Evolution of α_2 -macroglobulin

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

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The plaice (*Pleuronectes platessa* L.) papain-binding protein previously demonstrated to be homologous with human α_2 -macroglobulin, and designated plaice α_2 -macroglobulin homologue or α Mh, was shown to be a glycoprotein of $s_{20,w}$ 11.86S. In polyacrylamide-gel pore-limit electrophoresis under non-denaturing conditions plaice α Mh migrated to the same position as half-molecules of human α_2 -macroglobulin, and treatment with methylamine or a proteinase caused no change in its electrophoretic properties. Either denaturation in urea (4 M) or mild reduction by dithiothreitol (1 mM) partially dissociated plaice α Mh into half-molecules. Denaturation with reduction further dissociated the protein into quarter-subunits. In sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reducing conditions plaice α Mh dissociated into subunits of M_r 105 000 (I) and 90 000 (II). Approximately equal amounts of each subunit were formed, and peptide 'mapping' showed subunits I and II to be distinct polypeptide chains. Under alkaline denaturing conditions, a proportion of the I chains of α Mh were cleaved into fragments of M_r about 60 000 and 40 000. This cleavage was favoured by reducing conditions and prevented by prior inactivation of the α Mh with methylamine. [14 C]Methylamine allowed to react with α Mh became covalently linked to subunit I. These properties suggested the existence of an autolytic site on subunit I analogous to the autolytic site of human α_2 -macroglobulin. Reaction of α Mh with a proteinase resulted in cleavage of a fragment of M_r 10 000–15 000 from subunit I. A proportion of the proteinase molecules trapped by α Mh became covalently linked to the inhibitor. A scheme is proposed for the evolution of human α_2 -macroglobulin and plaice α Mh from a common ancestral protein, which may also have been an ancestor of complement components C3 and C4.

The preceding papers (Starkey & Barrett, 1982; Starkey *et al.*, 1982) have demonstrated that a variety of animal species comprising mammals, birds, reptiles and amphibians possess a papain-binding plasma protein similar to human α_2 -macroglobulin and of the same M_r (725 000), whereas all fish species tested, including representatives of the dipnoans, actinopterygians, elasmobranchs and

cyclostomes, have a papain-binding protein of M_r 360 000. Despite its lower M_r , however, the papain-binding protein of plaice (*Pleuronectes platessa* L.) plasma was shown to share so many of the distinctive inhibitory properties of human α_2 M that it must be considered homologous with the larger human protein.

Human α_2 M is a glycoprotein consisting of four identical polypeptide chains, M_r 182 000 (Barrett *et al.*, 1974). The quarter-subunits are linked in pairs by disulphide bonds, and the half-molecules associate by non-covalent interactions. Native α_2 M exists in two molecular conformations differing in their mobilities in polyacrylamide-gel electrophoresis and known as S ('slow')- and F ('fast')- α_2 M. Only S- α_2 M binds and inhibits proteinases (Barrett *et al.*, 1979).

Abbreviations used: α_2 M, α_2 -macroglobulin; α Mh, α -macroglobulin homologue; Dip-F, di-isopropyl phosphorofluoridate; Z-Arg-NNapOMe, benzyloxycarbonyl-L-arginine 2-(4-methoxy)naphthylamide; SDS, sodium dodecyl sulphate.

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Reaction of S- α_2 M with a proteinase is initiated by proteolytic attack of the enzyme on a particular region in the middle of the quarter-subunit polypeptide chain known as the 'bait' region (Barrett *et al.*, 1974). As a result of this cleavage, α_2 M changes conformation from the S- to the F-form, and the enzyme is physically trapped within the macroglobulin molecule. The bound enzyme retains its catalytic activity, though access of substrates, inhibitors and antibodies to the proteinase is sterically hindered (Barrett & Starkey, 1973; Starkey & Barrett, 1977).

