Interaction of Human Plasmin with Human α_2-Macroglobulin†

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ABSTRACT: The steady-state kinetic parameters of plasmin and the α_2-macroglobulin (α_2M)–plasmin complex toward the chromogenic substrate Val-Leu-Lys-*p*-nitroanilide (S-2251), in the presence and absence of plasmin competitive inhibitors, have been determined. At pH 7.4 and 22 °C, the K_m values for plasmin and α_2M–plasmin for S-2251 were 0.13 ± 0.02 mM and 0.3 ± 0.03 mM. The k_{cat} of this reaction, when catalyzed by α_2M–plasmin, was 6.0 ± 0.5 s^{-1}, a value significantly decreased from the k_{cat} of 11.0 ± 1.0 s^{-1}, determined when free plasmin was the enzyme. K_I values for benzamidine of 0.50 ± 0.05 mM and 0.23 ± 0.02 mM were obtained for S-2251 hydrolysis, as catalyzed by α_2M–plasmin and plasmin, respectively. When leupeptin was the competitive inhibitor, K_I values of 5.0 ± 0.65 μM and 1.0 ± 0.1 μM were obtained when α_2M–plasmin and plasmin, respectively, were the enzymes employed for catalysis of S-2251 hydrolysis. The comparative rates of reaction of the peptide inhibitor Trasylol (Kunitz basic pancreatic inhibitor) with plasmin and α_2M–plasmin were also determined. A concentration of Trasylol of at least 3 orders of magnitude greater for α_2M–plasmin than for free plasmin was required to observe inhibition rates on comparable time scales. Two different monoclonal antibodies, 10-H-2 and 10-V-1, to the kringle 1–3 region of human plasmin(ogen) and one monoclonal antibody, 10-F-1, to the kringle 4 region of this same enzyme were employed to analyze the topography of plasmin when complexed by α_2M. It is concluded that, while slight differences exist in the accessibility or conformation of the plasmin epitopes for these antibodies in the α_2M–plasmin complex, a significant portion of plasmin heavy chain is in contact with solvent in the α_2M–plasmin complex.

Human α_2-macroglobulin (α_2M) is a glycoprotein that exists in plasma as a tetramer of identical subunits, with a total molecular weight of 725 000 (Hall & Roberts, 1978). The molecular arrangement of subunits involves the noncovalent interaction of two pairs of half-molecules, each pair containing two monomers, covalently linked by disulfide bonds (Hall & Roberts, 1978; Harpel, 1973).

Human α_2M functions as an inhibitor of endoproteases of all four catalytic classes, i.e., carboxyl-, metallo-, seryl-, and sulfhydryl-proteases [see Barrett (1981) for a complete listing]. The amount of protease bound to α_2M depends upon its size. Proteases such as trypsin and chymotrypsin are bound in a 2:1 (mol/mol, protease to α_2M) stoichiometry (Bieth et al., 1970; Pochon et al., 1981). With larger proteases, such as plasmin, the results are not as clearly documented. Ratios of plasmin to α_2M (mol/mol) ranging from 1:1 (Ganrot, 1966; Pochon et al., 1981; Howell et al., 1983) to 2:1 (Straight & McKee, 1982) have been reported.

Many of these proteases, when complexed to α_2M, retain, to various degrees, their ability to hydrolyze small substrates but show greatly decreased activity toward larger substrates [see Barrett & Starkey (1973) for a review]. The binding of these endoproteases to α_2M occurs in several stages. After complex formation, the protease is believed to catalyze cleavage of a peptide bond in α_2M at a locus called the "bait region" (Barrett & Starkey, 1973). As a consequence of this event, a conformational change occurs in α_2M (Barrett et al., 1979; Bjork & Fish, 1982; Gonias et al., 1982) that is believed to result in tight binding of the protease to α_2M, which may be covalent (Barrett & Starkey, 1973) and exposure of sites on α_2M, which allow its uptake and degradation by macrophages (Debanne et al., 1975; Ohlsson, 1971). It has been proposed that proteases are physically entrapped within α_2M, a theory that provides a steric basis for the loss of ability of protease to react with large substrates and for the loss of protease reactivity with its antibodies (Katayama & Fujita, 1974; Mullertz, 1974; Geokas et al., 1977). However, some recent evidence, on the basis of electron microscopy of α_2M and its complexes with trypsin (Schramn & Schramn, 1982) and on the basis of the finding that proteases appear to be displaced from their complex with α_2M (Wang et al., 1983), argues against the necessity of invoking a molecular trap hypothesis. Also associated with proteolytic cleavage of the peptide bond in the bait region is the exposure of four sulfhydryl residues in α_2M, which appear to result from hydrolysis of labile thiol ester bonds in each of the monomer units (Sottrup-Jensen et al., 1980). The γ-carboxyl group of an activated glutamyl residue is also released as a result of this hydrolysis, which in turn covalently interacts with ε-amino groups of lysyl residues in the bound protease, leading to a covalent complex of protein components (Harpel & Rosenberg, 1976; Salvesen & Barrett, 1980).

These thiol ester bonds in α_2M are also cleaved by certain primary amines such as methylamine (Sottrup-Jensen et al., 1980; Swensen & Howard, 1979); a process that also leads to inactivation of this inhibitor (Steinbuch et al., 1968). This activity loss is believed to be a consequence of a primary amine induced conformational alteration in α_2M, similar to the change resulting from its interaction with proteases (Bjork & Fish, 1982; Gonias et al., 1972; Straight & McKee, 1982). Once the conformation of α_2M is perturbed by primary amines, the protein no longer retains the ability to interact with proteases.

