Biochimica et Biophysica Acta, 566 (1979) 200—210

BBA 68632

INTERACTIONS IN VITRO AND IN VIVO BETWEEN RAT SERUM PROTEASE INHIBITORS AND ANODAL AND CATHODAL RAT TRYPSIN AND CHYMOTRYPSIN

FRANCIS GAUTHIER [a], SVEN GENELL [b], HENRI MOURAY [a] and KJELL OHLSSON [b]

[a] *Laboratory of Biochemistry, Faculty of Medicine, 37200 Tours-Cedex, (France) and* [b] *Departments of Surgery and Clinical Chemistry, University of Lund, Malmö General Hospital, S-214 01 Malmö (Sweden)*

(Received June 1st, 1978)

Key words: Protease inhibitor; Trypsin; Chymotrypsin; (Anodal form, Cathodal form)

Summary

Reaction mixtures of increasing amounts of the pancreatic homologous proteases, anodal and cathodal chymotrypsin and trypsin, respectively, and normal rat serum were analyzed by immunoelectrophoretic methods in order to determine their distribution on serum protease inhibitors.

This paper concerns three proteins occurring in normal serum and capable of binding protease viz. α_1-macroglobulin, α_1-antitrypsin and α_1-inhibitor 3. The distribution of the enzymes among these protease inhibitors differed significantly from one protease to another.

The distribution of the proteases among the serum protease inhibitors following intravenous injection of ^{125}I-labelled proteases corresponded to that in vitro. Complexes formed with α_1-macroglobulin and α_1-inhibitor 3 were quickly eliminated irrespective of the enzyme species used, whereas those formed with α_1-antitrypsin persisted much longer in the circulation.

Introduction

The protease inhibitory effect of human serum is due mainly to α_1-antitrypsin and α_2-macroglobulin, although six other protease inhibitors with more or less broad specificity have been demonstrated [1—3]. In normal rat serum, three main inhibitors have been found [4], two of them, α_1-macroglobulin and α_1-antitrypsin, being homologous to the main serum inhibitors found in man. The third inhibitor, here called α_1-inhibitor 3, is possibly the homologue of the human serum inter-α-inhibitor [5]. A second type of macroglobulin with antiprotease properties [6,7] appears in rat serum in certain diseases [8—10], but can be disregarded in normal serum because of its very low concentration [11].

Rat pancreatic juice contains at least five proteolytic enzymes, which have

been recently isolated and described [12]. Two types of chymotrypsin and trypsin have been isolated and they are distinguished by their electrophoretic mobility, the anodal forms being more abundant. In most investigations of the interaction between serum and proteases in different species, the enzymes used were heterologous [13—16]. As pointed out by Bieth et al. [17] this could influence the characteristics of enzyme/inhibitor associations when comparing the use of human serum and homologous or heterologous enzymes.

The aim of this study was to show the interactions of the principal inhibitors of rat serum in the presence of increasing quantities of homologous proteases in order to elucidate the regulatory mechanisms of proteolytic enzymes in the circulation.

Materials

Rats. Blood was taken from male Sprague-Dawley rats (about 350-g) by cardiac puncture under mild diethyl ether anesthesia.

The serum obtained after coagulation at 4°C and centrifugation was used within the next 24 h.

Antibodies. Antisera against α_1-macroglobulin, α_1-antitrypsin and α_1-inhibitor 3 were prepared by conventional methods [4,5,18].

Proteases. Anodal and cathodal trypsin and chymotrypsin were isolated from rat pancreatic juice with the technique described by Genell et al. [12]. The purified enzymes were stored at —70°C.

The proteases were labelled with ^{125}I using a peroxidase method [19].

Excess iodine was eliminated by gel filtration on Sephadex G-25: the column (1.5 × 15 cm) was equilibrated in 0.05 M Tris-HCl buffer (pH 7.6), 0.15 M NaCl/0.01 M $CaCl_2$. The labelled enzyme had a specific activity of about 0.5 Ci/g. It was stored at —40°C until use.

Serum-protease interaction. 5-μl fresh serum fractions were incubated at room temperature with increasing amounts of enzyme (2—20 μg active enzyme) and the volumes were adjusted to 100 μl with 0.15 M NaCl. Samples were incubated for 15 min at room temperature and used within 24 h. These samples were analysed by crossed-immunoelectrophoresis [20] and electroimmunoassay [21] to determine the distribution of the enzyme among the various inhibitors.

