Selective Labeling of Pulmonary Surfactant Protein SP-C in Organic Solution

Inés Plasencia,* Antonio Cruz,* José Luis López-Lacomba,† Cristina Casals,† and Jesús Pérez-Gil* 1

*Departamento de Bioquímica, Facultad de Biología, and †Instituto Pluridisciplinar, Universidad Complutense, 28040 Madrid, Spain

Received February 2, 2001; published online August 2, 2001

Pulmonary surfactant protein SP-C has been isolated from porcine lungs and treated with dansyl isothiocyanate in chloroform:methanol 2:1 (v/v) solutions, under conditions optimized to introduce a single dansyl group covalently attached to the N-terminal amine group of the protein without loss of its native thioester-linked palmitic chains. The resulting derivative Dans-SP-C conserves the secondary structure of native SP-C as well as the ability to promote interfacial adsorption of DPPC suspensions and to affect the thermotropic behavior of DPPC bilayers. This derivative can be used to characterize lipid-protein and protein-protein interactions of a native-like SP-C in lipid/protein complexes.

Key Words: site-directed chemical modification; lipid-protein interactions; fluorescence spectroscopy; protein palmitoylation.

Pulmonary surfactant protein SP-C is a lipopeptide of 35 amino acids, including two palmitoylated cysteines, which is copurified with lipids after organic extraction of alveolar lavage from lungs (1, 2). It is widely assumed that SP-C has a role in the biophysical activity of surfactant, specially in facilitating formation of surface-active films at the air-liquid interface of the respiratory epithelium which are ultimately responsible for reducing surface tension and stabilizing the lungs during respiratory dynamics (3, 4). Numerous studies have approached the characterization of structure and lipid-protein interactions of SP-C in phospholipid bilayers (5–11) and monolayers (12–17) as an attempt to understand the role of the protein in the surfactant system from a molecular point of view. SP-C lacks intrinsic fluorophores such as tryptophan or tyrosine in its sequence, preventing the use of UV spectroscopy in the characterization of structure and dynamics of the native protein. This approach has been extensively used to study the other hydrophobic protein in surfactant, SP-B (18–20). An alternative is the site-selective introduction of an extrinsic fluorescent probe in SP-C, which may allow identification of structural features that could be directly assigned to local regions in the protein (21). Characterization of lipid-protein interactions of SP-C by other techniques such as epifluorescence microscopy (22, 23) or electron spin resonance (24) has also required introduction of extrinsic probes in proper locations of the protein, without producing significant alterations of the native conformation. However, the procedure to derivatize SP-C in organic solutions has not been optimized, specially to ensure preservation of the palmitic chains bound to the structure of SP-C via thioester linkages, and also to produce incorporation of single groups in selective sites.

In the present work we have optimized the chemistry to introduce a single fluorescent dansyl group covalently attached to the N-terminal end of porcine SP-C, without any further structural modification of the protein. The fluorescent properties of the dansyl group are critically dependent on the polarity of the environment, which makes it particularly useful for detecting and characterizing insertion of protein segments into membrane environments or their implication in protein-protein interactions (25, 26).

1 To whom correspondence should be addressed at Dept. Bioquímica, Fac. Biología, Universidad Complutense, 28040 Madrid, Spain. Fax: 34 91 3944672. E-mail: perejil@solea.quim.ucm.es.
EXPERIMENTAL PROCEDURES

Materials. Chloroform (Chl) and methanol (MeOH) were HPLC-grade solvents from Scharlau (Barcelona, Spain). The lipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), egg yolk phosphatidylcholine (PC), and 1-palmitoyl-2-lysophosphatidylcholine (LPC) were from Avanti Polar Lipids (Birmingham, AL). The fluorescent reagent dansyl isothiocyanate (dans-ITC) was obtained from Molecular Probes (Eugene, OR). Sephadex LH-20 and LH-60 chromatography gels were from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Protein purification. Surfactant protein SP-C was isolated from porcine lungs by a modification of the method published by Curstedt et al. (27), which has been discussed elsewhere (28). The protein was routinely checked for purity by SDS-PAGE, and for residual phospholipid contamination by phosphorus determination. Quantification of isolated protein was carried out by amino acid analysis as previously described (28), after acid hydrolysis during 24 h of phospholipid bilayers or micelles of 5.7 N HCl at 108°C, using the estimated content in Asx, Ala, Lys, and Arg and the amino acid composition reported for porcine SP-C (1). After isolation, SP-C was stored as chloroform:methanol (2:1, v/v) solution at −20°C until use.

