

Evidence of Hydrophobic Domains in Human Respiratory Mucins. Effect of Sodium Chloride on Hydrophobic Binding Properties[†]

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ABSTRACT: Hydrophobic binding properties of purified human respiratory mucins were studied by the fluorescence probe technique using mansylphenylalanine (Mns-Phe) as the fluorescent probe. Mucins were purified from tracheobronchial secretions of cystic fibrosis (CF) and asthmatic patients, as well as from individuals with normal lungs, according to a protocol earlier established in our laboratory. Purified mucins were subjected to reduction-alkylation and Pronase digestion to study the effects of these treatments on the hydrophobic properties of the mucins. In addition, the effects of increased NaCl concentration on the hydrophobic properties of native and reduced-alkylated mucins were also investigated. Native mucins showed evidence of a large number of low-affinity ($K_D \sim 10^{-5}$ M) binding sites for the hydrophobic ligand Mns-Phe and had between 40 and 50 binding sites/mg of mucin. Reduction of mucin using dithiothreitol in the presence of 6 M guanidine hydrochloride and subsequent alkylation with iodoacetamide apparently caused marked conformational changes in the mucin molecules as revealed by the presence of both high-affinity ($K_D \sim 10^{-6}$ M) and low-affinity ($K_D \sim 10^{-5}$ M) binding sites for the probe and an increase in the number of probe binding sites. Pronase digestion of the native and reduced-alkylated mucins almost completely eliminated binding of the fluorescent probe to the mucins, showing that the binding sites are on the nonglycosylated, Pronase-sensitive portion of the mucin molecules. Increasing NaCl concentrations (0.03–1.0 M) did not appreciably alter the native mucin-induced Mns-Phe fluorescence, while that of the reduced-alkylated mucin-induced Mns-Phe fluorescence was progressively increased. The results indicate that further unfolding of reduced-alkylated mucins at increased NaCl concentrations exposes additional hydrophobic domains. Also, the greater increase in the number of probe binding sites in the reduced-alkylated CF mucin as compared to reduced-alkylated non-CF mucins suggests possible differences in the three-dimensional structure of CF and non-CF mucins. Thus, this study provided evidence for the presence of hydrophobic domains in human respiratory mucins, and these properties, at least in part, may contribute to the polymeric structure of mucins through noncovalent interactions.

Mucins are high molecular weight glycoprotein components of mucus secreted by the epithelial cells lining the gastrointestinal, tracheobronchial, and genitourinary tracts. They contribute to a large extent toward the gelation and viscoelastic properties of the mucus. Mucins are essentially composed of oligosaccharide chains densely packed into clusters and covalently linked to the protein core (Carlstedt et al., 1985). The carbohydrate is comprised of five sugars, namely fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid. In addition, mucins contain sulfate groups (Boat & Cheng, 1980; Chace et al., 1985). The heavily glycosylated regions are interspersed with nonglycosylated "naked" stretches of the protein core. These exposed regions of the protein core are susceptible to proteolytic attack (Carlstedt & Sheehan, 1984) and thought to be partly responsible for the interaction of mucins with many biologically important compounds such as enzymes (Hao et al., 1977), cations (Deman et al., 1973; Forstner & Forstner, 1975), and proteins like serum albumin (List et al., 1978) and ovalbumin (Creeth et al., 1979). Earlier studies have shown the presence of hydrophobic domains in canine tracheal and ovine submaxillary mucins (Sachdev et

al., 1979), bovine gallbladder mucin (Smith & LaMont, 1984), and rat salivary mucin (Slomiany et al., 1988). In fact, hydrophobic bonding has been implicated to be primarily responsible for mucin-albumin interaction (List et al., 1978), resulting in the enhancement of mucus viscosity. Such interactions are perhaps more relevant in pathological states characterized by high albumin concentrations in mucus secretions (Knauff & Adams, 1968; Schachter & Dixon, 1965). However, to date, no direct evidence for the presence of hydrophobic domains in human respiratory mucins has been reported. It is important therefore to study hydrophobic interactions of the mucins since they may shed light on the altered rheological properties of respiratory mucus in disease states.

Fluorescent probes are small molecules that have characteristic emission properties and, when coupled to biological macromolecules, can reveal subtle aspects of their structure and dynamics (Azzi, 1973). Several *N*-arylamino-naphthalenesulfonates have been used in the past as probes for the hydrophobic regions in proteins (Weber, 1953; McClure & Feldman, 1966; Stryer, 1968; Brand & Gohlke, 1972; Sachdev et al., 1973, 1975, 1979). These probes exhibit virtually no fluorescence in aqueous solutions but fluoresce intensely in nonpolar solvents or when bound to certain proteins and exhibit altered emission properties.

In the present study we report the hydrophobic binding properties of purified human respiratory mucins using the

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fluorescent probe Mns-Phe and discuss the possible role of these hydrophobic domains in mucin polymeric structure. The influence of increasing NaCl concentrations on mucin hydrophobicity was evaluated, since our recent studies (Chace et al., 1989) have indicated that NaCl concentration exerts a marked effect on the aggregation properties of human respiratory mucins.

EXPERIMENTAL PROCEDURES

Collection of Human Respiratory Mucus Secretions. Tracheobronchial mucus secretions (sputum specimens) were collected from two cystic fibrosis (CF-4, CF-5) and two asthmatic (AS-1, AS-2) patients and from two individuals with normal lungs (N-3 and N-4), fitted with chronic tracheostomies required as respiratory support. Several specimens from the same patient were combined for the purification of mucins. All samples were collected on ice, diluted at least 5-fold with water containing gentamicin sulfate (100 µg/mL), and dialyzed exhaustively against a 0.02% sodium azide solution and then against deionized water. The retentate was lyophilized and stored at -20 °C (Chace et al., 1985).

Isolation and Purification of Native Respiratory Mucins. A detailed protocol for the purification of the respiratory mucins has been described earlier (Chace et al., 1985). In brief, the respiratory mucus secretions were solubilized in 0.1 M Tris-HCl buffer, pH 7.5, containing 0.22 M potassium thiocyanate and 0.02% sodium azide. Following solubilization treatment, the suspension was centrifuged at 27000g for 4 h. The clear supernatant was applied to a Bio-Gel A 5-m column (5 × 90 cm) preequilibrated with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.22 M potassium thiocyanate and 0.02% sodium azide and eluted by using the same buffer. The excluded fraction containing DNA and mucus glycoprotein was treated with DNase to digest DNA molecules. Subsequently, the mucus glycoprotein was separated from the DNA fragments by rechromatography on the Bio-Gel A 5-m column. The excluded fraction containing the DNA-free mucus glycoprotein together with small amounts of low molecular weight components was further fractionated on a hydroxylapatite column (2.5 × 30 cm) equilibrated with 0.01 M phosphate buffer, pH 6.8. The column was initially eluted with the equilibrating buffer and subsequently with a discontinuous gradient of 0.15, 0.30, and 0.50 M potassium phosphate buffer, pH 6.8. The major fraction corresponding to mucus glycoprotein did not bind to the column and was eluted with the equilibrating buffer. A minor mucin component and contaminating impurities eluted later with higher ionic strength buffer solution.

