Hydrogen/Deuterium Exchange and Aggregation of a Polyvaline and a Polyleucine α-Helix Investigated by Matrix-assisted Laser Desorption Ionization Mass Spectrometry*

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The membrane-associated pulmonary surfactant protein C (SP-C), containing a polyvaline α-helix, and a synthetic SP-C analogue with a polyvaline helix (SP-C(Leu)) were studied by hydrogen/deuterium exchange matrix-assisted laser desorption ionization (MALDI) mass spectrometry. SP-C, but not SP-C(Leu), formed abundant amyloid fibrils under experimental conditions. In CD3OD/D2O, 91:9 (v/v), containing 2 mM ammonium acetate, SP-C(Leu) and SP-C exchanged 40% of their exchangeable hydrogens within 1 min. This corresponds to exchange of labile side-chain hydrogen atoms, hydrogens on the N- and C-terminal heteratoms, and amide hydrogen atoms in the unstructured N-terminal regions. After ~300 h, four exchangeable hydrogen atoms in SP-C(Leu) and 10 in SP-C remained unexchanged. During this time period the ion current corresponding to singly charged SP-C decreased to <10% of the initial value due to the formation of insoluble aggregates that are not detected by MALDI mass spectrometry. In contrast, the ion current for SP-C(Leu) was maintained over this time period, although the peptides were incubated together. In combination, hydrogen/deuterium exchange and aggregation data indicate that the polyvaline peptide refolds into a helix after opening, while the unfolded polyvaline peptide forms insoluble β-sheet aggregates rather than refolding into a helix. The SP-C helix, but not the SP-C(Leu) helix, is thus in a metastable state, which may contribute to the recently observed tendency of SP-C and its precursor to misfold and aggregate in vivo. Molecular & Cellular Proteomics 1:592–597, 2002.

Aggregation and amyloid fibril formation of polypeptides is related to severe pathologic conditions, e.g. Alzheimer’s disease, systemic amyloidosis, type II diabetes mellitus, and Creutzfeldt-Jacob disease. For fibril formation to occur the peptides or proteins must lose their native structure and polymerize into a distinct cross-β-sheet (1). The fact that there are only about 20 different proteins that form disease-associated amyloid fibrils described to date suggests that the fibril formation process in vivo is selective. However, it has been shown that many peptides form fibrils in vitro and that virtually any polypeptide chain, including globular all-helical proteins, if kept under denaturing conditions for a prolonged time, can form fibrils (2). This suggests that fibrils are formed from partly unfolded species and that most proteins populate such conformations scarcely enough to practically avoid fibril formation. The proteins that form amyloid fibrils differ in size, location, and native three-dimensional structure and flexibility (3), suggesting that, despite the obvious similarities between the amyloid end-product in different diseases, different factors underlie fibril formation. In line with this assumption, mutations in lysozyme that are associated with systemic amyloidosis have been found to be destabilizing as detected by increased flexibility from hydrogen/deuterium exchange (HDX) and mass spectrometry measurements and by reduced melting temperatures (4). In contrast, most mutations that segregate with familial Creutzfeldt-Jakob disease do not destabilize the prion protein in vitro (5). We have found that several amyloid-forming proteins harbor an α-helix that is composed of an amino acid sequence that is predicted to form a β-strand structure, a phenomenon referred to as α-helix/β-strand discordance (6). By changing the amino acid sequences so that instead the helices were made of helix-prefering residues, in vitro fibril formation was inhibited, suggesting that α-helix/β-strand-discordant segments can influence the tendency to form fibrils (6). The possibility that specific protein regions are responsible for the conversion into fibrils has recently gained experimental support from the findings that mutations in only one of the two helices of acylphosphatase modulate its fibrillation potential (7, 8).

Surfactant protein C (SP-C) is a 35-amino acid lipopeptide, which is tightly associated with the lung surfactant that lowers the surface tension at the alveolar air/liquid interface. SP-C is exclusively found in the lung and is exceptionally hydrophobic because >80% of its amino acid residues have aliphatic side chains, including two palmitoylated cysteine residues. The NMR structure of SP-C in an aqueous organic solvent mixture

