Pulmonary surfactant-associated polypeptide C in a mixed organic solvent transforms from a monomeric α -helical state into insoluble β -sheet aggregates

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Abstract

In the 35-residue pulmonary surfactant-associated lipopolypeptide C (SP-C), the stability of the valyl-rich α -helix comprising residues 9–34 has been monitored by circular dichroism, nuclear magnetic resonance, and Fourier transform infrared spectroscopy in both a mixed organic solvent and in phospholipid micelles. The α -helical form of SP-C observed in freshly prepared solutions in a mixed solvent of $\text{CHCl}_3/\text{CH}_3\text{OH}/0.1 \text{ M HCl}$ 32:64:5 (v/v/v) at 10 °C undergoes within a few days an irreversible transformation to an insoluble aggregate that contains β -sheet secondary structure. Hydrogen exchange experiments revealed that this conformational transition proceeds through a transition state with an Eyring free activation enthalpy of about 100 kJ mol⁻¹, in which the polypeptide segment 9–27 largely retains a helical conformation. In dodecylphosphocholine micelles, the helical form of SP-C was maintained after seven weeks at 50 °C. The α -helical form of SP-C thus seems to be the thermodynamically most stable state in this micellar environment, whereas its presence in freshly prepared samples in the aforementioned mixed solvent is due to a high kinetic barrier for unfolding. These observations support a previously proposed pathway for *in vivo* synthesis of SP-C through proteolytic processing from a 21-kDa precursor protein.

Keywords: α -helix unfolding; hydrogen exchange; kinetic stability; surfactant-associated lipopolypeptide SP-C

Surfactant-associated lipopolypeptide C (SP-C) is a component of a surface-active material, “surfactant,” which is a complex mixture of phospholipids and polypeptides synthesized by the lung epithelium. Surfactant is excreted into the alveoli to reduce the surface tension at the air–liquid interface, thereby preventing alveolar collapse at low lung volumes (for a review, see Johansson & Curstedt, 1997). A low level of pulmonary surfactant in premature infants results in the respiratory distress syndrome, which can be treated with surfactant preparations containing phospholipids, mainly 1,2-

dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1 to 2% (w/w) of the surfactant-associated proteins SP-B and SP-C (e.g., Robertson & Taeusch, 1995). This clinical application has stimulated intense interest in the structural properties and the molecular biology of SP-B and SP-C (e.g., Weaver & Whitsett, 1991; van Golde et al., 1994). SP-C is a 4.2-kDa lipopolypeptide that is palmitoylated via thioester linkages at Cys5 and Cys6, and it is one of the most hydrophobic naturally occurring polypeptides known. Porcine SP-C contains 16 consecutive branched hydrophobic residues including twelve valines, and this valyl-rich polypeptide segment is conserved in all species investigated so far (Johansson et al., 1988, 1991; see Fig. 3 for the amino acid sequence of SP-C). SP-C is synthesized through proteolytic processing and post-translational modification from a 21-kDa precursor protein (proSP-C₂₁). Recent studies with epitope-specific antipeptide antibodies resulted in a detailed proposal for the pathway of processing of proSP-C₂₁ to mature SP-C (Beers et al., 1994; Beers & Lomax, 1995). The results of the present study now provide a possible rationale for the proposed *in vivo* processing of proSP-C₂₁, according to which the polypeptide corresponding to mature SP-C

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Abbreviations: 1D, one-dimensional; 2D, two-dimensional; CD, circular dichroism; DPC, dodecylphosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance; SP-C, surfactant protein C; SP-C^{*}, transition state of unfolding of α SP-C; α SP-C, α -helical SP-C in solution; β SP-C, aggregate of SP-C including β -sheet secondary structure; nhSP-C, nonhelical SP-C in solution; proSP-C₂₁, 21 kDa precursor protein of SP-C; TOCSY, total correlation spectroscopy; UV, ultraviolet.

forms transmembranous α -helical SP-C (α SP-C) before the proteolytic removal of the flanking regions.

This paper describes an investigation of the unfolding of the valyl-rich α -helix comprising residues 9 to 34 of the 35-residue SP-C dissolved in $\text{CHCl}_3/\text{CH}_3\text{OH}/0.1 \text{ M HCl}$, 32:64:5 (v/v/v) [or the corresponding deuterated solvent for nuclear magnetic resonance (NMR) studies] (Fig. 1) and its conversion into an aggregate containing β -sheet secondary structure. The study was stimulated by the observation, made during the determination of the NMR solution structure of native SP-C (Johansson et al., 1994) at 10 °C in the same mixed organic solvent (Fig. 1), that within approximately two weeks SP-C undergoes a transformation from the soluble state to a gel-like state for which no NMR signals could be detected. We have now characterized this transition by NMR, circular dichroism (CD), and Fourier transform infrared (FTIR) spectroscopy.

