The role of charged amphipathic helices in the structure and function of surfactant protein B

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Abstract: Surfactant protein B (SP-B) is essential for normal lung surfactant function. Theoretical models predict that the disulfide cross-linked, N- and C-terminal domains of SP-B fold as charged amphipathic helices, and suggest that these adjacent helices participate in critical surfactant activities. This hypothesis is tested using a disulfide-linked construct (Mini-B) based on the primary sequences of the N- and C-terminal domains. Consistent with theoretical predictions of the full-length protein, both isotope-enhanced Fourier transform infrared (FTIR) spectroscopy and molecular modeling confirm the presence of charged amphipathic α-helices in Mini-B. Similar to that observed with native SP-B, Mini-B in model surfactant lipid mixtures exhibits marked in vitro activity, with spread films showing near-zero minimum surface tensions during cycling using captive bubble surfactometry. In vivo, Mini-B shows oxygenation and dynamic compliance that compare favorably with that of full-length SP-B. Mini-B variants (i.e. reduced disulfides or cationic residues replaced by uncharged residues) or Mini-B fragments (i.e. unlinked N- and C-terminal domains) produced greatly attenuated in vivo and in vitro surfactant properties. Hence, the combination of structure and charge for the amphipathic α-helical N- and C-terminal domains are key to SP-B function.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; POPE, palmitoyloleylethanolamine; POPS, palmitoyloleoylphosphatidylserine; Chol, cholesterol; POPG, palmitoyloleoylphosphatidylglycerol; PBS, phosphate-buffered saline; HFIP, hexafluoroisopropanol; ATR, attenuated total reflectance; FTIR, Fourier transform infrared spectroscopy; 13C-FTIR, isotopically enhanced 13-Fourier transform infrared
Surfactant protein B (SP-B) is a small [79 amino acids; molecular weight (MW) approximately 8700], lipid-associating protein found in mammalian lung surfactant [1], with a conserved antigenic structure for approximately 300 million years [2]. Each SP-B monomer contains three intramolecular disulfide bridges [i.e. Cys-8 to Cys-77, Cys-11 to Cys-71, and Cys-35 to Cys-46; 3], and belongs to a class of “saposin-like” proteins that also includes saposins A-D and NK-lysin [4–7]. SP-B is essential for lung function, since mutations in the SP-B gene cause severe and fatal lung disease [8,9]. Surfactant preparations containing either synthetic peptides representing the N- and C-terminal domains of SP-B or full-length SP-B improve oxygenation and/or lung compliance in surfactant-deficient animal models [10–15].

Although detailed analyses of native SP-B structure have yet to be performed, models of SP-B were recently formulated based on the known three-dimensional [3D] structures of homologous proteins [e.g. NK-lysin and saposin-B] that share the “saposin” fold [16,17]. These extrapolated SP-B models predict a homodimeric structure characterized by amphipathic α-helices with hydrophilic (charged, neutral) residues facing solvent, and hydrophobic side chains forming a core stabilized by intramolecular disulfide bonds [18,19]. The high α-helical content predicted by these SP-B models has been verified by circular dichroism [CD] and Fourier transform infrared [FTIR] spectroscopy of native SP-B [6,20–23]. It is of interest that the SP-B model predicts a disk-like structure containing disulfide-linked, charged amphipathic helices [i.e. and N- and C-terminal domains], which may promote surfactant activity [18,19].

