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ORIGINAL PAPER

Self-aggregation of a recombinant form of the propeptide NH_2-terminal of the precursor of pulmonary surfactant protein SP-B: a conformational study

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Abstract A recombinant form of the peptide N-terminally positioned from proSP-B ($SP\text{-}B_N$) has been produced in *Escherichia coli* as fusion with the Maltose Binding Protein, separated from it by Factor Xa cleavage and purified thereafter. This protein module is thought to control assembly of mature SP-B, a protein essential for respiration, in pulmonary surfactant as it progress through the progressively acidified secretory pathway of pneumocytes. Self-aggregation studies of the recombinant propeptide have been carried out as the pH of the medium evolved from neutral to moderately acid, again to neutral and finally basic. The profile of aggregation *versus* subsequent changes in pH showed differences depending on the ionic strength of the medium, low or moderate, and the presence of additives such as L-arginine (a known aggregation suppressor) and Ficoll 70 (a macromolecular crowder). Circular dichroism studies of $SP\text{-}B_N$ samples along the aggregation process showed a decrease in α-helical content and a concomitant increase in *β*-sheet. Intrinsic fluorescence emission of $SP\text{-}B_N$ was dominated by the emission of Trp residues in neutral medium, being its emission maximum shifted to red at low pH, suggesting that the protein undergoes a pH-dependent conformational change that increases the exposure of their Trp to the environment. A marked increase in the fluorescence emission of the extrinsic probe bis-ANS indicated the exposure of hydrophobic regions of $SP\text{-}B_N$ at pH 5. The fluorescence of bis-ANS decreased slightly at low ionic strength, but to a great extent at moderate ionic strength when the pH was reversed to neutrality, suggesting that self-aggregation properties of the $SP\text{-}B_N$ module could be tightly modulated by the conditions of pH and the ionic environment encountered by pulmonary surfactant during assembly and secretion.

Keywords Self-aggregation · Pulmonary surfactant · Surfactant protein SP-B · N-terminal propeptide

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Introduction

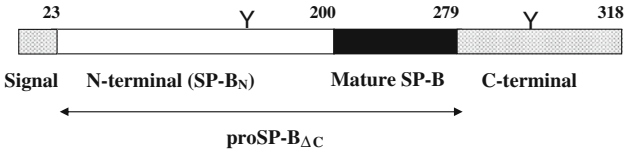
Protein aggregation is known to be a major medical, biotechnological and industrial problem nowadays. First, protein self-association in amyloid fibrils that accumulate in different tissues is the cause or is associated with several human disorders such as Alzheimer and Parkinson diseases and cataracts [44]. Second, the formation of inclusion bodies by proteins over-expressed in bacterial systems is the main bottleneck to optimize the production of recombinant proteins [43]. The manufacturing and exposure of proteins to physical and chemical stressing environments may enhance their ability to aggregate. If the aggregates are not removed, they will become part of the finished product and may cause adverse effects when administered to a patient [4, 24, 39]. Once the pharmaceutical is administered, the disassociation kinetics of the aggregates, if any, may be slowed and/or the size of aggregates may increase due to crowding effects (high macromolecular concentration in the patient fluids) and/or to a charge effect (due to changes in pH and ionic strength) causing immunogenicity [10, 27].

To develop strategies for preventing protein aggregation, the mechanism(s) and pathway(s) by which proteins aggregate must be first characterized. Protein aggregation may arise from association of partially folded proteins resulting

in non-regular amorphous structures caused mainly by intermolecular hydrophobic interactions [36]. This type of association exhibits, without exception, an increase in β-sheet content relative to the native conformation [43]. In contrast, amyloid fibrils display an ordered quaternary structure [36] with a high content of β-sheets forming a cross-β architecture core [43]. Aggregation-prone regions are short specific amino acid stretches known as "aggregation hot spots" whose side chains are usually hidden in the inner of the hydrophobic core or involved in the network of contacts that stabilizes the protein [43]. Moreover, using aggregation-prone proteins tagged with fluorophores, it has been proved that, in cells, protein aggregation is highly specific [26].

The characterization of the aggregation process requires studying not only the secondary structure of the protein, but also factors related to protein structural and physicochemical properties (thermodynamic stability, hydrophobicity and net charge) as well as the effect of environmental factors (pH, temperature, ionic strength, macromolecular crowding, etc.).