Autoradiography was used in combination with agarose gel electrophoresis and crossed-immunoelectrophoresis. The plates were exposed (Tri-X Pan Kodak film) for 48 h.

The distribution among the inhibitors of the different enzymes tested was determined by electroimmunoassay. The mixtures of serum and increasing amounts of labelled enzyme were analysed with the use of antiserum against each inhibitor. After precipitation the plates were washed several times in large volumes of 0.15 M NaCl. The precipitates were then cut out and the radioactivity of each precipitate was measured in a well-type scintillation detector. This permitted the plotting of saturation curves for each inhibitor as a function of the amount of added enzyme.

Enzyme injection. Rats (male and female) weighing about 250 g were anesthetized with Mebumal (3 ml intraperitoneal; 6 mg/l). The femoral vein and

artery were catheterized and 0.2—0.4 mg labelled enzyme in 1 ml were injected into the femoral vein during 2 min. 0.1-ml blood samples were taken from the femoral artery 3 min after the end of the enzyme injection and every 3—5 min for 20 min, then every 10 min for 2 h. The serum samples were analysed as before to assess the distribution of the enzyme among the inhibitors and the decrease in radioactivity with time.

Results

In vitro interaction between rat serum and rat chymotrypsins and trypsins

The serum samples incubated with increasing amounts of the various proteases were analysed by crossed-immunoelectrophoresis. The appearance of complexes is easily demonstrated with this technique because of a shift of the electrophoretic mobility of the inhibitors. When anodal proteases were used these shifts were less apparant, especially for α_1-antitrypsin complexes which could then be demonstrated by autoradiography (Fig. 1).

All the enzymes tested were bound by each of the inhibitors, though the patterns obtained showed differences characteristic of each inhibitor and enzyme (Fig. 1).

On crossed-immunoelectrophoresis of the serum samples incubated with increasing amounts of ^{125}I-labelled anodal chymotrypsin combined with autoradiography, α_1-macroglobulin and α_1-inhibitor 3 were gradually converted to components with slightly reduced electrophoretic mobilities (Fig. 1). These new components were shown by autoradiography to contain ^{125}I-labelled anodal chymotrypsin, suggesting that this enzyme is involved in complex formation with the two inhibitors. α_1-Antitrypsin did not change in electrophoretic mobility on addition of ^{125}I-labelled anodal chymotrypsin, but autoradiography revealed considerable binding also between this enzyme and inhibitor.

Electroimmunoassay of these samples permitted calculation of the capacity of each inhibitor to bind the enzyme at different concentrations of the latter and thus to plot the saturation curves for each inhibitor. When a very small quantity of ^{125}I-labelled anodal chymotrypsin was added to the serum sample, 68% of the radioactivity fixed by the inhibitors was precipitated by the anti-α_1-antitrypsin antibodies, while anti-α_1-macroglobulin and anti-α_1-inhibitor 3 precipitate 20 and 12% of the radioactivity, respectively (Fig. 2). However, with increasing additions of ^{125}I-labelled anodal chymotrypsin, the α_1-antitrypsin and the α_1-macroglobulin curves reached a plateau indicating saturation. Additional ^{125}I-labelled anodal chymotrypsin could not be precipitated by antiserum against α_1-antitrypsin or α_1-macroglobulin, but was precipitated by antiserum against α_1-inhibitor 3 (Fig. 2).

The distribution of cathodal chymotrypsin among the inhibitors was also studied with crossed-immunoelectrophoresis (Fig. 3). The enzyme was preferentially bound by α_1-macroglobulin, as indicated by the conversion of this inhibitor to a slightly slower component. Only when about 75% of α_1-macroglobulin was converted to this new component did α_1-inhibitor 3 and α_1-antitrypsin complexes with cathodal chymotrypsin appear in the immunoelectrophoretic patterns. α_1-Inhibitor 3 then showed signs of conversion to a new component with slightly reduced electrophoretic mobility and a new α_1-anti-