Interest in the results of the present study goes beyond the SP-C system, because α -helix to β -sheet transitions (Mihara & Takahashi, 1997) have been observed in a wide variety of processes, including the generation of novel supramolecular structures in materials research (e.g., Aggeli et al., 1997), and transformation of naturally occurring proteins into disease-associated aggregates. The latter includes, for example, the polyglutamine-containing proteins responsible for Huntington's disease (Stott et al., 1995), the leucine-rich repeat peptide of the *Drosophila* Toll protein (Gay et al., 1991), and the amyloid fibres linked to Alzheimer's disease (Kelly, 1996) and transmissible spongiform encephalopathies (Aguzzi & Weissmann, 1997; Harrison et al., 1997). Finally, the possibility of intermediate formation of α -helices in the folding of β -sheet-containing proteins attracts interest in α to β transitions also with regard to rational protein design (Carey, 1996).

Fig. 1. All-heavy atom representation of the NMR solution structure of α SP-C (Johansson et al., 1994). The amino acid residues forming an α -helix in freshly prepared solutions in $\text{CDCl}_3/\text{CD}_3\text{OD}/0.1 \text{ M DCl}$ 32:64:5 are depicted in blue and red, where red color is used for the residues 9–27, which maintain the helical conformation in the transition state to unfolding (see text). Cys5 and Cys6, which are palmitoylated in native SP-C, are indicated in magenta. Some side-chain positions are identified with the three-letter code and the sequence numbers (the complete amino acid sequence of SP-C is given in Fig. 3). The two different views of the molecule were obtained by a 180°-rotation about a vertical axis. The figure was generated using the program MOLMOL (Koradi et al., 1996).

Results

To characterize the transformation of α SP-C into insoluble aggregates, we monitored the cross-peak attenuation in 2D $[^1\text{H}, ^1\text{H}]$ -TOCSY during the aggregation of SP-C dissolved in the perdeuterated mixed solvent $\text{CDCl}_3/\text{CD}_3\text{OD}/0.1 \text{ M DCl}$ 32:64:5 (v/v/v). If amide proton exchange occurs within the lifetime of the soluble α SP-C, additional signal attenuation is expected for cross-peaks involving labile protons. Hydrogen exchange experiments (Hvidt & Nielsen, 1966) have a long tradition for probing protein conformational equilibria (for recent reviews, see Englander et al., 1996; Chamberlain & Marqusee, 1997; Clarke et al., 1997). The backbone conformation as well as the average degree of deuteration of the backbone amide groups of SP-C in the insoluble aggregate were analysed by CD and FTIR spectroscopy. These measurements in the organic solvent were complemented by a long-term observation of α SP-C in dodecylphosphocholine (DPC) micelles by CD spectroscopy.

α SP-C in the mixed organic solvent monitored by NMR spectroscopy

A series of eleven 2D $[^1\text{H}, ^1\text{H}]$ -clean-TOCSY spectra (Griesinger et al., 1988) was recorded for a 1.1 mM solution of α SP-C in $\text{CDCl}_3/\text{CD}_3\text{OD}/0.1 \text{ M DCl}$, 32:64:5 (v/v/v) over a time span of two weeks. Cross-peak integration yielded the signal attenuation as a function of time. A first key observation was that a mono-exponential decay was obtained for cross-peaks arising from carbon-bound, nonexchangeable protons (Fig. 2A). The concentration loss of α SP-C thus monitored exhibits first-order kinetics with an average decay rate constant, as obtained from observation of 29 well-resolved signals, of $k_n = (5.3 \pm 0.6) \cdot 10^{-5} \text{ min}^{-1}$. The first-order kinetics gives a first indication that the insoluble aggregate does not form by simple precipitation of α SP-C, because this would be expected to result in more complex kinetics.

Both the concentration loss of α SP-C and the amide proton exchange reactions are thus first-order processes, so that the resulting decay for labile protons must also be mono-exponential, with a rate constant equal to the sum of the rate constants of the two processes (Fig. 2B). This allows deconvolution of the decay contributions arising from concentration loss and H/D exchange, respectively. Figure 3 surveys the decay rate constants for cross-peaks with backbone amide protons. Comparison with the average decay rate for nonlabile protons, k_n , reveals two distinct groups of amide protons. Those of the polypeptide segment 13–27 behave like nonexchangeable protons, i.e., H/D exchange does not occur within the lifetime of soluble α SP-C, so that within the experimental error $k_{\text{dec}} = k_n$. In contrast, for the amide protons of the segments 2–12 and 28–35, a significant decay acceleration was observed due to H/D exchange superimposed on the formation of the aggregate, so that $k_{\text{dec}} > k_n$.

