The Separation of Alpha-2 Macroglobulin into Five Components with Differing Electrophoretic and Enzyme-Binding Properties

RUSSELL SAUNDERS, BARBARA J. DYCE, WILTON E. VANNIER, and
BERNARD J. HAVERBACK

From the Department of Medicine and Biochemistry, University of Southern
California School of Medicine, Los Angeles, California 90033

A B S T R A C T   The alpha-2 macroglobulins from human
serum and plasma were isolated by Bio-Gel P-300 and
A5m gel filtration. The material showed a single peak
on sedimentation velocity ultracentrifugation, a mol wt
of 650,000 by sedimentation equilibrium ultracentrifuga-
tion, and a major precipitin arc in the alpha-2 macro-
globulin region by immunoelectrophoresis against whole
human serum. Two bands were observed in the alpha-2
macroglobulin region when acrylamide gel electrophore-
sis was performed with a pH 8.9 running gel. When a
pH 7.8 gel was used, five electrophoretic species were
observed. In both cases, the preaddition of stoichiometric
amounts of trypsin or chymotrypsin added to alpha-2
macroglobulin resulted in disappearance of slower bands
leaving only one band on acrylamide gel electrophoresis
patterns.

Preparative acrylamide gel electrophoresis separated
alpha-2 macroglobulin obtained from Bio-Gel into five
closely-spaced species. Separation was sufficiently ade-
quate to show that those species of alpha-2 macroglobulin
which bound trypsin and chymotrypsin were represented
by slower moving species and that the fastest moving
material had lost virtually all of the ability to bind these
enzymes. Preparative acrylamide gel electrophoresis of
a mixture of alpha-2 macroglobulin-trypsin complex and
alpha-2 macroglobulin revealed that the fast moving
component was alpha-2 macroglobulin-trypsin complex
and that the slower moving material was unbound
alpha-2 macroglobulin. The naturally occurring amidase
activity of the alpha-2 macroglobulin using benzoyl-
arginine-p-nitroanilide (BAPNA) as substrate was in-
vestigated and unlike its trypsin-binding activity, ami-
dase activity was found to be of the same specific activity
in all electrophoretic fractions.

Binding of trypsin and chymotrypsin to alpha-2 macro-
globulin revealed that alpha-2 macroglobulin maximally
bound 2 moles of trypsin and 1 mole of chymotrypsin.
When the enzymes were added simultaneously there was
competition. Chymotrypsin added to alpha-2 macroglob-
ulin before the addition of trypsin prevented all trypsin
binding even though only one site was filled with chymo-
trypsin. These results were explained by the acrylamide
gels which showed that 1 mole of chymotrypsin was
sufficient to convert all the alpha-2 macroglobulin to a
species with the fastest mobility which no longer binds
additional enzyme.

INTRODUCTION

Although the function of the alpha-2 macroglobulin is
unknown, it is likely to be associated with the binding
of these proteins to a number of enzymes which have
similar properties. It is known for example, that the
serum alpha-2 macroglobulins bind trypsin (1), chymo-
trypsin (1), thrombin (2), and plasmin (3). Interest-
ingly, all these enzymes are endopeptidases with similar
molecular weights and active centers. Also, all hydrolyze
synthetic substrates such as benzoyl-arginine-p-nitro-
anilide (BAPNA).[1] When exogenously added trypsin is
bound to alpha-2 macroglobulin, the enzyme maintains
60–70% of its activity using BAPNA as a substrate and
20% of its proteolytic activity using casein as a sub-
strate (1). Ganrot's studies have shown that trypsin and
plasmin were competitive in that prior addition of plas-
min reduced the amount of trypsin bound to alpha-2
macroglobulin (3). As alpha-2 macroglobulins bind exo-
genously added trypsin, chymotrypsin, thrombin, and

[1] Abbreviations used in this paper: BAPNA, benzoyl-
arginine-p-nitroanilide.

plasmin, a search in our laboratory was made to determine whether or not a naturally occurring enzyme was bound to any of the alpha-2 macroglobulins. A naturally occurring enzyme bound to alpha-2 macroglobulin was found which had esterase activity (hydrolysis of tosyl-arginine-methyl ester), amidase activity (hydrolysis of BAPNA), and kallekrein activity (4).

