Biochem. J. (1982) **205**, 97–104
Printed in Great Britain

Evolution of α_2-macroglobulin

The purification and characterization of a protein homologous with human α_2-macroglobulin from plaice (*Pleuronectes platessa* L.) plasma

Phyllis M. STARKEY,*‡ Thelma C. FLETCHER† and Alan J. BARRETT*
**Department of Biochemistry, Strangeways Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K., and †N.E.R.C. Institute of Marine Biochemistry, St. Fittick's Road, Aberdeen AB1 3RA, Scotland, U.K.*

(*Received 2 November 1981/Accepted 29 March 1982*)

A papain-binding protein (PB-protein) was purified to homogeneity from the plasma of plaice (*Pleuronectes platessa* L.). PB-protein inhibited the activity of trypsin and pancreatic elastase (serine proteinases), thermolysin (a metalloproteinase) and papain (a cysteine proteinase). Presaturation of PB-protein with trypsin prevented the subsequent inhibition of thermolysin, and vice versa. Only catalytically active endopeptidases were bound by PB-protein. The catalytic activity of trypsin bound by PB-protein was inhibited by 95% against an insoluble protein substrate, but only by 38% against a low-molecular-weight synthetic substrate. The remaining activity of the bound trypsin was partially protected against further inhibition by soya-bean trypsin inhibitor. Trypsin bound by PB-protein showed a decrease of 67% in its reactivity with antibodies. The inhibitory activity of PB-protein was inactivated at pH 8.0 by methylamine (0.2M) or dithiothreitol (1mM). The inhibition of proteinases by plaice PB-protein shows the distinctive characteristics of inhibition by human α_2-macroglobulin, and it is concluded that the plaice protein is a homologue of the human macroglobulin.

In the preceding paper (Starkey & Barrett, 1982*a*) we have demonstrated the occurrence in a variety of vertebrate species of a protein resembling human α_2-macroglobulin (α_2M). This protein was detected in plasma samples after polyacrylamide-gel electrophoresis by its ability to bind papain in such a way that the bound enzyme retained activity against a low-molecular-weight chromogenic substrate. This ability is unique to α_2M among human plasma proteins (Barrett & Starkey, 1973).

In mammals, birds, reptiles and amphibians the papain-binding protein (PB-protein) appeared in polyacrylamide-gel pore-limit electrophoresis to have an M_r similar to that of human α_2M (725000). In contrast, the migration of the analogous fish protein suggested an M_r similar to that of half-molecules of human α_2M.

If the fish PB-protein, despite its smaller size, were found to be a true homologue of human α_2M, sharing its distinctive inhibitory properties, then its study might increase our understanding of the structure and function of the more complex human protein. We therefore isolated the PB-protein from fish plasma, and compared it with human α_2M. Since there appeared to be no major differences between the PB-protein of representative species of all the major classes of fish (Starkey & Barrett, 1982*a*), the species chosen for the purification (plaice, *Pleuronectes platessa* L., an actinopterygian) was selected solely on the grounds of availability.

Abbreviations used: α_2M, α_2-macroglobulin; PB-protein, papain-binding protein; Dip-F, di-isopropyl phosphorofluoridate; Pms-F, phenylmethanesulphonyl fluoride; Bz-Arg-NPhNO$_2$, benzoyl-L-arginine *p*-nitroanilide; RBB, Remazol Brilliant Blue.

‡ Present address: Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K.

Materials and methods

Materials

Sources of chemicals were as follows: thermolysin (type X), trypsin (bovine, 2× crystallized, type XII), papain (2× crystallized), Dip-F, Pms-F and soya-bean trypsin inhibitor (type II-S) were from Sigma Chemical Co.; Ultrogel AcA-34, agarose-EF and Ampholine (pH 3.5–9.5) for agarose electrofocusing were from LKB Instruments; hide powder was from Calbiochem; Remazol Brilliant Blue was from Hoechst Pharmaceuticals; Carbowax-20m poly(ethylene glycol) was from Raymond Lamb, London N.W.10, U.K.; GelBond was from Marine

0306-3275/82/070097-08$01.50/1

Colloids Division, FMC Corporation, Rockland, ME, U.S.A.; Bz-Arg-$NPhNO_2$ was from Bachem Feinchemikalien, Bubendorf, Switzerland; pancreatic elastase (pig) was a gift from Dr. J. Travis, University of Georgia, Athens, GA, U.S.A.; rabbit anti-(bovine trypsin) serum was a gift from Dr. E. Shapira, The Children's Hospital, Chicago, IL, U.S.A. Human α_2M and rabbit anti-(α_2M) serum were prepared in this laboratory as described by Barrett *et al.* (1979).

