The Interaction of α_2 -Macroglobulin with Proteinases

BINDING AND INHIBITION OF MAMMALIAN COLLAGENASES AND OTHER METAL PROTEINASES

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1. Experiments were performed to determine whether the specific collagenases and other metal proteinases are bound and inhibited by α_2 -macroglobulin, as are endopeptidases of other classes. 2. A specific collagenase from rabbit synovial cells was inhibited by human serum. The inhibition could be attributed entirely to α_2 -macroglobulin; α_1 -trypsin inhibitor was not inhibitory. α_2 -Macroglobulin presaturated with trypsin or cathepsin B1 did not inhibit collagenase, and pretreatment of α_2 -macroglobulin with collagenase prevented subsequent reaction with trypsin. The binding of collagenase by α_2 -macroglobulin was not reversible in gel chromatography. 3. The collagenolytic activity of several rheumatoid synovial fluids was completely inhibited by incubation of the fluids with α_2 -macroglobulin. 4. The collagenase of human polymorphonuclear-leucocyte granules showed time-dependent inhibition by α_2 -macroglobulin. 5. The collagenolytic metal proteinase of *Crotalus atrox* venom was inhibited by α_2 -macroglobulin. 6. The collagenase of *Clostridium histolyticum* was bound by α_2 -macroglobulin, and inhibited more strongly with respect to collagen than with respect to a peptide substrate. 7. Thermolysin, the metal proteinase of *Bacillus thermoproteolyticus*, was bound and inhibited by α_2 -macroglobulin. 8. It was shown by polyacrylamide-gel electrophoresis of reduced α_2 -macroglobulin in the presence of sodium dodecyl sulphate that synovial-cell collagenase, clostridial collagenase and thermolysin cleave the quarter subunit of α_2 -macroglobulin near its mid-point, as do serine proteinases. 9. The results are discussed in relation to previous work, and it is concluded that the characteristics of interaction of the metal proteinases with α_2 -macroglobulin are the same as those of other proteinases.

α_2 -Macroglobulin (α_2 M)* is a plasma glycoprotein of molecular weight about 725 000 (Jones *et al.*, 1972). The protein has been known for some time to bind and inhibit trypsin and various other 'serine proteinases'. [Proteolytic enzymes are classified in the present paper as was proposed by Hartley (1960), except that the term 'carboxyl' is adopted for the 'acid' proteinases; thus the four classes of endopeptidases are the 'serine', 'thiol', 'carboxyl' and 'metal' proteinases.] It has been established that proteinases of both 'thiol' and 'carboxyl' classes are also bound by α_2 M, and a general theory has been put forward about the nature of the interaction of endopeptidases with α_2 M (Starkey & Barrett, 1973; Barrett & Starkey, 1973). Briefly, it has been proposed that essentially any active proteinase can cleave

one of a series of peptide bonds in a sensitive region of the α_2 M molecule, and that this results in a conformational change in the macroglobulin such that the enzyme molecule is entrapped. The results of Harpel (1973) have provided further evidence for this possibility and indicate that a region sensitive to proteolysis exists near the mid-point of the polypeptide chain of each of the quarter subunits of α_2 M. The specific reaction of endopeptidases with α_2 M has certain general characteristics. (1) A molecule of α_2 M reacts irreversibly with a single proteinase molecule, so that saturation with one enzyme prevents subsequent binding of another. (2) The activity of the bound enzyme is more strongly inhibited with respect to large than to small substrate molecules. (3) The activity of the bound enzyme is resistant to other high-molecular-weight inhibitors, but is not protected against low-molecular-weight inhibitors.

Barrett & Starkey (1973) did not provide any evidence that the metal-dependent endopeptidases react with α_2 M in the same way as the serine, thiol and carboxyl enzymes, and yet the capacity to

* Abbreviations: α_2 M, α_2 -macroglobulin; IgG, immunoglobulin G; Dip-F, di-isopropylphosphorofluoridate; Pz-Pro-Leu-Gly-Pro-D-Arg, *p*-phenylazobenzyl-oxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine; Bz-Arg-NHC₁₀H₇, α ,*N*-benzoyl-DL-arginine 2-naphthylamide; Tos-LysCH₂Cl, 7-amino-1-chloro-3-tosylamido-L-heptan-2-one.

