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Analysis

Secondary Structure of Peptides

12. ^{13}C NMR CP/MAS Study of Amorphous Polypeptides Prepared by Solid State Polymerization of Amino Acid N-Carboxyanhydrides (NCAs)

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SUMMARY

In order to investigate the NMR spectroscopic characteristics of amorphous peptides and proteins, we have attempted to prepare amorphous polypeptides in three ways: first by polymerization of L-(or D) NCAs in the melt; second by polymerization of L-(or D-)NCAs in the solid state; third by oligomerization of D,L-NCAs in solution or in the solid state. Only the last two methods yielded disordered oligo- and polypeptides. Their ^{13}C NMR CP/MAS spectra display broad signals with line widths in the range of 5-10 ppm (350-750 Hz); yet their chemical shifts do not differ from those of highly ordered secondary structures. Upon reprecipitation amorphous poly(L-amino acid)s assume highly ordered conformations whereas oligo (D,L-amino acid)s do not significantly change their secondary structure.

INTRODUCTION

In previous papers of this series [1-8] we have reported that ^{13}C NMR CP/MAS spectroscopy enables a qualitative and quantitative analysis of the secondary structure of synthetic polypeptides and silk fibroins. It was demonstrated that ß-sheets are detectable in the presence of α-helices (or vice versa) [1-7], 3_1 helices in the presence of ß-sheets [2] or 10_3 helices (in the case of poly(proline)) [2] and a α-helix in the presence of both α-helix and ß-sheet structure [8]. However, in addition to these highly ordered secondary structures, proteins, and in particular the complex skleroproteins may contain considerably amounts of amorphous regions. Hence, prior to NMR spectroscopic investigations of such proteins the spectroscopic characteristics of amorphous regions need to be studied by means of model peptides.

RESULTS and DISCUSSION

The main difficulty of spectroscopic studies of amorphous peptides is their preparation. Due to hydrogen bonds, strong dipole-dipole interactions and severe steric interactions of the side chains peptides prefer only two or three energetically favourable conformations as demonstrated by Rachamandran diagrams [9]. Furthermore, the time required for conformational rearrangements of dissolved peptides is only on the order of 10^{-8}s. Hence, any synthesis of peptides in solution will yield a highly ordered secondary structure. For the same reasons reprecipitation of amorphous peptides will considerably increase the conformational order. Nonetheless, for two reasons oligo (D,L-amino acid)s with a random sequence of D,L-monomer units are the only exception of this rule. First, polymerization degrees (DPs) below 12 ± 1 (depending on the nature of the amino acid) prevent the formation of helices. Second, a random sequence of enantiomers prevents the formation of ordered ß-sheets.

On account of this situation we attempted to prepare steric ly uniform (isotactic) polypeptides with a low degree of conformational order by polymerization of L- or D-amino acid NCAs in the melt or in the solid state. When molten L-Phe-NCA, L-γ-OMe-Glu-NCA and L-Leu-NCA were polymerized at 145-150°C, the resulting polypeptides contain a substantial fraction (up to 20 %) of low molecular weight byproducts, which were extracted by means of ethanol. Regardless, whether the polypeptides were extracted or not the NMR spectra displayed the typical signal patterns of polypeptides with a high content (70-90 %) of α-helix structure. Neither the chemical shifts, nor the line widths differed from those of polymers prepared in solution, and after reprecipitation, the spectra were almost unchanged. Hence, it is obvious that polymerizations of molten L- or D-NCAs do not yield polypeptides with a substantial fraction of amorphous regions.