The addition of trypsin to serum was noted to cause a change in the mobility of proteins in the alpha-2 macroglobulin region. Plasmin and methylamine also have been reported to alter the electrophoretic properties of alpha-2 macroglobulin preparations (5, 6), yielding more of the components with faster mobility. Interestingly, methylamine is known to destroy trypsin-binding activity of alpha-2 macroglobulin (6). The purposes of these studies were to (a) isolate the alpha-2 macroglobulin using procedures least likely to cause denaturation of the protein or change its trypsin-binding activity, (b) determine whether alpha-2 macroglobulin was a simple protein or consisted of a family of similar proteins, (c) separate and isolate the components of the alpha-2 macroglobulin using acrylamide gel electrophoresis and determine naturally occurring amidase activity of each component, (d) investigate the nature of the sites which bind trypsin or chymotrypsin by the use of simultaneous addition of labeled enzymes, and (e) determine which components bind trypsin and study the relationship of electrophoretic mobility, enzyme-binding properties, and naturally occurring amidase activity, and (f) determine the maximal molar-binding ratio for trypsin and chymotrypsin.

METHODS

Isolation and characterization of alpha-2 macroglobulins

Isolation of alpha-2 macroglobulin. 15 ml of whole serum was applied to a Bio-Gel P-300 column (Bio-Rad Laboratories). The macroglobulin fractions (the first peak) were concentrated and reapplied to a column of Bio-Gel A5m. The second of three peaks was found to contain alpha-2 macroglobulin by immunoassay and trypsin-binding activity. This material was concentrated, reapplied, and only the fractions with the highest trypsin-binding activity pooled for further study. In all steps, 0.1M tris-HCl buffer at pH 7.65 was the eluant and column dimensions were 120 cm in length and 2.5 cm in diameter.

The concentration of alpha-2 macroglobulin was determined by absorbency measurements at 280 mμ using an $E^{1\%}_{280}$ value of 8.1 (7).

Trypsin-binding activity. To measure trypsin-binding activity of fractions of column eluants and those from preparative acrylamide gel electrophoresis, excess trypsin was added to a fraction followed by an incubation period of 15 min. Soybean trypsin inhibitor was then added to inactivate the unbound trypsin. Only the trypsin bound to the alpha-2 macroglobulin was protected from soybean trypsin inhibitor

(1, 8). The 35% inhibition of trypsin by alpha-2 macroglobulin was taken into consideration when calculating the amount of bound trypsin. To perform the assay, 100–200 μg of alpha-2 macroglobulin was incubated with 15 μg of Worthington 3x crystallized trypsin (Worthington Biochemical Corp.) and followed by 20 μg of Worthington soybean trypsin inhibitor. All solutions were in 0.1M tris-HCl buffer and the final reaction volume was brought to 3.0 ml with this buffer. 1 ml of a 1 mg/ml solution of BAPNA in distilled water was added and the rate of hydrolysis of BAPNA followed spectrophotometrically at 410 mμ.

Amidase activity. The ability of the material in a fraction to catalyze the hydrolysis of BAPNA is referred to as amidase activity. To perform these assays, 0.4 ml of a 1 mg/ml BAPNA solution in distilled water was added to 0.8 ml of the fraction in 0.1M tris-HCl buffer at pH 7.65. The rate of hydrolysis of BAPNA was determined by absorbency change at 410 mμ over a period of 24 hr. The rates were found to be linear for the alpha-2 macroglobulin amidase activity.

Immunological properties of alpha-2 macroglobulins. Immunodiffusion was performed in 0.8% ion agar (Colab) in 1% saline buffered at pH 8.4 with 10% v/v borate buffer. Antisera used in these experiments were goat anti-whole human sera. Tests for IgM concentration were performed using Hyland plates (Hyland Laboratories, Los Angeles, Calif.) containing anti-IgM and appropriate standards.

Analysis of alpha-2 macroglobulin by ultracentrifugation. Sedimentation velocity measurements were performed in a Spinco model E ultracentrifuge (Spinco Div., Beckman Instruments, Inc.) at 59,000 rpm and at 20°C. The protein concentration was 4.2 mg/ml. As sedimentation velocity primarily was used as a test for homogeneity, sedimentation coefficient were not extrapolated to zero concentration.

Sedimentation equilibrium measurements were carried out by the high speed method of Yphantis (9) using a synthetic boundary cell. The partial specific volume $\bar{\gamma}$ was assumed to be 0.733 as reported by Dunn and Spiro (10) and the buffer density, was determined by pyknometry to be 1.00313 for 0.1M tris-HCl buffer. Speeds in rpm ranged from 8,225 to 11,272. All photographs were taken after 24 hr which was found to be sufficient to reach equilibrium.

Analytical and preparative acrylamide gel electrophoresis of alpha-2 macroglobulins. The pH 8.9 analytical gels were prepared by the Davis method (11) but with modification of the gel composition to give 5% acrylamide. The buffer solution for the pH 7.8 gels was a solution of 0.48M HCl and 0.30M Tris containing 2.3 ml/liter of N,N,N',N'-tetra-methylethylenediamine. All other reagents to make the gels were the same as those used in the Davis method and the electrode solutions were identical.

