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Comparison of Physicochemical Properties of Purified Mucus Glycoproteins Isolated from Respiratory Secretions of Cystic Fibrosis and Asthmatic Patients†

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ABSTRACT: The major nonreduced mucus glycoproteins (mucins) from sputa of cystic fibrosis (CF) and asthmatic patients have been purified to electrophoretic homogeneity and subjected to physical and chemical characterization. The sputum specimens were solubilized in buffer containing 0.22 M KSCN and fractionated on Bio-Gel A-5m, followed by digestion with DNase, rechromatography on the same column, and chromatography on hydroxylapatite. Sodium dodecyl sulfate gel electrophoresis of purified mucins gave a single band. Carbohydrate analyses of the purified mucins showed no significant differences in the sugar components from the two mucins. However, the CF mucin contained substantially higher (11%) sulfate content than that observed for the asthmatic mucin (5.9%). Amino acid analyses indicated that the CF mucin had higher levels of serine plus threonine (35%) as compared to the asthmatic mucin (29%). In contrast, CF mucin contained a lower content of aspartate, glutamate, and glycine than that observed for the asthmatic mucin. Molecular weights of 3.8×10^6 and 3.5×10^6 were obtained for CF and asthmatic mucins, respectively, from light-scattering studies of mucins in the presence of 6 M guanidine hydrochloride. Reduction of the disulfide bonds of the two mucins did not alter their molecular weights. Liquid chromatographic studies on Sepharose CL2B showed that CF mucin forms aggregates sufficiently large to be excluded from the gel. As compared to the CF mucin, the asthmatic mucin formed fewer of these large aggregates under identical experimental conditions. Reduction and alkylation of the mucins resulted in their inability to form aggregates. The higher state of aggregation of CF mucin may influence the viscoelastic properties of the CF lung's mucus secretions.

Respiratory mucus secretions play an important role in the normal functioning of the lung airways. The thin mucus layer of the tracheobronchial tree of a normal individual protects the bronchial cells against airborn microorganisms and other foreign particles. This protection is accomplished through the continuous cephalad flow of mucus under the propelling action of ciliated epithelium, which helps in clearing the trapped particles and the microorganisms in the mucus lining the airways. Cystic fibrosis (CF) patients produce excessive amounts of viscous mucus secretions as compared to healthy controls (Lorin et al., 1972). The increased tenacity and viscosity of respiratory mucus in CF patients make it difficult for them to clear the secretions from the airways, thus causing pulmonary insufficiency, lung infection, and death (Gurwitz et al., 1979). The gelation and viscoelastic properties of the secretions are determined, to a large extent, by the presence of mucus glycoproteins (mucins) in the secretions (Yeager, 1971; Litt et al., 1974). Any changes in the chemical and/or physical properties of the mucins may alter the viscoelastic properties of the mucus secretions which, in turn, may influence the clearance of mucus secretions by ciliated epithelium (Chen & Dulfano, 1978; Gelman & Meyer, 1979; Giordano et al., 1978). Thus, isolation and characterization of the individual components from mucus are required to understand the relationship between mucin structure and the physical properties of the mucus.

While in recent years we have gained some knowledge from the isolation and characterization of mucins, it is not yet known

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whether there are any differences in the chemical and physical properties between native (nonreduced) CF respiratory mucin and native mucins purified from control respiratory mucus secretions. Although reduced–alkylated mucins have been purified and chemically characterized from respiratory mucus secretions of a CF patient, a chronic bronchitic patient, and a healthy individual with no history of lung disease (Boat et al., 1976), the reduction and alkylation of the mucus secretions precluded determination of the physical properties of the mucins.

Previous studies from this laboratory have been directed toward the isolation, purification, and characterization of mucin components from canine tracheal mucus (Sachdev et al., 1978, 1979), from tracheobronchial secretions of healthy human volunteers (Sachdev et al., 1980), and from CF respiratory mucus secretions (Chace et al., 1983). In this paper, we report the isolation, purification, and physicochemical properties of native (nonreduced) mucins isolated from respiratory mucus secretions of a CF and an asthmatic patient. In addition, the effects of disulfide bond reduction on the molecular weights of the mucins as well as on the aggregation properties of the mucins are reported.

Experimental Procedures

Collection of Respiratory Mucus Secretions. Sputum specimens from a CF patient and from an asthmatic patient were used as the starting material. The blood group status of these patients was determined, and they exhibited no detectable blood group A or B activity. The collected samples were immediately treated with an equal volume of gentamycin sulfate (100 μg/mL) and kept chilled in an ice bath. The specimens were exhaustively dialyzed at 4 °C against a 0.02% sodium azide solution and then against deionized water. Following dialysis, the specimens were lyophilized and stored at 4 °C. Several specimens from the same patient were combined for purification of mucins.

Solubilization of Mucus Secretions. The chaotropic agent potassium thiocyanate was used for the solubilization of the CF sputum specimens and the sputum specimens from the control patient. In each case, 400 mg of the sputum specimens was treated with 40 mL of 0.1 M tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) buffer, pH 7.5, containing 0.22 M potassium thiocyanate and 0.02% sodium azide. The suspension was gently shaken in an automatic shaker for 4 days at 4 °C. The solution was centrifuged at 27000*g* for 4 h at 4 °C to remove insoluble material. About 20% of the total starting weight of the specimens was present in the pellet. Both the pellet and supernatant were examined by sodium dodecyl sulfate (SDS) gel electrophoresis (see below) to assess the distribution of mucin components. The pellet material contained low molecular weight proteins but no high molecular weight mucins and hence was not studied further. Potassium thiocyanate has been used previously in the solubilization of canine tracheal mucus (Khan et al., 1976) and in the solubilization of respiratory mucus from a chronic bronchitic patient (Brown et al., 1981).