Structural and functional studies using synthetic peptides have indeed provided experimental support for the hypothesis that the charged, amphipathic helical N- and C-terminal regions in full-length SP-B participate in surfactant activities. For example, SP-B and a synthetic peptide representing the N-terminal domain of SP-B [i.e. SP-B1–25; residues 1–25] each increase the collapse pressure of lipid monolayers containing palmitic acid. This suggests that the cationic N-terminus of SP-B interacts with anionic lipids to remove the driving force for lipid squeeze-out from the surface film [24,25]. The critical insertion pressure, an index of the degree of protein association with lipid films, is also very high for both SP-B1–25 and the parent SP-B [21,26,27]. Moreover, SP-B1–25 and full-length SP-B each induce a coexistence of buckled and flat monolayers when added to surfactant lipids, leading to a low minimum surface tension and increased respreading of the surfactant monolayer [28,29]. These in vitro surfactant activities of SP-B1–25 are correlated with the improved oxygenation and lung compliance observed with this peptide in surfactant-deficient animal models [10–12]. As physical experiments indicate that N-terminal SP-B peptides in lipids or membrane-mimics show high α-helix content [26,30,31], the N-terminal domain of SP-B may participate in its in vitro and in vivo surfactant actions as a charged amphipathic α-helix. Interestingly, several non-natural analogs of SP-B1–25, which form α-helices in lipid environments also exhibit in vitro surfactant activities [32]. A charged helical C-terminus of SP-B may similarly participate in lung function, as synthetic peptides based on the C-terminal domain assume α-helical conformations [33,34] and also induce in vitro [13,14,34,35] and in vivo [14,14] surfactant activities that simulate those of the native protein. KL6α, a synthetic 21-residue peptide which more generally mimics the hydrophobic and hydrophilic repeats of SP-B, may achieve its surfactant activities [14,36] by folding as an α-helix [37].

In the present study, Mini-B, a synthetic construct [34-residues] based on the N- and C-terminal regions and the disulfide cross-linkages of native SP-B, was shown via isotope-enhanced FTIR spectroscopy and molecular modeling to share the topographical organization of these
domains in SP-B. Captive bubble surfactometry and animal models of respiratory distress indicate that the specific 3D structure and charge distribution of helices in Mini-B may be responsible for key in vitro and in vivo activities of full-length SP-B in lung surfactant.

Experimental Procedures

Materials

Peptide synthesis reagents, including Fmoc amino acids and coupling solvents, were obtained from Applied Biosystems (Foster City, CA, USA). Deuterium oxide was supplied by Aldrich Chemical Co. (Milwaukee, WI, USA) Fmoc-13C-carbonyl alanine, leucine, isoleucine, and valine were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Dipalmitoylphosphatidylcholine (DPPC), palmitoyloleoylphosphatidylglycerol (POPG), dioleoylphosphatidylcholine (DOPC), palmitoyloleylethanolamine [POPE], palm- itoyloleoylphosphatidylserine [POPS], and cholesterol [Chol; purity >99%] were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Solid phase peptide synthesis, purification, and characterization

The oxidized Mini-B peptide [34-residues; NH2-C14WLRALIKRIQAMIPKGRMLPQLVCRLVLRCS34-COOH] was synthesized using FastMoc™ (Applied Biosystems) chemistry with an ABI peptide synthesizer, and purified by reverse phase high-performance liquid chromatography (HPLC; 38). Mini-B residues 1–18 correspond to the N-terminal SP-B residues 8–25 (i.e. SP-B8–25) of the full-length human protein, while Mini-B residues 19–34 correspond to the C-terminal SP-B residues 63–78 (i.e. SP-B63–78). The disulfide linkages were directed by using acid labile triyl [Trt]- protecting groups at cysteine positions 1 and 33, while acid-resistant acetamidomethyl [ACM] side chain-protecting groups were employed at cysteine positions 4 and 27. The disulfide bond between Cys-1 and -33 was formed by air oxidation for 24 h trifluoroethanol : ammonium bicarbonate buffer (10 mM, pH 8.0) followed by iodine oxidation of the ACM-protected cysteines. The disulfide connectivity was confirmed by mass spectrometry of the enzyme-digested fragments (trypsin, chymotrypsin, Emory University Microchemical Facility). A reduced Mini-B peptide was similarly prepared, except that the oxidizing steps were omitted and cysteine residues 1 and 33 SH groups were blocked with N-ethylmaleimide. Mini-(thr), an oxidized variant of Mini-B with threonine replacing positively charged residues, was synthesized and purified following the protocol for oxidized Mini-B, using the sequence: NH2-C14WLCTALITTIQAMIPKGRMLPQLVCRLVLRCS34-COOH. The fragment peptides SP-Bα–35 (N-terminal 18-residues; NH2-C14WLRALIKRIQAMIPKGRMLPQLVCRLVLRCS34-COOH) and SP-B63–78 (C-terminal 16-residues; NH2-C63RMLPQLVCRLVLRCS78-COOH), numbered based on the full-length SP-B, were similarly synthesized. Purified native porcine SP-B protein was prepared [39] and identified by N-terminal sequence analysis (Emory University Microchemical Facility).