Protein labeling. One milligram of pure SP-C in chloroform:methanol 2:1 (vol/vol) was adjusted to an apparent pH of 7–7.2 by addition of the appropriate volume of a methanolic 50 mM Tris solution. This SP-C solution was incubated at 4°C overnight, in darkness, in the presence of Dans-ITC (0.6 mg/mL, final concentration). The reaction was stopped by addition of HCl until the pH decreased to 2. The unreacted probe was removed by Sephadex LH-20 chromatography, the elution profile of which was monitored by measuring absorbance of the fractions at 240 and 330 nm. Labeled protein was stored as chloroform:methanol (2:1, vol/vol) solutions, in darkness at −20°C, until use.

Electrophoresis. Purity of isolated pools of porcine SP-C and oligomerization state of SP-C after deacylation or dansyl treatment was checked by SDS electrophoresis using stacking and running gels of 4 and 18% acrylamide, respectively. Electrophoresis was performed in a Mini-Protean II (Bio-Rad) chamber and the protein bands were detected by silver staining.

Mass spectrometry. The state of acylation of native and derivatized SP-C was analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The laser desorption/ionization experiments were performed on a BIFLEX time-of-flight instrument (Bruker-Franzen Analytik, Bremen, Germany) operated in positive mode. A saturated solution of sinapinic acid in acetonitrile:water (1:2) with 0.1% TFA was used as the matrix. Equal volumes of the matrix and the sample solution were put together and vortexed, and 1 μl of the mixture was spotted on the target and air-dried. Samples were analyzed in the linear mode, and typically 100 laser shots were summed into a single mass spectrum. External calibration was performed, using myoglobin as the standard.

Lipid/protein reconstitution. To reconstitute SP-C and dans-SP-C in phospholipid bilayers or micelles of lysophospholipid, the lipids (DPPC, PC, LPC) were mixed with the appropriate amount of protein in Chl: MeOH 2:1 (v/v) and the mixture was dried under a N2 stream and then under vacuum overnight. The resulting dried films were then hydrated by addition of 1 ml of 50 mM Hepes buffer, pH 7, containing 150 mM NaCl, and incubated for 1 h, with eventual vortexing, at room temperature for PC and LPC or 50°C for DPPC. In the presence of phospholipids this treatment typically produces multilamellar vesicles (MLV). To obtain small unilamellar phospholipid vesicles (SUV) or lysophospholipid micelles the lipid or lipid/protein suspensions were sonicated in a Branson UP200S tip sonifier at 360 W/cm² during three cycles of 30 s at intervals of 30 s to avoid sample heating.

Surface activity. To compare biophysical activity of native and dansyl-labeled SP-C, 300 nmol of a DPPC multilamellar suspension, prepared in the absence or in the presence of 10% (w/w) of the respective protein, was dispersed with continuous stirring in 6 ml of a hypophase Hepes 50 mM, pH 7, containing 150 mM NaCl and 5 mM CaCl2. Interfacial adsorption of the samples was monitored over 45 min by measuring the changes in surface pressure with a Wilhelmy plate connected to a pressure transducer (29, 30).