Due to the in vivo importance of the interaction of human plasmin with α_2M (Harpel, 1981), we have decided to compare the kinetic properties of free plasmin with that of plasmin complexed to α_2M, in an attempt to probe important alterations in the plasmin active site in the inhibitor complex. Additionally, in an attempt to define the specific interactions of importance to complex formation and the topography of proteases within the complex, we have employed specific monoclonal antibodies to various regions of human plasmin in order to evaluate the regions of plasmin accessible to in-

† From the Department of Chemistry, University of Notre Dame, Notre Dame, Indiana 46556. *Received April 27, 1983.* This work was supported by Grants HL-13423 and HL-19982 from the National Institutes of Health. H.S.C. is the Paul A. Nicoll Fellow of the American Heart Association, Indiana Affiliate.

0006-2960/84/0423-0105$01.50/0

teraction with solvent molecules. The results of these investigations are presented in this paper.

Materials and Methods

Proteins. Human α_2M was prepared from fresh human plasma by modifications of existing methodology (Barrett, 1981). Trasylol (FBA Pharmaceuticals) was added to plasma to a level of 100 KIU/250 mL. The resulting solution was percolated over a column of Sepharose 4B–L-lysine to remove plasminogen (vide infra). The plasminogen-free plasma was adjusted to 5% in poly(ethylene glycol) (PEG), M_r 6000, by addition of an appropriate amount of a solution of 25% (w/v) PEG in H_2O. The solution was allowed to stand at room temperature for 30 min, after which time the precipitate was removed by centrifugation and discarded. Additional PEG was added to the resulting supernate until a final concentration of 12.5% was achieved. After 1 h at room temperature, the mixture was subjected to centrifugation and the supernate discarded. The precipitate was dissolved in a minimal volume of a buffer, consisting of 0.02 M phosphate/0.1 M NaCl, pH 7.4 (PBS). A 20-mL aliquot of this solution was chromatographed on a column (3.5 cm × 100 cm) of LKB Ultrogel AcA 22, equilibrated with PBS. Materials were eluted in this same buffer at a flow rate of 20 mL/h. The fractions containing α_2M were identified by sodium dodecyl sulfate gel electrophoresis and assay of their protective effect on hydrolysis of Bz-Arg-pNA by trypsin, in the presence of soybean trypsin inhibitor (Ganrot, 1966). The final pooled material was dialyzed against H_2O and any precipitate discarded. Approximately 95% of the total protein appeared as a single high molecular weight band in nonreduced sodium dodecyl sulfate gels. Upon reduction of the samples, again, approximately 95% of the protein existed as a single band of the expected size of the α_2M monomer.

Human plasminogen was eluted from the Sepharose–lysine column (see above) by a gradient of ϵ-aminocaproic acid, as previously described (Brockway & Castellino, 1972). For all studies reported herein, plasminogen variant 1 was employed.

Human plasmin was prepared by activation of human plasminogen with urokinase (UK). A solution of plasminogen at a concentration of 1–2 mg/mL, in 0.1 M phosphate/25% (v/v) glycerol, pH 8.0, was incubated with UK, as described by Morris et al. (1981). The procedure was modified such that incubation times of 6 h at room temperature and 18 h at 4 °C were allowed. The resulting solution was then diluted 2-fold and passed over a small column contining 1 mL of Sepharose–lysine. After being washed extensively with a buffer consisting of 0.1 M phosphate, pH 8.0, to remove UK, plasmin was eluted by adjusting the above buffer to 0.012 M in ϵ-aminocaproic acid. The concentration of active plasmin was determined by the *p*-nitrophenyl *p*-guanidinobenzoate burst assay of Chase & Shaw (1967).

Monoclonal antibodies, 10-V-1 and 10-F-1, to human plasminogen were prepared and characterized, as previously described (Ploplis et al., 1982). Antibody 10-H-2 was obtained from another clone of the same fusion.

Soybean trypsin inhibitor was purchased from Sigma Chemical Co. Urokinase was a gift of Dr. William H. Holleman of Abbott Laboratories.

When the binary α_2M–plasmin complex was required, the two proteins were combined in a 1:3–4 (α_2M to plasmin, mol/mol) ratio in PBS. Excess plasmin was removed by chromatography on Ultrogel AcA 22, as described above, with a 1.5 cm × 50 cm column. Unbound α_2M was separated from α_2M–plasmin by adsorption of the complex to Sepharose–lysine, essentially as described in the method for plasminogen purification (Brockway & Castellino, 1972). The α_2M–plasmin complex was eluted from the column upon addition of 0.012 M ϵ-aminocaproic acid to the column buffer.

Steady-State Kinetics of Plasmin and α_2M–Plasmin with the Substrate Val-Leu-Lys-p-nitroanilide (S-2251). The enzymatic activity of plasmin and the α_2M–plasmin complex toward S-2251 was employed to characterize their steady-state parameters. All experiments were performed at 22 °C with all components dissolved in a buffer consisting of 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/0.1 M NaCl, pH 7.4. For determination of the K_m and V_{max} for plasmin, each assay contained measured volumes of titrated enzyme, buffer, and substrate, such that the total volume was 0.8 mL. The substrate concentration range was 0.051–0.222 mM. Hydrolysis of S-2251 was monitored by absorption of liberated *p*-nitroanalide at 405 nm. K_I values for benzamidine hydrochloride and leupeptin (Ac-Leu-Leu-Arg-CHO) were obtained from steady-state initial rate inhibition studies in the usual fashion. In the case of the α_2M–plasmin complex, assays were performed in the same manner, except that soybean trypsin inhibitor was present in each assay, at a 2-fold molar excess over titratable plasmin, in order that any plasmin, not in complex with α_2M, would be inhibited (Ganrot, 1966). A substrate range of 0.1–1.5 mM was employed.