Fourier transform infrared spectroscopy

Infrared spectra were recorded at 25 °C using a Bruker Vector 22™ FTIR spectrometer (Bruker Optics, Billerica, MA, USA) with a DTGS detector, averaged over 256 scans at a gain of 4 and a resolution of 2 per cm. For SP-B peptides originally in 90% hexafluoropropanol (HFIP)/10% D2O, self-films were prepared by air-drying peptide solutions onto a 50 x 20 x 2 mm, 45° attenuated total reflectance (ATR) crystal for the Bruker (Pike Technologies, Madison, WI, USA) spectrometer (40,41). The overall secondary conformations were qualitatively assessed from the amide I band of conventional 13C-FTIR spectra of SP-B peptides [30,42]. The frequency limits for the different structures were: a-helix (1662–1645 per cm), β-sheet (1637–1613 and 1710–1682 per cm), β-turns (1682–1662 per cm), and disordered or random (1650–1637 per cm; 42).

Rationale for 13C-site-directed Mini-B substitutions

To more precisely explore secondary conformations within oxidized Mini-B, FTIR spectroscopy was performed with site-directed, isotope-enhanced peptides [30,40,41]. Separate peptides were prepared with ‘cassettes’ of multiple 13C-carbonyl substitutions that were staggered to sequentially cover the peptide: Mini-BL3–18/A6–17/L8–111 [13C=O at Leu-3, Ala-6, Leu-7, Ile-8, and Ile-11]; Mini-BL15/G18/G19/L22 [13C=O at Ile-15, Gly-18, Gly-19, and Leu-22]; and Mini-BL25/V26/L29/V30 [13C=O at Leu-25, Val-26, Leu-29, and Val-30]. As the stretching frequencies of the peptide backbone carbonyl groups are sensitive to local conformations, replacement of 12C with 13C should reduce the stretching frequency of an ‘isolated’ carbonyl oscillator by approxi-
mately 35 per cm (e.g. α-helix band shift from approximately 1657 to 1622 per cm, 43,44). For 13C=O substituted into an antiparallel β-sheet with elevated interactions between highly substituted 13C-labels in adjacent strands, a maximum frequency shift from approximately 1622 to 1585 per cm should be observed [43,45].

**Molecular modeling**

The oxidized and reduced Mini-B peptides were each modeled with the CHARMM-22 force field in the MODELLER 6v2 environment [46, City of Hope, Biomedical Informatics Core Facility]. The coordinates for 10 conformers of the oxidized Mini-B in the HFIP solution (i.e. 90% HFIP/10% D2O), together with a list of restraints, have been deposited in the Protein Data Bank (PDB) under the accession code 1SSZ.