Isolation of plaice PB-protein

Plaice (usually 200–300g) were caught throughout the year in shallow water off the Aberdeen coast, and transferred to tanks of aerated sea-water at 11–12°C. Within 2 days of capture bl d was taken from the caudal vein into cooled li· ım heparin-coated plastic tubes (L.I.P. Ltd., Shipley, West Yorkshire, U.K.). After centrifugation, the plasma was decanted with plastic pipettes, adjusted to 10mM-EDTA, 1mg of soya-bean trypsin inhibitor/ml and 0.1% NaN_3, freeze-dried, sent by post from Aberdeen to Cambridge and stored there at −20°C until used.

The freeze-dried plasma was reconstituted by stirring at 4°C overnight in the original volume (30ml) of 0.1M-sodium citrate buffer, pH6.5, containing 10% (v/v) glycerol, 5% (w/v) sucrose, 0.1mM-Pms-F and 1mM-Dip-F. Inhibitors were included to prevent the activation of plasma proteinases that might then react with PB-protein. Any insoluble material was removed by centrifugation, and the sample was applied to a column (5cm × 82cm; 1610cm³) of Ultrogel AcA-34 equilibrated with the same sodium citrate buffer as above but without sucrose or Dip-F, and eluted at a flow rate of 20ml/h. Fractions (14ml) were collected and scanned for protein by A_{280}, and for inhibitory activity against thermolysin and trypsin, and analysed by polyacrylamide-gel pore-limit electrophoresis. Fractions containing PB-protein were pooled and concentrated by dialysis against Carbowax to 15ml. Two such pools were combined, rechromatographed on Ultrogel AcA-34, and the fractions containing PB-protein at constant specific inhibitory activity were pooled, dialysed against 0.1M-sodium citrate buffer, pH6.5, containing 10% (v/v) glycerol to remove the added inhibitors, and concentrated to a protein concentration of about 5mg/ml. This final product was stored at −20°C in 0.1M-sodium citrate buffer, pH6.5, containing 30% (v/v) glycerol and 0.1% NaN_3. Unless otherwise stated, PB-protein solutions were diluted from the stock in 0.1M-sodium citrate buffer, pH6.5, containing 10% (v/v) glycerol.

Assay of proteinase-inhibiting activity

Activity was determined as the inhibition of proteolytic activity against the particulate substrate Remazol Brilliant Blue (RBB)–hide powder essentially as described by Barrett *et al.* (1979). The assay buffer for thermolysin and trypsin was 0.4M-Tris/HCl buffer, pH7.5, containing 40mM-$CaCl_2$ and 0.1% Brij 35, and for papain 0.1M-sodium/potassium phosphate buffer, pH6.0, containing 1.33mM-EDTA and 2.67mM-cysteine. The reaction was stopped with 1.0ml of 0.1M-EDTA for thermolysin, 5M-sodium formate buffer, pH3.0, for trypsin, or 5mM-mersalyl acid (2-{[3-(hydroxymercuri)-2-methoxypropyl]carbamoyl}phenoxyacetic acid) in 25mM-EDTA and 2% (w/v) Brij 35, pH4.0, for papain.

Other assay methods

Active-site titration of trypsin was by the method of Chase & Shaw (1967), and the activity of trypsin against Bz-Arg-$NPhNO_2$ was measured essentially as described by Erlanger *et al.* (1961).

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, or by absorption at 280nm by using $A_{280}^{1\%} = 13.1$ for purified B-protein (Starkey & Barrett, 1982*b*).

Gel electrophoresis

Unless otherwise stated, all polyacrylamide-slab-gel-electrophoresis systems were as described by Barrett *et al.* (1979). The pore-limit gels were run in 50mM-Tris/3mM-EDTA/62.5mM-boric acid buffer, pH8.0, however. Gels were stained for protein as described by Barrett *et al.* (1979).

Isoelectric focusing in agarose-EF

Isoelectric focusing in thin films of agarose-EF with a Pharmacia FBE3000 apparatus was performed by following the manufacturer's instructions, modified as suggested by Rosén *et al.* (1979) for high-M_r proteins.

Results

Assay of the inhibitory activity of PB-protein

Although PB-protein was originally detected in plaice plasma by its ability to bind papain, it was not convenient to use this enzyme in the test-tube assay for inhibitory activity. Being a cysteine proteinase, papain requires activation by thiols, but at relatively low concentrations these reagents inactivate plaice PB-protein (see below). PB-protein was therefore assayed by its ability to inhibit completely the activity of thermolysin against the insoluble substrate RBB–hide powder. Purified samples of PB-protein were also assayed for their inhibitory activity against trypsin. Thermolysin is a bacterial metalloproteinase, and α_2M is the only protein in human plasma to bind and inhibit this enzyme (Werb *et al.*, 1974). In contrast, trypsin is inhibited by several plasma proteins (Heimburger *et al.*, 1971).

