ACTA CHEMICA SCANDINAVICA 21 (1967) 602–608

Interaction of Plasmin and Trypsin with α_2-Macroglobulin

P. O. GANROT

Department of Clinical Chemistry, (University of Lund), Malmö General Hospital, Malmö, Sweden

The plasmin inhibiting activity was determined in gel filtration fractions of human serum containing α_2-macroglobulin. The ratio between the plasmin inhibition and α_2-macroglobulin concentration in five fractions was approximately constant and was about 1 mole of plasmin per mole of α_2-macroglobulin.

The ratio between the trypsin-binding and the plasmin-binding activity (both expressed in mole/ml) of a purified α_2-macroglobulin preparation was determined as 1.80.

The binding of plasmin to α_2-macroglobulin blocked about half of its trypsin binding capacity.

The findings suggest that one mole of α_2-macroglobulin binds one mole of plasmin and two moles of trypsin and that plasmin can be bound to one of the trypsin binding groups of the α_2-macroglobulin.

In 1952 Shulman [1] showed that addition of plasmin to serum reduces the trypsin inhibiting capacity of the serum by about 10 % and concluded that serum contains two trypsin inhibitors, one of which can also inhibit plasmin. Jacobson [2] showed that the latter inhibitor migrates with the α_2-fraction in the electrophoretic field and the other with the α_1-fraction. He also found that the α_2-inhibitor reacts rapidly with plasmin. Norman and Hill [3] demonstrated that also the α_1-fraction contains a plasmin inhibitor which, however, reacts only slowly with plasmin and that the rate of the reaction is very dependent on the ambient temperature. α_2-Macroglobulin (α_2-M) is identical with the trypsin and plasmin-binding α_2-inibitor,[4,5] while α_1-antitrypsin is responsible for the inhibiting capacity in the α_1-fraction.[4,6] One mole of α_2-M probably binds 2 moles of trypsin.[7] Compared with a corresponding amount of free trypsin the complex shows a largely unchanged esterase activity resistant to trypsin inhibitors such as α_1-antitrypsin and soy-bean trypsin inhibitor.[5,8] The molar activity of a plasmin inhibitor can be determined by comparing it with the plasmin inhibiting activity of the polyvalent protease inhibitor (PPI) from bovine lung.[9] This inhibitor binds trypsin equimolarly and can be standardised in terms of molar concentration against crystalline soy-bean trypsin inhibitor. In the present investigation this method was used for determining the molar binding quotient of the plasmin-α_2-M complex and the effect of plasmin on the trypsin binding capacity of α_2-M.

EXPERIMENTAL

Reagents. The same preparation of trypsin, ^{125}I-labelled trypsin, plasmin, PPI, soybean trypsin inhibitor, benzoyl-DL-arginine-*p*-nitroanilide-HCl (BAPNA) and casein were used as in a previous study.[9] Specific rabbit anti human α_2-macroglobulin was prepared in the way described before.[10]

Methods. The trypsin activity was determined with BAPNA as a substrate in the same way as in an earlier investigation.[11] The activity was expressed in terms of increase of optical density at 410 mμ. The trypsin-binding capacity of α_2-M was measured by a method based on the determination of the increase of the trypsin-like activity in the serum after saturation of the serum with trypsin when free trypsin is inactivated by excess of soybean trypsin inhibitor.[10] Under the conditions used this α_2-M-bound trypsin had a specific activity corresponding to 80 % of that of free trypsin.[12] The trypsin activity of the α_2-M-bound trypsin, expressed as increase of the optical density at 410 mμ, was therefore multiplied by 1.25 so that it could be compared with the optical density value found for a standard solution of free trypsin.

The plasmin activity was determined with casein as a substrate in the way described previously.[9] The activity was expressed as the increase of the optical density at 280 mμ in the supernatant obtained after precipitation with acid. The molar concentration of the plasmin solution used was measured as described in a previous study.[9]

α_2-M was determined immunochemically.[10] The determination was made against a standard serum whose concentration of α_2-M had been determined as 1.80 mg/ml [13] which corresponds to 2.20 nmole/ml, if the molecular weight of α_2-M is taken as 820 000.[14]

Partial purification of α_2-M was done by gel filtration of human pooled serum. That part of the macroglobulin fraction that was eluted first and that does not contain the somewhat later eluted inter-α trypsin inhibitor was collected.[15,16] Nor does the fraction contain any appreciable amounts of known plasmin inhibitors other than α_2-M. The concentration of α_2-M was about one fifth of that in serum. Other proteins in the fraction consisted mainly of low density lipoproteins, high molecular weight haptoglobins, and Ig M.