Fractionation of Solubilized Mucus Secretions. Initial separation of the high and low molecular weight components of the mucus secretions was carried out on a column (5 × 90 cm) of Bio-Gel A-5m. The material solubilized by potassium thiocyanate was applied to a column preequilibrated with Tris-HCl buffer, pH 7.5, and 0.02% sodium azide containing 0.22 M potassium thiocyanate. The column was then eluted with the same buffer. The column fractions from each peak were pooled, dialyzed exhaustively at 4 °C against deionized water, and then lyophilized.

DNase Digestion of the Bio-Gel A-5m Excluded Peak. The excluded fraction from the Bio-Gel A-5m column containing mucin and DNA molecules was digested with DNase for 6 h. Prior to digestion with DNase, contaminating proteolytic activity in the commercial DNase preparation was abolished by treatment with phenylmethanesulfonyl fluoride, a serine protease inhibitor (Gold, 1967). This allowed degradation of DNA molecules without affecting the integrity of the mucin component. The DNase digest was subjected to gel permeation chromatography on a Bio-Gel A-5m column (5 × 90 cm) in order to resolve the degraded DNA molecules from the intact mucins. Column fractions were monitored by measuring the absorbance at 260 and 280 nm and by determining the hexose content.

Hydroxylapatite chromatography was performed on a 2.5 × 9 cm column equilibrated with 0.01 M potassium phosphate buffer, pH 6.8. The DNA-free mucin fraction dissolved in equilibration buffer was applied to the column, and the column was initially eluted with the same buffer. The column was subsequently eluted with a discontinuous gradient of 0.15, 0.3, and 0.5 M potassium phosphate buffer, pH 6.8. The various fractions obtained were monitored for the optical density (OD) at 230 nm and for hexose content. The fractions from a given peak were pooled together, exhaustively dialyzed against deionized water, and lyophilized. The lyophilized fractions were examined by gel electrophoresis (see below). In some cases, the major mucin fraction was rechromatographed on the column and eluted with 0.01 M phosphate buffer, pH 6.8.

Reduction and Alkylation of Purified Native Respiratory Mucins. The native mucins purified from the sputum specimens of both patients were reduced with dithiothreitol (DTT) in the presence of 6 M guanidine hydrochloride (Sachdev et al., 1978). This step was carried out as follows: the purified mucin (0.3 mg/mL) was dissolved in 0.1 M Tris-HCl buffer, pH 8.5, containing 6 M guanidine hydrochloride. Nitrogen gas was bubbled through the solution for 30 min at 22 °C prior to the addition of DTT (final solution concentration 25 mM). After 2 h of incubation, additional amounts of DTT were added to give a solution concentration of 50 mM. The reduction was carried out for 4 h, and subsequently, iodoacetamide was added to the solution to give a concentration of 0.15 M. Incubation was carried out for an hour in the dark at 22 °C. The excess iodoacetamide was removed by adding DTT (final solution concentration 0.2 M). The reduced–alkylated mucins were exhaustively dialyzed against deionized water and lyophilized. Carbohydrate and amino acid compositions of the reduced–alkylated mucins were determined. The mucins were examined by SDS gel electrophoresis.

Analytical Methods. Neutral hexose was determined by the anthrone method (Carubelli et al., 1961), using a mixture of galactose and fucose (1:1 w/w) as the standard. Sialic acid was determined by the resorcinol method (Svennerholm, 1957) using *N*-acetylneuraminic acid as the standard. Fucose, galactose, deoxyribose, *N*-acetylgalactosamine, and *N*-acetylglucosamine were determined by gas–liquid chromatography as described previously (Sachdev et al., 1978; Wang et al., 1974). DNA was estimated by a fluorometric method using calf thymus DNA as the standard (Thomas & Farquhar, 1978). Sulfate was determined by the sodium rhodizonate method using sodium sulfate as the standard (Sachdev et al., 1978; Terho & Hartiala, 1976). Amino acid analyses were performed by the method of Spackman (1967) using a Beckman Model 119B amino acid analyzer following hydrolysis of the samples with constant-boiling hydrochloric acid at 110 °C for 22 h in evacuated sealed tubes.

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Protein was also determined from the amino acid composition data. The column effluents were monitored for protein by measuring the absorbance at 280 nm.

Gel Electrophoresis. Sodium dodecyl sulfate (SDS) gel electrophoresis of mucins was carried out on composite gels (2% acrylamide + 0.5% agarose) containing 0.1% SDS as described earlier (Sachdev et al., 1978; Holden et al., 1971). Duplicate gels were run for each sample, one of which was treated with the periodic acid–Schiff (PAS) reagent to stain for carbohydrates and the other with Coomassie Brilliant Blue for proteins, as described by Segrest & Jackson (1972).

Light-scattering studies were carried out on an instrument assembled in Dr. Olin Spivy's laboratory, Oklahoma State University, Stillwater, OK. Light-scattering studies of the mucin solutions were studied by using a laser light source (632.8 nm). For these experiments, mucin stock solutions were prepared by dissolving mucin (1–2 mg/mL) in 0.02 M Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide and either 0.15 M sodium chloride of 6 M guanidine hydrochloride. Final mucin concentrations were determined by means of the anthrone assay for neutral sugars (Carubelli et al., 1961). All buffer solutions used were filtered through a 0.22-μm filter to remove dust. After being allowed to stand at room temperature for 2 days, the mucin samples were centrifuged at 8700g for 3 min to sediment dust particles. The supernatant solution was then diluted to give the desired mucin concentrations. For experiments carried out in the buffer containing 6 M guanidine hydrochloride, the mucin sample concentrations ranged from 0.2 to 1.2 mg/mL. For experiments conducted in 0.15 M NaCl, the mucin sample concentration ranged from 0.1 to 0.5 mg/mL. Four different mucin concentrations were used for each light-scattering experiment. The scattering intensity of mucin solutions was measured at scattering angles ranging from 40° to 90°. The refractive index increment (dn/dc) was obtained by using a differential refractometer. Molecular weights of the mucins were obtained by extrapolation of the light-scattering data to zero angle and to zero concentration, using Zimm plots (Zimm, 1948). Radii of gyration of mucin molecules were also calculated from the Zimm plots.

