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Novel Complex Formed between a Nonproteolytic Cell Wall Protein of Group A Streptococci and α_2-Macroglobulin

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Binding of ^{125}I-labeled α_2-macroglobulin (α_2M) to streptococci belonging to serological groups A, B, C, and G was studied. Streptococci of groups A and G interacted only with native α_2M, and those of group C reacted only with α_2M-trypsin complex. Binding of α_2M to group A streptococci was saturable and reversible. The dissociation constant was 2.02×10^{-7} M, and the number of binding sites was calculated to be 18,000 per streptococcus. The α_2M-binding protein could be solubilized by treatment of group A streptococci with a murolytic enzyme and subsequently purified by affinity chromatography and high-pressure liquid chromatography. The purified protein was homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a molecular weight of 78,000. It possessed no proteolytic activity and interacted with native α_2M in Western blots (immunoblots). Interaction of purified binding protein with α_2M led to a change in the conformation of α_2M similar to that obtained by α_2M-protease complexes. Reversible binding of a nonproteolytic streptococcal component of α_2M is thus a novel feature of α_2M reactivity.

α_2-Macroglobulin (α_2M) is a plasma protein inhibitor with ability to inhibit nearly all endopeptidases (2). It is a glycoprotein of molecular weight 725,000 present in plasma at concentrations of 2 to 4 mg/ml (30). α_2M is composed of two pairs of identical subunits held together by disulfide bonds (1, 8). Each subunit contains a sequence of amino acids of 85,000 daltons termed the "bait" region (11). Cleavage of this region by proteinases leads to a change in conformation, resulting in irreversible binding of proteinase (2). The bound proteinase remains active toward small substrates, but activity toward large substrates is very much reduced. This unique complex formation involves covalent incorporation of proteinase into native α_2M (24, 26). Treatment of α_2M with methylamine also cleaves the thioester and produces a change in the conformation of the α_2M molecule which is similar to that observed after its reaction with proteinases (10).

The changed conformation of α_2M after its irreversible reaction with proteinase or amine leads to exposure of recognition sites with specific receptors on many cell types such as hepatocytes, macrophages, and fibroblasts (7, 13, 20). These cells do not recognize native α_2M. There is evidence that α_2M-proteinase complexes, but not native α_2M, are internalized by receptor-mediated endocytosis by macrophages (7, 12). The half-life of an α_2M complex in circulation is on the order of minutes as compared with hours for α_2M (21). α_2M is, therefore, considered to be an important defense mechanism because of the inhibition and removal of potentially harmful endoproteinases.

We have recently reported the binding of native α_2M to streptococci of serological groups A and G (5, 18). The reversibility of this interaction was a novel feature, and it was speculated that binding of α_2M to streptococci is different from its reaction with proteinases. The present study was undertaken with an aim to purifying the α_2M-binding site from group A streptococci for characterization of its complex formation with α_2M.

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MATERIALS AND METHODS

Streptococci and culture conditions. Ten cultures each of group A, B, C, and G streptococci were used. Of these, *Streptococcus pyogenes* reference strain A8189 was used for characterization of α_2M binding as well as purification of the α_2M-binding site. The streptococci were grown in Todd-Hewitt broth (GIBCO Europe, Karlsruhe, Federal Republic of Germany) for 18 h at 37°C and 60 rpm.

Isolation and radiolabeling of α_2M. α_2M was purified from human plasma by a two-step procedure. Plasma (20 ml) was dialyzed against 0.02 M K_2HPO_4 containing 0.02% sodium azide and passed through a column containing 100 ml of DEAE–Affi-Gel Blue (Bio-Rad, Munich, Federal Republic of Germany) equilibrated with the same buffer. After washing of the column, the bound α_2M was eluted with 0.4 M NaCl and further purified by zinc chelate chromatography by the method of Kurecki et al. (16). The activity of α_2M was determined by the method of Ganrot (9) by using α-benzoyl-L-arginine ethyl ester as a substrate. α_2M-trypsin complex (α_2M-T) was prepared by the method of Kaplan and Nielson (14), and α_2M-methylamine complex (α_2M-M) was prepared as described by Österberg and Malmensten (22). α_2M, α_2M-T, and α_2M-M were radiolabeled by the chloramine T method as described previously (3, 4). The specific activities were 1.2 mCi/mg for α_2M, 0.9 mCi/mg for α_2M-T, and 1.0 mCi/mg for α_2M-M.