**Captive bubble surfactometry**

For in vitro activity measurements with the captive bubble surfactometer, 0.1 μmol of protein or peptide was mixed with 35 mg total phospholipids consisting of 16 mg of DPPC, 10 mg of DOPC, 3 mg of POPG, 1 mg of POPE, 3 mg of POPS, and 2 mg Chol in 1 mL of phosphate-buffered saline (PBS). The surface activity of these surfactant preparations was determined with a captive bubble surfactometer, which has been described in detail elsewhere (47–49). In short, the instrument consists of a sample chamber cut from cylindrical glass tubing of high quality with an inner diameter of 1 cm. A Teflon piston with a tight O-ring seal is fitted into the glass tubing from the top end. A plug of buffered agarose gel is inserted between the piston and solution. The other end is fitted into a plate of stainless steel, which is provided with an inlet port in the center for adding solutions and the air bubble to the chamber. Chamber and piston are vertically mounted within a sturdy rack of steel whose height is regulated by a micrometer gear with minimal redundancy. For usual measurements, the chamber is filled with 10% sucrose Goerke’s buffer, to which 1 μl surfactant is added. The chamber content is stirred with a small magnetic bar, and its temperature is maintained at 37 °C. A small air bubble is introduced from beneath, whose volume and hence surface area can be changed by compression and decompression brought about by changing the height of the rack. The agarose plug’s ionic composition inhibits bubble adhesion to its surface, thus creating an uninterrupted surface area and a perfect bubble. During the compression and re-expansion cycles, bubble images are continuously recorded to the hard drive and on a video recorder. Selected single frames are stored in random access memory (RAM) for later image processing and analysis (50). Bubble areas and volumes are calculated by an original algorithm relating bubble height and diameter to areas of revolution, and the bubble surface tension is determined by using the method of Malcolm and Elliot [51].

**In vivo measurements with surfactant-deficient rats**

For in vivo measurements with surfactant-deficient rats, oxidized Mini-B surfactant was compared with porcine SP-B surfactant, reduced Mini-B surfactant (SP-B8–25 + SP-B63–78) surfactant, and mutant Mini-B(thr) surfactant, and lipids alone. The surfactants for the in vivo experiments had the same lipid and peptide (or protein) compositions as those used in the in vitro captive bubble surfactometry determinations. The animal experiments were performed with the approval of the Harbor-UCLA Research and Education Institute Animal Care and Use Committee. Anesthesia, surgery, lavage, ventilation, and monitoring used in this study are the same as previously described [12,52]. Briefly, six groups of eight adult male Sprague-Dawley rats weighing 200–225 g were anesthetized with 35 mg/kg pentobarbital sodium and 80 mg/kg ketamine by intraperitoneal injection, intubated, and ventilated with a rodent ventilator (Harvard Apparatus, Holliston, MA, USA) with 100% oxygen, a tidal volume of 7.5 mL/kg, a positive end-expiratory pressure (PEEP) of 3 cm H2O, and a rate of 60/min. An arterial line was placed in the abdominal aorta for monitoring blood pressure and blood gases. The rats were paralyzed with 1 mg/kg pancuronium bromide intravenously. Only animals with PaO2-values of >400 mmHg while ventilated with 100% oxygen were included in the experiments. Airway flow and pressures and tidal volume were monitored continuously with a pneumotachograph connected to the tracheal cannula and a pneumotach system [Hans Rudolph Inc., Kansas City, MO, USA]. Dynamic lung compliance was calculated by dividing tidal volume/kg body weight by changes in airway pressure (peak inspiratory pressure minus PEEP, mL/kg/cmH2O). The lungs of the rats were lavaged eight times with 8 mL of pre-warmed 0.9% NaCl, and was repeated after 15 min, and, if necessary, after 30 min until the PaO2 in 100% oxygen reached stable values of <100 torr. After reaching a PaO2 <100 torr,
For native Mini-B self-films dried from the HFIP solvent, the predominate peak at 1657 per cm in the conventional \(^{13}\)C-FTIR spectrum [Fig. 1A] is indicative of a major \(\alpha\)-helix component, while the small shoulder at approximately 1623 per cm suggests minor \(\beta\)-sheet. Because it is not possible from \(^{13}\)C-FTIR spectroscopy to attribute secondary conformations to specific residues, local conformations were probed using isotopically enhanced FTIR spectra of Mini-B labeled with \(^{13}\)C=O groups at neighboring, multiple sites. With Mini-B isotopically enhanced with \(^{13}\)C=O labeled at Leu-18, Ala-19, Leu-20, Ile-21, Ile-22, Ile-25, Val-26, Leu-29, and Val-30, there is a large decrease in the \(\alpha\)-helical peak centered at 1657 per cm, and a concurrent increase in a new peak at approximately 1622 per cm [Fig. 1A]. The isotopic shift of approximately 35 per cm is due to \(^{13}\)C=O-labeled Leu-3, Ala-6, Leu-7, Ile-8, and Ile-11 participating in an \(\alpha\)-helix [30]. The Mini-BL/I38/A6/L7/I11 spectrum [Fig. 1C] also shows a new peak at approximately 1622 per cm and reduced absorption at 1657 per cm, consistent with \(^{13}\)C=O-labeled Leu-25, Val-26, Leu-29, and Val-30 assuming an \(\alpha\)-helical conformation. Interestingly, the Mini-BL/I38/G18/G19/L22 spectrum [Fig. 1B] presents a distinct pattern with a new peak at approximately 1585 per cm and lowered absorption at approximately 1622 per cm, probably the result of the \(^{13}\)C=O-labeled residues [i.e. Ile-15, Gly-18, Gly-19, and Leu-22] participating in \(\beta\)-sheet which in turn produces a large shift in the new \(^{13}\)C=O peak. This shift is likely due to intermolecular transition dipole coupling interactions between \(^{13}\)C=O in adjacent strands when two Mini-B molecules form an antiparallel \(\beta\)-sheet in the loop region [41,45]. The \(\alpha\)-helices determined here for the N- and C-terminal regions of Mini-B compare favorably with those reported for peptide fragments based on the N-terminal [30,31] or C-terminal [33] domains of SP-B.