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1 The abbreviations used are: HDX, hydrogen/deuterium exchange; ES, electrospray; MALDI, matrix-assisted laser desorption ionization; SP-C, surfactant protein C; Aβ, amyloid β peptide.
shows a rigid \( \alpha \)-helix covering residues 9–34 and an unstructured N-terminal region covering residues 1–8 (9). Two positively charged residues are located in the proximal part of the helix (at positions 11 and 12), but otherwise the helix is made of exclusively nonpolar residues, predominantly Val (see “Materials and Methods” for amino acid sequence of SP-C). The size and structure of the SP-C helix is perfectly suited to span a fluid phospholipid bilayer. Freshly dissolved monomeric \( \alpha \)-helical SP-C in aqueous organic solvents converts over time to insoluble \( \beta \)-sheet aggregates, which show typical amyloid fibril appearance by electron microscopy (10, 11). SP-C aggregates and fibrils are also found in lung lavage fluid from patients suffering from pulmonary alveolar proteinosis, suggesting that SP-C can form amyloid fibrils in vivo (10). The SP-C helix contains a 16-residue \( \alpha \)-helix/\( \beta \)-strand discordant segment as expected from the very high \( \beta \)-strand propensity of Val (6). Interestingly, SP-C isolated from alveolar proteinosis patients is largely devoid of one or both of the palmitoyl groups, indicating that removal of the palmitoyl groups can promote fibril formation. This is supported by shorter half-lives of non- and monopalmitoylated SP-C compared with dipalmitoylated SP-C determined by mass spectrometry (12).

To circumvent the aggregation associated with SP-C the analogue SP-C(Leu) has been developed in which all valine residues in the SP-C helix have been replaced with leucine residues (see “Materials and Methods” for amino acid sequence of SP-C(Leu))). As predicted from the higher helical propensity of Leu versus Val, synthetic SP-C(Leu), in contrast to SP-C, readily forms a helical structure and can be refolded after acid-induced unfolding (13). Notably, SP-C(Leu) does not exhibit \( \alpha \)-helix/\( \beta \)-strand discordance, i.e. it is correctly predicted to be \( \alpha \)-helical, and does not form fibrils (6). SP-C and SP-C(Leu), although very similar in size, hydrophobicity, secondary structure, and biological activity, thus differ in terms of \( \alpha \)-helix \( \rightarrow \beta \)-strand conversion and fibril formation. In this study, we compared HDX and aggregation properties of SP-C and SP-C(Leu) to find explanations to their different behaviors.

While mass spectrometry has been thoroughly exploited in HDX studies (14–16), it has been used to a far lesser extent in the study of protein aggregation. A requirement for electro-spray (ES) and matrix-assisted laser desorption ionization (MALDI) is a soluble analyte. Insoluble species cannot be ionized by these methods and are undetected by mass spectrometry. Thus, aggregated species cannot be directly analyzed by ES or MALDI, although it is possible to observe soluble large multimeric complexes, which may constitute a pre-fibrillar state (17). Alternatively, fibrils can be dissolved prior to ES or MALDI analysis, and their monomeric constituents can be analyzed (18). A different approach to the study of protein aggregation via mass spectrometry is to measure the rate of disappearance of soluble protein from solution. Assuming insoluble protein species are “invisible” in an ES or MALDI experiment a decrease in protein \([M + \text{H}]^{+}\) ion current will be related to a decrease of protein concentration in solution and an increase in insoluble matter, i.e. aggregated protein (19). This procedure has been applied to the study of SP-C aggregation where it was found that dipalmitoylated SP-C was more stable than mono- or non-palmitoylated SP-C. It was also observed that SP-C tended to unfold in the ES process giving an artiﬁcially high HDX value (12).

MALDI is widely used in peptide analysis, generally providing higher sensitivity than ES, and furthermore, unlike in the ES process, SP-C does not unfold upon MALDI. SP-C and SP-C(Leu) are readily ionized by MALDI, and in the present study MALDI mass spectrometry was used in an HDX study of both SP-C and SP-C(Leu). Concurrently, the rate of decrease of protein \([M + \text{H}]^{+}\) ion current was measured for a solution containing the two proteins. These measurements provided data on the relative stability of the two \( \alpha \)-helices.

**MATERIALS AND METHODS**

**Peptides**—SP-C (LRIPC[pal(C)pal]PVNLKRLLVVNVVVLVVVVG-ALLMGL, pal = palmitoyl group) was purified from porcine lungs as reported previously (20) and stored at \(-20{\text{C}}\) in CHCl\(_3\)/MeOH, 1:1 (v/v). SP-C(Leu) (IPSSPVVKRLKLLLHLLLHLLLHALLMGL) was synthesized using an Applied Biosystems A430 peptide synthesizer utilizing tert-butloxycarbonyl (t-boc) chemistry. Cleavage with hydrogen fluoride, extraction, and purification by reversed-phase high performance liquid chromatography were performed as reported previously (13). SP-C(Leu) was stored in 98% ETOH. The purity of peptides was checked with MALDI and ES mass spectrometry.