The rate of inhibition of plasmin and α_2M–plasmin by Trasylol was determined by examination of the loss of activity of each enzyme toward S-2251. A solution of titrated plasmin at a final concentration of 24 nM was incubated with Trasylol at a final concentration of 48 nM at room temperature. Aliquots of the mixture were removed at various time intervals and added to a cuvette containing 0.21 mM S-2251. The hydrolysis rate of the substrate was monitored at 405 nm. In the case of the α_2M–plasmin complex, inhibition rates by Trasylol toward S-2251 hydrolysis were obtained at an α_2M–plasmin concentration of 18.5 mM, an S-2251 concentration of 0.21 mM, and a range of Trasylol concentrations of 6.7–67.2 μM.

Steady-state kinetic constants for S-2251 hydrolysis were obtained for plasmin–monoclonal antibody complexes and α_2M–plasmin–monoclonal antibody complexes. These experiments were performed as above, except that a 2-fold molar excess of the desired antibody was added to the enzyme, and allowed to incubate for 1 min prior to assay.

Rate of Incorporation of Plasmin into α_2M in the Presence and Absence of Monoclonal Antibodies. The rates of incorporation of plasmin and a plasmin–monoclonal antibody complex into α_2M were compared by using the substrate S-2251 to measure the bound plasmin. All experiments were performed at room temperature with all components dissolved in a buffer of 0.05 M Hepes/0.1 M NaCl, pH 7.4. Plasmin (final concentration 0.235 μM) was incubated with a 2-fold molar excess of α_2M. At desired time intervals (5 s–5 min), a 2-fold molar excess of soybean trypsin inhibitor over plasmin was added to selectively quench the plasmin not complexed to α_2M (Ganrot, 1966). The amount of plasmin bound to α_2M was determined by evaluation of the rate of hydrolysis of 0.52 mM S-2251. With knowledge of this latter initial reaction rate, the S-2251 concentration, and the K_m and V_{max} that characterize the hydrolysis of S-2251 by α_2M–plasmin, the amount of plasmin incorporated was calculated at each time interval. For similar experiments in the presence of various monoclonal antibodies, a 2–10-fold molar excess of antibody was added to plasmin and allowed to incubate for 1 min prior to addition of α_2M.

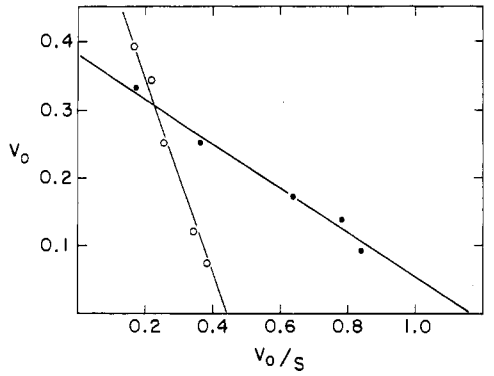

FIGURE 1: Steady-state amidase activity of plasmin (○) and α_2M–plasmin (●) toward Val-Leu-Lys-*p*-nitroanilide (S-2251). The initial velocities (V_0), obtained at pH 7.4 and 22 °C, are expressed as μmol of S-2251 hydrolyzed min^{-1} (nmol of plasmin)$^{-1}$.

Binding of Monoclonal Antibodies to α_2M–Plasmin Complex. A volume of 0.2 mL of goat anti-human α_2M (Miles), at a concentration of 15 μg/mL in a borate/saline buffer, pH 8.3, was added to separate wells of a 96-well poly(vinyl chloride) microtiter plate (Costar). After being allowed to stand for 1.5 h at room temperature, the supernate was removed, and the wells were washed 3 times with PBS. A total of 0.2 mL of a solution of the purified α_2M–plasmin complex (160 μg/mL) was added to each well and allowed to incubate for 1.5 h. After each well was washed 3 times with PBS, 0.2 mL of the desired monoclonal antibody solution, in PBS, at a concentration range of 0.025–0.5 μg/mL was incubated in the wells for 1.5 h. The wells were washed 3 times with PBS. Subsequent to this, excess molar quantities of rabbit anti-mouse IgG conjugated to alkaline phosphatase were added and allowed to incubate for 1.5 h, and the plates were washed as above. The presence of monoclonal antibody bound to the α_2M–plasmin complex was monitored by the hydrolysis of *p*-nitrophenyl phosphate, when added to the wells (Ploplis et al., 1982).

Iodination of Antibodies. The antibodies, 10-F-1, 10-H-2, and 10-V-1, were radiolabeled with ^{125}I with the Bolton–Hunter reagent (Bolton & Hunter, 1973) with a ratio of 1 mCi of ^{125}I/mg of protein. The ^{125}I-labeled antibody was separated from excess reagents by chromatography on a 1 cm × 50 cm column of Sephadex G-25, equilibrated and eluted with 0.05 M Hepes/0.1 M NaCl, pH 7.4. The final specific radioactivity varied between 100 and 150 cpm/ng of protein.

Sucrose Density Ultracentrifugation. The technique was employed in an attempt to confirm the formation of soluble α_2M–plasmin–antibody complexes. The components were combined at similar concentrations and in the same order and time intervals as were used in the kinetic studies. Stock solutions of plasmin, α_2M, and antibody in 0.05 M Hepes/0.1 M NaCl, pH 7.4, were prepared. A 2:1 (mol/mol) ratio of antibody to plasmin was incubated at room temperature for 1 min, followed by addition of a 2-fold molar excess of α_2M (over plasmin) for 5 min. The final concentrations of components were as follows: plasmin, 0.4 μM; antibody, 0.8 μM; α_2M, 0.8 μM. A volume of 100 μL of this solution was layered on 5 mL of a linear gradient of 5–20% sucrose in 0.05 M Hepes/0.1 M NaCl, pH 7.4. Centrifugation was performed at 4 °C in a Beckman SW 50.1 swinging-bucket rotor with a Beckman L5-65 ultracentrifuge at 38 000 rpm for 6 h. Simultaneously, other tubes containing ^{125}I-labeled antibody, alone, and plasmin + ^{125}I-labeled antibody, at the above concentrations, were subjected to the same procedure. At the conclusion of the centrifuge run, each tube was punctured at the bottom, and a 1-drop fraction was collected and analyzed for radioactivity in a Beckman Model 4000 gamma counter.