Despite the \(^{13}\)C-FTIR analysis in Fig. 1, the secondary conformation of a small number of residues in Mini-B were not determined experimentally. Molecular modeling was conducted to provide structural information on these ‘gap’ residues, and also to assess the overall conformational flexibility within Mini-B. Combining the \(^{13}\)C-FTIR findings in Fig. 1 with earlier results from residue-specific studies [see above] on N- and C-terminal fragments indicated the following preliminary conformation map for Mini-B in a...
hydrophobic environment: residues 1–15, α-helix; residues 18–22, β-sheet; residues 24–34, α-helix. To maintain these experimental structural elements, and also to accommodate the two disulfide linkages (Cys-1 to Cys-33, Cys-4 to Cys-27), the torsional angles of Pro-23 and Lys-24 were modified to form a hairpin turn. Seventy-five cycles of simulated annealing were performed on the Mini-B structure [46] and 10 conformers were selected for the final conformer and deposited in the PDB (accession code: 1SSZ; http://www.rcsb.org). The final conformer set was compatible with the original model, except that residues 32–34 no longer participated in the α-helix [Fig. 2A]. The Mini-B backbone folding [Fig. 2A] closely overlaps the corresponding domains of a SP-B model [Fig. 2B], obtained by templating the native sequence on to the known 3D structure of NK-lysin [16] and then performing dynamic energy minimizations [19]. Thus, Fig. 2B suggests that Mini-B faithfully reproduces the topographical organization of the charged, α-helical N- and C-terminal domains in full-length SP-B.

