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Evolution of α_2-macroglobulin

The demonstration in a variety of vertebrate species of a protein resembling human α_2-macroglobulin

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Plasma or serum samples from a large number of vertebrate species were screened for the presence of a papain-binding protein resembling human α_2-macroglobulin (α_2M). The screening method depended on the unique property of α_2M of binding proteinases in such a way that the enzyme retains partial activity against low-molecular-weight substrates. A papain-binding protein was detected in serum from members of all the major vertebrate taxa. In mammals, birds, reptiles and amphibians the protein had an M_r similar to that of human α_2M (725000), but in fish, including dipnoans, actinopterygians, elasmobranchs and cyclostomes, the papain-binding protein was of M_r about 360000. Of the invertebrate species tested, all of which were arthropods, two were negative, but the horseshoe crab, an arachnid, did possess a papain-binding protein, although this was heterogeneous in electrophoresis and differed from α_2M in resisting inactivation by methylamine. From the results, and a detailed study of the properties of the fish papain-binding protein described in an accompanying paper [Starkey, Fletcher & Barrett (1982) *Biochem. J.* **205**, 97–104], it seems that α_2M first appeared in an ancestor of all modern vertebrates as a protein of M_r 360000 and that the larger macroglobulin (M_r 725000) first appeared in an ancestor of the tetrapods.

Human α_2-macroglobulin (α_2M), a plasma glycoprotein of M_r 725000 (Jones *et al.*, 1972), has the unique property of binding and inhibiting the great majority of proteinases regardless of their catalytic mechanism (Barrett & Starkey, 1973). Apart from its broad specificity for a whole functional group of enzymes, the endopeptidases, α_2M is also unique among protein proteinase inhibitors in the molecular mechanism of its reaction with enzymes. Binding of an endopeptidase by α_2M is initiated by proteolytic attack of the enzyme on a particular region of the macroglobulin polypeptide chain, and the cleavage of a peptide bond in this 'bait' region results in a conformational change in the α_2M such that the proteinase becomes physically entrapped within the macroglobulin molecule. This 'trapping' mechanism is responsible for the distinctive characteristics of the interaction of proteinases with α_2M that distinguish the macroglobulin from other proteinase inhibitors. The active site of the enzyme is necessary for the initial interaction with α_2M, but is not involved in the maintenance of binding to the macroglobulin, and the bound enzyme thus retains partial reactivity with low-molecular-weight substrates and inhibitors, although it is sterically hindered from reacting with larger molecules (Barrett & Starkey, 1973; Starkey & Barrett, 1977).

In view of the important physiological role that seems to be played by α_2M in the clearance of active endopeptidases from the circulation (reviewed by Starkey & Barrett, 1977), it was decided to study the distribution in other animal species of proteins resembling the human protein. Similar studies have been undertaken previously, but these have been limited to mammalian species. Proteins resembling human α_2M have been isolated and characterized from rabbit (Picard & Heremans, 1963; Got *et al.*, 1965; Lebreton de Vonne & Mouray, 1968), dog (Ohlsson, 1971), rat (Ganrot, 1973; Nieuwenhuizen *et al.*, 1979), horse (Pepper, 1968; Lavergne & Raynaud, 1970), mouse (Greene *et al.*, 1971), pig (Jacquot-Armand & Guinand, 1967; Tsuru *et al.*, 1975), cow (Nagasawa *et al.*, 1970) and hedgehog (Picard *et al.*, 1966).

Comparative studies have also been described, but

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Abbreviations: IgG, immunoglobulin G; IgM, immunoglobulin M; α_2M, α_2-macroglobulin; Z-Arg-NNapOMe, benzyloxycarbonyl-L-arginine 2-(4-methoxy)naphthylamide.

these have relied on the demonstration of immunological cross-reactivity between species by use of antisera raised in one species against the macroglobulin of another (Bütler & Brunner, 1967; Berne *et al.*, 1971, 1972; Shortridge *et al.*, 1976). The results of such studies reflect the properties of the antisera as much as those of the cross-reacting antigens, and moreover the antisera recognize only proteins that are structurally very closely related. Such an approach does not permit one to survey a wide range of animal species.

