Biochimica et Biophysica Acta, 758 (1983) 24–29
Elsevier

BBA 21478

ACTIVATION OF BRONCHIAL MUCIN PROTEOLYSIS BY 4-AMINOPHENYLMERCURIC ACETATE AND DISULPHIDE BOND REDUCING AGENTS

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(Received February 1st, 1983)

Key words: Proteinase; Glycoprotein; Aminophenylmercuric acetate; Sulfhydryl reagent; (Bronchial mucin)

High molecular weight bronchial glycoproteins, as nearly native as possible, were treated with either 2-mercaptoethanol or 4-aminophenylmercuric acetate (APMA): analytical electrophoresis revealed that a decrease in molecular weight of glycoproteins coincided with the disappearance of some proteins associated with high molecular weight bronchial glycoproteins. These modifications were not observed if high molecular weight bronchial glycoproteins were incubated with paramethylsulphonyl fluoride and EDTA, two synthetic protease-inhibitors, prior to 2-mercaptoethanol or APMA action. These data suggest that protease-antiprotease complexes are associated with bronchial mucins and that reducing agents or APMA activate proteases.

Introduction

Human bronchial mucins represent the major constituents of bronchial secretions and play an important role in the viscoelastic properties of the mucus.

Previous studies on the physical and chemical properties of bronchial mucus glycoproteins have demonstrated the heterogeneity of these molecules. Unreduced native human bronchial mucins vary in molecular weight from $1.28 \cdot 10^6$ to $7 \cdot 10^6$ depending on the method of preparation [1–4]. Bronchial mucins prepared from the reduced mucus of a single subject are heterogeneous with regard to the average length of the carbohydrate chains, to their acidic character and to their peptide length as determined by electron microscopy [5]. Lamblin et al. [5] have suggested that this heterogeneity may be the result of different biosynthetic products and/or the result of enzymatic degradation at specific sites of the peptide backbone. The mucins prepared by reduction under non-dissociating conditions have a lower molecular weight and contain fewer amino acids and more carbohydrate than mucins reduced under dissociating conditions [6]. These data suggest that reduction under non-dissociating conditions probably activates a mucolytic enzymatic system.

In the present work, we report new evidence for the presence of mucolytic enzymatic systems induced by 2-mercaptoethanol or 4-aminophenylmercuric acetate.

Materials and Methods

Chemicals. Chemicals were purchased from the following sources: Sigma, paramethylsulfonyl fluoride (PMSF), 4-aminophenylmercuric acetate (APMA); Eastman, 2-mercaptoethanol; Touzart and Matignon, EDTA; Pharmacia, electrophoresis calibration kit.

Material. Sputum secreted by a patient suffering from chronic bronchitis was collected every day and kept frozen until used.

Abbreviations: APMA, 4-aminophenylmercuric acetate; PMSF, paramethylsulphonyl fluoride.

Solubilization of mucus secretions. The solubilization of mucus secretions was performed using a modification of the procedure described by Feldhoff et al. [2]. Mucus secretions (200 ml) were thawed and diluted 12 times with deionized water. Diluted material was stirred overnight at 4°C and then centrifuged at 3000 × *g* for 30 min. The pellet (125 mg) and the supernatant (3530 mg) referred to as bronchial soluble fraction were lyophilized separately. However, this fraction which was not dialyzed before Sepharose CL-2B chromatography contained about 50% of non-dialyzable material.

Isolation of bronchial mucins on Sepharose CL-2B. Bronchial mucins were isolated from this bronchial soluble fraction by Sepharose CL-2B gel chromatography. 100 mg of lyophilized bronchial soluble fraction, dissolved in 10 ml of 0.1 M Tris-HCl buffer containing 0.2 M NaCl, pH 8.0, were applied to a column of Sepharose CL-2B (2.5 × 48 cm). The chromatography was performed with the same buffer and 5 ml fractions were collected and analyzed for absorbance at 278 nm and for hexose by an automated orcinol assay [7].