Binding assay. The binding assay was performed with 4×10^7 streptococci and 10 ng of labeled α_2M, α_2M-T, or α_2M-M in phosphate-buffered saline (PBS) containing 0.05% Tween 20. After incubation for 1 h at room temperature, the streptococci were centrifuged at $10,000 \times g$ and washed with ice-cold PBS–0.05% Tween 20 (5). The radioactivity in the sediment was measured in a γ-spectrometer (Packard Instrument Co., Inc., Rockville, Md.). In chase experiments, the washed sediment was suspended in either PBS or 1 μM native α_2M in PBS. The radioactivity released in the supernatant was measured at different time intervals between 15 and 240 min. For equilibrium binding curves, the assay was

TABLE 1. Binding of labeled α_2M, α_2M-T, and α_2M-M

Organism (serological group)	Mean % binding of[a]:		
	α_2M	α_2M-T	α_2M-M
Streptococcus pyogenes (A)	51	2.5	1.5
Streptococcus agalactiae (B)	2.0	3.1	2.0
Streptococcus dysgalactiae (C)	1.8	48	53.5
Streptococcus sp. (G)	43	3.8	4.2

[a] The values are means from 10 cultures belonging to each group. Binding of less than 5% was considered negative; 100% was 34,000 cpm for α_2M, 32,500 cpm for α_2M-T, and 30,000 cpm for α_2M-M.

performed with 10 ng of labeled α_2M and various concentrations (3 to 830 pmol) of unlabeled α_2M. The bound and free fractions were then determined, and the data were plotted as described by Scatchard (25). The dissociation constant (K_d) and the number of binding sites were determined after analysis of equilibrium binding data by the LIGAND program of Munson and Rodbard (19) on a Cyber 134 computer.

Solubilization and purification of α_2M-binding protein. *S. pyogenes* reference strain A8189 (100 ml of a 10% [wt/vol] suspension in 0.01 M phosphate buffer, pH 7.0) was subjected to lysis by incubation with 50 μg of a lytic enzyme purified from *Streptomyces globisporus* (28) per ml. α_2M-binding activity in the lysate was determined by dot blotting with purified α_2M and anti-α_2M antibodies by the method of Hawkes et al. (12). α_2M-binding protein was purified from streptococcal lysate by a two-step procedure. A zinc chelate column (9 by 1.2 cm) was prepared as described by Kurecki et al. (16), and 10 mg of purified α_2M was absorbed on it. The column was equilibrated with 0.02 M phosphate buffer containing 0.15 M NaCl, pH 6.0. The lysate, after dialysis against the same buffer, was passed through a α_2M-zinc chelate column. After washing of the column with the buffer, the absorbed complex of α_2M and the binding protein was eluted with 0.02 M cacodylate buffer, pH 5.0 containing 0,15 M NaCl. For separation of binding protein from α_2M, sodium thiocyanate (final concentration, 1 M) was added to the complex, which was then subjected to high-pressure liquid chromatography (HPLC). The HPLC was performed on a Knauer system with a Zorbax Du Pont GF-250 column (9.4 by 250 mm). The eluant was 0.02 M cacodylate buffer, pH 5.0, containing 0.15 M NaCl and 1 M sodium thiocyanate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 12% gels by the method of Laemmli (17) with a Protean dual-slab cell (Bio-Rad). A low-molecular-weight kit (Pharmacia, Freiburg, Federal Republic of Germany) was used as marker proteins. For Western blotting (immunoblotting), the proteins from slab gels were transferred to a nitrocellulose membrane by using a trans-blot electrophoretic transfer cell (Bio-Rad). The binding sites for α_2M were then visualized by the method of Towbin et al. (29) with purified α_2M and anti-α_2M antibodies.

Proteolytic activity. Proteolytic activity was determined with azocasein (28), hide powder azure (23), or benzoyl-DL-arginine-*p*-nitroanilide (9) as a substrate.

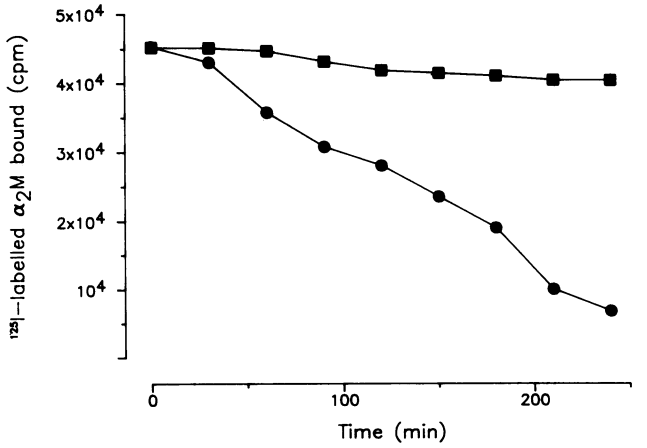

FIG. 1. Reversibility of α_2M binding to *S. pyogenes* A8189. Streptococci were incubated with ^{125}I-labeled α_2M for 1 h and, after washing, were suspended in PBS (■) or 1 μM α_2M solution in PBS (●). The radioactivity released in the supernatant was measured at different time intervals. The total radioactivity added to each assay was 95,000 cpm.

