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Chapter 1

Comparative Biology of the α_2-Macroglobulin-Based Immune System

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Contents

1 Introduction

Most multicellular organisms are subject during their lives to a nearly continual challenge by pathogenic microorganisms and multicellular parasites. In response, a variety of immune processes have evolved to suppress pathogens that have invaded the body. Some of these immune processes are phylogenetically unique. Perhaps the best-known example is the induced antibody response based on VDJ gene rearrangement of the immunoglobulin heavy and light chain genes, which apparently is present only in the vertebrates (Marchalonis and Schluter 1990). Other immune systems, such as the prophenoloxidase system (Söderhäll and Smith 1986), lysozyme (Engström et al. 1985), the pentraxins (Tennent et al. 1993), and α_2-macroglobulin (Armstrong and Quigley 1995), are of more ancient origin, because they are present in modern representatives of diverse animal phyla. This chapter reviews the role of the α_2-macroglobulin system in immunity.

α_2-Macroglobulin is the signature member of a protein family that includes C3, C4, and C5 (Tack 1983; Sottrup-Gensen 1987), which are important components of the vertebrate complement system, pregnancy-zone protein (Christensen et al. 1989; Devriendt et al. 1991), which is an acute phase protein of mammals,

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and the α_2-macroglobulin homologues found in arthropods (Armstrong and Quigley 1995) and mollusks (Armstrong and Quigley 1992; Bender et al. 1992; Thøgersen et al. 1992). Members of the α_2-macroglobulin family share peptide sequence identity (Spycher et al. 1987; Sottrup-Jensen et al. 1990a; Thøgersen et al. 1992) and most members possess a unique reactive internal thiol ester domain that is discussed in more detail later. The α_2-macroglobulins are soluble proteins that may be present at high concentration in the plasma[4] and that are also found in the whites of the eggs of birds and reptiles (Ikai et al. 1983, 1990; Nagase et al. 1983).

The first function ascribed to plasma α_2-macroglobulin was the binding and inhibition of proteases. The protease inhibitory mechanism of α_2-macroglobulin is unique amongst enzyme inhibitors, because it involves the physical entrapment

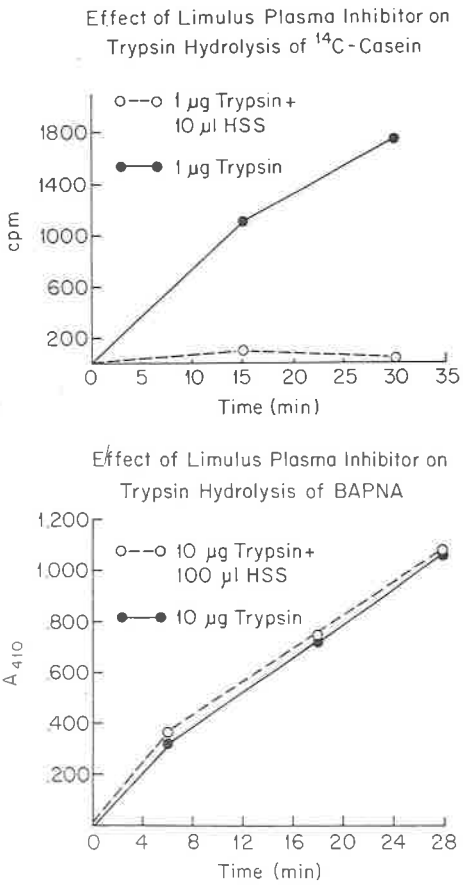

Fig. 1. Presence in *Limulus* plasma of a protease inhibitor that eliminates the caseinolytic activity of trypsin (*above*); but fails to inhibit the activity of trypsin against the low molecular mass amide substrate BAPNA (N^{α}-benzoyl-DL-arginine *p*-nitroanilide; *below*). Plasma was cleared of hemocyanin by ultracentrifugation and is referred to as HSS (high-speed supernatant). Hydrolysis of [^{14}C]-casein (*tp*) was determined by the release of acid-soluble [^{14}C]. Hydrolysis of BAPNA was determined by the increase in optical absorbance at 410 nm

[4] Members of the α_2-macroglobulin family of proteins comprise almost 5% of the plasma proteins of mammals, and α_2-macroglobulin is the third most abundant protein in the plasma of the American horseshoe crab, *Limulus polyphemus*.

Table 1. Comparison of α_2-macroglobulin with active site protease inhibitors

α_2-Macroglobulin	*Active site inhibitors*
Inhibits the proteolytic activity of proteases without inhibiting the hydrolysis of low molecular mass amide or ester substrates	Inhibits activity of target proteases against polypeptide and low molecular mass substrates
Reacts with endopeptidases of diverse catalytic mechanisms and substrate specificities	Reacts with a narrow spectrum of related proteases
Shields bound proteases from antibodies and high molecular mass active site inhibitors	Bound proteases remain reactive with antibodies
Presence of a unique internal reactive thiol ester group	Internal thiol ester is found only in proteins of the α_2-macroglobulin family

of the protease molecule in a molecular cage that forms a stearic barrier that prevents contact of the entrapped protease molecule with protein substrates and leaves the active site of the enzyme intact (Barrett and Starkey 1973; Starkey and Barrett 1977). Ester and amide substrates of the bound protease that are small enough to diffuse into the α_2-macroglobulin cage are readily hydrolyzed (Fig. 1; Bieth et al. 1970; Starkey and Barrett 1977). The protease-inhibitory mechanism of α_2-macroglobulin stands in marked contrast to all other protease inhibitors, which bind to the active site and inhibit the activity of the target protease against both protein and low molecular mass amide and ester substrates (Laskowski and Kato 1980; Travis and Salvesen 1983). This is one of several diagnostic properties (Table 1) that initially allowed us to characterize a protease inhibitory activity that we identified in the plasma of the American horseshoe crab, *Limulus polyphemus*, as the first known invertebrate homologue of α_2-macroglobulin (Quigley et al. 1982; Quigley and Armstrong 1983). Both the presence and function of the internal thiol ester group and the nature of the physical reorganization of α_2-macroglobulin during interaction with proteases have attracted the attention of physical biochemists. The physiological functions of α_2-macroglobulin have attracted the attention of immunologists and cell biologists.

2 Biochemistry of α_2-Macroglobulin

The plasma α_2-macroglobulins are monomers, dimers, and tetramers of a 180–185 kDa peptide, with the dimeric form (Fig. 2) being the most widely distributed (Starkey and Barrett 1982; Armstrong et al. 1991). All other protease inhibitors interact directly with the active site of the enzyme and inactivate its activity

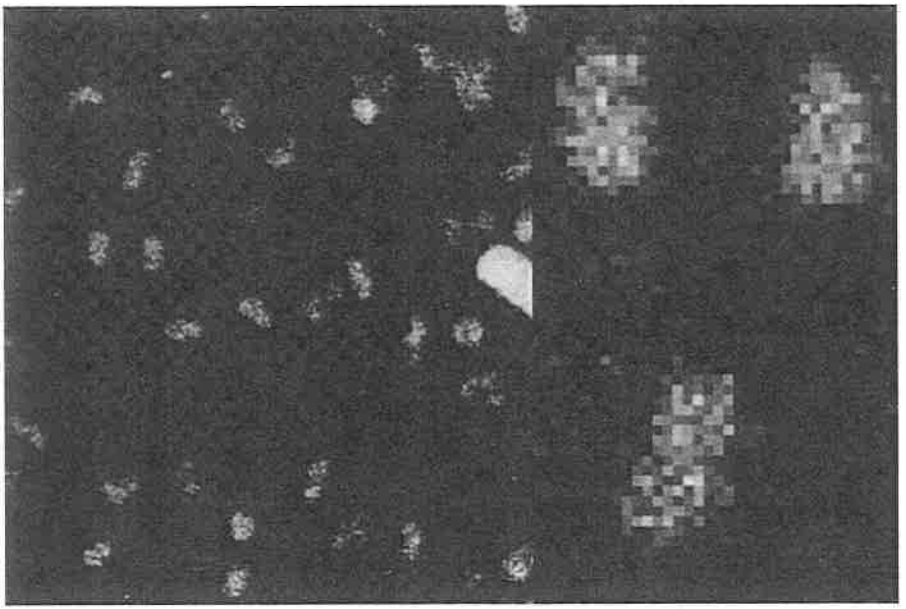

Fig. 2 Scanning transmission electron microscopy of freeze-dried, unstained, uncoated *Limulus* α_2-macroglobulin. **A** A low magnification view of a field of molecules; **B** a high magnification view of three α_2-macroglobulin molecules. The elongated structure of the individual α_2-macroglobulin molecules is consistent with a dimeric organization. The *cylindrical structure* in **A** is a tobacco mosaic virus particle, which was added to the sample as an internal calibration standard

against proteins and against low molecular mass ester and amide substrates (Laskowsky and Kato 1980; Travis and Salvesen 1983). Retention of the esterolytic and amidolytic activities of α_2-macroglobulin-bound proteases can be shown by their continued ability to hydrolyze low molecular mass substrates small enough to diffuse into the α_2-macroglobulin cage.