Isolation of PB-protein from plaice plasma

The results obtained when plaice plasma was subjected to gel chromatography on Ultrogel AcA-34 are shown in Fig. 1(*a*). A single peak of inhibitory activity against thermolysin was eluted at about 40% bed volume. This active material also demonstrated inhibitory activity against trypsin, and analysis by polyacrylamide-gel pore-limit electrophoresis showed the major protein species to be identical with the PB-protein previously detected by its papain-binding activity.

The major peak of inhibitory activity against trypsin was eluted at about 63% bed volume, but showed no inhibitory activity against thermolysin. The soya-bean trypsin inhibitor in the sample was eluted much later.

The combined concentrated pool of partially purified PB-protein run a second time on the same column of Ultrogel AcA-34 gave a single protein peak eluted at 40% bed volume (Fig. 1*b*), which coincided with the peak of inhibitory activity against trypsin. The specific inhibitory activity of the peak fractions was approximately constant. These fractions were pooled, concentrated and shown to contain PB-protein that was essentially homogeneous in isoelectric focusing in agarose, polyacrylamide-gel pore-limit electrophoresis and polyacrylamide-gel electrophoresis under alkaline conditions (Fig. 2). All the PB-protein used in the experiments described below had been purified by this method.

Typically, this purification scheme gave about 50mg of PB-protein from 60ml of plaice plasma,

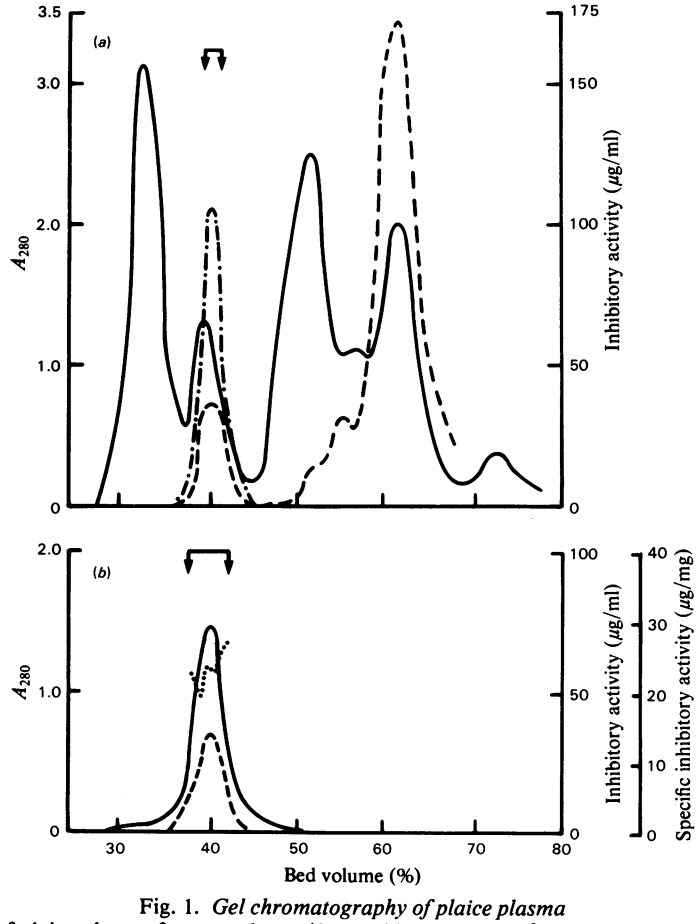

Fig. 1. *Gel chromatography of plaice plasma*

(*a*) Elution of 30ml of plaice plasma from a column (5cm × 82cm; 1610cm³) of Ultrogel AcA-34 equilibrated with 0.1M-sodium citrate buffer, pH6.5, containing 10% (v/v) glycerol and 0.1mM-Pms-F. (*b*) Rechromatography under the same conditions of the concentrated combined active pools from two 30ml samples of plasma run as in (*a*). Fractions (14ml) were assayed for A_{280} (——), inhibitory activity against thermolysin (–·–·–), and inhibitory activity (– – – –) and specific inhibitory activity (·····) against trypsin.