Table I: Steady-State Kinetic Parameters for Plasmin and α_2M-Plasmin toward Val-Leu-Lys-*p*-nitroanilide

enzyme	K_m (mM)	k_{cat} (s^{-1})
plasmin	0.13 ± 0.02	11.0 ± 1.0
α_2M-plasmin	0.30 ± 0.03	6.0 ± 0.5
α_2M-plasmin-10-F-1[a]	0.24 ± 0.03	6.4 ± 0.5

[a] Refers to the ternary complex of α_2-macroglobulin-plasmin-antibody (10-F-1).

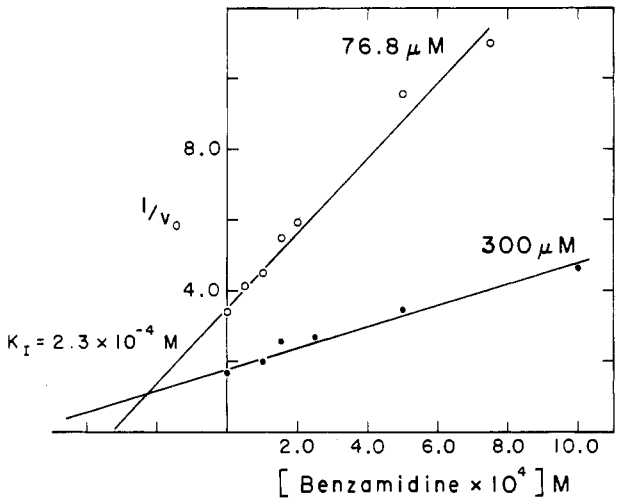

FIGURE 2: Inhibition by benzamidine hydrochloride of initial velocity (V_0) of S-2251 hydrolysis by plasmin, at pH 7.4 and 22 °C. The units of V_0 are expressed as in Figure 1. The V_0's for 76.8 (○) and 300 μM (●) S-2251, at varying levels of benzamidine, were obtained. The value of $-K_I$ for the inhibitor is equal to the point of intersection of the two lines. The concentration of plasmin employed was 24.9 nM.

Results

Figure 1 provides illustrations of Eadie–Hofstee plots of the effect of the concentration of S-2251 on its initial rate of hydrolysis by both plasmin and α_2M–plasmin. In the case of plasmin, the active site concentration was determined by titration with *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967). In order to determine the concentration of plasmin active sites in the α_2M–plasmin complex, a 2-fold molar excess of α_2M was added to a titrated level of plasmin and the amount of bound plasmin determined by assay with S-2251, after quenching the free plasmin with soybean trypsin inhibitor (Ganrot, 1966). From the data of Figure 1, a K_m of 0.13 ± 0.02 mM and a k_{cat} of 11.0 ± 1.0 s^{-1} can be calculated as characteristic of the reaction of S-2251 with plasmin, at 22 °C and pH 7.4. Similar calculations for the enzyme, α_2M–plasmin, lead to values of 0.30 ± 0.02 mM and 6.0 ± 0.5 s^{-1} for the K_m and V_{max}, respectively. These values are listed in Table I. By utilization of this latter data, the amount of plasmin bound to α_2M was calculated and ranged from 0.7 to 1.1 mol/mol in several different preparations.

The K_I for the plasmin inhibitor, benzamidine hydrochloride toward the α_2M–plasmin–S-2251 reaction was determined by analyzing the rates of hydrolysis of two different S-2251 concentrations at varying levels of benzamidine. The results are present in Figure 2 for plasmin and Figure 3 for α_2M–plasmin. In each case, benzamidine was found to be a competitive inhibitor of the enzyme, with K_I values of 0.23 ± 0.02 mM for plasmin and 0.50 ± 0.05 mM for α_2M–plasmin.

Similar experiments, aimed at evaluating the K_I for the plasmin inhibitor leupeptin (Ac-Leu-Leu-Arg-CHO), for both

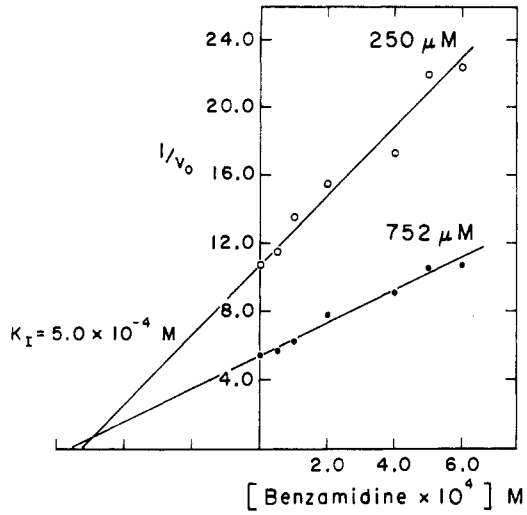

FIGURE 3: As in Figure 2, except that 12.4 nM plasmin, in the α_2M–plasmin complex, was employed with S-2251 concentrations of 250 (○) and 752 μM (●).