Reduction-Alkylation of Purified Respiratory Mucins. Reduction and carboxymethylation of purified mucins were performed as described by Chace et al. (1985). Mucin (10 mg) was dissolved in 0.1 M Tris-HCl buffer, pH 8.5, containing 6 M guanidine hydrochloride and 0.02% sodium azide, giving a final concentration of 0.3 mg/mL. Nitrogen gas was bubbled through the solution for 30 min at 22 °C prior to addition of dithiothreitol (final concentration 25 mM). After 2 h of incubation, additional amounts of dithiothreitol were added to give a final 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 done for an hour in the dark at 22 °C. The excess iodoacetamide was removed by the addition of dithiothreitol (final concentration 0.2 M). The reduced-alkylated mucins were exhaustively dialyzed against deionized water and lyophilized.

Desialylation of Purified Respiratory Mucins. Both native and reduced-alkylated mucins were enzymatically desialylated

by using neuraminidase (*Vibrio cholerae*; Calbiochem). Mucin (10 mg) was solubilized in 2 mL of 50 mM sodium acetate buffer, pH 5.5, containing 1 mM calcium chloride. An appropriate quantity (based on millimoles of sialic acid present in mucin) of neuraminidase was then added and the mixture incubated at 37 °C for 24 h. Following incubation samples were dialyzed exhaustively against deionized water at 4 °C and lyophilized.

Gel Electrophoresis. The purity of mucins was examined by gel electrophoresis on composite (2% acrylamide + 0.5% agarose) gels containing 0.1% sodium dodecyl sulfate (Sachdev et al., 1978; Holden et al., 1971). Duplicate gels were run for each sample, one of which was stained with the periodic acid-Schiff (PAS) reagent for carbohydrates and the other with Coomassie Brilliant Blue for proteins as described by Segrest and Jackson (1972). The gels were also examined by the silver stain method.

Pronase Digestion of Purified Native Respiratory Mucins. Proteolytic digestion of purified tracheobronchial mucin was performed as described by Smith and LaMont (1984) with slight modifications. Briefly, lyophilized mucin (2 mg/mL) was reconstituted in 0.1 M phosphate buffer, pH 6.8, containing 0.02% sodium azide and 100 µL of 100× PSN antibiotic mixture (Gibco Laboratories) per milliliter, containing 500 µg of penicillin, 500 µg of streptomycin, and 1 mg of neomycin. Protease (*Streptomyces griseus*, type XIV) was added in a ratio of 1:10 (w/w) to mucin at 0 and 24 h. The samples were incubated for 48 h at 37 °C, dialyzed for 72 h against deionized water at 4 °C, and then lyophilized. The treated mucins were examined by Superose 6 chromatography.

Lipid Analysis. Purified mucin (5 mg) was extracted for 30 min with 50 volumes each of chloroform/methanol (2:1 v/v, twice), chloroform/methanol (1:1 and 1:2 v/v), and chloroform/methanol/water (65:35:8, by volume) consecutively, as described by Slomiany et al. (1983). Also in a second method, samples were extracted with 4 mL of *n*-heptane/2-propanol (3:7 v/v) which contained 50 µg of cholesterol butyrate as the internal standard (Wang et al., 1982). The neutral lipids (triglycerides, cholesterol, and cholesterol esters) were determined by gas-liquid chromatography (GLC) as described by Kuksis et al. (1975). Total fatty acids in the extracted residue were determined following saponification with ethanol-KOH mixture and subsequently analyzed by GLC as their methyl esters prepared by treatment with BF₃/MeOH as described by McConathy et al. (1981). For determining the presence of covalently bound fatty acids in the purified mucins, the lyophilized, delipidated mucin was deacylated by treatment with 5 mL of 1 M hydroxylamine (pH 7.0) for 5 h at room temperature (Slomiany et al., 1983). The incubation mixture was extracted three times with hexane. The organic layers were evaporated to dryness and subjected to fatty acid analyses as described above.

Compositional Analysis of Purified Respiratory Mucins. Chemical and amino acid compositions were determined for purified native and reduced-alkylated respiratory mucins. Neutral hexose was determined by the anthrone method (Carubelli et al., 1961) using a mixture of fucose and galactose (1:1 w/w) as the standard. Sialic acid was estimated by the resorcinol method (Svennerholm, 1957) using *N*-acetylneuraminic acid as the standard. Fucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine were determined by gas-liquid chromatography as described previously (Wang et al., 1974; Sachdev et al., 1978). In addition, after hydrolysis (4 N HCl, 4 h at 100 °C), the sugar amines were also analyzed by using an amino acid analyzer. Amino acid analysis was

performed by the method of Spackman (1967) using a Durrum D-500 amino acid analyzer following hydrolysis of the sample with constant-boiling HCl at 110 °C for 22 h in evacuated sealed glass tubes. Sulfate was determined by the sodium rhodizonate method (Terho & Hartiala, 1971).

Superose 6 Chromatography. A Superose 6 (10/30) FPLC column was equilibrated with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.22 M potassium thiocyanate, 4 M guanidine hydrochloride, and 0.02% sodium azide. Native, reduced-alkylated, and Pronase-treated mucins were dissolved in the above buffer at a concentration of 2 mg/mL and eluted with the same buffer.

Preparation of Mucin Solution for Fluorescence Studies. Purified and lyophilized native, reduced-alkylated, and Pronase-treated mucins were dissolved in the appropriate buffer (2.5 mg/mL) and left on a rocker at 4 °C for 48 h. To remove dust particles, the solutions were then centrifuged at 12000g for 20 min, and the supernatant was used for fluorescence experiments.

Synthesis of Fluorescent Probe Mns-Phe. Mansylphenylalanine was essentially prepared as described earlier in detail (Sachdev et al., 1973). Aliquots from a 1.5 mM stock solution prepared by dissolving the probe in 20 mM Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide were diluted just prior to fluorescence studies to give a 200 μ M solution. The compound and the solution were protected from light prior to their use in fluorescence experiments.

