REASSEMBLY IN VITRO OF LUNG SURFACTANT LIPOPROTEIN

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Received December 22,1976

SUMMARY: This report describes a successful attempt to reassemble, in vitro, two fractions obtained from bovine lung surfactant lipoprotein. An apoprotein isolated by gel filtration in the presence of sodium deoxycholate was recombined with lipid extracts of the surfactant, in a highly alkaline buffer (pH 10) containing 10 mM sodium deoxycholate. Sonication, dilution 1 to 10, dialysis, and washing by means of centrifugation were used to produce a lipid-protein complex. Centrifugation in a continuous sucrose density gradient revealed that this material had a density of 1.081 gm/ml and a phospholipid/protein ratio respectively almost the same as those of the original lipoprotein.

The protein moiety of lung surfactant material was first isolated by King et al (1). It has immunological properies identical to the original surfactant lipoprotein, but has no lipid affinity (2). Recently we isolated an apoprotein from bovine lung surfactant material, by gel filtration in the presence of sodium deoxycholate. The molecular weight was higher than that of the apoprotein isolated by King, et al, suggesting that our apoprotein was closer to the native one (3). It seemes reasonable to determine whether our apoprotein had lipid affinity. Because our apoprotein was highly hydrophobic, the reassembly was performed by mixing the apoprotein with total lipid extracts in 10 mM sodium deoxycholate diluting the mixture 10 times with a buffer without sodium deoxycholate, after sonication, and then dialyzing it to remove sodium deoxycholate completely. The present report describes the procedures used in reassembly, and a comparison of the reassembled lipoprotein with the original one, using sucrose continuous density gradient centrifugation as a criterion.

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MATERIALS and METHODS

Preparation of Bovine Lung Surfactant: This was isolated from lung washings essentially following Frosolono's method (4). The lung washings were centrifuged in a Hitachi RPR 10-2 rotor at 1,000 rpm for 10 min to remove cells and cell debris as a sediment. The supernatant was then spun down in the Hitachi RPR 10-2 rotor at 10,000 rpm for 1 hour, and the sediment was recovered. The sediment was layered over 0.75 M sucrose dissolved in a buffer consisting of 0.01 M Tris·HCl-0.145 M NaCl-0.001 M EDTA (pH 7.4) and centrifuged in a Hitachi RPRS 40T rotor at 14,500 rpm for 1 hour. This buffer was used in all subsequent preparations unless otherwise indicated. The material concentrated at the interface was collected, suspended in the buffer and resedimented in a Hitachi RPR 20-3 rotor at 14,500 rpm for 1 hour. This washing by centrifugation was repeated three times. All the centrifugation was carried out at 4°C.

Lipid Removal by Gel Filtration: Samples of lung surfactant containing 4-5 mg of protein were dialyzed against 0.05 M NaCl-0.05 M sodium carbonate (pH 10) overnight at 4°C. In order to prevent protein degradation caused by the processes of solubilization and gel filtration, two kinds of proteinase inhibitors, phenylmethyl sulfonyl fluoride (PMSF, Sigma) and tosylamide phenylethyl chloromethyl ketone (TPCK, Sigma) were dissolved in dimethyl sulfoxide and were added to the sample solution in a final concentration of 10^{-4} M, and the mixture was incubated at 37°C for 15 min. 70 mg of sodium deoxycholate per mg of protein were added to solubilize the sample and the sample solution was examined by gel filtration. The gel filtration was performed with a column packed with Sephadex G-75 and equilibrated with 10 mM sodium deoxycholate-0.05 M NaCl-0.05 M sodium carbonate and 1 mM PMSF and TPCK (pH 10). The details of gel filtration have been reported previously (3).

Preparation of Lipid Extracts: This was performed following the method of Folch (5). The chloroform extracts were then dried by flash evaporation and stored under nitrogen at -20°C.

Reassembly Experiments: The protein fraction in the gel filtrate was collected and concentrated by ultrafiltration. 1.0 mg of protein and lipid extracts containing 5.0 μmol of phosphorus were mixed and sonicated briefly with a Branson Sonifier (50 watt, 1 min). The mixture was then diluted 10 times by rapid addition of the buffer, sonicated again with the same conditions as above, and dialyzed against the buffer overnight at 4°C. It was centrifuged in the Hitachi RPR 20-3 rotor at 16,000 rpm for 1 hour. The pellet was collected and washed three times by centrifugation.

Sucrose Continuous Density Gradient Centrifugation: 11 ml of a linear sucrose density gradient were prepared, ranging in sucrose concentration from 2 to 25 %. The sample pellet suspended in 1 ml of buffer was layered over the gradient and centrifuged in the Hitachi RPRS 40T rotor at 25,000 rpm for 16 hours. Fractionation by 0.4 ml was carried out starting from the top. Protein (6) and phosphorus (7) measurements were made on each sample.

RESULTS

The chemical compositions of bovine lung surfactant, including sodium dodecyl sulfate (SDS) disc gel electrophoresis pattern of apoprotein, and the lipid composition measured by thin layer chromatography were reported

Abbreviations:

PMSF (phenylmethyl sulfonyl fluoride).
TPCK (tosylamide phenylethyl chloromethyl ketone).
SDS (sodium dodecyl sulfate).