We are studying the lung surfactant proteins. Among these, the hydrophobic surfactant protein B (SP-B) is necessary together with lipids and other proteins to maintain the structural integrity of the alveolus during respiration [34] and is produced by the proteolytic processing of a precursor, proSP-B, which also liberates two polypeptides flanking the NH_2- and COOH-termini of the mature protein, respectively [46]. The 177 amino acid polypeptide flanking the NH_2-terminus of the mature peptide (SP-B_N, Scheme 1) is necessary and sufficient for targeting, processing and assembling of SP-B into surfactant complexes in vivo [19]. Deletion of SP-B_N results in accumulation of SP-B within the endoplasmic reticulum, suggesting that SP-B_N acts as an intra-molecular chaperone for the hydrophobic mature peptide [3, 20]. It seems that progressive acidification along the secretory pathway in pneumocytes triggers exposure of the SP-B module from its precursor, promoting its proteolytic processing and assembly in pulmonary surfactant membranes. Maturation of SP-B is likely therefore controlled by the properties of the SP-B_N module under the environmental conditions found by proSP-B along the secretory pathway of surfactant in the pneumocytes. Some evidences point that parts of SP-B_N may maintain association with surfactant membranes once cleaved from the whole precursor [3], opening the possibility of SP-B_N to play additional independent roles once secreted into the alveolar spaces. To get deeper into the study of the potential physiologically relevant properties of SP-B_N, as well as other possible biological activities, we have cloned the SP-B_N sequence of human proSP-B in pMAL-c2x and expressed it in *Escherichia coli* as a fusion with the Maltose Binding Protein. After cleavage of the fusion by protease FXa, non glycosilated SP-B_N was purified and characterized by mass spectrometry, tryptic digestion and Edman sequencing [23]. Our aim in the present study is to investigate the self-aggregation properties of recombinant SP-B_N, as a potentially relevant event in modulating processing and assembly of proSP-B derived polypeptides. Particularly, we have studied the influence on SP-B_N self-aggregation of pH and ionic strength, the effect of L-arginine as an aggregation suppressor and the effect of Ficoll 70 as an agent mimicking in vitro the macromolecular crowding conditions thought to exist in vivo. Changes in secondary structure and protein conformation associated with the aggregation process have been analysed by circular dichroism and intrinsic and extrinsic fluorescence spectroscopies.



Scheme 1 Protein structure of human preproSP-B. *Numbers* indicate the amino acid position at the end of the corresponding fragment. Potential glycosylation sites (Y) are Asn^{129} and Asn^{311}

Materials and methods

Production and purification of the propeptide NH_2-terminal of proSP-B

Cloning of the SP-B_N sequence of human preproSP-B cDNA in pMAL-c2x, expression of the fusion protein with the Maltose Binding Protein (MBP) in *E. coli* strain UT5600 and SP-B_N purification, once released from MBP by protease FXa, by anion exchange chromatography in an ÄKTA BASIC 10 system (GE-Amersham Biosciences) or stepwise elution, was described in detail in a previous paper [23]. Fractions containing SP-B_N (in 20 mM Tris–HCl buffer pH 7 to 400 mM NaCl) were dialyzed O/N towards the buffer needed in subsequent experiments.

SP-B_N self-aggregation assays

Self-aggregation of SP-B_N was studied by measuring the change in apparent absorbance at 360 nm due to light scattering in a Beckman DU 800 spectrophotometer at 1-min intervals. Both sample and reference cuvettes were filled with 800 µL of 5 mM Acetate, 5 mM MES, 5 mM Tris–HCl buffer (AMT buffer) pH 7, a buffer which maintains a constant ionic strength over a wide pH range. After some min equilibration, 200 µL of the same buffer or 200 µL of buffer containing 41 µg of SP-B_N was added to the reference

or the sample cuvettes, respectively. The effect of ionic strength on self-aggregation was checked by adding an aliquot of 4.5 M NaCl to both cuvettes to make solutions 150 mM NaCl. Later, another aliquot made both solutions 500 mM NaCl. The effect of pH on SP-B_N self-aggregation was carried out by applying the same protocol as above except that AMT buffer pH 7 was either 150 or 500 mM NaCl and that aliquots of 0.8 N HCl or 0.5 M NaOH were added to both cuvettes to get the desired pH (aliquots were previously determined in a Mettler-Toledo MP230 pH-meter provided with a microelectrode). As additives, L-arginine (Fluka) and Ficoll 70 (Mr ~70 kDa, Fluka) were employed.

Circular dichroism

Circular dichroism spectra of SP-B_N were recorded at 25 °C on a Jasco J-715 spectropolarimeter using thermostated quartz cells of 0.1-cm path length, at a scanning speed of 50 nm min^{-1} (1 s response time) for the far-UV (240–200 nm) spectral range, each spectrum being the accumulation of 5 scans. The spectra were obtained in 200 µL AMT buffer, 150 or 500 mM NaCl pH 7, at 0.115 mg mL^{-1} protein concentration. In some experiments, CD spectra were also obtained after the pH of the sample was shifted by addition of proper aliquots of HCl or NaOH. Mean molar residue ellipticities [θ] were calculated from the measured ellipticity taking into account the protein concentration (corrected for dilution), the molecular weight of SP-B_N (19902 Da, DNA Star program) and the number of amino acids per molecule (177). Estimations of the secondary structure content from the CD spectra were performed by using the CDPro program and the α-helix and β-sheet contents were calculated using three different methods, CONTIN/LL, SELCON3 and CDSSTR [40] employing their mean value in plots.

