Limulus α_2 -macroglobulin

First evidence in an invertebrate for a protein containing an internal thiol ester bond

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Intra-chain thiol ester bonds are present in a limited number of proteins. The thiol ester class of proteins includes vertebrate α_2 -macroglobulin and the complement proteins C3 and C4. We report here the first instance of a thiol ester protein from an invertebrate, the α_2 -macroglobulin proteinase-inhibitor homologue present in the plasma of the American horseshoe crab *Limulus polyphemus*. Our evidence is of three kinds: (1) the proteinase-binding activity of *Limulus* α_2 -macroglobulin is inactivated by the low-molecular-mass primary amine methylamine; (2) the native protein is subject to autolytic fragmentation during mild thermal denaturation, yielding fragments of approx. 125 kDa and 55 kDa, whereas the methylamine-treated protein is stable under these conditions of thermal treatment; (3) new thiol groups are generated rapidly during reaction of the protein with trypsin. The demonstration of the thiol ester bond in a protein from an ancient invertebrate provides evolutionary evidence for the importance of this bond in the function of plasma forms of the α_2 -macroglobulin-like proteinase inhibitors.

INTRODUCTION

One of the families of plasma proteins of vertebrates is the thiol ester family, which includes α_2 -macroglobulin and the complement components C3 and C4, and which is characterized by the presence of an internal thiol ester bond involving the thiol group of a cysteine residue and the γ -carboxy group of a nearby glutamic acid residue (Tack, 1983). Thiol ester proteins are activated by proteolytic attack at a defined site situated some distance from the thiol ester group (Harpel, 1973; Starkey & Barrett, 1977; Barrett *et al.*, 1979; Swenson & Howard, 1979a; Sottrup-Jensen *et al.*, 1981b). The activated thiol ester group can mediate covalent attachment to acceptor molecules by ester or amide bonding to the γ -carbonyl group of the glutamic acid residue (Swenson & Howard, 1979b; Law *et al.*, 1979, 1980a,b; Salvesen & Barrett, 1980; Campbell *et al.*, 1980; Pangburn & Müller-Eberhard, 1980; Sottrup-Jensen *et al.*, 1980, 1981a; Sim *et al.*, 1981; Salvesen *et al.*, 1981; Gadd & Reid, 1981; Howard, 1981; Van Leuven *et al.*, 1981; Wang *et al.*, 1981a,b; Wu *et al.*, 1981; Feinman *et al.*, 1985). The thiol ester group of the non-proteolysed native protein also is sensitive to nucleophilic attack at the γ -carbonyl group of the glutamic acid residue by small primary amines such as methylamine and ammonia (Harpel, 1976; Barrett *et al.*, 1979; Swenson & Howard, 1979b, 1980; Sottrup-Jensen *et al.*, 1980; Tack *et al.*, 1980; Howard, 1980, 1981; Salvesen *et al.*, 1981; Harrison *et al.*, 1981; Starkey *et al.*, 1982; Dangott & Cunningham, 1982; Gonias *et al.*, 1983; Feldman *et al.*, 1984; Larsson & Björk, 1984; Larsson *et al.*, 1985; Esnard *et al.*, 1985). Methylamine-treated samples are functionally inactive (Gordon *et al.*, 1926; Pillemer, 1943; Müller-Eberhard, 1961; Steinbuch *et al.*, 1968; Barrett *et al.*, 1979; Tack *et al.*, 1980; von Zabern *et al.*, 1981; Björk & Fish, 1982; Feldman &

Pizzo, 1984). Thiol ester-containing proteins undergo specific autolytic cleavage at elevated temperatures caused by nucleophilic attack by the amino group of the glutamic acid residue on the γ -carbonyl group, resulting in rupture of the peptide bond at this site (Barrett *et al.*, 1979; Harpel *et al.*, 1979; Janatova *et al.*, 1980b; Gorski & Howard, 1980; Howard, 1981; Ichihara *et al.*, 1981; Janatova & Tack, 1981; Seya & Nagasawa, 1981; Sim & Sim, 1981; Nelles & Schnebli, 1982; Esnard *et al.*, 1985). Samples pretreated with methylamine or proteinases are resistant to such autolytic fragmentation (Barrett *et al.*, 1979; Harpel *et al.*, 1979; Ichihara *et al.*, 1981; Janatova & Tack, 1981; Sim & Sim, 1981; Esnard *et al.*, 1985). An additional diagnostic characteristic of thiol ester proteins is that rupture of the thiol ester bond after exposure to proteinases or primary amines results in the appearance of the new free thiol group, which can be titrated with appropriate thiol-reactive reagents (Janatova *et al.*, 1980a,b; Tack *et al.*, 1980; Straight & McKee, 1982; Strickland & Bhattacharya, 1984; Larsson & Björk, 1984; Larsson *et al.*, 1985; Feinman *et al.*, 1985). In the present paper we report evidence that a functional homologue of α_2 -macroglobulin from the plasma of the American horseshoe crab *Limulus polyphemus* (Quigley & Armstrong, 1983, 1985) possesses an internal thiol ester bond.