Molecular Aggregation Studies. The aggregation behavior of the native (nonreduced) CF mucin, native asthmatic mucin, and corresponding reduced–alkylated mucins was studied by using gel permeation chromatography with a Sepharose CL-2B column (1.6 × 80 cm). Mucins (2–2.5 mg/mL), dissolved in 6 M guanidine hydrochloride, were applied to the column preequilibrated with 0.1 M Tris-HCl buffer, pH 7.5, containing 6 M guanidine hydrochloride. Elution was carried out by using the equilibration buffer, and the collected fractions were monitored for neutral hexose by the anthrone method (Carubelli et al., 1961).

RESULTS

Solubilization of CF and Asthmatic Mucus Secretions. The high molecular weight mucins present in the dialyzed and lyophilized mucus specimens could not be solubilized in aqueous buffer solutions even after prolonged stirring at room temperature. However, these sputum specimens could be slowly solubilized in the chaotropic agent potassium thiocyanate to provide solutions which are suitable for fractionation using column chromatographic procedures.

Fractionation of Respiratory Mucus Secretions. Chromatography of the solubilized CF mucus specimens on Bio-Gel A-5m (Figure 1) separated the material into an excluded

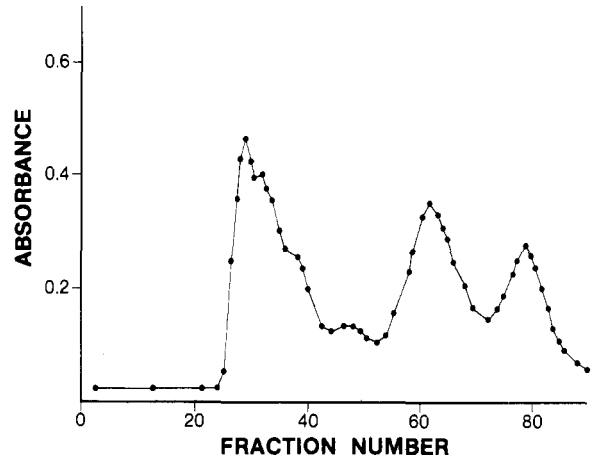

FIGURE 1: Bio-Gel A-5m chromatography of KSCN-solubilized CF mucus secretions. The column (5 × 90 cm) was operated at a flow rate of 65 mL/h, and 17-mL fractions were collected. The eluting buffer was 0.1 M Tris-HCl, pH 7.5, containing 0.22 M KSCN. The fractions were monitored at 280 nm. The fractions in peaks were combined for further study and designated as fractions I, II, and III, respectively.

fraction (peak I) and included fractions (peaks II and III). Results obtained from gel electrophoresis of the fractions indicated that peak I contained a high molecular weight (>1 × 10^6) mucin-type glycoprotein and a minor amount of low molecular weight proteins. Peaks II and III contained serum-type proteins and glycoproteins. The excluded fraction accounted for 75–85% of the total neutral sugar. By weight, however, the excluded fraction contained 55–75% of the total eluted material while fractions II and III contained 25–40% and <5%, respectively. In addition to mucus glycoprotein, peak I also contained DNA. Peaks II and III had no contaminating DNA. Chromatography of respiratory mucus secretions from an asthmatic patient also resulted in a similar chromatographic profile. In this case, the excluded fraction was also contaminated with DNA.

To separate mucus glycoprotein from contaminating DNA, fraction I (excluded peak) was treated with DNase and was rechromatographed on the Bio-Gel A-5m column (Figure 2). From the absorbance ratios (260 nm:280 nm) of the two peaks eluted, it was apparent that the excluded peak contained the native mucin component, while the included peak contained the degraded DNA. Analysis of the material in the excluded peak showed no detectable DNA. Results obtained from SDS gel electrophoresis showed that the excluded peak contained a high molecular weight mucin component together with small amounts of lower molecular weight protein components.

Further purification of the mucin in the excluded peak was achieved by chromatography on hydroxylapatite. The sample was applied to a column of hydroxylapatite preequilibrated with 0.01 M phosphate buffer, pH 6.8, and the elution was carried out with the same buffer. Subsequently, the column was eluted stepwise with 0.15, 0.3, and 0.5 M phosphate buffer, pH 6.8. The elution profile is shown in Figure 3. The fraction which eluted with equilibration buffer (peak I) contained only a high molecular weight glycoprotein (mucin) (>1 × 10^6), while that which eluted with 0.15 M PO_4 buffer (peak II) contained high molecular weight mucin as well as lower molecular weight proteins. The material eluting with 0.3 M PO_4 buffer (peak III) was comprised only of the low molecular weight glycoproteins and proteins. Rechromatography of peak II on hydroxylapatite separated the high molecular weight mucin from low molecular weight protein components. Peaks I, II, and III contained about 60%, 30%, and 10% of the total

