Interfacial Behavior of Spread SP-B and SP-C Layers and the Influence of Oligomerization and Secondary Structure

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Differences in the oligomerization state of the pulmonary surfactant protein SP-B as well as in the secondary structure of SP-C result in significant differences in the surface pressure/area isotherms. Different phase transitions in the surface pressure area isotherms are characteristic for these proteins indicated by kink-points. A new theoretical model was derived that takes into account a phase transition between the liquid expanded and condensed phase as well as several transitions caused by protein structure within the condensed phase of the spread layers. These transitions that are caused by the protein structure differ in the area/molecule. The experimental isotherms were satisfactory described in a wide range of surface coverage although the model takes into account only five independent parameters. Differences in the oligomerization state (dimers or trimers) of SP-B were reflected by a parameter that accounts for the number of kinetically independent units (fragments) in the molecule. This parameter was higher for SP-B samples with a large amount of trimers. The secondary structure (α-helical or β-structures) of SP-C was reflected by a parameter that accounts for the relative 2-dimensional compressibility of a condensed monolayer. The physical content of the model parameters is discussed.

Introduction

The surfactant proteins SP-B and SP-C are the most hydrophobic proteins in the alveolar lining fluid that could be obtained by lung washings. Therefore these proteins, SP-B and SP-C, decisively influence the interfacial behavior of the film that floats on the alveolar lining layer[1,2]

Recently, we showed that a difference in the oligomerization state of SP-B as well as in the secondary structure of SP-C significantly influences the surface pressure/area (Π/A) isotherms.[3] Oligomerization of SP-B and the secondary structure of SP-C depends on different protein/lipid concentrations and/or different solvents during the protein extraction procedure. The secondary structure of SP-B in surface layers is mainly α-helical, as shown by CD measurements at surfaces and by FTIR spectra. Differences, however, were observed in oligomerization, as measured by molecular weight determination. SP-C exists in α-helical or β-sheet conformation, and α-helix/β-sheet conversion of SP-C is caused by an increase of the summary protein/lipid concentration in solution. The Π/A isotherms determined for SP-C have more than two kink points.[3] The experimental data were roughly described by a model, which was able to fit discrete ranges of the isotherms.[4] This model is based on the assumption that molecules may occupy different definite areas for different phases at the interface. This assumption is quite reasonable for small molecules. However, for macromolecules and proteins the assumption of a large number of multiple phases seems to be improper.

In recent years mathematical models describing $\Pi-A$ isotherms were developed by us.[4−6] The earlier ones presume compressibility for the condensed phase of the monolayer,[5] whereas the newer model[4] agreed satisfactory with the experimental $\Pi-A$ isotherms possessing two or more critical points. In a recent work[6] the attempt was made to generalize the previous models.[4,5] The "new" model[6] was based on phase transitions for a monolayer for which the area per molecule in the condensed state exhibits either a linear decrease with increasing surface pressure or a sharp jump. The aim of the present paper is to generalize this model,[6] which was derived for insoluble monolayers such as lipids, for a protein layer. If such an approach would be suitable to describe the experimental data, we expect further information about processes taking place in adsorbed surface protein layers during compression and expansion. Furthermore, this paper should help to understand the physical meaning of the parameters used.

Materials and Methods

Materials. Chloroform (Ultra-Resi analyzed) was obtained from J. T. Baker (Griesheim, Germany). Methanol (LiChrosolv gradient grade) was obtained from Merck (Darmstadt, Germany). Water was purified by means of a Milli-Q Plus Water System (Millipore, Eschborn, Germany) and had a surface tension of 72.4 ± 0.2 mN/m at 23 °C, as determined by using the axisymmetric drop shape analysis for captive bubbles (ADSA-CB).[7] All glass vessels used for this study and the measuring cell were cleaned with KOH-saturated 2-propanol.

Isolation of SP-B and SP-C. The procedure of surfactant protein preparation and purification was described in detail earlier.[3] The main purification steps are given below to explain the differences between the protein samples that were used in this study.

