Eftink, M. R., & Jameson, D. M. (1982) *Biochemistry 21*, 4443-4449.
Foley, R., Poon, J., & Anderson, P. M. (1971) *Biochemistry 10*, 4562-4569.
Kasprzak, A. A., & Kochman, M. (1980) *Eur. J. Biochem. 104*, 443-450.
Kasprzak, A. A., & Kochman, M. (1981) *J. Biol. Chem. 256*, 6127-6133.
Kasprzyk, P. G., Anderson, P. M., & Villafranca, J. J. (1983) *Biochemistry 22*, 1877-1882.
Kasprzyk, P. G., Whalen-Pederson, E., Anderson, P. M., & Villafranca, J. J. (1985) *Bioorg. Chem. 13*, 98-109.
Kayne, F. J., & Price, N. C. (1972) *Biochemistry 11*, 4415-4420.
Malencik, D. A., & Anderson, S. R. (1988) *Biochemistry 27*, 1941-1949.
Matthews, S. L., & Anderson, P. M. (1972) *Biochemistry 11*, 1176-1183.
Powers, S. G., Meister, A., & Haschenmeyer, R. H. (1980) *J. Biol. Chem. 255*, 1554-1558.
Rajkowski, K. M., & Cittanova, N. (1981) *J. Theor. Biol. 93*, 691-696.
Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1978) *Biochemistry 17*, 5588-5591.
Raushel, F. M., Rawding, C. J., Anderson, P. M., & Villafranca, J. J. (1979) *Biochemistry 18*, 5562-5566.
Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1983) *Biochemistry 22*, 1872-1876.
Secrist, J. A., III, Barrio, J. R., Leonard, N. J., & Weber, G. (1972) *Biochemistry 11*, 3499-3506.
Teichberg, V. I., & Shinitzky, M. (1973) *J. Mol. Biol. 74*, 519-531.
Trotta, P. P., Burt, M. E., Haschenmeyer, R. H., & Meister, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2599.
Trotta, P. P., Estis, L. F., Meister, A., & Haschenmeyer, R. H. (1974) *J. Biol. Chem. 249*, 482-491.
Tyuma, I., Shimizu, K., & Imai, K. (1971) *Biochem. Biophys. Res. Commun. 43*, 423.
Weber, G. (1975) *Adv. Protein Chem. 29*, 1-83.

Protein Components of Human Tracheobronchial Mucin: Partial Characterization of a Closely Associated 65-Kilodalton Protein†

Nancy J. Ringler, R. Selvakumar,‡ H. D. Woodward,§ V. P. Bhavanandan, and E. A. Davidson*
Department of Biological Chemistry, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033

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ABSTRACT: A high-density mucin glycoprotein was isolated from human tracheobronchial secretions substantially free of contaminating protein, low-density glycoprotein, proteolytic enzymes, and lipid. A closely associated 65-kDa protein was discovered while investigating the effect of 2-mercaptoethanol treatment on the purified mucin glycoprotein. It has been established that the 65-kDa protein is neither α_1-antichymotrypsin nor human serum albumin, two proteins of similar molecular weight which are found in crude tracheobronchial secretions. This protein lacks cross-reactivity with antibodies directed against serum components and is presumably comparable to the 65-kDa protein similarly isolated from canine tracheal pouch secretions [Ringler et al. (1987) *Biochemistry 26*, 5322-5328]. Although both the presence of sulfhydryl groups and the ability to be reassociated with the mucin molecule have been established, it is not clear whether its association is due to direct disulfide bonding, hydrophobicity, or entrapment. It was found that ^{14}C-methylated methemoglobin was an inappropriate substrate for measurement of proteolytic activity in mucin preparations due to inherent entrapment and clearance capabilities of mucin molecules.

Secretory products of the upper airway epithelial cells provide the components necessary for control of the physical properties of the respiratory mucus. Since this material must be translocated in an upward direction for clearance, and simultaneously provide a coating on cellular surfaces, its rheological properties must be tightly regulated. These are largely defined by a mucin-type glycoprotein with a covalent molecular weight in the range of 10^6. The ability of this macromolecule to aggregate is an important feature of the overall system as is its capacity to bind lipid and cations. Detailed studies of the chemical structure of the glycoprotein have been performed in several laboratories, and a composite analytical profile has been defined (Roberts, 1976; Creeth et al., 1977; Sachdev et al., 1980; Rose et al., 1984; Slayter et al., 1984; Woodward et al., 1982, 1987). A recent study of material isolated from canine tracheal mucus indicated that a nonglycosylated protein of M_r 65K was closely associated with the mucin glycoprotein (Ringler et al., 1987). The present report details the properties of a comparable protein from human secretions and the role of disulfide bonds in the associative behavior of the two macromolecules.

Materials and Methods

Isolation of Delipidated Tracheobronchial Mucin. Tracheobroncial aspirates were collected from patients hospitalized for nonpulmonary illness, and the major mucin glycoprotein was isolated as before with minor modifications (Woodward et al., 1982). Briefly, upper respiratory tract aspirates were

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* Correspondence should be addressed to this author at the Department of Biochemistry, Georgetown University, Washington, DC.
‡ Present address: Department of Clinical Biochemistry, Christian Medical College, Vellore, India.
§ Present address: Department of Biochemistry and Molecular Biology, M. D. Anderson Hospital and Tumor Institute, Houston, TX.

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immediately made 0.02% with sodium azide and 0.1 mM with α-toluenesulfonyl fluoride and stored at −20 °C till workup. The accumulated material was dialyzed and lyophilized prior to chromatography on Sepharose CL-4B. The void volume material was further fractionated by density gradient centrifugation, and the high-density fraction (1.45–1.60 g/mL) was extracted with three sequential 10-h chloroform–methanol treatments [twice with 2:1 and once with 1:2 (v/v) chloroform–methanol]. The resulting mucin glycoprotein was pelleted by centrifugation at 12000*g* for 20 min and the remaining solvent removed under nitrogen. Mucin preparations were stored lyophilized at −20 °C.