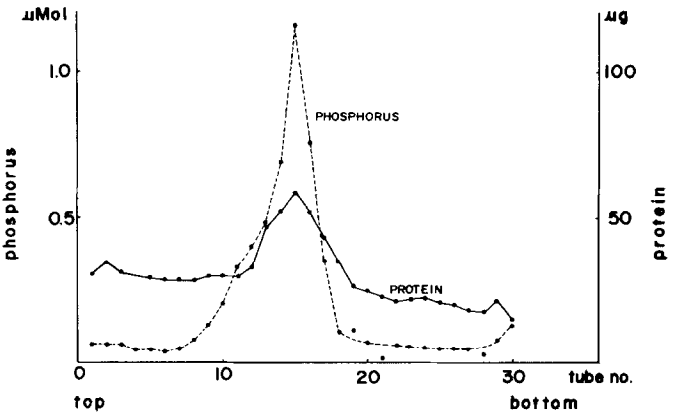

Fig. 1. Sucrose continuous density gradient centrifugation of the reassembled lipid-protein complex, a phosphorus and protein distribution in a 0.4 ml fractionated sample. Linear gradient ranging from 2.0 to 25 % sucrose concentration in the buffer. Centrifugation at 25,000 rpm for 16 hours.

previously (8). The phosphorus:protein ratio was 4.9 ± SD 0.62 µmol/mg (mean of 5 samples).

Reassembly Studies: After a series of reassembly processes, a white pellet similar in appearance to the original surfactant lipoprotein was obtained. It contained 3.34 µmol of phosphorus and 0.27 mg of protein (mean of 3 experiments). The phosphorus:protein ratio was 11.4 µmol/mg; protein recovery was 29 % and phospholipid recovery was 67 %.

Continuous Density Gradient Centrifugation: Sucrose continuous density gradient centrifugation of the reassembled lipid-protein complex showed one band visible at a density of 1.081 g/ml. The protein and phosphorus distribution are shown in fig. 1, for comparison with those of the original surfactant lipoprotein (fig. 2). 0.4 ml fractionation from the top showed a peak of both protein and phosphorus at tube number 15, corresponding to a band at a density of 1.081 g/ml. The phosphorus:protein ratio of this peak was 19.3 µmol/mg in the reassembled lipid-protein comples and 20.2 µmol/mg in the original lipoprotein.

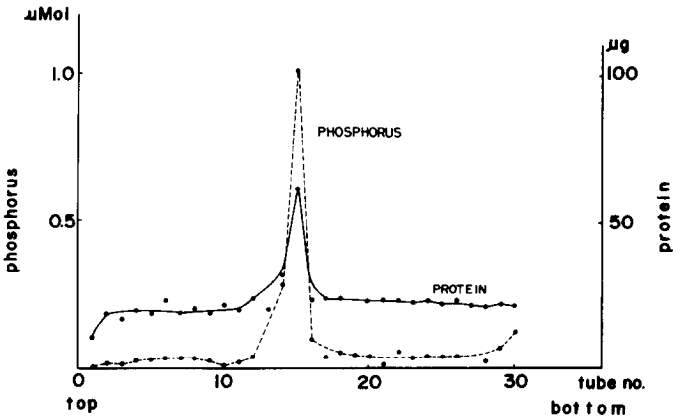

Fig. 2. Sucrose continuous density gradient centrifugation on the original surfactant lipoprotein, under the same conditions of centrifugation as in fig. 1.

DISCUSSION

From the results of sucrose continuous density gradient centrifugation, the reassembled lipid-protein complex has a density identical to that of the original lipoprotein (d=1.081 g/ml). The phospholipid:protein ratios at the peak, resulting from density gradient centrifugation, are very close to each other. Apoprotein isolated by gel filtration in the presence of SDS also was tested in reassembly studies in the same way as the apoprotein obtained by gel filtration in the presence of sodium deoxycholate. This was unsuccessful: centrifugation, after dialysis against the buffer, yielded only aggregated protein, and the lipids remained in the supernatant. These facts suggest that only apoprotein isolated by gel filtration in the presence of sodium deoxycholate has an affinity to lipids, and the resultant lipid-protein complex has a lipid and protein composition and density identical to the original lipoprotein.

The protein recovery was low (29 %) compared with the lipid recovery (67 %). A part of the apoprotein may be degraded through the processes of isolation and reassembly, in spite of the existence of proteinase inhibitors, and lose its affinity for lipids. The addition of more apoprotein would

result in a lower phosphorus:protein ratio in the lipid-protein complex sedimented for washing by centrifugation. In any case, the final peak phospholipid:protein ratio after continuous density gradient centrifugation is very close to that of the original lipoprotein.

The initial sonication was found to be very important: dilution 10 x with the buffer without previous sonication resulted in a very rapid aggregation of the apoprotein, and therefore reassembly was unsuccessful.

The question remains unsolved as to whether the reassembled lipid-protein complex really is the same as the original lipoprotein. Physicochemical studies to examine this question are in progress in our laboratory. At present, we would like to limit this report to the conclusion that the apoprotein isolated by gel filtration in the presence of sodium deoxycholate has lipid affinity, and that the resultant lipid-protein complex has a density and chemical composition very close to those of the original surfactant lipoprotein.

ACKNOWLEDGEMENT

We thank Dr. Tsunehiko Katsunuma, Department of Biochemistry, Tokai University School of Medicine for his suggestions in accomplishing his work.

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