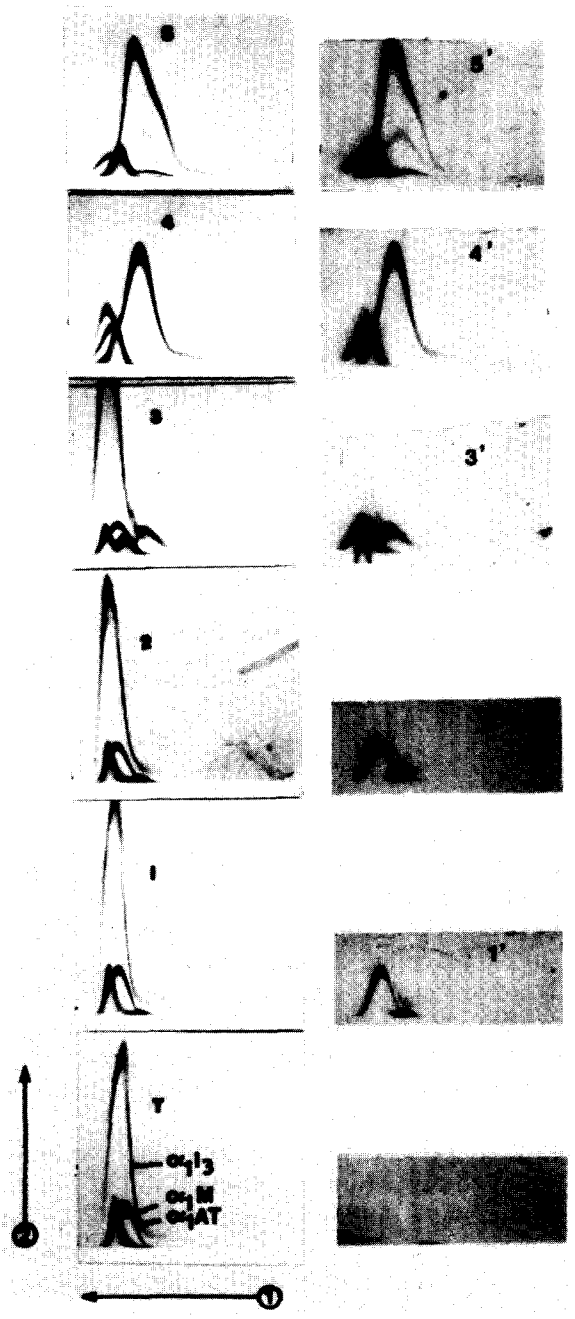

Fig. 1. Crossed immunoelectrophoretic patterns of rat serum (5 μl) reacted with increasing amounts (2—20 μg) of ^{125}I-labelled anodal chymotrypsin. A mixture of anti-α_1-macroglobulin, anti-α_1-antitrypsin and anti-α_1-inhibitor 3 antibodies was added to the agarose for the second step of electrophoresis. T denotes the serum without enzyme, 1—5 sera with increasing amounts of enzyme and 1′—5′ the corresponding autoradiographs.

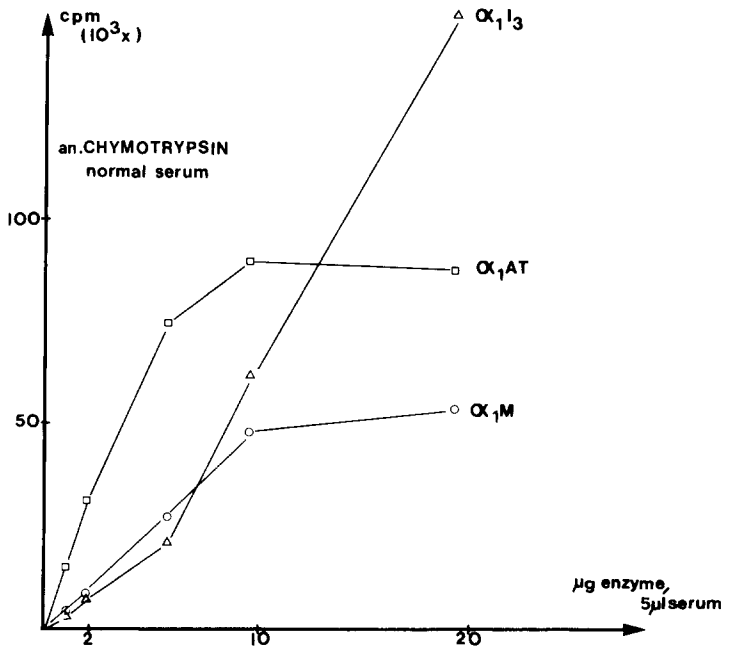

Fig. 2. Saturation curves for protease inhibitors from normal rat serum after addition of ^{125}I-labelled anodal chymotrypsin. The values are expressed as the radioactivity of the proteinase inhibitor specific antibodies precipitates after reaction of constant amounts of serum with increasing amounts of labelled enzyme.

trypsin component appeared in the β_2-region (Fig. 3).