Polyacrylamide gel electrophoresis. Analytical electrophoresis of bronchial mucins was carried out on polyacrylamide gels using the technique described by Kerckaert [8]. SDS-polyacrylamide gel electrophoresis was performed on a 5–15 p. 100 gradient using the buffer system of Laemmli [9]. Before electrophoresis, mucin samples (10 mg/ml) were mixed with an equal volume of buffer containing 1% SDS and heated for 5 min in a boiling water bath.

After electrophoresis, gels were stained for protein with Coomassie brilliant blue according to Laemmli [9] and for carbohydrate by the periodate-Schiff method of Zaccharius et al. [10]. Molecular weights were determined in SDS-polyacrylamide gel electrophoresis using the Pharmacia low molecular weight calibration kit.

Reduction of bronchial mucins. Reduction of bronchial mucins by 2-mercaptoethanol in phosphate buffer was performed as described previously [11].

Action of APMA on mucins. 25 mg of mucins were dissolved by stirring for 4 h in 10 ml of 0.1 M Tris-HCl buffer (pH 8.0); 70 mg 4-aminophenylmercuric acetate (APMA) was added to obtain a final concentration of 0.2 mM. After equilibration for 10 min at 40°C, the reaction mixture was incubated for 24 h at 37°C. Incubation was stopped by freezing and the treated mucins were studied by chromatography on Sepharose CL-2B as described earlier.

Inhibition of the mucolytic effects induced by reducing agent or APMA. 25 mg of mucins were first dissolved by stirring for 4 h in 2.5 ml 0.05 M Tris-HCl buffer, pH 7.5, and then PMSF (10 mM final concentration in 250 µl methanol) was added. After stirring overnight at 4°C, EDTA was added to yield a final concentration of 12.5 mM. After stirring for another 4 h at room temperature, 2-mercaptoethanol or APMA were added and the mixture was incubated at 37°C for different periods of time. Incubation was stopped by freezing.

Results

Isolation and characterization of high molecular weight bronchial glycoproteins

Bronchial soluble fraction (100 mg) was fractionated on a column of Sepharose CL-2B into

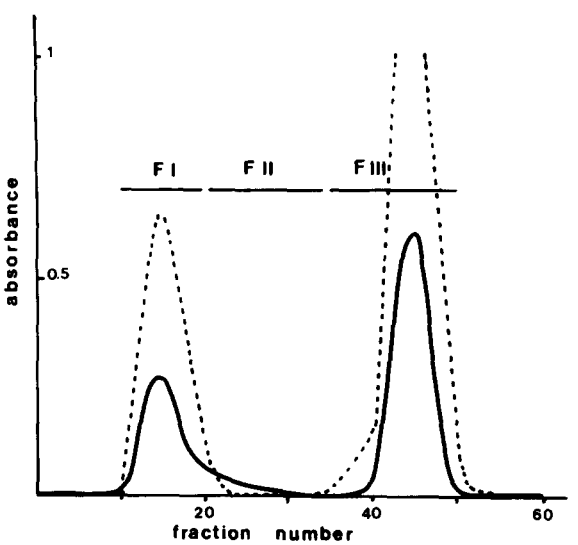

Fig. 1. Chromatography of bronchial soluble fraction (100 mg) on Sepharose CL-2B column (2.5 × 48 cm). The Sepharose CL-2B column was eluted by 0.1 M Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl. Collected fractions (5 ml) were analyzed for hexose (solid line) and for absorbance at 278 nm (dashed line). Fractions indicated by bars were pooled, dialyzed and lyophilized.

three fractions FI (9 mg), FII (6 mg) and FIII (16 mg) (Fig. 1). The three fractions were dialyzed against water, lyophilized, and then studied in analytical gel electrophoresis. The high molecular weight fraction, FI, contained a major glycoprotein component which did not enter the gel and some low molecular weight protein components (M_r < 94000) which were dissociated from the glycoprotein by SDS-gel electrophoresis (Fig. 3, I, slot 1).

Fraction FII, which contained a mixture of glycoproteins with lower molecular weights and fraction FIII which contained essentially low molecular weight proteins were not studied further.

All subsequent studies were carried out on fraction FI, also referred to as high molecular weight bronchial glycoproteins.