Fluorescence spectroscopy

The intrinsic fluorescence emission spectra of SP-B_N were recorded at 25 °C in a SLM-Aminco AB2 spectrofluorimeter using a 1-cm quartz cell with excitation (275 or 290 nm) and emission slits set at 4 nm and a scan speed of 2 nm s^{-1}. The sample contained 41 µg of protein in 1 mL of AMT buffer, 150 mM NaCl pH 7. The spectrum obtained upon excitation at 290 nm was normalized multiplying it by a factor to determine the contribution of tryptophan residues to the total fluorescence spectrum of the protein. This factor is the ratio between the fluorescence intensities using $\lambda_{exc} = 275$ and $\lambda_{exc} = 290$ nm at wavelengths higher than 380 nm where no tyrosine contributes [6]. The tyrosine contribution to the total emission spectrum was calculated by subtracting from the emission spectrum obtained with $\lambda_{exc} = 275$ nm the normalized emission spectrum due to tryptophans.

Extrinsic fluorescence of bis-ANS probe (4,4′-bis-1-phenylamine-8-naphftalene sulfonate, obtained from Molecular Probes) was determined by adding 7 µL of a 0.2 mM probe stock solution in methanol to 600 µL of AMT buffer 150 mM NaCl containing 29 µg protein at pH 7 or 5. Samples were incubated at 25 °C for 5 min, and afterwards, the emission spectrum of bis-ANS was obtained with excitation at 395 nm, the spectra being recorded between 400 and 600 nm. Proper blanks were also recorded from samples without protein. Other conditions were as described above.

Analytics

The purity of SP-B_N samples was routinely checked by electrophoresis and amino acid analysis. Amino acid analysis of SP-B_N (1–2 nmol) was determined on a Beckman 6300 automatic amino acid analyser after hydrolysis with 6 N HCl at 105 °C for 24 h in sealed tubes under vacuum. The protein concentration was calculated from their absorbance at 280 nm using 20,790 M^{-1} cm^{-1} as experimental molar absorption coefficient at 280 nm which is quite similar to the theoretical one at the same wavelength (20,830 M^{-1} cm^{-1}, DNA Star program).

Results

Effect of ionic strength, pH and additives on SP-B_N self-aggregation

To check the influence of the ionic strength of the medium on SP-B_N self-aggregation, the absorbance at 360 nm of the solution (AMT buffer pH 7) was recorded and it did not change when physiological (150 mM NaCl) or moderate ionic strength (500 mM NaCl) was reached after NaCl addition from a stock solution (not shown).

The effect of pH on SP-B_N aggregation, in the presence of either 150 or 500 mM NaCl or in the presence of additives such as arginine or Ficoll, has been studied and the results are depicted in Fig. 1. The decrease of pH from neutral to mildly acidic (pH 5) caused a substantial increase in turbidity of SP-B_N solution at 150 mM NaCl. Attempts to reverse self-aggregation by neutralizing the medium not only failed but caused a further increase in turbidity and aggregation that was only partially reverted after ulterior alkalization (Fig. 1a). When the solution contained 500 mM NaCl the increase in turbidity by acidification to pH 5 was more limited than observed with low salt and partial reversion was obtained at neutral pH whereas subsequent alkalization caused no further changes (Fig. 1b).