Fluorescence Studies. Fluorescence measurements were made on a Shimadzu RF-5000 fluorescence spectrophotometer. The excitation source was a 150-W xenon lamp. Bandwidths of 10 and 5 nm for excitation and emission spectra, respectively, were used. No corrections were introduced for changes in the sensitivity of the detection system at different wavelengths. Prior to each set of experiments, the instrument was calibrated as follows: the cuvette was filled with a standard solution of quinine sulfate in 0.1 N H₂SO₄ (1 μ g/mL) and, by use of an excitation wavelength of 350 nm, the emission at 450 nm was set to read 200 arbitrary fluorescence units with the sensitivity set at "high". All samples in these experiments had a final volume of 0.5 mL, and their fluorescence was studied in 0.5-cm² cuvettes. Mns-Phe was prepared from stock as a 200 μ M solution in 20 mM Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide alone or in buffer containing appropriate additions of NaCl. Lyophilized mucin preparations were reconstituted in 20 mM Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide alone or in buffer containing appropriate additions of NaCl. All fluorescence measurements were conducted at 25 °C and spectra recorded within 30 min of preparation.

Binding of Mns-Phe by Respiratory Mucin. Respiratory mucin binding of Mns-Phe was studied by measuring mucin-induced alterations in the fluorescence spectra of the hydrophobic ligand. The excitation and emission spectra of Mns-Phe (12.5 μ M) were measured alone and in the presence of purified mucin preparation (500 μ g/mL).

To evaluate the affinity of Mns-Phe binding by the respiratory mucin preparations, a Scatchard plot (Scatchard, 1949) was constructed in the following way: The limiting fluorescence, F_{\max} , was first determined by titrating a fixed amount of probe (5 μ M) with increasing mucin concentrations (0.06–0.50 mg/mL) and measuring the fluorescence after each increment. A plot of $1/P$ versus $1/F$ gave a straight line, extrapolation of which to zero abscissa gave the reciprocal of F_{\max} . Titration of a fixed mucin concentration (0.5 mg/mL) against increasing probe concentration (4.8–66.6 μ M) yielded

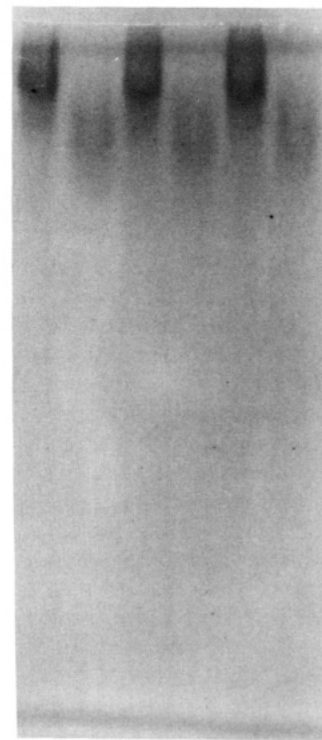


FIGURE 1: SDS gel electrophoresis of purified native and reduced-alkylated mucins. Composite gels (2% acrylamide + 0.5% agarose) containing 0.1% SDS were used and stained with PAS reagent. Lanes 1, 3, and 5 correspond to 60 μ g each of native CF, asthmatic, and normal mucin and lanes 2, 4 and 6 correspond to 60 μ g each of reduced-alkylated CF, asthmatic, and normal mucins, respectively.

values of bound and free probe concentrations. A plot of $[\text{Mns-Phe}]_{\text{bound}}$ per milligram of mucin versus $[\text{Mns-Phe}]_{\text{bound}}$ per milligram of mucin/ $[\text{Mns-Phe}]_{\text{free}}$ gave a straight line. Extrapolation of the line to zero ordinate gave the number of binding sites (n) per milligram of mucin and the extrapolation to zero abscissa gave n/K_D from which K_D (dissociation constant) values were calculated. Ten separate fluorescence titrations were performed by using from 0.06 to 0.50 mg/mL of purified respiratory mucin. Mns-Phe concentration was varied from 1.5 to 12.5 μ M to determine high-affinity binding (HAB) sites and from 4.8 to 66.6 μ M to determine low-affinity binding (LAB) sites in each experiment. The method of least squares was used to calculate linear regression plots of the data. In all cases the correlation coefficient (r) value was ≥ 0.92 .

Effect of NaCl Concentration on the Hydrophobic Binding Properties of Respiratory Mucins. The effect of NaCl concentration on the hydrophobic binding properties of mucins was studied by dissolving mucin preparations (0.5 mg/mL) in 20 mM Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide plus the desired NaCl concentration. The probe concentration was kept constant at 12.5 μ M and the relative fluorescence measured. Fluorescence titrations were also performed with the native and reduced-alkylated mucins dissolved in buffer containing different NaCl concentrations to determine the effect of NaCl on the probe binding properties of the mucins.

RESULTS

Purification of Mucins. The mucins isolated from respiratory mucus secretions were examined for their purity by SDS composite gel electrophoresis. The CF, asthmatic, and normal mucins gave a single broad band when stained with the PAS reagent (Figure 1). No low molecular weight components were detected when the gels were stained with Coomassie

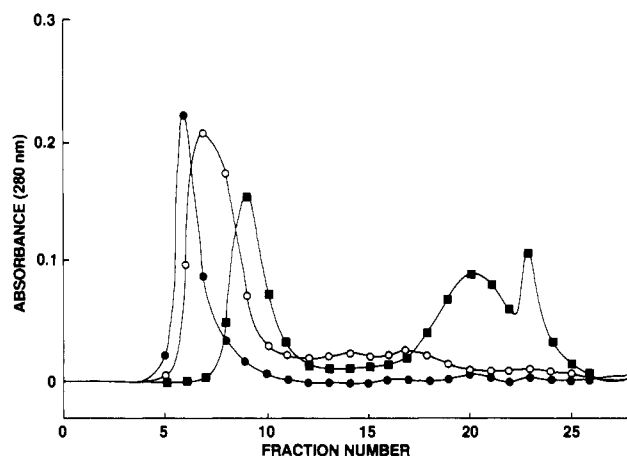


FIGURE 2: Superose 6 column chromatography of native (●), reduced-alkylated (○), and Pronase-digested (■) respiratory mucins. A Superose 6 (FPLC, Pharmacia) analytical column was eluted with 0.1 M Tris-HCl buffer, pH 7.4, containing 4 M guanidine hydrochloride and 0.02% sodium azide at a flow rate of 0.3 mL/min. Samples (1 mg in 0.5 mL) were loaded and 1.0-mL fractions collected.