Pulmonary surfactant was obtained from cell free sheep lung lavage. After differential centrifugation a pellicle was washed and homogenized in 4 mL of water and the hydrophobic surfactant components were extracted either into 1-butanol [8] or into chloroform/methanol.[9] The first residue contained the hydrophobic components of pulmonary surfactant from butanol

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TABLE 1: Isolation and Purification Procedures Used for SP-B and SP-C

protein sample	extraction solvent/isolation method/ volume of lipid extracts	protein storage concn (mg/mL)	oligomerization state	secondary structure at the interface
SP-B-1	butanol/LH 60 column/ 5 mL	0.04−0.06	dimers	α-helix
SP-B-2	chloroform- methanol/HPLC column/ 5 mL	0.8−1.0	trimers	α-helix
SP-B-3	chloroform-methanol/HPLC column/ 1.5 mL	0.8−1.0	trimers	α-helix
SP-C-1	butanol/LH 60 column/ 5 mL	0.04−0.06		α-helix
SP-C-2	chloroform-methanol/HPLC column/ 5 mL	0.8−1.0		α-helix + β-sheet
SP-C-3	chloroform-methanol/HPLC column/ 1.5 mL	0.8−1.0		antiparallel β-sheet

extraction (lipids, SP-B-1 and SP-C-1). Surfactant proteins were isolated using gel exclusion chromatography. SP-B-1 and SP-C-1 fractions were collected, and aliquots of the solutions with final concentrations of 0.04−0.06 mg/mL were stored at −20 °C. The final concentration was determined using the HPLC method as described elsewhere.[8] The second residue contained the hydrophobic components of pulmonary surfactant from chloroform/methanol extraction (lipids, SP-B-2, and SP-C-2). Surfactant proteins were isolated using a semipreparative HPLC column. SP-B-2 and SP-C-2 fractions were collected, the solvent was evaporated, and the purified proteins were weighted and then redissolved in chloroform/methanol (1:1, v/v) to give a final concentration of ∼0.8−1.0 mg/mL. Aliquots were stored at −20 °C. The third residue contained the hydrophobic components of pulmonary surfactant from chloroform/methanol extraction (lipids, SP-B-3, and SP-C-3). It was purified as described above. It should be emphasized that the only difference between residues 2 and 3 is the concentration of their protein/lipid extracts. Table 1 summarizes the three extraction and purification procedures that were used to obtain the various SP-B and SP-C samples.

Measurements of the Π/*A* Isotherms. The Π/*A* isotherms were determined by using a captive bubble device as a kind of microfilm balance and ADSA-CB. Methodical details are reported elsewhere.[3]

Theory

The equation of state for monolayers in the fluid (gaseous (G) or liquid-expanded (LE)) state is represented by Volmer's equation, see refs 4−6:

$$\Pi = \frac{nkT}{A - \omega} - \Pi_{coh} \tag{1}$$

In the phase coexistence region (LE and liquid-condensed (LC) state for $A < A_c$), the generalized Volmer's equation leads to

$$\Pi = \frac{nkT\alpha\beta}{A - \omega[1 + \epsilon(\alpha\beta - 1)]} - \Pi_{coh} \tag{2}$$

In these two equations, Π is the surface pressure, k is the Boltzmann constant, T is the temperature, ω is the partial molecular area for monomers (or the limiting area of molecule in the gaseous state), A is the area per molecule, A_c is the molecular area which corresponds to the onset of the phase transition (i.e., at $\Pi = \Pi_c$), Π_{coh} is the cohesion pressure, which accounts for the intermolecular interaction, and n is the number of kinetically independent units (fragments or ions) in a molecule.[4] The parameter α expresses the dependence of the aggregation constant on the surface pressure, and β is the fraction of the monolayer free from aggregates:

$$\alpha = \frac{A}{A_c} \exp\left[-\epsilon \frac{\Pi - \Pi_c}{kT} \omega\right] \tag{3}$$