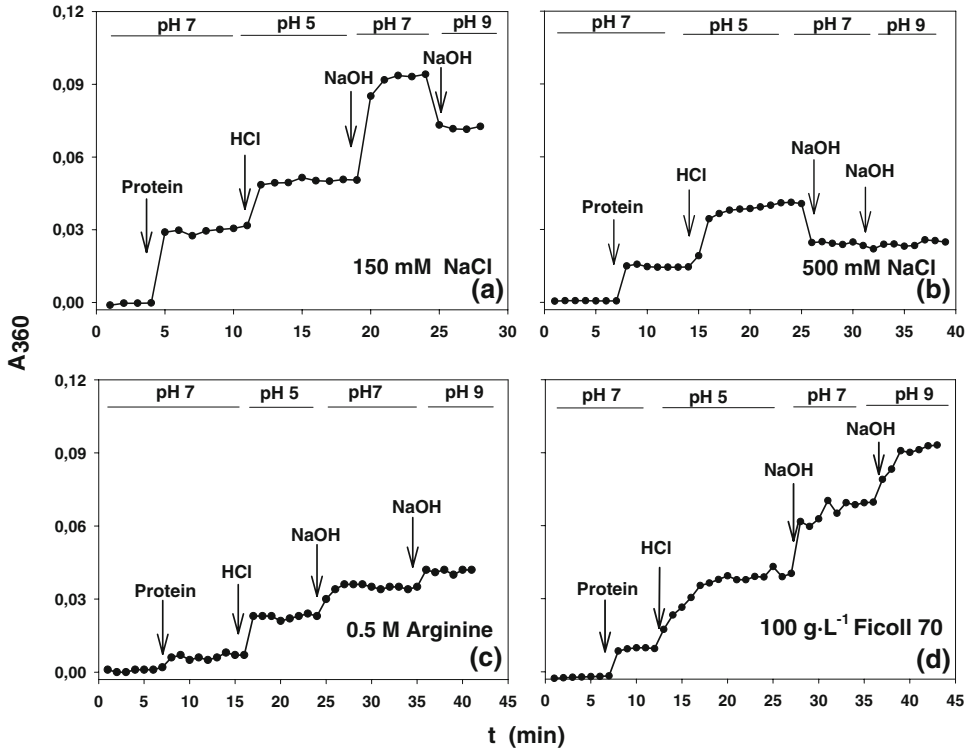


Fig. 1 pH induced SP-B_N self-aggregation. Aggregation was carried out in 800 µL AMT buffer pH 7 containing either 150 mM (**a**) or 500 mM (**b**) NaCl. Protein (41 µg) in 200 µL of the same buffer or just buffer was added to sample and reference cuvettes, respectively. The addition of an aliquot of 0.8 N HCl to cuvettes lowered pH to 5, addition of 0.5 M NaOH restored it to 7 and additional NaOH increased it to 9. In **c**, both cuvettes contained L-arginine from a 2 M stock solution in AMT buffer, 150 mM NaCl pH 7. Protein (41 µg) or buffer was added and pH changes were carried out as in **a**. Final arginine concentration was 0.5 M. In **d**, both cuvettes contained AMT buffer, 150 mM NaCl pH 7 plus 125 g L^{-1} of Ficoll 70. Addition of protein and pH changes as in **a**. The experiments were repeated thrice and the same results were obtained so that the profiles depicted are representative of them

In addition to the effect of pH on the protein, we checked the effect of two types of additives, one known to prevent and another to promote aggregation. Among well-known protective additives such as sugars, polyols, polymers and amino acids, L-arginine has been widely used since it prevents protein aggregation by reducing protein–protein interactions [41]. Figure 1c illustrates the effect that 0.5 M arginine has on the pH-induced SP-B_N aggregation in the presence of 150 mM NaCl. The increase in turbidity upon protein addition was almost negligible and a small increase in it followed pH acidification, neutralization and alkalization, respectively, so that no reversion of aggregation was observed.

On the other hand the combined effect of modifying pH when SP-B_N is in a crowded medium is illustrated in Fig. 1d, where pH-induced protein aggregation has been analyzed in the presence of 100 g L^{-1} of Ficoll 70, a concentration thought to mimic the range of total macromolecular concentration inside the cell [42]. The profile of turbidity *versus* time is quite similar to that shown in the presence of arginine in Fig. 1c but aggregation is both far greater and slower than in the presence of arginine.

Circular dichroism studies

The effect that changes in pH (from neutral to acid, again neutral and alkaline pH thereafter) have on the secondary structure of SP-B_N in AMT buffer containing 150 mM NaCl, was studied by far-UV circular dichroism (CD). Figure 2a shows that, at pH 7, the CD spectrum of the protein displays two minima in molar ellipticity at 208 and 220 nm, a typical behaviour of proteins with a main α-helical conformation. Upon acidification, the negative ellipticity was reduced to an extent that ulterior changes in pH could not restore completely. CD data were processed using the SELCON3, CONTIN/LL and CDSSTR programs and we found that the calculated percent proportions of turn (21.4 ± 0.4) and random (28.5 ± 0.5) in the secondary structure of the protein at initial pH 7 did not practically change upon pH variations ($21.4 \pm 0.2\%$ and $29.3 \pm 0.9\%$ were their mean values along the whole process). In contrast Fig. 2b shows how the mean value calculated for α-helix and β-sheet content in SP-B_N changes as a function of pH. The experiment was repeated in the presence of 500 mM NaCl and results are also depicted in Fig. 2b. The