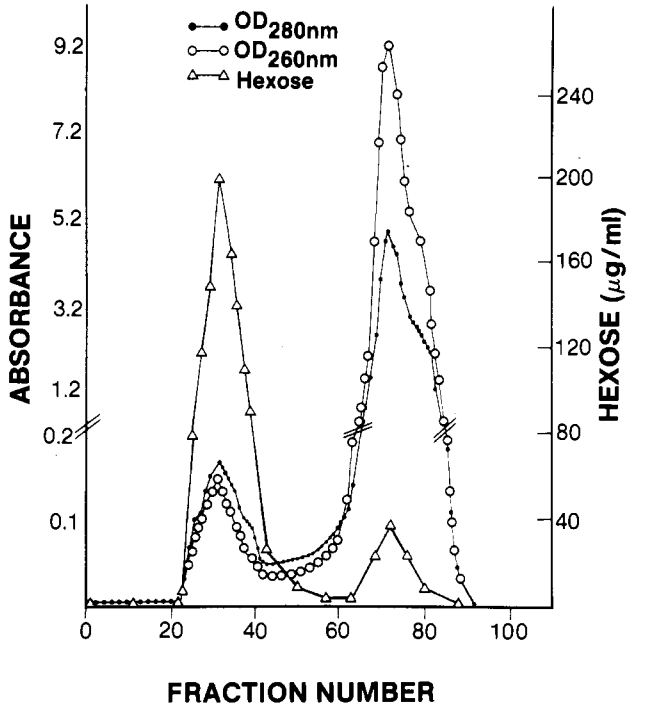

FIGURE 2: Chromatography of DNase-treated fraction I on a Bio-Gel A-5m column (5 × 90 cm). The flow rate was 65 mL/h; the fraction volume was 17 mL. Fractions were monitored for the absorbance at 260 and 280 nm and also for neutral hexose. Fractions showing an absorbance greater than 2.0 were diluted to get the correct absorbance.

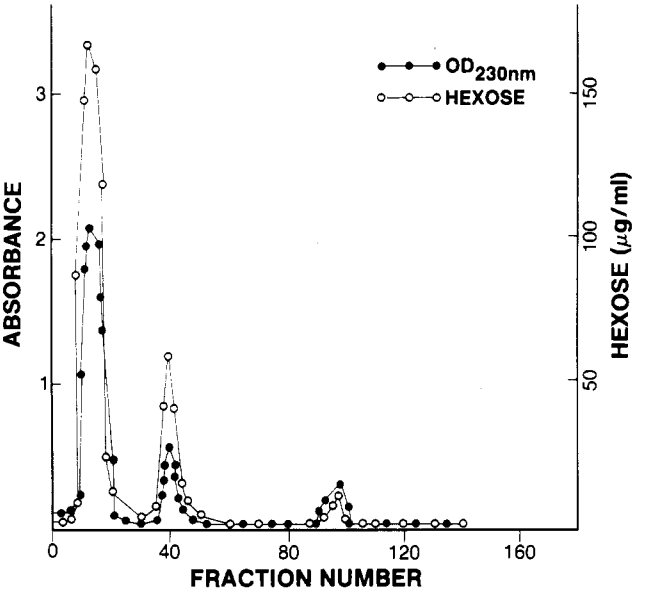

FIGURE 3: Hydroxylapatite chromatography of mucin fraction. The column (2.5 × 9.0 cm) was operated at a flow rate of 15 mL/h, and 2.5-mL fractions were collected. The fractions were monitored for the absorbance at 230 nm and for neutral hexose content. Peak I (fractions 8–20), eluted with 0.01 M phosphate buffer, pH 6.80, peak II (fractions 36–45), eluted with 0.15 M phosphate buffer, pH 6.8, and peak III (fractions 95–100), eluted with 0.3 M phosphate buffer, pH 6.8, were dialyzed and lyophilized.

eluted material, respectively. No differences in the elution profile were observed between CF and asthmatic respiratory mucus secretions.

SDS gel electrophoresis of the material eluting from the hydroxylapatite column, peak I, on composite gels of polyacrylamide (2%) and agarose (0.5%) showed the presence of a single PAS-positive band. Treatment of duplicate gels with Coomassie Brilliant Blue showed the absence of any contaminating proteins in the purified preparations.

Table I: Chemical Composition of Purified Nonreduced Mucins Isolated from Respiratory Mucus Secretions of CF and Asthmatic Patients

component	% dry wt of mucus glycoprotein	
	CF	asthmatic
protein[a]	18.0	18.2
fucose[b]	9.2	11.3
galactose[b]	18.9	26.8
N-acetylgalactosamine[b]	8.5	5.4
N-acetylglucosamine[b]	17.0	14.3
sialic acid[c]	17.3	18.1
sulfate[d]	11.0	5.9

[a] Calculated from amino acid composition. [b] Assayed by gas–liquid chromatography of alditol acetates. [c] Assayed by the resorcinol reaction using synthetic *N*-acetylneuraminic acid as the standard. [d] Assayed by the sodium rhodizonate method; expressed as SO_4.

Table II: Amino Acid Composition of Nonreduced Purified Mucins from Respiratory Mucus Secretions of CF and Asthmatic Patients

amino acid	residues/1000 residues	
	CF	asthmatic
aspartic acid	47	66
threonine	233	177
serine	119	113
glutamic acid	60	71
proline	118	103
glycine	53	86
alanine	107	97
valine	58	65
methionine	5	13
isoleucine	29	31
leucine	59	58
tyrosine	13	15
phenylalanine	14	20
histidine	22	25
lysine	26	24
arginine	36	36
serine + threonine + proline + glycine + alanine	630	575

Chemical Composition of the Purified Mucus Glycoproteins. The chemical analyses of the electrophoretically homogeneous native (nonreduced) mucus glycoprotein isolated from respiratory mucus secretions of a CF patient and an asthmatic patient are shown in Table I. In both cases, carbohydrate analyses by gas–liquid chromatography revealed the presence of galactose, fucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine. In addition, the purified mucins contained sialic acid. The absence of mannose and deoxyribose in both purified mucins suggested an absence of serum glycoprotein(s) and DNA. The overall carbohydrate content of the native (nonreduced) mucus glycoproteins from the CF patient and the control patient ranged from about 71% to 76% of the dry weight of the mucins. Compared to the asthmatic purified mucin, the purified mucin from the CF patient contained larger amounts of sulfate. Other than sulfate content, only minor differences were observed in the other chemical parameters of the purified mucins.