$$\beta = 1 + \omega(1 - \epsilon)(\alpha - 1)/A \tag{4}$$

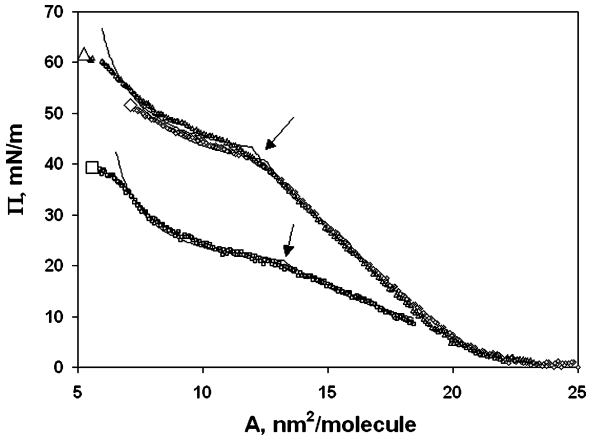

Figure 1. Π/*A* isotherms of the SP-B-(1,2,3). Experimental results shown by symbols: (□) SP-B-1; (◇) SP-B-2; (△) SP-B-3. The theoretical curves (lines) were calculated by using eqs 1−5, and the corresponding parameters are given in Table 2.

where $\epsilon = 1 - \omega_{cl}/\omega$, and ω_{cl} is the area per one monomer in a cluster.

The area per molecule in the cluster can differ from the limiting area per molecule in the gaseous state. This fact is taken into account by the parameter $\epsilon = \epsilon_0 + \eta\Pi$:

$$\omega_{cl} = \omega(1 - \epsilon) = \omega(1 - \epsilon_0 - \eta\Pi) \tag{5}$$

where ϵ_0 is the relative jump of the area per molecule, η is a relative 2-dimensional compressibility of the condensed monolayer.[6]

The theoretical model expressed by eqs 1−5 for protein monolayers leads to high values for n, because protein molecules exhibit a high value of kinetically independent fragments. Notice, the theory for spread monolayers and adsorption layers is quite similar. The equation of state for adsorption layers given in ref 10 for the range of one- and two-phase transitions of monolayers approximately follows eqs 1 and 2, whereas, however, $n = \omega/\omega_0$, with ω being the area per protein and ω_0 the area per solvent molecule, which is approximately the area per amino acid residues. Therefore eqs 1−5 may be used to analyze protein surface films with one or more phase transitions, which are connected with molecular area changes or different stages of deformation of the 2-dimensional structures.

Results and Discussion

The surface pressure/area isotherms of SP-B-(1,2,3) are presented in Figure 1. These isotherms have only one (main) phase transition point (shown by the arrows). The theoretical curves calculated by using of eqs 1−5 and the values of the parameters given in Table 2 agree quite well with the experimental data. The values of the parameters for layers of SP-B in different forms only slightly differ. The exception is the parameter n, the number of kinetically independent units (fragments or ions) in a molecule, which are 60 for SP-B-1 but increase to 90 for SP-B-2 and SP-B-3. The higher n, the higher

TABLE 2: Characteristics of the SP-B-(1,2,3) Monolayers

parameters	SP-B-1	SP-B-2	SP-B-3
ω, nm^2	5.0	5.3	5.0
A_c, nm^2	13.2	12.5	12.0
Π_c, mN/m	21.0	40.8	43.2
η, m/mN	0.00	0.00	0.00
n	60	90	89
Π_{coh}, mN/m	8.26	10.3	8.35