Methyl-^{14}C-methylated Methemoglobin Protease Assay. Initially, mucin samples were assayed for proteolytic activity by the procedure of Williams and Lin (1971) using [^{14}C]-methemoglobin (New England Nuclear, Boston, MA; 30 μCi/mg) as substrate. Apparent positive results were further checked as follows: Mucin samples (250 μg) were dissolved by vortexing in 240 μL of 50 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl),[1] pH 8.0, buffer containing 0.02% sodium azide and protease inhibitors (1 mM benzamidine hydrochloride, 1 mM α-toluenesulfonyl fluoride, 2 mM *N*-ethylmaleimide, 5 mM EDTA, 100 mM 6-aminohexanoic acid, 5 μg/mL leupeptin, 5 μg/mL pepstatin, and 100 KIU/mL aprotinin). Blank and positive control consisted of 240 μL of the above buffer and 240 μL of a trypsin solution (2 mg/mL in 50 mM Tris-HCl, pH 8.0, containing 0.02% sodium azide), respectively. [^{14}C]Methemoglobin (11 100 dpm in 10 μL) was added to all samples prior to overnight incubation at 37 °C. Bovine serum albumin (100 μL of 10 mg/mL) and ice-cold 10% trichloroacetic acid (350 μL) were added at the end of the incubation period, and the samples were vortexed briefly. The samples were kept in an ice bath for 1 h to complete precipitation prior to centrifugation in a Beckman microfuge at 4 °C for 15 min. The supernatant (500 μL) was assayed for radioactivity by liquid scintillation spectrometry. In additional experiments to check for the presence of protease, the mucin samples were either heated at 100 °C for 15 min prior to incubation, trichloroacetic acid precipitated immediately after the addition of [^{14}C]methemoglobin (in effect, having no incubation period), or incubated with [^{14}C]methemoglobin at 0–4 °C rather than 37 °C.

Azocoll Protease Assay. Mucin (250 μg) was dissolved in 750 μL of 50 mM Tris-HCl, pH 8.0, containing 0.02% sodium azide. Buffer alone and trypsin (400 μg) in 750 μL of the buffer were used as blank and positive control, respectively. After the addition of 10 mg of Azocoll (Calbiochem, San Diego, CA), the samples were incubated with end-over-end mixing at 37 °C overnight. The samples were then centrifuged in a Beckman microfuge at 4 °C for 15 min, and the presence of proteolytic activity was determined by measuring the absorbance of the supernatant at 520 nm (Calbiochem-Behring, San Diego, CA; technical information).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Mucin samples were electrophoresed on a Hoefer Model 220 vertical slab gel unit. The separating and stacking gels (1.5 mm thick) routinely contained 10% and 5% (w/v) acrylamide, respectively. The gels were prepared according to the method of Haas and Kennett (1979), with two alterations. First, *N,N'*-methylenebis(acrylamide) (1 part for 37.5 parts of acrylamide) was used to cross-link the gel rather than *N,N'*-diallyltartardiamide because the latter is unsuitable for staining with periodic acid. Second, the sample buffer (0.063 M Tris-HCl, pH 6.8, containing 2.3% SDS and 10% glycerol) did not routinely contain 2-mercaptoethanol unless specifically noted. Gels were stained with Coomassie blue, destained, and subsequently stained for glycoprotein by the periodic acid–Schiff procedure (Fairbanks et al., 1971). Radiolabeled samples were visualized by fluorography (Bonner & Laskey, 1974).

Preparative gel electrophoresis was carried out by the method of Hager and Burgess (1980) with the following modifications: a 5-mm slice was removed from one edge of the gel and either stained or exposed to X-ray film to determine the position of the sample on the remaining gel; bovine serum albumin was omitted from the elution and dilution buffers, and sodium dodecyl sulfate was removed from the eluted sample by chromatography on Extracti-gel D (Pierce, Rockford, IL) rather than by acetone precipitation.

Cesium Bromide Density Gradient Centrifugation. Samples were dissolved overnight at 4 °C in 16.7 mM sodium phosphate, pH 6.8, containing 4 M guanidinium chloride, 33 mM NaCl, 0.02% sodium azide, and 42% (w/w) cesium bromide at a concentration of 1–2 mg/mL. When specified, 20 mM 2-mercaptoethanol was included in the solubilization buffer to effect disulfide bond reduction. Centrifugation was carried out for 72 h at 14 °C and 42 000 rpm in a Beckman 60 Ti rotor (Creeth et al., 1977). The gradients were fractionated into approximately 1-mL fractions and analyzed for protein, neutral sugar, and density.

Column Chromatography. The initial crude mucus was fractionated by Sepharose CL-4B chromatography. Lyophilized mucus (200 mg) was dissolved in 20 mL of 50 mM Tris-HCl, pH 8.0, containing 0.02% sodium azide and 0.1 mM α-toluenesulfonyl fluoride by stirring at 4 °C for approximately 2 h. The solution was centrifuged at 12000*g* for 20 min, and the supernatant was applied to a 5 × 95 cm column. Fractions (20 mL) were collected, and 0.5-mL aliquots were analyzed for neutral sugar and protein.

Analytical Sepharose CL-4B chromatography was performed on a 1.4 × 45 cm column. Routinely, 10 mg of sample was dissolved in 2.0 mL of 50 mM Tris-HCl, pH 8.0, containing 6 M urea, 0.02% sodium azide, and 0.1 mM α-toluenesulfonyl fluoride. Fractions (1 mL) were collected and analyzed for protein and neutral sugars.

Analytical Sephacryl S-500 chromatography was performed on a 1.5 × 40.5 cm column equilibrated with 50 mM Tris-HCl, pH 8.0, containing 6 M urea, 0.02% sodium azide, and 0.1 mM α-toluenesulfonyl fluoride (with or without 20 mM 2-mercaptoethanol, as indicated). Unless otherwise noted, 10-mg samples in 2.0 mL of the above buffer were chromatographed, and 1.0-mL fractions were collected.

Ouchterlony Immunodiffusion Precipitation. Ouchterlony immunodiffusion was performed according to the method of Williams and Chase (1971). The low-density proteins and high-density mucin fractions (F_1 and F_3, respectively), obtained during the initial density gradient fractionation, and the 65-kDa protein isolated by preparative SDS–PAGE were dissolved in 0.15 M sodium phosphate, pH 7.2, at a concentration of 1.0 mg/mL, placed in immunodiffusion wells, and allowed to diffuse against rabbit anti-human serum albumin (Miles-Scientific, Naperville, IL) and goat anti-human serum (Bionetics, Charleston, SC) for 48 h at 20 °C. Human serum albumin (500 μg/mL) was run in parallel as a positive control.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFMS, trifluoromethanesulfonic acid; F_3, high-density fraction obtained after CsBr density gradient centrifugation of human tracheobronchial mucus; dF_3, delipidated F_3; βF_3, dF_3 after treatment with β-mercaptoethanol and reisolation by CsBr density gradient centrifugation.

Similarly, unfractionated mucus, Sepharose CL-4B void and included fractions, and the 65-kDa protein were allowed to diffuse against sheep anti-human α_1-antichymotrypsin (Berman et al., 1986) (generously given to us by Dr. R. A. Stockley, The General Hospital, The University of Birmingham, Birmingham, United Kingdom). Normal human plasma was run as a positive control.