For six N-terminal and three C-terminal residues, the H/D exchange was too fast to be registered with 2D NMR spectroscopy, indicating a lower limit of 0.01 min^{-1} for the corresponding rate constants (Johansson et al., 1994). To establish a reference value for amide proton exchange rates of random coil structures in the presently used mixed organic solvent, we studied three oligopeptides. The thus-obtained reference rate constants were between two and five times larger than the estimated lower limit. In particular, we obtained the following values of $k_{\text{HD}}^{\text{int}}$ for the underlined residues (in min^{-1}): 0.06 ± 0.01 and 0.09 ± 0.01 for Gly-Gly-Val-

Fig. 2. Signal decay observed in a series of 2D $[^1\text{H}, ^1\text{H}]$ -clean-TOCSY spectra recorded at $T = 10^\circ\text{C}$ for α SP-C dissolved in the mixed organic solvent described in Figure 1. The intensity of the cross peak volume, I , is plotted versus time. **A:** Cross-peak between the α -proton and one of the δ -methyl groups of Leu35 (Johansson et al., 1994). The decay rate constant, k_{dec} , is indicated. **B:** Cross-peak between the α -proton and the backbone amide proton of Leu32. The value for k_{dec} as well as the rate constant for the H/D exchange, k_{HD} , are indicated. k_{HD} was calculated as the difference between the k_{dec} value measured for this cross-peak and the average of the decay constants measured for well-resolved intense cross-peaks involving only nonexchangeable protons, i.e., $k_n = (5.3 \pm 0.6) \cdot 10^{-5} \text{ min}^{-1}$.

Ala, 0.02 ± 0.01 and 0.03 ± 0.01 for Gly-Leu-Leu-Gly, and 0.05 ± 0.01 for Gly-palmitoyl-Cys-Phe. Hence, we may approximate the intrinsic H/D exchange rate constant, k_{HD}^{int} , by the average value of 0.05 min^{-1} , which is very close to the exchange rate constant of 0.1 min^{-1} for backbone amide protons in water at pH 2.3 (Wüthrich, 1986).

The polypeptide segment 28–32 shows accelerated amide proton exchange when compared with the central segment 13–27, indicating substantial fraying of the penultimate helical turn. Moreover, the high protection of the backbone amide protons of Lys11 and Arg12 suggests that the α -helix comprises also Pro7 and Val8, so that Pro7 might act as helix initiator (Richardson, 1981). Although the (ϕ, ψ) angle ranges obtained from the DIANA structure calculation (see Fig. 7 in Johansson et al., 1994) previously led to the conclusion that the helix starts at Asn9, $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ NOEs were observed for both Pro7 and Val8 (see Fig. 1 in Johansson et al., 1994). The protection observed for the amide proton of Leu10 may well be due to a hydrogen-bonding interaction with the C=O group of the palmitoyl moiety attached to the side chain of the N-capping residue Cys6. Such side-chain–

backbone interactions are common local structural motifs stabilizing the N-termini of α -helices (Richardson & Richardson, 1988).

Analysis of the SP-C aggregate by CD and FTIR spectroscopy

A fraction of the insoluble aggregate of SP-C that was obtained from the NMR sample after the measurement was finished was dissolved in DPC micelles. The subsequently recorded CD spectrum lacks α -helical characteristics and, instead, corresponds to SP-C exhibiting an extended β -sheet conformation (Fig. 4), indicating that α SP-C unfolds and forms an aggregate composed of β -sheets (β SP-C) during gel formation. An FTIR spectrum of the aggregate confirms this conclusion. The shape and the frequency of the amide I band ($1,600$ – $1,700 \text{ cm}^{-1}$), which is mainly associated with the $\nu(\text{C}=\text{O})$ vibration of the peptide bond and is sensible to the polypeptide chain conformation (for recent reviews, see Surewicz et al., 1993; Goormaghtigh et al., 1994) was used to determine the content of secondary structure. Comparison of the FTIR spectra of α SP-C and the aggregate (Fig. 5) shows a shift of the maximum of the amide I band from $1,656 \text{ cm}^{-1}$ (Fig. 5, peak A) to $1,623 \text{ cm}^{-1}$ (Fig. 5, peak B), i.e., a transition from a helical to a β -sheet like structure upon aggregation. In agreement with the CD experiment, analysis of the amide I band indicates a β -sheet content of more than 80%.