**HDX Experiments**—Incubation solutions were made by mixing 4 \( \mu l \) of SP-C(Leu) stock solution (2.5 mg/ml) and 20 \( \mu l \) of SP-C stock solution (0.77 mg/ml), and solvents were evaporated to near dryness under a stream of nitrogen. Then 160 \( \mu l \) of premixed 91% CD\(_3\)OD (99.8% deuterium, Aldrich), 9% D\(_2\)O (99.8% deuterium, Sigma) was added, and to this solution 0.16 \( \mu l \) of 2 \( M \) ammonium acetate was added. This component ratio of SP-C(Leu) to SP-C was found to give almost equivalent \([M + \text{H}]^{+}\) ion signals by MALDI mass spectrometry. All sample handling was made in a closed box flushed with dry \( \text{N}_2 \) and \( \text{N}_2 \) flushing was performed for 5 min preceding any operation. Spotting of the sample onto the MALDI target plate was also performed under a stream of nitrogen. Then 160 \( \mu l \) of premixed 91% CD\(_3\)OD (99.8% deuterium, Aldrich), 9% D\(_2\)O (99.8% deuterium, Sigma) was added, and to this solution 0.16 \( \mu l \) of 2 \( M \) ammonium acetate was added. This component ratio of SP-C(Leu) to SP-C was found to give almost equivalent \([M + \text{H}]^{+}\) ion signals by MALDI mass spectrometry. All sample handling was made in a closed box flushed with dry \( \text{N}_2 \) and \( \text{N}_2 \) flushing was performed for 5 min preceding any operation.

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RESULTS

HDX of SP-C and SP-C(Leu)—An inherent problem in the study of SP-C is that its monomeric form gradually disappears. In preliminary experiments it was noted that in the acidic solvents previously used for studies of SP-C HDX (12), the exchange rate was too slow to allow near-complete exchange in SP-C(Leu) within the time period that SP-C remains detectable. For sufficiently rapid HDX it was necessary to supplement the CD3OD/D2O solvent with 2 mM ammonium acetate. Under this condition the palmitoyl groups are cleaved from the cysteine residues of SP-C resulting in a mixture of di-, monopalmtoylated and dipalmitoylated SP-C. Thus, when a mixture of SP-C and SP-C(Leu) is exposed to the deuterated solvent containing 2 mM ammonium acetate, depalmitoylation will proceed in addition to HDX as shown in Fig. 1. By recording MALDI spectra over a period of 500 h it was possible to monitor HDX in SP-C(Leu) and SP-C. The number of exchangeable hydrogens in the singly charged forms of SP-C(Leu) and monopalmtoylated and dipalmitoylated SP-C are 43, 48, and 47, respectively. In [SP-C(Leu) + H]⁺ there are two N-terminal hydrogens, 29 amide hydrogens, 10 side-chain exchangeable hydrogens, and one C-terminal and one added (charge) proton. In dipalmitoylated SP-C there are 32 amide hydrogens, 12 side-chain exchangeable hydrogens, two N-terminal hydrogens (the C terminus is methylated), and one added proton. Monopalmtoylated SP-C has one more exchangeable hydrogen than dipalmitoylated SP-C. The rate of HDX can be represented in terms of a plot of the number of shielded hydrogens (i.e. the number of hydrogen atoms bound to heteroatoms that have not exchanged with deuterium) against time. Such a curve is shown in Fig. 2 where the rates of HDX for SP-C(Leu) and monopalmtoylated SP-C are represented. As dipalmitoylated SP-C is degraded with time into the monopalmtoylated form, the HDX curve for dipalmtoylated SP-C terminates after 82 h (reflector mode) or 174 h (linear mode), and the curve for monopalmtoylated SP-C starts at 90 min. It should be noted that weak but detectable signals corresponding to protonated molecules of di-, mono-, or non-palmtoylated peptide were observed throughout the 22-day period of investigation. Previous studies have shown that the HDX kinetics for di-, mono-, and non-palmtoylated SP-C are essentially similar (12). There are three discernible regions of the HDX rate curves shown in Fig. 2: (i) an initial very rapid uptake of deuterium following the addition of the deuterated solvent (this is reflected in the high degree of HDX during the time elapse preceding the first MALDI analysis (Fig. 1)), (ii) a period of reduced exchange up to about 100 h, and (iii) finally a period of very slow deuterium incorporation. When the degree of deuterium incorporation has reached a maximum the number of shielded hydrogens in SP-C(Leu) is 4, and in monopalmtoylated SP-C it is 10.