Western Blotting. Blotting was performed according to the procedure of Towbin et al. (1979). The 65-kDa protein (100 μg) was dissolved in 100 μL of electrophoresis sample buffer containing 5% (v/v) 2-mercaptoethanol, heated at 100 °C for 10 min, and subjected to SDS–PAGE before transfer to nitrocellulose and treatment with sheep anti-human α_1-antichymotrypsin followed by rabbit anti-sheep IgG-conjugated horseradish peroxidase (Accurate Chemical & Scientific Corp., Westbury, NY).

Radioimmunoassay. Cross-reactivity of the 65-kDa protein with α_1-antichymotrypsin antibody was checked by radioimmunoassay. The production of rabbit anti-65-kDa protein and rabbit anti-deglycosylated reduced human tracheobronchial mucin was also monitored by radioimmunoassay. The cross-reactivity of the rabbit antisera with various samples was determined by competitive radioimmunoassay.

Amino Acid Analysis. Analyses were performed on a Dionex MBF/SS amino acid analyzer after hydrolysis in vacuo in 6 N hydrochloric acid for 24 h at 110 °C. Glucosamine and galactosamine were determined after hydrolysis in vacuo in 4 N hydrochloric acid for 8 h at 100 °C. Cysteine was determined as cysteic acid after hydrolysis in 6 N hydrochloric acid containing 0.25 M dimethyl sulfoxide (Spencer & Wold, 1969).

Radioiodination. Bovine serum albumin (25 μg), 65-kDa protein (10 μg), and deglycosylated reduced human tracheobronchial mucin (50 μg) were separately ^{125}I labeled by Chloramine-T-catalyzed iodination (Greenwood et al., 1963).

Disulfide Bond Reduction. Routinely, samples were treated by stirring overnight in buffer containing 20 mM 2-mercaptoethanol at 4 °C prior to fractionation (in the same buffer) by density gradient centrifugation or column chromatography.

Subunit Reassociation. Reduced mucin subunits were obtained by incubating 10 mg of glycoprotein (fraction $dF_3 4B_1$, Figure 8) in 2 mL of 50 mM Tris-HCl, pH 8.0, containing 6 M urea, 0.02% sodium azide, 0.1 mM α-toluenesulfonyl fluoride, and 20 mM 2-mercaptoethanol overnight at 4 °C before chromatography on Sephacryl S500. The fractions containing the reduced mucin and the reduced 65-kDa protein were pooled separately and stored frozen (without dialysis or lyophilization) until needed for recombination experiments.

To obtain radiolabeled material, the 65-kDa protein was isolated from fraction βF_1 (Figure 4) by preparative SDS–PAGE and subsequently iodinated. To effect reassociation, ^{125}I-labeled protein (50 000 cpm) was mixed with approximately 2 mg of reduced mucin and 200 μg of reduced 65-kDa protein in a total volume of about 6 mL (a stoichiometry analogous to that present prior to disulfide bond reduction). The sample was concentrated to 2 mL and dialyzed against 50 mM Tris-HCl, pH 8.0, containing 6 M urea, 0.02% (w/v) sodium azide, and 0.1 mM α-toluenesulfonyl fluoride using an Amicon ultrafiltration device. Oxygen was bubbled through the concentrated mixture for 1 h prior to rechromatography on Sephacryl S500. Fractions (1.0 mL) were collected and analyzed for protein, neutral sugar, and radioactivity when appropriate. Reduced mucin subunits containing 50 000 cpm of iodinated bovine serum albumin, or reduced mucin subunits alone, were run in parallel as control experiments.

S-Alkylation. Briefly, fraction $dF_3 4B_1$ (500 μg) was dissolved in 5 mL of 0.3 M Tris-HCl, pH 8.3, containing 6 M urea, by stirring at 20 °C for 1 h under nitrogen. The solution was made 0.1 M in iodoacetamide and incubated under nitrogen at 37 °C for 4 h in the dark (Tabachnik et al., 1981). The excess iodoacetamide was removed from the resulting alkylated mucin by dialysis, and 1 μmol of dithiothreitol was added. The solution was flushed with nitrogen and incubated for 4 h in the dark at 37 °C; 0.05 mCi (2 μmol) of iodo-[^{14}C]acetamide (New England Nuclear, Boston, MA) was added, and incubation in the dark under nitrogen was continued for 4 h. The resulting radiolabeled reduced sample was recovered by exhaustive dialysis and lyophilization.

Deglycosylation of Mucin for Antibody Production. High-density reduced, dilipidated mucin (fraction βF_3, Figure 4) was partially deglycosylated by the method of Edge et al. (1981) with modifications. The sample was first treated at 37 °C with a mixture of *Vibrio cholerae* and *Arthrobacter ureafaciens* neuraminidase (Behring Diagnostics, San Diego, CA) to enzymatically cleave sialic acid residues. The asialomucin was separated from the enzymes by chromatography on Sepharose CL-4B and treated with TFMS–anisole (2:1) at 0 °C, twice, for a total exposure time of 9 h and the apoprotein recovered as described (Woodward et al., 1987). As previously reported (Woodward et al., 1987), changes in the amino acid composition were minimal, no amino termini were detected by the dansylation procedure, and the bulk of the carbohydrate was removed.

Antibody Production. Polyclonal antibodies were raised in female New Zealand white rabbits (Hazleton Research Animals, Reston, VA). Deglycosylated reduced human tracheobronchial mucin (1 mg) was dissolved in 250 μL of 0.9% NaCl, to which 500 μL of Freunds complete adjuvant was added. The suspension was injected subcutaneously at multiple sites. The rabbit was boosted after approximately 3 weeks and again at 5 weeks, with 500 μg of antigen dissolved in 100 μL of 0.9% NaCl, to which 200 μL of Freunds incomplete adjuvant was added. In some cases, Ribi's adjuvant was used instead of Freunds. Serum was monitored biweekly. Antibody was detected by radioimmunoassay at the seventh week, and antisera collection, by ear venapuncture, was done at approximately 2-week intervals. Additional 250-μg booster injections were continued as needed. Similarly, rabbit 65-kDa protein antisera were obtained. In this case, immunization was accomplished by using an initial inoculum of 500 μg, followed by 250-μg inoculations at 3 weeks and again at 7 weeks.

Additional Methods. Neutral sugar was measured by the phenol–sulfuric acid method (Dubois et al., 1956). Protein was followed by absorbance at 280 nm, and density was determined by weight.