Table I: Chemical Composition of Purified CF, Asthmatic, and Normal Respiratory Mucins^a

component	CF		asthmatic		normal	
	CF-4	CF-5	AS-1	AS-2	N-3	N-4
protein ^b	14.0	15.6	28.1	28.3	24.6	23.0
fucose ^c	10.2	13.1	7.1	5.0	11.5	9.1
galactose ^c	23.0	21.3	19.1	23.8	23.2	20.7
<i>N</i> -acetylgalactosamine ^d	6.5	5.2	9.8	7.6	7.9	10.6
<i>N</i> -acetylglucosamine ^d	18.8	19.4	16.3	18.6	17.0	18.2
sialic acid ^e	20.2	17.8	13.8	10.8	9.3	13.0
sulfate ^f	7.3	7.6	5.4	5.5	6.0	5.2

^aThe results are expressed as a percentage of the dry weight of mucin specimens. The data were normalized to reflect at 100%. ^bCalculated from amino acid composition data. ^cAssayed by gas-liquid chromatography of alditol acetate of sugars. ^dAssayed by using amino acid analyzer. ^eAssayed by the resorcinol method using synthetic *N*-acetylneuraminic acid as the standard. ^fAssayed by the sodium rhodizonate method; expressed as SO₄.

Brilliant Blue or by the silver stain method, thus indicating the absence of any low molecular weight protein/glycoprotein in the purified preparations.

Superose 6 Chromatography of Respiratory Mucins. Purified native human respiratory mucin eluted at the void volume of the Superose 6 column as a single peak (Figure 2). Our earlier studies (Chace et al., 1985, 1989) using static light scattering technique have shown an average M_r of about 5×10^6 for the native mucins. The reduced-alkylated mucin eluted close to the void volume as a broad peak, indicating a slight decrease in molecular weight. The Pronase-digested mucin fragments eluted in the M_r range of around 7×10^5 with two low molecular weight fractions of $M_r \sim 30,000$.

Chemical Composition of the Purified Respiratory Mucins. The chemical compositions of the purified CF, asthmatic, and normal mucins are shown in Table I. In all cases, carbohydrate analyses by gas-liquid chromatography revealed the presence of fucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine. In addition, all mucins contained sialic acid and sulfate. No mannose or deoxyribose in all mucins showed the absence of serum glycoprotein(s) and DNA. The overall carbohydrate content of the native mucins ranged from about 70% to 75% of the dry weight of the mucins, while the sialic acid and sulfate content was higher for CF mucins as compared to asthmatic and normal mucins. The average protein content varied from 14.8% in CF mucins to 26% in non-CF mucins. Only minor differences were found in the

Table II: Amino Acid Composition of Purified CF, Asthmatic, and Normal Human Respiratory Mucins

amino acid	residues/1000 residues					
	CF-4	CF-5	AS-1	AS-2	N-3	N-4
aspartic acid	30	38	53	52	54	58
threonine	289	283	222	224	247	258
serine	143	141	141	120	115	116
glutamic acid	55	47	61	65	67	62
proline	139	118	119	103	129	123
glycine	63	69	74	81	75	73
alanine	92	98	83	97	88	94
valine	40	49	57	47	48	48
methionine	7	8	8	8	7	6
isoleucine	19	20	19	28	16	16
leucine	42	47	43	67	52	50
tyrosine	6	5	16	15	10	10
phenylalanine	13	7	22	12	14	14
histidine	22	22	21	23	25	23
lysine	15	17	27	25	21	17
arginine	25	31	34	33	32	32

other chemical parameters of the purified mucins studied in this work.

Lipid extraction and analyses were performed on the purified mucins. Only trace amounts of triglycerides and cholesterol esters were detected, while cholesterol amounted to about 0.03% of the dry weight of the sample. Total neutral lipid content was estimated to be about 0.1% of the dry weight of the sample. Both extraction procedures gave similar results. Total fatty acid content was about 0.4% of the dry weight of the specimens. This is consistent with the low levels of neutral lipids in the samples. Since the total fatty acid content of the samples was quite low, we did not attempt to quantitate the phospholipids and glycolipids in the samples. On the basis of these data, we believe that the purified mucins had less than 1% of noncovalently bound lipids in them. Also, no covalently bound lipids could be detected following deacylation of the mucins with hydroxylamine.

Amino acid compositions of the purified CF, asthmatic, and normal mucins are shown in Table II. Comparison of the data revealed some differences between the CF and non-CF mucins. The CF mucin had a higher combined content of serine and threonine (~43.0%) as compared to non-CF mucins (~36.0%). In addition, the CF mucin had a higher amount of proline and lower aspartate, glutamate, and glycine content. For the CF mucin, threonine, serine, proline, glycine, and alanine comprised 72% of the total amino acid residues, while the amount of these five amino acids for the asthmatic mucin was 57% and that for the normal mucins was 66% of the total amino acid residues. All mucins had a low content of aromatic amino acid residues.

Chemical and amino acid compositions of the native and reduced-alkylated mucins were compared (data not shown) and no significant differences found for all the mucins.

Binding of Fluorescent Probe with Respiratory Mucins. The excitation and emission spectra of unbound Mns-Phe (12.5 μ M) showed excitation maxima at 325 nm and emission maxima at 465 nm. When bound to human respiratory mucin (500 μ g/mL), however, the emission maxima shifted to 445 nm. The fluorescence intensity of the Mns-Phe-mucin complex was about 4-fold higher as compared to that observed for Mns-Phe alone, while upon binding to reduced-alkylated mucins, the fluorescence intensity increased about 10-fold (Figure 3). In contrast, upon binding to Pronase-digested native and reduced-alkylated mucins, no enhancement of Mns-Phe fluorescence was observed.

Effect of NaCl Concentration on Hydrophobic Binding Properties of Human Respiratory Mucins. We examined the

Table III: Binding of Mns-Phe with Purified CF, Asthmatic, and Normal Human Respiratory Mucins in 20 mM Tris Buffer, pH 7.4, Containing 0.02% Sodium Azide^a

mucin	native		reduced-alkylated			
	<i>n</i>	<i>K_D</i> (M)	<i>n</i> (HAB)	<i>K_D</i> (M)	<i>n</i> (LAB)	<i>K_D</i> (M)
CF-4	47	1.84×10^{-5}	17	1.13×10^{-6}	53	2.94×10^{-5}
CF-5	46	2.12×10^{-5}	21	2.18×10^{-6}	35	1.62×10^{-5}
AS-1	48	2.89×10^{-5}	25	1.38×10^{-6}	39	1.92×10^{-5}
AS-2	46	2.51×10^{-5}	26	1.64×10^{-6}	34	1.76×10^{-5}
N-3	40	4.12×10^{-5}	13	1.18×10^{-6}	32	2.42×10^{-5}
N-4	50	2.74×10^{-5}	18	1.09×10^{-6}	37	2.05×10^{-5}

^a*n*, number of binding sites per milligram of mucin. *K_D* (M), dissociation constant. HAB, high-affinity binding sites. LAB, low-affinity binding sites.

