Biochimica et Biophysica Acta 1858 (2016) 3113–3119

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Stability of an amphipathic helix-hairpin surfactant peptide in liposomes

Alan J. Waring [a,b,*], Monik Gupta [b], Larry M. Gordon [b], Gary Fujii [c], Frans J. Walther [b,d]

[a] Department of Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, United States
[b] Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA, United States
[c] Molecular Express Inc., Rancho Dominguez, CA, United States
[d] Department of Pediatrics, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, United States

ARTICLE INFO

Article history:
Received 15 July 2016
Received in revised form 13 September 2016
Accepted 19 September 2016
Available online 21 September 2016

Keywords:
Surfactant protein B (SP-B)
Super Mini-B (SMB)
Captive bubble surfactometry
FTIR spectroscopy
Homology modeling
Surfactant lipids
Lyso-phospholipids

ABSTRACT

Surfactant protein B (SP-B; 79 residues) is a member of the saposin superfamily and plays a pivotal role in lung function. The N- and C-terminal regions of SP-B, cross-linked by two disulfides, were theoretically predicted to fold as charged amphipathic helices, suggesting participation in surfactant activities. Previous studies with oxidized Super Mini-B (SMB), a construct based on the N- and C-regions of SP-B (i.e., residues 1–25 and 63–78) joined with a designer turn (-PKGG-) and two disulfides, indicated that freshly prepared SMB in lipids folded as a surface active, α-helix-hairpin. Because other peptides modeled on α-helical SP domains lost helicity and surfactant activity on storage, experiments were here performed on oxidized SMB in surfactant liposomes stored at ~2–8 °C for ≤5.5 years. Captive bubble surfactometry confirmed low minimum surface tensions for fresh and stored SMB preparations. FTIR spectroscopy of fresh and stored SMB formulations showed secondary structures compatible with the peptide folding as α-helix-hairpin. A homology (I-TASSER) model of oxidized SMB demonstrated a globular protein, exhibiting a core of hydrophobic residues and a surface of polar residues. Since mass spectroscopy indicated that the disulfides were maintained on storage, the stability of SMB may be partly due to the disulfides bringing the N- and C-α-helices closer. Mass spectroscopy of stored SMB preparations showed some methionine oxidation, and also partial deacylation of surfactant phospholipids to form lyso-derivatives. However, the stable conformation and activity of stored SMB surfactant suggest that the active helix-hairpin resists these chemical changes which otherwise may lead to surfactant inhibition.

1. Introduction

Lung surfactant is a mixture of lipids and proteins that is pivotal for normal breathing because of its ability to prevent alveolar collapse during expiration by reducing alveolar surface tension to extremely low values. Surfactant is synthesized and secreted into the alveolar fluid by alveolar type II cells and consists of approximately 80% phospholipids, 10% neutral lipids, and 10% proteins [1]. Although dipalmitoyl phosphatidylcholine (DPPC) and phosphatidylglycerol (PG) are the principal phospholipid components in lung surfactant, its biophysical activity largely depends on the presence of the hydrophobic surfactant protein B (SP-B) and, to a lesser degree, the extremely hydrophobic surfactant protein C (SP-C) [1,2]. Human SP-B is a 79 amino acid, lipid-associating monomer (MW ~8.7 kDa) that is found in the lung as a covalently linked homodimer. Each SP-B monomer consists of 4–5 α-helices with 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 the saposin protein superfamily [4]. The helical bundle for SP-B is folded into two leaves, with one leaf having α-helices 1 (N-terminal helix), 5 (C-terminal helix) and 4 and the second composed of α-helices 2 and 3 [5,6].

Super Mini-B (SMB) is a 41-residue construct, based on the primary sequence, secondary structure and tertiary folding of the 79-residue SP-B, which seeks to mimic the high surfactant activity of the parent protein [6]. SMB reproduces the topology of the N- and C-terminal domains of SP-B, as it contains the N-terminal α-helix (~residues 8–25) and C-terminal α-helix (~residues 63–78) joined with a custom turn –PKGG– to form an α-helix hairpin. The oxidized SMB was shown to have two vicinal disulfide bonds (i.e., Cys-8 to Cys-77 and Cys-11 to Cys-71) that further covalently link the N- and C-terminal α-helices. Lastly, the hydrophobic N-terminal insertion sequence (i.e., residues 1–7; FPIPLPY) may insert into the lipid bilayer as an anchor for oxidized SMB attachment. Various experimental techniques were used to confirm the above structural model for oxidized SMB, including conventional ^{12}C-FTIR spectroscopy, mass spectroscopy and