On addition of ^{125}I-labelled anodal trypsin to rat serum α_1-macroglobulin was rapidly converted to a component with a slightly lower rate of migration (Fig. 4). Autoradiography showed this new component to contain ^{125}I-labelled antitrypsin. With increasing addition of ^{125}I-labelled anodal trypsin also part of α_1-inhibitor 3 showed lower electrophoretic mobility and blackening of the autoradiograph indicating complexation. α_1-Antitrypsin did not show any change in the electrophoretic mobility but the autoradiograph of the immunoprecipitate showed intense blackening indicating binding of ^{125}I-labelled anodal trypsin.

Electroimmunoassay analyses (Fig. 5) revealed that on addition of minute amounts of ^{125}I-labelled anodal trypsin to rat serum, α_1-macroglobulin bound 77% of the fixed radioactivity, α_1-inhibitor 3 18% and α_1-antitrypsin 5%. On continued addition of ^{125}I-labelled anodal trypsin, α_1-macroglobulin reached a plateau indicating saturation. Additional ^{125}I-labelled anodal trypsin was precipitated by antiserum against α_1-antitrypsin and α_1-inhibitor 3 (Fig. 5).

On addition of increasing amounts of cathodal trypsin to rat serum, crossed immunoelectrophoresis showed that α_1-macroglobulin was gradually converted to a component with reduced electrophoretic mobility (Fig. 6) indicating that complexation of this inhibitor was primary. On further addition of enzyme, binding by α_1-inhibitor 3 was suggested by the appearance of a new and