When S- α_2 M is incubated in denaturing conditions a certain proportion of the quarter-subunits undergo cleavage at a point one-third of the way from the C-terminus. The proportion cleaved increases as the temperature and/or the pH increases, but is decreased by prior reduction of the α_2 M. F- α_2 M is not cleaved. The cleavage is not due to contaminating proteinases, but is a property of the α_2 M itself, and has been described as 'autolytic cleavage' (Barrett *et al.*, 1979; Harpel *et al.*, 1979; Howard *et al.*, 1980; Sim & Sim, 1981). It is now believed that the susceptible bond is adjacent to a glutamic acid residue that forms a thiol ester with a nearby cysteine residue. A chemical mechanism for the autolytic cleavage of the peptide bond has been proposed (Salvesen *et al.*, 1981). In the unchanged α_2 M the thiol ester is protected within the molecule, but on conversion into F- α_2 M or denaturation it becomes exposed and reacts with any nucleophilic species present. When S- α_2 M reacts with a proteinase, a proportion of the trapped enzyme molecules become covalently linked to the α_2 M through reaction of a lysine side chain with the autolytic site. The proportion of proteinase molecules covalently bound varies between enzymes, but covalent binding is not essential to the trapping and inhibition of proteinases by α_2 M, and can be inhibited by competing low-molecular-weight nucleophiles without affecting the trapping and inhibition of proteinases by the macroglobulin. Methylamine is able to react with the thiol ester in unchanged S- α_2 M and in so doing becomes covalently attached to the glutamic acid residue, preventing subsequent autolytic cleavage (Swenson & Howard, 1979; Howard *et al.*, 1980; Salvesen & Barrett, 1980; Salvesen *et al.*, 1981).

In the present paper we have studied the structure of the plaice α_2 -macroglobulin homologue (α Mh) in the hope of increasing our knowledge of the mechanism of action of human α_2 M and providing some insight into its evolution.

Materials and methods

Materials

Sources of chemicals, and methods in addition to those mentioned in the preceding papers (Starkey &

Barrett, 1982; Starkey *et al.*, 1982), were as follows: *Staphylococcus aureus* V8 proteinase was from Miles Laboratories; Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) was from Pierce and Warriner, Chester, Cheshire, U.K., and Na¹²⁵I (essentially carrier-free) and [¹⁴C]methylamine were from The Radiochemical Centre. Plaice α Mh, unless otherwise stated, was in 0.1 M-sodium citrate buffer, pH 6.5, containing 10% (v/v) glycerol, and was inactivated with methylamine by incubation at 5 mg/ml (in citrate buffer adjusted to pH 8.0 by the addition of 0.1 M-Tris/HCl buffer, pH 9.0) with 0.2 M-methylamine for 2 h.

Carbohydrate analyses

Carbohydrate analyses were kindly performed by Professor J. Clamp, Department of Medicine, University of Bristol, Bristol, U.K., as described by Clamp (1977).

Sedimentation-velocity ultracentrifugation

Sedimentation-velocity ultracentrifugation of α Mh (4.3 mg/ml) was kindly done by Mr. Neville Buttress, A.R.C. Institute of Animal Physiology, Babraham, Cambridge, U.K. in a Beckman model E analytical ultracentrifuge:

Radiochemical methods

Pancreatic elastase was labelled with ¹²⁵I by the Iodogen method of Fraker & Speck (1978) as modified by Salvesen *et al.* (1981). Radioactivities of samples were counted in a Packard BPGD Auto-gamma counter.

Gel electrophoresis

Conditions for SDS/polyacrylamide-slab-gel electrophoresis were as described by Barrett *et al.* (1979), except that samples were as a routine incubated for 30 min at 37°C.

Peptide 'mapping' in polyacrylamide gels (15% total, 2.6% cross-linked) of α Mh subunits subjected to limited proteolysis with *Staphylococcus aureus* V8 proteinase was as described by Salvesen & Barrett (1980).

Gels were stained for glycoprotein by the method of Zacharius *et al.* (1969). Densitometry of stained gels was as described by Salvesen & Barrett (1980). For radioautography, polyacrylamide gels dried on to filter paper (Whatman 3MM) were exposed to Fuji Rx-medical X-ray film for 4–7 days. The film was developed with Kodak DX-80 developer in accordance with the manufacturer's instructions. In all Figures of electrophoresis gels, migration was downwards, towards the anode.

Results and discussion

Physical properties of plaice α Mh

Carbohydrate content. Plaice α Mh was shown to be a glycoprotein by its positive reaction in the

periodic acid-Schiff procedure after polyacrylamide-gel electrophoresis under non-denaturing conditions. Carbohydrate analysis of the purified protein showed it to contain 54 residues of mannose, 40 residues of galactose, 5 residues of glucose, 49 residues of *N*-acetylglucosamine and 29 residues of sialic acid per molecule, taking an M_r for α Mh of 390 000 (see below). No lipid was detected.