The average extent of H/D exchange for both α SP-C and the aggregate can be estimated from the relative intensities of the amide II band, which represents N–H, and the amide II' band, which represents N–D (Fig. 5). The sensitivity of this band to the H/D exchange results from the fact that about 60% of its energy is associated with the $\delta(\text{N}–\text{H})$ vibration of the peptide bond. From the ratio of the areas of the amide bands II and II', it is estimated that more than 90% of the amide protons are replaced with deuterons in the aggregated end product. To investigate whether the H/D exchange occurred in solution or from the aggregate, we back-exchanged D to H by incubation of the deuterated aggregate in the protonated mixed organic solvent for 50 days. A FTIR spectrum then showed that about 40% of the deuterons had been exchanged back during this time period, which exceeds the duration of the exchange studies by NMR about fourfold. Hence, at least half of the amide protons exchange extremely slowly in the aggregate. To assess the fraction of rapidly exchanging amide protons in the aggregate, the sample on the FTIR plate was flushed with D_2O -saturated nitrogen, and spectra were recorded after different time intervals. After 210 min, the initial situation was reached, i.e., more than 90% of the amide protons were again deuterated. The most likely explanation is that rapid exchange in the aggregate is limited to parts of the polypeptide chains that are located near the surface of the aggregate. Provided that no significant rearrangements take place in the β -sheet aggregate once it is formed, the previously obtained final deuteration level of $>90\%$ thus implies that H/D exchange must have taken place prior to aggregation, while the unfolding SP-C was still in solution.

Long-term stability of SP-C in dodecylphosphocholine (DPC) micelles monitored by CD spectroscopy

Based on the length of the α -helix of α SP-C and data from infrared spectroscopy (Pastrana et al., 1991; Vandenbussche et al., 1992), it has been concluded that the α SP-C spans a phospholipid bilayer in vivo (Johansson et al., 1995b). It was, thus, desirable to assess

Fig. 3. Logarithmic plot of the decay rate constants, k_{dec} , vs. the amino acid sequence of SP-C obtained for 2D [^1H , ^1H]-clean-TOCSY cross-peaks involving the backbone amide protons. The rate constants (in min^{-1}), which reflect the signal attenuation arising from both the amide proton exchange and the formation of the β -sheet aggregate, are indicated by black horizontal lines, and the 99% confidence intervals (corresponding to 2.5σ) are represented by vertically hatched bars. Whenever more than one intense cross-peak with the amide proton was resolved, the average value is given. Due to spectral overlap the same averaged value is given for Val17 and Val18. For the residues where the exchange is too fast for the cross-peaks to be seen in the first spectrum (identified by arrows), the rate constants were estimated to be higher than 10^{-2} min^{-1} (Johansson et al., 1994). The average decay rate constant calculated from measurements of 29 well-resolved intense cross-peaks involving only carbon-bound, nonexchangeable protons, $k_n = (5.3 \pm 0.6) \cdot 10^{-5} \text{ min}^{-1}$, is indicated by the horizontal line at $\log k_{\text{dec}} = -4.27$, and the corresponding 99% confidence interval covers the area between the two dotted lines. Above the amino acid sequence of SP-C, the α -helix reported for the NMR structure of SP-C in solution (Johansson et al., 1994) is represented by a gray bar, where the segment 9–27, which retains a helical conformation with protection of the amide protons in the transition state to unfolding, SP-C[#], are boxed. The cross-hatched bar indicates an extension of the α -helix to Pro7 and Val8 when compared with the published NMR structure (see text). The palmitoyl moieties of Cys5 and Cys6 are also indicated.

stability differences of α SP-C when dissolved in the mixed organic solvent and when embedded in a closer mimic of the phospholipid bilayer, for which we used DPC micelles. CD spectra of α SP-C at about 30- μM concentration in DPC micelles were recorded after incubation for two weeks at 10 °C and seven weeks at 50 °C (Fig. 6). No significant conformational changes could be detected, suggesting that α SP-C in DPC micelles is thermodynamically stable. To check whether α SP-C exhibits long-term stability in DPC micelles also at the concentrations used here to monitor the unfolding in the mixed organic solvent by NMR, α SP-C was incubated at a concentration of 1.6 mM in DPC micelles for five weeks at 10 °C. Again, no detectable formation of non-helical peptide was observed, as judged by CD. These data strongly suggest that the α -helical conformation represents the global free enthalpy minimum when α SP-C is embedded in a phospholipid environment. This view is in full agreement with the work of Li and Deber (1994), who showed that Val, Leu, and Ile rank among the best helix promoters in a membrane environment. It also agrees with the fact that algorithms for the identification of transmembrane

regions (e.g., Hofmann & Stoffel, 1993) predict an α -helix region of residues 13–33 in SP-C.

