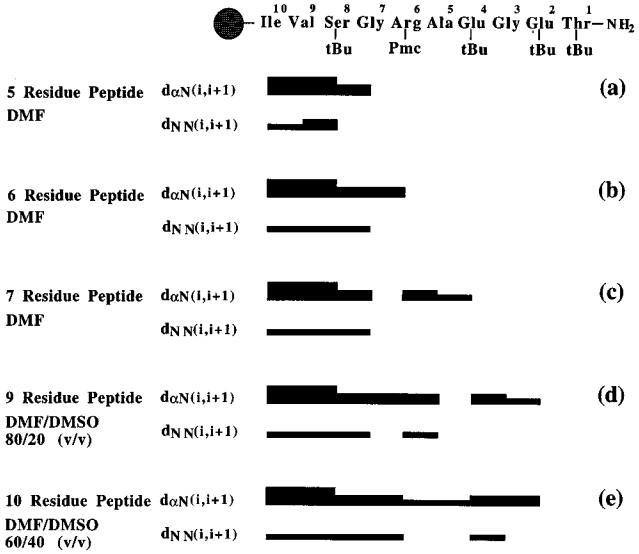

Figure 4. Summary of sequential NOEs for the 5- (a), 6- (b), and 7- (c) residue pepdidyl-resins swollen in DMF-d_7 and for the 9- (d) and 10- (e) residue peptidyl-resins swollen in a solvent mixture of DMF-d_7/DMSO-d_6 (80/20, v/v) and DMF-d_7/DMSO-d_6 (60/40, v/v) respectively.

shift values. In the second stage, secondary structures are delineated and subsequently identified on the basis of the values and "densities" of these chemical shift indices. This method was applied using the Hα, Cα, and Cβ chemical shifts (Figure 5a,b). The main chemical shift deviations were identified for the ^{13}C shifts: we observed a local density of consecutive -1's and $+1$'s for the Cα and Cβ indices, respectively, significant of an extended structure. Good agreement exists between the secondary structure predicted by the NOE analysis and the one derived from the CSI method. Whereas the applicability of the latter method is potentially hampered by the absence of random coil chemical shifts for resin-bound amino acids in organic solvents, the good agreement with the NOE data supports the model of an extended structure for the peptide. It should be noted that for peptides in the solid state, a similar approach of conformational classification in α-helices and β-sheets has been proposed based on ^{13}C chemical shift displacements.[30] Further efforts of our laboratory will go toward establishing a database of chemical shift values for resin-bound amino acids in a random coil conformation.

(30) (a) Saitô, H. *Magn. Reson. Chem.* **1986**, *24*, 835−852. (b) Heller, H.; Kolbert, A.; Larsen, R.; Ernst, M.; Bekker, T.; Baldwin, M.; Prusiner, S.; Pines, A.; Wemmer, D. *Protein Sci.* **1996**, *5*, 1655−1661.

NMR Analysis of 9- and 10-Residue Peptidyl-Resins. Upon using the same experimental stategy for the 9- and 10-residue peptidyl-resins—combining swelling of the resin beads in DMF with MAS NMR spectroscopy—we ran into problems due to increased ^{1}H line widths. We have previously shown that adequate swelling conditions are absolutely crucial for obtaining high-quality spectra and that the most relevant parameter to evaluate the swelling is the diameter of the individual beads.[17] We therefore have first studied the effect of the solvent composition on the bead size of the 9- and 10-residue peptidyl-resins as a macroscopic parameter indicative of the space volume available to the peptide chains. The bead mean diameter of the 9- and 10-residue peptidyl-resins increases significantly upon the incorporation of DMSO into the swelling solvent (Table 1A). For the 10-residue peptidyl-resin an optimum is reached for 40% DMSO, whereas the beads tend to shrink again upon further addition of DMSO (Table 1B).

For the 9-residue peptidyl-resin, addition of 20% DMSO to the DMF (v/v) improved the spectral quality to some extent (data not shown). For the 10-residue peptidyl-resin the use of DMSO proved to be highly critical for the NMR analysis (Figure 6). In DMF the ^{1}H NMR spectrum displayed a broad resonance envelope, attributed to decreased local motions of the peptide chain. Increasing amounts of DMSO led to sharper line widths with an optimal result (average line width of 20 Hz) for a 40% DMSO versus DMF (v/v) solvent mixture. At this ratio 2D spectra could also be obtained for the 10-residue peptidyl-resin (Figure 7) and led to a full ^{1}H sequential assignment. When we further increased the DMSO concentration the spectral quality was again degraded, indicating an optimal solvation and mobility at a 60%/40% DMF/DMSO solvent mixture.