The enfolding of the targeted protease by the α_2-macroglobulin polypeptide chain is initiated by a proteolytic cleavage of α_2-macroglobulin at a specialized region, the bait region (Harpel 1973; Hall and Roberts 1978; Sottrup-Jensen et al. 1981, 1989). Most forms of α_2-macroglobulin subsequently undergo a significant compaction as a manifestation of the trapping process (Barrett et al. 1979; Bjork and Fish 1982; Gonias et al. 1982; Armstrong et al. 1991). All active-site protease inhibitors are limited in the range of proteases that they inhibit (Laskowski and Kato 1980; Travis and Salvesen 1983), but α_2-macroglobulin is unique amongst protease inhibitors for its ability to interact with endopeptidases of diverse catalytic mechanisms and substrate specificities (Starkey and Barrett 1977). The bait region contains an ensemble of peptide bonds that provide in the aggregate at least one suitable cleavage site for most endopeptidases. This is the basis for the broad spectrum of reactivity of α_2-macroglobulin against all manner of proteases

(Enghild et al. 1989c; Sottrup-Jensen et al. 1989). The promiscuous reactivity equips α_2-macroglobulin with the ability to bind proteases of endogenous and exogenous origin including exoproteases released by microbial and multicellular parasitic pathogens.

Immediately following the cleavage of the α_2-macroglobulin chain at the bait region, an internal thiol ester bond situated downstream of the bait region is activated to generate a new cysteinyl thiol and a reactive γ-carbonyl of a glutamyl residue:

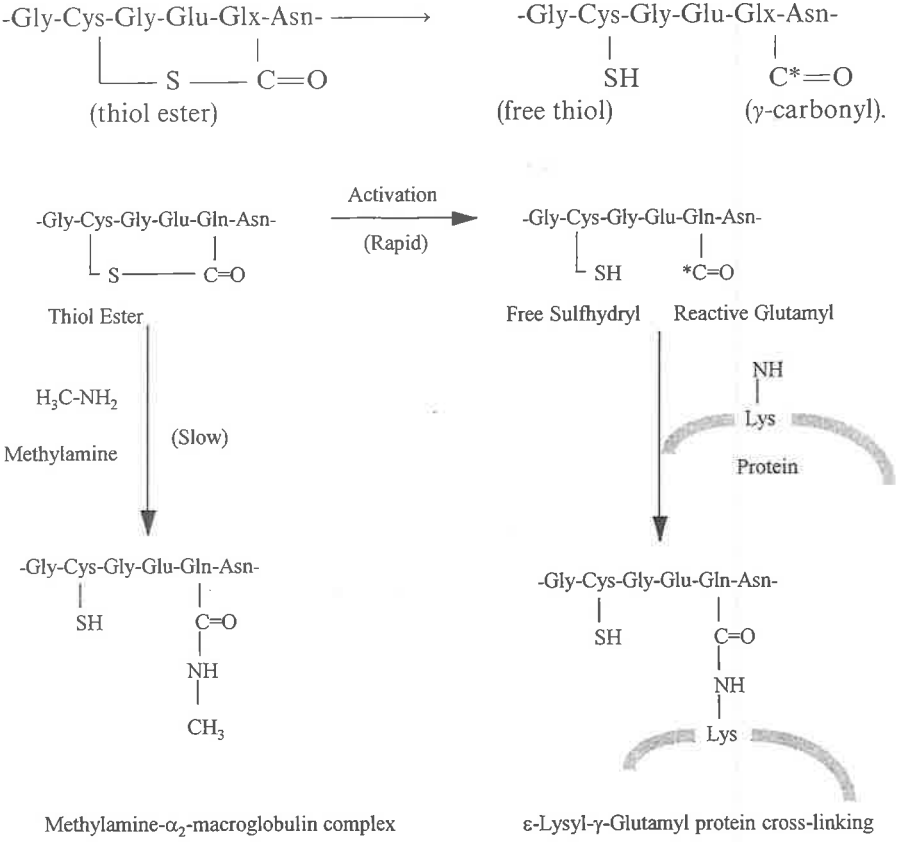

Fig. 3. Activation and cleavage of the internal thiol ester of α_2-macroglobulin expose a new thiol group on the cysteine and a reactive γ-carbonyl on the glutamyl residue. The reactive internal thiol ester of members of the α_2-macroglobulin protein family is cleaved following proteolysis at the distantly located protease bait region of the protein. Thiol ester cleavage generates an activated γ-carbonyl at the glutamyl residue and a free thiol at the cystenyl residue (*top line* of figure). The reactive glutamyl can form amide linkages with proteins (*right side* of figure). The thiol ester can also react slowly with small primary amines, such as methylamine (*left side* of figure), even in the absence of proteolytic cleavage at the bait region. Methylamine treatment eliminates many of the functional activities of α_2-macroglobulin in parallel with its inactivation of the thiol ester. Sensitivity of a molecular function to treatment with methylamine is a useful test for the possibility that that function is dependent on the activity of a protein of the α_2-macroglobulin family

This internal thiol ester is unique to the members of the α_2-macroglobulin family of proteins (Tack 1983; Sottrup-Jensen 1987). Prior to reaction with proteases, the thiol ester is protected from reaction with most nucleophilic substrates. However, small amines such as methylamine and ammonium can access the thiol ester and react to form covalent derivatives of the glutamyl residue (Fig. 3). This destroys the subsequent ability of the molecule to react with proteases (Barrett et al. 1979; Bjork and Fish 1982; Armstrong and Quigley 1987). Following the normal reaction with proteases, the thiol ester is exposed and the glutamyl residue reacts with nucleophiles in its immediate environment (Fig. 3; Tack et al. 1980; Sottrup-Jensen et al. 1980). Reaction with ε-amino and hydroxyl residues on the reacting protease allows α_2-macroglobulin to establish covalent γ-glutamyl isopeptide bonds linking the protease to the peptide bearing the thiol ester domain (Fig. 3; Sottrup-Jensen et al. 1990b; Chen et al. 1992). C3 and C4 of the vertebrate complement system also experience activation of the internal thiol ester following proteolytic cleavage. Apparently, the thiol ester of C3 and C4 is directed away from the proteolytic cleavage site, because in this case, the isopeptide bonds link with external proteins and carbohydrate moieties, rather than with the reacting protease (Law and Levine 1977). The isopeptide bonding of C3 and C4 to external acceptors is essential for attachment to target particles such as invading microbes. Covalent binding of C3 marks foreign particles as targets for immune destruction by cytolysis and phagocytosis (Law and Reid 1988). Monomeric forms of plasma α_2-macroglobulin, such as α_1 inhibitor-3, also require isopeptide bonding, this time with the reacting protease, to bind protease (Enghild et al. 1989a). Although α_2-macroglobulin cannot bind proteases if its thiol ester has been reacted with methylamine (Steinbuch et al. 1968; Swenson