The gel reagents and electrode solutions used in the preparative electrophoresis experiments were the same as those used for the analytical gels at pH 7.8. The internal diameter of the gel tube was 4.5 cm and the gel length was 5.3 cm. 2–10 mg of the protein in 2 ml of 15% sucrose was layered over the gel and electrophoresis performed at 34°C for 6 hr at 80–90 ma. After electrophoresis, the gel was sliced in 0.4-cm sections and eluted with 0.1M tris-HCl buffer at pH 7.65.

Each of the alpha-2 macroglobulin fractions obtained from preparative gel electrophoresis was further purified by repeated preparative gel electrophoresis. For these experiments, the gel length was 9 cm and the diameter 1.8 cm and electrophoresis was performed for 4 hr at 50 ma. Tryp-

sin binding and amidase activity of these fractions was determined as previously described for gel filtration eluant fractions.

Electrophoretic mobility of isolated components of alpha-2 macroglobulin. Mobility values of the different alpha-2 macroglobulins were calculated using as a reference the slowest moving component of purified alpha-2 macroglobulins. Relative electrophoretic mobility of each species was assigned as the ratio of the distance of the individual components to the distance of the slowest band. Each of the separately isolated five species of alpha-2 macroglobulins were electrophorsed in analytical acrylamide gels in duplicate, simultaneously with duplicates of whole alpha-2 macroglobulin purified by gel filtration. The electrophoresis was performed for 1½ hr at 2.5 ma per tube.

Enzyme-binding studies of alpha-2 macroglobulins

Reversibility of alpha-2 macroglobulin-trypsin complex. To determine the reversibility of the alpha-2 macroglobulin-trypsin complex, Worthington 3x crystallized bovine trypsin was labeled with [131]Iodine (^{131}I) by the method of Hunter and Greenwood (12). The ^{131}I activity was 2μCi per mg of trypsin. Unlabeled and labeled trypsin were mixed to give an activity of 100,000 cpm for 300 μg of trypsin. The labeled and unlabeled trypsin (300 μg) was incubated with 3 mg of alpha-2 macroglobulin for 15 min and the excess enzyme separated by gel filtration on Sephadex G-100. Two peaks of ^{131}I activity were found: one in the alpha-2 macroglobulin and the other in the trypsin fractions. The alpha-2 macroglobulin-trypsin-^{131}I complex was incubated with 300 μg of unlabeled trypsin for 48 hr and gel filtration performed. The amount of bound and free trypsin-^{131}I was determined. A corallary experiment to determine the displacement of cold trypsin by trypsin-^{131}I also was performed. The concentration of alpha-2 macroglobulin was determined by absorbency at 280 mμ. As alpha-2 macroglobulin appears to bind no more than 2 moles of enzyme per mole of alpha-2 macroglobulin, the contribution to absorbency by trypsin or chymotrypsin (mol wt 24,000) was negligible compared to absorbency due to alpha-2 macroglobulin (mol wt 650,000).

Trypsin-binding activity using gel filtration. Experiments to accurately determine the amount of trypsin that could be bound to alpha-2 macroglobulin were performed by incubating excess trypsin (300 μg) with 3 mg of alpha-2 macroglobulin. Separation of the complex from free trypsin was accomplished by gel filtration on a column of Sephadex G-100. The amount of trypsin bound was determined by measuring the catalysis of BAPNA hydrolysis by the material in the fractions (corrected for 35% inhibition).

Bound trypsin was also measured by labeling the trypsin with ^{131}I as described above before proceeding with the incubation with alpha-2 macroglobulin. To determine the value of micrograms of enzyme bound per millgrams of alpha-2 macroglobulin, three gel filtration experiments were performed. Two alpha-2 macroglobulin fractions were selected from each gel filtration experiment for measurement of bound enzyme. The data was recorded as the mean of these six values.

Chymotrypsin-binding activity. 300 μg of Worthington 3x crystallized chymotrypsin was incubated for 15 min with 3 mg of alpha-2 macroglobulin, free chymotrypsin was separated from the alpha-2 macroglobulin-chymotrypsin complex by gel filtration on Sephadex G-100 as described

above. The amount of bound chymotrypsin was measured by the ability of the fractions in the first peak to catalyze the hydrolysis of the chymotrypsin substrate glutaryl-1-phenylalanine p-nitroanilide as described by Ehrlanger (13). Because of the interesting and as yet inexplicable finding of a 15% increase in chymotrypsin activity on synthetic substrates when complexed with alpha-2 macroglobulin, the values were corrected for this increase in activity. Bound chymotrypsin also was measured by the ^{131}I-labeling technique.