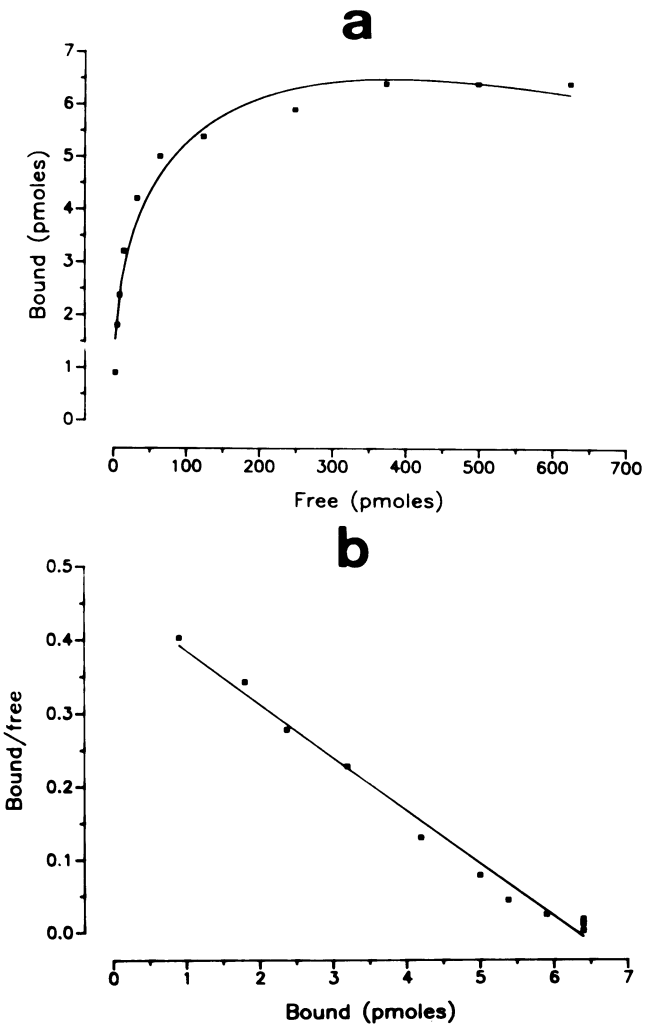

FIG. 2. Equilibrium binding curve (a) and Scatchard plot (b) of α_2M binding to *S. pyogenes* A8189. Analysis of data by the LIGAND program on a Cyber 134 computer gave K_d values of 2.02×10^{-7} M.

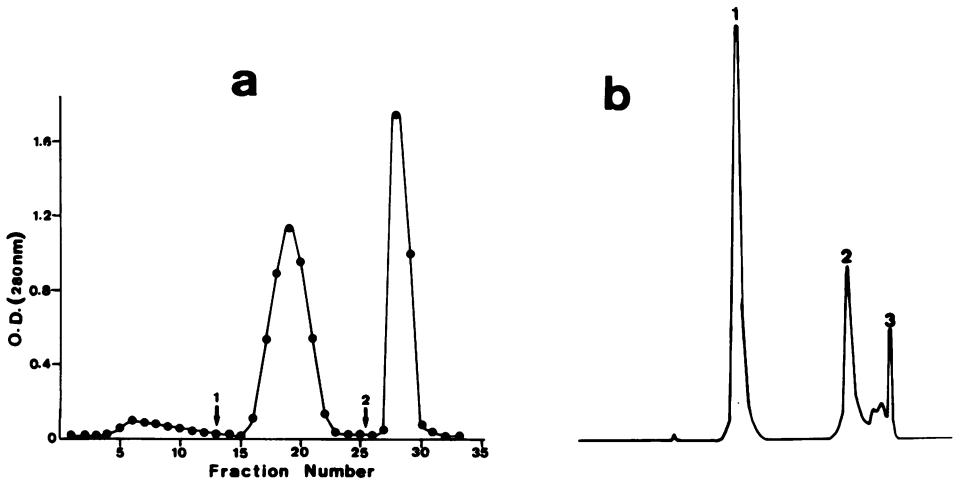

FIG. 3. α_2M-zinc chelate chromatography (a) of binding protein solubilized from *S. pyogenes* A8189. At arrow 1, the streptococcal lysate was passed through the column, and after washing, the bound complex of α_2M with the binding protein was eluted with cacodylate buffer, pH 5.0 (arrow 2). O.D., Optical density. The streptococcal protein was then separated from α_2M by HPLC (b). Peak 1 contained α_2M, and peak 2 contained its binding protein from streptococci. Peak 3 contained a number of small fragments from either α_2M or its binding protein.