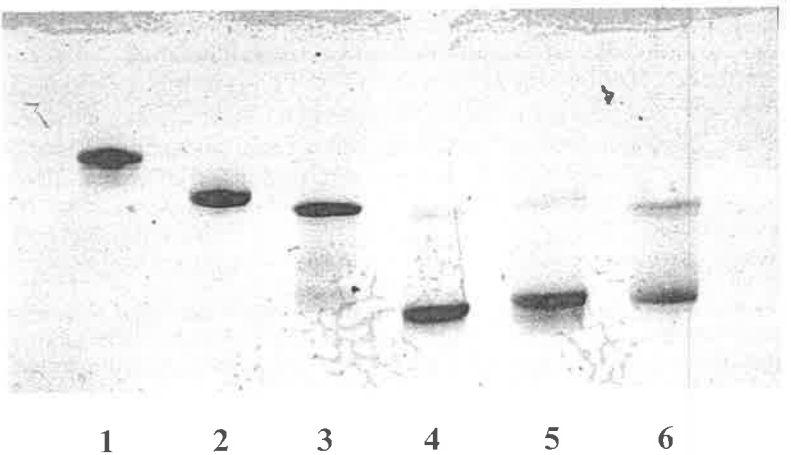

Fig. 4. Pore-limit polyacrylamide gel electrophoresis of human and *Limulus* α_2-macroglobulin. Unreacted tetrameric human α_2-macroglobulin (*lane 1*) shows a slower mobility in the gel than trypsin-reacted human α_2-macroglobulin (*lane 2*). Similarly, unreacted dimeric *Limulus* α_2-macroglobulin (*lane 3*) shows a slower mobility than after reaction with trypsin (*lane 4*), chymotrypsin (*lane 5*), or methylamine (*lane 6*)

and Howard 1979; Nielsen et al. 1994), the physiological function of thiol ester-mediated isopeptide bonding of multimeric forms of plasma α_2-macroglobulin is less clear, because variant forms of α_2-macroglobulin that lack the thiol ester, such as chicken ovomacroglobulin, bind proteases perfectly well (Kitano et al. 1982; Nagase and Harris 1983; Feldman and Pizzo 1984; Nielsen et al. 1994), and proteases such as pancreatic elastase, which lacks ε-amino groups, and that consequently is unable to establish isopeptide bonds with the γ-carbonyl of the activated thiol ester is, nevertheless, successfully bound by multimeric forms of α_2-macroglobulin (Salvesen et al. 1981; Van Leuven et al. 1981). Also, *Limulus* α_2-macroglobulin establishes isopeptide bonds exclusively within the peptide chains of α_2-macroglobulin itself, and fails to bond covalently with the enfolded protease (Quigley et al. 1991).

The refolding of α_2-macroglobulin following cleavage at the bait region results in a significant compaction of the molecule that has been documented as

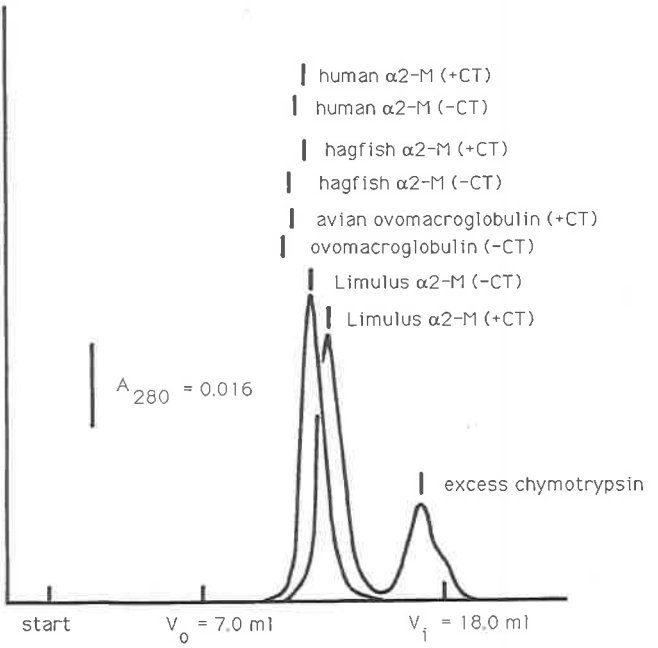

Fig. 5. Gel-filtration chromatography of unreacted and chymotrypsin-reacted α_2-macroglobulin. Samples of unreacted and chymotrypsin-reacted *Limulus* α_2-macroglobulin were applied to a TSK G4000SW gel permeation column and eluted with 0.05 M phosphate buffer. The protease-reacted sample was significantly retarded, indicative of a compaction of the molecule. The *calibration bars* above the figure indicate the elution positions of native and chymotrypsin-reacted tetrameric (human α_2M and ovomacroglobulin) and dimeric (hagfish α_2M) forms of α_2-macroglobulin. The column was further calibrated with tetrameric, dimeric, and monomeric ovomacroglobulin. The dimeric forms of α_2-macroglobulin (e.g., *Limulus* and hagfish α_2-macroglobulin) show a more pronounced compaction as estimated by the separation of the elution profiles of native and reacted forms than do the tetrameric varieties

an increase in electrophoretic mobility by pore-limit electrophoresis (Fig. 4; Barrett et al. 1979; Armstrong et al. 1991), by a retardation of the molecule by gel filtration chromatography (Fig. 5; Armstrong et al. 1991), by electron microscopy (Fig. 6; Armstrong et al. 1991; Delain et al. 1992), and by a variety of physical techniques including small angle X-ray scattering (Arakawa et al. 1994). Interestingly, dimeric forms of α_2-macroglobulin characteristically experience a more

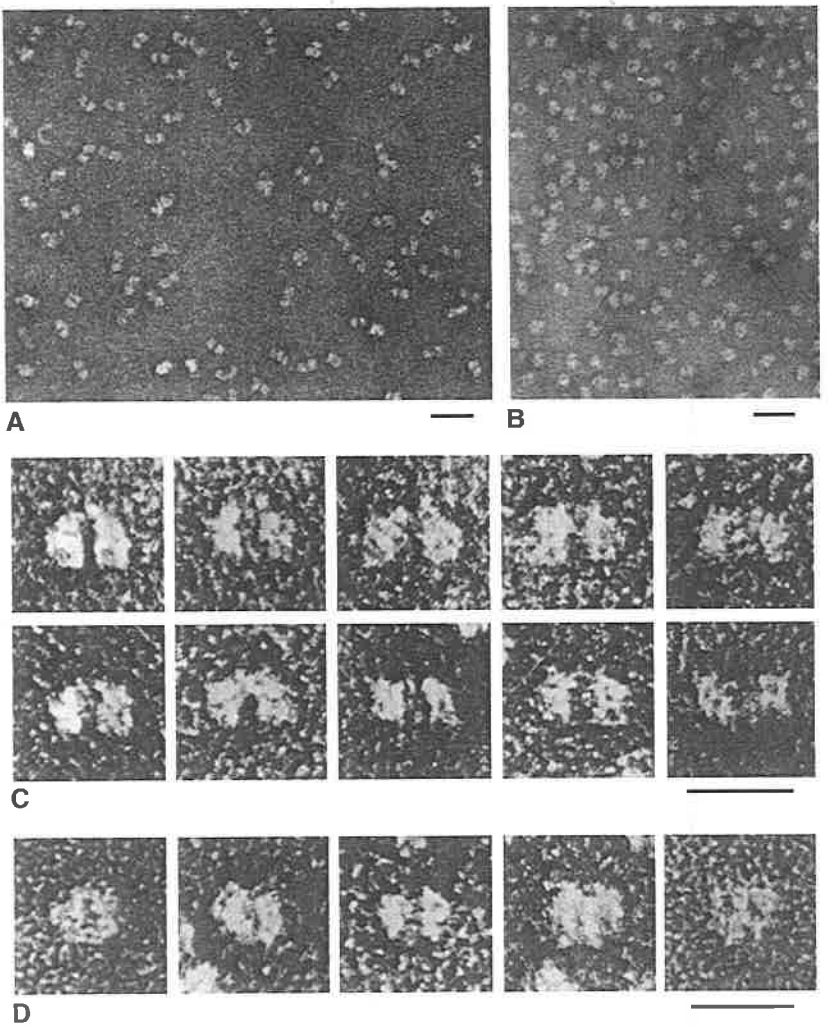

Fig. 6A-D. Transmission electron microscopy of individual molecules of negatively stained *Limulus* α_2-macroglobulin. **A, B** Low-magnification views, respectively, of fields of native and chymotrypsin-reacted *Limulus* α_2-macroglobulin molecules. **C, D** High-magnification views, respectively, of individual molecules of native **(C)** and chymotrypsin-reacted **(D)** *Limulus* α_2-macroglobulin. The native molecule of *Limulus* α_2-macroglobulin is relatively extended and resembles a butterfly, whereas the chymotrypsin-reacted molecule is significantly more compact.

profound compaction than tetrameric α_2-macroglobulin (Fig. 5; Armstrong et al. 1991). Characterization at the atomic level of the configurational changes of proteolyzed α_2-macroglobulin is not yet available. Recent success in crystallizing the protein indicates that the high-resolution picture available from X-ray diffraction analysis is possible (Andersen et al. 1991, 1994).