Optical absorption coefficient. The protein concentrations of three separate preparations of α Mh were determined by the method of Lowry *et al.* (1951) and the mean $A_{280}^{1\%}$ was found to be 13.1.

Sedimentation coefficient. The major sedimenting species in the α Mh preparation, which constituted more than 90% of the protein in the sample, had $s_{20,w}$ 11.86S.

Polyacrylamide-gel pore-limit electrophoresis of α Mh in non-denaturing conditions

α Mh before and after treatment with a proteinase or methylamine. α Mh (50 μ g) was incubated under the conditions described previously for plasma (Starkey & Barrett, 1982), with 0, 2.5, 5.0 or 7.5 μ g of papain; 5 μ g of papain gave 100% saturation of the α Mh. The α Mh/papain samples were analysed by polyacrylamide-gel pore-limit electrophoresis; the gel was then cut in half, and one half was stained for protein and the other for activity against Z-Arg-NNapOMe. Acid-treated α_2 M was included as a source of half-molecules of the human protein.

The results (Fig. 1) showed α Mh to migrate to the

same position as half-molecules of human α_2 M (M_r 360 000). After reaction with papain, there was no evidence of a change in electrophoretic behaviour, and the band of activity staining coincided exactly with the single protein band, regardless of the amount of papain used.

Methylamine-inactivation of α Mh also had no effect on its migration in this system.

α Mh native or dissociated with urea and/or dithiothreitol. α Mh in 50 mM-Tris/HCl buffer, pH 8.0, containing 10% (v/v) glycerol was either incubated with 4M-urea at 20°C for 4h, or reduced in 1 mM-dithiothreitol for 30 min and free thiol groups then alkylated by addition of iodoacetic acid to a final concentration of 3 mM. A sample of the urea-denatured α Mh was also reduced with dithiothreitol, and alkylated as above, and a sample of the reduced α Mh was incubated with urea. α Mh (116 μ g), both treated and untreated, was analysed by polyacrylamide-gel pore-limit electrophoresis.

Denaturation in 4M-urea or reduction with 1 mM-dithiothreitol partially dissociated α Mh into half-molecules, whereas denaturation with reduction (the result was the same whether urea was added before or after the dithiothreitol) further dissociated α Mh into quarter-subunits.

Subunit structure of plaice α Mh

Polyacrylamide-gel electrophoresis of α Mh in denaturing conditions. Samples of plaice α Mh denatured by incubation with SDS in either reducing or non-reducing conditions were analysed by polyacrylamide-gel electrophoresis. Human α_2 M denatured but not reduced, and α_2 M incubated for 5 min with a less-than-saturating concentration of pancreatic elastase, inhibited with Dip-F and then denatured and reduced, were analysed at the same time for comparison.

As is shown in Fig. 2, human α_2 M denatured in non-reducing conditions gave a single band representing the dissociated half-molecules of M_r 360 000 (Barrett *et al.*, 1979). Under denaturing and reducing conditions the α_2 M/elastase mixture gave some intact quarter-subunits, M_r 182 000, as well as the 111 000- M_r and 98 000- M_r fragments produced by the specific cleavage of the α_2 M subunits in the 'bait' region.

Plaice α Mh denatured without reduction gave a major band with a mobility slightly less than that of the α_2 M quarter-subunits. The sample also contained some high-molecular-weight material. Under denaturing and reducing conditions the plaice protein gave two major bands, designated I and II, of M_r 105 000 and 90 000 respectively. A densitometer trace of such a gel indicated that band I consisted of two components, the minor component, designated Ia, having the lower mobility, and suggested that band II might also consist of two

Fig. 1. Polyacrylamide-gel pore-limit electrophoresis of plaice α Mh

Samples were (a) acid-treated human α_2 M, (b) plaice α Mh (35 μ g) and (c and d) plaice α Mh (35 μ g) preincubated with papain (3.5 μ g). (a)–(c) were stained for protein, and (d) was stained for activity against Z-Arg-NNapOMe. The arrow indicates the position of the half-molecule of α_2 M.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of human α_2M and plaice αMh