Influence of the Solvent Composition on the Coupling Yield at Critical Synthesis Steps. The above results indicate a clear relationship between solvent condition and NMR spectral quality. As the line width is directly related to chain mobility, and as the lack of mobility due to aggregation is a potential source of synthesis problems, we have evaluated the chemical yield in the same DMF/DMSO solvent mixtures as used for the NMR analysis. Two different synthesis conditions were chosen. The first condition corresponded to a low coupling yield, chosen to amplify any existing difference in yield. The colorimetric picric acid test was used to evaluate the coupling efficiency. The second condition corresponded to actual conditions of synthesis, where only yields superior to 98%, as measured by the quantitative ninhydrin reaction, were considered as satisfactory. Fmoc-Thr with a *t*-Bu as side-chain protection

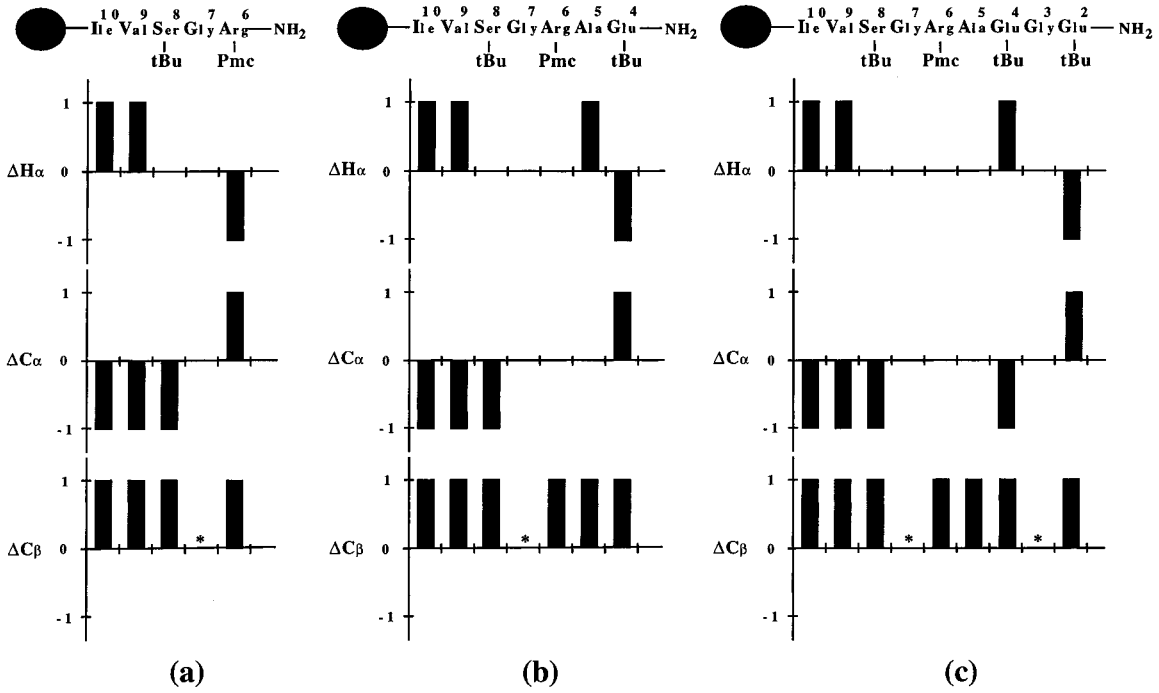

Figure 5. Summary of ^{1}Hα, ^{13}Cα, and ^{13}Cβ chemical shift indices according to the method of Wishart for the 5- (a) and 7- (b) residue peptidyl-resins swollen in DMF-d_7 and the 9- (c) residue peptidyl-resin swollen in a solvent mixture of DMF-d_7/DMSO-d_6 (80/20, v/v). The glycine residues on the ^{13}Cβ axis are noted with an asterisk (*). The ^{1}H and ^{13}C chemical shifts were measured relative to TMS.