3 Physiological Function of α_2-Macroglobulin

As emphasized previously, one of the principal functions of α_2-macroglobulin is to bind and inhibit a diverse array of endopeptidases that appear in the body fluids (Sottrup-Jensen 1987). Proteases play important roles in a variety of immune processes, including blood clotting and clot resolution (Furie and Furie 1992; Iwanaga et al. 1992), complement activation (Reid and Porter 1981), inflammation (Cohn 1975; Haverman and Janoff 1978), and tissue remodeling (Werb 1993). Proteases, whether of endogenous or exogenous origin, have the potential for serious destructive effects on the surrounding tissues after their release into the tissue spaces. Proteases contribute to a variety of pathological conditions such as tumor dissemination (Testa and Quigley 1990) and a variety of degenerative connective-tissue disorders, which are directly traceable to the activities of proteases present in the wrong places and at the wrong times (Perlmutter and Pierce 1989). Additionally, proteases are important agents facilitating the invasion of parasites (Breton et al. 1992; McKerrow et al. 1993). The unique ability of α_2-macroglobulin to react productively with all manner of endopeptidases enables it to back up the array of more specialized active-site inhibitors that may be present in the circulation and to capture endopeptidases for which the animal may lack a reactive active-site inhibitor. For example, the horseshoe crab appears to lack circulating active-site inhibitors of serine proteases (Quigley and Armstrong 1983). Instead, in this organism, α_2-macroglobulin serves as the principal barrier to the persistence of serine proteases in the blood (Melchior et al. 1995).

In mammals (Van Leuven 1984) and in *Limulus* (Melchior et al. 1995), the interaction of proteases with α_2-macroglobulin initiates a receptor-mediated clearance pathway that rapidly removes the α_2-macroglobulin-protease complex from the plasma. In mammals, α_2-macroglobulin is but one of a diverse array of protease inhibitors in the plasma (Laskowski and Kato 1980; Travis and Salvesen 1983), whereas in *Limulus* it is only plasma inhibitor involved in protease clearance (Quigley and Armstrong 1983; Melchior et al. 1995). Both in mammals and in *Limulus*, the cell surface receptors ignore native unreacted α_2-macroglobulin, but rapidly bind protease-reacted α_2-macroglobulin. The conformational changes of α_2-macroglobulin following proteolysis of the bait region result not only in an enfolding of the protease molecule, but also expose a previously hidden recognition domain close to the carboxyl terminus of the α_2-macroglobulin chain that is recognized by the cell surface receptor (Sottrup-Jensen et al.

1986; Van Leuven et al. 1986; Enghild et al. 1989b; Thomsen and Sottrup-Jensen 1993). The clearance time of active proteases introduced into the circulation of mammals is short, typically 5–10 min (Ohlsson 1971). Clearance times are longer in *Limulus*, with half clearance times of 20–25 min, but this appears primarily to be a consequence of a more sluggish circulation of the blood in that animal and a consequently slower initial mixing of the injected protease with the blood (Melchior et al. 1995). In mammals, clearance is primarily to hepatocytes (Davidsen et al. 1985; Van Dijk et al. 1992; Ogata et al. 1993), whereas the blood cells are the principal cells involved in clearance in *Limulus* (Melchior et al. 1995). Both in *Limulus* and in mammals, the receptor-bound α_2-macroglobulin-protease complex is internalized and degraded in secondary lysosomes (Van Leuven 1984; Melchior et al. 1995). Based on these observations, it has been suggested that one of the primary functions of α_2-macroglobulin is to serve as a protease carrier molecule with the capabilities of binding endopeptidases of all possible enzymatic mechanisms and from all possible endogenous and exogenous sources to thereby render the bound proteases sensitive to removal from the body fluids by the receptor-mediated endocytotic pathway. In this sense, α_2-macroglobulin is thought to serve as a broad-spectrum recognition system for the identification and marking of proteases for their subsequent endocytosis and destruction in secondary lysosomes.

The mammalian cell surface receptor for α_2-macroglobulin has recently been identified as low-density lipoprotein receptor-related protein (LRP), a transmembrane protein related to the well-characterized low-density lipoprotein receptor (Kristensen et al. 1990; Strickland et al. 1990). Interestingly, this protein is a receptor for a variety of dissimilar ligands including plasminogen activator/plasminogen activator inhibitor-1 complexes, lipoprotein lipase, lactoferrin, *Pseudomonas* exotoxin A, and apolipoprotein E-rich chylomicron remnant (Hussain et al. 1991; Moestrup et al. 1993; Nykjaer et al. 1993; Andreasen et al. 1994). LRP is, like α_2-macroglobulin itself, an ancient protein that was present prior to the great evolutionary radiation that established the divergent deuterostome and protostome superphyla because it has been found in modern representatives of lineages that diverged at the time of the Precambrian radiation, notably in vertebrates and the nematode, *Caenorhabditis elegans* (Yochem and Greenwald 1993). It has not been established whether invertebrate homologues of LRP participate in the clearance of α_2-macroglobulin-protease complexes, but a protein has been identified in detergent extracts of *Limulus* blood cells that has recognition capabilities that makes it a likely candidate for the α_2-macroglobulin receptor (Aimes et al. 1995).

In addition to its activity as a protease-binding molecule, α_2-macroglobulin is also capable of binding a variety of other proteins, most notably several of the peptide mitogens (James 1990; Borth 1992; Gonias 1992). In some cases, the binding of peptide mitogens to α_2-macroglobulin has been shown to require the activation of the thiol ester and involves the establishment of a disulfide linkage via the exposed thiol of the cysteinyl residue of the activated thiol ester (Borth and Luger 1989). The binding of cytokine growth factors has proven interesting

because certain cytokines have been found to be complexed with α_2-macroglobulin in the serum of mammals, suggesting that the interaction is physiologically significant (Huang et al. 1984; O'Connor-Court and Wakefield 1987). Perhaps binding to α_2-macroglobulin represents one of the several buffering systems that regulate and restrain cytokines. In some situations, the binding to α_2-macroglobulin inhibits the mitogenic activities of the growth factor and in other cases it does not (Borth 1992). Also, it must be remembered that serine proteases, which are primary targets of α_2-macroglobulin, are potent mitogens, which interact with a dedicated G-protein-coupled surface receptor (Vu et al. 1991). Although the best-described interaction of the different growth factors with the target cells is via specific cytokine receptors (Sporn and Roberts 1990), the frequent association of circulating cytokines and mitogenic proteases with α_2-macroglobulin established the possibility that productive mitogenic interaction might also occur via the LRP/α_2-macroglobulin receptor. Thus, when exploring the functions of α_2-macroglobulin in invertebrates, it is important to extend our vision beyond the specifics of α_2-macroglobulin-protease interaction and to consider the possibility that proteases may not be the only important molecules whose functions are modulated by binding to α_2-macroglobulin. Indeed, as will be described below, protease-reacted forms of α_2-macroglobulin function as negative regulators of a plasma-based cytolytic system in *Limulus*.

One of the important immune defense strategies of higher animals is to kill invading pathogenic organisms by inducing their cytolysis in the blood. In vertebrates, cytolysis is mediated by the complement system, which is comprised of an ensemble of serially activated effector proteins and a number of receptors and regulatory proteins (Law and Reid 1988). The key event in the activation of the mammalian complement system is the covalent binding of C3 to the surfaces of target cells, thereby marking them for destruction by cytolysis and phagocytosis. Binding involves the covalent bonding of the γ-carbonyl of the glutamyl residue of the reactive internal thiol ester of the C3 molecule with hydroxyl and amino residues at the surface of the target particle (Law and Levine 1977).