Samples were (a) human α_2M (10 μg) and (b) plaice αMh (10 μg), both denatured without reduction, (c) plaice αMh (10 μg), and (d) human α_2M (10 μg) partially saturated with pancreatic elastase (2 μg), both denatured and reduced. The M_r values of the α_2M components are indicated.

components of slightly different M_r . The αMh sample shown in Fig. 2 was isolated from pooled plasma, but similar patterns were obtained with preparations of αMh isolated separately from three different individual plasmas, indicating that the complexity of the pattern was not due to variation between individuals. Analysis of five different samples of αMh gave a ratio of bands I/II of 0.8–1.1:1.0. Band Ia comprised 26–29% of band I, although because of the overlap between protein bands these values are only approximate. It thus appears that the plaice αMh molecule contains four polypeptide chains, two I chains and two II chains.

The amino acid sequences of the I and II chains were compared by peptide 'mapping' with *Staphylococcus aureus* V8 proteinase. αMh was subjected to electrophoresis under SDS/reducing conditions, slices of gel (2 mm deep by the width of the sample lane) were cut from that region of the gel containing bands I and II and applied to the top of a second polyacrylamide gel (15% total, 2.6% cross-linked) together with a sample of the *Staphylococcus aureus* V8 proteinase, and electrophoresed as described by Salvesen & Barrett (1980).

The results (Fig. 3) indicate that bands I and II represent two different polypeptide chains, each with its own distinctive amino acid sequence. In order to have sufficient protein on the second-dimension gel, it was necessary to use a very high protein concentration on the first-dimension gel, so that

inevitably the separation between bands I and II was incomplete. Nevertheless, it is clear that the upper gel sections, which contained mainly protein from band I, and the lower sections, which contained mainly band II protein, gave different 'mapping' patterns. This is particularly well illustrated with reference to the bands marked A and B, which can be considered as markers for chains I and II respectively.

Autolytic cleavage of αMh . To investigate whether αMh contains an autolytic cleavage site with the characteristics of that in α_2M , we examined in more detail the behaviour of the plaice αMh under denaturing conditions.

αMh or αMh previously inactivated with methylamine was incubated in SDS under the standard reducing or non-reducing conditions, at 37°C for 30 min or 100°C for 15 min. Samples were also treated at both temperatures with SDS alone, and reduced with 2-mercaptoethanol at the end of the incubation. All samples were then electrophoresed alongside mixtures of standard proteins.

Under non-reducing conditions at 37°C both the untreated (Fig. 4) and the methylamine-inactivated αMh (results not shown) gave a major product, designated band 1a, with a smaller amount of band 1b. At this high loading, several other minor bands were also visible. At 100°C the untreated αMh again gave 1a and 1b as the major bands, with the relative amount of band 1b being perhaps slightly less, but

Fig. 3. Peptide 'mapping' of subunits I and II of plaice α Mh

α Mh subunits I and II were partially separated by electrophoresis under SDS/reducing conditions. The respective gel sections were cut out, incubated with *Staphylococcus aureus* V8 proteinase and the peptide fragments run in a 'mapping' gel system. The brackets indicate the distribution of subunits I and II in the first-dimension gel.

Fig. 4. Autolytic cleavage of plaice α Mh under denaturing conditions

Samples (a)–(c) were incubated under SDS/non-reducing conditions, (d) and (e) under SDS/reducing conditions. Samples were (a) α Mh incubated at 37°C for 30 min, (b) and (e) α Mh incubated at 100°C for 15 min, (c) methylamine-inactivated α Mh incubated at 100°C for 15 min, and (d) methylamine-inactivated α Mh incubated at 37°C for 30 min.

more striking was the appearance of several new protein species. These were band 2, a single discrete fragment of M_r 132 000, and a group of smaller fragments, of which the most abundant, 3a and 3b, had M_r 65 000 and 63 000 respectively. In contrast, the methylamine-inactivated α Mh heated at 100°C showed no evidence of fragments 2, 3a or 3b. At this higher temperature, however, the methylamine-inactivated α Mh gave an increased proportion of the 1b component, compared either with the pattern given by the same α Mh sample incubated at 37°C or with that of untreated α Mh incubated at 100°C.