Results and Discussion

The treatments employed to isolate the mucin glycoprotein from tracheobronchial secretions, sequential gel filtration on Sepharose CL-4B, density gradient centrifugation in 42% (w/w) cesium bromide–4 M guanidinium chloride, and extraction in chloroform–methanol (Figure 1), substantially separate the resulting high-density, high molecular weight fraction (mucin) from noncovalently bound materials (Woodward et al., 1982, 1987). Proteolytic enzymes, which may be present in crude mucus secretions, are not completely separated from the mucin by exclusion chromatography (presumably some have become entrapped within the mucin molecule) but are dissociated during density gradient cen-

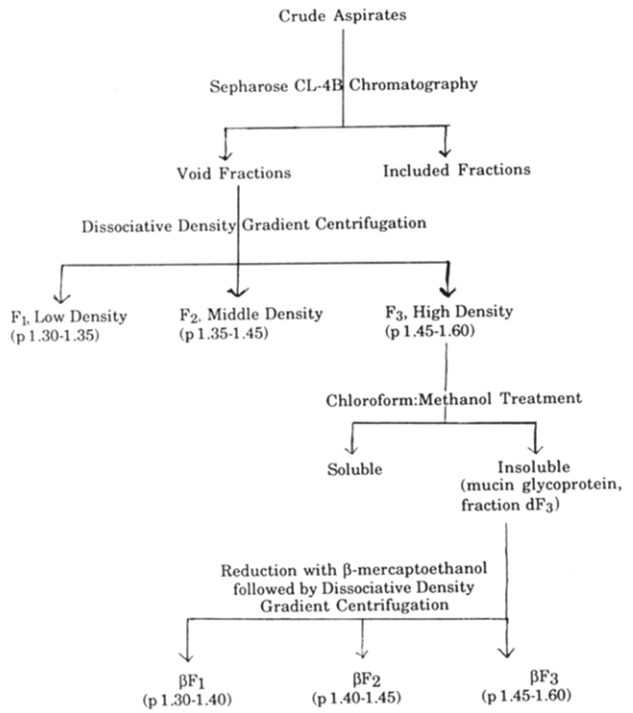

FIGURE 1: Schematic representing the purification of human tracheobronchial mucin.

trifugation (see below). Accordingly, precautions are taken throughout the fractionation procedure to minimize proteolysis.

Methyl-^{14}C-methylated methemoglobin has been routinely used by this laboratory as a substrate for assaying contaminating protease activities in glycosidase preparations. In the case of a solution with no protease contamination, all radioactivity should be trichloroacetic acid precipitable. However, in every test we performed with mucin samples, including the controls (heat treatment prior to incubation, a decrease in temperature during incubation, or a decrease in the time of incubation), nonprecipitable radioactivity was present. Further, no consistent correlation between time or temperature of incubation with nonprecipitable radioactivity could be seen. It was thus concluded that due to the inherent entrapment and clearance capabilities of the mucin molecule, hemoglobin-like molecules were inappropriate substrates for the assessment of proteolytic activity in mucin preparations.

After assay conditions were established in control experiments, crude mucus, Sepharose CL-4B void and included fractions, and density gradient fractions F_1, F_2, and F_3 (Figure 1) were assayed for proteolytic activity using Azocoll as substrate. The results indicate that the bulk of the proteolytic enzymes (76.5% of that in the initial crude mucus) are not tightly associated and are separable during exclusion chromatography. The remainder are separable from the mucin molecule only after dissociative density gradient centrifugation. F_3, the high-density mucin glycoprotein fraction, contains no measurable amount of protease, but fractions F_1 and F_2 had 12.1% and 11.4%, respectively, of the initial protease activities.

As ascertained by SDS–PAGE, the cesium bromide–guanidinium chloride density gradient fractionation cleanly separated the high-density mucin glycoprotein from contaminants. Analysis of fraction F_3 showed an apparently homogeneous high molecular weight glycoprotein (Figure 2, lane 2) while fractions F_1 and F_2 contained several proteins and glycoproteins (Figure 2, lane 1). Reapplication of fractions F_3 and delipidated F_3 to the density gradient also yielded profiles indicative of a homogeneous high-density component (Figure 3).

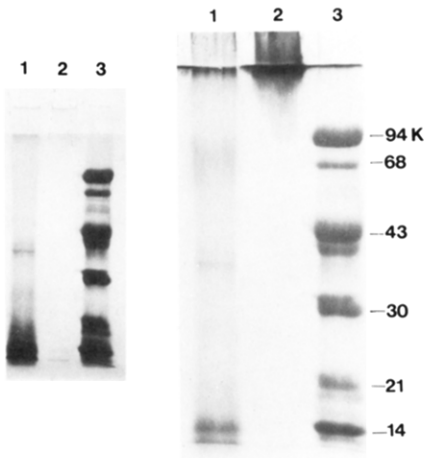

FIGURE 2: SDS–PAGE of cesium bromide–guanidinium chloride density gradient centrifugation fractions. The gel was stained with Coomassie blue (left panel), and the same section was then stained with periodic acid–Schiff reagent (right panel). Lane 1, 500 μg of the combined low-density fractions (F_1 and F_2); lane 2, 500 μg of the high-density fractions (F_3); lane 3, molecular weight standards, 20 μg of each.

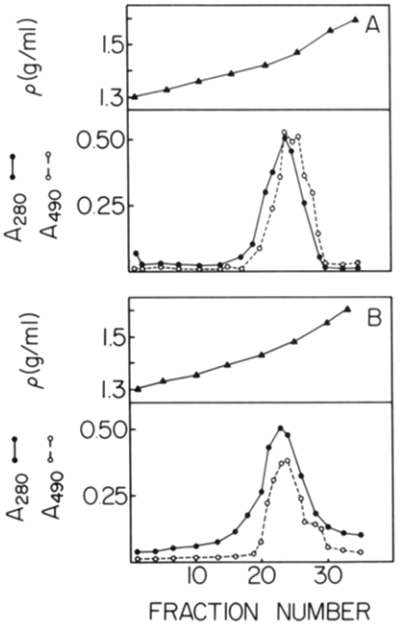

FIGURE 3: Analytical density gradient centrifugation of fraction F_3 (panel A) and delipidated F_3 (panel B). Approximately 10 mg of each sample was dissolved by stirring overnight at 4 °C in 35 mL of buffer, as described in the text, prior to centrifugation. After 72 h at 42 000 rpm and 14 °C, the gradients were fractionated and analyzed for protein (●), neutral sugar (○), and density (▲).

Ouchterlony immunodiffusion with fraction F_1 showed precipitin bands for both anti-human serum and anti-human serum albumin; results for both antibodies with fraction F_3 were negative.

Disulfide bonds in the delipidated high-density mucin (fraction dF_3) were reduced by treatment with 20 mM 2-mercaptoethanol for 18 h, and the products were fractionated by density gradient centrifugation in the presence of 2-mercaptoethanol. In addition to a high-density mucin fraction, βF_3 (ρ 1.45–1.60), which was 80–85% by weight of the original material, two other distinct fractions were evident, βF_2 (ρ 1.40–1.45) and βF_1 (ρ 1.30–1.40). βF_2 was approximately 10% by weight, and βF_1 was approximately 5% by weight of the original mucin.