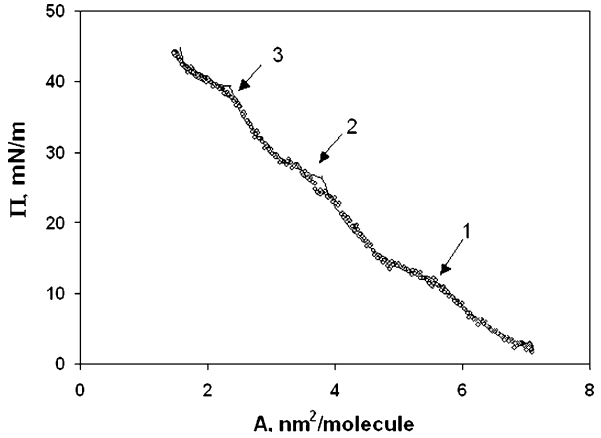

Figure 2. Π/*A* isotherms of the SP-C-1. Experimental results shown by symbols (○). Theoretical curves (lines) were calculated by using eqs 1−5, and the corresponding parameters are given in Table 3. Further symbols are explained in the text.

becomes the slope of the isotherm in the range of the LE phase. The molecular area of a kinetically independent fragment of a protein molecule in the LE phase, i.e., ω_0, ranges between 0.083 and 0.056 nm^2 for SP-B. This is approximately twice as much as an area calculated for one amino acid group.[3] This means that not all amino acid groups are kinetically independent. Gel chromatography data and HPLC chromatograms suggest that the SP-B-1 sample consists mostly of dimers and probably few trimers, whereas the SP-B-2 and SP-B-3 samples are mostly trimers (30% dimers).[3] The present data of the model parameters show that the amount of kinetically independent fragments in dimers is obviously lower than in trimers.

Another result derived from applying the theoretical model on surface pressure/area isotherms of SP-B-(1,2,3) is given by Π_c. This surface pressure at nearly identical surface coverage for all SP-B samples (see Table 2) is nearly twice as much for SP-B-2 and SP-B-3 compared to SP-B-1 and probably also due to the higher amount of kinetically independent units in trimers.

There are different phase transitions for SP-C-(1,2,3). Using SP-C-1 three phase transition points were obtained (one main phase transition and two different ones related to the 2-dimensional structures). SP-C-2 has two phase transitions, one main phase transition and one kink point, which distinguishes two 2-dimensional structures. Possibly there is also a third phase transition at higher surface pressure. However, because of data scattering this wing of the isotherm was not evaluated. SP-C-3 has only one main phase transition and differs strongly from SP-C-1 and SP-C-2.

The experimental and the calculated isotherms are given in Figures 2−4. The parameters for eqs 1−5 are collected in Table 3. Figure 2 shows the experimental and the calculated isotherms of SP-C-1. The arrows show the phase transition points. The theoretical curves agree well with the experimental data.

For the first phase transition (main) the value of the relative 2-dimensional compressibility $\eta = 0$. For the second structure-determined transition $\eta = 0.002$ m/mN. For $\Pi_c = 28.2$ mN/m the area per molecule decreases from 2.9 to 2.74 nm^2, i.e., by

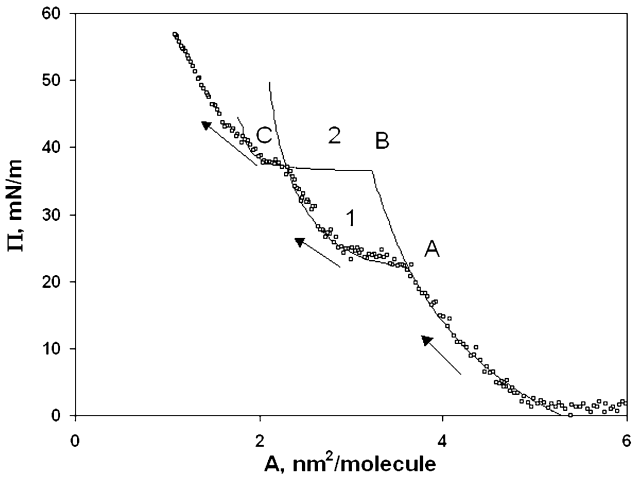

Figure 3. Π/*A* isotherms of the SP-C-2. Experimental results shown by symbols (□). Theoretical curves (lines) were calculated by using eqs 1−5, and the corresponding parameters are given in Table 3. Further symbols are explained in the text.