eliminate these enzymes could well be an important aspect of the proposed physiological function of α_2 M in the control of extracellular proteolytic activity. Not only do the specific collagenases of mammalian tissues have the characteristics of metal proteinases (M. C. Burleigh, unpublished work), but the less specific collagenases of some snake venoms and of various bacteria also fall into this group. Since the general hypothesis for the mechanism of interaction of α_2 M with proteinases (Barrett & Starkey, 1973) requires that α_2 M serve as a substrate for the enzyme in the first stage of the interaction, it seemed that the highly specific mammalian collagenases would represent a critical test of the general nature of the reaction with endopeptidases; these collagenases are not generally thought to attack any protein other than collagen. Some previous workers have reported that various collagenases are resistant to inhibition by serum, or purified α_2 M. The evidence has been re-examined, and on the basis of further experiments the present authors conclude that the mechanism of interaction of the metal proteinases with α_2 M is the same as that of proteinases of the other classes. A preliminary report of part of the present work has been published (Werb, 1973).

Materials and Methods

[14 C]Glycine-labelled collagen, for use as a substrate for collagenase, was prepared as described by Werb & Burleigh (1974). Assays of collagenolytic activity with the radioactive substrate in the form of reconstituted fibrils or in solution, were as described by Werb & Burleigh (1974). One unit of collagenase activity rendered soluble 1 μ g of reconstituted 14 C-labelled collagen fibrils/min at 35°C.

Specific collagenases were partially purified from the 3-day serum-free culture medium of rabbit synovial fibroblasts and of rabbit skin. The media were dialysed against 100mM-Tris-HCl buffer containing 5mM-CaCl₂ (Tris-Ca²⁺ buffer), concentrated by ultrafiltration in a Diaflo 50 apparatus fitted with a PM-10 membrane (Amicon Ltd., High Wycombe, Bucks., U.K.) and partially purified by gel filtration on Sephadex G-100 as described by Werb & Burleigh (1974).

For the preparation of polymorphonuclear-leucocyte granules, the cells were isolated from 2 litres of pooled human blood as described by Lazarus *et al.* (1968) and homogenized in 10vol. of 0.34M-sucrose by 15s bursts in a Polytron homogenizer (The Northern Media Supply Ltd., Hull, U.K.). The homogenate was centrifuged at 10000g for 15min at 4°C, and the pellet was resuspended in 5vol. of 0.34M-sucrose and frozen and thawed five times to disrupt the granules. The preparation was clarified by centrifugation at 10000g for 10min and used immediately.

Synovial fluids removed by needle aspiration from knee joints, and samples of synovial tissue, from patients with rheumatoid arthritis, were kindly provided by Dr. J. Cosh, Royal National Hospital for Rheumatoid Diseases, Bath, U.K.; the fluids were stored frozen until used. The synovial tissue was cultured as described for rabbit skin (Werb & Burleigh, 1974).

Chromatographically purified collagenase from *Clostridium histolyticum* was grade CLSPA (250 units/mg) from Worthington Chemical Corp., obtained through Cambrian Chemicals Ltd., Croydon CR9 6AG, U.K. Twice-crystallized bovine trypsin (type III), venom from the rattlesnake *Crotalus atrox* and thermolysin were obtained from Sigma (London) Chemical Co. Ltd., Kingston-on-Thames, Surrey KT2 7BH, U.K.

The synthetic substrate Pz-Pro-Leu-Gly-Pro-D-Arg (Collagenase Chromophor Substrat) made by Fluka A.-G. was purchased from Fluorochem Ltd., Glossop SK13 9NU, U.K., and used in assays as described by Werb & Burleigh (1974).

The metal proteinase of *Crotalus atrox* venom was assayed with Azocoll (dyed hide powder) (Calbiochem Ltd., 10 Wyndham Place, London W1H 1AS, U.K.) as substrate. Reaction mixtures (4ml) contained 50mM-Tris-HCl buffer, pH 7.8, with 10mM-CaCl₂, and 5mg of Azocoll; the incubations were for 2h at 37°C with gentle agitation. CaCl₂ was omitted from reaction mixtures for tests of inhibition by chelating agents. At the end of the incubation period, the reaction mixtures were filtered and E_{520} of the filtrates was measured. E_{520} was related to enzymic activity by use of a standard curve.

Thermolysin was assayed in reaction mixtures (1ml) containing 15mg of azocasein (Charney & Tomarelli, 1947) in 25mM-Tris-HCl buffer, pH 7.6, containing 5mM-CaCl₂. After 10min at 40°C the reactions were stopped by addition of 5ml of 5% (w/v) trichloroacetic acid, and the E_{366} of the filtrates was measured.

Trypsin was labelled with [3 H]acetic anhydride, and assayed with Bz-Arg-NHC₁₀H₇ as substrate, as described by Barrett & Starkey (1973). The operational molarity of solutions of trypsin was determined by active-site titration (Chase & Shaw, 1967). Human cathepsin B1 was assayed and purified as described by Barrett (1972, 1973).