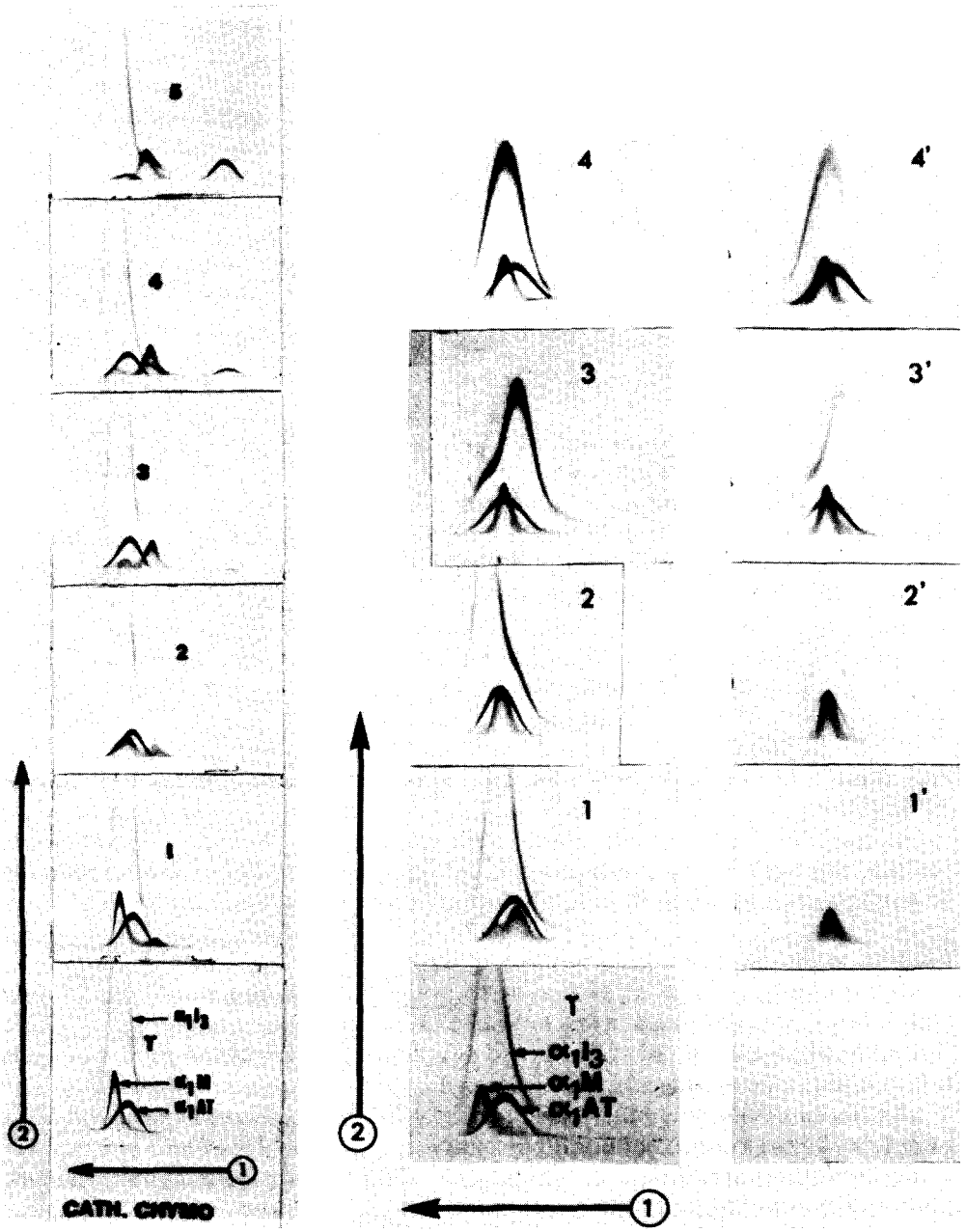

Fig. 3. Crossed immunoelectrophoretic patterns of 5 μl normal rat serum reacted with increasing amounts (2—20 μg) of cathodal chymotrypsin (1—5). T denotes the serum without enzyme. For the second step agrose gel contains the same mixture of antibodies as in Fig. 1.

Fig. 4. Precipitation patterns obtained by antigen antibody crossed electrophoresis of 5 μl rat serum reacted with increasing amounts (2—20 μg) of ^{125}I-labelled anodal trypsin (1—4) using a mixture of anti-α_1-macroglobulin, anti-α_1-antitrypsin and anti-α_1-inhibitor 3 antibodies for the second step. 1′—4′ are the corresponding autoradiographic patterns of 1—4.

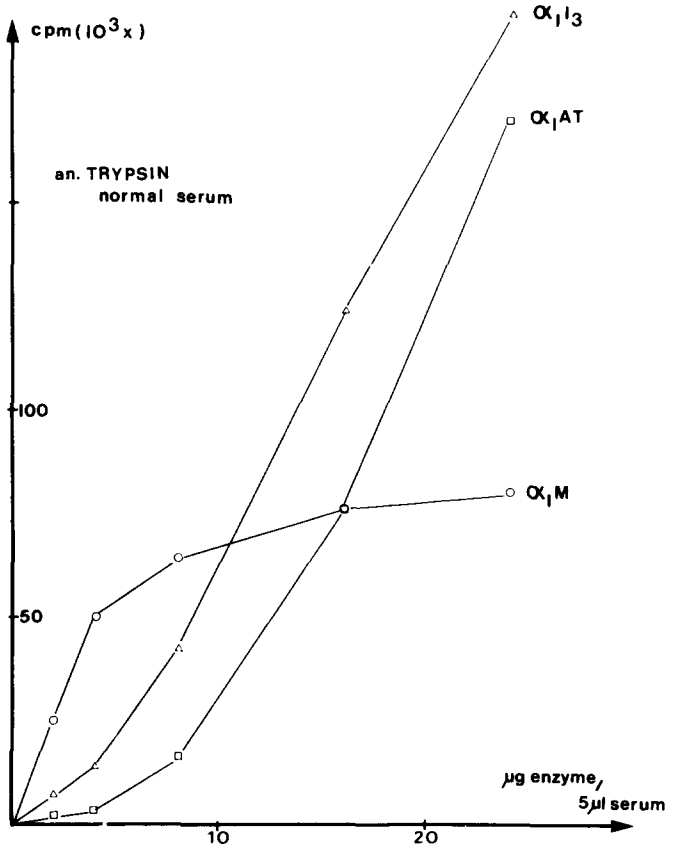

Fig. 5. Saturation curves for proteinase inhibitors of normal rat serum after addition of ^{125}I-labelled anodal trypsin.

slightly less mobile component as well as by α_1-antitrypsin by the appearance of a new α_1-antitrypsin component in the β_1-region (Fig. 6).

Enzyme injection

The samples obtained at different intervals after the injection of antitrypsin and anodal chymotrypsin were analysed, as were those prepared in vitro in order to determine the distribution of the enzymes among the inhibitors, and the catabolic rate of the complexes formed.