This unsatisfactory result prompted us to investigate the time consuming solid state polymerization of several L- or D-NCAs. It is well-known that NCAs are not stable on storage and slowly polymerize even when stored in a closed flask below $0^{o}C$. Thereby the monomer crystals do not change their shape or size. Thus, the chain growth must proceed in the crystal lattice of the monomers, and under these conditions, disordered secondary structure of the peptide chains are expected. When D-Ala-NCA was polymerized at $-15^{o}C$, the NMR spectrum (Fig. 1 A) displayed three broad signals with line widths of 5 ppm for the α-C or ß-C signal and 9 ppm for the CO signal. The chemical shift of the CO-signal was intermediate between those of the α-helix and ß-sheet structures of poly (D-alanine), whereas the α-C and ß-C signals were nearly identical with those of α-helical poly(L-alanine) [2]. After reprecipitation all signals displayed the two peaks of the α-helix and ß-sheet structure with the usual line widths of ca. 3 ppm (Fig. 1 B). When D,L-Ala NCA was polymerized at $-15^{o}C$ line widths of ca. 6 ppm were found for the α-C and ß-C signals and 10 ppm for the CO signal. However, in this case reprecipitation did not cause any spectroscopic change (Fig. 1 C). These results indicate that the solid state polymerization of D-Ala-NCA result in a thermodynamically instable secondary structure with high content of unfavourable conformations, whereas D,L-Ala-NCA yields a likewise disordered, but thermodynamically stable structure. From the chemical shifts of the amorpous poly(L-alanine) one may conclude that most conformations are similar to those of α-helix and ß-sheet structure or between these two extremes in agreement with the partially allowed conformational regions of Ramachandran diagrams [9,10].

Similar results were obtained with the solid state polymerizations of L-Leu-NCA, Phg-NCA and L-Phe-NCA. Poly(L-leucine) prepared from crystalline L-Leu-NCA at $-15^{o}C$ shows signals with line widths around 5 ppm and chemical shifts that fully agree with those of the α-helical structure [2,6] (Fig. 2 A). After reprecipitation the α-C and ß-C signals split into the peaks of the ß-sheet and α-helical fractions (Fig. 2 B) with line

widths around 3 ppm. Broad signals (line widths ca. 5-7 ppm) with chemical shifts between those of the α-helix and ß-sheet peaks were also found when crystalline L-Phe-NCA was polymerized at -15°C. These spectra (and those of poly (D-Alanine)) allow the following conclusions. First, solid state polymerizations of L- or D-NCAs of α-helix forming amino acids yield thermodynamically unstable secondary structures with a high content of unfavourable conformations. Second, this amorphous character of the peptide results in greater line widths, but not in new signals exceeding the shift range known from the α-helix and ß-sheet peaks [2]. Third, after reprecipitation thermodynamically stable secondary structures are obtained which contain more than 50 % ß-sheets. Fourth, the average degrees of polymerization ($\overline{DPs}$) are low, presumably ≤ 20. The last conclusion deserves a short comment. When L-Ala-NCA and L-Leu-NCA were polymerized in solution [4,6] the α-helix/ß-sheet ratio was found to increase with increasing DP. By extraction of oligomers we were able to demonstrate that the thermodynamically stable ß-sheet fraction results from the presence of oligomers which are too short to assume the α-helical conformation. Hence, we may conclude that also the ß-sheet fractions of the reprecipitated solid state polymers (Figs. 1 B and 2 B) originate from oligomers with $\overline{DPs} < 12$.

The fourth monomer polymerized in the solid state was D-phenylglycine-NCA. In contrast to phenylalanine (Phe) phenylglycine (Phg) is a helix destabilizing amino acid so that its peptides exclusively adopt a ß-sheet structure. In agreement with this property the poly(D-phenylglycine) obtained from crystalline Phg-NCA at -15°C did not display any new signals or signal splittings in its ^{13}C NMR CP/MAS spectrum after reprecipitation (Figs. 3 A, B). However, the line widths decreased again from ca. 6 ppm ("as polymerized" sample) to 3.0-3.5 ppm (reprecipitated sample). Thus, also these measurements confirm that amorphous regions of polypeptides increase the line widths and do not cause new signals as it was observed for technical polymers such as poly(ethylene) or poly(propylene) [11-13]. In this connection, it is worth mentioning that the amide I and II

bands in the IR spectra of our "as polymerized" samples also possess an increased band width, when compared with entirely helical samples.