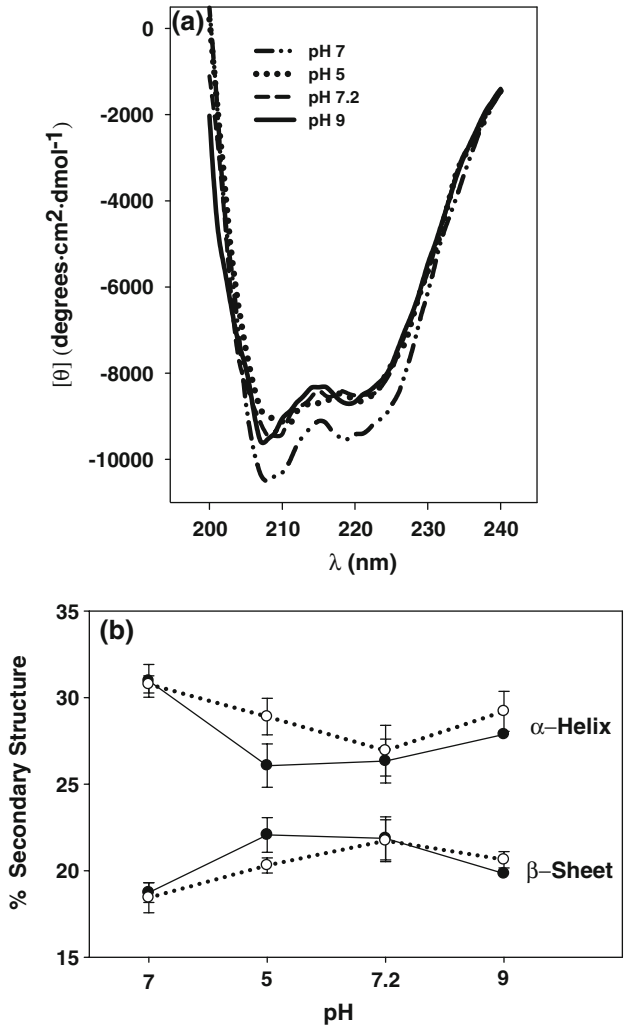


Fig. 2 Circular dichroism spectra of SP-B_N. Spectra of 0.115 mg mL^{-1} protein were recorded in AMT buffer 150 mM NaCl pH 7 before and after subsequent pH shifts to 5, 7.2 and 9 by successive additions of 0.8 N HCl or 0.5 M NaOH aliquots (**a**). Variation of the α-helix and *β*-sheet percentual content of SP-B_N, estimated from the dichroism spectra, as a function of pH at 150 mM NaCl (*solid line*) and 500 mM NaCl (*dotted line*) (**b**)

initial (pH 7) α-helical content (30.9 ± 0.9) of SP-B_N decreased and that of *β*-sheet (18.7 ± 0.5) increased to a different extent upon pH acidification, both in the presence of physiological and moderate ionic strength. After neutralization, no further changes occurred in 150 mM NaCl and the tendency to increase *β*-sheet at the expense of decreasing α-helix was maintained with 500 mM NaCl. The ulterior alkalization of the solution caused a small recovery of the initial secondary structure parameters values disregarding the salt concentration. The changes in α-helix and *β*-sheet content upon acidification parallel well the turbidity data depicted in Fig. 1a, b at low and medium salt but did not correlate with aggregation data at the regained neutral and ulterior alkaline pH.

Fluorescence studies

The fluorescence emission of a SP-B_N sample at pH 7 was recorded in AMT buffer containing 150 mM NaCl. The total fluorescence emission spectrum of the protein (Fig. 3a) is dominated by the contribution of Trp residues, showing a maximum at 335 nm when excited at 275 nm, which is conserved in the normalized spectrum upon excitation at 290 nm. The blue-shifting of the maximum with respect to the emission of Trp in aqueous solution (350 nm) is typical of a protein with buried tryptophanyl residues in a hydrophobic microenvironment indicating that SP-B_N is folded. A small contribution of Tyr residue emission with a maximum around 303 nm is also present. This contribution, lower than expected for a Trp:Tyr ratio of 3:2, is also typical of a properly folded protein and is probably due to either the existence of resonance energy transfer (FRET) of the Tyr fluorescence to nearby Trp or the quenching of Tyr fluorescence by other close side chains. Next, the pH of the sample was acidified to 5, allowed to stabilize for a few min and neutralized thereafter being then the emission spectrum recorded and depicted in Fig. 3b. The spectrum at the regained neutral pH shows a shifting of the maximum to the red (340 nm). Also two shoulders show up at 324 and 355 nm, respectively, either upon excitation at 275 or in the normalized spectrum of Trp emission. This seems to indicate that as a consequence of pH changes Trp residues became more exposed to the solvent and to a different extent as if corresponding to different Trp populations. Also the Tyr emission spectrum shows a displacement of the shoulder to reach 308–309 nm which is typical of Tyr entering a less polar environment since the maximum wavelength of Tyr in proteins is shifted from 303 to 309 in dimethylsulfoxide (dielectric constant 36) and is displaced 4 nm in ethanol (dielectric constant 24.6) [17]. Moreover, the relative contribution of Tyr with regard to Trp to the overall fluorescence emission spectrum has increased as is noted around 300 nm, where Trp fluorescence intensity, which equalizes the Tyr one in Fig. 3a, has fallen notably as shown in Fig. 3b. All these features suggest that as a consequence of the pH changes, the structure of SP-B_N has been partially unfolded, producing a partial exposure of tryptophans to the aqueous environment.

To evaluate the possibility that acidic pH could induce exposure of hydrophobic patches of SP-B_N to the medium, we have analyzed the effect of pH on the emission fluorescence of the extrinsic probe bisANS, in the presence of the protein. This probe is typically used to map accessible hydrophobic pockets in proteins due to its ability to bind to hydrophobic residues surrounded by positive charges [11] of partially folded but not of unfolded proteins [18] which is associated with a dramatic change of its fluorescence emission properties. Since bis-ANS has apolar rings and

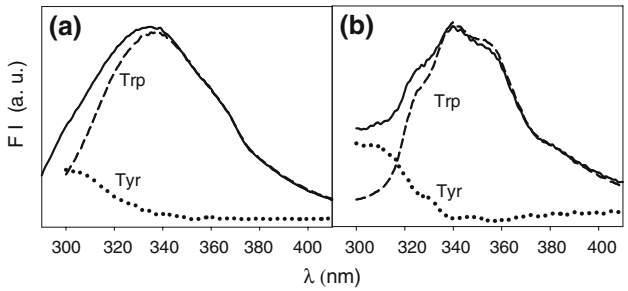


Fig. 3 Intrinsic fluorescence emission spectra of SP-B_N. The emission spectrum was obtained from 41 μg protein in AMT buffer 150 mM NaCl pH 7 upon excitation at 275 nm (*solid line*), and the normalized contribution of Trp (*dashed line*) and Tyr (*dotted line*) residues was calculated as described in "Materials and methods" (**a**). The pH was lowered to 5 upon addition 0.8 N HCl and after few min equilibration was increased to 7 by adding 0.5 M NaOH. Then the spectrum was recorded and normalized as in **a** (**b**)