These results suggest that two separate processes occur when α Mh is denatured in SDS without reduction, both occurring more readily at 100°C than at 37°C. These processes are firstly the conversion of component 1a into component 1b, which is apparently unaffected by the pre-inactivation of the α Mh with methylamine, and secondly the formation of components 2, 3a and 3b, which is completely prevented by methylamine.

In reducing conditions all samples gave the major bands I and II, but α Mh incubated at 100°C showed extra fragments that were present at only very low concentrations in α Mh incubated at 37°C. These were fragments III, IV, V and VI of M_r 63 000, 58 000, 43 000 and 39 000 respectively. At either 37°C or 100°C pre-inactivation of the α Mh with methylamine prevented the generation of fragments III–VI, and at both temperatures α Mh denatured by incubation with SDS alone, and reduced just before electrophoresis, generated higher concentrations of the fragments than did α Mh reduced and denatured at the beginning of the incubation. The generation of fragments III–VI therefore shows the same characteristics as the autolytic cleavage of human α_2 M: it is favoured by denaturation at alkaline pH and by high temperature, disfavoured by prior reduction, and prevented by inactivation with methylamine. Further evidence that the cleavage is autolytic and not due to contamination with proteinases was given by the fact that cleavage was unaffected by the presence or absence of 1 mM-Dip-F, and incubation with added proteinases gave quite different products (see below).

In an attempt to relate the pattern given by α Mh denatured in non-reducing conditions to that obtained in reducing conditions, samples were first prepared under the standard SDS/non-reducing conditions, electrophoresed and the protein bands detected by brief staining as for the 'mapping' gels. The desired sections of gel were cut out, incubated for 30 min at 20°C in upper-reservoir buffer (1.1 ml) containing SDS and 10 mM-dithiothreitol, and then placed in the sample slots of a fresh polyacrylamide gel and electrophoresed under denaturing conditions as usual.

The results obtained with α Mh denatured without reduction at 100°C are shown in Fig. 5. In the

Fig. 5. SDS/polyacrylamide-gel electrophoresis in reducing conditions of components of α Mh generated by denaturation without reduction

Samples had all been incubated in SDS/reducing conditions and were (a) component 1a, (b) component 1b, (c) component 2 and (d) component 3, all derived from α Mh treated at 100°C for 15 min in SDS without reduction. (e) was α Mh not pretreated.

second dimension, band 1a, when reduced, gave all the fragments normally seen with α Mh denatured and reduced at 100°C, i.e. bands I–VI, and band IV could now be seen to be a doublet. Band 1b gave an almost identical pattern though fainter, presumably because band 1b on the first-dimension gel had contained less protein than had band 1a. Band 2 gave bands II, V and VI only, with no detectable subunit I. Band 3 (3a and 3b having been cut out together) gave only bands III, IVa and IVb. Of the other samples, band 1a from α Mh incubated at 37°C gave a pattern identical with that given by band 1a or 1b from α Mh incubated at 100°C. In contrast, both bands 1a and 1b from methylamine-inactivated α Mh incubated at 100°C gave only bands I and II, with no evidence of any smaller fragments.

These results show that, for a given sample of α Mh, bands 1a and 1b contain the same protein species, despite their different electrophoretic mobilities. Components 1a and 1b each represent half-molecules, I–II dimers linked by disulphide bonds (see Fig. 8), and both bands 1a and 1b can consist of a mixture of intact and autolytically cleaved half-molecules. The difference between components 1a and 1b is not due to denaturation to dissimilar half-molecules of I–I and II–II dimer or to autolytic cleavage, but to some other chemical change (loss of amides or carbohydrate, cleavage of disulphides?) at the higher temperature.

Bands 2, 3a and 3b, on the other hand, are generated by autolytic cleavage, and the finding that band 2 comprised intact II chains linked by disulphide bonds to fragments V or VI strongly suggested that the autolytic cleavage site is located on subunit I, dividing the polypeptide chain into fragments of M_r about 40000 and 60000, corresponding to fragments V or VI, and III or IV. More direct evidence for this hypothesis was sought by attempting to link [^{14}C]methylamine covalently to αMh .

αMh (98 μg in 40 μl) was incubated overnight at 20°C at pH 8.0 with 15 μl of [^{14}C]methylamine (25 mM, 1 mCi/ml) before being analysed by electrophoresis under SDS/reducing conditions.