Differential scanning calorimetry. Dried samples of 1 mg of DPPC in the absence or the presence of 2, 5, or 10% (w/w) of SP-C or Dans-SP-C were dispersed in 1 ml of Hepes 50 mM, pH 7, containing 150 mM NaCl, to form multilamellar suspensions. The amount of 0.47 ml of this suspension was loaded in the sample cell of a microcalorimeter DASM-4, with buffer in the reference cell. Three calorimetric scans were collected from each sample between 25 and 60°C, at a rate of 0.5°C/min, as previously described (31).
Circular dichroism. Far-UV circular dichroism spectra of lipid/protein samples were recorded as described in a Jasco 715 spectropolarimeter equipped with a xenon lamp (32). Amounts of 50–75 μg of protein were reconstituted in different phospholipids to a final protein:lipid ratio of 1:5 (w/w), in 0.7 mL of buffer, Hepes 50 mM, pH 7, 150 mM NaCl, to form multilamellar suspensions which were then sonicated to form SUVs, in order to minimize problems with scattering. The final protein concentration in each sample was checked by amino acid analysis. All the spectra were recorded in a 0.15 mL cell of 0.1-cm optical path. Ellipticity was calculated taking 106 as the mean molecular weight per residue in SP-C.

All the data presented in this work are representative of two repeated experiments carried out with a single batch of purified and labeled SP-C. Labeling and characterization of structure and surface activity were repeated with three different batches of protein, and the results were qualitatively and quantitatively comparable.

RESULTS

Derivatization of amine groups in SP-C requires treatment of the protein at mild alkaline pH, to ensure deprotonation and reactivity of the amine functions (21–24). On the other hand, incubation of the protein at basic pH may produce cleavage of the labile thioester bonds that attach palmitic chains to the two cysteines in the N-terminal segment of the protein (2, 13). The main objective of the present study was to optimize the attachment of an extrinsic fluorescent probe in SP-C, via amine derivatization, but in a manner that would preserve the state of acylation of the protein. Figure 1A presents the effect of incubating different concentrations of purified SP-C in organic solution, for 1 h at 30°C, after adding a trace of bicarbonate buffer that shifts the apparent pH to 9.5. Before alkaline treatment, pure SP-C migrates in electrophoresis gels as a broad band with mobility corresponding to around 5–5.5 kDa (Fig. 1A, lane 9). Samples of SP-C, adjusted to alkaline pH at protein concentrations lower than 8 μg/mL, migrate in the gels as a main band of 4 kDa, probably corresponding to the deacylated monomer. If SP-C undergoes alkalinization at protein concentrations higher than 12 μg/mL, increasing amounts of bands corresponding to n-mers of the protein show up in the gels, probably as a consequence of covalent intermolecular disulfide oligomerization following deacylation. We have analyzed the dependence of deacylation on pH by quantitating the amount of dimer formed after treatment of a solution of SP-C 30 μg/mL at different apparent pH's (Fig. 1B). Deacylation of SP-C seems to occur under our conditions with an apparent pK of about 8. A conclusion for this experiment is that chemical modification of amines in SP-C should be performed at pH below 8, to prevent simultaneous deacylation of the protein. Figure 2A shows the electrophoretic behavior of a sample of SP-C treated with 1.6 mM dansyl isothiocyanate at pH 7.5 and 4°C, over-
night. The treated protein has a decreased electrophoretic mobility corresponding to an apparent 7- to 7.5-kDa band that was fluorescent when observed under UV illumination (Fig. 2A, lane 3). This indicates that SP-C incorporates covalently bound dansyl groups. The relative fluorescence incorporated in SP-C as a function of pH is shown in Fig. 2B. The experimental data could be reasonably well fitted to a theoretical curve in which 50% of the maximum labeling would be incorporated with $pK_1 = 6.5 \pm 0.2$ and further 50% with $pK_2 = 7.7 \pm 0.1$. These two $pK$ values can probably be assigned to the apparent ionization $pK$'s of the N-terminal amine and the $e$-amine of lysine 11, respectively, which are the two unique groups in SP-C susceptible to modification by the isothiocyanate. The relatively low value determined for these $pK$'s is probably a consequence of the low polarity of the protein environment in the organic solution.

Comparison of the pH-dependence curves for labeling and deacylation suggests that chemical derivatization at pH 7 could be the most favorable condition to label SP-C without loss of its acyl chains.