Table 1. Bead Mean Diameter and Coupling Yields

A. 9-Residue Peptidyl-Resin (next amino acid to couple: *N*-α-Fmoc-*O*-*tert*-butylthreonine)

			yield (%)	
	bead diameter (μm)[a]	standard deviation (μm)	1 equiv/20 min[b]	4 equiv/60 min[c]
dry	68	6		
DMF	110	8	58	99.1
DMF/DMSO (80/20)	121	7	67	99.4

B. 10-Residue Peptidyl-Resin (next amino acid to couple: *N*-α-Fmoc-isoleucine)

			yield (%)	
	bead diameter (μm)[a]	standard deviation (μm)	1 equiv/20 min[d]	4 equiv/60 min[e]
dry	71	6		
DMF	94	5	36	98.1
DMF/DMSO (80/20)	108	5	46	98.4
DMF/DMSO (60/40)	123	8	61	99.2
DMF/DMSO (40/60)	110	10	60	99.3

[a] Measured under a light microscope on a population of 250 beads/solvation condition. [b] 1 molar excess of *N*-α-Fmoc-*O*-*tert*-butylthreonine, HOBt, and HBTU and 2 molar excess of DIEA; reaction time 20 min; coupling yield measured by the quantitative picric acid test. [c] 4 molar excess of *N*-α-Fmoc-*O*-*tert*-butylthreonine, HOBt, and HBTU and 8 molar excess of DIEA; reaction time 45 min; coupling yield measured by the quantitative ninhydrin test. [d] 1 molar excess of *N*-α-Fmoc-isoleucine, HOBt, and HBTU and 2 molar excess of DIEA; reaction time 20 min; coupling yield measured by the picric acid test. [e] 4 molar excess of N-α-Fmoc-isoleucine, HOBt, and HBTU and 8 molar excess of DIEA; reaction time 45 min; coupling yield measured by the quantitative ninhydrin test.

and Fmoc-Ile have been coupled to the 9- and 10-residue peptidyl-resins, respectively. For both the 9- and 10-residue peptidyl-resins the coupling yields increase upon addition of DMSO. For the 10-residue peptidyl-resin, the efficiency improved when using 40% DMSO and stayed constant to within experimental error when the DMSO concentration was further increased (Table 1B).

Discussion

The combined swelling of the resin and the MAS NMR technique have led to the complete ^{1}H and ^{13}C resonance assignments of the peptide chains during solid-phase peptide synthesis. It should be stressed here that the full assignment could be obtained without any isotopic labeling, giving the method a decisive advantage over other spectroscopic techniques, such as IR-FT, EPR, or ^{13}C gel-phase NMR, and making its routine use during an actual synthesis completely feasible. The chemical shift information was combined with the distance information from homonuclear NOE spectra and indicated an extended structure for the peptide through its elongation. The anomalous chemical shift for the Hα resonance of the last coupled amino acid with a free amino group points toward a very specific conformation of this residue or to a local or global pH effect. We are still in the process of elucidating its structural and chemical origin but should already point out that the chemical shift anomaly can be advantageously used to monitor the correct deprotection of the chains.

While the swelling with DMF proved to be satisfactory for the peptidyl-resin with eight or less residues, the NMR analysis of the 9- and 10-residue peptidyl-resins was nearly impossible

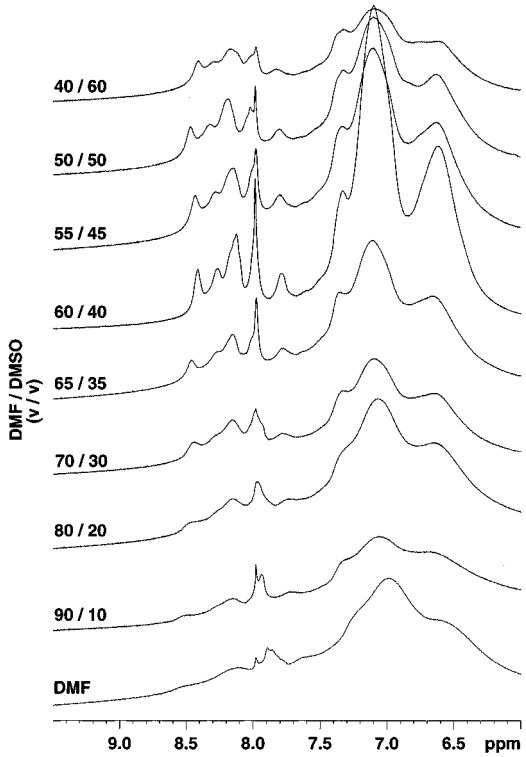

Figure 6. ^{1}H 1D MAS (4 kHz) spectra of the 10-residue peptidyl-resin (resin-Ile-Val-Ser(O*t*-Bu)-Gly-Arg(Pmc)-Ala-Glu(O*t*-Bu)-Gly-Glu(O*t*-Bu)-Thr(O*t*-Bu)-NH_2) swollen in various solvent mixtures of DMF-d_7/DMSO-d_6.