Action of 2-mercaptoethanol on high molecular bronchial glycoproteins

Time course of action of 2-mercaptoethanol studied by polyacrylamide gel electrophoresis. High molecular weight bronchial glycoproteins fractions (2 mg) were dissolved in 0.1 ml of 0.05 M Tris-HCl buffer (pH 7.5) and then incubated at 37°C with 1 µl of 2-mercaptoethanol. Incubation was stopped at different times by freezing, and treated glycoproteins were studied by analytical polyacrylamide gel electrophoresis (Fig. 3). Coomassie blue staining showed the progressive disappearance as a function of time of the proteins with molecular weights between 30000 and 94000. Periodate-Schiff staining of the gel showed a progressive decrease in molecular weight of the glycoproteins in fraction FI (high molecular weight bronchial glycoproteins) until almost complete penetration of the glycoproteins into the gel occurred after 4 h of incubation.

Sepharose CL-2B chromatography of high molecular weight bronchial glycoproteins after 24 h reduction. Freeze dried high molecular weight bronchial glycoproteins (25 mg) were reduced by 2-mercaptoethanol during 24 h. The reduced material was fractionated by chromatography on a Sepharose CL-2B column into three fractions F1a, F2a and F3a (Fig. 2a). The major glycoprotein fraction, F2a, was retained on the column; that proved a degradation of high molecular weight bronchial glycoproteins, after reduction with mercaptoethanol.

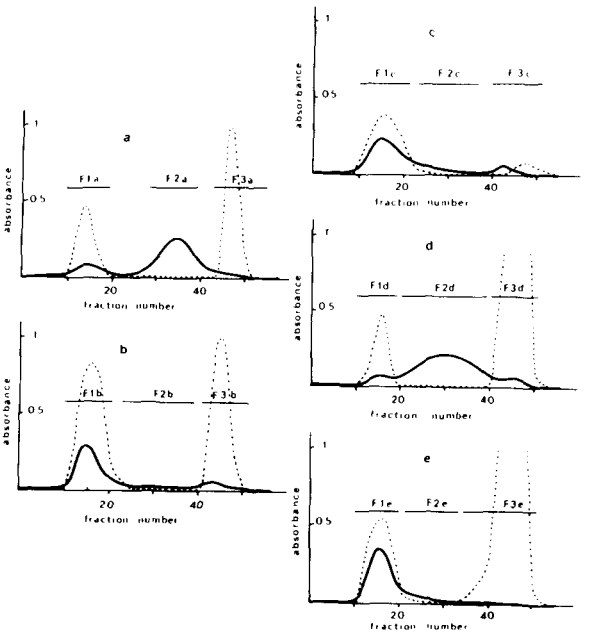

Fig. 2. Chromatography on Sepharose CL-2B column (2.5 × 48 cm) of: a, fraction FI or high molecular weight bronchial glycoproteins (25 mg) reduced by 2-mercaptoethanol; b, high molecular weight bronchial glycoproteins (25 mg) treated by PMSF and EDTA then reduced by 2-mercaptoethanol; c, high molecular weight bronchial glycoproteins (25 mg) after 24 hours of incubation at 27°C with stirring; d, high molecular weight bronchial glycoproteins (25 mg) treated by APMA; e, high molecular weight bronchial glycoproteins (25 mg) treated by PMSF and EDTA then APMA. In every case, the column of Sepharose CL2B was eluted with 0.1 M Tris-HCl buffer pH 8.0, containing 0.2 M NaCl. Collected fractions (5 ml) were analyzed for hexose (solid line) and for absorbance at 278 nm (dashed line). Fractions indicated by bars were pooled, dialyzed and lyophilized.

Action of APMA on high molecular weight bronchial glycoproteins

The chromatographic profiles of high molecular weight bronchial glycoproteins (25 mg) incubated for 24 h with or without APMA were compared (Fig. 2). After 24 h at 37°C, high molecular weight bronchial glycoproteins had an unchanged behaviour on Sepharose CL-2B: they were still excluded from the gel (Fig. 2c). But after treatment with APMA, the molecular weight of most glycoproteins decreased (Fig. 2d); their elution from the column of CL-2B Sepharose was retarded (F2d).