Finally, it is noteworthy that also the X-ray powder patterns we measured of our "as polymerized" samples indicate their amorphous character. However, it must be emphasized that "amorphous" X-ray patterns of polypeptides and proteins, on the one hand, and technical polymers like poly(ethylene), poly(propylene), poly(propyleneoxide) or poly(caprolactone) do not have the same meaning. The X-ray pattern yields information on the long range order (or disorder) of the polymer, which in the case of most synthetic polymers is closely connected to the short range (or local) order of the polymer chains. Local order means here the conformations of individual monomers in short chain segments. However, copolypeptides and proteins may possess a high degree of local order combined with a low degree of long range order. In such a case most chain segments possess the energetically most favourable conformation; yet this conformation varies with the nature and sequence of the amino acid units. The chain segments having different conformations are linked to each other by one, two or more amino acids with unfavourable conformations. Thus the entire chain possesses a long range disorder, although more than 90 % of all monomer units exist in highly ordered local conformations. Furthermore, the packing of several polypeptide chains may be disordered despite a high degree of conformational order (e.g. packing of helices). Thus, it may happen that CP/MAS NMR spectra, which mainly sum up the conformations of amino-acid, di- and tripeptide units indicate a highly ordered secondary structure, whereas the X-ray pattern manifests a predominantly amorphous character of the sample. These two levels of information must be taken into account, when both methods are combined for the characterization of copolypeptides and proteins.

EXPERIMENTAL

Polymerizations: Three different polymerization methods were applied: A) the twice recrystallized NCAs were stored in closed

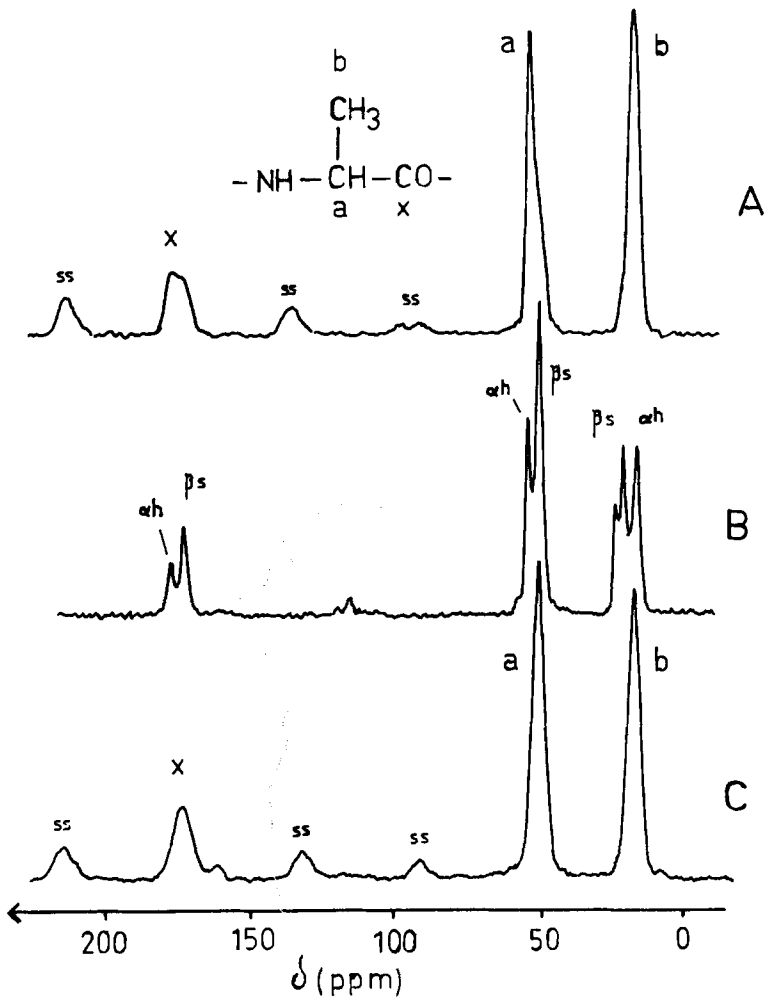

Figure 1: 75.4 MHz ^{13}C NMR CP/MAS spectra of: A) crystalline D-alanine-NCA polymerized at -15°C; B) the same poly(D-alanine) after reprecipitation from TFA/H_2O; C) crystalline D,L-alanine-NCA polymerized at -15°C and reprecipitated from TFA/diethyl-ether.