The screening method that we selected for detecting proteins resembling human α_2M was designed to identify proteins with similar binding characteristics rather than similar structure, so as to allow a survey of all the major vertebrate groups. Thus we screened plasma or serum samples for the presence of proteins sharing with human α_2M the capacity to form complexes with a proteinase such that the bound enzyme retained activity against low-molecular-weight substrates (Barrett & Starkey, 1973). The proteinase we chose to use was papain, a plant cysteine proteinase. This was considered preferable to a serine proteinase such as trypsin, for two main reasons: firstly, because α_2M is quantitatively the major protein in human plasma that binds cysteine proteinases (Barrett & Starkey, 1973; Ryley, 1979), whereas several inhibitors of serine proteinases are present (Heimburger *et al.*, 1971), and, secondly, because we have developed a sensitive colour reaction for locating cysteine proteinases after polyacrylamide-gel electrophoresis (Starkey & Barrett, 1973). Samples were accordingly incubated with papain and analysed by pore-limit electrophoresis; the gels were then stained to locate papain-binding proteins by use of a low-molecular-weight chromogenic substrate.

Materials and methods

Materials

Papain (2× crystallized) was from Sigma Chemical Co., and Z-Arg-NNapOMe was from Bachem Feinchemikalien, Bubendorf, Switzerland.

The serum samples, except those mentioned below, were pooled samples from groups of animals at the London Zoo, and were a gift from Dr. D. E. Bidwell, Nuffield Institute of Comparative Medicine, The Zoological Society of London, London N.W.1, U.K. Other gifts were as follows: elasmobranch plasma samples were from Mr. Nigel Downing, Department of Zoology, University of Cambridge, Cambridge, U.K., plaice plasma was from Dr. T. C. Fletcher, N.E.R.C. Institute of Marine Biochemistry, Aberdeen, Scotland, U.K., hagfish and lamprey sera were from Mr. Finn Walvig, Biologisk Stasjon, Universitetet i Oslo, Dröbak, Norway, and *Limulus polyphemus* haemolymph was from Dr. G. W. Warr, Department of Biochemistry, Medical University of South Carolina, Charleston, SC, U.S.A. All the serum and plasma samples were stored at −20°C before use.

Detection of papain-binding protein

A sample (25 μl) of serum or plasma was mixed with 25 μl of 0.1 M-Tris/HCl buffer, pH 7.0, containing 2 mM-EDTA, 1.25 mM-cysteine and 6.25 μg of papain. The papain had been preactivated for 5 min at 1 mg/ml in the same buffer containing 5 mM-cysteine. The mixture was incubated at 20°C for 15 min, in parallel with control samples containing no papain.

Samples were electrophoresed in slab gels containing concave gradients of polyacrylamide concentration (4–26%) as described by Barrett *et al.* (1979), except that the buffer was 50 mM-Tris/3 mM-EDTA/62.5 mM-boric acid buffer, pH 8.0.

After electrophoresis, gels were stained for protein (Barrett *et al.*, 1979) or for activity against Z-Arg-NNapOMe as described by Starkey & Barrett (1973), except that the colour reagent was prepared by diluting 1.1 ml of freshly diazotized Fast Garnet (4-amino-2′,3-dimethylazobenzene) (Barrett, 1976) to 50 ml with 5 mM-mersalyl acid (2-{[3-(hydroxymercuri)-2-methoxypropyl]carbamoyl}phenoxyacetic acid) in 25 mM-EDTA, pH 4.0.

Preparation of acid-treated α_2M

Human α_2M prepared as described by Barrett *et al.* (1979) (15 mg/ml) was incubated for 30 min at 20°C in 50 mM-sodium citrate buffer, pH 6.5, with an equal volume of 1 M-glycine/NaOH buffer, pH 2.0. Before use the acid-treated α_2M was diluted with water to 1.5 mg/ml.

Results

Serum or plasma samples from a wide range of vertebrate species were incubated with papain, subjected to polyacrylamide-gel pore-limit electrophoresis and the gels stained for enzymic activity. The results are summarized in Table 1, and some obtained with representative species of the major vertebrate taxa are shown in Fig. 1.

A papain-binding protein was detected in the plasma of species from all the major vertebrate taxa: mammals, birds, reptiles, amphibians, dipnoans, actinopterygians (bony fish), elasmobranchs (cartilaginous fish) and cyclostomes (lamprey and hagfish). In the first four of these taxa, comprising the tetrapods, the papain-binding protein appeared to have an M_r very similar to that of human α_2M (725 000). The papain-binding protein demonstrated in all the fish species tested was smaller, however, corresponding to an M_r about half that of human α_2M (Fig. 1).