SDS–PAGE performed under reducing conditions (Figure 4) indicated that while fraction βF_2 (lane 2) contained a

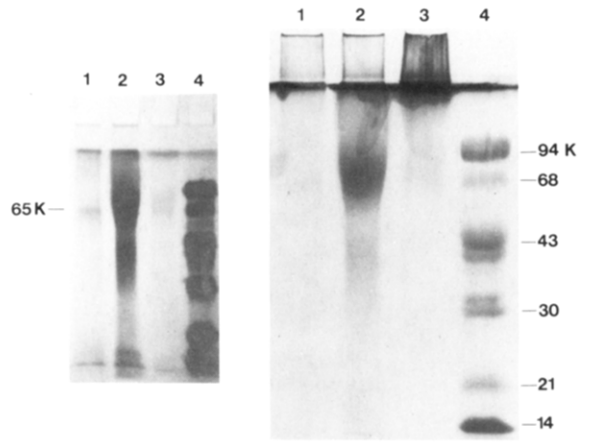

FIGURE 4: SDS–PAGE under reducing conditions. Samples were reduced prior to gel electrophoresis by heating at 100 °C for 15 min in electrophoresis buffer containing 5% (v/v) 2-mercaptoethanol. Gels were stained with Coomassie blue (left panel) to visualize protein prior to visualization of glycoprotein by the periodic acid–Schiff method (right panel). Lane 1, 500 μg of fraction βF_1; lane 2, 500 μg of fraction βF_2; lane 3, 500 μg of fraction βF_3; lane 4, molecular weight standards, 20 μg of each.

mixture of several proteins and fraction βF_1 (lane 1) contained predominantly a single protein having a molecular weight of 65 000, fraction βF_3 (lane 3) contained mostly high molecular weight mucin, with trace amounts of lower molecular weight materials. The predominant 65-kDa protein was often paired with a protein having a molecular weight of approximately 60 000. Although the proteins as a pair were readily isolated by preparative SDS–PAGE, their proximity to one another made attempts to completely separate them unsuccessful. In the subsequent studies, although an attempt was made to focus on and isolate the predominant 65-kDa protein, the 60-kDa protein remained a trace contaminant.

Amino acid analyses of the fractionation products (Table I) demonstrate that the 65-kDa protein may be distinguished from the mucin fraction (βF_3) and from human serum albumin (having a similar molecular weight) by its amino acid composition. The absence of hexosamines indicates that this protein is not glycosylated, a result in agreement with analysis of the comparable protein from canine tracheal mucin (Ringler et al., 1987).

Ouchterlony immunodiffusion indicated that neither goat anti-human serum, rabbit anti-human serum albumin, nor sheep anti-human α_1-antichymotrypsin antibodies had cross-reactivity with the 65-kDa protein. Rabbit anti-human α_1-antichymotrypsin was found to cross-react with unfractionated tracheobronchial mucus [in agreement with the results of Stockley and co-workers (Berman et al., 1986)] and Sepharose CL-4B included fractions, but not with Sepharose CL-4B void volume fractions. Radioimmunoassay and Western blotting also confirmed that the 65-kDa protein did not cross-react with sheep anti-human α_1-antichymotrypsin (data not shown).

The original mucin fraction (delipidated F_3) was reexamined by SDS–PAGE. The sample was heated for 15 min in buffer containing 2-mercaptoethanol to effect complete disulfide bond reduction prior to electrophoresis. The results confirmed the presence of the proteins apparent in Figure 5 which had previously been undetected when the mucin was electrophoresed in its native configuration (data not shown). Evidence suggested that the native delipidated mucin molecule contained one or more proteins which were not readily dissociated by chaotropic agents, but were separable by disulfide bond disruption.

The βF_3 fraction still containing the 65-kDa protein (Figure 4, lane 3) was retreated with 2-mercaptoethanol, and the reduction products were separated by density gradient centrifugation to yield three fractions. Fraction βF_{3b} was approximately 92%, fraction βF_{2b} approximately 7%, and fraction βF_{1b} approximately 1% by weight of the original sample. Examination by SDS–PAGE (Figure 5) of the fractionation products indicated that the βF_{1b} fraction (lane 1) contained mostly the 65 000 and 60 000 molecular weight proteins, and the βF_{2b} fraction (lane 2) contained these proteins plus higher molecular weight glycoprotein material. These results were consistent with the previous SDS–PAGE analysis of fraction βF_3, the original reduced mucin sample (Figure 4, lane 3). βF_{3b}, the major mucin-containing fraction after the second reduction, contained only a high molecular weight glycoprotein species (Figure 5, lane 3). Repeated disulfide bond reduction and fractionation by density gradient centrifugation enabled

Table I: Amino Acid Analysis of Human Tracheobronchial Preparations and Albumin[a]

amino acid	F_3[b]	βF_3[c]	βF_2	βF_1	65 kDa[d]	HSA[e]
$CySO_3H$	50	41	70	42	32	63
Asp	62	40	94	79	113	97
Thr	205	248	111	61	52	53
Ser	142	173	108	146	121	40
Glu	74	55	102	181	116	149
Gly	95	135	91	158	201	21
Ala	95	113	93	71	65	113
Val	51	32	56	35	63	70
Met	16	6	17	6	15	10
Ile	19	12	22	21	64	15
Leu	56	39	56	38	22	109
Tyr	19	6	31	17	7	32
Phe	26	13	34	21	24	53
His	30	34	27	54	16	28
Lys	33	30	44	52	57	104
Arg	27	23	44	18	31	41
$GlcNH_2$	10.7	11.5	5.5	2.5	<0.1	0
$GalNH_2$	4.1	4.4	3.0	1.0	<0.1	0

[a] Amino acids are expressed as residues per 1000 residues, and hexosamines are expressed as weight percent. [b] Fraction F_3 is high-density lung mucin glycoprotein. [c] βF_3, βF_2, and βF_1 are the high, middle, and low buoyant density products, respectively, obtained after delipidated mucin was treated with 2-mercaptoethanol and refractionated by density gradient centrifugation. [d] 65 kDa is the 65 000-dalton protein isolated by preparative SDS–PAGE from a βF_1 preparation. This preparation may have had a trace of the 60 000-dalton protein still present. [e] The composition of human serum albumin is included for comparison.