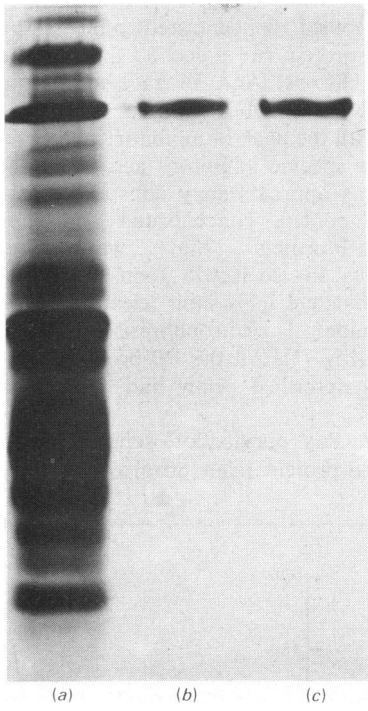

Fig. 2. *Polyacrylamide-gel electrophoresis of PB-protein samples under non-denaturing conditions*

Samples (20 μl) were (*a*) plaice plasma (diluted with an equal volume of water), (*b*) the pool of PB-protein from the first Ultrogel AcA-34 column (7.8 μg of protein) and (*c*) the final pool of PB-protein after the second Ultrogel AcA-34 column (7.7 μg of protein). Electrophoresis was under the standard non-denaturing conditions in gels of acrylamide concentration 5% total, 2.6% cross-linked. Migration was downwards towards the anode.

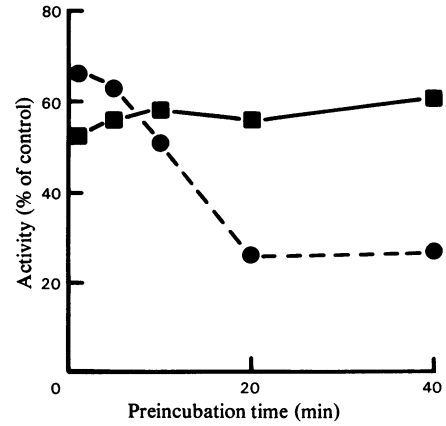

Fig. 3. *Rate of reaction of PB-protein with trypsin and thermolysin*

Trypsin (0.2 μg) (■) was preincubated with PB-protein (1.26 μg) in 0.7 ml of the standard Tris/Ca^{2+} assay buffer, pH 7.5, for 1–40 min before addition of the substrate. Thermolysin (0.2 μg) (●) was preincubated under the same conditions except that the amount of PB-protein was 0.63 μg. Controls contained enzyme but no PB-protein. Activity against RBB–hide powder was determined by the standard method.

a recovery of approx. 28% of the inhibitory activity of plasma against thermolysin.

Characteristics of the reaction of proteinases with PB-protein

PB-protein was found to inhibit the activity against RBB–hide powder of trypsin and elastase (serine proteinases), thermolysin (a metalloproteinase) and papain (a cysteine proteinase). The extent of inhibition increased with increasing concentration of PB-protein to a maximum of 95–98%. The rate of reaction of PB-protein with trypsin was fast (1 min; see Fig. 3), but the reaction of PB-protein and thermolysin was much slower, being complete under these conditions only after 20 min. It was difficult to assess the rate of reaction of PB-protein with papain because the inhibitor was unstable in the cysteine/EDTA/phosphate assay buffer, and the extent of inhibition actually decreased as the preincubation time increased. Maximal inhibition of papain was obtained with a preincubation time of 5 min or less.

For trypsin, 1 mg of PB-protein inhibits 26.6 μg of active enzyme. Assuming an M_r of 390000 for PB-protein (Starkey & Barrett, 1982*b*), and taking the M_r of trypsin to be 23300 (Barman, 1969), this represents a binding ratio of 0.45 mol of trypsin/mol of PB-protein.

To investigate whether proteinases of different catalytic classes react with PB-protein in the same way, PB-protein presaturated with trypsin was tested for its inhibitory activity against thermolysin, and the trypsin-inhibitory activity of PB-protein presaturated with thermolysin was also determined.

PB-protein (1.26 μg in 0.1 ml of assay buffer) was incubated with an excess of trypsin (1 μg in 0.1 ml of the same buffer) for 5 min at room temperature. The sample was adjusted to 2 mM-Pms-F to inhibit the trypsin and then assayed for inhibitory activity against thermolysin by the standard method. Controls were as above but assayed directly without the addition of thermolysin, to check that the trypsin was inactivated by the Pms-F, and as above but with trypsin omitted, to check that Pms-F had no effect on the thermolysin.

The opposite experiment was done by incubating PB-protein (2.5 μg in 0.1 ml of assay buffer), with

thermolysin (2μg in 0.1ml of the same buffer) for 5min or 20min, then adjusting the sample to 10mM-EDTA to inhibit the thermolysin and assaying for inhibitory activity against trypsin by the standard method. Controls were as above but assayed directly without added trypsin, and as above but without the thermolysin.