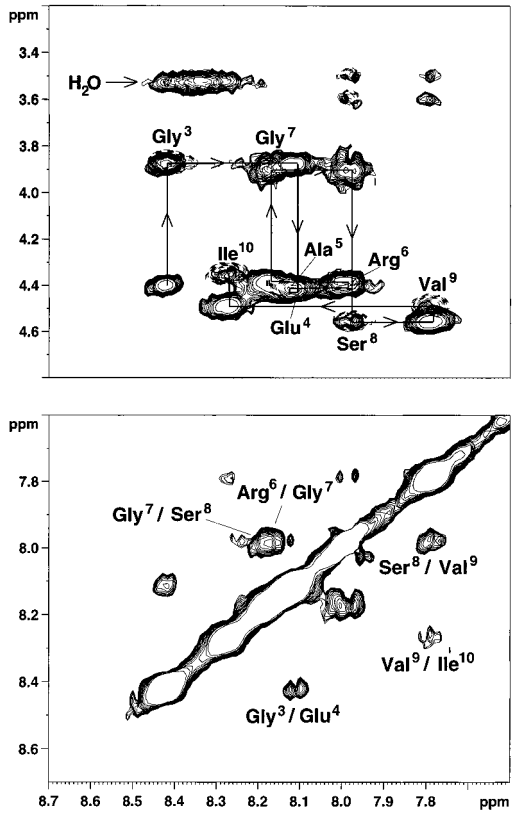

Figure 7. Stretch of the ^{1}H sequential assignment of the 10-residue peptidyl-resin (resin-Ile^{10}-Val^9-Ser^8(O*t*-Bu)-Gly^7-Arg^6(Pmc)-Ala^5-Glu^4-(O*t*-Bu)-Gly^3-Glu^2(O*t*-Bu)-Thr^1(O*t*-Bu)-NH_2) swollen in a solvent mixture of DMF-d_7/DMSO-d_6 (60/40, v/v). The top of the figure shows the superimposition of the NH−Hα domain of the MAS (4 kHz) TOCSY (70 ms) spectrum and the MAS (4 kHz) NOESY (150 ms) spectrum. Labels are added to the (dashed lines) intraresidue NH−Hα TOCSY cross-peaks. Full lines connect the interresidue sequential NOE cross-peaks. The bottom of the figure shows the NH−NH domain of the MAS NOESY spectrum.

due to excessive ^{1}H line broadening. We and others[17,18] previously have shown that correct swelling of the beads is crucial for the applicability of the technique. Similarly, it has since been long known in peptide chemistry that satisfactory coupling yields cannot be obtained without a good solvation of both the cross-linked polymer and the growing peptide chains. The level of solvation which determines the accessibility of the peptide chains to the deprotecting and acetylating reagents inside the polymeric network is not necessarily constant throughout the peptide elongation. According to the Merrifield strategy, solid-phase peptide synthesis begins in an apolar environment due to the polystyrene matrix, and this environment evolves gradually toward a more polar nature when the peptide chains grow. If the nature of the solvents does not evolve in a parallel way, aggregation and consequently additional cross-linking into the peptidyl-resin complex (depending on the sequence, the side-chain protection and the resin loading) may result.[9]

When coupling difficulties arise in solid-phase peptide synthesis, both allowing a longer reaction time and speeding up the reaction kinetics by increasing the reagent concentration and/or the temperature[31] are used to improve the coupling yield. The repetition of the reactions or the use of a low-resin substitution[9] is also often applied to increase reaction efficiency. Another strategy is aimed directly toward diminishing the aggregation of the chains, by the incorporation of either amino acids with a secondary α-amino group,[32] where the nitrogen can no longer act as a hydrogen bond donor, or an oxazolidine-protected pseudo-proline[33] that can additionally introduce a kink in the growing peptide chain. An alternative strategy to improve the chain solvation is the use of chaotropic salts such as LiCl or LiBr[34] or aprotic polar solvents as efficient hydrogen bond acceptors.[5,9,10,35] The latter strategy also seemed suitable to extend the applicability of the MAS NMR analysis to the longer peptides. We therefore have done both a structural and chemical evaluation of the two critical coupling steps in different solvent mixtures, in an attempt to examine at the molecular level the origin of the coupling difficulties. Evaluation of the individual swollen bead diameters was previously shown to be an easy and rapid technique to predict the success of the MAS NMR results. For the 9-residue peptide, addition of 20% DMSO increased the bead diameter by 10%, which is equivalent to a volume increase of some 30%. An extensive titration of DMSO with the 10-residue peptidyl-resin showed an almost 30% increase of bead diameter for the optimal DMF/DMSO ratio of 60/40 (v/v), whereas for both lower and higher DMSO concentrations, the beads again shrink.