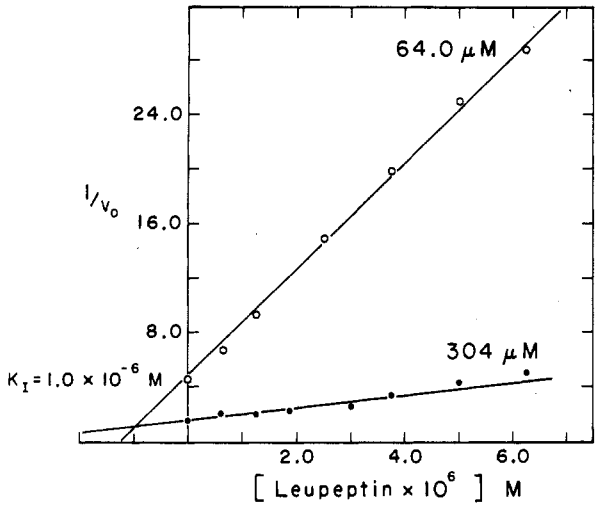

FIGURE 4: Inhibition by leupeptin of initial velocity (V_0) of S-2251 hydrolysis by plasmin at pH 7.4 and 22 °C. The units of V_0 are as expressed in Figure 1. The V_0's for 64.0 (○) and 304 μM (●) S-2251, at varying levels of leupeptin, were obtained. The concentration of plasmin was 24.1 nM. The value for K_I for leupeptin was calculated as in Figure 2.

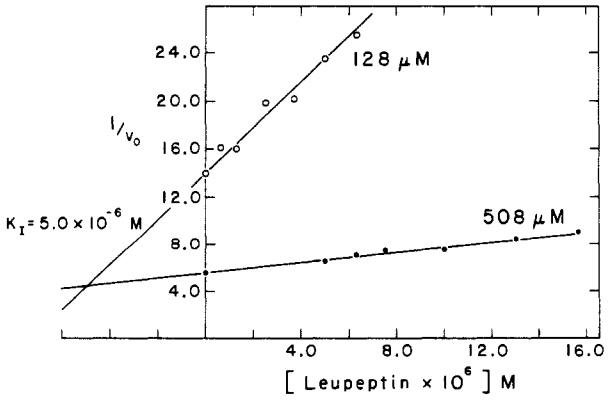

FIGURE 5: As in Figure 4, except that 12.0 nM plasmin, in the α_2M–plasmin complex, was employed with S-2251 concentrations of 128 (○) and 508 μM (●).

plasmin and α_2M–plasmin, with S-2251 as the substrate are shown in Figures 4 and 5, respectively. Again, leupeptin was found to be a competitive inhibitor for both enzymes, characterized by a K_I of 1.0 ± 0.1 μM for plasmin and 5.0 ± 0.6 μM for α_2M–plasmin. All K_I values obtained are summarized in Table II.

Table II: K_I Values for Plasmin, α_2M-Plasmin, Plasmin-Antibody Complexes, and α_2M-Plasmin-Antibody Complexes toward Plasmin Inhibitors

enzyme	K_I benzamidine (mM)	K_I leupeptin (μM)
plasmin	0.23 ± 0.02	1.0 ± 0.1
α_2M-plasmin	0.50 ± 0.05	5.0 ± 0.6
plasmin-10-F-1	0.15 ± 0.02	1.0 ± 0.1
α_2M-plasmin-10-F-1	0.48 ± 0.04	4.8 ± 0.5
plasmin-10-H-2	0.20 ± 0.02	1.4 ± 0.1
α_2M-plasmin-10-H-2	0.40 ± 0.02	5.6 ± 0.5
plasmin-10-V-1	0.19 ± 0.02	1.2 ± 0.1
α_2M-plasmin-10-V-1	0.48 ± 0.02	5.6 ± 0.5

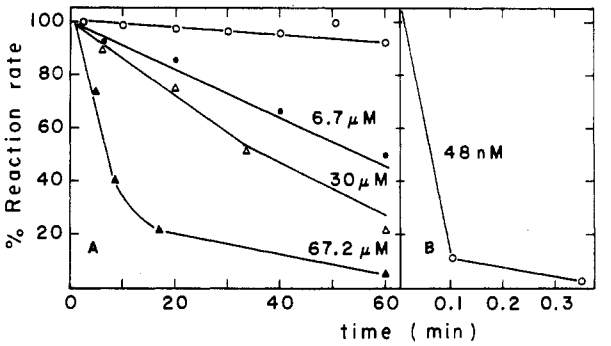

FIGURE 6: Inhibition by Trasylol of S-2251 amidase activity of plasmin and α_2M–plasmin, at pH 7.4 and 22 °C. The percent of the initial reaction rate remaining as a function of time of incubation of Trasylol and the enzyme was determined. (A) The enzyme was α_2M–plasmin at a concentration of 18.5 nM. Trasylol concentrations of (○) none, (●) 6.7 μM, (△) 30 μM, and (▲) 67.2 μM were employed. The S-2251 concentration for all assays was 210 μM. (B) The enzyme was plasmin at a concentration of 24.0 nM. The Trasylol concentration was 48.0 nM, and the S-2251 concentration was 210 μM.

A series of experiments were designed to determine the accessibility of the plasmin active site in the α_2M–plasmin complex toward a progressively larger inhibitor, Kunitz basic pancreatic inhibitor (Trasylol). Figure 6 illustrates data obtained by measurement of the initial rate of 0.21 mM S-2251, as a function of time, in the presence of various levels of Trasylol. As can be seen from Figure 6B, in the presence of a 2-fold molar excess of Trasylol, plasmin was essentially completely refractory toward S-2251 hydrolysis at the first time point (6 s) that could be measured. On the other hand, the data of Figure 6A show that much larger concentrations of Trasylol are necessary to inhibit plasmin when bound to α_2M. At a concentration of 67.2 μM Trasylol, approximately 8 min is required to produce 50% inhibition of the initial rate of S-2251 hydrolysis by α_2M–plasmin.