According to these results the conditions described under Experimental Procedures were selected to label a batch of protein. Quantitation of the amount of label bound to SP-C was performed after amino acid analysis by spectroscopic determination (molar extinction coefficient of dansyl determined in Chl:MeOH (2:1, v/v), $\epsilon^{330} = 9.23 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$), yielding 0.6–0.8 mol of dansyl per SP-C molecule. Protein sequence analysis by Edman degradation of SP-C treated with Dans-ITC could liberate only 24% of the amino terminal residue of Dans-SP-C, confirming that around 75% of the SP-C molecules treated had a dansyl label attached to the N-terminal amine group. The amount of Dans-ITC used in the labeling reaction corresponded to a protein/label molar ratio of 1/70. Labeling with 1 mol of dansyl per mole of protein was optimal when using above 1/20 protein/probe molar ratio. We also compared dansyl incorporation to SP-C when the reaction mixture was incubated at 4°C or at 25°C and found that incubation at 4°C in darkness produced the highest fluorescence incorporation into the protein bands as observed by PAGE (data not shown). Protection of dansylated protein from light during labeling and storage was essential to minimize probe photobleaching.

Mass spectrometry analysis of SP-C treated with Dans-ITC at pH 7 (Fig. 3) confirms that this treatment does not cause deacylation. The mass profile shows a peak at 4553 Da corresponding to native dipalmitoylated SP-C (2) plus a single extra dansyl group of 383 Da, a peak at 4170 Da (dipalmitoylated SP-C), and another peak at 3960 Da (monopalmitoylated protein). The dansylated fraction, consisting of 75% of the protein, produces apparently lower peaks in the spectrograms than the nonlabeled species, indicating that the dansylated protein must be less efficiently volatilized than nonlabeled SP-C under the conditions used to carry out the MALDI mass analysis. The large shift observed in electrophoresis mobility upon dansylation of the protein (seen in Fig. 2) is more probably due to effects of the incorporated probe in conformation and/or charge/mass ratio of the protein/SDS complexes than to the real difference in mass between labeled and native proteins.

Figure 4 compares the fluorescence emission spectrum of Dans-SP-C in Chl:MeOH (2:1, v/v) with that of the free probe in the reagent Dans-ITC. Both spectra are virtually identical, with fluorescence emission maxima at 515 nm, suggesting that the fluorophore in

![FIG. 3. MALDI mass spectra of porcine SP-C after treatment with Dans-ITC under the conditions described under Experimental Procedures. The peak at 4553 corresponds to the monodansylated dipalmitoylated protein. The major peak of 4170 Da is from the dipalmitoylated native protein. The peaks at 3960 and 3957 Da correspond to partially deacylated SP-C forms (see main text).](image)

![FIG. 4. Fluorescence emission spectrum of the dansyl group of Dans-ITC (A) and Dans-SP-C (B) in chloroform:methanol 2:1, vol/vol. The spectra were obtained at a fluorophore concentration of 12 $\mu$M. Fluorescence was measured at 25°C upon excitation at 330 nm and presented in arbitrary units (a.u.).](image)
Dans-SP-C is completely exposed to the organic environment. This feature is expected considering that in the NMR structure determined for SP-C in chloroform solution (33), the N-terminal segment of the protein is flexible and conformationally disordered.