Competition between trypsin and chymotrypsin for alpha-2 macroglobulin-binding sites. Competition between exogenously-added trypsin and chymotrypsin for sites on the alpha-2 macroglobulin were performed in the following ways: (a) 300 μg of both enzymes were added simultaneously to 3 mg of alpha-2 macroglobulin and incubated for 15 min, (b) 300 μg of one enzyme was incubated with alpha-2 macroglobulin for 15 min before incubation with 300 μg of the other enzyme, and (c) 90 μg of trypsin was incubated with 3 mg of alpha-2 macroglobulin for 15 min before incubation with 300 μg of additional trypsin. All complexes were separated from free enzymes by gel filtration on Sephadex G-100. The amount of bound trypsin was determined by ^{131}I activity or enzymatic activity corrected for the effect of alpha-2 macroglobulin on catalytic activity.

RESULTS

Isolation and characterization of alpha-2 macroglobulin

Properties of alpha-2 macroglobulin purified by gel filtration. The alpha-2 macroglobulin pool obtained from the second Bio-Gel A5m fractionation of serum showed a single Schlieren peak at a protein concentration of 4 mg/ml. Immunoelectrophoresis against goat anti-human serum showed a single arc with slight contamination by IgM. The concentrations of IgM varied depending upon the alpha-2 macroglobulin preparation from 2 to 10%. The trypsin-binding activity of the preparations averaged 55 μg of trypsin per mg of alpha-2 macroglobulin. The naturally occurring enzyme associated with the alpha-2 macroglobulin was able to catalyze the hydrolysis of BAPNA (0.33 mg/ml) at a rate which gave an absorbency change at 410 mμ of 0.075/hr per mg of alpha-2 macroglobulin. This rate is low compared with the rate when a similar amount of alpha-2 macroglobulin is saturated with trypsin.

Electrophoretic separation of alpha-2 macroglobulins. The alpha-2 macroglobulins prepared by gel filtration displayed two major bands when the Davis method was employed using a running gel of 5% acrylamide at pH 8.9. When a running gel of 5% acrylamide at pH 7.8 gel was used, five major electrophoretic species were observed (Fig. 1–1 and 2–1). Electrophoresis in 4% gel with 10% sucrose showed a similar pattern with greater separation of the bands (Fig. 2–2); however, at times, diffusion prevented sharp resolution of the five bands. The mobility of IgM was found to be considerably less
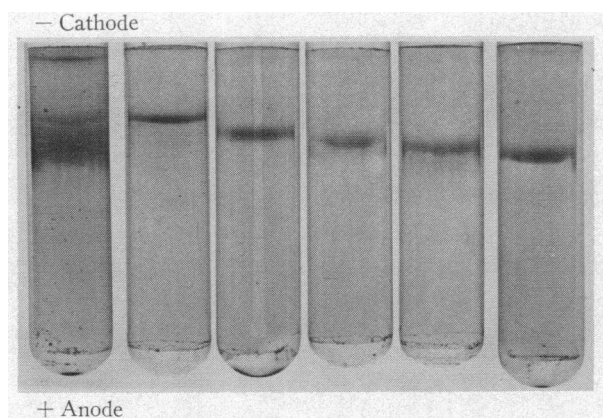
FIGURE 1 Analytical acrylamide gel electrophoresis at pH 7.8 of 1, whole alpha-2 macroglobulin and 2–6 isolated species of alpha-2 macroglobulin having different mobilities and described in Table I as gel numbers 2–6.
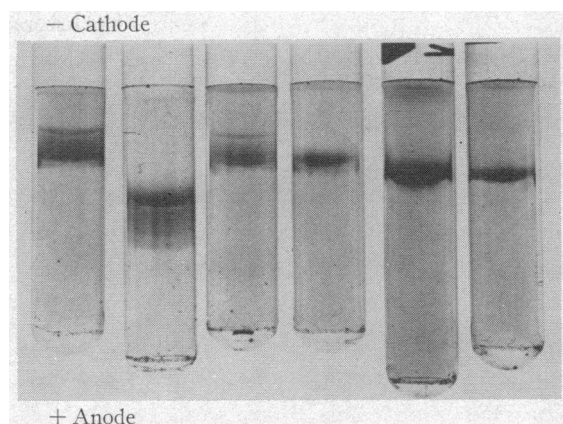

FIGURE 2 Analytical acrylamide gel electrophoresis of alpha-2 macroglobulin at pH 7.8. The gels from left to right are for (1) serum alpha-2 macroglobulin, (2) serum alpha-2 macroglobulin (a 4% gel in 10% sucrose), (3) untreated serum alpha-2 macroglobulin, (4) alpha-2 macroglobulin incubated for 15 min with 29.3 μg of trypsin per mg of alpha-2 macroglobulin, (5) alpha-2 macroglobulin incubated for 15 min with 29.3 μg of chymotrypsin per mg of alpha-2 macroglobulin, and (6) alpha-2 macroglobulin dialyzed for 12 hr against 0.2 M methylamine.

than that of alpha-2 macroglobulin and the IgM band can be observed at the top of the gels in Fig. 1–1 and Fig. 2.