The distribution of anodal trypsin and anodal chymotrypsin among protease inhibitors 3 min after injection of the enzyme is given in Table I.

These results corroborate the conclusion of the in vitro investigation, namely that as soon as the enzymes react with the plasma proteins, anodal trypsin shows a predilection for α_1-macroglobulin and anodal chymotrypsin for α_1-antitrypsin. The radioactivity of each proteinase-proteinase inhibitor complex precipitated at electroimmunoassay was calculated at different intervals after the injection of the labelled enzyme.

It was found that α_1-macroglobulin complexes of all the enzyme species were rapidly eliminated. During the first half hour the half-life of the complexes was 6—8 min, after which it became longer. Furthermore, when larger amounts of

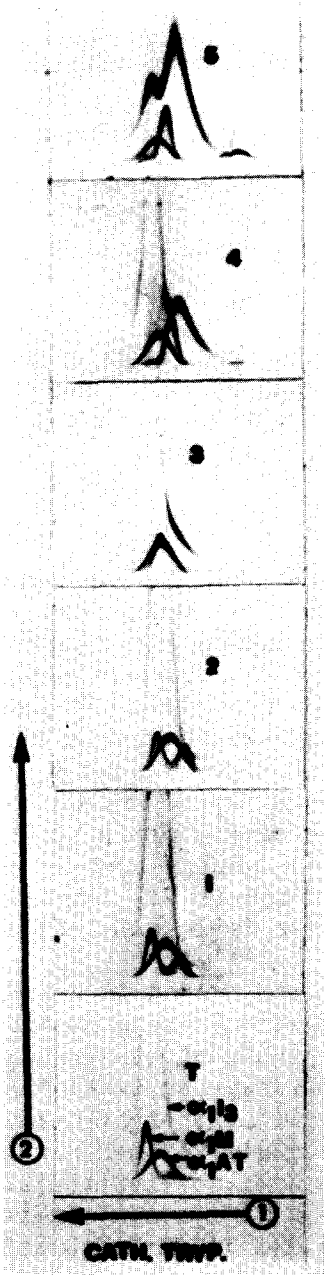

Fig. 6. Antigen antibody precipitation patterns of normal rat serum (5 μl) reacted with increasing amounts (2–20 μg) of cathodal trypsin (1—5). T denotes the serum without enzyme. The same mixture of antibodies as before was used in the second step.

enzymes were injected a decrease in the macroglobulin seric concentration was observed parallel to the decrease in radioactivity. This suggests that the whole complex is eliminated unchanged from the circulation. Enzyme-bound α_1-inhib-

TABEL I

DISTRIBUTION OF MINUTE AMOUNTS OF ANODAL TRYPSIN AND ANODAL CHYMOTRYPSIN AMONG PROTEASE INHIBITORS OF NORMAL SERUM 3 MIN AFTER INJECTION OF LABELLED ENZYME

	α_1-Macroglobulin (%)	α_1-Antitrypsin (%)	α_1-Inhibitor$_3$ (%)
^{125}I-labelled anodal trypsin	80	5	15
^{125}I-labelled anodal chymotrypsin	9	73	18

itor 3 also disappears quickly since the decrease in radioactivity of antigen-antibody precipitates corresponds to a half-life of about 15 min. Complexes formed with α_1-antitrypsin, however, persist much longer in the circulation; their half-life was found to be 110 min with anodal chymotrypsin.

Discussion

At least three proteins in normal rat serum can form complexes with homologous pancreatic trypsins and chymotrypsin, but in ratios differing from one enzyme to another. The two quantitatively most important enzymes are anodal trypsin and anodal chymotrypsin. On addition of minute amounts of enzyme to serum, anodal trypsin is complexed mainly by α_1-macroglobulin (77%) and anodal chymotrypsin by α_1-antitrypsin (68%). A similar distribution has been demonstrated between trypsin and α_1-macroglobulin the homologue of human α_2-macroglobulin, and between chymotrypsin and α_1-antitrypsin in other species [22,23]. This suggests that α_1-antitrypsin is a biologically more important inhibitor of chymotrypsin than of trypsin. The term α_1-protease inhibitor has been proposed as a more appropriate name for α_1-antitrypsin [24]. This term, however, is difficult to use in rats, where the 3 inhibitors demonstrated all have α_1-mobility.