Table 1. *Distribution of a papain-binding protein in the serum (or plasma) of vertebrate species*
The classification of species is based on that of Wiley (1979), and the size of the papain-binding protein is indicated by L (M_r approx. 725000) or S (M_r approx. 360000).

Classification	Species	Size of papain-binding protein
Mammals	Man (*Homo sapiens*)	L
	Chimpanzee (*Pan troglodytes*)	L
	Rhesus monkey (*Macaca mulatta*)	L
	Night monkey (*Aotes trivergatus*)	L
	Hedgehog (*Erinaceus europaeus*)	L
	Squirrel (*Sciurus vulgaris*)	L
	Tiger (*Panthera tigris*)	L
	Asian elephant (*Elephas maximus*)	L
	Camel (*Camelus ferus*)	L
	Wallaby (*Wallabia rufogrisea*)	L
Birds	Canada goose (*Branta canadensis*)	L
Crocodiles and alligators	Chinese alligator (*Alligator sinensis*)	L
Lizards and snakes	Nile monitor (*Varanus niloticus*)	L
	Boa constrictor (*Boa constrictor*)	L
	Indian python (*Python molurus*)	L
Amphibians	American bullfrog (*Rana catesbeiana*)	L
	European frog (*Rana temporaria*)	L
Dipnoans	African lungfish (*Protopterus amphibius*)	S
Actinopterygians	*Reinhardtius hippoglossoides*	S
	Brosme brosme	S
	Halibut (*Hippoglossus hippoglossus*)	S
	Mora moro	S
	Plaice (*Pleuronectes platessa*)	S
Elasmobranchs	Guitar fish (*Rhinobatus cerniculas*)	S
	Sawfish (*Pristis pectinatus*)	S
	Dogfish (*Squalus acanthus*)	S
Cyclostomes	Hagfish (*Myxine glutinosa*)	S
	Lamprey (*Petromyzon marinus*)	S

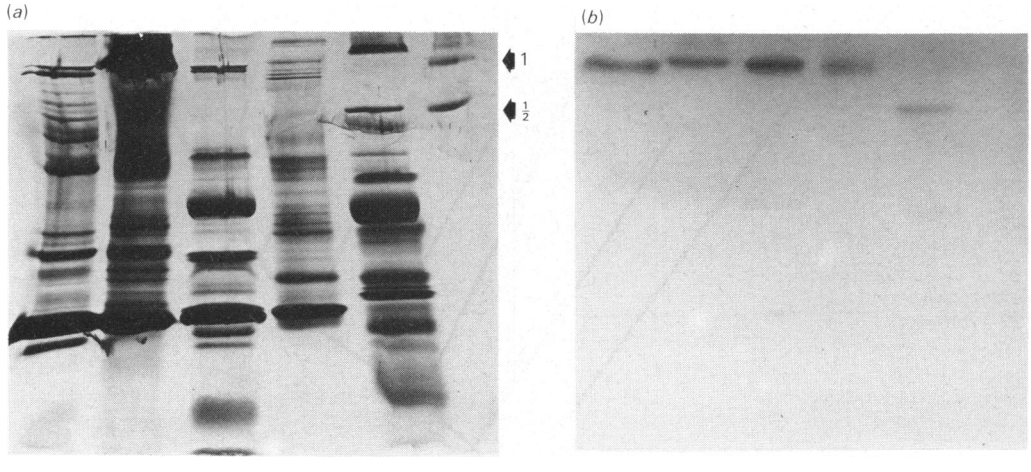

Fig. 1. *Polyacrylamide-gel pore-limit electrophoresis of plasma samples incubated with papain*
Electrophoresis was in gels of increasing acrylamide concentration (4–26%) and migration was downwards towards the anode. Samples were (i) human plasma, (ii) alligator plasma, (iii) Canada-goose plasma, (iv) frog plasma and (v) plaice plasma, each incubated with papain as described in the Materials and methods section, and (vi) acid-treated human α_2M. The arrows indicate the bands due to whole molecules (1) and half-molecules ($\frac{1}{2}$) of human α_2M. (*a*) shows staining for protein, and (*b*) staining for activity against Z-Arg-NNapOMe.