Aggregation Analysis of SP-C and SPC(Leu) by MALDI Mass Spectrometry—MALDI mass spectrometry is not generally regarded as a quantitative technique. Furthermore the ionization efficiency of different peptides varies markedly (21, 22). However, it can be shown that for a mixture of SP-C and SP-C(Leu) changes in the relative concentrations of the pep-
tides ([SP-C(Leu)]/[SP-C]) are reflected in changes in the relative ion currents ([ISP-C(Leu)]/[ISP-C]) generated by MALDI. In fact [SP-C(Leu)]/[SP-C] is approximately proportional to [ISP-C(Leu)]/ISP-C in the concentration range of the present experiment (see Fig. 3, inset). Thus the ratio [ISP-C]([ISP-C(Leu)] reflects the in-solution concentration ratio of the two proteins. Shown in Fig. 3 is a plot of the ion current ratio for the two peptides varies with their concentration ratio.

**DISCUSSION**

In this study HDX in combination with MALDI mass spectrometry has been used for the analysis of flexibility and aggregation of two peptides composed of α-helical poly-Val (SP-C) and poly-Leu (SP-C(Leu)) segments, respectively. The results of this study explain the differences observed previously between these two polypeptides in their tendencies to form insoluble β-sheet aggregates and amyloid fibrils (6).

With the HDX MALDI mass spectrometry approach 10 hydrogens in SP-C were found not to exchange during the period of observation (about 22 days). In a previous study, using HDX in combination with NMR, 15 hydrogens of SP-C were found not to exchange during 10 days (11). Previous determination of HDX of SP-C dissolved in the acidic mixture ethanol/chloroform/methanol/trifluoroacetic acid, 3:2:2:1 (by volume), using MALDI were in good agreement with the NMR data (12). Therefore, the most likely reason for the difference in the number of protected hydrogens now detected by MALDI and previously by NMR is the difference in the solvents used. In the NMR study an acidic mixture, chloroform/methanol/0.1 M HCl, 32:64:5 (by volume), was used, while in this study 2 mM ammonium acetate in methanol/water, 91:9 (v/v), was used. Therefore to make a meaningful comparison of HDX of these two peptides it is necessary to analyze the two peptides dissolved together in the one solution.

The HDX kinetics for both the peptides are similar in the early stages of the reaction, i.e. ~100 h. During this period HDX proceeds at the exchangeable side-chain hydrogens and at amide hydrogens outside the central helical sections. HDX within the helical section proceeds at a slower rate in both peptides. Shown in Fig. 4 is a schematic potential energy diagram for the unfolding of SP-C and SP-C(Leu). Initial rapid HDX will proceed from the ground state (A) to an unfolded intermediate of SP-C (B) which reverts to the helical conforma-

![Fig. 3. Plot of [ISP-C]/[ISP-C(Leu)] against time. The inset shows how the ion current ratio for the two peptides varies with their concentration ratio.](image)

![Fig. 4. Schematic potential energy diagram for the unfolding SP-C (dashed line) and SP-C(Leu) (solid and dotted line). A represents the helical state, B represents the unfolded/open state, and C represents the β-sheet aggregates of both peptides. In the current MALDI mass spectrometry experiments only peptides in the A state are observed. SP-C has been experimentally observed to populate all three states, while for SP-C(Leu) β-sheet aggregates have not been observed, and this part of the line is therefore dotted. See text for details.](image)
C(Leu) can be rationalized as shown in the energy diagram of Fig. 4. SP-C(Leu) exhibits no change in overall secondary structure over the time period now studied (13), indicating that no significant accumulation of peptides (8) with open/unfolded conformations takes place. The potential energy of the open conformation of SP-C(Leu) is therefore higher than for the α-helical form. In contrast, for SP-C the helical form disappears without accumulation of detectable amounts of open peptide, and analysis of SP-C that has been incubated for 3–10 days shows β-sheet aggregates and amyloid fibrils (10, 11). This implies that open SP-C rapidly converts into aggregates, which are not observed by MALDI mass spectrometry, and that for SP-C the energetic barrier going from open to helical conformation is higher then the barrier between open and β-sheet aggregates. It is assumed that SP-C(Leu), like virtually any protein (2), can form β-sheet aggregates. Since formation of SP-C(Leu) aggregates were not detected it is concluded that for SP-C(Leu) the energetic barrier going from open to helical conformation is lower then the barrier between open and β-sheet aggregates and/or that β-sheet aggregates are destabilized relative to the helical state.