Discussion

Kinetics of the global unfolding of α SP-C and its transition to β SP-C

This investigation shows that amide proton exchange in the polypeptide segment 13–27 does not occur measurably from α SP-C, and that in the aggregated β SP-C only partial exchange takes place. This indicates that the near-complete exchange observed during the transformation of the α - into the β -form must involve an unfolded form of SP-C with much lower protection factors than either α SP-C or β SP-C. This would be expected if neither the α -helix nor the β -sheet hydrogen bonds were highly populated, and we, therefore, refer to this kinetic intermediate as “nonhelical SP-C” or nhSP-C. The following kinetic scheme can then be derived:

Fig. 4. CD spectrum of the SP-C aggregate obtained after two weeks of incubation at $T = 10^\circ\text{C}$ in $\text{CDCl}_3/\text{CD}_3\text{OD}/0.1\text{ M DCl } 32:64.5$ (v/v/v). The aggregate was dissolved in 10 mM DPC/50 mM sodium phosphate buffer at pH 6.0 and $T = 10^\circ\text{C}$. The SP-C concentration was $10\text{ }\mu\text{M}$ as determined by amino acid analysis.

where k_{uf} , k_{uf}^{-1} , k_{ag} , $k_{\text{HD}\alpha}$, k_{HDnh} , and $k_{\text{HD}\beta}$ represent the rate constants for unfolding of $\alpha\text{SP-C}$, refolding of $\alpha\text{SP-C}$ from nhSP-C, aggregation of SP-C, and NH to ND exchange in the α -helical state, the nonhelical state, and the β -sheet-containing aggregate, respectively. $\alpha\text{SP-C(H)}$, nhSP-C(H), and $\beta\text{SP-C(H)}$ denote the different forms of SP-C with ^1H at the amide positions of residues 13–27 (Fig. 3), and the ^2H -containing SP-C is identified accordingly. In the following we denote the concentrations of the species in Scheme (1) by brackets.

In Scheme 1, the aforementioned amide proton exchange data show that $k_{\text{HD}\alpha}$ and $k_{\text{HD}\beta}$ are very small, and the dominant reaction pathway can be characterized by the following observations: (a) because no separate NMR signals were detected that could be attributed to nhSP-C, [nhSP-C] during the aggregation process must be small compared to the [$\alpha\text{SP-C}$] range monitored by NMR (Fig. 2), i.e., $[\text{nhSP-C}] \ll 0.1\text{ mM}$ throughout the aggregation process. (b) The NH to ND exchange is irreversible because a deuterated solvent was used. (c) The aggregation to $\beta\text{SP-C}$ is irreversible because $\alpha\text{SP-C}$ is transformed quantitatively into $\beta\text{SP-C}$. (d) $d[\alpha\text{SP-C(D)}]/dt = k_{uf}^{-1} \cdot [\text{nhSP-C(D)}] \approx 0$ (Fig. 3). (e) $d[\beta\text{SP-C(H)}]/dt = k_{ag} \cdot [\text{nhSP-C(H)}] \approx 0$. Hence, only the reaction steps indicated by black arrows in Scheme 1 were registered by the experiments used. Assuming that $k_{\text{HDnh}} \sim k_{\text{HD}}^{\text{int}}$ and with $k_{\text{HD}}^{\text{int}} \gg k_{uf}$, two limiting regimes are consistent with our experimental data. (1) $\alpha\text{SP-C}$ is metastable with respect to nhSP-C and the refolding process is much slower than the unfolding process, i.e., $k_{uf}^{-1} \ll k_{uf}$. Because no accumulation of nhSP-C was observed, the aggregation to $\beta\text{SP-C}$

Fig. 5. Attenuated total reflection FTIR spectra of $\alpha\text{SP-C}$ freshly dissolved in $\text{CHCl}_3/\text{CH}_3\text{OH}/0.1\text{ M HCl } 32:64:5$ (v/v/v) (broken line) and the SP-C aggregate obtained after two weeks of incubation at $T = 10^\circ\text{C}$ in this solvent (solid line). A and B indicate the amide I band in the two forms of SP-C, and the amide II and II' bands are also indicated.

must be at least as fast as the unfolding reaction of $\alpha\text{SP-C}$. (2) $\alpha\text{SP-C}$ is thermodynamically more stable than nhSP-C, i.e., $k_{uf}^{-1} > k_{uf}$, but rapid aggregation to $\beta\text{SP-C}$ prevents refolding of

Fig. 6. CD spectra of a freshly prepared solution of porcine SP-C (concentration $28\text{ }\mu\text{M}$ as determined by amino acid analysis) in 10 mM DPC/50 mM sodium phosphate buffer at pH 6.0 and $T = 10^\circ\text{C}$ (solid line), and of the same sample after incubation for two weeks at $T = 10^\circ\text{C}$ and seven weeks at $T = 50^\circ\text{C}$ (dotted line).

nhSP-C to α SP-C. Two lines of evidence speak in favor of regime 1. First, a synthetic peptide with the primary structure of depalmitoyl-SP-C does not spontaneously adopt the α -helical conformation in any solvent tried so far (Johansson et al., 1995a). Second, the secondary structure prediction algorithm PHDsec (Rost & Sander, 1994) yields a β -conformation for the segment 12–27 with nearly maximal score.