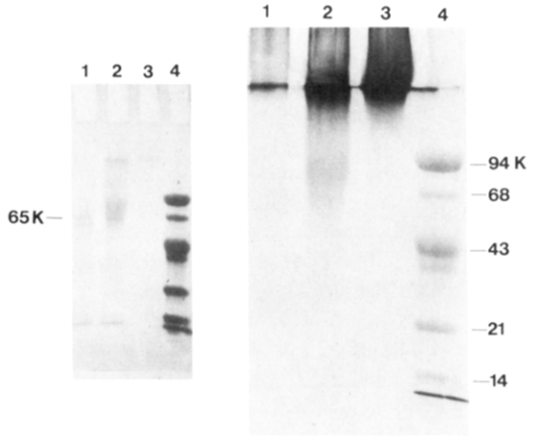

FIGURE 5: SDS–PAGE of the fractions obtained from the second reducing gradient. Lane 1, 500 μg of fraction βF_{1b}; lane 2, 500 μg of fraction βF_{2b}; lane 3, 500 μg of fraction βF_{3b}; lane 4, molecular weight standards, 20 μg of each. The samples were dissolved in electrophoresis sample buffer containing 5% (v/v) 2-mercaptoethanol and heated at 100 °C for 15 min prior to electrophoresis. The gel was stained with Coomassie blue (left panel), and the same section was then stained with periodic acid–Schiff reagent (right panel).

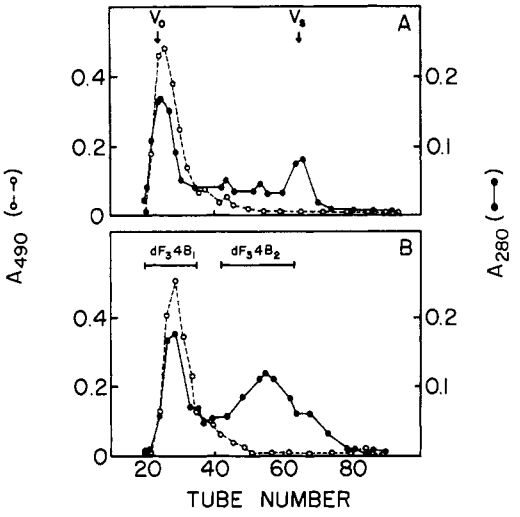

FIGURE 6: Sepharose CL-4B column (1.4 × 45 cm) chromatography of density gradient fraction F_3, native high-density lung mucin (panel A), and fraction F_3 after prolonged treatment with chloroform–methanol (panel B). The column was eluted with 50 mM Tris-HCl, pH 8.0, containing 6 M urea. 1-mL fractions were collected and analyzed for protein (●) and neutral sugar (○). Fractions $dF_3 4B_1$ and $dF_3 4B_2$ were pooled as indicated in panel B.

complete dissociation of the mucin 65-kDa protein complex.

To assess the involvement of hydrophobicity in the mucin–protein relationship, a sample of native high-density mucin (Figure 1, fraction F_3) was treated for 120 h in chloroform–methanol [twice for 48 h in (2:1) and once for 24 h in (1:2) chloroform–methanol]. The insoluble material was isolated by centrifugation, dissolved in 50 mM Tris-HCl, pH 8.0, containing 6 M urea, 0.02% sodium azide, and 0.1 mM α-toluenesulfonyl fluoride, and chromatographed on Sepharose CL-4B (Figure 6). A distinct protein-rich included fraction which was not present prior to the lengthy chloroform–methanol treatment became resolvable. Fraction $dF_3 4B_2$ accounted for 27% by weight of the original sample. Both fraction $dF_3 4B_2$ and the void volume fraction, $dF_3 4B_1$, were analyzed by gel electrophoresis. SDS–PAGE indicated that while fraction $dF_3 4B_2$ was enriched in the 65-kDa protein (in addition to smaller molecular weight proteins), the protein had not been entirely separated from the mucin. This is in agreement with our observation that gel exclusion chromatography in the presence of SDS did not resolve the 65-kDa component of the canine material.

It was thus concluded that the hydrophobic pockets in the mucin molecule help maintain the integrity of the protein–mucin association, and possibly shield proteins from the effect of routine chaotropic agents such as urea and guanidinium chloride. The minute amount of protein remaining associated with the mucin molecule after the chloroform–methanol treatment (a) had been initially dissociated during treatment, and association reoccurred when the sample was returned to an aqueous environment prior to gel filtration, or (b) due to the involvement of disulfide bonds, was inherently more tightly associated with the mucin than the readily separable protein(s).

To study the association of the tightly bound protein to the mucin macrostructure, and the role of disulfide bonds, we needed a system which could differentiate the intact mucin molecule (fraction $dF_3 4B_1$) from its reduced state (βF_3) and the 65-kDa protein. After examinination of several gel matrices, including Sephacryl S1000 and Sepharose CL-4B (neither of these yielded clear separation of fractions βF_3 and $dF_3 4B_1$), Sephacryl S500 was chosen as the appropriate column material.

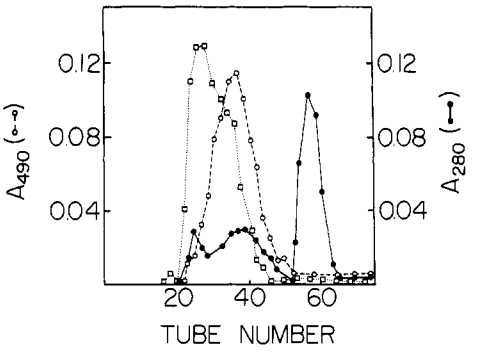

FIGURE 7: Sephacryl S500 column (1.5 × 40.5 cm) chromatography of mucin fractions $dF_3 4B_1$ and reduced $dF_3 4B_1$. 1.0-mL fractions were collected and analyzed for neutral sugar [$dF_3 4B_1$ (□); reduced $dF_3 4B_1$ (○)] and protein [reduced $dF_3 4B_1$ (●)]. The reduced mucin fractions (20–45) and 65-kDa protein fractions (52–65) were pooled separately and recovered.