flask at -15/-20°C for several month until the IR spectra showed the complete absence of monomers. B) The recrystallized NCAs were heated in a loosely stoppered Erlenmeyer flask for ca. 30 min. to 145-150°C, whereby solid foams of polypeptides were obtained. C) D,L-NCAs were oligomerized in dioxane and acetonitrile at 20°C by means of benzylamine (monomer/initiator ratio = 10 : 1). For reprecipitation 1 g polypeptide was dissolved in

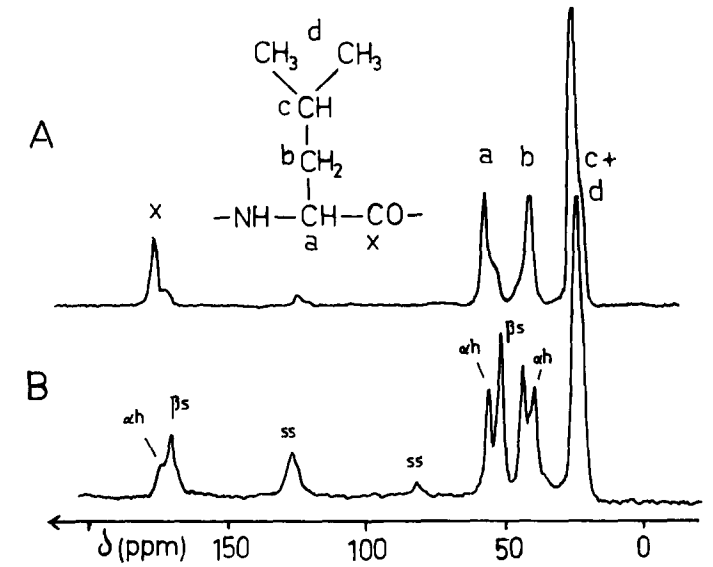

Figure 2: 75.4 MHz ^{13}C NMR CP/MAS spectra of
A) L-Leu-NCA polymerized at -15°C in the solid state
B) the same poly(L-leucine) after reprecipitation from TFA/H_2O

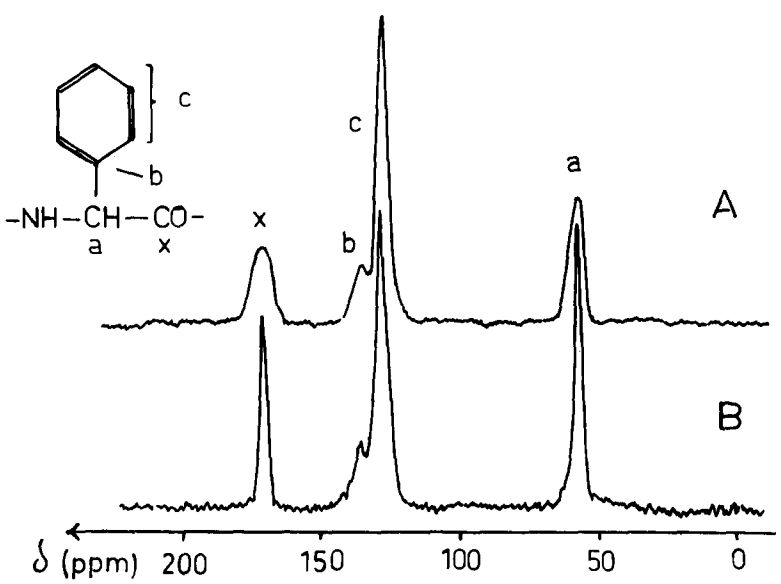

Figure 3: 75.4 MHz ^{13}C NMR CP/MAS spectra of
A) D-phenylglycine-NCA polymerized at -15°C in the solid state
B) the same poly(D-phenylglycine) after reprecipitation from TFA/H_2O

15 ml of trifluoroacetic acid (+ 5 % methane sulfonic acid in the case of poly(L-leucine) and poly(L-phenylalanine)) and precipitated into 150 ml of cold water or diethylether.

NMR Measurements: 75.4 MHz ^{13}C NMT CP/MAS spectra were measured as described in previous papers [2,3]. A contact time of 1 ms and pulse repetition time of 4 s was used for all measurements.

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