RESULTS

Inhibition of plasmin activity with purified α_2-M. The inhibition of plasmin by α_2-M was studied by mixing 25 μl of a plasmin solution with an increasing amount of a purified α_2-M preparation. After 15 min incubation of the mixture

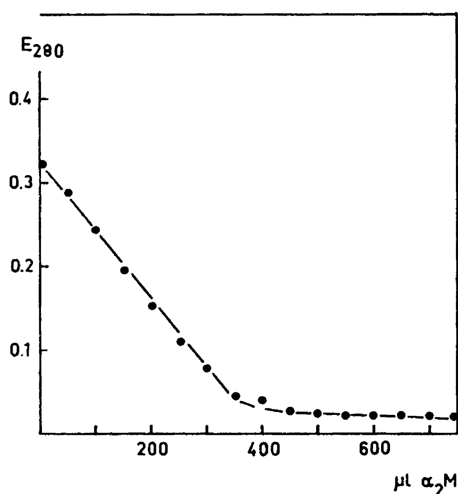

Fig. 1. Inhibition of plasmin activity by α_2-M. Ordinate: Plasmin activity expressed as optical density at 280 mμ (−blank). Abscissa: Amount of purified α_2-M added.

at 37°C the remaining plasmin activity was determined with casein as a substrate. The activity decreased linearly with the amount of the added α_2-M, down to about 10 % of the uninhibited activity (Fig. 1). Further addition of α_2-M had barely any affect on the residual activity. The binding of plasmin to α_2-M appeared to occur rapidly at 37°C. The same degree of inhibition was obtained when the substrate was added immediately after the plasmin had been mixed with α_2-M as when the plasmin and α_2-M had been allowed to react for 15 min.

Determination of the molar combining ratio of α_2-M and plasmin. 5 ml of pooled serum was fractionated by gel filtration in Sephadex G 200. The plasmin inhibiting capacity was measured in each of the five fractions that contained the bulk of α_2-M. 100 μl of each fraction was incubated with 50 μl plasmin in tris buffer for 15 min before addition of substrate, after which the residual plasmin activity was determined. The activity was determined simultaneously in the same amount of plasmin without inhibitor, and the same amount of macroglobulin fractions was added after the end of the determination time. The difference between the inhibited and uninhibited activity (expressed as optical density at 280 mμ) was taken as a measure of the plasmin inhibiting capacity of the macroglobulin fractions. The inhibition produced by the five fractions ranged from 12 to 27 %. The concentration of the plasmin had been determined as 5.9 nmole/active plasmin/ml.[9] 50 μl gave an increase of optical density of 0.430. The plasmin inhibiting capacity of the fractions in nmole/ml was calculated by multiplying the inhibition by the fractions, expressed as the difference in optical density/100 μl, by $5.9 \times 0.05 \times 10/0.430$. The result is given in Table 1. In the same fraction immunochemical determinations were also made of the α_2-M-concentration in relation to a standard serum

Table 1. Results of determination of the plasmin inhibiting capacity, of the α_2-M concentration and of the ratio between these in fractions of serum obtained by gel filtration.

Fraction No.	Difference in optical density	Inhibition of plasmin (nmole/ml)	Concentration of α_2-M (nmole/ml)	Ratio Plasmin:α_2-M
20	0.059	0.40	0.374	1.07
21	0.093	0.64	0.651	0.98
22	0.115	0.79	0.716	1.10
23	0.092	0.64	0.606	1.05
24	0.053	0.36	0.347	1.03

containing 2.20 nmole α_2-M/ml (Table 1). The number of moles of plasmin inhibited per mole α_2-M was calculated for each fraction and all gave a value very close to 1.

Determination of the ratio between the trypsin-binding capacity and the plasmin-binding capacity of α_2-M. Determining the ratio between moles of trypsin inhibited and moles of plasmin inhibited by an α_2-M-preparation does not require knowledge of the α_2-M-concentration of the preparation. Nor is any absolute standardization of enzyme preparations necessary. The ratio