Previous theoretical and physical experiments on peptides based on the N- and C-terminal domains suggest that Mini-B partitions into the polar headgroup region of lipids. Electron spin resonance (ESR) and fluorescence spectroscopy of SP-B1–25 indicate a surface location in lipid bilayers for the N-terminal helix [i.e. Mini-B residues 1–15; 38], while two-dimensional nuclear magnetic resonance (2D-NMR) of the C-terminal SP-B63–78 in SDS micelles showed that its helix [i.e. Mini-B residues 24–34] was restricted to the water interface [33]. These results agree with predictions using hydrophobic moment plots that the N- and C-terminal helical domains of SP-B are ‘surface membrane-seeking’ [53]. Because the disulfide-linked N- and C-helical domains probably assume similar compact structures in both Mini-B and full-length SP-B [Fig. 2], the surface location for native SP-B in lipid bilayers [21] may be due to the resistance of these charged amphipathic domains to deep penetration. Interestingly, analogous ‘mini-B’ configurations have been reported for the N- and C-terminal domains of other proteins [e.g. NK-lysin, saposin-B and -C] that share the saposin fold [16,17,54], and may also participate in the association of those proteins with membrane lipid bilayers [16]. Hydrophobic moment analysis of the N- and C-terminal regions for NK-lysin, saposin-B and -C confirms that these domains are surface-seeking helices [data not shown].

The overall secondary conformations of several Mini-B variants and fragments were analyzed using conventional 12C FTIR spectroscopy. The FTIR spectrum of reduced Mini-B [i.e. no intrachain disulfide linkages] in the HFIP solvent was nearly identical to that of oxidized Mini-B [Fig. 3A], suggesting that the oxidized and reduced forms share similar secondary structures. Unlike the compact oxidized Mini-B that was found to be conformationally inflexible due to two disulfide bridges [Fig. 2A], dynamic energy minimizations of reduced Mini-B show that the N- and C-terminal α-helices ‘flare’ about the central core [data not shown]. The corresponding 12C FTIR spectrum of Mini-B[thr] [i.e. an oxidized Mini-B variant with positively charged residues replaced by threonine] in the HFIP solvent also showed a predominant α-helical component [Fig. 3B]. However, the small shift in the Mini-B[thr] spectrum to lower wave numbers indicate that the introduction of threonines probably enhanced random/β-sheet structures by slightly fraying the α-helices of the N- and/or C-terminal regions. Interestingly, the FTIR spectrum of the SP-B8–25
and SP-B_{65-78} fragments in HFIP solvent [Fig. 3C] closely mimicked that of the oxidized Mini-B spectrum, even though the fragments were not covalently linked. The high \( \alpha \)-helical levels found here for the mixture of SP-B_{8-25} and SP-B_{63-78} in the HFIP solvent are consistent with previous FTIR and 2D-NMR studies on the separate N- and C-terminal peptides of SP-B in membrane-mimics \( [30,31,33] \).

**Captive bubble surfactometry of SP-B, oxidized Mini-B, and related peptides**

As Mini-B mimics the 3D organization of the N- and C-terminal regions in the SP-B protein [Fig. 2B], Mini-B should also reproduce key in vitro and in vivo activities of native SP-B if this structure is important for SP-B function. As has been noted \( [55] \), several criteria must be met for a surfactant preparation to be functional, including rapid adsorption to the air–water interface, attainment of near-zero surface tension upon film compression, and fast and efficient respreading of the surfactant on expansion of the interface. Using captive bubble surfactometry \( [49] \), in vitro film formation and dynamics were studied for surfactant preparations containing SP-B, oxidized Mini-B or related peptides. The adsorption of surfactant preparation in the subphase to a clean interface of the air bubble in the chamber is readily determined from changes in the bubble shape. Lipid surfactants, either with or without protein/peptides, were injected into the chamber. A bubble was then created and adsorption was allowed for 5 min. The chamber was then sealed and the initial bubble volume (approximately 0.03–0.05 cc) measured. The surface tensions at this time are reported (see cycle 0 in Fig. 4). Except for the mutant Mini-B(thr) preparation, surfactants containing oxidized Mini-B, reduced Mini-B and \( [\text{SP-B}_{8-25} + \text{SP-B}_{63-78}] \) each exhibit similar activity compared with the protein-free sample (approximately 36–39 mN/m vs. approximately 57 mN/m). However, these values for the Mini-B peptides are significantly higher than that for native SP-B (approximately 25 mN/m; Fig. 4), indicating that these peptides only partly reproduce the surface tension-lowering properties of the native protein at cycle 0. A similar partial mimicking of the initial surface activity of native SP-B was observed in captive bubble surfactometry of peptides based solely on the N-terminal domain of SP-B \( [29] \), and here Veldhuizen *et al.* \( [29] \) suggested that the N-terminal peptides are more resistant than the full-length SP-B in penetrating an existing, relatively tight-packed lipid layer at cycle 0. Interestingly, the mutant Mini-B(thr) preparation showed a very high surface tension approaching that of the protein-free lipid sample [Fig. 4], suggesting that positive-charged \( \alpha \)-helices are a necessary requirement for in vitro surface activity.