While complexes formed with α_1-antitrypsin completely inhibit the enzyme [25], those formed with α_1-macroglobulin retain the activity of an esterase and the proteolytic activity which, although substantially inhibited, is nevertheless real [26]. The in vivo experiments, however, showed that complexes formed with α_1-macroglobulin are rapidly eliminated. As its half-life is only 6—8 min, the retention of enzymic activity might be of minor importance. Rapid elimination of α_2-macroglobulin-enzyme complexes has also been shown in man [27] and dog [28,29] where 80—85% of the radioactivity of the injected complexes was found in the reticulo-endothelial system 30 min after injection. This rapid decrease in plasma radioactivity is due to the elimination of the whole complex and not of the enzyme alone, as there is a proportional decrease in serum inhibitor concentration. A similar observation has been made in the dog after injection of bovine trypsin [30]. Such a rapid decrease can be explained by the confirmational change of the macroglobulin molecule [31] induced by the trapping of the enzyme, which makes it immediately identifiable by the reticulo-endothelial cells [28,29]. This confirmational change also induces a modification of the net charge of complex, which can be visualized on crossed immu-

noelectrophoresis. This variation of charge is rather similar for all enzymes and the complexed forms always have a similar mobility. In contrast, the electrophoretic mobility of α_1-antitrypsin enzyme complexes is essentially a function of the charge of the respective enzyme; thus cathodal enzymes give the complex a β-mobility, whereas anodal enzymes cause barely any modification on the mobility (Figs. 1—3).

As far as α_1-inhibitor 3 is concerned, the reduction of mobility on binding of enzyme is similar to that of α_1-macroglobulin and may have a similar explanation. Furthermore, the half-lives of α_1-inhibitor 3 protease complexes in the circulation are also comparable to those of α_1-macroglobulin. Preliminary results showed that the α_1-inhibitor 3 molecule, like that of α_1-macroglobulin, cannot completely inhibit enzyme activity [5]. There must, however, be more than one trapping mechanism as trypsin retains its immunoreactivity when bound by α_1-inhibitor 3 but not by α_1-macroglobulin.

The immunologic techniques used in this investigation permit only studies of those plasma protease inhibitors to which antibodies are available. However, the three inhibitory proteins studied seem to represent the main inhibitors in rat serum, at least for the quantitative point of view. However, in some preliminary experiments using homologous trypsin, we have found evidence of an another inhibitor in rat serum. Identification of this inhibitor must abide further research. α_1-Macroglobulin appears to play a key role in protease neutralization and elimination from the circulation. Most enzymes in the serum are bound mainly by this inhibitor and the complexes are then eliminated via the reticuloendothelial cells. Furthermore, it has been demonstrated that in dog serum protease α_1-antitrypsin complexes can separate and the enzyme is then immediately trapped by the α-macroglobulin and eliminated [29,30,32]. This transfer from one inhibitor to another might be explained by differences in half-life between complexed α_1-macroglobulin and α_1-antitrypsin favouring a shift in balance towards the α_1-macroglobulin. The rate of dissociation of α_1-antitrypsin complexes is comparable to that in the dog [29] and in man [27]; but differs significantly from what Aubry et al. [33] found in vitro. These authors have demonstrated the extreme stability of complexes formed between human α_1-antitrypsin and proteases, particularly those of human origin. To explain this discrepancy one might suppose that the spontaneous dissociation is very slow in vitro and that in vivo some active mechanism must be involved. Recent investigations [24,34] have shown that α_1-antitrypsin is inactivated by protease inhibition because of a partial proteolysis by the enzyme.

α_1-Antitrypsin seems to be a carrier or transitory protease inhibitor [34] since because of its low molecular weight it intervenes in both the circulation and in the extravascular spaces. α_2-Macroglobulin acting essentially in the intravascular space would then permit elimination of the proteolytic enzyme. The α_1-inhibitor 3 mode of action is still unknown, even though this protein binds all the protease varieties tested here.

Acknowledgements

This investigation was supported by the Swedish Medical Research Council (project no. B78-17X-03910-06B), the Medical Faculty, University of Lund,

the Foundation of Albert Pahlsson, the Foundation of Thorsten and Elsa Segerfalk, Bayer AG and DGRST no. 75 70 189.