can be calculated if the concentrations of the plasmin and trypsin are expressed in the same relative units. The activity of the trypsin, respectively plasmin, solutions used was determined in the presence of increasing amounts of a PPI-solution. The concentration of the PPI-solution was taken to be *a* nmole/ml. The concentration of the trypsin solution was then calculated as 0.148 *a* nmole/ml and that of the plasmin solution as 0.056 *a* nmole/ml. The trypsin-binding capacity of α_2-M was determined in a purified α_2-M-preparation. The difference found in optical density was multiplied by 1.25 to correct for the lower specific activity of α_2-M-bound trypsin compared with that of free trypsin. A corrected optical density value of 0.234 was obtained for 250 μl of the fraction. At the same time 50 μl of the standardized trypsin preparation showed an optical density difference of 0.529. The trypsin-binding capacity of the α_2-M-preparation was then calculated as 0.01315 *a* nmole/ml ($0.148\ a \times 0.05 \times 0.235/0.25 \times 0.529$). The plasmin-binding capacity of the α_2-M-preparation was determined by mixing 50 μl of the standardized plasmin preparation with 100 μl of the α_2-M-preparation. The difference between the residual plasmin activity in the mixture and the activity in a corresponding amount of plasmin without inhibitor was, on the average, 0.057 (four determinations). The activity of 50 μl of the standardized plasmin without inhibitor showed an optical density difference of 0.219. From this the plasmin-binding capacity of the α_2-M-preparation was calculated as 0.00729 *a* nmole/ml ($0.056\ a \times 0.05 \times 0.057/0.1 \times 0.219$). The ratio between the molar trypsin-binding capacity and the molar plasmin-binding capacity of the α_2-M-preparation was finally calculated as 1.80 ($0.01315\ a/0.00729\ a$).

Reduction of trypsin-binding capacity of α_2-M by plasmin. In order to find out whether plasmin and trypsin compete for the same binding site on α_2-M it was checked whether plasmin reduces the trypsin-binding capacity of α_2-M. 100 μl (0.066 nmole) of a purified α_2-M-preparation was mixed at 0°C with different amounts of plasmin. 10 min later trypsin (1.66 nmole) was added, and after a further 10 min, soybean trypsin inhibitor in excess. The

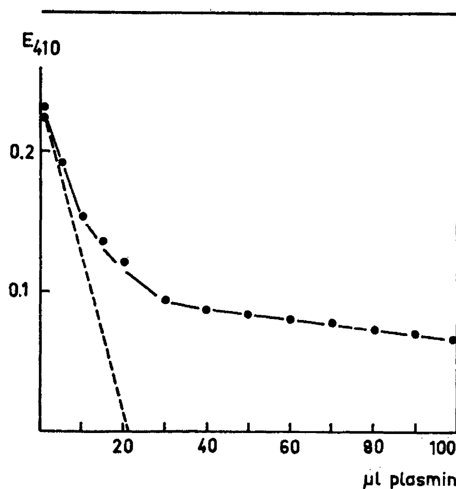

Fig. 2. Blocking of the trypsin binding capacity of α_2-M by plasmin. Ordinate: Activity of trypsin bound to α_2-M expressed as optical density at 410 mμ (−blank). Abscissa: Amount of plasmin added before trypsin. Broken line denotes calculated activity assuming that each molecule of plasmin added prevents the binding of one molecule of trypsin to α_2-M.

α_2-M-bound trypsin activity was then determined after further addition of so much plasmin that the final amount of plasmin in all samples was equal. The activity of the α_2-M-bound trypsin decreased markedly after the first addition of plasmin. The decrease following addition of further amounts was increasingly smaller (Fig. 2). The initial decrease found corresponded to that expected if it is assumed that one molecule of α_2-M binds 2 molecules of trypsin with the same specific activity and assuming that each plasmin molecule added prevents the binding of one molecule of trypsin. The slight reduction noted on addition of plasmin in excess was practically linearly dependent on the amount of plasmin in the interval of 3—10 fold molar excess of plasmin in relation to the trypsin-binding capacity of α_2-M. The small reduction was in the order of 0.015 mole α_2-M/mole plasmin or 0.03 mole trypsin-binding activity/mole plasmin.

In separate experiments determinations were made of the rate at which α_2-M binds plasmin and trypsin at 0°C. The degree of inhibition of plasmin was found to be the same whether the α_2-M was allowed to react with excess of plasmin for 5 min or 15 min. When trypsin was added to α_2-M saturated with plasmin variation of the interval from 5 to 15 min between the addition of trypsin and STI had no effect on the activity of the bound trypsin. Under the prevailing conditions of the determination the amount of plasmin used had no demonstrable hydrolytic effect on BAPNA and no inhibiting effect on the α_2-M-bound trypsin.

The influence of plasmin on the binding of trypsin to α_2-M was studied also directly with ^{125}I-labelled trypsin. 500 μl (0.33 nmole) of a purified α_2-M-preparation was mixed at 0°C with 200 μl plasmin (1.2 nmole). After 15 min about 1.5 nmole ^{125}I-labelled trypsin was added. In a control experiment the trypsin was mixed with the α_2-M before addition of the plasmin. The two mixtures were then fractionated by gel filtration in Sephadex G 200 and the radioactivity of the fractions was determined in a γ-spectrometer. When plasmin was added before trypsin the radioactivity of the macroglobulin fraction was found to be 35.8 % of that when the order of addition was the reverse.