RESULTS

Binding studies. All streptococcal cultures of groups A and G interacted with ^{125}I-labeled native α_2M with mean binding percentages of 51 and 43%, respectively. Group A and G streptococci did not react with α_2M-T or α_2M-M. On the other hand, the streptococcal cultures of group C reacted only with α_2M or α_2M-M, with mean binding percentages of 48 and 53%, respectively. The streptococci of group B reacted with neither α_2M nor its derivatives (Table 1).

Binding of native α_2M to group A streptococci was specific because other plasma proteins such as fibrinogen, albumin, immunoglobulins, transferrin, and β_2-microglobulin did not influence the binding. The binding was reversible, as indicated by chase experiments. About 80% of the labeled α_2M bound to streptococci could be displaced by 1 μM unlabeled α_2M within 4 h (Fig. 1). The saturability of binding was established by the equilibrium binding curve (Fig. 2a). For the saturation analysis data, when plotted as described by Scatchard, a linear plot was obtained (Fig. 2b). Analysis of the data by the LIGAND program gave a K_d value of 2.02×10^{-7} M and a binding capacity of 2.4×10^{-8} M, from which the number of binding sites was calculated to be 18,000 per streptococcus.

Purification of α_2M-binding protein. Lysis of group A streptococci with a murolytic enzyme from *S. globisporus* proved to be most suitable for solubilized of α_2M-binding protein. The solubilized protein retained its α_2M-binding activity as determined by dot blotting. For purification, α_2M absorbed to zinc chelate was more effective than α_2M immobilized on Sepharose. The solubilized binding protein was selectively absorbed on α_2M-zinc chelate. All other proteins present in the lysate, none of which had α_2M-binding activity, flowed through the column (Fig. 3a). The α_2M-binding protein was then eluted from the column with 0.02 M cacodylate buffer, pH 5.0. α_2M-binding protein was then effectively separated from α_2M by HPLC in the presence of 1 M sodium thiocyanate (Fig. 3b). The first peak had all of the α_2M activity, whereas the second peak contained purified α_2M-binding protein. The yield of purified protein was 0.3 mg/g (wet weight) of streptococci.

Properties of purified α_2M-binding protein. Purified α_2M-binding protein from group A streptococci was homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4a). The molecular weight, as calculated from the calibration curve with marker proteins, was 78,000. Western blot analysis of lysate, as well as purified binding protein, gave a distinct band at 78,000 with native α_2M (Fig. 4b). The purified binding protein possessed no proteolytic activity, irrespective of the substrate used, thus excluding the possibility of an α_2M-proteinase type of interaction.

To evaluate the change in conformation of α_2M as a result of its binding to streptococcal protein, we assayed the binding activity of the complex to *Streptococcus dysgalactiae* cultures, which bind only α_2M-T and not native α_2M.

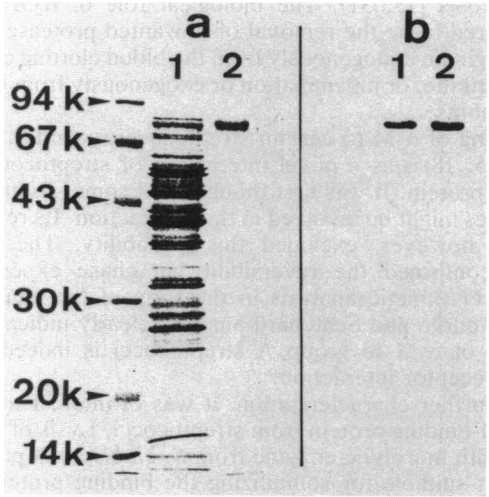

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel stained with Coomassie blue (a) and Western blot of purified α_2M and anti-α_2M antibodies (b). Lane 1 contained streptococcal lysate (200 μg of protein), and lane 2 contained purified streptococcal protein (20 μg). The lane at the extreme left contained molecular weight marker proteins. Molecular weights (10^3) are indicated on the left.