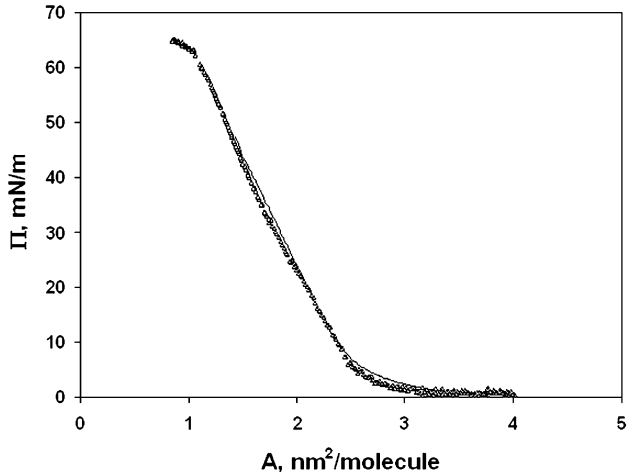

Figure 4. Π/*A* isotherms of the SP-C-3. Experimental results shown by symbols (△). Theoretical curves (lines) were calculated by using eqs 1−5, and the corresponding parameters are given in Table 3.

TABLE 3: Characteristics of the SP-C-(1,2,3) Monolayers

parameters	SP-C-1	SP-C-2	SP-C-3
ω, nm^2	2.9/1.7[b]	2.0	2.5
A_c, nm^2	5.53/4.62[a]/3.66[b]	3.62/3.22[a]	4.0−5.0
Π_c, mN/m	12.1/28.2[a]/38.9[b]	22.2/36.6[a]	0.0−1.0
η, m/mN	0.0/0.002[a]/0.0023[b]	0.0004/0.0025[a]	0.01
n	18	18	16
Π_{coh}, mN/m	14.8/18.3[b]	21.6	26.2−42.0

[a] Parameters of the second (structural) phase transition [b] Parameters of the third (structural) phase transition.

0.16 nm^2. This is the reason for the second phase transition of SP-C-1. For the third phase transition $\eta = 0.0023$ m/mN and negligibly differs from that of the second phase transition. At the same time, however, the area per molecule SP-C-1 strongly decreases (the extrapolated area for $\Pi_c = 0$ becomes 1.7 nm^2). Therefore, the reason the phase transitions observed for SP-C-1 as well as that for SP-C-2 (see below) is a decrease of the area per molecule in the condensed state and the corresponding coefficient of the 2-dimensional compressibility of the protein molecules.

A Π/*A* isotherm of the sample SP-C-1 was previously described by using a more simple model.[4] When the parameters of these two models are compared, some differences in the

model parameters appear. The values of A_c are in good agreement with those reported previously.[4] The reason for the differences of the other parameters is that in the previous work we chose a limited fitting interval due to the theoretical model, from 4.1 to 8 nm^2. Therefore the resulting parameter ω became correspondingly high, i.e., 4.1 nm^2. In contrast, in the present approach we describe with only one set of parameters (ω, n, and Π_{coh}) the whole range of the LE monolayer, 2.4−8 nm^2. Only in the range of small areas per molecule, less than 2.4 nm^2, was it necessary to choose a lower value for ω. This is an advantage of the present approach.

The surface pressure/area isotherm for SP-C-2 is presented in Figure 3. In addition to the main phase transition (indicated by the point A, curve 1), another (structural) phase transition appears, indicated by point C. To describe the second phase transition for SP-C-2, a value $\eta = 0.0025$ m/mN for the relative 2-dimensional compressibility of the condensed monolayer was assumed.

Both theoretical curves describing the second phase transition by using the model parameters given in Table 3 agree well with the experimental data.