DISCUSSION

On the basis of personal experiments Norman and Hill [3] believed the reaction between plasmin and α_2-anti-plasmin to be readily reversible. The experiments were carried out on plasmin, purified by acid extraction according to Kline.[17] Such plasmin has an intact active centre but is partially denatured and is not readily soluble in water at neutral pH.[18] This partial denaturation may, perhaps, severely affect the binding of the enzyme to α_2-M or the dissociation of the complex. The plasmin used in the present investigation had been prepared essentially according to Wallén and Bergström [19] by ion exchange chromatography of Cohn's fraction III and by autoactivation of the purified plasminogen in 50 % glycerol. The plasmin activity in the plasmin preparation used appeared to consist of two parts, a major one inhibited stoichiometrically by α_2-M, and a minor one (about 8 %) resistant to α_2-M. The sharp knee of the curve and the high resistance of the residual activity to α_2-M does not argue for a dissociation equilibrium of the α_2-M-plasmin complex being a cause of

incomplete inhibition. On incubation of the α_2-M-plasmin complex with trypsin in excess before the addition of STI the plasmin was not replaced by trypsin in any demonstrable amount during 15 min, which should have occurred if the rate of dissociation of the plasmin-α_2-M-complex had been high. The plasmin therefore appeared to be relatively firmly bound to α_2-M under the experimental conditions used.

Both trypsin and thrombin are bound to α_2-M in such a way that the active centre of the enzyme is not completely inhibited.[8,20] It would therefore not appear unreasonable to assume that plasmin is bound in an analogous way and that the residual activity is in reality the activity of the α_2-M-plasmin complex. One might, however, also imagine that the residual activity of the plasmin preparation in the presence of excess α_2-M represents plasmin molecules, that have in some way been changed outside their active centres so that the binding to α_2-M is prevented or in such a way as markedly to increase the dissociation rate of the complex. PPI is probably bound to the active centre of the plasmin molecules. If so, it would explain why the plasmin preparation when tested against PPI appeared homogeneous.[9]

In the calculations set forth above it was assumed that the plasmin binding capacity of α_2-M is equal to the plasmin inhibiting capacity with casein as a substrate (expressed in mole). This is probably correct provided that the inhibition is determined at a relatively low degree of inhibition, and that the residual activity discussed is due to a change in some of the plasmin molecules. If on the other hand, the plasmin-α_2-M-complex really possesses enzyme activity against casein, the true plasmin binding capacity should exceed the plasmin inhibiting capacity measured by about 9 %. The difference is, however, not large enough to invalidate the following conclusions.

The results suggest that the plasmin is bound to α_2-M equimolarly. The ratio found between the trypsin binding and the plasmin binding capacity of α_2-M corroborates also the conclusion in a previous paper that one mole of α_2-M binds 2 moles of trypsin.[7] The decrease in trypsin activity bound to α_2-M when plasmin was added to α_2-M before trypsin is due to reduction of the binding of trypsin to α_2-M and not to a decreased specific activity of the α_2-M bound trypsin on simultaneous binding of plasmin. Plasmin and trypsin thus appear to compete for one of the two trypsin binding sites of α_2-M. If only one of these binds plasmin, it does not mean that they are different from one another. The binding of one plasmin molecule to α_2-M can be a steric hindrance for the binding of a further molecule. For the much smaller trypsin molecules this hindrance need not occur.

If there are two equivalent binding sites the fact that only one mole of active plasmin appears to be bound per mole of α_2-M could be explained if it is assumed that the plasmin preparation contains a large amount of changed inactive plasmin molecules with the ability to be bound firmly to α_2-M and half of the plasmin binding sites of α_2-M be occupied by such an enzyme. This assumption appears less likely because only half of the trypsin binding capacity in α_2-M was inhibited by the plasmin preparation. This would require that the changed plasmin molecules are dissociated so readily that they are immediately replaced by trypsin. This would, however, imply that in the presence of plasmin preparation in excess they would be rapidly replaced by active

plasmin molecules with the ability to be bound more firmly and then the plasmin inhibition of α_2-M would be 2 moles/mole α_2-M.

The weak inhibiting effect on the remaining trypsin binding group of α_2-M after incubation with excess of plasmin is probably due to some contaminant of the plasmin, which is readily bound at the trypsin binding site of α_2-M. If it is assumed that the plasmin itself is bound to this second binding site, this binding must either occur relatively slowly or the dissociation constant of the complex must be high. In the former case the degree of inhibition would presumably have increased with time which it did not, and in the latter case the plasmin molecules would be rapidly replaced by trypsin, which is bound firmer and then this binding of plasmin would not cause any inhibition of the binding of trypsin.

Acknowledgements. This investigation was supported by grants from the *Medical Faculty, University of Lund* and the *Swedish Medical Research Council*, (Project No. 13X-581-02A). AB Kabi, Stockholm, kindly supplied the plasmin preparation used.

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Received December 1, 1966.