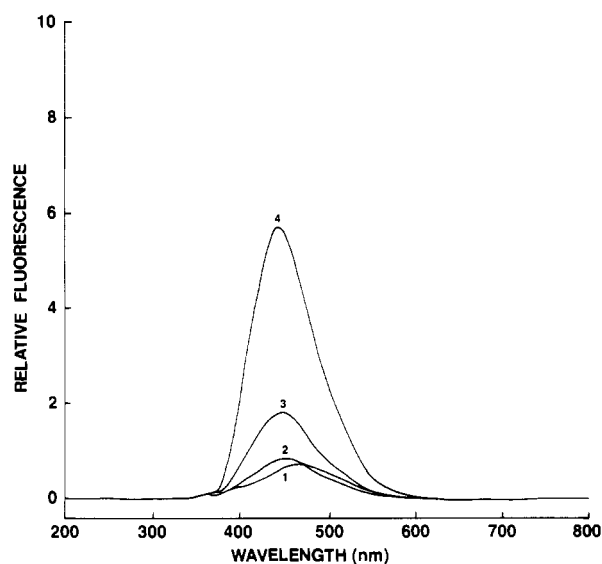


FIGURE 3: Fluorescence emission spectra of free and bound Mns-Phe. Fluorescence emission spectra of Mns-Phe (12.5 μ M) in 20 mM Tris-HCl buffer, pH 7.4 (1), and in the presence of buffer containing 500 μ g/mL of Pronase-digested (2), native (3), and reduced-alkylated (4) respiratory mucin are shown. All measurements were made at 25 $^{\circ}$ C, and the excitation wavelength was 325 nm.

effect of increasing NaCl concentration on the mucin-induced fluorescence of Mns-Phe, a negatively charged probe. Increasing NaCl concentration only slightly increased the native mucin-induced probe fluorescence (data not shown). However, reduced-alkylated mucin-induced probe fluorescence was considerably affected in the presence of increased NaCl concentration. The effect was more marked in the case of CF mucins (Figure 4) than in the case of normal mucins (Figure 5). In addition, the effect of increasing NaCl concentrations on the fluorescence emission of desialylated native and reduced-alkylated mucins was investigated. For both CF and non-CF mucins the increase in fluorescence emission was considerably dampened upon desialylation. The dampening effect was more marked in the case of desialylated, reduced-alkylated CF mucins (Figure 6) as compared to that in desialylated and reduced-alkylated non-CF mucins (Figure 7). In separate experiments the higher ionic strength buffer alone had no effect on Mns-Phe fluorescence.

Binding Affinity and Number of Binding Sites upon Interaction of Mns-Phe with Mucins. Scatchard plots of binding of Mns-Phe with native mucin showed the *K_D* values to be of the order of 10^{-5} M for both CF and normal mucins in Tris buffer alone (Table III) as well as in buffer containing up to 1.0 M NaCl (Table IV). The numbers of binding sites per milligram of CF and normal mucin were 47 and 50, respectively, and did not significantly change with increasing NaCl concentration. The number of binding sites (*n*) and *K_D* values

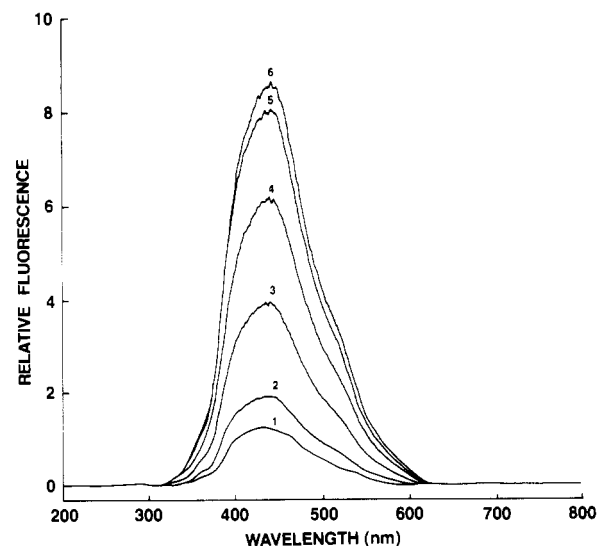


FIGURE 4: Effect of increasing NaCl concentration on CF respiratory mucin-induced fluorescence of Mns-Phe. Reduced-alkylated CF mucin (200 μ g/mL) was incubated with Mns-Phe (40 μ M) in 20 mM Tris-HCl buffer, pH 7.4, containing no NaCl (1) and containing 0.03 (2), 0.1 (3), 0.15 (4), 0.5 (5), and 1.0 M (6) NaCl. Excitation and emission wavelengths of 325 and 445 nm were used, respectively.

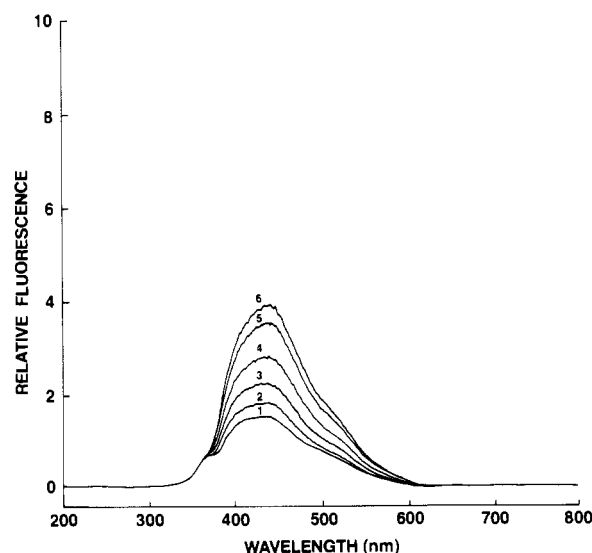


FIGURE 5: Effect of increasing NaCl concentration on normal respiratory mucin-induced fluorescence of Mns-Phe. Reduced-alkylated normal mucin (200 μ g/mL) was incubated with Mns-Phe (40 μ M) in 20 mM Tris-HCl buffer, pH 7.4, containing no NaCl (1) and containing 0.03 (2), 0.1 (3), 0.15 (4), 0.5 (5), and 1.0 M (6) NaCl. Excitation and emission wavelengths of 325 and 445 nm were used, respectively.

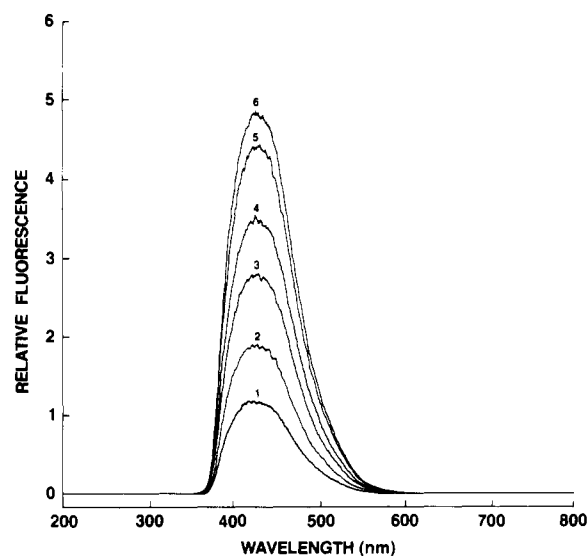
for each of the mucins at different NaCl concentrations are given in Table IV.