Normal human serum, plasma and blood were kindly supplied by Dr. C. Entwistle, Immunohaematology Unit, Addenbrooke's Hospital, Cambridge, U.K. Human α_2 M was prepared by Method II of Barrett & Starkey (1973), and its concentration in solution was determined by single radial diffusion on Partigen plates supplied by Hoechst Pharmaceuticals Ltd., Brentford, Middx., U.K. Rabbit anti-(human α_2 M) and anti-(human serum protein) immunoglobulins were obtained

from Mercia Diagnostics Ltd., Watford, Herts., U.K. Immunoelectrophoresis and double immunodiffusion were performed as described by Starkey & Barrett (1973).

Polyacrylamide-gel electrophoresis of proteins in the presence of sodium dodecyl sulphate was carried out essentially as described by Weber & Osborn (1969) except that the phosphate buffer of the gels and reservoirs was replaced by one consisting of 0.10M-Tris with 0.10M-Bicine [*N,N*-bis(2-hydroxyethyl)-glycine] (pH 8.1). The heavy chain of rabbit myosin (kindly given by Dr. A. Weeds, M.R.C. Molecular Biology Laboratory, Cambridge, U.K.) (mol. wt. 200000) and horse transferrin (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) (mol. wt. 75000) served as molecular-weight markers.

Results

Collagenase from rabbit synovial fibroblasts

Inhibition by human serum fractions. Normal human serum (1.5 ml) was run on a column (1.5 cm $\times$ 85 cm, 150 cm³) of Sephadex G-200 in 100 mM-Tris-HCl

buffer, pH 7.6, at 4°C. Fractions (1.5 ml) were assayed for inhibitory activity against trypsin and synovial-cell collagenase, 50 μ l samples being used with 0.1 unit of collagenase in the assay with reconstituted collagen fibrils, and 100 μ l samples with 25 μ g of trypsin assayed against Bz-Arg-NHC₁₀H₇.

The results are presented in Fig. 1. A single peak of inhibitory activity against collagenase was obtained in the fractions containing high-molecular-weight proteins including α_2 M. A peak of inhibitory activity against trypsin was also associated with the distribution of α_2 M, but a second much larger peak corresponded to α_1 -trypsin inhibitor. The complex of trypsin with α_2 M is known to retain about 80% activity against low-molecular-weight substrates (Ganrot, 1966), whereas α_1 -trypsin inhibitor causes complete inhibition.

Samples (50 μ l) from the two peaks of inhibitory activity against trypsin were tested for the property of inhibiting the action of collagenase on collagen in solution (see the Materials and Methods section). As shown in Plate 1, the α_2 M-rich samples inhibited the cleavage of collagen, whereas the α_1 -trypsin-inhibitor-rich peak had no effect.

Fig. 1. Distribution of collagenase inhibitor after gel chromatography of human serum

Distribution of protein (····) and inhibitory activity against rabbit synovial fibroblast collagenase (----) and trypsin (—) in effluent fractions obtained by gel chromatography of normal human serum on Sephadex G-200 (see the text for details). The distribution of various serum proteins, determined by immunoelectrophoresis and double diffusion against antisera specific for α_2 M and α_1 -trypsin inhibitor, is indicated by the horizontal bars: (a) α_2 M, (b) haptoglobin, (c) IgG, (d) α_1 -trypsin inhibitor, (e) albumin, (f) transferrin.

The collagenase was not inhibited by sheep IgG (0.27 mg/ml) or crystalline bovine serum albumin (20 mg/ml).

Dose-response of inhibition by human serum and purified α_2 M. Various quantities of normal human serum and purified α_2 M were tested for the capacity to inhibit collagenase activity against reconstituted fibrils. The concentration of α_2 M in both serum and the purified preparation was determined by radial immunodiffusion (see the Materials and Methods section) and the results are expressed in terms of the amount of α_2 M in each incubation mixture (Fig. 2). It is clear that all the inhibitory activity of serum is attributable to α_2 M.

Identity of the collagenase-inhibiting site of α_2 M with that active against other endopeptidases. It has previously been shown that a molecule of α_2 M has the capacity to react with one proteinase molecule only, so that it may be saturated with one enzyme and is then unable to react with another (Barrett & Starkey, 1973). Experiments were done to establish whether α_2 M reacts with collagenase in the same way.