References

1 Heimburger, N. (1974) in Bayer Symposium V, Proteinase Inhibitors (Fritz, H., Tschesche, H., Greene, L.J. and Truscheit, E., eds.), pp. 14—22, Springer-Verlag, Berlin
2 Collen, D. (1976) Eur. J. Biochem. 69, 209—216
3 Sasaki, M., Minakata, K., Yamamoto, M., Niwa, M., Kato, T. and Ito, N. (1977) Biochem. Biophys. Res. Commun. 76, 917—924
4 Gauthier, F., Genell, S., Mouray, H. and Ohlsson, K. (1978) Biochim. Biophys. Acta 526, 218—226
5 Gauthier, F. and Ohlsson, K. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 987—992
6 Ganrot, K. (1973) Biochim. Biophys. Acta 322, 62—67
7 Gauthier, F. and Mouray, H. (1975) in Protides of the Biological Fluids, 23rd Colloquium (Peeters, H., ed.), pp. 139—143, Pergamon Press, Oxford
8 Beaton, G.H., Selby, A.E., Veen, M.J. and Wright, A.M. (1961) J. Biol. Chem. 236, 2005—2008
9 Heim, W.G. (1962) Nature 193, 491
10 Heim, W.G. and Lane, P.H. (1964) Nature 203, 1077—1078
11 Ganrot, K. (1973) Biochim. Biophys. Acta 295, 245—251
12 Genell, S., Gustafsson, B.E. and Ohlsson, K. (1977) Scand. J. Gastroenterol. 12, 811—820
13 Haverback, B.J., Dyce, B., Bundy, H.F., Wirtschafter, S.K. and Edmonson, H.A. (1962) J. Clin. Invest. 41, 972—980
14 Ganrot, P.O. (1967) Arkiv. Kemi 26, 577—582
15 Ohlsson, K. (1977) Scand. J. Clin. Lab. Invest. 28, 5—11
16 Bieth, J., Metais, P. and Warter, J. (1969) Clin. Chim. Acta 20, 69—80
17 Bieth, J. Aubry, M. and Travis, J. (1974) in Bayer Symposium V, Proteinase Inhibitors (Fritz, H., Tschesche, H., Greene, L.J. and Truscheit, E., eds.), pp. 53—62, Sringer-Verlag, Berlin
18 Gauthier, F., Leng, M. and Mouray, H. (1974) C.R. Acad. Sci. (Paris) 279, 1409—1411
19 Thorell, J.I. and Johansson, B.G. (1971) Biochim. Biophys. Acta 251, 363—370
20 Ganrot, P.O. (1972) Scand. J. Clin. Lab. Invest. 29, Suppl. 124, 39—47
21 Laurell, C.B. (1966) Anal. Biochem. 15, 45—52
22 Bundy, H. and Mehl, J. (1959) J. Biol. Chem. 234, 1124—1128
23 Ohlsson, K. (1971) Clin. Chim. Acta 32, 215—220
24 Johnson, D.A. and Travis, J. (1976) Biochem. Biophys. Res. Commun. 72, 33—39
25 Laurell, C.B. (1965) Scand. J. Clin. Lab. Invest. 17, 271—274
26 Gauthier, F., Lebreton de Vonne, T. and Mouray, H. (1975) Comp. Biochem. Physiol. 50, 549—554
27 Ohlsson, K. and Laurell, C.B. (1976) Clin. Sci. Mol. Med. 51, 87—92
28 Ohlsson, K. (1971) Acta Physiol. Scand. 81, 269—272
29 Ohlsson, K. (1974) in Bayer Symposium V, Proteinase Inhibitors (Fritz, H., Tschesche, H., Greene, L.J. and Truscheit, E., eds.), pp. 96—105, Springer-Verlag, Berlin
30 Ohlsson, K., Ganrot, P.O. and Laurell, C.B. (1971) Acta Chim. Scand. 137, 113—121
31 Bloth, B., Chasebro, B. and Svehag, S.-E. (1968) J. Exp. Med. 127, 749—763
32 Balldin, G., Laurell, C.B. and Ohlsson, K. (1978) Hoppe-Seyler's Z. Physiol. Chem. (1978) 359, 699—708
33 Aubry, M. and Bieth, J. (1977) Clin. Chim. Acta 78, 371—380
34 Baumstark, J.S., Tinglee, C. and Luby, R.J. (1977) Biochim. Biophys. Acta 482, 400—411
35 Oda, K., Laskowski, Sr, M., Kress, L.F. and Kowalski, D. (1977) Biochem. Biophys. Res. Commun. 76, 1062—1070