The maximum and minimum surface tensions \( (\gamma_{\text{max}} \text{ and } \gamma_{\text{min}}) \) during cycling with these surfactant mixtures are illustrated in Fig. 4. The oxidized and reduced Mini-B preparations have increased activity compared with the control lipid samples, with 10th cycle \( \gamma_{\text{min}} \)-Values of

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**Figure 3.** Conventional \(^{13}\)C-Fourier transform infrared (FTIR) spectra of the oxidized Mini-B peptide, and Mini-B variants and fragments, for peptides in 90% hexafluoroisopropanol (HFIP)/10% D\(_2\)O. Peptides were initially suspended at 510 \( \mu \text{M} \) and dried on the attenuated total reflectance [ATR] plate. [A] Reduced Mini-B [solid line], oxidized Mini-B [dashed line]. [B] Oxidized Mini-B[thr] [solid line], oxidized Mini-B [dashed line]. [C] Mixture of SP-B_{8-25} and SP-B_{63-78} peptide fragments [solid line], oxidized Mini-B [dashed line]. The SP-B_{8-25} and SP-B_{65-78} peptides are numbered according to the 1–78 sequence of the full-length SP-B protein, and correspond to residues 1–18 and 20–34, respectively, of the Mini-B construct (Experimental Procedures). See Results and Discussion for spectral assignments of secondary structure.


≤1 mN/m for both oxidized and reduced mini-B [vs. approximately 16–17 mN/m for protein-free samples], while the respective γ_{max}-values are approximately 42 and 57 mN/m [vs. approximately 61 mN/m for the protein-free samples; Fig. 4]. Interestingly, the 10th cycle γ_{min} for native SP-B (approximately 2 mN/m) agrees to within experimental error with the corresponding γ_{min}-values for the oxidized and reduced Mini-B preparations (Fig. 4), while the ranking for the 10th cycle γ_{max}-values is SP-B [approximately 35 mN/m] < oxidized Mini-B [approximately 43 mN/m] < reduced Mini-B [approximately 50 mN/m; Fig. 4]. These data indicate that oxidized Mini-B accurately mimics the in vitro cycling surface activity of the full-length SP-B. While the reduced Mini-B also promotes near-zero surface tension upon film compression (Fig. 4), its relatively high 10th cycle γ_{max}-value suggests that disulfide linkages are necessary for Mini-B to express optimal in vitro surfactant activity. It should be also noted that the physical contiguity and positive-charge distribution of the α-helical N- and C-terminal SP-B domains are critical for in vitro surface activity, as [SP-B_{8-25} + SP-B_{63-78}] and mutant Mini-B{[thr]} each show high surface tensions upon expansion and compression (Fig. 4).