Samples of α_2 M (13.8 μ g, 19 pmol) were treated with human cathepsin B1 (0.25 or 4.0 μ g, 10 or 160 pmol), or trypsin (10 μ g, 256 pmol), or left untreated. The mixtures (100–240 μ l) in 50 mM-potassium phosphate buffer, pH 6.0, with 2 mM-cysteine for the cathepsin B1 samples, were incubated at 4°C for 30 min. The reaction mixtures were adjusted to 0.1 mM-Tos-LysCH₂Cl, to inactivate the cathepsin B1 and trypsin, and 0.12 unit of collagenase was then added to each tube. The complete mixtures were dialysed against Tris-Ca²⁺ buffer (see the Materials and Methods section) before being

assayed for collagenase activity. Complete inhibition of the collagenase was produced by untreated α_2 M and also by that treated with 10 pmol (0.52 molar ratio) of cathepsin B1. Very little inhibition (12%) was produced by the α_2 M that had reacted with 160 pmol of cathepsin B1 (an 8.4-fold molar excess), and only 1% inhibition was produced by the sample that had been exposed to 256 pmol (a 13.5-fold excess) of trypsin. A sample of the α_2 M that had been treated with 8.4 molecular weight equivalents of cathepsin B1 was treated with ³H-labelled trypsin (0.5 mg) and run on Sepharose 6B as described by Barrett & Starkey (1973). None of the radioactivity ran with the α_2 M, confirming that saturation had indeed been achieved with cathepsin B1. Tos-LysCH₂Cl is an inhibitor of both cathepsin B1 and trypsin, but not of collagenase (Werb & Burleigh, 1974), and it was used to establish that the former enzymes were not interfering in the assay of collagenases. Identical results were obtained without the use of Tos-LysCH₂Cl, however.

In another experiment the effect of pretreatment of α_2 M with collagenase on the subsequent inhibition of trypsin was determined. Samples (in quadruplicate) of α_2 M (69 μ g, 95 pmol) were treated with rabbit fibroblast collagenase (0.61 or 11 units), or were left untreated, in a total volume of 50 μ l of Tris-Ca²⁺ buffer, for 30 min at 4°C. Duplicate samples from each group were assayed for collagenase activity in the assay with reconstituted collagen fibrils, or for the capacity to inhibit the action of trypsin (320 pmol) on Bz-Arg-NHC₁₀H₇. The samples treated with 0.61 unit of collagenase had no collagenase activity, and had 89% of the inhibitory capacity for trypsin of the untreated α_2 M. The samples treated with 11 units of collagenase retained 5.4 units of collagenase activity, and had no inhibitory capacity for trypsin. Collagenase alone had no effect on tryptic activity, and it was concluded from this experiment that 11 units, but not 0.61 unit, of collagenase saturated 95 pmol of α_2 M, preventing subsequent reaction with trypsin.

Irreversibility of binding of collagenase by α_2 M. Collagenase, α_2 M and mixtures of collagenase and α_2 M such that either enzyme or inhibitor was in excess were run on a column (1.5 cm $\times$ 28 cm, 50 cm³) of Sephadex G-200 in 50 mM-Tris-HCl buffer, pH 7.5, containing 5 mM-CaCl₂ and 200 mM-NaCl. Effluent fractions (2% of bed vol.) were analysed for collagenase activity.

When collagenase alone (6.2 units) was run on the column, 6.0 units were recovered in the effluent fractions (peak, 63% of bed vol.), whereas no collagenase was detected after chromatography of α_2 M (138 μ g) alone (peak, 37% of bed vol.). When a mixture of α_2 M (138 μ g) with 1.25 units of collagenase was run, negligible activity was recovered, whereas 11.7 units were recovered from a similar mixture containing 22 units of collagenase. The

Fig. 2. Inhibition of rabbit synovial-fibroblast collagenase by α_2 M

The collagenase (0.3 unit) was treated with α_2 M as the purified protein (----) or as the equivalent amount of normal human serum (—).

EXPLANATION OF PLATE I

Disc electrophoresis of fragments produced by the action of rabbit synovial-cell collagenase on collagen in solution at 24°C

The direction of migration was downwards, towards the cathode. The single α chains and cross-linked dimers (β) of collagen are degraded to the specific cleavage products β^A , α^A and α^B by the active enzyme (Werb & Burleigh, 1974). The staining due to serum proteins in two samples is marked 'S'. (i) Control incubation without enzyme, (ii) with collagenase (0.5 unit), (iii) with collagenase and partially purified α_2 M (about 10 μ g), (iv) with collagenase and α_1 -trypsin-inhibitor-rich material (about 10 μ g of α_1 -trypsin inhibitor on the basis of inhibition of trypsin), and (v) collagen without collagenase, but with α_1 -trypsin inhibitor.