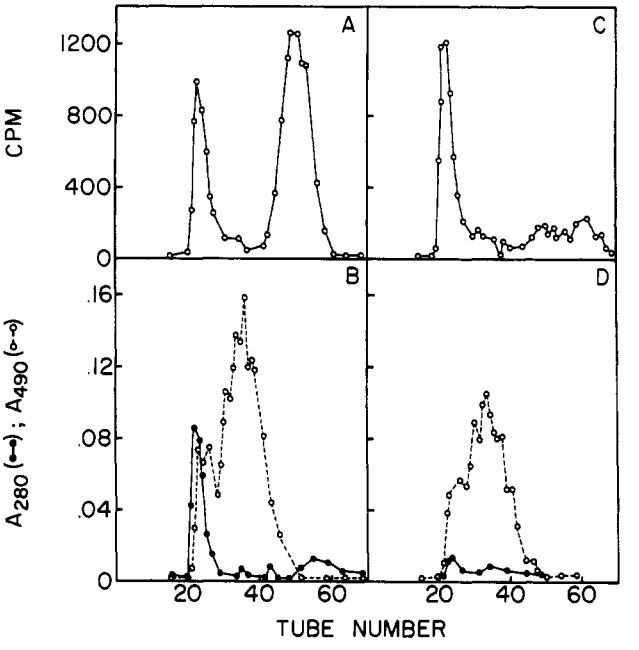

FIGURE 8: Sephacryl S500 column (1.5 × 40.5 cm) chromatography of mucin subunit reassociation experiments. (Panels A and B) 50 000 cpm of ^{125}I-labeled 65-kDa protein was added to approximately 2 mg of reduced mucin (estimated weight, Figure 7 fractions 20–45) and 200 μg of reduced 65-kDa protein (estimated weight, Figure 7 fractions 52–65). Oxidation was allowed to occur, and the sample was rechromatographed in a nonreducing buffer. 1.0-mL fractions were collected and analyzed for protein [panel B (●)], neutral sugar [panel B (○)], and radioactivity [panel A (○)]. (Panel C) 50 000 cpm of ^{125}I-labeled bovine serum albumin was added to 2 mg of reduced mucin. Oxidation was allowed to occur, and the sample was treated as in panel A [radioactivity (○)]. (Panel D) 2 mg of reduced mucin was allowed to oxidize prior to chromatography. Conditions as in panel A [neutral sugar (○); protein (●)].

To obtain reduced mucin subunits, fraction $dF_3 4B_1$ was treated overnight at 4 °C with 20 mM 2-mercaptoethanol prior to chromatography on Sephacryl S500 in buffer containing 2-mercaptoethanol (Figure 7). Reassociation was attempted by recombining the reduced mucin fractions with the reduced protein fractions and simultaneously concentrating and dialyzing the mixture against a nonreducing buffer. ^{125}I-Labeled 65-kDa protein was added to the mixture during dialysis and concentration. The sample was then rechromatographed on Sephacryl S500 (Figure 8, panels A and B). Control samples containing either reduced mucin and ^{125}I-labeled bovine serum albumin or only reduced mucin were run in parallel (Figure 8, panels C and D, respectively). The formation of a carbo-

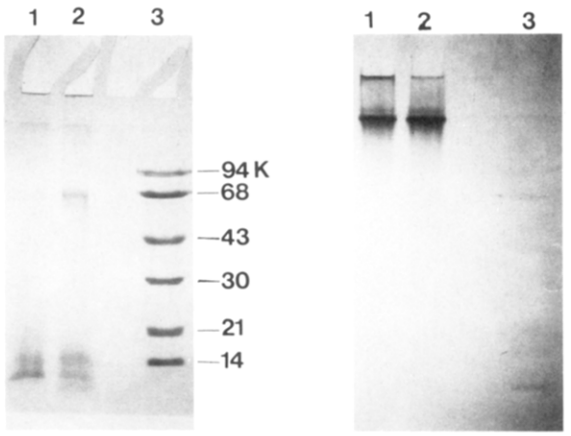

FIGURE 9: SDS–PAGE of sample dF_34B_2 (unreduced 65-kDa protein). Lane 1, 250 μg of dF_34B_2 dissolved in electrophoresis buffer and applied directly. Lane 2, 250 μg of dF_34B_2 dissolved in electrophoresis buffer and heated at 100 °C for 10 min prior to application. Lane 3, molecular weight standards, 20 μg of each. The gel was stained with Coomassie blue (left panel), and the same section was then stained with periodic acid–Schiff reagent (right panel).

hydrate-rich peak at the void volume of the Sephacryl S500 column profile in Figure 8 (panel B) indicates that reduced mucin subunits are capable of undergoing reassociation under oxidizing conditions; furthermore, radiolabeled 65-kDa protein can be incorporated into the mucin macrostructure during the reassociation procedure (panel A). It appears (panel D) that the 65-kDa protein is not necessary for reassociation to occur; however, because of the inherent problems of entirely removing the initial reduced mucin molecule from the 65-kDa protein, the stoichiometry of the reassociation is unclear.

The inclusion of the ^{125}I-labeled 65-kDa protein during re-formation of the mucin suggests that the protein must either become entrapped within the mucin macromolecular structure during reassociation (as does bovine serum albumin; Figure 8, panel C) or become a part of that structure through direct formation of disulfide bonds.

To assess the ability of the 65-kDa protein to undergo disulfide bond formation, fraction dF_34B_1 was alkylated by treatment with iodoacetamide, reduced with dithiothreitol, and radiolabeled by alkylation with iodo[^{14}C]acetamide. In this manner, the readily accessible –SH groups were alkylated initially, then internal disulfide bonds were disrupted, and the newly formed –SH groups became available for radiolabeling during the second alkylation. SDS–PAGE of the ^{14}C-labeled mucin sample indicated the presence of *S*-[^{14}C][(carboxymethyl)amido]cysteine groups on the 65-kDa protein, as well as additional material which was not detectable previously by Coomassie blue staining. Although the incorporation of label into the 65-kDa protein was indicative of the presence of sulfhydryl groups, it did not distinguish disulfide bonds between the protein–mucin complex from disulfide bonds internal to the 65-kDa protein.

To explore these possibilities, fraction dF_34B_2 (i.e., the unreduced 65-kDa protein preparation isolated after chloroform–methanol treatment and chromatography on Sepharose CL-4B) was electrophoresed under nonreducing conditions. dF_34B_2 (500 μg) was dissolved by vortexing in SDS–PAGE sample buffer (0.063 M Tris-HCl, pH 6.8, containing 2.3% SDS and 10% glycerol). Half of the sample was heated for 10 min at 100 °C before application (Figure 9, lane 2) while the remainder was directly applied to the gel (Figure 9, lane 1). Neither sample had been exposed to reducing conditions at any time. The heat-treated sample showed the same profile on SDS–PAGE as the protein preparation isolated and electrophoresed previously under reducing conditions (fraction βF_1; Figure 4, lane 1). Therefore, the native 65-kDa protein apparently did not contain internal disulfide bonds, and thus the formation of the *S*-[^{14}C]carboxymethylamide bonds took place on –SH groups which initially were either present in disulfide bonds with the mucin subunits or sterically hidden from the first alkylation (prior to reduction). There was insufficient material available to explore this latter possibility in detail.

Table II: Antisera Cross-Reactivity[a]

antisera	protein/glycoprotein	competition
65-kDa protein	reduced human tracheobronchial mucin	+
	deglycosylated human tracheal mucin	+
	canine tracheal mucin	–
	deglycosylated canine tracheal mucin	–
	glycophorin	–
	α_1-acid glycoprotein	–
	ferritin	–
	human serum albumin	–
deglycosylated reduced mucin	65-kDa protein	+
	reduced human tracheobronchial mucin	–
	canine tracheal mucin	–
	deglycosylated canine tracheal mucin	–
	65-kDa canine tracheal protein	–
	glycophorin	–
	α_1-acid glycoprotein	–
	ferritin	–
	human serum albumin	–

[a] Cross-reactivity was determined by competitive radioimmunoassay as described in the text.