The results of this experiment are shown in Fig. 4, and demonstrate that pretreatment of PB-protein with an excess of trypsin almost completely abolished the ability of the PB-protein subsequently to inhibit thermolysin [compare (*c*) with (*b*) in Fig. 4]. Similarly, PB-protein preincubated with an excess of thermolysin for 5min or 20min lost 87% or 98% respectively of its inhibitory activity against trypsin [compare (*g*) with (*f*) and (*k*) with (*j*) in Fig. 4]. That the loss of inhibitory activity was not due to degradation of the inhibitor was shown by the unchanged mobility of the PB-protein in non-denaturing electrophoresis after incubation with excess of enzyme. Thus trypsin (a serine proteinase) and thermolysin (a metalloproteinase) react with PB-protein in the same way (and presumably in the same binding ratio). The results after 5 and 20min preincubation with thermolysin confirm that its rate of reaction with PB-protein is slow, and that the increase in inhibition up to 20min is not due to non-specific binding of excess thermolysin.

To investigate whether proteinases need to be catalytically active to be bound by PB-protein, a further experiment was performed with the amounts of reagents and conditions described above, but in which PB-protein was pretreated with Pms-F-inactivated trypsin or EDTA-inactivated thermolysin before being assayed for inhibitory activity against thermolysin or trypsin respectively. The results (Fig. 4) show that neither inactive proteinase decreased the inhibitory activity of the PB-protein against active proteinases added subsequently.

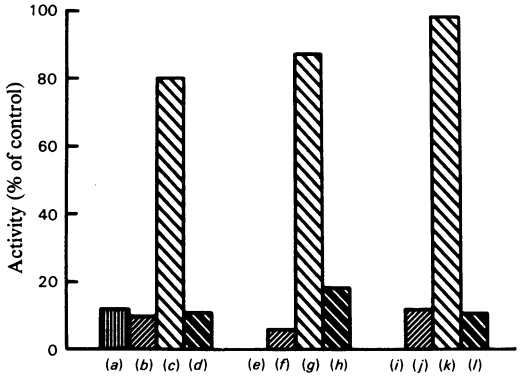

Fig. 4. *Competition between trypsin and thermolysin for binding to PB-protein*

Samples were (*a*) PB-protein incubated with an excess of trypsin for 5min and then with 2.5mM-Pms-F to inactivate the trypsin, (*b*) PB-protein incubated with Pms-F alone for 5min and then with 0.2μg of thermolysin, (*c*) PB-protein incubated with an excess of trypsin for 5min, then with Pms-F and finally with 0.2μg of thermolysin, (*d*) PB-protein incubated with Pms-F-inactivated trypsin and then with 0.2μg of thermolysin, (*e*) PB-protein incubated with an excess of thermolysin for 5min and then with 10mM-EDTA to inactivate the thermolysin, (*f*) PB-protein incubated with EDTA alone for 5min and then with 0.2μg of trypsin, (*g*) PB-protein incubated with an excess of thermolysin for 5min, then with EDTA and finally with 0.2μg of trypsin, and (*h*) PB-protein incubated for 5min with EDTA-inactivated thermolysin and then with 0.2μg of trypsin. (*i–l*) were identical with (*e–h*) except that the initial incubation was for 20min. All mixtures were assayed for activity against RBB–hide powder and activities are expressed as percentages of the activity of 0.2μg of thermolysin (*a–d*) or trypsin (*e–l*) alone. Further details are given in the text.

Properties of proteinases bound to PB-protein

From the results given in the preceding paper (Starkey & Barrett, 1982*a*) it was already known that papain bound to PB-protein retained at least partial activity against a low-molecular-weight synthetic substrate. To investigate further the reactivity of the catalytic sites of enzymes bound to PB-protein, the activity of trypsin against the low-molecular-weight substrate Bz-Arg-NPhNO$_2$ was measured in the presence of increasing amounts of PB-protein. The susceptibility of the bound enzyme to further inhibition by soya-bean trypsin inhibitor (M_r 22500; Vogel *et al.*, 1968) was also tested.

Quadruplicate mixtures of 0.9ml of 0.1M-Tris/HCl buffer, pH8.0, containing 10mM-$CaCl_2$, 2μg of trypsin and 0–140μg of PB-protein were incubated for 10min at 20°C. To half the tubes was added 0.1ml of buffer containing 4μg of soya-bean trypsin inhibitor; the remaining mixtures received buffer alone. Controls contained PB-protein but no trypsin or soya-bean trypsin inhibitor. After a 5min incubation at 40°C, 1ml of the Tris/Ca^{2+} buffer was added to each tube, and the mixtures were assayed at 40°C for activity against Bz-Arg-NPhNO$_2$ by the standard assay method. In a parallel experiment, identical mixtures of trypsin (2μg) and PB-protein (0–140μg) were incubated in a total volume of 1ml for 5min at 20°C as described above. Samples (100μl) were then removed and assayed for activity against RBB–hide powder.