The behavior of any thermodynamic system is determined by its stability condition. The relevant criterion for monolayers is $d\Pi/dA \leq 0$;[11] i.e., not only the surface pressure should decrease with area per molecule, but also this dependence should possess a minimum slope to be thermodynamically stable. Arrows in Figure 3 show the thermodynamically stable course of the SP-C-2 isotherm. The arrow at the right of the point A corresponds to the fluid (LE) state of the monolayer. Of the two possible routes in point A, the system follows the route along curve 1 across the phase coexistence region, i.e., the route characterized by the lower slope of the isotherm. If the system would follow route 2 it would conserve invariable numbers of components in the monolayer $N_0 = \text{const}$. However, the system follows route 1 with an invariable chemical potentials of components at point C, i.e., $\mu = \text{const}$. This is in accordance with the theory, i.e., $d\Pi/dA_{No=const} \leq d\Pi/dA_{\mu=const}$. Special cases may occur where route 2 describes the thermodynamic behavior. Such a case may occur when the monolayer is compressed faster than the speed of nucleation and a new 2-dimensional phase appears. Then thermodynamically unstable states can arise. In such cases the system, when reaching point A, follows curve 2 (the state of the monolayer corresponds to a layer that is oversaturated with monomers up to point B, which arises due to the finite nucleation rate and growth of the nuclei). Then the system returns to curve 1. Examples of such dependencies were reported by Vollhardt et al.[12] In point C the system also begins to proceed along the branch corresponding to the condensed state of larger density, which possesses the lower slope.

The area per SP-C-2 molecule is 1.87 nm^2, in agreement with eq 5 for $\eta = 0.0025$ m/mN and $\Pi_c = 36.6$ mN/m. For the first phase transition $\eta = 0.0004$ m/mN and the area increases to 1.97 nm^2. Therefore the reason for the second phase transition is a change in the 2-dimensional monolayer structure, which leads to a decrease of the area per molecule in the condensed state by 0.1 nm^2 corresponding to the large compressibility for this state.

There is no plateau observed for SP-C-3 (Figure 4). The shape of the curve is typical for a coexistence of LE-LC phases in a wide range of surface coverage with starting condensation at considerable high area per molecule, approximately at 4−5 nm^2 (Table 3). Only one (main) phase transition and a high monolayer compressibility coefficient ($\eta = 0.01$ m/mN) is characteristic for the condensed SP-C-3. Therefore the area per SP-C-3 molecule in a condensed layer decreases from 2.5 to 1 nm^2 when the surface pressure increases from 0 to 60 mN/m. The cohesion pressure of SP-C-3 in β-sheet conformation is higher than the cohesion pressure of SP-C-1 in α-helical conformation (Table 3), which is consistent with the higher tendency of this protein to aggregate.

The parameter n is obviously nearly the same for all modifications of SP-C, i.e., $n = 16-18$. This leads to an area per kinetically independent fragment in the LE monolayer (ω_0 in eq 2) in the range between 0.16 and 0.11 nm^2. This value is twice as much as that found for SP-B and 2−3 times higher than the area calculated per amino acid residue. Therefore less than half of the amino acid groups of SP-C are kinetically independent and influences the surface pressure.

The new introduced relative 2-dimensional compressibility parameter of the condensed monolayer has a surface rheological meaning. In recent works we reported experimental results for the surface dilatational elasticity for SP-B[13] and SP-C,[6] with surface dilatational elasticity roughly equal a reciprocal compressibility. These samples of surfactant proteins were extracted into chloroform/methanol and therefore correspond to SP-B-2 and SP-C-1 used here; i.e., all proteins mainly exhibit α-helical structure.