MATERIALS AND METHODS

Adult horseshoe crabs were bled by cardiac puncture under sterile endotoxin-free conditions into chilled tubes (Armstrong, 1985). The cells were immediately removed by centrifugation and the plasma was recovered and stored at 4 °C until further processing. These conditions prevent contamination of the plasma by the materials

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released from the blood cells, which will undergo exocytosis if whole blood is allowed to stand (Armstrong & Rickles, 1982; Armstrong & Quigley, 1985). Haemocyanin was removed from the plasma by ultracentrifugation, and the α_2 -macroglobulin homologue was purified by differential precipitation with poly(ethylene glycol) and gel-permeation chromatography with Sephacryl S-300 (Pharmacia) as previously described (Quigley & Armstrong, 1985).

Proteolytic activity of trypsin was determined with [14 C]methyl- α -casein as substrate (Quigley & Armstrong, 1985), and the amidolytic activity of trypsin was determined with N^α -benzoyl-DL-arginine p -nitroanilide (Sigma Chemical Co.) as substrate (Erlanger *et al.*, 1961). Protection of trypsin's ability to hydrolyse N^α -benzoyl-DL-arginine p -nitroanilide from the macromolecular active-site inhibitor soya-bean trypsin inhibitor (Bieth *et al.*, 1981; Wang *et al.*, 1981b; Björk *et al.*, 1985), was used to quantify the amount of α_2 -macroglobulin activity (Ganrot, 1966; Armstrong *et al.*, 1985). The fractional activity of trypsin (T-8003, lot no. 25F-8120; Sigma Chemical Co.) was determined by titration with p -nitrophenyl p' -guanidinobenzoate hydrochloride as described by Chase & Shaw (1970).

Thiol groups of α_2 -macroglobulin were quantified with 4,4'-dipyridyl disulphide by monitoring the increase in absorbance at 324 nm (Feinman *et al.*, 1985). The molar absorption coefficient of the reaction product of 4,4'-dipyridyl disulphide with cysteine was determined to be $1.68 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

RESULTS AND DISCUSSION

Limulus α_2 -macroglobulin incubated with 0.2 M-methylamine in 0.06 M-Tris/HCl buffer, pH 8.2, for 5–18 h at 22 °C lost reactivity with trypsin. Methylamine-treated α_2 -macroglobulin failed to inhibit hydrolysis of [14 C]methyl- α -casein (Quigley & Armstrong, 1983, 1985) and failed to protect the amidolytic activity of trypsin from the macromolecular active-site inhibitor soya-bean trypsin inhibitor. In this latter regard, a sample of haemocyanin-free *Limulus* plasma capable of protecting 2.87 ± 0.04 nmol of enzymically active trypsin/ml retained the ability to protect only 0.11 ± 0.02 nmol/ml after treatment with methylamine. This is a 96% inactivation of the trypsin-binding activity of the *Limulus* α_2 -macroglobulin. With mammalian thiol ester proteins, methylamine disrupts the thiol ester bond by nucleophilic attack, yielding γ -glutamylmethylamine (Swenson & Howard, 1979b), and destroying the activity of the treated protein (Steinbuch *et al.*, 1968; Barrett *et al.*, 1979; Tack *et al.*, 1980; von Zabern *et al.*, 1981). Inactivation of the *Limulus* protein by methylamine is consistent with the proposal that it contains an internal thiol ester bond necessary for activity.

During mild heat denaturation, the amino group of the thiol ester glutamic acid residue serves as a nucleophilic group that reacts with the thiol ester bond, forming an amide bond with the γ -carbonyl group and breaking the polypeptide chain at this site (Sim & Sim, 1983). Human and *Limulus* α_2 -macroglobulins were incubated in parallel at 80 °C for 2 h in 0.05 M-Tris/HCl buffer, pH 8.0, and then analysed by SDS/polyacrylamide-gel electrophoresis in the presence of the reducing agent 2-mercaptoethanol. Under reducing conditions, the subunit polypeptide chains of native human