Invertebrates also have plasma- or hemocyte-based cytolytic systems (Canicatti 1990), but none have been shown unequivocably to resemble the vertebrate complement system at the molecular level. The plasma cytolytic system of the horseshoe crab, *Limulus polyphemus*, shows a superficial similarity to the vertebrate system because it employs members of two protein families involved in the vertebrate complement system, namely, α_2-macroglobulin and a member of the pentraxin protein family (Enghild et al. 1990; Armstrong et al. 1993). As mentioned previously, C3 and α_2-macroglobulin are members of the same family of proteins, based on peptide sequence homology and the presence of the reactive thiol ester (Tack 1983; Sottrup-Jensen 1987). Inactivation of the thiol ester of C3 by treatment with methylamine inactivates the cytolytic activity of vertebrate plasma and a similar inactivation of α_2-macroglobulin by methylamine treatment significantly reduces the cytolytic activity of *Limulus* plasma (Enghild et al. 1990; Armstrong et al. 1993). Activity is restored to methylamine-treated *Limulus* plasma by addition of *Limulus* α_2-macroglobulin (Fig. 7).

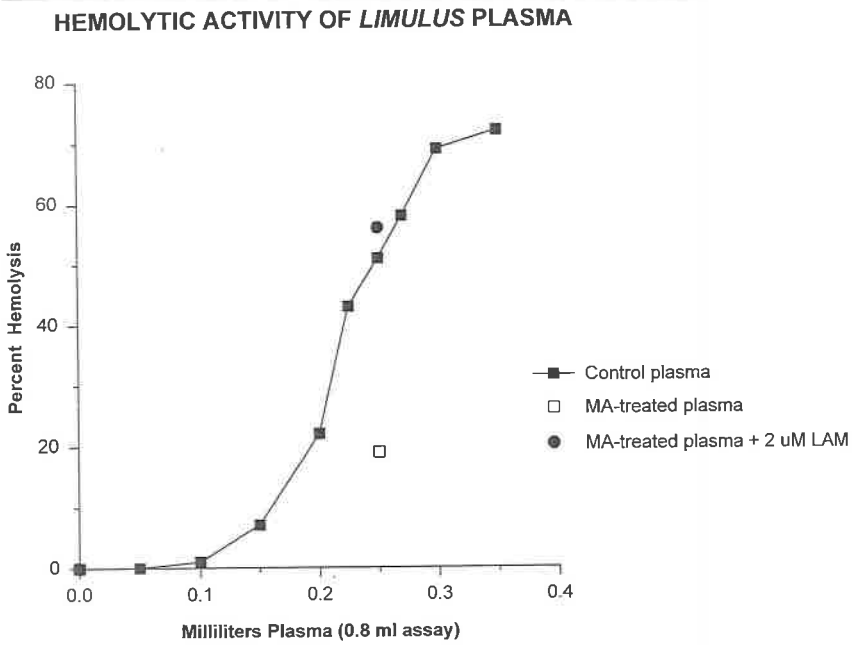

Fig. 7. Cytolytic activity of *Limulus* plasma that has been depleted of hemocyanin by ultracentrifugation (120 000 × g, 16 h). Washed sheep erythrocytes were incubated with increasing amounts of *Limulus* plasma at room temperature for 4 h, and the extent of hemolysis was determined by measuring the quantity of hemoglobin released into the incubation buffer (optical absorbance at 412 nm). Untreated plasma (*filled squares*) shows a dose-responsive hemolytic activity that is diminished in plasma pretreated with methylamine (*open square*) to inactivate α_2-macroglobulin. The hemolytic activity of methylamine-treated plasma is restored by addition of 2 μM *Limulus* α_2-macroglobulin (*circle*)

The initial interpretation of these observations was that α_2-macroglobulin plays an essential role as an effector of cytolysis, perhaps similar to the role of C3 in the mammalian complement pathway (Enghild et al. 1990). This, however, does not appear to be the case. The *Limulus* cytolytic system does not show the absolute requirement for α_2-macroglobulin in the manner that the vertebrate system does for C3 (Armstrong et al. 1993). Instead, the actual cytolytic protein of the *Limulus* system is a member of the pentraxin protein family, namely, the lectin limulin (Srimal et al. 1993; Armstrong et al. 1994a). In mammals, members of the pentraxin protein family can activate the classical pathway of the complement system (Miyazawa and Inoue 1990; Jiang et al. 1991). Highly purified limulin is cytolytic alone at physiological concentrations and limulin-free plasma is without cytolytic activity (Armstrong et al. 1994a).Unactivated *Limulus* α_2-macroglobulin does not increase the cytolytic activity of limulin. Instead, protease- or methylamine-activated forms of α_2-macroglobulin function in the *Limulus* cytolytic system as inhibitors of the cytolytic activity of limulin (Armstrong et al. 1994a). Although *Limulus* α_2-macroglobulin does not serve as a functional equivalent of C3, the inhibitory role of protease-activated α_2-macroglobulin may

be of importance in restraining the cytolytic activities of limulin to prevent unwanted damage to host tissues. It is to be remembered that the vertebrate complement system includes a spectrum of regulatory factors that serve exactly that function. Thus, *Limulus* employs molecular homologues to components of the mammalian complement system in its immune defense, but without their serving as exact functional homologues.

4 Evolutionary Considerations

Systems of immunity can be characterized as adaptive or innate. The former are characterized by inducibility and selectivity for the inducing challenge and often have the property of immunologic memory. The latter are expressed constitutively and are of broader reactivity. Probably the best-characterized immune system is the adaptive, immunoglobulin-based immune system of vertebrates. In many people's minds, immunology is solely about the study of the immunoglobulin-based immune system and functionally connected systems such as the complement system. In truth, serious compromise of the adaptive antibody-based immune system of vertebrates is typically followed by illness and death, often at the hands of an indolent pathogen that would be of little threat to the uncompromised individual (Waldman 1988). Although characteristic immunoglobulin protein motifs are of ancient origin, being present in invertebrates and vertebrates (Grenningloh et al. 1990), the system of immunoglobulin gene rearrangement to construct a large number of immune proteins from a small number of genes is unique to vertebrates (Marchalonis and Schluter 1990). That the immunoglobulin-based immune system has not been unambiguously identified in an invertebrate suggests that this system evolved after the Precambrian divergence of the evolutionary lineages leading to the vertebrates and the diverse invertebrate phyla. In this context, it is interesting that invertebrates survive, often in highly septic environments, without this particular system of immunity. Although the life cycle of many invertebrates is completed in only weeks or months, individuals of other species may live for many years. 17-Year cicadas live for 17 years, horseshoe crabs live at least 20 years, lobsters may live for 50 or more years, and freshwater clams live for 80–100 years (Comfort 1961; Finch 1990)—and without antibodies or lymphocytes. It is clearly possible to fashion a highly effective immune system without resorting to the complex and sometimes self-destructive antibody-based system that we see in the vertebrates. Instead, invertebrates depend mostly on a variety of innate immune systems to defend against pathogenic parasites.

Interestingly, several elements of the innate immune system are common to invertebrates and vertebrates. α_2-Macroglobulin is certainly an ancient component of internal defense because it is present in all classes of vertebrates (Starkey and Barrett 1982; Osada et al. 1986), mandibulate and chelicerate arthropods (Armstrong and Quigley 1995), and cephalopod, gastropod, and

bivalve mollusks (Armstrong and Quigley 1992; Thøgersen et al. 1992). The forms of α_2-macroglobulin found in invertebrates share many of the unique features of vertebrate α_2-macroglobulin including the presence of the reactive internal thiol ester (Armstrong and Quigley 1987; Spycher et al. 1987; Sottrup-Jensen et al. 1990a), the ability to interact with proteases of various catalytic mechanisms (Quigley and Armstrong 1985), and an interaction that involves the physical envelopment of the protease molecule by α_2-macroglobulin that leaves the enzymatic site intact (Quigley and Armstrong 1883, 1985; Armstrong et al. 1985; Armstrong and Quigley 1987). Because the vertebrate, arthropod, and molluscan lineages diverged in evolution approximately 550 million years ago (Sepkoski 1978; Runnegar 1982), the presence of α_2-macroglobulin in modern representatives of these three classes of animals indicates its antiquity and demonstrates that the molecule must have evolved prior to the Precambrian evolutionary radiation of the modern phyla of higher animals. The preservation of this unique molecule for so extended a period of evolution in forms as distinctively different as arthropods, mollusks, and vertebrates indicates that it plays an essential role in the survival of higher animals.