The results with RBB–hide powder (Fig. 5) showed that equivalence was reached at 105–140μg of PB-protein. At this point, the activity of the bound trypsin against the protein substrate was inhibited by 95%, whereas activity against Bz-Arg-NPhNO$_2$ was inhibited by only 38%, and this activity of the bound

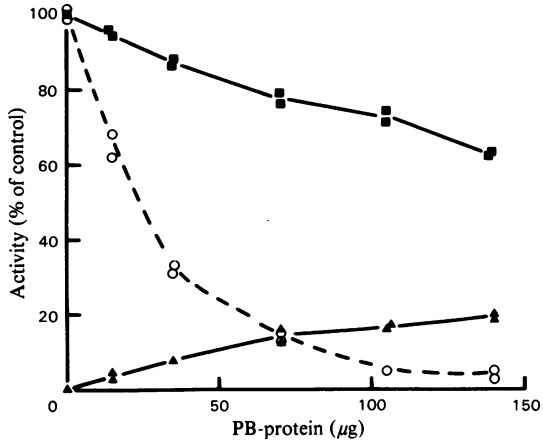

Fig. 5. *Reactivity of the active site of trypsin bound to PB-protein*

Trypsin (2μg) was preincubated for 10min with PB-protein (0–140μg) and then for 5min with (▲) or without (■) soya-bean trypsin inhibitor (4μg) before being assayed for activity against Bz-Arg-$NPhNO_2$. In a parallel experiment, portions containing 0.2μg of trypsin (0–14μg of PB-protein) and no soya-bean trypsin inhibitor were assayed against RBB–hide powder (○). Duplicate samples were tested throughout. Further details are given in the text.

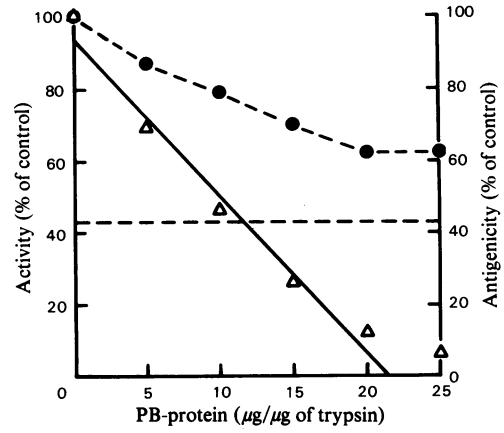

Fig. 6. *Antigenicity of trypsin bound to PB-protein*

Trypsin (0.4μg) was incubated for 10min with 0–9.8μg of PB-protein, and then inactivated with 10mM-Dip-F. The antigenicity of the samples against anti-(trypsin) antibody was measured by radial immunodiffusion and is expressed as a percentage of the control value given by trypsin alone (●). The horizontal broken line indicates the percentage of trypsin found by active-site titration to be catalytically inactive and therefore unable to react with PB-protein. In a parallel experiment, trypsin (0.2μg) was incubated for 10min with PB-protein (0–4.9μg) and then assayed for activity against RBB–hide powder (△). The activity was expressed as a percentage of the control value and the linear regression was computed by least-squares analysis.

enzyme could then be inhibited by a further 68% by added soya-bean trypsin inhibitor. The relatively weak protection of the bound trypsin against soya-bean trypsin inhibitor in this experiment may be compared with values of only 40–57% inhibition by this inhibitor of trypsin bound to other preparations of PB-protein, and may perhaps reflect a change that occurs during storage.

These results showed that the active site of the bound trypsin is accessible to low-molecular-weight substrates such as Bz-Arg-$NPhNO_2$ while being partially protected against medium-molecular-weight inhibitors such as soya-bean trypsin inhibitor, and is completely prevented from reacting with high-molecular-weight proteins such as RBB–hide powder. It was therefore decided to investigate whether the interactions of other parts of the bound enzyme molecule with antibodies were also hindered. Trypsin (2μg in 10μl) was incubated for 10min at 20°C with 10μl of 0.1M-sodium citrate buffer, pH6.5, containing 0–49μg of PB-protein, the mixtures were then adjusted to 10mM-Dip-F and incubated for a further 30min. Duplicate portions of each mixture (5μl) were analysed by radial immunodiffusion (Mancini *et al.*, 1965) in a gel of agarose (1% in 0.1M-sodium citrate buffer, pH6.5, containing 0.1% NaN_3) containing 2% (v/v) of antiserum against bovine trypsin. The diameters of the zones of precipitation were measured after 5 and 7 days.

To determine the equivalence of the samples of trypsin and PB-protein used, trypsin (0.2μg in 0.1ml) was incubated for 10min at 20°C with 0.1ml of 0.1M-sodium citrate buffer, pH6.5, containing 0–4.9μg of PB-protein, and the activity of these mixtures was measured against RBB–hide powder. The proportion of active trypsin in the stock solution was 57%.