and *Limulus* α_2 -macroglobulins had estimated molecular masses of 180 kDa (Fig. 1, lane 5) and 185 kDa (Fig. 1, lane 1) respectively. Human α_2 -macroglobulin generated cleavage products of approx. 125 kDa and 67 kDa (Fig. 1, lane 6). The corresponding cleavage products of *Limulus* α_2 -macroglobulin were approx. 125 kDa and 55 kDa (Fig. 1, lane 3). Prior treatment of both human (results not shown) and *Limulus* (Fig. 1, lane 4) α_2 -macroglobulin with methylamine prevented fragmentation. The similarity of autolytic heat fragmentation patterns of human (Barrett *et al.*, 1979; Harpel *et al.*, 1979; Janatova *et al.*, 1980b; Gorski & Howard, 1980; Howard, 1981; Ichihara *et al.*, 1981; Janatova & Tack, 1981; Seya & Nagasawa, 1981; Sim & Sim, 1981; Nelles & Schnebli, 1982; Esnard *et al.*, 1985) and *Limulus* α_2 -macroglobulin is also indicative of the presence of an internal thiol ester bond in the *Limulus* protein.

The reaction of thiol ester proteins with proteinases results in cleavage at a defined site some distance from

Fig. 1. Heat treatment of *Limulus* α_2 -macroglobulin: comparison with human α_2 -macroglobulin and protection by methylamine treatment

Purified *Limulus* α_2 -macroglobulin ($L\alpha_2 M$) and methylamine-treated *Limulus* α_2 -macroglobulin ($MA-L\alpha_2 M$) were incubated in parallel at 80 °C for 2 h in 0.05 M-Tris buffer, pH 8.0. Control samples of *Limulus* α_2 -macroglobulin and methylamine-treated *Limulus* α_2 -macroglobulin were left at room temperature (20 °C) for 2 h (Untreated). After incubation, the samples were dissolved in SDS sample buffer containing 2-mercaptoethanol at 20 °C and subjected to electrophoresis on 6.5% polyacrylamide gels. A sample of human α_2 -macroglobulin ($H\alpha_2 M$) was similarly treated and analysed in parallel lanes in the polyacrylamide gel (Untreated). The cleavage products resulting from heat treatment of human α_2 -macroglobulin (125 kDa and 67 kDa) are consistent with reported values for cleavage at a methylamine-sensitive bond (Harpel *et al.*, 1979; Howard, 1980). A similar cleavage results with *Limulus* α_2 -macroglobulin (125 kDa and 55 kDa), but does not occur with methylamine-treated *Limulus* α_2 -macroglobulin.

the thiol ester group (Sottrup-Jensen *et al.*, 1983). Proteolysis of human α_2 -macroglobulin results in fragments of approx. 100 and 85 kDa that may co-electrophorese at 85 kDa (Harpel, 1973; Hall & Roberts, 1978; Barrett *et al.*, 1979; Swenson & Howard, 1979a; Nelles *et al.*, 1980; Sottrup-Jensen *et al.*, 1981b; Howell *et al.*, 1983) and also in products revealed by reducing SDS/polyacrylamide-gel electrophoresis that are larger than the single chain and appear to result from covalent bonding between proteinase and α_2 -macroglobulin (Salvesen *et al.*, 1981; Sottrup-Jensen *et al.*, 1981d; Van Leuven *et al.*, 1981; Wu *et al.*, 1981; Harpel & Brower, 1983; Straight & McKee, 1984; Wang *et al.*, 1984; Feinman *et al.*, 1985). A similar cleavage pattern has been seen with *Limulus* α_2 -macroglobulin with products of approx. 100 and 80 kDa and several products larger than 185 kDa (J. P. Quigley & P. B. Armstrong, unpublished work). Proteolysis results in activation and hydrolysis of the thiol ester bond, with the appearance of a cysteine thiol group (Janatova *et al.*, 1980a,b; Sottrup-Jensen *et al.*, 1980; Tack *et al.*, 1980; Feinman *et al.*, 1985). The appearance of new thiol groups during reaction with proteinases is indicative of the presence of an internal thiol ester bond. Thiol groups were titrated spectrophotometrically with 4,4'-dipyridyl disulphide. The trypsin-binding activity of α_2 -macroglobulin was determined by the soya-bean-trypsin-inhibitor protection assay. The amidolytic activity of trypsin bound to *Limulus* α_2 -macroglobulin is the same as that of free trypsin (Quigley & Armstrong, 1983). The fractional activity of the trypsin preparation as determined by titration with *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride was 51%. Protein concentrations were determined by the Bradford (1976) methods, with bovine serum albumin as the standard. A preparation containing 3.0 mg of human α_2 -macroglobulin/ml bound 8.0 nmol of enzymically active trypsin/ml and generated 16.3 nmol of thiol groups/ml during the reaction with trypsin (Table 1). A preparation containing 1.9 mg of purified *Limulus* α_2 -macroglobulin/ml bound 4.0 nmol of trypsin/ml and generated 10.5 nmol of thiol groups/ml (Table 1). The appearance of the new thiol groups was rapid for both forms of α_2 -macroglobulin, being complete at the earliest determination (10 s). Neither preparation reacted with the thiol-reactive titrant before reaction with trypsin, indicating that the untreated α_2 -macroglobulins lack accessible free thiol groups. The generation of new thiol groups concomitant with reaction of the *Limulus* molecule with trypsin adds support for the hypothesis that this protein contains an internal thiol ester bond. The molar ratio of trypsin bound to new thiol groups generated was 1:2.04 for human α_2 -macroglobulin and was 1:2.6 for *Limulus* α_2 -macroglobulin. The reason for this difference is not known. Although, owing to the inaccuracies of the Bradford (1976) method, the concentrations of α_2 -macroglobulin were not determined with high accuracy, this should not affect the ratios of trypsin bound to thiol groups released for the two forms of α_2 -macroglobulin, since this ratio was determined at saturating concentrations of trypsin and should be independent of the concentration of α_2 -macroglobulin. It has been suggested that the *Limulus* homologue is a homotrimer (Quigley & Armstrong, 1985). If *Limulus* α_2 -macroglobulin binds 1 mol of trypsin/mol of α_2 -macroglobulin, and if this is accompanied by activation of all three thiol ester groups, then a ratio of 1:3 would