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References

Aimes RT, Quigley JP, Swarnakar S, Strickland DK, Armstrong PB (1995) Preliminary investigations on the scavenger receptors of the amebocyte of the American horseshoe crab, *Limulus polyphemus*. Biol Bull 189 (in press)

Andersen GR, Jacobsen L,Thirup S, Nyborg J, Sottrup-Jensen L (1991) Crystallization and preliminary X-ray analysis of methylamine-treated α_2-macroglobulin and 3 α_2-macroglobulin-proteinase complexes. FEBS Lett 292: 267–270

Andersen GR, Thirup S, Nyborg J, Dolmer K, Jacobsen L, Sottrup-Jensen L (1994) Low-resolution X-ray diffraction data obtained from hexagonal crystals of methylamine-treated α_2-macroglobulin. Acta Cryst D50: 298–301

Andreasen PA, Sottrup-Jensen L, Kjøller L, Nykjaer A, Moestrup SK, Petersen CM, Gliemann J (1994) Receptor-mediated endocytosis of plasminogen activators and activator/inhibitor complexes. FEBS Lett 338: 239–245

Arakawa H, Urisaka T, Tsuruta H, Amemiya Y, Kihara H, Ikai A (1994) The kinetics of conformational changes of α_2-macroglobulin determined by time resolved X-ray solution scattering. FEBS Lett 337: 171–174

Armstrong PB, Quigley JP (1987) *Limulus* α_2-macroglobulin. First evidence in an invertebrate for a protein containing an internal thiol ester bond. Biochem J 248: 703–707

Armstrong PB, Quigley JP (1992) Humoral immunity: α_2-macroglobulin activity in the plasma of mollusks. Veliger 35: 161–164

Armstrong PB, Quigley JP (1995) Immune function of α_2-macroglobulin in invertebrates. Prog Mol Subcell Biol. Springer, Berlin Heidelberg New York (in press)

Armstrong PB, Rossner MT, Quigley JP (1985) An α_2-macroglobulin-like activity in the blood of chelicerate and mandibulate arthropods. J Exp Zool 236: 1–9

Armstrong PB, Mangle WF, Wall JS, Hainfield JF, Van Holde KE, Ikai A, Quigley JP (1991) Structure of *Limulus* α_2-macroglobulin. J Biol Chem 266: 2526–2530

Armstrong PB, Armstrong MT, Quigley JP (1993) Characterization of a complement-like hemolytic system in the arthropod, *Limulus polyphemus*: involvement of α_2-macroglobulin and C-reactive protein. Mol Immunol 30: 929–934

Armstrong PB, Misquith S, Srimal S, Melchior R, Quigley JP (1994a) Identification of limulin as a major cytolytic protein in the plasma of the American horseshoe crab, *Limulus polyphemus*. Biol Bull 187: 227–228

Barrett AJ, Brown MA, Sayers CA (1979) The electrophoretically "slow" and "fast" forms of the α_2-macroglobulin molecule. Biochem J 181: 401–418

Barrett AJ, Starkey PM (1973) The interaction of α_2-macroglobulin with proteinases. Characteristics and specificity of the reaction and a hypothesis concerning its molecular mechanism. Biochem J 133: 709–724

Bender RC, Fryer SE, Bayne CK (1992) Proteinase inhibitory activity in the plasma of a mollusc. Evidence for the presence of α_2-macroglobulin. Comp Biochem Physiol B102: 821–824

Beith J, Pichoir M, Metais P (1970) The influence of α_2-macroglobulin on the elastolytic and esterolytic activity of elastase. FEBS Lett 8: 319–321

Bjork I, Fish WW (1982) Evidence for similar conformational changes in α_2-macroglobulin on reaction with primary amines or proteolytic enzymes. Biochem J 207: 347–356

Borth W (1992) α_2-Macroglobulin, a multifunctional binding protein with targeting characteristics. FASEB J 6: 3345–3353

Borth W, Luger TA (1989) Identification of α_2-macroglobulin as a cytokine binding plasma protein. Binding of interleukin-1β to "F" α_2-macroglobulin. J Biol Chem 264: 5818–5825

Breton CB, Blisnick T, Jouin H, Barale JC, Rabilloud T, Langsley G, Pereira da Silva LH (1992) *Plasmodium chabaudi* p68 serine protease activity required for merozoite entry into mouse erythrocytes. Proc Natl Acad Sci USA 89: 9647–9651

Canicatti C (1990) Hemolysins: pore-forming proteins in invertebrates. Experientia 46: 239–244

Chen BJ, Wang D, Yuan AI, Feinman RD (1992) Structure of α_2-macroglobulin-protease complexes. Methylamine competition shows that proteases bridge two disulfide-bonded half molecules. Biochemistry 31: 8960–8966

Christensen U, Simonsen M, Harrit N, Sottrup-Jensen L (1989) Pregnancy zone protein, a proteinase-binding macroglobulin. Interactions with proteinase and methylamine. Biochemistry 28: 9324–9331

Cohn Z (1975) The role of proteases in macrophage physiology. In: Reich E, Rifkin DB, Shaw E (eds) Proteases and biological control. Cold Spring Harbor Conf Cell Proliferation, vol 2. Cold Spring Harbor, New York, Cold Spring Harbor Press, pp 483–893

Comfort A (1961) The life span of animals. Sci Am 205 (2): 108–119

Davidsen O, Christensen EI, Gliemann J (1985) The plasma clearance of human α_2-macroglobulin-trypsin complexes in the rat is mainly accounted for by uptake into hepatocytes. Biochim Biophys Acta 846: 85–92

Delain E, Pochon F, Barray M, Van Leuven F (1992) Ultrastructure of alpha 2-macroglobulins. Electron Microsc Rev 5: 231–281

Devriendt K, van den Berghe H, Cassiman J-J, Marynen P (1991) Primary structure of pregnancy zone protein. Molecular cloning of a full length PZP cDNA clone by the polymerase chain reaction. Biochim Biophys Acta 1088: 95–103

Enghild JJ, Salvesen G, Thøgersen IB, Pizzo SV (1989a) Proteinase-binding and inhibition by the monomeric α-macroglobulin rat α_1-inhibitor-3. J Biol Chem 264: 11428–11435

Enghild JI, Thøgersen IB, Roche PA, Pizzo SV (1989b) A conserved region in α-macroglobulins participates in binding to the mammalian α-macroglobulin receptor. Biochemistry 28: 1406–1412

Enghild JJ, Salvesen G, Brew K, Nagase H (1989c) Interaction of human rheumatoid synovial collagenase (matrix metalloproteinase 1) and stromolysin (matrix metalloproteinase 3)with human α_2-macroglobulin and chicken ovostatin. Binding kinetics and identification of matrix metalloproteinase cleavage sites. J Biol Chem 264: 8779–8785

Enghild JJ, Thøgersen IB, Salvesen G, Fey GH, Fegler NL, Gonias SL, Pizzo SV (1990) α_2-Macroglobulin from *Limulus polyphemus* exhibits proteinase inhibitory activity and participates in a hemolytic system. Biochemistry 29: 10070–10080

Engström Å, Xanthopoulos K, Boman HG, Bennich H (1985) Amino acid and cDNA sequences of lysozyme from *Halophora cecropia*. EMBO J 4: 2119–2122

Feldman SR, Pizzo SV (1984) Comparison of the binding of chicken α_2-macroglobulin and ovomacroglobulin to the mammalian α_2-macroglobulin receptor. Arch Biochem Biophys 235: 267–275

Finch CE (1990) Longevity, senescence and the genome. The University of Chicago Press, Chicago, 922 pp + xv

Furie B, Furie BC (1992) Molecular and cellular biology of blood coagulation. N Engl J Med 326: 800–806