The stained gel showed the usual pattern of protein bands for αMh , and a radioautograph of the dried gel demonstrated a single band of radioactivity corresponding to subunit I; no radioactivity was associated with subunit II. It was concluded that αMh contains a site on subunit I that forms an SDS-stable (probably covalent) link with methylamine. Since such a covalent-linking site has previously been described only in human $\alpha_2\text{M}$ and complement components C3 and C4 (Swenson & Howard, 1979; Tack *et al.*, 1980; Janatova & Tack, 1981), where it is identical with the autolytic site, and since αMh , in common with these proteins, is prevented from autolytic cleavage by reaction with methylamine, it seems almost certain that the methylamine covalent-linking site and the autolytic site of αMh are identical.

Specific cleavage of αMh by a proteinase

Since αMh binds only catalytically active proteinases (Starkey *et al.*, 1982) it seemed likely that, as for $\alpha_2\text{M}$, the interaction of the plaice protein with a proteinase was initiated by specific proteolytic cleavage of the inhibitor.

SDS/polyacrylamide-gel electrophoresis under reducing conditions of αMh before and after saturation with pancreatic elastase suggested that the enzyme might be cleaving a fragment from subunit I of the inhibitor, so altering its mobility to that of subunit II. To test this hypothesis, αMh was labelled specifically in subunit I by overnight incubation with [^{14}C]methylamine as described above. Portions of the [^{14}C]methylamine/ αMh mixture were then incubated at 20°C for 10 min with saturating amounts of either pancreatic elastase or thermolysin (each 50 $\mu\text{g}/\text{mg}$ of αMh). The enzymes were then inhibited by 1 mM-Dip-F or 10 mM-EDTA and the mixtures were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions. Control samples were as above but without the enzymes, or as above but with incubation of the enzymes with the appropriate inhibitor for 10 min before the addition of αMh .

The results (Fig. 6) confirmed that with unchanged αMh the [^{14}C]methylamine became covalently linked to subunit I. On reaction with saturating amounts of elastase or thermolysin the protein band due to subunit I became much fainter, and the radioautograph of the dried gel showed the major band of radioactivity in these samples to have an increased mobility comparable with that of subunit II; elastase gave a smaller increase in mobility than did thermolysin. Controls with inactivated enzymes gave the same pattern as the unchanged αMh . It therefore appears that reaction of a proteinase with αMh involves proteolytic cleavage of subunit I such that a fragment of M_r 10000–15000 is cleaved from one end of the polypeptide chain. In this experiment the labelled αMh had of course been inactivated by its reaction with methylamine, but the fact that inactivated human $\alpha_2\text{M}$ is still cleaved specifically by proteinases in the 'bait' region (Barrett *et al.*, 1979), and that, with the exception of small amounts of additional low-molecular-weight fragments, methylamine-inactivated αMh and active αMh when incubated with proteinases both gave the same pattern of bands in SDS/polyacrylamide-gel electrophoresis, suggest that these results can be applied to the reaction of native αMh with a proteinase.

Fig. 6. SDS/polyacrylamide-gel electrophoresis of [^{14}C]methylamine-labelled αMh with and without a proteinase

Samples contained [^{14}C]methylamine-labelled αMh (16 μg) alone (a and b), with 2 μg of thermolysin (c and d) or with 2 μg of pancreatic elastase (e and f). (a), (c) and (e) were stained for protein; (b) (d) and (f) are the corresponding radioautographs.

Covalent binding of the trapped proteinase to α Mh

The ability of α Mh to form a covalent link with some of the trapped proteinase molecules was investigated by incubating α Mh (168 μ g) with 125 I-labelled elastase (5 μ g) at 20°C for 5 min, inactivating the enzyme with 10 mM-Dip-F for 30 min, and then subjecting the mixture to electrophoresis under standard non-denaturing conditions in a 5% polyacrylamide gel. Protein bands were detected by brief staining, and the α Mh band was cut out, rinsed in upper-reservoir buffer and incubated for 30 min at 37°C in 1 ml of modified SDS/reducing sample buffer containing no glycerol. The gel section was then placed in a sample slot of a 7% polyacrylamide gel and run under standard SDS/reducing conditions simultaneously with a control sample of α Mh incubated with Dip-F-inactivated 125 I-labelled elastase.