Amino acid analyses of the purified nonreduced mucins from a CF patient and an asthmatic patient are shown in Table II. Comparison of the data revealed some differences in the amino acid composition between the two mucins. The purified CF mucin had a higher combined content of serine and threonine (35.2%) as compared to that observed in the asthmatic mucin (29.0%). In addition, the CF mucin contained a higher proline content and lower asparate, glutamate, and glycine contents. For the CF mucin, threonine, serine, proline, glycine, and alanine comprised 63.0% of the total amino acid residues, while

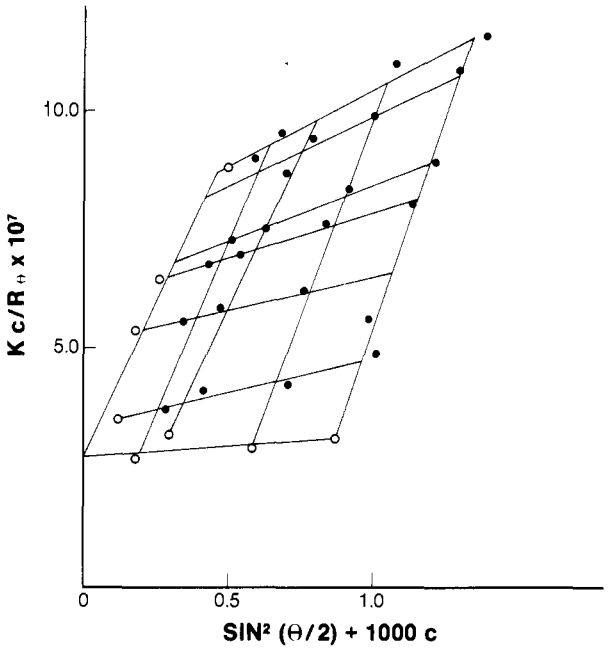

FIGURE 4: Zimm plot for the light-scattering data of the CF mucin solutions in 0.02 M Tris-HCl buffer, pH 7.4, containing 6 M guanidine hydrochloride. Mucin concentrations used were 0.175, 0.291, 0.582, and 0.873 mg/mL, and scattering angles were 40°, 50°, 60°, 70°, 80°, and 90°. The optical constant K was 8.08×10^{-8}, c is the mucin concentration (in grams per milliliter), and R_θ is the Rayleigh ratio of the mucin solution $[=(r^2i/I_0)(1 + \cos^2\theta)]$, where i is the intensity of the scattering light at the scattering angle θ. I_0 is the intensity of the incident light, and r is the distance of the photomultiplier tube from the scattering solution.

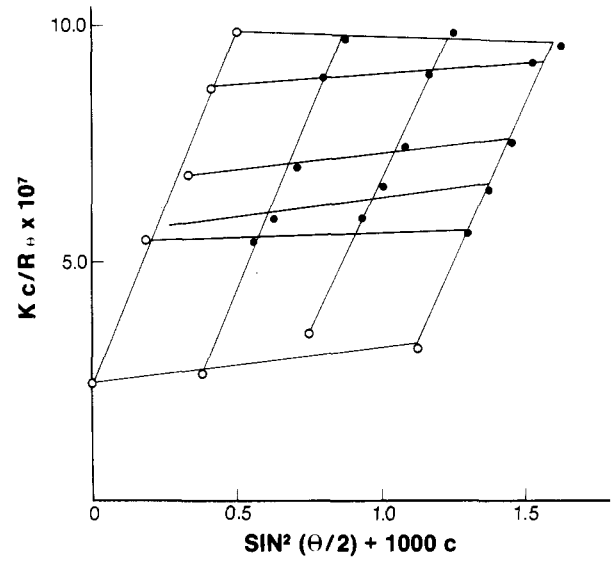

FIGURE 5: Zimm plot of the light-scattering data of the reduced–S-carboxymethylated CF mucin in 0.02 M Tris-HCl buffer, pH 7.4, containing 6 M guanidine hydrochloride. The mucin concentrations used were 0.187, 0.373, and 0.560 mg/mL, and the scattering angles were 50°, 60°, 70°, 80°, and 90°. Axes are labeled as in Figure 4.

the content of these five amino acids for the asthmatic mucin was 57.6% of the total amino acid residues. Both CF and asthmatic mucins had low levels of aromatic amino acids (phenylalanine and tyrosine), i.e., 2.7% and 3.5% of the total amino acid residues.

The carbohydrate composition and also the amino acid composition of the CF and asthmatic mucins studied in this work were not altered as a result of reduction and S-carboxymethylation (data not shown). Results obtained from SDS gel electrophoresis of the nonreduced and reduced–alkylated CF and asthmatic mucins showed only a slight difference in the mobility of the mucins; the reduced mucins showed a slight increase in mobility over the nonreduced mucin. Similarly, there was no difference between the gel electrophoretic mobility of reduced–alkylated CF mucin and reduced–alkylated asthmatic mucin.

The molecular weight and radii of gyration of the nonreduced and reduced–S-carboxymethylated CF and asthmatic mucins were determined by the technique of static light scattering. The experiments were conducted in buffer solution containing 0.15 M NaCl or 6 M guanidine hydrochloride. The Zimm plots for the nonreduced and reduced–S-carboxymethylated CF mucins in 6 M guanidine hydrochloride shown in Figures 4 and 5, respectively. In the presence of 6 M guanidine hydrochloride, the nonreduced (native) CF mucin had a molecular weight of 3.8×10^6 and a radius of gyration (R_G) of 134 nm. Under identical experimental conditions, the reduced–alkylated CF mucin had a similar molecular weight (4.1×10^6) and a radius of gyration of 151 nm. When dissolved in a buffer solution containing 0.15 M NaCl, the nonreduced CF mucin had a molecular weight of 5.1×10^6 and an R_G value of 113 nm. The observed increase in molecular weight (5.1×10^6) of nonreduced CF mucin in the buffer containing 0.15 M NaCl over that observed in buffer containing 6 M guanidine hydrochloride (3.8×10^6) is due to the apparent inability of the mucin molecules to dissociate to monomer molecules in the presence of 0.15 M NaCl, even at the low mucin concentrations used in our work. This results in a higher weight-average molecular weight of the CF mucin in the presence of 0.15 M NaCl compared to that observed in 6 M guanidine hydrochloride. The greater R_G value of the CF mucin in the presence of 6 M guanidine hydrochloride (134 nm) compared to that observed (113 nm) in the presence of 0.15 M NaCl suggests that the mucin molecules are expanded in the presence of this denaturant.