We then analyzed if the labeling reaction altered somehow the structure of the native protein. Figure 5 compares the far-UV circular dichroism spectra of native SP-C and the fluorescent derivative Dans-SP-C. The spectra of the two proteins are virtually indistinguishable when reconstituted in either lysophosphatidylcholine micelles or PC vesicles. All the spectra are dominated by the \(\alpha\)-helical contribution to the secondary structure which was around 60% for the two proteins in LPC and about 70% in phospholipid vesicles, as previously reported (28). The labeling reaction has not therefore perturbed the overall conformation of the protein, which is very sensitive to environmental perturbations during manipulation such as changes in solvent polarity, pH, or protein concentration (5, 18, 34), but also to chemical deacylation (5, 35). Dans-SP-C also conserves the ability of the native protein to promote interfacial adsorption of phospholipids, as seen in Fig. 6. Native or dansylated SP-C produced quantitatively similar adsorption kinetics of DPPC multilamellar suspensions to an open air-liquid interface, and were also comparable to others previously shown (14, 29, 36). Finally, to find out whether the mode and extent of interaction of Dans-SP-C with phospholipids were comparable to those of the native protein, we have compared the effect of Dans-SP-C and native SP-C on the thermotropic behavior of DPPC bilayers. Figure 7 presents differential scanning calorimetry thermograms obtained from DPPC multilayer suspensions prepared in the absence and in the presence of 2, 5, or 10% of either native or dansylated SP-C. Both proteins produced similar effects on the phase transition of DPPC bilayers, including removal of the pretransition, progressive broadening of the main transition peak, and a concomitant decrease in the associated enthalpy.

**FIG. 5.** Far-UV CD spectra of porcine SP-C before (closed circles) and after (open circles) treatment with Dans-ITC under the conditions described under Experimental Procedures and reconstituted in micelles of LPC (left panel) or egg yolk PC vesicles (right panel). Symbols represent the experimental data, resulting from averaging four spectra.

**FIG. 6.** Interfacial adsorption isotherms for DPPC bilayers in the absence and in the presence of 10% by weight of native porcine SP-C or Dans-SP-C prepared after treatment of the native protein with Dans-ITC under the conditions described under Experimental Procedures. Dispersions containing 300 nmol of DPPC were injected into 6 mL of subphase Hepes 50 mM, pH 7, containing NaCl 150 mM and 5 mM CaCl\(_2\), under continuous stirring. Temperature was 24°C.

**FIG. 7.** Differential scanning thermograms of DPPC suspensions in the absence or presence of 2, 5, or 10 wt% of either native porcine SP-C (left panel) or Dans-SP-C (right panel) prepared after treatment of the native protein with Dans-ITC under the conditions described under Experimental Procedures. Scans were obtained on heating at 0.5°C/min. The scans presented were the third after recording three consecutive scans from 25 to 60°C from each sample, all of them being qualitatively similar.
measured as the area under the main peak. Neither of the two proteins produced a significant shift of the phase-transition temperature of DPPC multilayers. Table 1 summarizes the parameters of the calorimetric thermograms of DPPC in the absence and presence of either SP-C or Dans-SP-C. The reduction of the enthalpy as a function of the DPPC/Dans-SP-C molar ratio (Fig. 8) was similar to that previously published for native SP-C (10).

**DISCUSSION**

The two amine groups in the sequence of the N-terminal segment of porcine SP-C, the N-terminal amine of Leu1 and the ε-amine of Lys11, are probably the most susceptible groups to chemical derivatization. In the past we used amine-reactive reagents to introduce different groups in SP-C, such as visible-emission fluorescent (22, 23) or spin (24) probes. Derivatization of amines is critically dependent on pH as they are good nucleophiles only in their nonprotonated state. This feature allows a certain selectivity of derivatization as one can in principle selectively label the N-terminal amine of the protein at less alkaline pH than that of the amine of lysine side chain. This is a common procedure for derivatizing proteins and peptides in aqueous solutions but it is not trivial when labeling proteins in organic solvents. We have labeled amines in SP-B and SP-C by adding traces of buffer to the protein solution in chloroform/methanol, so allowing a certain control of apparent pH (22–24). However, alkaline pH can produce deavage of the labile thioester bonds which maintain the two cysteines of SP-C palmitoylated (2, 22) and, in fact, we could not avoid total or partial depalmitoylation of the protein in previous studies. A proper characterization of the structure and lipid-protein interactions of the N-terminal segment of SP-C requires maintaining the protein in the palmitoylated form. Here, we present a method that produces a dipalmitoylated form of SP-C which has a dansyl group covalently attached to the N-terminal amine. The efficiency of label incorporation is limited, but an increase of labeling efficiency cannot be achieved without partial deacylation. The secondary structure of the protein is not affected by the derivatization. In addition, some important properties of native SP-C, such as the ability of the protein to promote interfacial adsorption of phospholipids and the effect of the protein on the thermotropic properties of phospholipid bilayers, remain unaltered after the dansylation process. Therefore we can assume that the Dans-SP-C derivative prepared here can be confidently used to find out information about parameters governing lipid-protein and protein-protein interactions by fluorescence spectroscopy.