Preparative acrylamide gel electrophoresis of the alpha-2 macroglobulin gave partial separation of the five species. The major components in fractions from several preparative electrophoresis experiments were purified further using a semipreparative gel, and the analytical acrylamide gel electrophoresis patterns of the purest of the isolated species are shown in Fig. 1. Table I describes the characteristics of the isolated species (gels 2 through 6) as compared with the alpha-2 macroglobulin preparation which had not been separated into compo-

nents on preparative gel (gel 1). The trypsin-binding activity was greater in those fractions with the lower mobility and fell to virtually negligible levels in the fastest fraction. The slowest moving material bound 72 μg of trypsin per mg of alpha-2 macroglobulin and the fastest moving band bound only 5 μg. The naturally occurring amidase activity was found to be similar in all five species. This value being 38.41 absorbency change/

TABLE I

Properties of Alpha-2 Macroglobulin Species Separated by Acrylamide Gel Electrophoresis

Gel number*	1	2	3	4	5	6
Relative mobility, mm	—	1.04 ±0.10	1.18 ±0.05	1.28 ±0.07	1.40 ±0.10	1.56 ±0.0
Specific trypsin-binding activity, μg trypsin/mg alpha-2 macroglobulin	53	72	68	60	40	5
Amidase activity, Absorbency change, at 410 mμ per mg alpha-2 macroglobulin	40	38	41	38	39	38
Mol wt/1000	620–680	620–680	620–680	620–680	620–680	620–680

Relative mobility was computed as the ratio of the distance of the individual species to the distance of the slowest moving band. Samples were run for 1½ hr at 2.5 ma/tube. The results are averages of four experiments or eight values. Amidase activity is presented as absorbency change at 410 mμ per mg alpha-2 macroglobulin due to hydrolysis of BAPNA (0.33 mg/ml) at pH 7.65, 25°C. Gel 1 represents alpha-2 macroglobulin purified by gel filtration, gels 2–6 represent species of alpha-2 macroglobulin isolated by repeated acrylamide gel electrophoresis.
* See Fig. 2.

hour per mg of alpha-2 macroglobulin. The average molecular weight of the pooled alpha-2 macroglobulin from the second Bio-Gel fractionation (corresponding to gel *1*) was found to be in the range of 620–680,000 at a protein concentration of 0.2–1.0 mg/ml as determined by sedimentation equilibrium untracentrifugation. The ln y₁ vs. r₁² plots for these preparations, however, displayed slight curvature at the bottom of the cell at lower speeds indicating contamination by material of a higher molecular weight. The average molecular weight of each of the five species purified by preparative gel electrophoresis was found to be in the range of 620–680,000 as determined by sedimentation equilibrium ultracentrifugation. Linear plots were obtained at speeds of 8,225–11,274 indicating the homogeneity by weight of these protein fractions, prepared by acrylamide gel electrophoresis.

Enzyme-binding studies of alpha-2 macroglobulin

Reversibility of the complex. The high irreversibility of the alpha-2 macroglobulin-trypsin complex was shown in experiments in which the unlabeled trypsin-alpha-2 macroglobulin complex was incubated with trypsin-131I for 48 hr. After fractionation by gel filtration, it was found that there was no significant exchange of labeled trypsin (0.006 ±0.001 μg/mg of alpha-2 macroglobulin). This indicated that the dissociation constant or rate of dissociation of the enzyme from alpha-2 macroglobulin-enzyme complex was minimal and the small amount of

TABLE II
Maximum Binding of Trypsin and Chymotrypsin to Alpha-2 Macroglobulin

Enzyme added to form complex	Amount of trypsin bound	Amount of chymotrypsin
μg/mg alpha-2 macroglobulin		
Trypsin-131I	90	53.6 ±2.2	—
Trypsin	90	53.0 ±1.3	—
Chymotrypsin-131I	90	—	25.5 ±1.2
Chymotrypsin	90	—	25.5 ±1.6

The complexes were formed by incubation for 15 min with enzyme and separated from free enzyme by gel filtration. Bound enzyme was determined by 131I activity or catalysis of the substrates BAPNA or GPNA. The results are average values calculated from six separate determinations. Alpha-2 macroglobulin purified by gel filtration was used in these experiments. As this preparation contains species of alpha-2 macroglobulin which bind and which also do not bind trypsin or chymotrypsin, it is clear that the amount of enzyme bound would be higher if only the former species were used (see Table I). Considering the mol wt of the slowest moving species of alpha-2 macroglobulin to be 650,000, it is clear that 2 moles of trypsin or 1 mole of chymotrypsin can be bound to a mole of the slowest species of alpha-2 macroglobulin.