Table IV: Binding of Mns-Phe with Purified Native CF, Asthmatic, and Normal Human Respiratory Mucins in 20 mM Tris Buffer, pH 7.4, Containing 0.02% Sodium Azide and Different Concentrations of NaCl^a

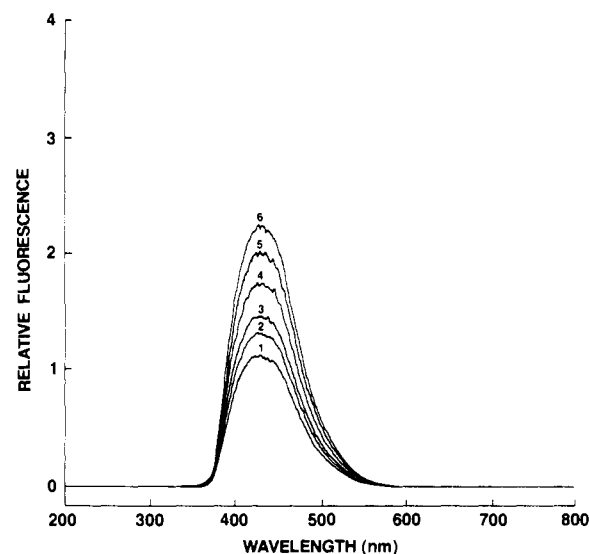
mucin	NaCl concentration									
	0.03 M		0.10 M		0.15 M		0.50 M		1.0 M	
	<i>n</i>	<i>K_D</i> (M)	<i>n</i>	<i>K_D</i> (M)	<i>n</i>	<i>K_D</i> (M)	<i>n</i>	<i>K_D</i> (M)	<i>n</i>	<i>K_D</i> (M)
CF-4	42	1.10×10^{-5}	43	1.19×10^{-5}	48	1.26×10^{-5}	55	1.25×10^{-5}	56	1.04×10^{-5}
CF-5	36	0.66×10^{-5}	38	0.62×10^{-5}	45	0.49×10^{-5}	56	0.70×10^{-5}	55	0.73×10^{-5}
AS-1	34	1.30×10^{-5}	37	1.48×10^{-5}	41	1.82×10^{-5}	45	1.66×10^{-5}	47	1.80×10^{-5}
AS-2	37	1.70×10^{-5}	40	1.65×10^{-5}	45	1.79×10^{-5}	45	1.73×10^{-5}	47	1.80×10^{-5}
N-3	34	3.77×10^{-5}	35	3.88×10^{-5}	35	3.93×10^{-5}	36	3.71×10^{-5}	38	3.80×10^{-5}
N-4	50	1.23×10^{-5}	49	1.04×10^{-5}	47	0.80×10^{-5}	45	0.76×10^{-5}	46	0.83×10^{-5}

^a *n*, number of binding sites per milligram of mucin. *K_D* (M), dissociation constant.Table V: Binding of Mns-Phe with Purified, Reduced-Alkylated CF, Asthmatic, and Normal Human Respiratory Mucins in 20 mM Tris Buffer, pH 7.4, Containing 0.02% Sodium Azide and Different Concentrations of NaCl^a

		NaCl concentration									
		0.03 M		0.10 M		0.15 M		0.50 M		1.0 M	
mucin		<i>n</i>	<i>K_D</i> (M)	<i>n</i>	<i>K_D</i> (M)	<i>n</i>	<i>K_D</i> (M)	<i>n</i>	<i>K_D</i> (M)	<i>n</i>	<i>K_D</i> (M)
CF-4	HAB	33	7.22×10^{-6}	48	1.65×10^{-5}	53	1.82×10^{-5}	68	2.34×10^{-5}	76	2.33×10^{-5}
	LAB	107	8.48×10^{-5}	152	1.60×10^{-4}	172	1.80×10^{-4}	200	2.14×10^{-4}	249	1.78×10^{-4}
CF-5	HAB	23	6.50×10^{-6}	40	1.25×10^{-5}	50	1.56×10^{-5}	55	1.69×10^{-5}	70	2.15×10^{-5}
	LAB	112	9.18×10^{-5}	130	1.26×10^{-4}	160	1.56×10^{-4}	205	1.97×10^{-4}	250	2.35×10^{-4}
AS-1	HAB	23	1.39×10^{-6}	33	1.95×10^{-6}	50	2.68×10^{-6}	63	3.38×10^{-6}	85	4.80×10^{-6}
	LAB	57	1.74×10^{-5}	67	2.16×10^{-5}	110	3.20×10^{-5}	132	3.84×10^{-5}	165	5.15×10^{-5}
AS-2	HAB	28	1.48×10^{-6}	36	1.98×10^{-6}	44	2.31×10^{-6}	58	3.05×10^{-6}	90	4.73×10^{-6}
	LAB	52	1.66×10^{-5}	64	2.08×10^{-5}	81	2.50×10^{-5}	104	3.24×10^{-5}	165	4.44×10^{-5}
N-3	HAB	27	1.48×10^{-6}	33	2.20×10^{-6}	37	2.60×10^{-6}	43	3.01×10^{-6}	51	3.57×10^{-6}
	LAB	35	1.27×10^{-5}	37	1.42×10^{-5}	42	1.69×10^{-5}	47	1.95×10^{-5}	57	2.35×10^{-5}
N-4	HAB	25	2.43×10^{-6}	30	2.75×10^{-6}	36	3.00×10^{-6}	40	3.33×10^{-6}	42	3.10×10^{-6}
	LAB	55	1.99×10^{-5}	60	2.26×10^{-5}	72	2.46×10^{-5}	78	2.68×10^{-5}	83	1.66×10^{-5}

^a *n*, number of binding sites per milligram of mucin. *K_D* (M), dissociation constant. HAB, high-affinity binding sites. LAB, low-affinity binding sites.FIGURE 6: Effect of increasing NaCl concentration on desialylated CF respiratory mucin-induced fluorescence of Mns-Phe. Reduced-alkylated and desialylated CF mucin (200 μ g/mL) was incubated with Mns-Phe (40 μ M) in 20 mM Tris-HCl buffer, pH 7.4, containing no NaCl (1) and containing 0.03 (2), 0.1 (3), 0.15 (4), 0.5 (5), and 1.0 M (6) NaCl. Excitation and emission wavelengths were 325 and 445 nm, respectively.