Using the values of η given in Table 3 to estimate a corresponding elasticity, these calculated elasticities become very high. They exceed the experimental dilatational elasticities found for SP-C-1[6] strongly. Actually, the relation between surface rheological parameters is complex. The problem is that these parameters are not constants of matter but depend on the stress or strain regime. In particular, the surface behavior of protein layers is mainly viscoelastic and therefore cannot be adequately described by using only one parameter, i.e., elasticity or viscosity. Furthermore, the time scales of different processes are not easily comparable. The experimental dilatational parameters of SP-B and SP-C were determined for a sinusoidal oscillation of the surface with small amplitude in a finite frequency range. In contrast, the Π/A isotherms used in the present paper are determined by a linear deformation of the surface under quasi-equilibrium conditions. Principally, the parameters determined for one deformation regime can be transformed into another, but only when the frequency dependence of these parameters is well-known even for border conditions. Especially for proteins there are different relaxation processes that influence the parameters determined for different time scales. Therefore the experimentally determined dilatational elasticity of SP-B-2 ranges between 50 and 220 mN/m and is nearly independent of the frequency between 6×10^{-3} and 2×10^{-1} Hz and a surface pressure between 10 and 55 mN/m.[13] Nevertheless, with the present model we were able to describe the whole Π/A isotherms by neglecting any influence of a relative 2-dimensional compressibility of the condensed monolayer.

For SP-C-1 the experimentally determined dilatational elasticity ranges between 40 and 90 mN/m in the frequency range 6×10^{-3} and 2.5×10^{-2} Hz and surface pressures between 10 and 35 mN/m.[4] At the same time we used model compressibility parameters for the condensed phase between 0.002 and 0.0023 m/Nm. The reciprocal values of these parameters are much higher than the experimentally determined elasticities. Therefore the independently determined experimental elasticities cannot be straightforward used to confirm our model. Vice versa, compressibilities calculated by using Π/A isotherms cannot be used to simulate a definite dynamical process, for instance breathing.

When comparing the compressibility parameters of the three SP-C samples those of SP-C-1 and SP-C-2 are much lower than that of SP-C-3. The general slope of the isotherm and considering β-sheet conformation for SP-C-3 would have expected the opposite. This finding shows that the relative 2-dimensional compressibility parameter of the condensed phase in a monolayer does not contain the whole complexity of the compression behavior but is related to a definite range of the isotherm. Therefore, the use of this compressibility parameter is limited. The necessity of using a compressibility parameter to describe SP-C in contrast to SP-B explains in principle that the experimentally determined maximum elasticities for SP-B are in general higher than those obtained for SP-C. The proposed model correctly reflects this experimental result.

Conclusions

A new theoretical model was applied to fit experimental surface pressure/area isotherms of different samples of the surfactant proteins SP-B and SP-C. The basic idea of the model is that the protein molecules at the interface can occupy different areas per molecule depending on the kind of phase, liquid expanded or liquid condensed, but also depending on their conformation, which in turn is influenced by oligomerization and secondary structure of the protein molecules at the interface. We have shown previously that oligomerization of SP-B and the secondary structure of SP-C are influenced by the protein extraction and purification procedures. Three different samples of each protein were characterized concerning oligomerization and secondary structure and used in this study.

The secondary structure of SP-B in spread layers was always α-helical. SP-B-1 consisted of mainly dimers and SP-B-2 and SP-B-3 were dominantly trimers. The surface pressure at nearly identical surface coverage of the liquid-condensed phase is approximately twice as much for SP-B-2 and SP-B-3 when compared to SP-B-1. The presented model reflects this effect by a higher amount of kinetically independent units in SP-B samples that contain trimers.

The SP-C samples differed only in their secondary structure. SP-C-1 was almost α-helical, SP-C-3 showed only β-sheet structure, and SP-C-2 was a mixture of α-helical and β-sheet structure. The interfacial behaviors of the samples SP-C-1 and SP-C-2 were characterized by one main phase transition (LE/LC transition) and several transitions caused by conformational changes of the protein. In contrast, SP-C-3 had only one main phase transition. The differences in the secondary protein structure at the interface were best reflected by the relative 2-dimensional compressibility parameter of the condensed monolayer that increases with surface pressure. This compressibility parameter contains some features that explain the higher surface elasticities of SP-B compared with SP-C. Nevertheless, this parameter does not directly correlate with the experimentally determined dilatational elasticities.

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