Gliemann J, Larsen TR, Sottrup-Jensen L (1983) Cell association and degradation of α_2-macroglobulin-trypsin complexes in hepatocytes and adipocytes. Biochim Biophys Acta 756: 230–237

Gonias SL (1992) α_2-Macroglobulin: a protein at the interface of fibrinolysis and cellular growth regulation. Exp Hematol 20: 302–311

Gonias SL, Reynolds JA, Pizzo SV (1982) Physical properties of human α_2-macroglobulin following reaction with methylamine and trypsin. Biochim Biophys Acta 705: 306–314

Grenningloh G, Bieber AJ, Rehm EJ, Snow PM, Tarquina ZR, Hortsch M, Patel NH, Goodman CS (1990) Molecular genetics of neuronal recognition in *Drosophila*. Evolution and function of immunoglobulin superfamily cell adhesion molecules. Cold Spring Harbor Symp Quant Biol 55: 327–340

Hall M, Söderhäll K, Sottrup-Jensen L (1989) Amino acid sequence around the thiolester of α_2-macroglobulin from plasma of the crayfish, *Pacifastacus leniusculus*. FEBS Lett 254: 111–114

Hall PK, Roberts RC (1978) Physical and chemical properties of human plasma α_2-macroglobulin. Biochem J 173: 27–38

Harpel PC (1973) Studies on human plasma α_2-macroglobulin-enzyme interactions. Evidence for proteolytic modification of the subunit chain structure. J Exp Med 138: 508–521

Havermann K, Janoff A (1978) Neutral proteases of human polymorphonuclear leukocytes. Urbran and Schwarzenberg, Baltimore

Hergenhahn HG, Söderhäll K (1985) α_2-Macroglobulin-like activity in plasma of the crayfish *Pacifastacus leniusculus*. Comp Biochem Physiol 81B: 833–835

Hergenhahn HG, Aspan A, Söderhäll K (1987) Purification and characterization of a high-Mr proteinase inhibitor of prophenoloxidase activation from crayfish plasma. Biochem J 248: 223–228

Hergenhahn HG, Hall M, Söderhäll K (1988) Purification and characterization of an α_2-macroglobulin-likeproteinase inhibitor from plasma of the crayfish *Pacifastacus leniusculus*. Biochem J 255: 801–806

Huang JS, Huang SS, Deuel TF (1984) Specific covalent binding of platelet derived growth factor to human plasma α_2-macroglobulin. Proc Natl Acad Sci USA 81: 342–347

Hussain MM, Maxfield FR, Más-Oliva J, Tabas I, Ji Z-S, Innerarity TL, Mahley RW (1991) Clearance of chylomicron remnants by the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor. J Biol Chem 266: 13936–13940

Ikai A, Ditamoto T, Nishigai M (1983) Alpha-2-macroglobulin-like protease inhibitor from the egg white of Cuban crocodile (*Crocodylas rhombifer*). J Biochem (Tokyo) 93: 121–127

Ikai A, Kiruchi M, Nishigai M (1990) Interval structure of ovomacroglobulin studied by electron microscopy. J Biol Chem 265: 8280–8284

Iwanaga S, Miyata T, Tokunaga F, Muta T (1992) Molecular mechanism of hemolymph clotting system in *Limulus*. Thromb Res 68: 1–32

James K (1990) Interactions between cytokines and α_2-macroglobulin. Immunol Today 11: 163–167

Jiang H, Siegel JN, Gewurz H (1991) Binding and complement activation by C-reactive protein via the collagen-like region of C1q and inhibition of these reactions by monoclonal antibodies to C-reactive protein and C1q. J Immunol 146: 2324–2330

Kitano T, Nakashima M, Ikai A (1982) Hen egg white ovomacroglobulin has a protease inhibitory activity. J Biochem 92: 1679–1682

Kristensen T, Moestrup SK, Gliemann J, Bendtsen L, Sand O, Sottrup-Jensen L (1990) Evidence that the newly cloned low-density-lipporotein receptor related protein (LRP) is the α_2-macroglobulin receptor. FEBS Lett 276: 151–155

Laskowski M, Kato I (1980) Protein inhibitors of proteases. Annu Rev Biochem 49: 593–626

Law SK, Levine RP (1977) Interaction between the third complement protein and cell surface macromolecules. Proc Natl Acad Sci USA 74: 2701–2705

Law SK, Reid KBM (1988) *Complement*. IRL Press, Oxford, 72 pp

Liang Z, Lindblad P, Beauvais A, Johansson MW, Latage J-P, Hall M, Cerenius L, Söderhäll K (1992) Crayfish α_2-macroglobulin and 76 kDa protein; their biosynthesis and subcellular localization of the 76kDa protein. J Insect Physiol 38: 987–995

Marchalonis JJ, Schluter SF (1990) Origins of immunoglobulins and immune recognition molecules. BioScience 40: 758–768

McKerrow JH, Sun E, Rosenthal PJ, Bouvier J (1993) The proteases and pathogenicity of parasitic protozoa. Annu Rev Microbiol 47: 821–853

Miyazawa K, Inoue K (1990) Complement activation induced by human C-reactive protein in mildly acidic conditions. J Immunol 145: 650–654

Melchior R, Quigley JP, Armstrong PB (1995) α_2-Macroglobulin-mediated clearance of proteases from the plasma of the American horseshoe crab, *Limulus polyphemus*. J Biol Chem (in press)

Moestrup SK, Holtet TL, Etzerodt M, Thøgersen HC, Nykjaer A, Andreasen PA, Rasmussen HH, Sottrup-Jensen L, Gliemann J (1993) α_2-Macroglobulin-proteinase complexes, plasminogen activator inhibitor type-1-plasminogen activator complexes, and receptor-associated protein bind to a region of the α_2-macroglobulin receptor containing a cluster of eight complement-type repeats. J Biol Chem 268: 13691–13696

Nagase H, Harris ED (1983) Ovostatin: a novel proteinase inhibitor from chicken egg white II. Mechanism of inhibition studied with collagenase and thermolysin. J Biol Chem 258: 7490–7498

Nagase J, Harris ED, Woessner JF, Brew K (1983) Ovostatin: a novel proteinase inhibitor from chicken egg white I. Purification, physicochemical properties and tissue distribution of ovostatin. J Biol Chem 258: 7481–7489

Nielsen KL, Sottrup-Jensen L, Nagase H, Thøgersen HC, Etzerodt M (1994) Amino acid sequence of hen ovomacroglobulin (ovostatin) deduced from cloned cDNA. DNA Sequencing 5: 111–119

Nykjaer A, Bengtsson-Olivecrona G, Lookene A, Moestrup SK, Petersen CM, Weber W, Beisiegel U, Gliemann J (1993) The α_2-macroglobulin receptor/low density lipoprotein receptor-related protein binds lipoprotein lipase and β-migrating very low density lipoprotein associated with the lipase. J Biol Chem 268: 15048–15055

O'Connor-Court MD, Wakefield LM (1987) Latent transforming growth factor-β in serum. J Biol Chem 262: 14090–14099

Ogata H, Kouyoumdjian M, Borges DR (1993) Comparison between clearance rates of plasma kallikrein and of plasma kallikrein-α_2-macroglobulin complexes by the liver. Int J Biochem 25: 1047–1051

Ohlsson K (1971) Elimination of [^{125}I]-trypsin α_2-macroglobulin complexes from blood by the reticuloendothelial cells in dog. Acta Physiol Scand 81: 269–272

Osada T, Nishigai M, Ikai A (1986) Open quaternary structure of the hagfish proteinase inhibitor with similar properties to human α_2-macroglobulin. J Ultrastruct Mol Struct Res 96: 136–145

Perlmutter DH, Pierce JA (1989) The α_1-antitrypsin gene and emphysema. Am J Physiol 257: L147–L162

Quigley JP, Armstrong PB (1983) An endopeptidase inhibitor, similar to mammalian α_2-macroglobulin, detected in the hemolymph of an invertebrate, *Limulus polyphemus*. J Biol Chem 258: 7903–7906

Quigley JP, Armstrong PB (1985) A homologue of α_2-macroglobulin purified from the hemolymph of the horseshoe crab *Limulus*. J Biol Chem 260: 12715–12719