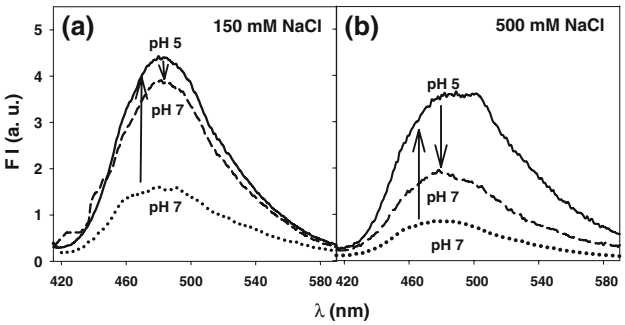


Fig. 4 Fluorescence emission of the extrinsic probe bis-ANS in the presence of SP-B_N. Emission spectra of bis-ANS in AMT buffer pH 7 either in 150 mM NaCl (**a**) or 500 mM NaCl (**b**), containing 29 μg of protein upon excitation at 395 nm. The spectra were again recorded at pH 5 after the addition of 0.8 N HCl and again at pH 7 after 0.5 M NaOH addition

negative charges (sulfonate groups) it stabilizes low molecular weight intermediates through both hydrophobic and electrostatic interactions [8] so that it often blocks their aggregation [5]. Figure 4 shows the fluorescence emission spectrum of the extrinsic probe bis-ANS in AMT buffer, 150 mM NaCl pH 7 in the presence of SP-B_N (Fig. 4a). Once pH was acidified to 5, the fluorescence of bis-ANS showed a noteworthy increase in intensity, which indicates that hydrophobic regions of SP-B_N were exposed to the medium and bound the probe. The maximum fluorescence intensity of bis-ANS was centred at 482 nm, blue-shifted relative to 533 nm, where the emission takes place in water as found in other studies [35]. The ulterior pH neutralization of the sample could not reverse the conformational change the protein suffered at acidic pH, as monitored by the extrinsic probe. This result can explain the turbidity profile seen in Fig. 1a, where acid-induced turbidity could not be reverted upon neutralization. The protein seems to maintain an altered conformation at the regained neutral pH thus hindering the reversion of protein self-aggregation. When the experiment was repeated in the presence of 500 mM NaCl (Fig. 4b) the extrinsic fluorescence emission profiles were similar except for some differences such as lower basal fluorescence intensity at the highest ionic strength (initial pH 7). After acidification, the fluorescence intensity of the probe was also lower than that observed in low salt (Fig. 4a) and the spectrum was broadened showing almost a plateau at the highest intensity values (482–500 nm), being the maximum centred at ~489 nm (Fig. 4b). This profile is indicative of the probe bound to both exposed domains which are partially hydrophilic (emission at 500 nm) and also to really very hydrophobic sites (emission at 482 nm) as has been found for other proteins such as transthyretin [9]. When neutral pH was regained in mild salt the fluorescence emission of the probe was decreased and the plateau was vanished giving rise to a clear maximum at 478 nm, suggesting that partial reversibility of the conformational changes has taken place, at the expense of losing the probe the binding to partial hydrophilic domains.

SP-B_N self-aggregation studies at basic pH

A last objective was to check whether protein aggregation at the regained neutral pH that followed acidification in Fig. 1a was also produced if neutral pH was regained following exposure to alkaline pH. The experiment was carried out at physiological ionic strength by adding first aliquots of NaOH to the protein solution and neutralizing thereafter. Results depicted in Fig. 5 show that pH 9 caused no protein aggregation although ulterior neutralization did it as well as the subsequent acidification of the medium.

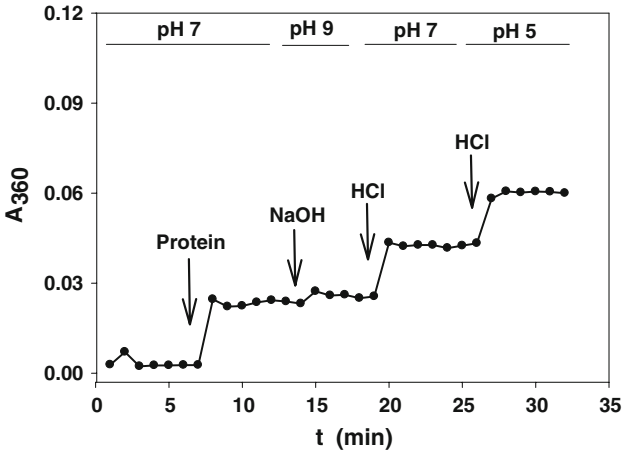


Fig. 5 Effect of alkaline pH on SP-B_N self-aggregation. SP-B_N aggregation was assessed under the same conditions described for Fig. 1a except that neutral pH of the medium was first alkalinized by addition of NaOH aliquots, then neutralized with HCl and acidified adding more HCl