TABLE III
Binding of Trypsin and Chymotrypsin When Both Are Added Simultaneously to Alpha-2 Macroglobulin

Enzymes added to form complex	Amount of trypsin bound	Amount of chymo-trypsin bound
μg/mg alpha-2 macroglobulin		
Trypsin-131I and chymotrypsin	90 and 90	41.0 ±2.8	12.6 ±2.1
Trypsin and chymotrypsin-131I	90 and 90	43.0 ±3.3	12.0 ±2.0
Trypsin and chymotrypsin	90 and 90	42.6 ±3.2	13.2 ±3.5
Chymotrypsin and trypsin	900 and 90	25.5 ±2.1	26.0 ±3.9

The complexes were formed by incubation of the alpha-2 macroglobulin for 15 min with excess trypsin and chymotrypsin and isolated by gel filtration. It is clear from these results that competitive binding occurs between trypsin and chymotrypsin for what appears to be one binding site on alpha-2 macroglobulin.

trypsin-131I bound may have been due to nonspecific absorption. This contention was supported by experiments in which a trypsin-131I-alpha-2 macroglobulin complex was formed first. Addition of unlabeled trypsin followed by gel filtration showed that any displacement of the trypsin-131I was not measureable at the concentrations used. The marked affinity of the enzymes for the alpha-2 macroglobulin and the low enzyme dissociation made the study of dissociation constants impractical but greatly facilitated the study of binding ratios by gel filtration.

Trypsin and chymotrypsin-binding ratios. Trypsin or chymotrypsin was incubated for 15 min with alpha-2 macroglobulin and separated by gel filtration from the complex. The amount of bound trypsin or chymotrypsin was determined by kinetic assay or 131I activity. The alpha-2 macroglobulin preparation from the second Bio-Gel A5m fractionation bound 53 μg of trypsin per mg of alpha-2 macroglobulin and 25 μg of chymotrypsin per mg of alpha-2 macroglobulin (Table II). However, when the enzymes were added simultaneously, each in threefold molar excess, 41 μg of trypsin and 12 μg of chymotrypsin were bound (Table III). The simultaneous addition of a 10-fold molar excess of chymotrypsin over trypsin to the alpha-2 macroglobulin gave equal binding of the two enzymes (25 μg of each enzyme per mg of alpha-2 macroglobulin) as shown in Table III. When a threefold molar excess of chymotrypsin was added first to alpha-2 macroglobulin followed in 15 min by trypsin, chymotrypsin was able to fill one site (25 μg/mg alpha-2 macroglobulin) on the alpha-2 macroglobulin (Table IV). The trypsin binding was much lower than expected as only 2–4 μg of trypsin was bound per mg of alpha-2 macroglobulin (Table IV). The binding of chymotrypsin appeared to destroy the additional trypsin-binding sites.

To determine if trypsin in half saturation amounts added to alpha-2 macroglobulin could prevent further trypsin binding, alpha-2 macroglobulin was incubated

TABLE IV

Inability to Dissociate Alpha-2 Macroglobulin-Enzyme Complex by Subsequent Addition of Either Trypsin or Chymotrypsin and Blocking of Second Trypsin Site by Chymotrypsin

Enzymes added to form complex		Second enzyme		Amount of trypsin bound	Amount of chymotrypsin bound
		µg/mg alpha-2 macroglobulin			
Trypsin	90	Chymotrypsin-131I	90	52.0 ±2.9	1.2 ±0.8
Trypsin	90	Chymotrypsin	90	51.8 ±1.5	1.8 ±1.1
Chymotrypsin	90	Trypsin-131I	90	4.5 ±1.0	25.3 ±1.3
Chymotrypsin	90	Trypsin	90	2.1 ±0.6	27.0 ±2.1

The first complex was formed by incubation of alpha-2 macroglobulin with an excess of one enzyme. This was followed by addition of an excess of the other enzyme and the free enzymes separated from the complex by gel filtration. In addition to the failures of each enzyme to displace the other when complexed to alpha-2 macroglobulin, prior addition of chymotrypsin-blocked binding at the trypsin site.

with unlabeled trypsin in amounts approximated to be equimolar to the amount of alpha-2 macroglobulin. This was followed by an excess of 131I-labeled trypsin. The results shown in Table V indicate that although the unlabeled trypsin binding was not sufficient to fill half the available sites, further trypsin-binding activity was greatly reduced and apparently the second-trypsin-binding site was destroyed or inhibited.