Whereas the swollen bead diameter is a macroscopic parameter indicating the space available to the peptide chains, the NMR spectra provide a direct measure of their residual mobility.

(31) Yamashino, D.; Blake, J.; Li, C. H. *Tetrahedron Lett.* **1976**, *18*, 1469−1472.

(32) Bedford, J.; Hyde, C.; Johnson, T.; Jun, W.; Owen, D.; Quibell, M.; Sheppard, R. C. *Int. J. Pept. Protein Res.* **1992**, *40*, 300−307.

(33) (a) Mutter, M.; Nefzi, A.; Sato, T.; Sun, X.; Wahl, F.; Waohr, T. *Pept. Res.* **1995**, *8*, 145−153. (b) Haack, T.; Mutter, M. *Tetrahedron Lett.* **1992**, *33*, 1589−1592.

(34) (a) Hendrix, J. C.; Halverson, K. J.; Jarrett, J. T.; Lansbury, P. T. *J. Org. Chem.* **1990**, *55*, 4517−4518. (b) Thaler, A.; Seebach, D.; Cardinaux, F. *Helv. Chim. Acta* **1991**, *74*, 628−643.

(35) (a) Pugh, K. C.; York, E. J.; Stewart, J. M. *Int. J. Pept. Protein Res.* **1992**, *40*, 208−213. (b) Cilli, E. M.; Oliviera, E.; Marchetto, R.; Nakaie, C. R. *J. Org. Chem.* **1996**, *61*, 8992−9000. (c) Oliveira, E.; Marchetto, R.; Jubilut, G. N.; Paiva, A. C. M.; Nakaie, C. R. In *Peptides: Chemistry, Structure and Biology; Proceedings of the 12th American Peptide Symposium*; Smith, J., Rivier, J., Eds.; ESCOM: Leiden, 1992; pp 569−570.

Addition of DMSO increased the mobility of the peptide chains, resulting in an important line narrowing of the proton lines in the MAS NMR spectra. As the chemical yields increased simultaneously, we can conclude that chain mobility is a determining factor for both spectral quality and chemical optimization. The structural results of the 9- and 10-residue peptidyl-resins give also important insights into the molecular nature of the coupling difficulties. Whereas we do not have direct evidence about the structural nature of the 9- and 10-residue peptides in DMF, because of the poor line widths, the latter parameter does indicate reduced chain mobility due to interchain aggregation. When the NMR analysis became possible due to the addition of DMSO, the peptide was found in an extended conformation, analogous to the shorter peptides in DMF (Figures 4d,e and 5c). These results substantiate, at the difficult synthesis steps, the formation of peptide chain clustering owing to interchain association favored by the extended nature of the structure on resin beads. The aggregation can lead to an incomplete solvation of the peptidyl-resin, resulting in a shrinkage of the gel matrix, a reduced reagent penetration, and finally a decrease in coupling yield. The hydrogen bond-accepting solvent DMSO, when added to DMF, worked as an aggregation disruptive additive in the polymeric network of the peptidyl-resin. The need to increase the polarity of the swelling solvent to analyze the peptidyl-resin when spectral degradation arises does reinforce the assumption of a hydrogen-bonded interchain association, rather than an aggregation of the chains stabilized by apolar contacts between protected side chains.[10] The disruption of peptide aggregates allowed the remaining residual free amino functions to be more accessible for the deprotecting and acylating reagents inside the polymeric network resulting in better coupling efficiency at critical synthesis steps.

Conclusion and Perspectives

The MAS NMR technique is a sensitive and nondestructive analytical method to completely characterize peptides while still bound to the solid support. Coupling difficulties and aggregation of the peptides on the beads were correlated by performing chemical analysis and MAS NMR spectroscopy in varying swelling solvent mixtures. The structural features of the peptide on beads showed that the main limiting factor to the synthesis is the hydrogen-bonded interchain association inside the polymeric network. We have shown that MAS-NMR offers a simple and fast method to evaluate the mobility and thus the solvation of the peptide chains into the peptidyl-resin complex. The 1D ^{1}H NMR spectrum taken on a sample of pepidyl-resin previously swollen in a suitable solvent mixture is sufficient to determine that this mixture allows an efficient solvation of the peptide chains in the polymer and the formation of an open gel system in which there is free access of reagents to active sites.

In this report we have focused on the identification of synthesis problems during elongation of difficult sequences. However, the structural analysis by MAS NMR can also be applied to resin-bonded peptides that adopt biologically relevant conformations. We are currently studying the applicability of the method to the field of biological screening with various ligand binding assays.

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