Quigley JP, Armstrong PB, Gallant P, Rickles FR, Troll W (1982) An endopeptidase inhibitor, similar to vertebrate α_2-macroglobulin, present in the plasma of *Limulus polyphemus*. Biol Bull 163: 402

Quigley JP, Ikai A, Arakawa H, Osada T, Armstrong PB (1991) Reaction of proteinases with α_2-macroglobulin from the American horseshoe crab, *Limulus*. J Biol Chem 266: 19426–19431

Reid KBM, Porter RR (1981) The proteolytic activation systems of complement. Annu Rev Biochem 50: 433–464

Runnegar B (1982) The Cambrian explosion: animals or fossils? J Geol Soc Aust 29: 395–411

Salvesen GS, Sayers CA, Barrett AJ (1981) Further characterization of the covalent linking reaction of α_2-macroglobulin. Biochem J 195: 453–461

Sepkoski JJ (1978) A kinetic model of phanerozoic taxonomic diversity I. Analysis of marine orders. Paleobiology 4: 223–251

Söderhäll K, Smith VJ (1986) Prophenoloxidase-activating cascade as a recognition and defense system in arthropods, In: Gupta AP (ed) Hemocytic and humoral immunity in arthropods. Wiley, New York, pp 251–285

Sottrup-Jensen L (1987) α_2-Macroglobulin and related thiol ester plasma proteins. In: Putnam FW (ed) The plasma proteins. Structure, function, and genetic control, 2nd edn, vol 5. Academic Press, Orlando, pp 191–291

Sottrup-Jensen L, Petersen TE, Magnusson S (1980) A thiol-ester in α_2-macroglobulin cleaved during proteinase complex formation. FEBS Lett 121: 275–279

Sottrup-Jensen L, Lonblad PB, Stephanik TM, Peterson TE, Magnusson S, Jornvall H (1981) Primary structure of the "bait" region for proteinases in α_2-macroglobulin. Nature of the complex. FEBS Lett 127: 167–173

Sottrup-Jensen L, Gliemann J, Van Leuven F (1986) Domain structure of human α_2-macroglobulin. Characterization of a receptor-binding domain obtained by digestion with papain. FEBS Lett 205: 20–24

Sottrup-Jensen L, Sand O, Dristensen L, Fey GH (1989) The α_2-macroglobulin bait region. Sequence diversity and localization of cleavage sites for proteinases in five mammalian α_2-macroglobulins. J Biol Chem 264: 15781–15789

Sottrup-Jensen L, Borth W, Hall M, Quigley JP, Armstrong PB (1990a) Sequence similarity between α_2-macroglobulin from the horseshoe crab, *Limulus polyphemus*, and proteins of the α_2-macroglobulin family from mammals. Comp Biochem Physiol 96B: 621–625

Sottrup-Jensen L, Hansen JF, Pedersen HS, Kristensen L (1990b) Localization of lysyl-γ-glutamyl cross-links in five human α_2-macroglobulin-proteinase complexes.Nature of the high molecular weight cross-linked products. J Biol Chem 265: 17727–17737

Sporn MB, Roberts AB (1990) Peptide growth factors, vols I and II. Springer, Berlin Heidelberg New York

Spycher SE, Arya S, Isenman DE, Painter H (1987) A functional, thioester-containing α_2-macroglobulin homologue isolated from the hemolymph of the American lobster (*Homarus americanus*). J Biol Chem 262: 14606–14611

Srimal S. Quigley JP, Armstrong PB (1993) Limulin and C-reactive protein from the plasma of *Limulus polyphemus* are different proteins. Biol Bull 185: 325

Starkey PM, Barrett AJ (1977) α_2-Macroglobulin, a physiological regulator of proteinase activity. In: Barrett AJ (ed) Proteinases in mammalian cells and tissues. Elsevier, Amsterdam, pp 663–696

Starkey PM, Barrett AJ (1982) Evolution of α_2-macroglobulin. The demonstration in a variety of vertebrate species of a protein resembling human α_2-macroglobulin. Biochem J 205: 91–95

Steinbuch M, Pejaudier L, Quentin M, Martin V (1968) Molecular alteration of α_2-macroglobulin by aliphatic amines. Biochim Biophys Acta 154: 228–231

Stöcker W, Breit S, Sottrup-Jensen L, Zwilling R (1991) α_2-Macroglobulin from the haemolymph of the freshwater crayfish *Astacus astacus*. Comp Biochem Physiol 98B: 501–509

Strickland DK, Ashcom JD, Williams S, Burgess WH, Migliorini M, Argraves WS (1990) Sequence identity between the α_2-macroglobulin receptor and low density lipoprotein

receptor-related protein suggests that this molecule is a multifunctional receptor. J Biol Chem 265: 17401–17404

Swenson RP, Howard JB (1979) Characterization of alkylamine-sensitive site in α_2-macroglobulin. Proc Natl Acad Sci USA 76: 4313–4316

Tack BF (1983) The β-Cys-γ-Glu thiolester bond in human C3, C4 and α_2-macroglobulin. Springer Semin Immunopathol 6: 259–282

Tack BF, Harrison RA, Janatova J, Thomas ML, Prahl JW (1980) Evidence for presence of an internal thiolester bond in third component of human complement. Proc Natl Acad Sci USA 77: 5764–5768

Tennent GA, Butler PJG, Hutton T, Woolfitt AR, Harvey DJ, Rademacher TW, Pepys MB (1993) Molecular characterization of *Limulus polyphemus* C-reactive protein. 1. Subunit composition. Eur J Biochem 214: 91–97

Testa JE, Quigley JP (1990) The role of urokinase-type plasminogen activator in aggressive tumor cell behavior. Cancer Metast Rev 9: 353–367

Thøgersen IB, Salvesen G, Brucato FH, Pizzo SV, Enghild JJ (1992) Purification and characterization of an α-macroglobulin proteinase inhibitor from the mollusc *Octopus vulgaris*. Biochem J 285: 521–527

Thomsen NK, Sottrup-Jensen L (1993) α_2-Macroglobulin domain structure studied by specific limited proteolysis. Arch Biochem Biophys 300: 327–334

Travis J, and Salvesen GS (1983) Human plasma proteinase inhibitors. Annu Rev Biochem 52: 655–709

Van Dijk MCM, Boers W, Linthorst C, van Berkel TJC (1992) Role of the scavenger receptor in the uptake of methylamine-activated α_2-macroglobulin by rat liver. Biochem J 287: 447–455

Van Leuven F (1984) Human α_2-macroglobulin. Primary amines and the mechanisms of endoprotease inhibition and receptor-mediated endocytosis. Mol Cell Biochem 58: 121–128

Van Leuven F, Cassiman J-J, Van Den Berghe J (1981) Functional modifications of α_2-macroglobulin by primary amines II. Inhibition of covalent binding of trypsin to α_2M by methylamine and other primary amines. J Biol Chem 256: 9023–9027

Van Leuven F, Marynen P, Sottrup-Jensen L, Cassiman J-J, Van Den Berghe H (1986) Receptor-binding domain of human α_2-macroglobulin. Isolation after limited proteolysis with a bacterial proteinase. J Biol Chem 261: 11369–11373

Vu T-K, Hung DT, Wheaton VI, Coughlin SR (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64: 1057–1068

Waldman TA (1988) Immunodeficiency diseases. In: Samter M, Talmage DW, Frank MM, Austen KF, Claman HN (eds) Immunological diseases, 4th edn. Little, Brown & Co, Boston, pp 411–465

Werb Z (1993) Proteases and matrix degradation. In: Kelley WN, Harris ED, Ruddy S, Sledge CB (eds) Textbook of rheumatology, 4th edn. Saunders, Philadelphia, pp 300–321

Wu K, Wang D, Feinman RD (1981) Inhibition of proteases by α_2-macroglobulin. The role of lysyl amino groups of trypsin in covalent complex formation. J Biol Chem 256: 10409–10414

Yochem J, Greenwald I (1993) A gene for a low density lipoprotein-related protein in the nematode *Caenorhabditis elegans*. Proc Natl Acad Sci USA 90: 4572–4576