The radioautograph of the stained gel (Fig. 7) showed that under denaturing and reducing conditions most of the radioactivity of the α Mh-

125 I-labelled elastase complex ran just behind the dye front with the mobility of free enzyme. However, about 5% of the radioactivity (measured by counting the radioactivities of sections of the dried gel) was localized in a discrete band corresponding to a very faint protein band of mobility between that of the α Mh subunits and that of α Mh half-molecules. No such band was present in the α Mh/inactivated elastase mixture, showing that its appearance was dependent on complex-formation. The band presumably represents enzyme covalently linked to the methylamine-binding autolytic site on subunit I. The fact that only a very small proportion of the enzyme trapped in the α Mh-elastase complex was covalently bound to the α Mh, 95% being released from the complex by denaturation, shows that, as with α_2 M, the covalent binding of the proteinase to the inhibitor is not essential for the trapping of enzyme by α Mh.

Conclusions

From the results obtained by gel electrophoresis of α Mh in both non-denaturing and denaturing systems, we propose a model for the structure of the plaice protein that is depicted schematically in Fig. 8. In the native protein, subunits I and II are linked in heterologous dimers by disulphide bonds, and these dimers associate in pairs by non-covalent interactions. It has not been possible to determine whether reduction with dithiothreitol dissociates the molecule to a mixture of I-I and II-II dimers, or to I-II dimer only, and for this reason we have not specified whether the non-covalent interactions are between homologous or heterologous subunits.

The multiplicity of fragments generated by autolytic cleavage of subunit I under denaturing conditions is a little puzzling. One possibility is that there are two autolytic sites separated by about 20 amino acid residues, but this seems unlikely. A second, more plausible, explanation may be that different α Mh molecules contain variable amounts of carbohydrate. Such heterogeneity would lead to multiple bands in SDS/polyacrylamide-gel electrophoresis, and may be responsible for the difference between subunits 1a and 1b. It should be noted that, with human α_2 M, component III generated by autolytic cleavage also runs as a doublet (Barrett *et al.*, 1979).

We have been unable to obtain direct evidence that α Mh undergoes a conformational change; unlike α_2 M (Barrett *et al.*, 1979), it exhibits no change in migration in polyacrylamide-gel pore-limit electrophoresis after reaction with a proteinase or methylamine. We find it difficult to envisage a mechanism by which the active site of the proteinase is essential for the initial interaction with α Mh, and in which the bound enzyme retains its catalytic activity though being sterically hindered, without implicating a conformational change in the α Mh,

Fig. 7. SDS/polyacrylamide-gel electrophoresis of α Mh- 125 I-labelled elastase complex

Samples were (a and b) plaice α Mh (13.7 μ g) incubated with Dip-F-inactivated 125 I-labelled elastase (5 μ g) and (c and d) α Mh- 125 I-labelled elastase complex, isolated electrophoretically as described in the text, from a mixture containing 50 μ g of α Mh. (a) and (c) were stained for protein; (b) and (d) are the corresponding radioautographs.

Fig. 8. *Proposed subunit structure of plaice α Mh*

The upper part of the Figure depicts the structure of the native protein. The actual number of disulphide bonds linking subunits I and II in heterologous dimers is unknown, but two only are shown for convenience. The non-covalent interactions linking the dimers in pairs are indicated by the stippled area, as it is not known whether these interactions occur between homologous subunits, heterologous subunits or both. In the lower part of the Figure, the subunits and fragments seen in SDS/polyacrylamide-gel electrophoresis of the denatured protein are depicted. These are drawn to scale, based on their M_r values as determined in SDS/polyacrylamide-gel electrophoresis (M_r shown in parentheses), and the sites of specific proteinase cleavage and autolytic cleavage are indicated.

however. The selective clearance of α Mh-proteinase complexes from the circulation *in vivo* (Starkey *et al.*, 1982) is also most easily explicable in terms of a conformational change.