Haemolymph samples from a limited number of invertebrate species were tested in the same way for the presence of a papain-binding protein. Two, shore crab (*Carcinus maenus*) and silk moth (*Bombyx mori*), contained no detectable papain-binding protein. In contrast, haemolymph samples from the horseshoe crab (*Limulus polyphemus*) did show papain-binding activity. The staining was rather diffuse and extended from a region corresponding to that of intact human α_2M to that of half-molecules. This diffuse staining was not an artifact due to the ionic strength of the haemolymph, since it was unaffected by prior dialysis of the sample. Control samples of haemolymph alone showed no activity staining, and neither did haemolymph incubated with papain in the presence of 10mM-$HgCl_2$ to reversibly inhibit the enzyme, showing that the papain-binding protein of *Limulus* bound only active enzyme.

Human α_2M is unusual in being readily inactivated by incubation with methylamine at alkaline pH values (Barrett *et al.*, 1979), and the sensitivity of the *Limulus* protein to methylamine was therefore tested. *Limulus* haemolymph (25μl) previously dialysed against 50mM-sodium citrate buffer, pH 6.5, was mixed with 25μl of 0.4M-methylamine in 0.1M-Tris/HCl buffer, pH 8.0 (final pH of mixture 7.9), and incubated at 20°C for 2h. A control sample of haemolymph was incubated with the Tris/HCl buffer without methylamine. Methylamine-treated and control samples were then separately incubated with activated papain and electrophoresed under the standard conditions. Both control and methylamine-treated samples showed the usual diffuse patterns of activity staining, indicating that the *Limulus* papain-binding protein, unlike human α_2M, is resistant to inactivation by methylamine.

Discussion

The method that we have used to detect proteins resembling human α_2M relies on the unique property of the macroglobulin of binding proteinases in such a way that the bound enzyme retains at least partial activity against low-molecular-weight substrates. Proteinase molecules complexed with any other known proteinase inhibitor (apart from specific antibodies) are catalytically inactive. Thus, although an inhibitor specific for cysteine proteinases is present in human plasma (Sasaki *et al.*, 1977; Ryley, 1979), this would not have affected our screening method. Confirmation of the specificity of the method was given by the subsequent characterization of the fish papain-binding protein: the protein from plaice (*Pleuronectes platessa* L.) was shown to have inhibitory properties almost identical with those of human α_2M, indicating that, despite its smaller molecular size, the fish protein resembles α_2M so strongly that it must be considered as a homologue of the human macroglobulin (Starkey *et al.*, 1982).

The lowest species in which we could detect a protein closely resembling human α_2M were the

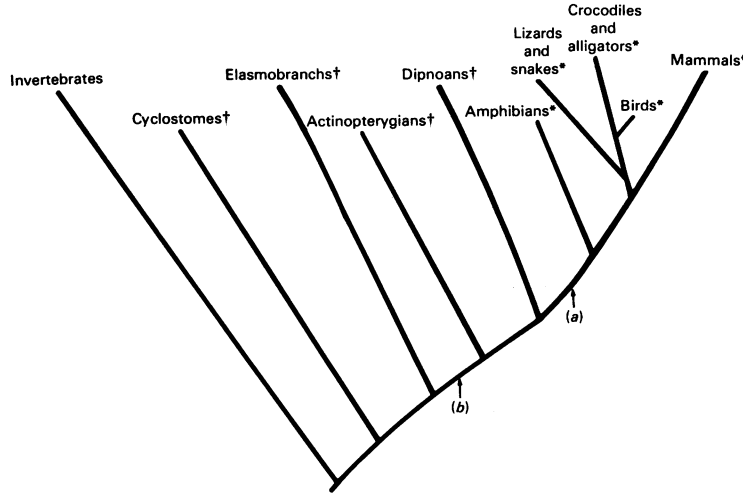

Fig. 2. *Cladogram of the major vertebrate taxa showing the distribution of the papain-binding protein*
This cladogram is based on the classification of vertebrate species proposed by Wiley (1979). Taxa marked † include species possessing a papain-binding protein of M_r approx. 360000, and taxa marked * include species with a papain-binding protein of M_r approx. 725000. (*a*) and (*b*) are points at which the ancestral coelacanth might have diverged from the other taxa (see the text).

cyclostomes, hagfish and lamprey. The appearance of a papain-binding protein in the arachnid invertebrate *Limulus* could be of significance in connection with the controversial view that the arachnids are the living invertebrate taxon most closely related to the vertebrates (Løvtrup, 1977). Further examination of the protein would be needed to show whether it has a genuine relationship to α_2M.