Both the presence of external sulfhydryl groups on the 65-kDa protein and the apparent inclusion of the protein during the re-formation of the intact mucin molecule indicate the possibility that the protein may undergo disulfide bond formation with the mucin subunits.

Interestingly, the sample which was not heat treated showed no protein bands detectable by Coomassie blue (Figure 9, lane 1). The periodic acid–Schiff stain indicated that both samples had comparable material present at the interface of the stacking and separating gel (presumably having arisen from contaminating dF_34B_1 mucin). The unheated sample contained slightly more insoluble material which had remained in the application well. Apparently, heat treatment facilitates both the solubilization of the 65-kDa protein and denaturation of the mucin–protein complex, whereas utilization of detergent alone has little effect.

To determine the cross-reactivity of the 65-kDa protein with a few specific serum components and mucin-type glycoproteins, competitive radioimmunoassays were performed using rabbit anti-65-kDa protein antisera. Concurrently, rabbit anti-deglycosylated reduced human tracheobronchial mucin antisera were isolated and similarly tested. The results are summarized in Table II. Of the samples tested, the 65-kDa protein antiserum cross-reacted only with reduced human tracheobronchial mucin and deglycosylated tracheobronchial mucin. In contrast, the deglycosylated, reduced mucin antiserum cross-reacted with the 65-kDa protein, but not with nondeglycosylated reduced mucin.

In summary, the 65-kDa protein is unusually cryptic in the mucin–glycoprotein complex. Neither immunological probes nor SDS gel electrophoresis reveals this component prior to dissociation (either by disulfide bond reduction, chloroform–methanol treatment, deglycosylation, or heat denaturation in the presence of SDS). Reassociation studies show that both the 65-kDa protein and nonrelated molecules such as serum albumin can bind to the mucin to form complexes not resolved by exclusion chromatography. However, albumin and other

serum-derived components are completely resolved by the dissociative gradient centrifugation step, a result which suggests some level of specificity in the interaction between the 65-kDa protein and the mucin. The presence of a component with similar physical properties in canine tracheal mucin supports the possibility of a functional role for this macromolecule.

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REFERENCES

Berman, G., Afford, S. C., Burnett, D., & Stockley, R. A. (1986) *J. Biol. Chem. 261*, 14095–14099.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Creeth, J. M., Bhaskar, K. R., Horton, J. R., Das, I., Lopez-Vidriero, M. T., & Reid, L. (1977) *Biochem. J. 167*, 557–569.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E., & Weber, P. (1981) *Anal. Biochem. 118*, 131–137.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114–123.

Haas, J. B., & Kennett, R. H. (1979) in *Monoclonal Antibodies* (Kennett, R. H., McKearn, T. J., & Bechtol, K. B., Eds.) pp 407–411, Plenum, New York and London.

Hager, D. A., & Burgess, R. R. (1980) *Anal. Biochem. 109*, 76–86.

Ringler, N. J., Selvakumar, R., Woodward, H. D., Simet, I. M., Bhavanandan, V. P., & Davidson, E. A. (1987) *Biochemistry 26*, 5322–5328.

Roberts, G. P. (1976) *Arch. Biochem. Biophys. 173*, 528–537.

Rose, M. C., Voter, W. A., Brown, C. F., & Kaufman, B. (1984) *Biochem. J. 222*, 371–377.

Sachdev, G. P., Myers, F. J., Horton, F. O., Fox, O. F., Wen, G., Rogers, R. M., & Carubelli, R. (1980) *Biochem. Med. 24*, 82–94.

Slayter, H. S., Lamblin, G., LeTreut, A., Galabert, C., Houdret, N., Degand, P., & Roussel, P. (1984) *Eur. J. Biochem. 142*, 209–218.

Spencer, R. L., & Wold, F. (1969) *Anal. Biochem. 32*, 185–190.

Tabachnik, N. F., Blackburn, P., & Cerami, A. (1981) *J. Biol. Chem. 256*, 7161–7165.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Williams, C. A., & Chase, M. W. (1971) *Methods Immunol. Immunochem. 3*, 146–160.

Williams, H. R., & Lin, T.-Y. (1971) *Biochim. Biophys. Acta 250*, 603–607.

Woodward, H., Horsey, B., Bhavanandan, V. P., & Davidson, E. A. (1982) *Biochemistry 21*, 694–701.

Woodward, H. D., Ringler, N. J., Selakumar, R., Simet, I. M., Bhavanandan, V. P., & Davidson, E. A. (1987) *Biochemistry 26*, 5315–5322.

Unfolding Free Energy Changes Determined by the Linear Extrapolation Method. 1. Unfolding of Phenylmethanesulfonyl α-Chymotrypsin Using Different Denaturants†

Marcelo M. Santoro and D. W. Bolen*

Departments of Chemistry and Biochemistry and Medical Biochemistry, Southern Illinois University, Carbondale, Illinois 62901

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ABSTRACT: Characteristics and properties of the unfolding free energy change, $\Delta G°_{N-U}$, as determined by the linear extrapolation method are assessed for the unfolding of phenylmethanesulfonyl chymotrypsin (PMS-Ct). Difference spectral measurements at 293 nm were used to define PMS-Ct unfolding brought about with guanidinium chloride, urea, and 1,3-dimethylurea. All three denaturants were shown to give identical extinction coefficient differences ($\Delta\epsilon_{N-U}$) between native and unfolded forms of the protein in the limit of zero concentration of denaturant. The independence of $\Delta\epsilon_{N-U}$ on denaturant supports the linear extension of pre- and postdenaturational base lines into the transition zone, allowing evaluation of unfolding equilibrium constants based on the two-state assumption. An expression, based on the linear extrapolation method, was used to provide estimates of $\Delta G°_{N-U}$ for the three denaturants using nonlinear least-squares fitting of the primary data, $\Delta\epsilon$ versus [denaturant]. The three $\Delta G°_{N-U}$ values were identical, within error, suggesting that the free energy change is a property of the protein system and independent of denaturant. It is suggested that the error in $\Delta G°_{N-U}$ determined from use of the linear extrapolation method is significantly larger than commonly reported in the literature.

Of numerous agents known to denature proteins, guanidinium chloride, urea, and like compounds are believed to cause the most complete unfolding (Tanford, 1968). Many soluble globular proteins can be reversibly unfolded by these agents and exhibit two-state behavior, a condition upon which analyses of unfolding free energy measurements have been based (Saito

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