The energies of the open conformations of SP-C and SP-C(Leu) are likely to be similar given the similarity between Val and Leu side chains and the flexible nature of the open conformation. Furthermore, the time required for near-complete exchange of SP-C(Leu) is similar to the time required to reach near-complete aggregation of SP-C. The rate-limiting step for both these phenomena is assumed to be helix unfolding (Fig. 4), and hence the rate of helix unfolding and the corresponding activation energy are similar for SP-C and SP-C(Leu). This together with the notion that α-helical SP-C likely is at a higher energy level than α-helical SP-C(Leu) (due to restriction in Val side-chain rotation, compared with Leu, upon helix formation) suggests that the refolding barrier is lower for SP-C(Leu) then for SP-C (Fig. 4).

Recently it has been shown that regions that determine folding and aggregation can be localized to different regions of the polypeptide chain (7, 8). Using muscle acylphosphatase, a two-helix protein, it was found that mutations in mainly one of the α-helices influence the aggregation properties, while mutations in the other helix affect the folding properties (8). A key question then is what identifies an aggregation-determining region and a folding-determining region, respectively. The differences in refolding and aggregation between SP-C and SP-C(Leu) in combination with their relatively simple structures afford a suitable system for addressing this question. There is no major difference between SP-C and SP-C(Leu) in size, polarity, or structure of the native state. In contrast, the secondary structure preferences derived from the fractional occurrence of residues in determined three-dimensional structures (Chou-Fasman propensities) differ between Leu and Val. The ratio of the α-helix and β-strand Chou-Fasman propensity values is 1.2 for Leu and 0.46 for Val. Using these propensity values SP-C is strongly, but incorrectly as regards the native state, predicted to form a β-strand, while SP-C(Leu) is strongly predicted to form an α-helix (6). Consequently, there is an excellent agreement between the Chou-Fasman secondary structure propensities and the fates of the open conformation of SP-C and SP-C(Leu) (Fig. 4); unfolded SP-C forms β-sheet aggregates, while SP-C(Leu) reforms an α-helix. This supports the observation that partitioning between aggregation and folding can be attributed to conformational preferences of the denatured polypeptide chain (7).

From host-guest studies of alanine-based peptides dissolved in SDS micelles or butanol, Deber and coworkers (23, 24) have derived residual secondary structure propensities for unpolar environments. Using these propensities the helical structure of SP-C isolated from lung tissue is correctly predicted. However, also in the unpolar environments methanol/water (this study), chloroform/methanol/water (11, 12), or do-decylphosphocholine micelles (25) SP-C preferentially forms β-sheet aggregates. In contrast, SP-C(Leu) forms helical structures in all these solvents (Ref. 13 and this study). This suggests that folding of the SP-C poly-Val segment into a helical conformation requires more than simple partitioning of the polypeptide chain into a hydrophobic environment. Intracellular misprocessing and accumulation of mutant pro-SP-C has been observed in familial interstitial lung disease (26) and in cell culture systems (27), and overexpression of SP-C in mice leads to intracellular aggregation of SP-C and altered lung development (28). Misfolding (β-sheet formation) and aggregation of the SP-C poly-Val region may in part cause these effects.

The present study as well as previous NMR and ES mass spectrometry studies show that α-helical SP-C dissolved in aqueous organic solvents is in a metastable state and will quantitatively convert into β-sheet aggregates via a high energy transition state (activation energy ~100 kJ/mol) (11). Interestingly, it was recently found that the mainly α-helical conformation of the prion protein is likewise kinetically stabilized relative to a β-rich isoform with an energetic barrier of ~80 kJ/mol (29). SP-C and the prion protein thus retain an α-helical fold because of high energetic barriers to unfolding.

Amyloid fibrils formed from lysozyme mutants do not include wild-type lysozyme, indicating sequence specificity in fibril formation (4). However, co-aggregation of Aβ(1–40) and Aβ(1–42) has been observed (30, 31), suggesting that fibrils can accommodate slightly different peptides and/or that the C-terminal part of Aβ is not directly involved in fibril formation. A significant finding in this study is that there is no detectable co-aggregation of SP-C and SP-C(Leu), showing that aggregates of SP-C do not promote SP-C(Leu) aggregation. In conclusion, the results of the present study show that α-helices composed of similar amino acids differ in their ability to refold and that the inability of the polyvaline helix to reform causes it to form β-sheet aggregates and amyloid fibrils.
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