Table III: Weight-Average Molecular Weights and Radii of Gyration of Mucins Isolated from Tracheobronchial Secretions of Cystic Fibrosis and Asthmatic Patients[a]

mucin	treatment	solvent	mol wt ($\times 10^{-6}$)	radius of gyration, R_G (nm)
CF	–[b]	0.15 M NaCl	5.1	113
	–	6 M guanidine hydrochloride	3.8	134
	+[c]	6 M guanidine hydrochloride	4.1	151
asthma	–	0.15 M NaCl	4.1	112
	–	6 M guanidine hydrochloride	3.5	165
	+	6 M guanidine hydrochloride	3.6	145

[a] Data obtained from Zimm plots (for details, see Experimental Procedures). [b] The minus sign indicates untreated (i.e., native) mucin. [c] The plus sign indicates reduced and S-carboxymethylated mucin.

A comparison of the molecular weight and radius of gyration data for CF and asthmatic mucins is shown in Table III. The nonreduced and reduced–alkylated asthmatic mucins had slightly lower molecular weights than those observed for the CF nonreduced and reduced–alkylated mucins. This was true for data observed in the presence of 0.15 NaCl or 6 M guanidine hydrochloride. Interestingly, the observed R_G values of the CF mucin in buffer containing 0.15 M NaCl (113 nm) were the same as those observed for the asthmatic mucin (112 nm) under similar experimental conditions. Also, as observed for CF mucin, the reduction of the asthmatic mucin did not affect its molecular weight.

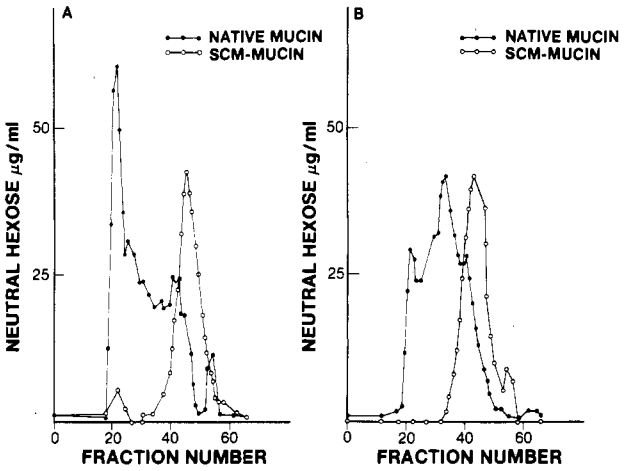

FIGURE 6: Sepharose CL2B chromatography of the nonreduced and reduced–alkylated CF (A) and asthmatic mucins (B) in 0.02 M Tris-HCl buffer, pH 7.4, containing 6.0 M guanidine hydrochloride. Two milliliters of the mucin sample was applied to the column (1.6 × 80 cm) which was operated at a flow rate of 6.7 mL/h. Two-milliliter fractions were collected and monitored for their hexose content by the anthrone reaction.

The molecular aggregation behavior of nonreduced and reduced–alkylated purified respiratory mucins from CF and asthmatic patients was studied by using liquid chromatography on Sepharose CL2B. In each case, mucin (2.0–2.5 mg/mL) dissolved in Tris-HCl buffer, 0.02 M, pH 7.5, containing 6 M guanidine hydrochloride was applied to a Sepharose CL2B column equilibrated with the same buffer system. The elution profiles of the nonreduced and reduced–alkylated CF and asthmatic mucins are shown in Figure 6. In comparison to the nonreduced respiratory mucins from both CF and asthmatic patients, which elute as complex mixture of peaks (Figure 6A,B), the reduced–alkylated CF and asthmatic mucins elute as a single peak (Figure 6A,B). The majority of the CF mucin was excluded from the Sepharose CL2B column (Figure 6A) while the majority of the asthmatic mucin was included in the column (Figure 6B) under identical experimental conditions. This suggests that the CF nonreduced mucin exists in solution in a higher state of aggregation than the nonreduced asthmatic mucin. However, this difference in aggregation behavior of the CF and asthmatic mucins was abolished upon reduction of the disulfide bonds of the mucins (Figure 6A,B). Even though the molecular weights of the nonreduced mucins and reduced mucins are similar (light-scattering studies), the observed elution profiles for nonreduced mucins and reduced mucins are different on Sepharose CL2B. These observations can be explained if nonreduced mucins, but not the reduced mucins, form aggregates in the buffer system containing 6 M guanidine hydrochloride. However, in a buffer solution containing 0.15 M NaCl, even the reduced–alkylated mucins formed aggregates. The elution profiles of reduced–alkylated CF mucin from Sepharose CL2B columns equilibrated with buffer solution containing 0.15 M NaCl, and from the same column equilibrated with buffer solution containing 6 M guanidine hydrochloride, are shown in Figure 7. Clearly, reduced–alkylated CF mucin elutes earlier with buffer containing 0.15 M NaCl than when the elution is carried out with the buffer solution containing 6 M guanidine hydrochloride. Also, there is a slight difference in the elution pattern of nonreduced mucin on the Sepharose CL2B column depending upon whether the elution is carried out with buffer containing 0.15 M NaCl or with 6 M guanidine hydrochloride (Figure 8). Different proportions of the mucin are excluded from the Sepharose CL2B column in the two