Abbreviations: ATR-FTIR, attenuated-total-reflectance Fourier-transform infrared; CBS, captive bubble surfactometer; DPPC, dipalmitoyl-phosphatidylcholine; DTGS, deuterium triglyceride sulfate; MD, molecular dynamics; MLVs, multi-lamellar vesicles; PBS, phosphate buffered saline; POPC, palmitoyl-oleoyl-phosphatidylcholine; POPG, palmitoyl-oleoyl-phosphatidylglycerol; RDS, respiratory distress syndrome; SMB, Super Mini-B.

* Corresponding author at: Los Angeles Biomedical Research Institute, Harbor-UCLA Medical Center, 1124 West Carson Street, Torrance, CA 90502, United States.
E-mail address: awaring@labiomed.org (A.J. Waring).

http://dx.doi.org/10.1016/j.bbamem.2016.09.014
0005-2736/

Molecular Dynamics (MD) simulations in lipid mimics and lipid bilayers [6,7]. Importantly, oxidized SMB has shown excellent surface activity as single surfactant peptide in a lipid mixture that mimics the composition of native lung surfactant [6]. This synthetic lipid mixture consists of DPPC, palmitoyl-oleoyl-phosphatidylcholine (POPC) and palmitoyl-oleoyl-phosphatidylglycerol (POPG) at a weight ratio of 5:3:2 [6,8].

Intratracheal instillation of animal-derived lung surfactant extracts, which contain only polar lipids and native SP-B and SP-C, has greatly improved the survival of premature infants with neonatal respiratory distress syndrome (RDS) due to surfactant-deficiency and lung immaturity [9]. Package inserts of the porcine surfactant Curosurf® and the bovine surfactants Infasurf® and Survanta® mention a shelf life of 18 months if the vials are stored upright at a temperature of 2° to 8 °C (36° to 46 °F) since freezing would inactivate the dispersion and protected from light, and state that warming and returning an unopened vial to the refrigerator is acceptable practice. However, aging and stability studies of these clinical surfactant preparations have not been reported. The latter is also true for synthetic lung surfactant formulations containing peptide mimics. For example, two commercial preparations (i.e., synthetic surfactant preparations with the α-helical SP-B mimic KL4 (Surfaxin®) and the α-helical SP-C mimic recombinant SP-Cff (Venticute®)) have fallen out almost entirely because they were unstable on storage.

In this study, we measured the stability and shelf life of synthetic lung surfactant preparations consisting of 3% oxidized SMB in DPPC:POPC:POPG 5:3:2 (wt:wt:wt), in which fresh samples are compared to those stored at temperature of ~2°–8 °C for 1 to 5.5 years. This surfactant preparation has been found to be highly surface active in both biophysical and animal studies [6,10–12]. Unlike stored KL4 and recombinant SPCff formulations, captive bubble surfactometry confirmed low minimum surface tensions for fresh and stored SMB preparations. Furthermore, FTIR spectra of fresh and stored SMB formulations indicated secondary conformations compatible with SMB folding as a surface-active, α-helix-hairpin. Another contribution to SMB stability on prolonged storage may be the 'stapling' of the helix-hairpin with two disulfide bridges, as confirmed by both mass spectroscopy and I-TASSER homology modeling [13]. Lastly, mass spectroscopy indicated some oxidation of SMB-methionine and partial deacylation of lipids on storage. Nevertheless, the stable conformation and activity for stored SMB suggest that the active helix-hairpin resists these chemical changes by folding as a tight, globular structure in lipid.