Because the unfolding of α SP-C represents the rate-determining step in both regimes, we arrive at the conclusion that $k_{uf} = k_n$, i.e., α SP-C and nhSP-C are not interconverted within the lifetime of α SP-C ($\tau_{1/2} = \ln 2/k_n = 13,000$ min). The corresponding free activation enthalpy for unfolding, $\Delta G_{uf}^\ddagger$, may then be estimated in the framework of Eyring's transition state theory (Eyring, 1935) according to $\Delta G_{uf}^\ddagger = -RT \ln(hk_{uf}/kT)$. With $k_{uf} = 8.9 \cdot 10^{-7} \text{ s}^{-1}$, $h = 6.63 \cdot 10^{-34} \text{ J s}$, $k = 1.38 \cdot 10^{-23} \text{ J K}^{-1}$ and $T = 283 \text{ K}$, one obtains $\Delta G_{uf}^\ddagger = 102 \text{ kJ mol}^{-1}$. Also, when assuming a pre-exponential factor of only 10^6 to account for diffusional processes during the unfolding process (Eaton et al., 1996), we obtain a value of 60 kJ mol^{-1} . This is apparently the largest kinetic barrier for the unwinding of a single helix in solution that has so far been observed. Although kinetic schemes with partial unfolding as the rate determining step cannot be excluded, it is tempting to speculate that the α SP-C $\rightarrow$ nhSP-C transition occurs via a helical transition state (SP-C ‡) in which the network of helical ($i, i + 4$) hydrogen bonds in the segment 9–27 remains largely intact (Fig. 1). This view is consistent with the similarity of the exchange rate constants observed throughout this segment (Fig. 3), indicating that the transition is highly cooperative, and it is also compatible with a recent molecular dynamics simulation (Kovacs et al., 1995), which indicates that the hydrogen bonds are protected by the tightly interlocked isopropyl moieties of the valines.

Thermodynamics of local processes:

Fraying of the helical termini

The fraying of the helical termini is evidenced by increased amide proton exchange rates (Fig. 3). With $k_{HD}^{\text{int}} = 0.05 \text{ min}^{-1}$, the exchange reaction occurs in the almost universally observed EX2 limit (Hvidt & Nielsen, 1966; Roder et al., 1985). The equilibrium constant describing the local equilibrium is then given as $K_{\text{loc}} = k_{HD}/k_{HD}^{\text{int}}$, and the corresponding apparent free enthalpy, ΔG_{loc} , can be determined from $\Delta G_{\text{loc}} = -RT \ln(k_{HD}/k_{HD}^{\text{int}})$. This yields ΔG_{loc} values of 19 and 17 kJ mol^{-1} for the residues 11 and 12, respectively, and values between 12 and 17 kJ mol^{-1} for the residues 28–32 (Table 1). The free enthalpy for the opening of the putative N-cap of the helix, as derived from the exchange rate constant of Leu10, is about 14 kJ mol^{-1} . Because k_{HD} is derived from the difference $k_{\text{dec}} - k_n$, and the two quantities exhibit an error of about $0.5 \cdot 10^{-5} \text{ min}^{-1}$ each (Fig. 3), the minimal exchange rate constant that we may detect in our experiment is about $1 \cdot 10^{-5} \text{ min}^{-1}$ (corresponding to about 20 kJ mol^{-1}). The detection of possible pre-equilibria, which would have $\Delta G_{\text{loc}} > 20 \text{ kJ mol}^{-1}$, is thus prevented by the limited lifetime of α SP-C.

Free energy diagram of the α SP-C to β SP-C transition

Figure 7 affords a sketch of a free energy diagram for the unfolding of α SP-C and transformation into β SP-C. The ensemble of initially present α SP-C molecules covers the conformational space around a narrow local minimum in which the ruggedness of the surface visualizes local unfolding and refolding processes at the helix ends.

Table 1. Backbone amide H/D exchange rate constants, k_{HD} , and apparent free enthalpies for local unfolding processes in α SP-C, ΔG_{loc}^a

Residue	k_{HD} (10^{-5} min^{-1}) ^b	ΔG_{loc} (kJ mol^{-1}) ^c
Leu10	15 ± 3	13.7 ± 1.0
Lys11	2.7 ± 0.8	18.6 ± 1.1
Arg12	3.3 ± 0.9	17.2 ± 1.1
Val28	3.3 ± 0.7	17.2 ± 1.1
Gly29	5.2 ± 0.7	16.2 ± 0.9
Ala30	32 ± 1	11.9 ± 0.9
Leu31	11 ± 0.6	14.4 ± 1.0
Leu32	26 ± 2	12.4 ± 1.0

^aErrors are calculated according to the Gaussian law of error propagation.