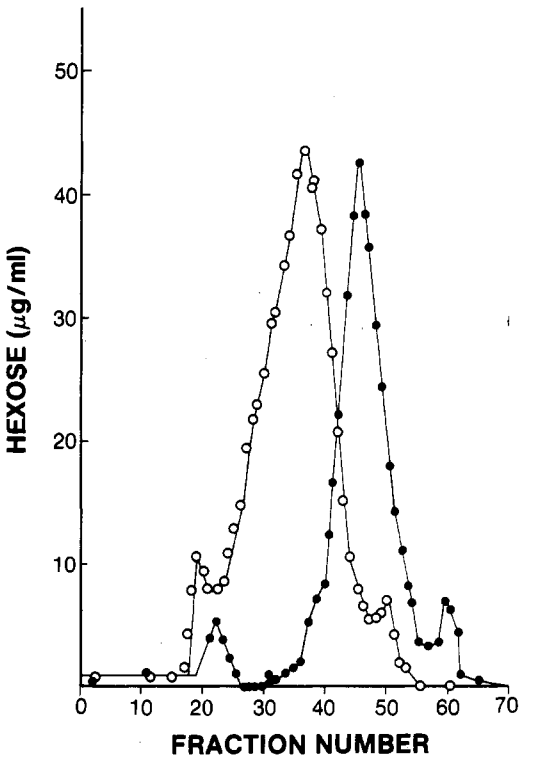

FIGURE 7: Sepharose CL2B chromatography of reduced–S-carboxymethylated CF mucin in 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl (○) and in the buffer solution containing 6 M guanidine hydrochloride (●). Two milliliters of the mucin samples was applied to the column (1.6 × 80 cm) which was operated at a flow rate of 6–7 mL/h. Two-milliliter fractions were collected and monitored for hexose content by the anthrone reaction.

cases. For example, a larger proportion of the applied mucin (48%) appeared in the excluded peak (fractions 16–22) when 0.15 M NaCl was used as the eluant, as compared to only 34% in the excluded peak (fractions 18–24) when eluted with 6 M guanidine hydrochloride. This indicates that in the presence of 0.15 M NaCl the nonreduced CF mucin forms more of aggregates that are excluded from the Sepharose CL2B column than those present in the presence of 6 M guanidine hydrochloride.

Discussion

Physicochemical characterization of the respiratory mucus glycoproteins present in gellike mucus secretions requires a solubilization step prior to their analysis. To obtain mucin preparations in as nearly native form as possible, solubilization with 0.22 M potassium thiocyanate was used, as it is known to provide mucin preparations which are rheologically active (Khan et al., 1976, Brown et al., 1981). In several previous studies, however, reduction of the disulfide bonds and subseuqnt alkyltion of the free thiol groups have been used to solubilize the bronchial mucus secretions (Boat et al., 1976; Roussel et al., 1975; Roberts, 1974, 1976; Havez et al., 1970; Sachdev et al., 1978). However, it has not been shown whether the reduction of the disulfide bonds of the respiratory mucins affects their molecular weights and/or other physical characteristics.

The purification scheme used in this work provided electrophoretically homogeneous native (nonreduced) mucins. In our experience, it is essential to remove proteolytic activity from commercial preparations of DNase in order to isolate intact respiratory mucins. The hydroxylapatite chromatographic step in our purification scheme is important as it provided mucin components totally free of the entrapped low

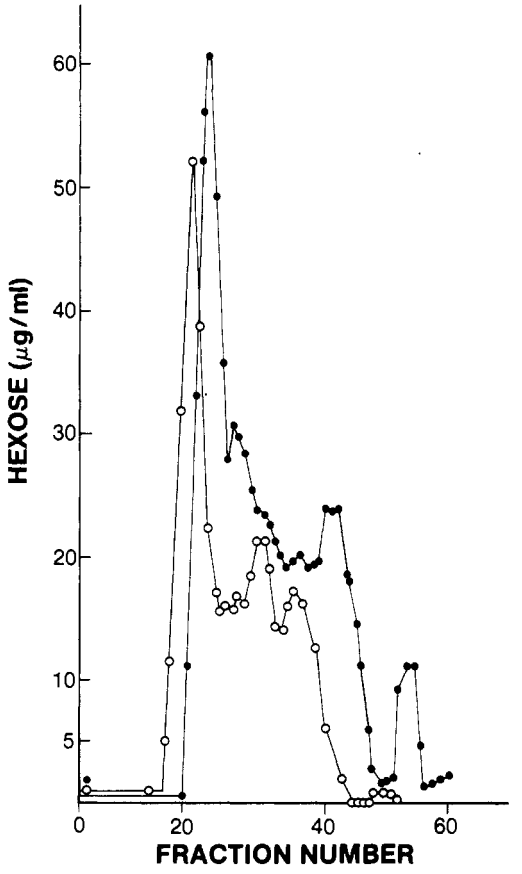

FIGURE 8: Sepharose CL2B chromatography of the nonreduced CF mucin in 0.02 M Tris-HCl, pH 7.4, containing 0.15 M NaCl (○) and in the buffer solutions containing 6 M guanidine hydrochloride (●). Two milliliters of the mucin samples was applied to the column (1.6 × 80 cm) which was operated at a flow rate of 6.7 mL/h. Two-milliliter fractions were collected and monitored for hexose content by the anthrone reaction.

molecular weight components. Since different investigators in the past have used different methods to isolate mucins from the respiratory secretions of patients and normal individuals (Harding & Creeth, 1983; LeTreut et al., 1981; Snyder et al., 1982; Woodward et al., 1982; Williams et al., 1982; Sachdev et al., 1980), it has been difficult to ascertain whether there are differences in the physicochemical properties of the CF native (nonreduced) mucin and the respiratory mucin isolated from non-CF subjects. In this paper, we have used identical experimental conditions to isolate purified mucins from respiratory secretions of a CF patient and an asthmatic patient, and their physicochemical properties have been compared.