From the assays for inhibitory activity, it was calculated that for these samples of PB-protein and trypsin equivalence was reached at a ratio of 21:1 (w/w). The radial-immunodiffusion assays showed that, as the ratio of PB-protein to trypsin increased, the areas of the zones of precipitation progressively decreased (Fig. 6). There was no significant difference between the diameters of the zones of precipitation measured after 5 or 7 days, so that the decrease in antigenicity was not attributable to the lower rate of diffusion of the trypsin–PB-protein complex compared with that of the free enzyme. At equivalence, the reactivity of trypsin with its anti-

body had been decreased by 38%, but, since PB-protein only binds active trypsin, which constituted 57% of the trypsin in the sample, whereas the antibodies presumably recognize both active and inactive enzyme, the maximum amount of trypsin bound to the PB-protein could only represent 57% of the trypsin present. Thus the decrease in antigenicity of bovine trypsin bound to PB-protein can be calculated to be 67%.

Sensitivity of PB-protein to methylamine and dithiothreitol

Human α_2M is unusual in being inactivated by methylamine and low concentrations of dithiothreitol, and so the sensitivity of PB-protein to these reagents was tested. PB-protein (252μg in 0.2ml of sodium citrate buffer adjusted to pH 8 with 20μl of 2M-Tris, pH 9.0), was incubated at 20°C in 0.2M-methylammonium chloride. A control mixture contained no methylammonium salt. After 30min, samples from each mixture were diluted with Tris/Ca^{2+} assay buffer and assayed for inhibitory activity against trypsin in the standard RBB–hide powder assay. The undiluted methylamine-treated PB-protein was dialysed overnight at 4°C against 1 litre of the citrate/glycerol buffer, and then a further portion was removed and assayed for inhibitory activity as described above.

Incubation with methylamine under these conditions was found to abolish 98% of the inhibitory activity of the PB-protein against trypsin. Overnight dialysis to remove the methylamine did not reverse the inactivation.

In a similar experiment, PB-protein (126μg in 0.1ml of the citrate/glycerol buffer adjusted to pH 8) was adjusted to 1mM-dithiothreitol and incubated at 20°C for 30min, before being assayed for inhibitory activity as above. Under these conditions, dithiothreitol abolished 40% of the inhibitory activity of the PB-protein against trypsin, as compared with a control incubated without dithiothreitol.

Discussion

Human α_2M is unique amongst plasma proteinase inhibitors in its mechanism of action (reviewed by Starkey & Barrett, 1977). The reaction of a proteinase with α_2M is initiated by proteolytic attack of the enzyme on a particular region of the macroglobulin polypeptide chain. This cleavage results in a conformational change in the α_2M such that the proteinase becomes physically entrapped within the macroglobulin molecule. As a consequence of this remarkable mechanism, the interaction of proteinases with α_2M has certain characteristic properties that distinguish it from the interaction of proteinases with other inhibitors: (i) endopeptidases of all four catalytic classes are trapped by α_2M, but the enzymes must be catalytically active; (ii) saturation of α_2M with any proteinase prevents subsequent trapping of another proteinase; (iii) all reactions of the trapped proteinase molecule are sterically hindered; (iv) the proteinase molecule is irreversibly bound.

The results described in the present paper show that plaice PB-protein shares many of these distinctive properties. (i) PB-protein inhibits papain (a cysteine proteinase), trypsin and elastase (serine proteinases) and thermolysin (a metalloproteinase). (Its ability to inhibit aspartic proteinases was not tested.) Only catalytically active forms of trypsin and thermolysin react. (ii) Saturation of PB-protein with trypsin prevented subsequent inhibition of thermolysin, and vice versa. (iii) For trypsin bound to PB-protein, the catalytic site of the bound enzyme displayed steric hindrance, being almost completely inhibited against a high-molecular-weight protein substrate, but only 38% inhibited against a low-molecular-weight synthetic substrate. Similarly, the remaining activity of the bound trypsin was 32% protected against further inhibition by soya-bean trypsin inhibitor. That the steric hindrance was not limited to the catalytic site, but affected the interactions of the whole molecule, was shown by the decreased antigenicity of the bound trypsin to antibodies directed against it. The plaice protein thus exhibits three of the four distinctive properties of α_2M. We obtained no direct evidence that the trapping of proteinases by PB-protein is irreversible, but the fact that PB-protein can be prevented by saturation with one proteinase from inhibiting another proteinase, and that proteinase–PB-protein complexes may be isolated electrophoretically (Starkey & Barrett, 1982*a*,*b*), is strong indirect evidence that the binding of the enzyme by PB-protein is irreversible or at least extremely tight.