On the basis of the model proposed for α Mh in Fig. 8, there are two mechanisms by which the larger human α_2 M might have evolved from an ancestral protein similar to α Mh. The first requires that the structural genes for subunits I and II of α Mh be adjacent and that a deletion of the 'stop sequence' between the two genes resulted in their being transcribed as a single mRNA, and translated as a single polypeptide chain corresponding to the quarter-subunit of α_2 M. The second explanation requires that the α Mh subunits be synthesized as a precursor of M_r 195 000 that is then post-translationally cleaved by a specific proteinase to give the separate I and II subunits. A mutation that resulted in a change in the amino acid sequence recognized by this specific proteinase could then give a precursor protein that was not cleaved but persisted as the 182 000- M_r subunit of the human protein.

This second explanation seems the most probable, and receives support from recent work on the complement proteins C3 and C4. These, despite their different physiological role, share important properties with α_2 M. Thus all three proteins undergo a conformational change as a result of specific proteolytic attack, all are inactivated by amines, all

undergo an autolytic cleavage in denaturing conditions that is prevented by prior inactivation by methylamine, and all are capable of forming covalent links with other proteins (Porter & Reid, 1979; Campbell *et al.*, 1980; Howard *et al.*, 1980; Janatova & Tack, 1981; Salvesen *et al.*, 1981; Sim & Sim, 1981). Moreover, the same sequence of seven amino acids occurs around the reactive thiol ester bond in complement proteins C3 and C4 and α_2 M, and additional sequence homologies have also been reported (Swenson & Howard, 1979; Tack *et al.*, 1980; Campbell *et al.*, 1981). In the light of these results it was first suggested by Sim & Sim (1981) that α_2 M and complement proteins C3 and C4 might share a common evolutionary origin. Each complement protein is synthesized as a single-chain precursor of M_r about 200 000 that is post-translationally cleaved to two chains in protein C3 and three in protein C4 (Brade *et al.*, 1977; Gigli, 1978; Goldberger & Colten, 1980). If the quarter-subunit of α_2 M is aligned with the precursor forms of complement proteins C3 and C4 as shown in Fig. 9, it can be seen that in all three the site of specific proteolytic cleavage is roughly half way and the autolytic site two-thirds of the way from the *N*-terminus. By making assumptions for plaice α Mh about which end of subunit I is the *N*-terminus, it is possible to align the α Mh subunits as on a putative precursor molecule to fit the same pattern.

Fig. 9. Comparison of the subunits of human α_2M , plaice αMh and human complement components C3 and C4. This Figure, based on the scheme given by Sim & Sim (1981) with the addition of the αMh data, depicts the quarter-subunit of human α_2M , and the polypeptide chains of plaice αMh and human complement proteins C3 and C4 drawn to scale on the basis of the M_r values indicated by SDS/polyacrylamide-gel electrophoresis. The polypeptide chains of proteins C3 and C4 are aligned as they occur in the single-chain precursor molecules, and they and the quarter-subunit of α_2M are orientated with the *N*-terminus at the left. The orientation and order of the αMh subunits in its putative precursor molecule are speculative. The positions of the sites of specific proteolytic cleavage and autolytic cleavage on each molecule are indicated by arrows.

We therefore propose that the larger α_2M quarter-subunit arose from the two αMh subunits by loss of the post-translational cleavage site, this mutation probably occurring in an early tetrapod (Starkey & Barrett, 1982). It is likely that the mutation to the larger single type of subunit made possible new subunit interactions that led to the formation of the tetrameric 725000- M_r molecule in place of the smaller fish molecule. However, in the absence of any structural studies on the macroglobulins of species other than mammals and actinopterygians, it cannot be ruled out that the changes in subunit size and molecular size occurred at different stages of evolution. The increased subunit interactions possible in the larger α_2M may well have facilitated the conformational change on reaction with a proteinase and thus contributed to the apparently greater efficiency of the human protein compared with plaice αMh in trapping proteinases (Starkey *et al.*, 1982).

It is known that elasmobranchs and actinoptery-

gians contain amine-sensitive complement components that are presumably homologues of proteins C3 and/or C4 (Gigli & Austen, 1971), and Nonaka *et al.* (1981*a,b*) have reported the isolation from rainbow-trout plasma of homologues of proteins C3 and C5. In view of their apparent common evolutionary origin, it would clearly be of great interest to compare the structure of αMh and of homologues of proteins C3 or C4 in elasmobranchs or in the most primitive living vertebrates, the cyclostomes.

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