Analytical polyacrylamide gel electrophoresis revealed that untreated high molecular weight bronchial glycoproteins, (fractions Flc and F2c), did not enter the gel as shown by periode-Schiff staining (Fig. 3) whereas the major fraction of high molecular weight bronchial glycoproteins treated by APMA, F2d, was heterogeneous and contained two bands, one entering the gel and the other, of higher molecular weight, remaining in the starting gel (Fig. 3). This fraction F2d contained fewer low molecular weight proteins stained by Coomassie blue than the control fractions Flc and F2c (Fig. 3).

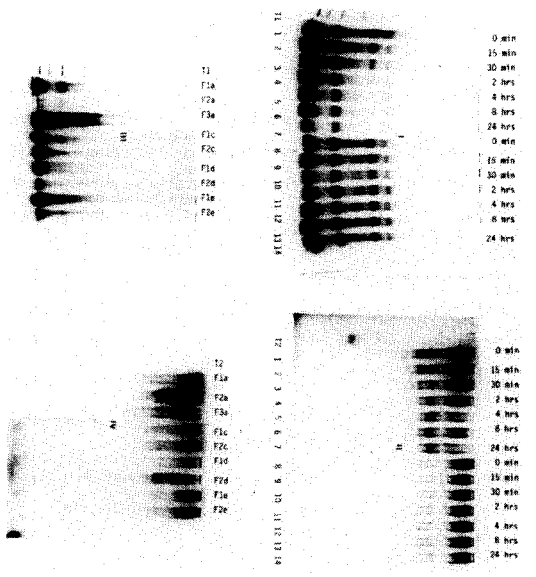

Fig. 3. Analytical gel electrophoresis of high molecular weight bronchial glycoproteins subfractions obtained in different conditions. I and II: action of 2-mercaptoethanol on high molecular weight bronchial glycoproteins according to time. Gel I was stained by Coomassie blue, gel II stained by periodate-Schiff method. The time of incubation is indicated above every slot. Slots 1–7 correspond to samples incubated in the absence of inhibitors. Slots 8–14 correspond to samples incubated in the presence of PMSF and EDTA. III and IV: different fractions obtained on Sepharose CL-2B column. Gel III was stained by Coomassie blue, gel IV by periodate-Schiff method. Fractions (obtained under various conditions as described in Fig. 2) studied are indicated above every slot. T1 is a molecular weight markers mixture containing phosphorylase B (94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100), α-lactalbumin (14400). T2 contains α_1 acid glycoprotein (41000).

Inhibition of the mucolytic action of 2-mercaptoethanol by PMSF and EDTA

Time course of mucolytic action studied by polyacrylamide gel electrophoresis. Samples of high molecular weight bronchial glycoproteins (25 mg) treated with PMSF and EDTA were divided in fractions of 0.1 ml and then incubated with 1 μl of 2-mercaptoethanol for different periods of time. Incubation was stopped as previously described and all fractions were studied by electrophoresis (Fig. 3). No change was observed in the mobility of proteins, as shown by Coomassie blue staining, or in that of glycoproteins during the 24-h period of incubation.

Sepharose CL-2B chromatography of high molecular weight bronchial glycoproteins after action of PMSF and EDTA. 25 mg of high molecular weight bronchial glycoproteins treated by PMSF and EDTA were incubated for 24 h at 37°C with 2-mercaptoethanol as already described. The mixture was then fractionated by chromatography on Sepharose CL-2B into three fractions Flb, F2b, F3b (Fig. 2b). The large molecular weight glycoprotein fraction Flb, which was excluded from the gel, was still present showing the lack of effect of 2-mercaptoethanol.

Both EDTA and PMSF were necessary to inhibit the effect of mercaptoethanol. When they were tested separately, there were a weak inhibition with PMSF and no effect with EDTA (data not shown).

Inhibition of the action of APMA on high molecular weight bronchial glycoproteins by PMSF and EDTA

25 mg of high molecular weight bronchial glycoproteins treated with PMSF and EDTA as described under Materials and Methods, were subsequently submitted to the action of APMA. The mixture was fractionated by chromatography on Sepharose CL-2B into three fractions Fle, F2e and F3e (Fig. 2e). The major fraction Fle was excluded from the gel. A minor glycoprotein fraction F2e was eluted later.