2. Materials and methods

2.1. Materials

HPLC grade chloroform, methanol, and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA 15275), TFA from Sigma Chemical Co (Saint Louis, MO 63103) and Sephadex LH-20 chromatography gel from Pharmacia (Uppsala, Sweden). Phospholipids were supplied by Avanti Polar Lipids (Alabaster, AL 35007) and oleic acid was obtained from NU-CHECK PREP, Inc. (Elysian, MN 56028). Sodium Dodecyl Sulfate detergent was purchased from Sigma Chemical Co (Saint Louis, MO 63103).The Super Mini-B (SMB) peptide (amino acid sequence: NH2-FPIPLPYCWLCRALIKRIQAMIPKGGRMLPQLVCRLVLRCS-COOH) was synthesized employing a standard Fmoc protocol with a Symphony Multiple Peptide Synthesizer (Protein Technologies, Inc., Tucson, AZ 85714) or a CEM Liberty microwave synthesizer (CEM Corporation, Mathews, NC 28104), cleaved-deprotected and purified using reverse phase HPLC as described previously [6]. This protocol included folding of the peptide in a structure promoting trifluoroethanol-buffer solvent system to enhance the oxygen mediated disulfide linkages between Cys-8 and Cys-40 and a second linkage between Cys-11 and Cys-34 [6]. This covalently stabilized connectivity gave the peptide a helix-hairpin conformation, similar to that observed for the N-terminal and C-terminal helical domains of the saposin family of proteins [4].

2.2. Formulation and isolation of proteins and lipids from surfactant dispersions

Peptide and lipids were formulated as lipid-peptide dispersions to have a total of 3% by mole fraction of SMB and 35 mg of total lipid per ml of dispersion. The peptide was dissolved in 10 ml of trifluoroethanol and co-solvated with the lipid in chloroform, followed by removal of the solvents with a stream of nitrogen gas and freeze drying of the resulting lipid-peptide film to remove residual solvent. The film was then dispersed with Phosphate Buffered Saline and the sample flask containing the hydrated film was rotated for 1 h at 60 °C to produce a solution of multi-lamellar vesicles (MLVs) [6]. This dispersion was then stored at 4 °C for various periods of time prior to structure and compositional measurements and functional surface activity determinations. In order to determine the molecular mass of peptides formulated with lipids as a function of time, the SMB was separated from the lipid using normal phase chromatography with Sephadex LH-20 [14].

2.3. Analysis of surface activity by captive bubble surfactometry

Surface activity of the surfactant preparations was checked with a captive bubble surfactometer (CBS), which has been described in detail elsewhere [6,15]. Adsorption and surface tension lowering ability of surfactant preparations were measured at physiological cycling rate, area compression, temperature, and humidity. In brief, after inserting the bubble into the surfactant sample, adsorption of the surfactant to the bubble's air-liquid interface is monitored. After adsorption, the bubble chamber is sealed and quasi-static compression and expansion of the bubble is performed in discrete steps (at a rate of ~5% of bubble volume every 10 s) for 10 cycles. Images of the changes in bubble area are recorded during each experiment and the bubble shapes are analyzed with custom-designed software. The surface tension of the bubble is calculated on the basis of shape of the air bubble and minimum and maximum surface tension values are plotted for the first 10 cycles. We routinely insert surfactant samples of 1 μL (35 mg phospholipids/ml) into the bubble chamber with a volume of ~1.5 ml, i.e. at a concentration of ~20 μg/ml, and perform all measurements in quadruplicate.

2.4. MALDI TOF MS and HPLC analysis of SMB

Purified SMB samples were analyzed using an AB SCIEX TOF/TOF 5800 System (Sciex, Framingham, MA 01701). Samples (~50 pmol/μL) were co-solvated with either α-cyano-4-hydroxycinnamic acid or sinapic acid (10 mg matrix/ml water:acetonitrile 1:1, v:v with 0.3% TFA) by mixing 24 μL of matrix solution with 1 μL of peptide solution. Two μL of this mixture was then deposited onto a metal Maldi sample plate and allowed to air dry before mass spectral measurement. Mass spectra were collected using the instrument in positive linear mode. The resulting mass spectra were analyzed with AB SCIEX Analyst and Data Explorer software.

SMB peptide samples were analyzed using a Jasco binary preparative HPLC (Jasco, Easton, MD 21601) with PU-2087 pumps and a MX-2080-32 mixer. The instrument was fitted with a Vydac 219TP DiPhenyl 250 mm by 4.6 mm reverse phase analytical column (Grace Inc., Columbia, MD 21044) and run in the analytical mode with a flow rate of 3 ml/min. The samples were chromatographed with a linear elution gradient from water to 100% acetonitrile using 0.1% trifluoroethanol (v:v) as an ion pairing agent over a 1 h period. The elution of the peptide was monitored at 280 nm with a Jasco UV-2075 detector. The HPLC control and data collection was facilitated by a PC computer interfaced to the system using the Jasco ChromNAV Chromatography Data system software.