Discussion

$SP\text{-}B_N$ follows the secretory pathway in lung cells while flanking mature SP-B [19] although its destiny once released from SP-B is still being explored. On the one hand, fragments of the propeptide of less than 19 kDa were found in the bronchoalveolar lavage fluid of children suffering diffuse chronic lung disease, although they were not determined in healthy children due to shortage of lavage [12]. On the other hand, ~5 kDa fragments of the propeptide were found in isolated rat lamellar bodies [3]. It is known that aggregation at mildly acidic pH, often enhanced by Ca^{2+}, is a property shared by many proteins that are stored in granules [31] since their environment becomes more acidic as they progress through the secretory pathway. The pH drops from neutral in the endoplasmic reticulum to ~6 in the lumen of the *trans*-Golgi layer and to ~5.2–5.5 in secretory granules [48]. Most members of the SAPLIP family (saposin-like proteins), to which $SP\text{-}B_N$ belongs [49], are targeted to acidic compartments, with the saposins themselves being a good example of targeting and processing coupled to pH acidification [13]. Other surfactant proteins such as SP-A have been also described to be sorted towards secretory pathways following acidic-pH self-aggregation [29]. Aggregation of saposin-like proteins is also important in the context of membrane-protein interactions mediating antipathogenic activities, which are shared by many of the members of these protein family and that are important in the context of the respiratory environment [30].

We have set about studying acidic self-aggregation of the recombinant $SP\text{-}B_N$ at physiological ionic strength (150 mM NaCl). The propeptide aggregates at pH 5 but when neutrality was restored, turbidity was not only decreased but increased further suggesting that protein–protein interactions at acidic pH, disregarding the type, allow or promote new contacts when neutralization takes place. Several secreted proteins such as rat prolactin in GH4C1 cells have also shown lack of aggregation reversion when acidic intracellular compartments are neutralized [16], and it has been proposed that irreversible pH-dependent effects are important in directing assembly of large supramolecular complexes. Two important features should be taken into consideration to evaluate the potential significance of $SP\text{-}B_N$ aggregation properties in the physiological context. On one hand, the $SP\text{-}B_N$ module likely encounters acidic pH along the secretory pathway of type II cells while flanked by the other proSP-B modules, particularly the surface active mature SP-B domain. It has been proposed that $SP\text{-}B_N$ is necessary and sufficient to protect the highly hydrophobic SP-B domain from interaction with cellular structures until the precise moment when SP-B has to assemble into pulmonary surfactant complexes [1], presumably in the transition between multivesicular bodies (MVB) and the lamellar bodies (LB) [47]. On the other hand, we have preliminary data showing that a His-tagged recombinant form of $proSP\text{-}B_{\Delta C}$ ($SP\text{-}B_N$ plus the mature SP-B domain, pI ~6) shows also acidic pH-promoted self-aggregation, which is in this case apparently reversed after neutralization [33]. Exposure of hydrophobic regions of $SP\text{-}B_N$ could therefore produce, in the context of $proSP\text{-}B_{\Delta C}$, different protein-protein interactions, presumably involving the mature SP-B module, than the observed in the isolated N-terminal flanking domain. It should be noted, however, that processing of proSP-B is coupled in the pneumocyte with assembly of SP-B into surfactant lipid-protein complexes, thought to occur in parallel with acidification of the medium in the transition from MVB to LB [46, 47]. Acidification-promoted exposure of the hydrophobic SP-B domain likely promotes irreversible association of the protein with surfactant phospholipids, liberating the $SP\text{-}B_N$ domain upon cleavage of the precursor. The irreversible self-association of the independent $SP\text{-}B_N$ polypeptide observed in this study could therefore mimic the behaviour of this protein module in vivo, once cleaved from the precursor. Recent data suggest that at least part of $SP\text{-}B_N$, i.e. its type-B saposin-like domain, could be secreted and is detectable at the alveolar spaces (Dr. Timothy Weaver, personal communication) suggesting that this protein could play its own role, still to be determined, in the respiratory surface. The study of the environment-dependent structural properties of $SP\text{-}B_N$ gains in this context considerable interest.