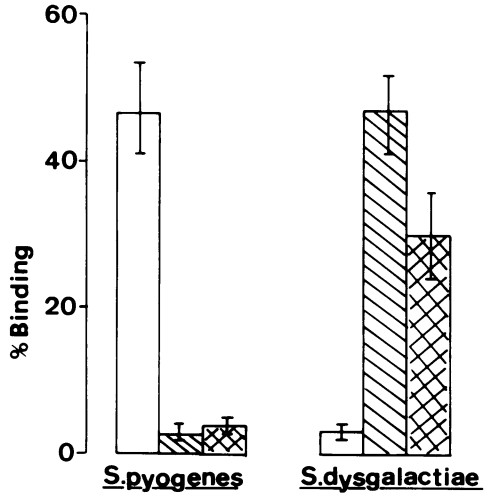

FIG. 5. Binding of ^{125}I-labeled native α_2M (open bars), α_2M-T (hatched bars) and α_2M complexed with purified group A streptococcal protein (cross hatched bars) to *S. pyogenes* (group A streptococci) and *S. dysgalactiae*. The values represent means from five cultures of each species.

The complex of labeled α_2M and streptococcal binding protein showed moderate binding to *S. dysgalactiae* (Fig. 5), indicating that this complex formation also leads to changes in the conformation of the α_2M molecule. This complex, just like α_2M-T, did not bind to group A streptococci (Fig. 5).

DISCUSSION

α_2M is one of the major components in the circulation of vertebrates and is well known for its capacity to inhibit endoproteases (30). Native α_2M contains an internal thioester (27) which is probably involved in the formation of irreversible covalent complexes with proteases. These complexes bind to specific receptors on macrophages and fibroblasts and are ultimately internalized by receptor-mediated endocytosis (15, 31). The biological role of α_2M is thus considered to be the removal of unwanted proteases which may originate endogenously from the blood clotting cascade, injured tissue, or inflammation or exogenously from bacteria and venoms.

Binding of α_2M to certain streptococci recently reported by us (5, 18) was a novel interaction of streptococci with plasma protein. It was first thought that some streptococcal proteases might be involved in this interaction. Its reversible nature, however, excluded this possibility. The present study confirmed the reversibility in chase experiments. Moreover, kinetic analysis in the form of the equilibrium binding curve and Scatchard analysis clearly indicated that binding of α_2M to group A streptococci is indeed a true ligand-receptor interaction.

For further characterization, it was of interest to isolate the α_2M-binding protein from streptococci. Lysis of streptococci with murolytic enzyme from *S. globisporus* proved to be most suitable for solubilizing the binding protein in its active form. Other solubilization methods, such as trypsin and papain extraction or ultrasonication, resulted in considerable loss of α_2M-binding activity. Attempts to purify the binding protein by affinity chromatography with α_2M coupled to agarose were unsuccessful. Perhaps α_2M immobilized on agarose differs slightly from native α_2M, which is absolutely essential for interaction with the binding protein from group A streptococci. α_2M coupled to zinc chelate, however, was extremely effective in forming a complex selectively with the binding protein present in the lysate. Separation of the complex could then be achieved by HPLC, which further confirmed the reversibility of such complex formation. Lack of proteolytic activity in the purified binding protein further confirmed that its complex with α_2M is a novel feature which is different from α_2M-proteinase or α_2M-M complex formation.

Does formation of a complex between streptococcal protein and α_2M results in a change in conformation similar to that observed with α_2M-T or α_2M-M? We could answer this question by binding studies with *S. dysgalactiae* cultures which bind only the changed conformation of α_2M and not native α_2M. Binding of a complex between α_2M and streptococcal protein to *S. dysgalactiae* indicated that this interaction also leads to a change in conformation which may be similar to those obtained with proteinase or methylamine (6, 8, 32). Reversibility of the interaction, however, may lead to speculation that the mechanism by which the streptococcal protein changes the conformation of α_2M is different from the mechanism of interaction between α_2M and proteinase or methylamine.

Because of the importance of α_2M in the removal of unwanted proteinases and its unique mechanism of inhibiting proteinases, there has been considerable interest in elucidation of structural and functional aspects of α_2M (8, 22). This novel reversible complex formation between α_2M and a nonproteolytic streptococcal protein might be of interest in such an elucidation. The biological significance of this interaction has yet to be elucidated. Since macrophages recognize only α_2M complexes for receptor-mediated endocytosis, it could be postulated that interaction of α_2M with group A streptococci plays a role in pathogenicity.

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