In addition to these striking similarities in inhibitory properties between α_2M and PB-protein, the plaice protein also shares the unusual sensitivity of α_2M to inactivation by low concentrations of dithiothreitol and by methylamine (Barrett *et al.*, 1979). Apart from α_2M, only the human complement proteins had previously been reported to be sensitive to inactivation by amines (Gordon *et al.*, 1926; Ratnoff *et al.*, 1954; von Zabern *et al.*, 1980) and low concentrations of dithiothreitol (R. B. Sim, unpublished work cited by Sim & Sim, 1981).

Plaice PB-protein thus shares so many of the distinctive properties of human α_2M that it seems reasonable to conclude that the two proteins share a common ancestor, i.e. that PB-protein is an α-macroglobulin homologue.

Since PB-protein has an M_r about half that of α_2M (Starkey & Barrett, 1982*b*), each molecule might be expected to trap half as many enzyme molecules as does a molecule of α_2M, i.e. one enzyme molecule

per molecule of PB-protein. However, the molar ratio determined experimentally for the trapping of trypsin by PB-protein was 0.45. For this molar ratio to be correct, two molecules of PB-protein would have to co-operate to trap each enzyme molecule in complexes of M_r about 800000. This unlikely scheme is at variance with the observed occurrence of complexes of M_r 400000 only (Starkey & Barrett, 1982*b*). More reasonable explanations of the low trapping ratio might be that the true ratio is equimolar, and that either the PB-protein is only 45% active or the efficiency of the trapping reaction is less than 100%, i.e. not every enzyme molecule that cleaves PB-protein is trapped by the inhibitor. A combination of the two possibilities seems most likely. We have no way of determining the concentration of inactive PB-protein in our preparations because, unlike α_2M, PB-protein shows no change in electrophoretic mobility on reaction with a proteinase or becoming inactivated (Starkey & Barrett, 1982*b*). However, trapping ratios determined with three different preparations of PB-protein were very similar, and, though it is possible that all preparations were inactivated to the same extent, it seems most probable that plaice PB-protein has a trapping efficiency of about 50% under the conditions that we used.

Preliminary studies (T. C. Fletcher & P. M. Starkey, unpublished work) with an antiserum against plaice PB-protein have shown this inhibitor to be a major plasma protein. Plaice were found to have a plasma protein concentration of about 28 mg/ml, of which 3 mg/ml was PB-protein, about 10% of the total. Experiments performed *in vivo* have demonstrated that intravenous injection into plaice of active thermolysin (600 μg of enzyme/100 g body wt.) caused plasma PB-protein concentrations to fall by 66% in 5 h and by 75% in 30 h. Control injections of saline or EDTA-inactivated thermolysin caused no decrease. This clearance of PB-protein from the circulation after the injection of active enzyme further supports the idea that the plaice protein is an α-macroglobulin homologue and fulfils the same physiological role as α_2M in binding active endopeptidases and removing them from the circulation.

References

Barman, T. E. (1969) *Enzyme Handbook*, vol. 2, p. 618, Springer-Verlag, Berlin

Barrett, A. J. & Starkey, P. M. (1973) *Biochem. J.* **133**, 709–724

Barrett, A. J., Brown, M. A. & Sayers, C. A. (1979) *Biochem. J.* **181**, 401–418

Chase, T. & Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* **29**, 508–514

Erlanger, B. F., Kokowsky, N. & Cohen, W. (1961) *Arch. Biochem. Biophys.* **95**, 271–278

Gordon, J., Whitehead, H. R. & Wormall, A. (1926) *Biochem. J.* **20**, 1028–1035

Heimburger, N., Haupt, H. & Schwick, H. G. (1971) *Proc. Int. Res. Conf. Proteinase Inhibitors 1st* (Fritz, H. & Tschesche, H., eds.), pp. 1–22, Walter de Gruyter, Berlin

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235–254

Ratnoff, O. D., Lepow, I. H. & Pillemer, L. (1954) *Bull. Johns Hopkins Hosp.* **94**, 169–179

Rosén, A., Ek, K. & Åman, P. (1979) *J. Immunol. Methods* **28**, 1–11

Sim, R. B. & Sim, E. (1981) *Biochem. J.* **193**, 129–141

Starkey, P. M. & Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 663–696, North-Holland, Amsterdam

Starkey, P. M. & Barrett, A. J. (1982*a*) *Biochem. J.* **205**, 91–95

Starkey, P. M. & Barrett, A. J. (1982*b*) *Biochem. J.* **205**, 105–115

Vogel, R., Trautschold, I. & Werle, E. (1968) *Natural Proteinase Inhibitors*, pp. 9–29, Academic Press, New York

von Zabern, I., Nolte, R. & Vogt, W. (1980) *J. Immunol.* **124**, 1543

Werb, Z., Burleigh, M. C., Barrett, A. J. & Starkey, P. M. (1974) *Biochem. J.* **139**, 359–368