^bThe values of k_{HD} are calculated from $k_{HD} = k_{\text{dec}} - k_n$ (see Fig. 3).

^cCalculated with $k_{HD}^{\text{int}} = 0.05 \pm 0.02 \text{ min}^{-1}$.

SP-C ‡ represents an ensemble of transition states appearing around a local maximum, and nhSP-C represents the ensemble of unfolded SP-C molecules in solution that are presumably distributed over a large volume of conformational space. Amide proton exchange takes place when nhSP-C meanders through the latter subspace. With respect to the conclusions drawn above, the energy of nhSP-C is set below the one of α SP-C, implying that the latter species is metastable relative to the unfolded form. Finally, β SP-C is represented by a global minimum with a width that allows for possible conformational polymorphism of SP-C in the aggregated state.

Conclusions

Native α SP-C in vivo is generated from a 21-kDa precursor, proSP-C₂₁ (Glasser et al., 1988), through a sequence of proteolytic events and post-translational modifications that take place in different subcellular compartments (Beers et al., 1994; Beers & Lomax, 1995). The following pathway has been suggested (see Fig. 7 in Beers et al., 1994). ProSP-C₂₁ is synthesized in the endoplasmic

Fig. 7. Hypothetical free energy diagram displaying the variation of the free enthalpy, G , along the "reaction coordinate" (RC) during α SP-C unfolding and its aggregation to β SP-C. α SP-C denotes α -helical SP-C in solution, SP-C ‡ indicates the transition state of unfolding in which the α -helical ($i, i + 4$) hydrogen bonds of the polypeptide segment 9–27 are potentially preserved (see text and Fig. 1), nhSP-C represents nonhelical SP-C in solution, and β SP-C indicates aggregated SP-C containing predominantly β -sheet secondary structure.

reticulum and then transferred to the Golgi complex, where the polypeptide is palmitoylated. After translocation into the lamellar body, palmitoylated proSP-C₂₁ is membrane inserted so that the polypeptide segment corresponding to α SP-C spans the phospholipid bilayer. Cleavage of the C-terminal and N-terminal flanking regions of residues 1–23 and 59–197 occurs only after the valyl-rich α -helix has been embedded in the bilayer.

The results presented in this paper enable the following comments on this hypothetical mechanism. Considering the limited stability of α SP-C in solution (Fig. 7), the free enthalpy of folding of the fivefold larger precursor protein may be the driving force for formation of the valyl-rich α -helix. The kinetic stability of α SP-C might play an important role in maintaining the helical conformation when the molecule is transiently not embedded in a bilayer, for example, during the formation of the surface-active DPPC monolayer at the air/liquid interface. This points at the relevance of kinetic considerations for the conformation adopted by native proteins (Baker & Agard, 1994). Because α SP-C is thermodynamically stable in a micellar environment, protection by the precursor protein is probably only needed until α SP-C is embedded in a phospholipid bilayer, i.e., in vivo proteolytic cleavage takes place once the polypeptide segment corresponding to mature α SP-C is brought into a thermodynamically stable state.

The structural transition of α SP-C into β -sheet aggregates resembles the transitions observed for amyloidogenic proteins (Kelly, 1996). Perhaps thermodynamic metastability, caused either by point mutations or by transfer of a protein from surroundings in which it is thermodynamically stable into an environment where it is metastable, may be a factor in the generation of pathogenic amyloid deposits. In this context it is intriguing that the Alzheimer β -peptide found in the plaques formed during the progress of Alzheimer's disease (Glennner & Wong, 1984) is derived from the transmembrane region of a precursor protein (Kang et al., 1987).

Finally, the implicated metastability of α SP-C in solution might explain the large variations in helicity (between 50–90%) so far reported for SP-C (Pastrana et al., 1991; Baatz et al., 1992; Vandenbussche et al., 1992; Pérez-Gil et al., 1993; Shiffer et al., 1993; Johansson et al., 1995a, 1995b), because the helix content measured for any particular sample may be strongly affected by the solvent mixtures used and the duration of storage.