Analytical electrophoresis of the two glycoprotein fractions confirmed that they still had high molecular weights as shown by periodate-Schiff staining of the polyacrylamide gel (Fig. 3). The associated proteins were still present, although the Coomassie blue staining of fraction F2e was weaker than that of fraction Fle (Fig. 3).

Discussion

In order to study the mechanism of reduction of bronchial mucins, we have tried, as have many other authors [2,12–15], to prepare mucins in a state as nearly native as possible. According to Feldhoff et al. [2] bronchial glycoproteins solubilized by stirring mucus after dilution in water appeared as high molecular weight material. A similar procedure was applied to the whole secretion of a patient suffering from chronic bronchitis: after such a treatment, more than 90% of the non-dialyzable material was soluble. Chromatography of this solubilized mucus on Sepharose CL-2B, under non-dissociating conditions allowed to prepare a fraction of high molecular weight bronchial glycoproteins which only represented 10% of the solubilized mucus. This poor recovery was due, partly to the use of undialyzed solubilized mucus (about 50% dialyzable material) and partly to some binding to the gel under non-dissociating conditions (about 40% of the non-dialyzable material was lost).

Electrophoretic characterization of high molecular weight bronchial glycoproteins proved the heterogeneity of these molecules, contrary to Feldhoff et al.'s report [2]. Several proteins of relatively low molecular weight were still associated with the glycoproteins by non-covalent bonds.

Until now, the reduction of bronchial mucins has been studied by gel filtration, followed by the determination of molecular weight and of chemical composition of the resulting fractions [5,16–19]. Analytical polyacrylamide gel electrophoresis revealed another aspect of high molecular weight bronchial glycoproteins reduction, namely, the progressive disappearance of some proteins as a function of time, and the concomitant decrease in molecular weight of glycoproteins as visualized by their mobility into the gel (Fig. 2). Several observations have already been reported supporting the idea that 2-mercaptoethanol activates mucolytic enzymatic systems: the heterogeneity of molecular weight and of length of reduced mucins has been described [5]; the effect of heat denaturation or PMSF treatment before reduction of native mucins [20]; molecular weights and chemical compositions of mucins reduced in 6 M guanidine [6]. Moreover Carlstedt et al. [21] and Williams et al. [22] have recently demonstrated the resistance of mucins to dithiothreitol after urea treatment, and found no evidence for disulphide bond-linked glycoprotein subunits in mucus from healthy subjects. The present data present new evidences of a mucolytic activation mechanism.

These enzyme-inhibitor complexes may correspond to the low molecular weight proteins associated with high molecular weight bronchial glycoproteins and would only be dissociated by polyacrylamide gel electrophoresis in the presence of SDS. The progressive disappearance of some protein fractions during polyacrylamide gel electrophoresis after the action of 2-mercaptoethanol or of APMA can be explained by the dissociation of such enzyme-inhibitor complexes leading to the liberation and possibly to the progressive destruction of active proteases able to degrade proteins associated with mucins.

In conclusion we suggest that 4-aminophenylmercuric acetate activates enzymes by dissociation of covalently or non-covalently-bound enzyme-inhibitor complexes, whereas 2-mercaptoethanol acts by modifying the conformation of an inhibitor non-covalently associated with an enzyme-inhibitor complex. Both effects would liberate active enzymes, capable of breaking the polypeptide chains of bronchial mucins and associated proteins, and sensitive to serine protease and metalloprotease inhibitors.

Thus, it is very important to elucidate the role of such complexes in bronchial mucus and to determine whether a defect in the synthesis or degradation of the constituents of these complexes influence the rheological properties of bronchial mucus, especially in chronic bronchial diseases.

Acknowledgements

The authors thank M. Luyckx, B. Gillet and D. Vandeperre for their technical assistance. This work was supported by contract 803025 from INSERM and by la Fondation pour la Recherche Médicale Française.

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