We currently have at our disposal three monoclonal antibodies to different epitopes on the heavy chain of human plasmin and felt that these could be productively employed to evaluate the accessibility of this portion of plasmin in the complex for solvent molecules, a study that bears upon the question of whether the protease is completely entrapped within α_2M. Initially, we designed experiments to determine the effect of each antibody on the rate of reaction of plasmin with α_2M. Here, at various times of reactions of α_2M and plasmin, in the presence of antibody, free plasmin was quenched by addition of soybean trypsin inhibitor, and plasmin (or plasmin–antibody complexes) bound to α_2M was measured by hydrolysis of S-2251. Control experiments demonstrated that both plasmin and the plasmin–antibody complex were similarly

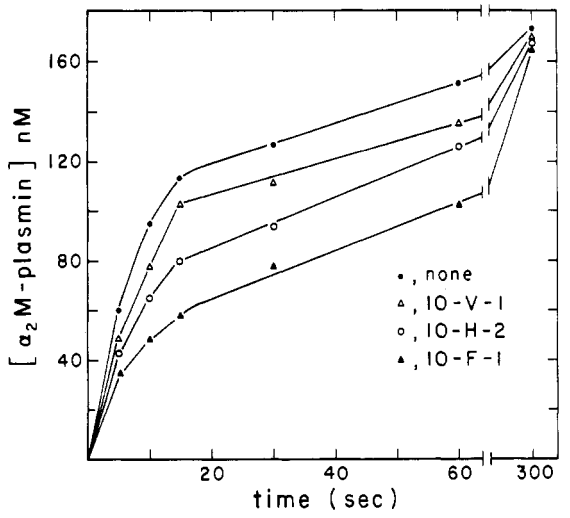

FIGURE 7: Rate of incorporation of plasmin into α_2M, in the absence and presence of monoclonal antibodies. Human plasmin, at a final concentration of 235 nM, was mixed with the antibody of interest, the latter at a final concentration of 470 nM. This solution was added to α_2M (final concentration 470 nM). Aliquots were removed as a function of time, and the free antibody–plasmin complex was neutralized with a 2-fold excess of soybean trypsin inhibitor. The concentration of α_2M–plasmin was determined from the initial velocity of its catalysis of 0.52 mM S-2251, with knowledge of the K_m and V_{max} for this substrate. The antibodies employed were (●) none, (△) 10-V-1, (○) 10-H-2, and (▲) 10-F-1.

inhibited by soybean trypsin inhibitor. The results of the experiment are presented in Figure 7. Compared to the reaction rate obtained in the absence of antibodies, the rate of plasmin incorporation into α_2M is progressively decreased upon addition of 10-V-1 > 10-H-2 > 10-F-1. In all cases, an equal amount of plasmin was incorporated into α_2M at the conclusion of the reaction.

The data of Figure 7 do not allow a firm conclusion to be reached regarding the ability of the plasmin–antibody complex to be incorporated into α_2M, since the antibody may dissociate from plasmin prior to its incorporation. We have addressed this point in several different fashions. Kinetic studies have shown that preincubation of a 2–10-fold molar excess of each of the antibodies to plasmin, prior to reaction with α_2M, does not lead to progressive differences in the rate or extent of reaction with α_2M over that antibody concentration range. In all cases, the data of Figure 7 are obtained. This observation suggests that the plasmin–antibody complex is incorporated into α_2M and that prior dissociation of the complex need not occur. In addition, we have examined the ability of various monoclonal antibodies to bind to performed α_2M–plasmin complex by two methods. By employment of a plate binding assay, individual wells were coated with anti-α_2M, followed by adsorption of the complex. Various levels of monoclonal antibody were then added. The bound monoclonal antibody was then labeled with an excess of anti-mouse IgG, which was covalently coupled with alkaline phosphatase. The amount of alkaline phosphatase present was evaluated by its initial rate of hydrolysis of *p*-nitrophenyl phosphate. These latter rates, as a function of monoclonal antibody added, are plotted in Figure 8. Control experiments, performed as above, with α_2M adsorbed to anti-α_2M-coated wells were employed to determine nonspecific monoclonal antibody binding. Clearly, the epitopes on plasmin for the particular antibodies employed herein are exposed on plasmin to varying degrees. The 10-V-1 epitope on plasmin appears to be best retained by plasmin in the α_2M–plasmin complex, followed in order by the 10-H-2 and 10-F-1 epitopes.

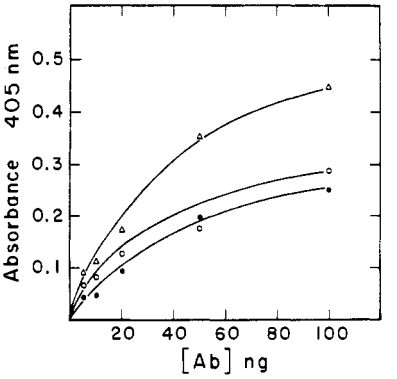

FIGURE 8: Ability of various monoclonal antibodies to react with preformed α_2M–plasmin complex. The assay, which is described under Materials and Methods, consists of determination of the amount of monoclonal antibody (IgG) bound to the anti-α_2M–α_2M–plasmin complex by analysis of the initial rate of *p*-nitrophenyl phosphate hydrolyzed (absorbance 405 nm) by alkaline phosphatase covalently attached to anti-mouse IgG, which in turn is adsorbed to the anti-α_2M–α_2M–plasmin–monoclonal antibody complexes. The monoclonal antibodies employed were (△) 10-H-2, (○) 10-V-1, and (●) 10-F-1.