**Conclusions**

Our results show that Mini-B, a synthetic construct [34-residues] that includes the α-helical N- and C-terminal domains and closely mimics important components of the saposin-folding pattern of SP-B, also reproduces key in vitro and in vivo functions of full-length SP-B. Reducing the positive charge of Mini-B diminishes the ability of this peptide to promote low surface tension on film compression and reproducible respreadng, both of which appear to be important for proper in vivo performance. Similar to native SP-B (56), Mini-B probably interacts through its cationic residues [i.e. lysine, arginine] with anionic lipids such as phosphatidylglycerol (57), thereby counteracting the driving force for lipid squeeze-out from the surface film. The relative in vitro surfactant activities of Mini-B and its variants (Fig. 4) correlate well with the relative oxygenation and lung compliance in surfactant-deficient animals

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**In vivo performance of SP-B, oxidized Mini-B, and related peptides**

Rats demonstrated a rapid recovery of oxygenation [PaO_2] and dynamic compliance (tidal volume/kg body weight) after rescue treatment with oxidized Mini-B, native SP-B, reduced Mini-B, and [SP-B_{8-25} + SP-B_{63-78}], but not with mutant Mini-B{[thr]} surfactant or lipids alone (Fig. 5). The average PaO_2-values following lung lavage were 13% of the pre-lavage values and increased after treatment with oxidized Mini-B, native SP-B, reduced Mini-B, (SP-B_{8-25} + SP-B_{63-78}), mutant Mini-B{[thr]}, and lipids alone to 99%, 87%, 71%, 40%, 22% and 20% of the pre-lavage values, respectively. Postlavage dynamic compliance dropped to 62% of the pre-lavage values and increased after treatment with oxidized Mini-B, native SP-B, reduced Mini-B (SP-B_{8-25} + SP-B_{63-78}), mutant Mini-B{[thr]}, and lipids alone to 85%, 79%, 76%, 73%, 70% and 69% of the pre-lavage values, respectively. PaO_2-values for oxidized Mini-B and native SP-B were significantly higher than those of the other surfactant preparations or lipids alone when tested with one-way ANOVA (P < 0.001 and P < 0.05, respectively). Dynamic compliance values for oxidized Mini-B surfactant and native SP-B surfactant exceeded those of the other surfactant preparations and lipids alone, but only the differences with lipids alone were statistically significant (P < 0.001 and P < 0.05, respectively). The differences between oxidized Mini-B and native SP-B surfactant were not statistically significant.

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**Figure 4.** Quasi-static captive bubble curves of the six surfactant preparations used in the experiments. About 1 μL of each of the surfactant preparations was injected into the subphase and allowed to adsorb to the interface for 5 min. Maximum and minimum surface tension values during cycling (0.17–0.44 cycles/min for the various surfactant preparations) of adsorbed films are indicated. Surface tension values shown are the average of at least four separate experiments (±SEM).
treated with the same surfactants (Fig. 5). Furthermore, the similar in vitro and in vivo activities reported here for both Mini-B and native, full-length SP-B (Figs 4 and 5) support the hypothesis that surfactant functions of SP-B are at least partially localized to a disk-like structure containing the disulfide-linked, charged amphipathic N- and C-terminal domains (18,19).

Both in vitro and in vivo activities (Figs 4 and 5) show that the function of Mini-B (and hence native SP-B) is dependent on its 3D structure and charge distribution. Either reduction of the disulfide linkages of Mini-B or complete disconnection of the N- and C-terminal helical domains yielded abnormal compression–expansion curves (Fig. 4) and reduced oxygenation in surfactant-deficient rats (Fig. 5). Consequently, our results suggest that the saposin-folding pattern, observed for both Mini-B and SP-B (Fig. 2), participates in the optimal expression of surfactant activity. This structure–function model explains why peptides based on only the N- or C-terminal domains are not able to fully reproduce the surfactant activities of full-length SP-B, as the topographical organization (i.e. saposin fold) of the disulfide-linked, N- and C-terminal regions is only partially mimicked. Of more general interest is the finding that comparable Mini-B configurations have been observed in the N- and C-terminal domains of other saposin proteins (16,17,54,58). Consistent with earlier proposals that the disulfide-linked N- and C-terminal domains of various saposins (e.g. NK-lysin, saposin-A and -C) insert into lipid bilayers (16,59), our present findings with the Mini-B derived from SP-B confirm that the membrane–lipid interaction properties of other saposin proteins may be generally due to respective ‘Mini-B’ domains.

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