Table 1. Titration with 4,4'-dipyridyl disulphide of free thiol groups in α_2 -macroglobulin after reaction with trypsin

The concentration of α_2 -macroglobulin given is the final concentration in the reaction cuvette. For measurement of the increase in A_{324} the spectrophotometer was set at a wavelength of 324 nm and was 'blanked' with buffer. Then 0.60 ml (trial 1) or 0.54 ml (trial 2) of a 1.9 mg/ml (*Limulus*) or 3.0 mg/ml (human) solution of purified α_2 -macroglobulin was added to 0.22 ml of 0.1 M-phosphate buffer, pH 8.0, and 0.2 ml of 1.5 mM-4,4'-dipyridyl disulphide in a spectrophotometer cuvette and the absorbance was recorded. A 2-fold excess (0.4 mg) of trypsin was added and the absorbance was again recorded. The increase in A_{324} was the difference between the two values, corrected for the increase in volume with the addition of trypsin. The final reaction volume was 1 ml.

Trial	Species	[α_2 -Macro-globulin] (mg/ml)	Increase in A_{324}
1	<i>Limulus</i>	1.1	0.096
	Human	2.3	0.140
2	<i>Limulus</i>	1.0	0.095
	Human	2.1	0.148

be expected, reasonably close to the observed ratio of 1:2.6. The observed ratio for human α_2 -macroglobulin is consistent with the reported maximum stoichiometry of proteinase binding to tetrameric α_2 -macroglobulin (Ganrot, 1966; Barrett *et al.*, 1979; Swenson & Howard, 1979a; Sottrup-Jensen *et al.*, 1980; Pochon *et al.*, 1981; Howell *et al.*, 1983; Gonias & Pizzo, 1983; Björk, 1984; Björk *et al.*, 1984; Straight & McKee, 1984) and the report that all thiol ester groups are activated during reaction with proteinase (Björk *et al.*, 1984; Feinman *et al.*, 1985).

The presence of a protein functionally and structurally homologous to α_2 -macroglobulin in an arthropod suggests that the protein appeared in evolution before the divergence of the arthropod and vertebrate lineages 0.55×10^9 years ago. The presence of a thiol ester bond in the two proteins suggests that this group appeared early in the evolution of higher animals and is characteristic of the ancestral form of α_2 -macroglobulin. Variants of α_2 -macroglobulin have been described that apparently lack the thiol ester bond but that retain the ability to bind proteinases (Nagase *et al.*, 1983). The retention of the thiol ester bond in plasma forms of α_2 -macroglobulin throughout 0.55×10^9 years of evolution suggests that this bond does play an important role in the function of the forms of α_2 -macroglobulin that are found in the blood. These functions include the covalent linkage of α_2 -macroglobulin to target proteinases (Swenson & Howard, 1979b; Salvesen & Barrett, 1980; Sottrup-Jensen *et al.*, 1980, 1981c; Salvesen *et al.*, 1981; Howard, 1981; Van Leuven *et al.*, 1981; Wu *et al.*, 1981), a function that is distinct from the physical entrapment of proteinase (Barrett & Starkey, 1973). The covalent binding following activation is important for the activity of complement components C3 and C4 (Porter & Sim, 1981). It will be interesting to determine if the complement-like activities described for *Limulus* plasma (Noguchi, 1903; Day *et al.*, 1970; Gewurz *et al.*, 1970) are mediated by the α_2 -macroglobulin homologue.

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