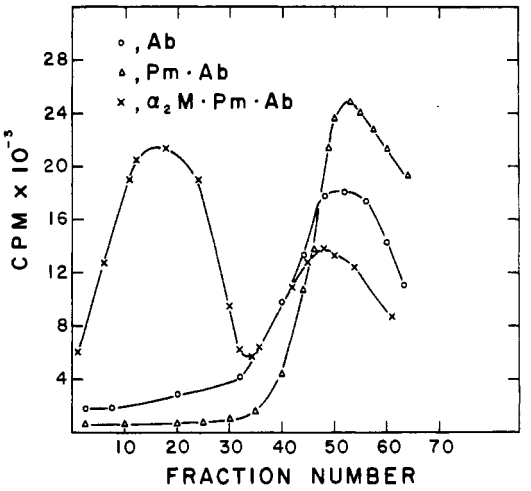

FIGURE 9: Sucrose density gradient analysis of formation of an α_2M–plasmin–antibody complex. (○) [^{125}I]-10-H-2, (△) [^{125}I]-10-H-2–plasmin, and (×) [^{125}I]-10-H-2–plasmin–α_2M were simultaneously subjected to sedimentation analysis in separate tubes at 38 000 rpm for 6 h at 4 °C. Fraction 1 represents the tube bottom. The samples were prepared as described under Materials and Methods.

In order to confirm the existence of a ternary α_2M–plasmin–antibody complex for each antibody under the conditions of enzymatic assays, we have performed sucrose density gradient analysis with ^{125}I-labeled antibodies. The data presented in Figure 9, shown with antibody 10-V-1, are typical of the results with all antibodies. Here, compared to the sedimentation position at a fixed time of ^{125}I-labeled antibody and ^{125}I-labeled antibody–plasmin, alone, there is a large increase in the migration of labeled antibody when the plasmin–^{125}I-labeled antibody complex is added to α_2M, strongly suggesting the presence of an α_2M–plasmin–^{125}I-labeled antibody complex. The data in this figure do not show a large separation between plasmin and ^{125}I-labeled antibody–plasmin. However, this is due to the experimental conditions chosen. The short sedimentation times and low rotor speeds employed were not designed to allow extensive migration of the antibody or the antibody–plasmin complex, thereby affecting their resolution. The conditions chosen were designed to allow α_2M resolution from antibody and plasmin–antibody complexes, without migration of α_2M to the cell bottom. Thus, the conditions of Figure 9 were chosen to clearly show whether antibody was bound to the α_2M–plasmin complex.

Finally, we wished to examine the integrity of the plasmin active site in the ternary α_2M–plasmin–monoclonal antibody complex toward plasmin substrates and inhibitors. These experiments were conducted similarly to those presented in Figures 2–6, and the results are listed in Tables I and II. The K_m and k_{cat} values for the ternary α_2M–plasmin–10-F-1 complex toward S-2251 were not significantly altered from those found when the α_2M–plasmin complex was used as the enzyme. Also, it can be seen that none of the antibodies on plasmin or α_2M–plasmin significantly affected the K_I values obtained in the absence of antibody, for benzamidine and leupeptin. These results suggest that antibody is not present in a location in the α_2M–plasmin complex that affects diffusion of substrate and inhibitors into the active site. Further, in agreement with this view, when Trasylol is employed as the inhibitor in the α_2M–plasmin–antibody complex, the rate of its incorporation into the plasmin active site is virtually identical with its rate of incorporation into α_2M–plasmin, in the absence of antibody.

Discussion

The results presented in this paper confirm and extend earlier conclusions that proteases that are bound to α_2M are altered in their catalytic abilities toward hydrolysis of various substrates. It is known that the trypsin-catalyzed hydrolytic rates of small substrates are decreased when trypsin is bound to α_2M. This is expressed by changes in K_m, V_{max}, pH optimum, and effect of ionic strength on the activity of trypsin in the α_2M–trypsin complex, compared to those of trypsin alone (Rinderknecht et al., 1975). Catalytic rates of proteases bound to α_2M are more dramatically reduced when large protein substrates are considered. A greatly decreased caseinolytic activity of α_2M–trypsin is observed, compared to that of trypsin alone (Rinderknecht et al., 1975). In a study that is of particular importance to blood coagulation and fibrinolytic phenomena, it has been found that the fibrinogenolytic activities of the α_2M–trypsin (Rinderknecht et al., 1975), α_2M–thrombin (Switzer et al., 1983), and α_2M–plasmin (Harpel & Mosesson, 1973) complexes are reduced to approximately 1–5% of the activity of the protease in the absence of α_2M. The nature of the substrate, exclusive of its size, appears to also play a role in its ability to be hydrolyzed by α_2M–protease complexes. For example, the α_2M–thrombin complex possesses 50% of the activity of free thrombin toward activation of a large substrate, factor VIII (Switzer et al., 1983).

While the rapid uptake of α_2M–protease complexes by various cells may be a mechanism for clearance of such enzymes, the low level of enzymatic activity retained by the proteases in these same complexes may play a regulatory role in preserving their function for limited time periods against rapid inactivation by other plasma protease inhibitors. Therefore, we felt it important to evaluate the integrity of the active site of the protease when bound to α_2M. Detailed kinetic analyses of α_2M–protease complexes have not been extensively reported in the literature. Due to our long-standing interest in plasmin function, we have undertaken a study of its activity and topography in the α_2M complex.

With regard to the tripeptide substrate S-2251, the lower enzymatic activity expressed by the α_2M–plasmin complex is a result of both 2.3-fold increase in the K_m and a k_{cat} that is approximately 0.55 of that of plasmin. This indicates that the plasmin active site in the α_2M–plasmin complex is different in its catalytic ability to that of plasmin alone. Whether the magnitude of this difference is sufficiently sensitive to the size of the low molecular weight substrate employed can best be addressed by examination of the binding of various competitive inhibitors of the substrate. When benzamidine hydrochloride was employed as the inhibitor, a 2-fold weaker binding to α_2M–plasmin occurred, compared to plasmin alone (Figures 2 and 3, Table II). When a tripeptide competitive inhibitor, leupeptin, was employed in the comparative study (Figures 4 and 5 and Table II), approximately 5-fold weaker binding to the α_2M–plasmin complex occurred, compared to that of plasmin. Therefore, the altered site of plasmin in the complex more effectively binds small, rather than large, substrates. The ability of even larger inhibitors to react with plasmin and α_2M–plasmin was assessed by evaluation of rate of interaction of a large peptide inhibitor (Trasylol) with each enzyme (Figure 6). At merely a 2-fold molar excess of Trasylol to plasmin, the enzyme was virtually completely inhibited as quickly as the measurement could be made without employing rapid-time techniques. However, easily measurable inhibition rates were obtained at approximately a 3600-fold molar excess of Trasylol to the α_2M–plasmin complex. Whether this indicated a slow diffusion into the active site in the complex followed by a rapid inhibition of the active site or whether the diffusion was rapid and the reactivity with the altered active site was slow cannot be definitely stated. Although authors of several previous published reports have concluded that reduced rates of large substrate hydrolysis by α_2M–protease complexes were due to their reduced abilities to diffuse into the putative molecular trap in α_2M–protease complexes, other interpretations are possible, since, at least in the case of α_2M–plasmin, the enzyme also appears to bind large inhibitors (and, perhaps, substrates) less efficiently than smaller such compounds.