Materials and methods

NMR spectroscopy

Protonated SP-C purified from porcine lungs (2.2 mg) (Curstedt et al., 1987) was dissolved in 500 μ L of CDCl₃/CD₃OD/0.1 M DCl, 32:64:5 (v/v/v), yielding an SP-C concentration of about 1.1 mM. A series of eleven 2D [¹H, ¹H]-clean-TOCSY experiments (Griesinger et al., 1988; mixing time of 80 ms) was started 0.1, 24.1, 47.7, 71.2, 94.7, 118, 141.2, 216, 239, 263, and 286.3 h after sample preparation. The spectra were recorded at $T = 10^\circ\text{C}$ on a Bruker AMX600 spectrometer operating at a ¹H resonance frequency of 600 MHz. The 512(t_1) $\times$ 1,024(t_2) complex points were accumulated, with $t_{1,\text{max}} = 85$ ms, $t_{2,\text{max}} = 127$ ms, and a measurement time of about 24 h per spectrum. Prior to Fourier transformation the data matrix was multiplied with a cosine window in t_1 , and a sine window shifted by 70° in t_2 (DeMarco & Wüthrich, 1976). The digital resolution after zero filling was 5.9 Hz along ω_1 and 3.9 Hz along ω_2 . The spectra were processed and integrated using the programs PROSA (Güntert et al., 1992) and XEASY

(Bartels et al., 1995), respectively. A least-squares fit of a single exponential function to the peak volumes yielded the decay rate constants. These were obtained for 49 resolved cross-peaks involving backbone amide protons and 29 cross-peaks that correlate pairs of carbon-bound, nonlabile protons. The ¹H resonance assignment of SP-C was taken from Johansson et al. (1994).

To determine the intrinsic H/D exchange rate constants of polypeptide backbone amide protons in CDCl₃/CD₃OD/0.1 M DCl, 32:64:5 (v/v/v), at $T = 10^\circ\text{C}$, 500 μ L of 5 to 10 mM solutions of the oligopeptides H-Gly-Gly-Val-Ala-OH, H-Gly-Leu-Leu-Gly-OH, and H-Gly-palmitoyl-Cys-Phe-OH were prepared in CDCl₃/CD₃OH/0.1 M HCl 32:64:5 (v/v/v). 2D 2QF-[¹H, ¹H]-COSY (Rance et al., 1983) spectra, and 2D [¹H, ¹H]-ROESY (Bothner-By et al., 1984) spectra with 200 ms mixing time were recorded to obtain sequence-specific assignments of backbone amide resonances for H-Gly-Leu-Leu-Gly-OH and H-Gly-palmitoyl-Cys-Phe-OH. Subsequently, the samples were diluted by adding 250 μ L of fully deuterated solvent. Immediately after dilution, a series of 1D ¹H NMR spectra was recorded on a Bruker DRX500 spectrometer in about 1.5 h. The exchange rate constants were then obtained by a least-squares fit of a single exponential function to the decay of the amide proton peak intensities.

CD spectroscopy

Approximately 50 μ g of native SP-C, purified from porcine lungs, was dissolved in 150 μ L of 20 mM DPC/50 mM sodium phosphate buffer at pH 6.0, and then diluted with buffer to 10 mM DPC. DPC was synthesized from dodecanol and choline acetate (Johansson et al., 1995a). After removal of insoluble material by centrifugation, CD spectra were recorded at $T = 10^\circ\text{C}$ using a Jasco J-720 spectropolarimeter at three different times, i.e., immediately after sample preparation, after two weeks of incubation at $T = 10^\circ\text{C}$ and after seven weeks of incubation at $T = 50^\circ\text{C}$. The scan speed was 10 nm/min (184–260 nm), the response time 2 s, and the resolution 2 data points/nm. The temperature of the sample was adjusted by a thermostat-controlled water bath.

A CD spectrum with identical experimental settings was recorded for the SP-C aggregate formed during the NMR measurements. The supernatant was discarded, the aggregate was dried under a stream of nitrogen, dissolved in 20 mM DPC/sodium phosphate buffer, and diluted with buffer to 10 mM DPC. Peptide concentrations for calculating the mean residue ellipticity were determined by quantitative amino acid analysis.

FTIR spectroscopy

The insoluble aggregate obtained after the NMR measurements was taken from the CDCl₃/CD₃OD/0.1 M DCl solution (see CD spectroscopy) and analyzed by FTIR spectroscopy (Goormaghtigh et al., 1994). Spectra were recorded at room temperature on a Perkin-Elmer 1720X FTIR spectrometer with a nominal resolution of 2 cm⁻¹. A total of 128 scans was averaged for each measurement. Films of protein were spread on a germanium crystal by slowly evaporating the sample under a stream of nitrogen. To assess possible H/D exchange occurring when the aggregate is exposed to the solvent, the deuterated solvents were subsequently removed and the product was incubated in CHCl₃/CH₃OH/0.1 M HCl, 32:64:5 (v/v/v), at $T = 10^\circ\text{C}$. Then, FTIR spectra were recorded at different times. Contents of secondary structure elements and the degree of hydrogen to deuterium exchange were calculated as described by Vandenbussche et al. (1992).

Supplementary material in electronic appendix

The decay rate constants, k_{dec} , for the amide protons of residues Leu10–Leu32 determined from the 2D [^1H , ^1H]-TOCSY spectra are available as supplementary material.

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