There was no significant difference in the sugar components between the CF mucin and asthmatic mucin. There was, however, a striking difference in the sulfate content of the CF mucin (11%) compared to that observed for the asthmatic mucin (5.9%). Comparative studies using reduced–alkylated mucins (Boat et al., 1976) also showed that the CF sputum contains larger amounts of a higher sulfated mucin component than "normal" or bronchitic sputum. The sulfate groups are probably esterified to galactose (Roussel et al., 1975). Since the sialic acid content is similar in both CF and asthmatic mucins, the increased sulfate content observed in the CF mucin may impart a more anionic character to the mucin and alter the physical properties of the CF mucin. Recent studies on chicken tracheal mucin have shown that solutions of highly sulfated mucins are more viscous than solutions of less sulfated mucin (Mian et al., 1982). However, similar studies with CF mucin remain to be done.

Amino acid analysis of the purified CF mucin and asthmatic mucin showed differences in the overall composition; CF mucin had increased levels of hydroxyamino acids, serine and threonine, and lower amounts of asparate, glutamate, and glycine. The purified mucins isolated from normal individuals also have a considerably lower content of the hydroxyamino acids serine and threonine (Woodward et al., 1982; Sachdev et al., 1980) than has been observed for the CF mucin. These observed differences in the amino acid composition of the CF and asthmatic mucins may be due to differences in the proportion of different types of mucins present in the two respiratory secretions. It is possible that the isolated purified mucins from CF and asthmatic secretions are comprised of two or more mucins secreted from different cell types in the tracheobronchial tree (e.g., mucins are secreted by goblet cells as well as by mucus glands). It has been reported that there is an antigen present in purified mucin from rabbit tracheobronchial mucus that is detected in only 10% of the mucin-producing cells of the bronchial tree (St. George et al., 1984). In addition, two immunochemically distinct mucins have been reported to be present in human colonic mucin (Gold et al., 1981). Also, rat goblet cell mucin appears to be heterogeneous when examined by gel electrophoresis at very low mucin concentrations (Fahim et al., 1983). Thus, the observed difference in amino acid composition between CF and asthmatic mucins may be due to the presence of different proportions of mucins secreted from different cell types in the tracheobronchial tree. In order to further substantiate these findings, it will be necessary to study the amino acid composition of mucins isolated from sputa of several CF and asthmatic patients.

One of the interesting observations of this work is the finding that reduction of the disulfide bonds of the respiratory CF and asthmatic mucins does not alter the molecular weights of the mucin molecules (Table III). These results indicate that the polymeric structure of the respiratory mucin is not based on interdisulfide bonds. Respiratory mucins appear to differ from the gastric and cervical mucins in this respect. For example, pig gastric mucin has a molecular weight of 2×10^6; the reduction of its disulfide bonds with dithiothreitol resulted in four equal subunits with a molecular weight of 500 000 (Pearson et al., 1981). Similarly, reduction of human cervical mucin (Carlstedt et al., 1983) and pig submaxillary mucin (Shrogren et al., 1983) resulted in a considerably smaller molecular weight of the mucin.

Another important observation of this work is the finding that at concentrations greater than 0.5 mg/mL the respiratory native (nonreduced) mucins formed aggregates even under strong denaturation conditions, such as that provided by the presence of 6 M guanidine hydrochloride (Figures 6A,B). However, under similar experimental conditions, the CF native mucin formed larger aggregates (the majority of the CF mucin was excluded from the Sepharose CL2B column) as compared to that observed with the native asthmatic mucin (the majority of the asthmatic mucin was included in the Sepharose CL2B column). This aggregation behavior of the native mucins is apparently dependent on the intramolecular disulfide bonds in the mucins, as the reduction with dithiothreitol dramatically changed the elution pattern on Sepharose CL2B, while not altering the molecular weights of the mucins (Table III). Thus, it appears that the presence of an intact three-dimensional structure in the native mucin, which in part may be dependent on intradisulfide bonds, is necessary for aggregation to occur in these mucins. Disulfide bonds are important to the structure of some mucins; e.g., in the goblet cell mucin from

small intestine, the antigeneity of the nonglycosylated regions of the molecule is lost upon reduction of the disulfide bonds (Mantle et al., 1984).

The self-aggregation of the mucins to form large aggregates may be an important factor in determining the viscoelastic and gelation properties of the mucins. The nature of the molecular interactions responsible for aggregate formation is not known. Carbohydrate moieties have been implicated in the aggregation behavior of ovine submaxillary mucin (Hill et al., 1977; Rose et al., 1984). Also, canine mucins have been shown to have hydrophobic regions (Sachdev et al., 1979) which may affect the aggregation behavior of mucins. The higher state of aggregation of CF mucin may alter the viscoelastic properties of the secretions, which, in turn, may influence the clearance of the CF lung mucus secretions by the ciliated epithelium.

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Registry No. L-Fucose, 2438-80-4; D-galactose, 59-23-4; D-*N*-acetylgalactosamine, 1811-31-0; D-*N*-acetylglucosamine, 7512-17-6; sulfate, 14808-79-8; L-aspartic acid, 56-84-8; L-threonine, 72-19-5; L-serine, 56-45-1; L-glutamic acid, 56-86-0; L-proline, 147-85-3; glycine, 56-40-6; L-alanine, 56-41-7; L-valine, 72-18-4; L-methionine, 63-68-3; L-isoleucine, 73-32-5; L-leucine, 61-90-5; L-tyrosine, 60-18-4; L-phenylalanine, 63-91-2; L-histidine, 71-00-1; L-lysine, 56-87-1; L-arginine, 74-79-3.

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