A topographical study of plasmin in the α_2M complex, in regard to the exposure of certain regions of plasmin to solvent probes, was performed by employing monoclonal antibodies, specific for various loci on plasmin. Three antibodies were employed, two of which recognized different epitopes on the kringle 1–3 region of plasmin(ogen) (residues Tyr_{79}-Val_{337}/Val_{353}) and another which recognized an epitope on the kringle 4 (Val_{354}-Ala_{439}) region (Ploplis et al., 1982; H. S. Cummings et al., unpublished results). None of those epitopes are in linear proximity to the active site serine (residue 740).

The data of Figure 7 show that the rate of incorporation of plasmin into α_2M is influenced by these antibodies. A slight inhibition is seen with the antibody 10-V-1, and progressively increasing inhibition is observed with antibodies 10-H-2 and 10-F-1. An approximate 4-fold increase in the time required for incorporation of plasmin in the presence of antibody 10-F-1, compared to that of plasmin alone, is seen. With time, all plasmin–antibody complexes, as well as free plasmin, reached the same level of incorporation, showing that antibodies to the plasmin heavy chain do not preclude complex formation.

We next designed experiments to attempt to probe the accessibility of various regions of plasmin to solvent, when plasmin is incorporated into α_2M. Here, after adsorption of preformed α_2M–plasmin to insolubilized α_2M antibodies, various levels of monoclonal antibodies were added, and the quantity of the latter adsorbed to the complex was evaluated by an enzyme-linked immunosorbant assay. The results are shown in Figure 8. The amount of total antibody required to produce 50% binding decreases through the series 10-F-1 > 10-H-2 > 10-V-1. These data, when correlated to the time study of Figure 7, suggest the following: (1) the 10-V-1 epitope of kringle 1–3 does not significantly affect movement of plasmin into the bait region of α_2M, and when plasmin alone is bound, the epitope is exposed; (2) the 10-H-2 epitope on

kringle 1-3 has an inhibitory effect on movement of plasmin into the bait region of α_2M but yet has its epitope exposed in the α_2M-plasmin complex to some degree; (3) the 10-F-1 epitope on kringle 4 is positioned such that it hinders to the greatest extent the movement of plasmin into the α_2M bait region and is more shielded when the α_2M-plasmin complex is formed. It is, of course, possible that the differing reactivities of plasmin antibodies with α_2M-plasmin result from minor localized conformational changes and not steric effects, in the various regions in plasmin, when incorporated into α_2M. It is not possible at this juncture to evaluate whether differing epitope shielding or differing conformational alterations in the plasmin antibody epitopes are responsible for the variations in the reactivity of the antibodies with α_2M-plasmin. However, it definitely appears as though the ternary α_2M-plasmin-antibody complexes are formed.

The fact that antibodies toward a region of human plasmin do react with this protease in the α_2M-plasmin complex raises some question as to the nature of a molecular trap, if it is assumed that this trap is encapsulating the entire protease. The main reasons for invoking a total-trap hypothesis revolve around the limited reactivity of large substrates with proteases bound to α_2M, the conformational change in α_2M accompanying its interaction with proteases, and the lack of binding of antibodies to proteases bound to α_2M (Barrett & Starkey, 1973). Our work, presented in this paper, addresses two of these points. Regarding the reactivity of bound proteases with large substrates, alterations in active site specificity, rather than accessibility, may be sufficient to explain this effect, at least in regard to human plasmin. With respect to past studies of the lack of antibody interaction with α_2M-bound proteases, it should be pointed out that all previous studies on this matter were performed with polyclonal antibodies. It is conceivable that the particular population of monoclonal antibodies in the polyclonal pools therein used were indeed raised against regions of the protease that were shielded after complex function with α_2M. Our approach with monoclonal antibodies to defined regions of human plasmin is much more powerful in terms of assessing the particular regions of plasmin exposed to solvent molecules. We conclude that, at least with human plasmin, the molecule is not completely encapsulated within α_2M in the α_2M-plasmin complex. Other recent studies cast additional doubt on the existence of a molecular trap. Wang et al. (1983) have shown that certain proteases are dissociable from the α_2M complex, and Schram & Schram (1982) suggest from analysis of electron micrographs of α_2M-trypsin complexes that the bound protease may not be encapsulated by α_2M. In addition to the above, we show herein that α_2M can bind to a human plasmin-antibody preformed complex. It is unlikely that a molecular trap would be capable of accommodating a complex of such large size.

Finally, the kinetic parameters of the ternary α_2M-plasmin-antibody complexes are not significantly different from those of the α_2M-plasmin complex. These results argue strongly that the plasmin active site is situated close to the surface in the α_2M complex and that the antibodies are bound to a region of the protease remote from the active site.

Registry No. S-2251, 62354-43-2; plasmin, 9001-90-5; benzamidine, 618-39-3; Trasylol, 9087-70-1; plasminogen, 9001-91-6.

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