The distribution of proteins resembling human α_2M throughout the major vertebrate taxa is summarized in Fig. 2. The evolution of the large (M_r 725000) papain-binding protein from the small (M_r 360000) vertebrate protein must have occurred in a species that was a direct ancestor of all the existing tetrapods, but was not an ancestor of the dipnoans or the other fish. It would obviously be of interest to determine whether the coelacanth (crossopterygian) papain-binding protein is large or small, as opinion is divided as to whether the crossopterygians diverged from the tetrapods at the point marked (*a*) in Fig. 2 (Romer & Parsons, 1977) or from the lineage leading to the dipnoans and tetrapods at point (*b*) (Wiley, 1979). The advantages of the doubling in molecular size can only be guessed at, but it seems probable that the increased number of possible subunit interactions in the larger protein gives greater stability, and may, by co-operation between subunits, increase the efficiency of the conformational change that leads to the trapping of a proteinase by the macroglobulin.

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References

Barrett, A. J. (1976) *Anal. Biochem.* **76**, 374–376

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

Berne, B. H., Dray, S. & Knight, K. (1971) *Proc. Soc. Exp. Biol. Med.* **138**, 531–535

Berne, B. H., Greene, N. D. & Damian, R. T. (1972) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **31**, 839

Bütler, R. & Brunner, E. (1967) *Haematologia* **1**, 375–385

Ganrot, K. (1973) *Biochim. Biophys. Acta* **295**, 245–251

Got, R., Mouray, H. & Moretti, J. (1965) *Biochim. Biophys. Acta* **107**, 278–285

Greene, N. D., Damian, R. T. & Hubbard, W. J. (1971) *Biochim. Biophys. Acta* **236**, 659–663

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

Jacquot-Armand, Y. & Guinand, S. (1967) *Biochim. Biophys. Acta* **133**, 289–300

Jones, J. M., Creeth, J. M. & Kekwick, R. A. (1972) *Biochem. J.* **127**, 187–197

Lavergne, M. & Raynaud, M. (1970) *Ann. Inst. Pasteur Paris* **119**, 27–49

Lebreton de Vonne, T. & Mouray, H. (1968) *C. R. Hebd. Séances Acad. Sci.* **266**, 1076–1079

Løvtrup, S. (1977) *The Phylogeny of Vertebrata*, John Wiley and Sons, London

Nagasawa, S., Hoon, B. H., Sugihara, H. & Suzuki, T. (1970) *J. Biochem. (Tokyo)* **67**, 821–832

Nieuwenhuizen, W., Emeis, J. J. & Hemmink, J. (1979) *Biochim. Biophys. Acta* **580**, 129–139

Ohlsson, K. (1971) *Biochim. Biophys. Acta* **236**, 84–91

Pepper, D. S. (1968) *Biochim. Biophys. Acta* **156**, 327–339

Picard, J. J. & Heremans, J. F. (1963) *Biochim. Biophys. Acta* **71**, 554–561

Picard, J. J., Vandenbroek, G., Heremans, J. F. & Defossé, G. (1966) *Biochim. Biophys. Acta* **117**, 111–114

Romer, A. S. & Parsons, T. S. (1977) *The Vertebrate Body*, 5th edn., W. B. Saunders, Philadelphia

Ryley, H. C. (1979) *Biochem. Biophys. Res. Commun.* **89**, 871–878

Sasaki, M., Minakata, K., Yamamoto, H., Niwa, M., Kato, T. & Ito, N. (1977) *Biochem. Biophys. Res. Commun.* **76**, 917–924

Shortridge, K. F., Belyavin, G. & Bidwell, D. E. (1976) *Comp. Biochem. Physiol. A* **54**, 319–321

Starkey, P. M. & Barrett, A. J. (1973) *Biochem. J.* **131**, 823–831

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., Fletcher, T. C. & Barrett, A. J. (1982) *Biochem. J.* **205**, 97–104

Tsuru, D., Tomimatsu, M., Fujiwara, K. & Kawahara, K. (1975) *J. Biochem. (Tokyo)* **77**, 1305–1312

Wiley, E. O. (1979) *Zool. J. Linn. Soc.* **67**, 149–179