Self-aggregation of $SP\text{-}B_N$ is associated with pH-dependent conformational changes. Conformational analysis of the propeptide from CD spectra obtained at pH 5 indicates a decrease in α-helix and an increase in the content of *β*-sheet structure. This conformational change agrees with the known general fact that, upon aggregation proteins adopt new non-native structures and, without exception up today, show an increase in *β*-sheet concomitant to α-helix structure content decrease [43]. Surfactant protein SP-A also displays this general behaviour as pH is progressively reduced from 7 to 4.7 [29]. The intrinsic fluorescence emission profile of $SP\text{-}B_N$ at neutral pH indicates that the protein is folded and the spectrum obtained upon acidification and ulterior neutralization points to changes in the microenvironment of the Trp residues. An only partial unfolding is expected since the propeptide, as a member of the SAPLIP family, probably displays a characteristic pattern of disulphide bridges [14]. We could not titrate any of its ten Cys in the presence of 5.7 M guanidinium hydrochloride, so that 5 disulphide bridges were formed (data of a manuscript in preparation). Besides, disulphide bridges are a typical feature of secreted proteins and are considered to contribute to their overall stability [21]. In addition to the structural

changes described above, the exposure to the medium of SP-B_N hydrophobic patches induced at pH 5 was detected with the probe bis-ANS at 150 mM NaCl. The bis-ANS probe is considered to be a "detector" of protein molten globule states, i.e. a state in which most of the secondary and some of the tertiary structure is still formed but supported in a much more dynamic or fluid-like state than provided by the fully folded protein [32]. The probe emitted in our experiments at acidic pH upon binding to certain exposed domains of SP-B_N, which were not hided again after subsequent neutralization. A similar behaviour was described when bis-ANS bound to hemagglutinin of influenza virus exposed to acidic pH [15].

By increasing the ionic strength to moderate values (500 mM NaCl) some effects were detected on both protein structure and aggregation. Protein acid aggregation was less marked, its secondary structure was less affected and the extrinsic fluorescence intensity emission of bis-ANS was decreased with respect to the values obtained at physiological ionic strength. After neutralization, and in contrast to the experiments at low salt, aggregation was partially reverted and the probe emission decreased but there was no reversion of the changes in secondary structure. In other pertinent examples, the level of SP-A aggregation was lower in the presence than in the absence of salts [28] and RNase T1 (an acidic protein) is stabilized by the presence of salts [22]. Modulation of SP-B_N self-aggregation by the ionic conditions could be relevant in the context of LB, where high concentrations of certain ionic species and limited hydration are expected.

Human proSP-B has potential glycosilation sites in vivo both in N- and C-terminal propeptides (Scheme 1) and it is known that N-linked glycans, besides other effects, can hinder protein aggregation. The glycosilation sites at the SP-B_N domain differ, however, in different protein polymorphisms [45] making of interest the study of the behaviour of non-glycosylated variants. Since recombinant SP-B_N lacks glycosilation as produced in *E. coli*, we tried to simulate the possible effect of glycosilation on the aggregation properties of the protein by assaying the effect of the addition of L-arginine as a potential aggregation suppressor. We found that arginine really decreased aggregation either at acid or neutral but not at alkaline pH (Fig. 1c). Arginine exerts its effect through its guanidinium group in a way independent of the size or the pI of the protein [37]. Prevention of lysozyme aggregation by arginine is due to electrostatic interactions since NaCl abolished this protection and arginine acted as suppressor of aggregation at neutral pH (positive charge at its amino group) but not at pH 10 (no charge, pK ~9.2) pointing to the relevance of the additive state of charge [38]. Arginine is efficient to prevent SP-B_N self-aggregation at acidic pH but not that much at alkaline pH, probably due to its weak charge at this pH. However, there is another theory, the "gap effect", which tries also to explain the role of arginine as a suppressor of protein self-aggregation. If the additive is much larger than the solvent, as is the case, it will be excluded from the gap between the protein molecules for steric reasons resulting in an increase of the free energy associated to the protein-protein encounter complex [2].

Another additive we have assessed is Ficoll 70, a macromolecular crowder used to mimic in vitro the high concentration of macromolecules existing in vivo so that a part of the cell volume is physically occupied and is not available to other molecules. This is called "the excluded volume" and has the consequence that the thermodynamic activity (effective concentration) of any protein inside the cell is greater than its actual concentration. Thermodynamic approaches predict that proteins will move ~100 times slower and that the equilibrium constants for protein–protein association will be increased 2–3 orders of magnitude under such a crowded environment. However, crowders only enhance the inherent tendency of proteins to associate but do not create per se such a tendency. Therefore both the folded and the partially unfolded state of a protein are probably affected, being stabilized the first and enhanced the tendency to aggregate of the second [7]. Following acidification, SP-B_N (likely partially unfolded) aggregates in the presence of Ficoll 70 to a greater extent, although at an apparent slower rate than in its absence, and about the same occurs upon neutralization. The aggregation properties of SP-B_N could then be particularly exacerbated by the particularly crowded conditions concurring in the environment of LB, full of highly packed surfactant structures [25].

A combined analysis of the results presented in Figs. 1 and 5 leads to the conclusion that the propeptide aggregates at mildly acidic but not alkaline pH, as expected according to its origin, cell location and possible physiological role per se. It aggregates herewith at neutral pH following acid pH (except at higher than physiological ionic strength) as well as in a macromolecular crowded medium and in the presence of an aggregation suppressor. Moreover, the tendency of SP-B_N to aggregate at neutral pH is maintained when the precedent medium was alkaline. The full meaning of this reaffirmed tendency is still to be explored, but optimization of the experimental conditions preserving structure and stability of SP-B_N will be our next objective, which will allow a further exploration of its possible role in the context of the pulmonary surfactant complexes spread along the alveolar spaces.

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