In the case of reduced-alkylated mucins the Scatchard plots showed evidence of the existence of both high- and low-affinity binding sites for Mns-Phe. Representative Scatchard plots for reduced-alkylated CF and normal mucins are shown in Figures 8 and 9, respectively. In Tris buffer alone the reduced-alkylated CF mucin had 17 high-affinity binding sites

FIGURE 7: Effect of increasing NaCl concentration on desialylated normal respiratory mucin-induced fluorescence of Mns-Phe. Reduced-alkylated and desialylated normal mucin (200 μ g/mL) was incubated with Mns-Phe (40 μ M) in 20 mM Tris-HCl buffer, pH 7.4, containing no NaCl (1) and containing 0.03 (2), 0.1 (3), 0.15 (4), 0.5 (5), and 1.0 M (6) NaCl. Excitation and emission wavelengths of 325 and 445 nm were used, respectively.

($K_D = 1.13 \times 10^{-6}$ M) and 53 low-affinity binding sites ($K_D = 2.94 \times 10^{-5}$ M). The reduced-alkylated normal mucin, on the other hand, had 18 high-affinity binding sites ($K_D = 1.09 \times 10^{-6}$ M) and 37 low-affinity binding sites ($K_D = 2.05 \times 10^{-5}$ M). In the presence of buffer containing 1.0 M NaCl, the reduced-alkylated CF mucin showed 76 high-affinity ($K_D =$

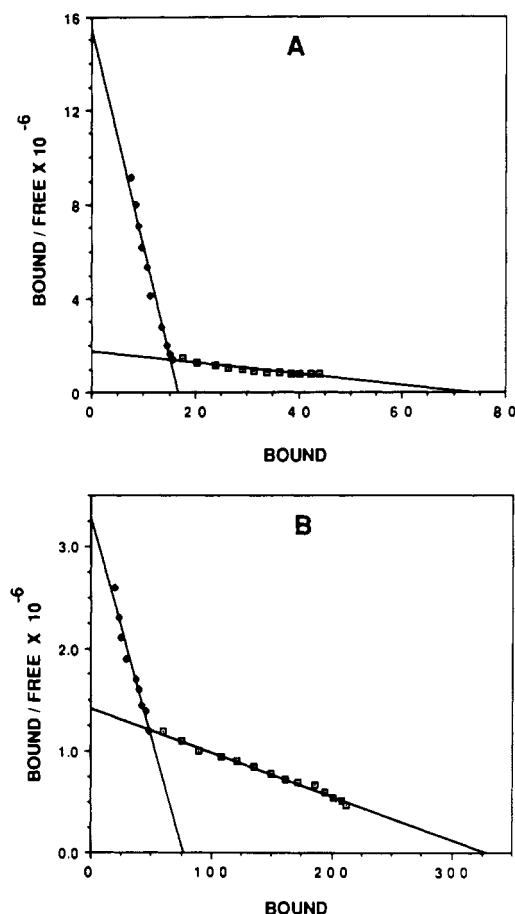


FIGURE 8: Scatchard plots of binding of fluorescent probe Mns-Phe with reduced-alkylated CF mucin. Binding of Mns-Phe with reduced-alkylated CF mucin in 20 mM Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide (A) and in the same buffer containing 1 M NaCl (B) is shown.

2.33×10^{-5} M) and 249 low-affinity binding sites ($K_D = 1.78 \times 10^{-4}$ M). In contrast, the normal reduced-alkylated mucin had 42 high-affinity ($K_D = 3.10 \times 10^{-6}$ M) and 83 low-affinity ($K_D = 1.66 \times 10^{-5}$ M) binding sites. A summary of binding sites and K_D values at different NaCl concentrations for each of the reduced-alkylated mucins is given in Table V.

DISCUSSION

The mucins purified by the protocol established in our laboratory (Chace et al., 1985) gave a single broad band when gels were stained with periodic acid-Schiff reagent which stains glycoproteins. Also, no low molecular weight components were detected when the gels were stained with Coomassie Brilliant Blue and by the silver staining method. In addition, both CF and non-CF mucins gave identical elution profiles upon Superose 6 (FPLC) chromatography in buffer containing 4 M guanidine hydrochloride. In both experiments, a single peak corresponding to the mucus glycoprotein eluted at the void volume of the column. The absence of other absorbance peak(s) in the elution profile, in strongly dissociative conditions, further supports the purity of the mucin preparations. Furthermore, molecular weight determination of CF and non-CF mucins in 6 M guanidine hydrochloride by static laser light scattering technique showed no differences in molecular weights between CF and non-CF mucins (Chace et al., 1989). Recently we have successfully deglycosylated both CF and normal mucins using chemical (trifluoromethanesulfonic acid) and enzymatic (α -N-acetylgalactosaminidase) treatments (Sachdev et al., 1989). Both the CF and normal apomucins were found to have identical molecular mass ($\sim 100,000$

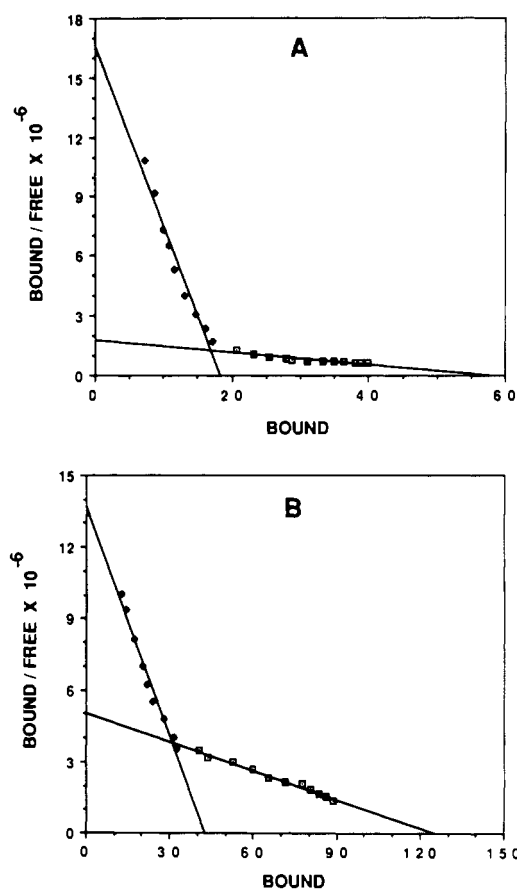


FIGURE 9: Scatchard plots of binding of fluorescent probe Mns-Phe with reduced-alkylated normal mucin. Binding of Mns-Phe with reduced-alkylated normal mucin in 20 mM Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide (A) and in the same buffer containing 1 M NaCl (B) is shown.

daltons). Thus, all the above-cited observations strongly suggest the absence of any notable degradation of the CF mucin.