Enzyme-binding effect on electrophoretic mobility. When increasing amounts of trypsin were added, the four slower bands gradually disappear and assume the mobility of the fastest band. As the trypsin concentration approached 30 µg of trypsin per mg of alpha-2 macroglobulin, only the fastest species was observed indicating conversion of all bands to this species (Fig. 2-4). This also was true for chymotrypsin (Fig. 2-5). The alpha-2 macroglobulin in 0.2M methylamine also appeared as a single fast band (Fig. 2-6).

DISCUSSION

Previous studies have shown that alpha-2 macroglobulin binds enzymes such as trypsin, chymotrypsin, thrombin, and plasmin, all of which are similar in molecular weight, structure, and active centers (1). Methods previously used to isolate alpha-2 macroglobulin involve the use of ammonium sulfate which decreases the trypsin-binding activity of alpha-2 macroglobulin (14). It was for this reason that ammonium sulfate fractionation was avoided in this study and also ion exchange methods were found to be unnecessary as the preparations after gel filtration were sufficiently pure for direct acrylamide gel electrophoresis. By using small volumes of serum relative to the size of the columns, relatively pure preparations of alpha-2 macroglobulin were obtained by gel filtration.

A pool of alpha-2 macroglobulin from several preparations had the ability to bind 53 µg of exogenous trypsin per mg of alpha-2 macroglobulin. This preparation of alpha-2 macroglobulin had naturally occurring enzyme activity which is referred to as amidase activity because of its ability to catalyze the hydrolysis of BAPNA. 1 mg of purified alpha-2 macroglobulin catalyzed the hydrolysis of BAPNA at a rate which gave an 0.040 absorbency change at 410 mµ/hr. This rate is contrasted with a rate of 5.800 absorbency change at 410 mµ/hr when 1 mg of alpha-2 macroglobulin is bound to capacity with trypsin. As naturally occurring enzymatic activity is small compared with that achieved by complexing exogenous trypsin to purified alpha-2 macroglobulin, it is clear that the naturally occurring activity would not interfere appreciably with the trypsin-binding determinations. The relatively low hydrolytic activity associated with the naturally occurring enzyme for BAPNA gives no indication of absolute amounts as other synthetic or natural substrates may be more specific for the natural enzyme. The physiological function or pathological role of this naturally occurring amidase remains obscure.

The preparations from gel filtration primarily consisted of alpha-2 macroglobulin as judged by ultracentrifugation and immunological analysis and only one band was obtained when the protein was electrophoresed in starch gels, cellulose acetate, or 3½% acrylamide gels. However, in a 5% acrylamide gel, five major bands appeared. That these five species were all forms of alpha-2 macroglobulin was demonstrated by preparative acrylamide gel electrophoresis. Separation from IgM easily was accomplished and partial separation of the slow from the fast moving species of alpha-2 macroglobulin was accomplished by preparative electrophoresis.

TABLE V

Binding of Trypsin to an Alpha-2 Macroglobulin-Trypsin Complex Formed by Incubation with Equimolar Amounts of Trypsin

Enzymes added to form complex		Second enzyme		Bound unlabeled trypsin	Bound labeled trypsin
		µg/mg alpha-2 macroglobulin			
Trypsin	30	Trypsin-131I	90	22.0 ±3.1	3.3 ±1.2
Trypsin	30	Trypsin	90	21.8 ±4.2	—

The first complex was formed by incubation of alpha-2 macroglobulin with slightly less than equimilar amounts of unlabeled trypsin. This was followed by addition of excess-labeled trypsin and separation of the free enzyme from the complex by gel filtration. It is clear that the binding of one molecule of trypsin prevented the binding of a second molecule when added subsequently even though both molecules of trypsin will bind when added simultaneously.

The Separation of Alpha-2 Macroglobulin into Five Components   2381

Further resolution of the five alpha-2 macroglobulins was accomplished by repetitive preparative acrylamide gel electrophoresis. From these experiments, it was established (Table I) that the slowest moving species had the greatest trypsin-binding activity and the fastest moving species had lost the ability to bind trypsin. The three intermediate species had levels of trypsin-binding between the slowest and fastest moving components. Whether each of the intermediate species had a progressive decrease in trypsin-binding activity or whether each had trypsin-binding activity comparable to the slowest species but represented mixtures of the intermediate species and fastest component remains unanswered presently.

The high purity of the preparative acrylamide gel fractions allowed a direct molecular weight determination by a sedimentation equilibrium method, the value being between 620,000 and 680,000. This value is more acceptable than the previously published value of 820,000 in which sedimentation velocity (15) methods were used and where the required value of the diffusion coefficient may have been a source of error. The slowest moving alpha-2 macroglobulin (highest trypsin-binding activity) had trypsin-binding activity of 72 μg of trypsin per mg of alpha-2 macroglobulin. Using a a mol wt of 650,000, as established in our experiments, a molar-binding ratio of 2:1 was established.