Dansyl-derivatives have been widely used to monitor and characterize the interaction and insertion of proteins and peptides into membrane environments (25, 26, 37, 38). Both, the quantum yield and the wavelength of the emission maximum of dansyl are very much affected by the polarity of the environment. Immersion of a dansyl-bearing segment of a protein into nonpolar membrane regions can be followed by the concomitant changes in the fluorescence emission spectrum of dansyl. Dansylated protein is also suitable to follow lipid-protein interactions by monitoring fluorescence energy transference from protein dansyl groups to rhodamine-labeled lipids. In this regard, another interesting advantage of using dansyl as an extrinsic fluorescence probe in proteins is that the absorption spectrum of dansyl largely overlaps with the fluorescence emission spectrum of tryptophan. This feature permits detection of protein-protein interactions by the possible occurrence of radiationless fluorescence energy transfer from tryptophan to dansyl provided that the two fluorophores are close enough on the molecular scale (39, 40).

**TABLE 1**

<table>
<thead>
<tr>
<th>Protein content (by weight)</th>
<th>Tm (°C)</th>
<th>(ΔH) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>41.5</td>
<td>8.33</td>
</tr>
<tr>
<td>DPPC/SP-C 2%</td>
<td>41.4</td>
<td>7.82</td>
</tr>
<tr>
<td>DPPC/SP-C 5%</td>
<td>40.9</td>
<td>7.18</td>
</tr>
<tr>
<td>DPPC/SP-C 10%</td>
<td>41.0</td>
<td>5.08</td>
</tr>
<tr>
<td>DPPC/Dans-SP-C 2%</td>
<td>41.3</td>
<td>8.07</td>
</tr>
<tr>
<td>DPPC/Dans-SP-C 5%</td>
<td>41.0</td>
<td>6.69</td>
</tr>
<tr>
<td>DPPC/Dans-SP-C 10%</td>
<td>40.8</td>
<td>5.18</td>
</tr>
</tbody>
</table>

![FIG. 8](image-url) Dependence of the DPPC phase-transition enthalpy (megacalories per mole of protein) on the phospholipid/protein molar ratio, for samples containing native porcine SP-C (closed triangles) or Dans-SP-C (open triangles). The data plotted are the enthalpies determined from the DSC scans in Fig. 6, whose values are summarized in Table 1. The line is the behavior for the native porcine protein previously determined by Shiffer et al. (10).
The derivative Dans-SP-C prepared in the present study can be therefore used to gain information about parameters governing the interaction of the N-terminal segment of SP-C with bilayers of relevant surfactant phospholipids or with other surfactant proteins such as SP-A or SP-B. Studies in this respect have been carried out successfully (to be published elsewhere). A method similar to that described here could also be used to introduce other probes covalently attached to the N-terminal amine group of SP-C, facilitating a versatile characterization of the protein by alternative techniques.

ACKNOWLEDGMENTS

We are grateful to Alicia Prieto, from CIB, CSIC, for carrying out the MALDI mass spectrometry analysis of samples, and to Dr. Derek Marsh, from Max-Planck-Institut für Biophysikalische Chemie in Göttingen, Germany, for his suggestions and critical reading of the manuscript. This work was supported by grants from DGICYT (BIO2000-0929 and PB98-0769) and CAM (07B/0017/99). I. Plasencia is recipient of a FPI grant and Dr. A. Cruz of a postdoctoral grant, both from Comunidad Autonoma de Madrid.

REFERENCES