The reduced-alkylated mucins also gave a single broad band in the gels stained with PAS reagent and had slightly greater mobility as compared to that of the native mucins. This observation is in agreement with the results obtained by Superose 6 chromatography which also showed a slight decrease in molecular weight for the reduced-alkylated mucins.

Purified CF, asthmatic, and normal respiratory mucins had chemical compositions in agreement with those reported earlier (Chace et al., 1985, 1989) for other mucins. The major advantage of our purification protocol is that the purified mucin preparations had only very small amounts of noncovalently bound lipids ($<1\%$ of the dry weight of the mucins) and no detectable amounts of covalently bound lipids. While Mantle and Forstner (1986) have reported less than 5% by weight of noncovalently bound lipids in the purified human intestinal mucin, purified bovine gallbladder mucin was found to have trace levels of lipid (Smith & LaMont, 1984). Slomiany et al. (1981, 1983) have reported the presence of large amounts of noncovalently and covalently bound lipids in purified gastrointestinal mucins, as have Woodward et al. (1982) in respiratory mucin. The reason for the presence of very low levels of lipids in the mucins we purified is perhaps due to the different purification protocol we employed.

The mansyl group has been shown earlier (Cory et al., 1968; Turner & Brand, 1968) to be a sensitive fluorescent hydrophobic probe. Mansyl peptides have been synthesized and used to study enzyme-substrate interactions (Sachdev et al., 1973).

Mansyl compounds have also been found to be more sensitive probes as compared to analogous dansyl compounds (Sachdev et al., 1973). Our earlier studies on the hydrophobic binding sites of canine tracheal and ovine submaxillary mucins (Sachdev et al., 1979) using the fluorescent probes mansyl-phenylalanine and sodium mansate have shown mansyl-phenylalanine to be a better probe than sodium mansate to investigate binding affinity and hydrophobic binding sites of ovine submaxillary mucin. Hence, in the present study mansylphenylalanine was used as the probe to determine the hydrophobic binding properties of the respiratory mucins.

Human respiratory mucin contained a large number of hydrophobic binding sites for Mns-Phe which appeared to be in the nonglycosylated portion of the protein core. This is supported by the observation that probe binding was almost completely eliminated after Pronase digestion of the mucins. These observations are in agreement with those obtained earlier for bovine gallbladder mucin (Smith & LaMont, 1984), bovine cervical mucin (Bhushana Rao & Masson, 1977), and rat salivary mucin (Slomiany et al., 1988). While Slomiany et al. (1988) have suggested that associated and covalently bound lipids contribute to the hydrophobic characteristics of salivary mucins, data from our present study show that the hydrophobic properties of human respiratory mucins are primarily due to the protein core.

Our earlier studies (Chace et al., 1985) have shown that, unlike the gastric and cervical mucins, the human respiratory mucins upon reduction of disulfide bonds undergo only a slight change in molecular weight, implying that the polymeric structure of the mucin may not be based on interdisulfide bonds alone. This is further supported by the gel electrophoretic data reported here for native and reduced mucins (Figure 1). Noncovalent interactions between mucin molecules may be partly responsible for the polymeric structure as demonstrated recently for bovine gallbladder mucin (Smith et al., 1989). We have previously shown that hydrophobic domains also exist in canine tracheal and ovine submaxillary mucins (Sachdev et al., 1979).

Reduction-alkylation of disulfide bonds in the native mucins probably causes profound changes in the three-dimensional structure of the mucin molecule, resulting in an unfolded conformation that exposes additional hydrophobic domains. The existence of both high- and low-affinity hydrophobic domains, not observed in the native mucins, lends support to this hypothesis. The unfolding is perhaps progressively increased with increasing NaCl concentration, thus explaining the observed increase in binding of the hydrophobic probe to the mucin molecule at higher salt concentrations. A relatively higher number of hydrophobic sites observed in the reduced-alkylated CF mucins as compared to the reduced-alkylated asthmatic or normal mucins in the presence of different NaCl concentrations (Table V) may be due to the fact that the CF mucin by virtue of being more sulfated and having a higher sialic acid content is more anionic and thus more susceptible to change in conformation mediated by the charge-shielding effects of increased NaCl concentration. To test this hypothesis, the effects of increasing NaCl concentrations on the fluorescence emission of desialylated native and reduced-alkylated CF and non-CF mucins were studied. The results obtained (Figures 6 and 7) clearly indicate that the reduction in fluorescence emission upon desialylation is more marked in the case of reduced-alkylated CF mucins as compared to non-CF mucins. This shows that the greater anionic charge on the CF mucins is more responsive to the charge-shielding effects of increasing NaCl concentrations. It would be in-

teresting to study if removal of sulfate groups from the desialylated mucins further dampened the effects of increasing NaCl concentrations on fluorescence. However, in the absence of any known sulfohydrolases that can preferentially remove sulfate from the mucins, these studies are not feasible at this time.

The differences in the observed number of binding sites and the K_D values obtained also suggest that the three-dimensional structures of CF and non-CF mucins may be different. The CF mucins are likely to have a more extended structure than non-CF mucins due to increased repulsion of like charges (anionic). This would account for greater access of the probe to hydrophobic binding sites upon neutralization of the charge. Interestingly, Mantle and Stewart (1989), on the basis of immunochemical studies, reported the possibility of differences in the three-dimensional configuration of CF and non-CF intestinal mucins. Our recent studies (Sachdev et al., 1989) using monoclonal antibodies raised against the CF apomucin also suggested possible differences in the three-dimensional structures of CF and non-CF mucins. Also, Rose (1988) found that meconium mucins from CF patients upon digestion with trypsin-treated TPCK yielded peptides whose hydrophobic properties differed from those obtained in the same way from mucins isolated from non-CF individuals. This was interpreted to indicate that naked peptide regions of CF mucins are different from those of non-CF mucins.

On the basis of chemical compositional data (Table I) one may assume that an apparently lower net protein content in the case of CF mucins as compared to non-CF mucins may mean a correspondingly lower number of hydrophobic probe binding sites in the CF mucins. On the contrary, however, it must be pointed out that, rather than net protein content alone, it is the three-dimensional configuration of the protein molecule that determines the affinity of binding and accessibility of hydrophobic domains for the probe. The observed differences in K_D values, number of binding sites, and effect of increasing NaCl concentration on the binding of the probe with the mucins strongly suggest differences in the three-dimensional structure of CF and non-CF mucins.

To the best of our knowledge, this is the first evidence for the presence of hydrophobic domains in human respiratory mucins. At least two different types of hydrophobic regions may be present on the mucin molecule revealed upon conformational changes induced by reduction and alkylation. On the basis of the results from this work it seems very likely that hydrophobic and other noncovalent interactions may also play a part, in addition to covalent linkages, in the total polymeric structure of the human respiratory mucins. Thus, hydrophobic interactions may play an important role in the viscoelastic and gelation properties of the respiratory mucus.

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