The binding of trypsin to alpha-2 macroglobulin appears to be highly irreversible since labeled trypsin did not displace bound trypsin and vice versa. The alpha-2 macroglobulin prepared by gel filtration had a binding activity of 53 μg of trypsin per mg of alpha-2 macroglobulin (Table II). It was clear that the molar-binding ratio with this preparation was between 1:1 and 2:1. The reason for this intermediate ratio is that alpha-2 macroglobulin prepared by gel filtration contains species of alpha-2 macroglobulin which bind trypsin but also species which do not bind trypsin. When only the slowest moving species was used, 73 μg of trypsin bound to 1 mg of alpha-2 macroglobulin giving a molar-binding ratio of 2:1 (Table I). Table III indicates that the enzymes trypsin and chymotrypsin compete when added simultaneously to alpha-2 macroglobulin. Trypsin binding is reduced by 25% and chymotrypsin binding by 50%. These studies indicate that chymotrypsin binds at only one site and also that trypsin and chymotrypsin compete with approximately equal affinity at the chymotrypsin site. These hypothesis are substantiated by the experiments wherein a tenfold excess of chymotrypsin is added simultaneously with trypsin. In these experiments, chymotrypsin fills one site completely, but trypsin was still able to fill the other site (Table III). Addition of chymotrypsin to alpha-2 macroglobulin before the addition of trypsin prevents trypsin binding at the chy-

motrypsin site and chymotrypsin binding was equivalent to binding achieved in experiments where no trypsin was added (Table IV). Even more striking is the nearly complete inhibition of trypsin binding by chymotrypsin although one-half the available sites should be free. A similar phenomenon occurs when half saturation amounts of trypsin are added to alpha-2 macroglobulin (Table V). It would appear, therefore, that preincubation with chymotrypsin or equimolar amounts of trypsin causes a blocking of the trypsin site on alpha-2 macroglobulin that is time dependent. Although steric hindrance and a low dissociation rate may be suitable explanations for these results, the binding of trypsin and chymotrypsin to alpha-2 macroglobulin is likely to be more complex. A change in chemical or physical structure of alpha-2 macroglobulin probably occurs when bound to an enzyme, as the complex becomes more electronegative when bound to trypsin despite the fact that the latter substance is an electropositive molecule. We could postulate that highly electropositive portions of the alpha-2 macroglobulin could be removed or displaced by trypsin and cause a change in the mobility of the alpha-2 macroglobulin. The liberated portion would likely have a low molecular weight in relation to alpha-2 macroglobulin, as the weight of alpha-2 macroglobulin appears to be relatively unaltered when trypsin is added. A more likely explanation is that the alpha-2 macroglobulin has undergone a conformational change with enzyme binding which results in a change of charge, or shape, or both as reflected by a change in electrophoretic mobility. The change in alpha-2 macroglobulin structure, in fact, may be responsible for the high degree of irreversibility shown by the alpha-2 macroglobulin-enzyme complex.

With the binding of trypsin, the five species of alpha-2 macroglobulin were converted to a single form with a mobility similar to the fastest moving alpha-2 macroglobulin. Interestingly, complete conversion of the five bands to one occurred when the amount of trypsin was sufficient to fill only half the trypsin-binding sites on alpha-2 macroglobulin. Furthermore, alpha-2 macroglobulin which was found to bind only half as much chymotrypsin was converted to a single fast species by this enzyme. The fact that addition of equimolar amounts of trypsin or chymotrypsin converted all of the slower moving species to the fast moving speices (which do not bind trypsin) explains why this preincubation for a given period of time prevented further binding of trypsin although its maximal molar-binding ratio is 2:1 (Tables IV and V).

It was also observed that the naturally occurring amidase activity in all the five species of alpha-2 macroglobulin of varying mobility was constant. The trypsin-binding activity of a species appeared to be unrelated to

the activity of the naturally bound enzyme. The type of association of the naturally occurring amidase and alpha-2 macroglobulin can be speculated upon as follows: (a) the enzyme is present in small amounts and is bound to only some of the alpha-2 macroglobulin molecules. It was found that all five species contained similar amidase activity, regardless of mobility. As the addition of trypsin to an alpha-2 macroglobulin molecule converts it to the fast moving species, it would appear that the amidase is bound by a different mechanism and possibly at a different site from trypsin. (b) The amidase is present in greater amounts on each of the five species of alpha-2 macroglobulin than that indicated by hydrolysis of BAPNA. Were this the case, and if the binding site were similar to that for trypsin, it would be likely that a relationship between amidase activity and trypsin binding would exist. This was not the case. (c) The amidase enzyme is bound to or is a part of the alpha-2 macroglobulin in such a manner which does not interfere with trypsin binding.

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