EXPLANATION OF PLATE 2

Electrophoresis of α_2 M and the products of interaction with trypsin, thermolysin and synovial-cell collagenase, after reduction in the presence of sodium dodecyl sulphate

The direction of migration was downwards, towards the anode; all gels were loaded with $6\mu\text{g}$ of α_2 M or products derived from it. The calibration is in terms of the mobility of the Bromophenol Blue dye front. (i) Control α_2 M untreated with proteinases (showing minor bands due to impurities running ahead of the quarter subunit of α_2 M), (ii) α_2 M treated with $0.18\mu\text{g}$ and (iii) $0.36\mu\text{g}$ of trypsin; α_2 M treated with (iv) $0.18\mu\text{g}$ and (v) $0.36\mu\text{g}$ of thermolysin; α_2 M treated with (vi) 0.36 unit and (vii) 0.90 unit of synovial-cell collagenase. The enzyme treatments were for 30 min at 40°C , as described in the text. Bands due to the enzyme protein ran near the front and were barely visible.

activity recovered from mixtures with α_2 M was at the range of elution volume characteristic of free collagenase. It was concluded that free collagenase could be eluted in good yield from the Sephadex G-200 column, but that when 1.25 units were run with an excess of α_2 M (so that the mixture showed no activity) a negligible amount of activity was detectable after gel filtration. When collagenase was in excess (so that 54% of activity remained directly detectable, as shown above) 53% of the activity was detectable after gel filtration. Thus there was no reversal of inhibition in gel filtration, and it was concluded that the α_2 M-collagenase complex had no detectable tendency to dissociate under these conditions. In a separate experiment it was found that collagenase activity could be partially recovered from the α_2 M-collagenase complex by denaturation of the α_2 M with 3M-NaSCN as described by Abe & Nagai (1972a).

Collagenase from rabbit skin in tissue culture

Collagenase partially purified from rabbit skin was rapidly and completely inhibited by human and rabbit serum and by purified α_2 M, but not inhibited by serum fractions rich in α_1 -trypsin inhibitor, these results were obtained in assays with both reconstituted collagen fibrils and with collagen in solution. The enzyme from skin thus resembled that from synovial cells.

Collagenase from human synovial tissue in tissue culture

Small amounts of human collagenase were also obtained from the medium of human synovial tissue maintained in tissue culture, as described for rabbit skin. This enzyme was completely inhibited by α_2 M without preincubation.

Collagenase from human synovial fluid

Synovial fluids from ten patients with rheumatoid arthritis were tested for collagenase activity by using reconstituted collagen fibrils, and three were found to be active, containing 3.0, 3.8 and 16.6 units/ml respectively. The assays were made in the presence of 20mM-CaCl₂, rather than the 2mM concentration usually used, because this gave greater activity. Samples (60 or 80 μ l) of the synovial fluids showing collagenolytic activity were mixed with α_2 M (200–500 μ g) and tested in the assay with reconstituted collagen fibrils during a 20h incubation. It was found that 35% (range 26–48%) of the original activity remained detectable, and this percentage was independent of the amount of α_2 M used. In further experiments with only 4h incubation,

82 $\pm$ 3.2% (S.E.M., $n = 6$) of activity persisted, and this suggested that inhibition might be occurring progressively during the incubation period.

The effect of time on the interaction of synovial fluid collagenase with α_2 M was further examined in an experiment in which mixtures of individual synovial fluids with an excess of α_2 M were assayed with variable periods of preincubation and of incubation with the substrate (Fig. 3). The lines $\cdots \cdots$ show that the enzyme remained fully active in the absence of α_2 M; the lines $—$ show the rate of inactivation by interaction with α_2 M during preincubation, being defined by samples with various times of preincubation, and the line $----$ shows the rate of interaction under assay conditions, since the duration of the assay was variable and there was no preincubation. These representative results, obtained with a single fluid, show that the half-time for inhibition during the preincubation under these experimental conditions was about 1.7h,

Fig. 3. Rate of interaction of synovial-fluid collagenase with α_2 M in the presence or absence of collagen

Samples of synovial fluid were preincubated in 50mM-Tris-HCl buffer (pH 7.6) containing 30mM-CaCl₂ with or without α_2 M; they were then assayed for collagenase activity in the assay with reconstituted collagen fibrils. $\circ$, 20 μ l of synovial fluid preincubated for various periods without α_2 M, and then assayed for 4h; Δ , 5 μ l samples preincubated without α_2 M and assayed for 20h; $\bullet$, 20 μ l samples preincubated with 720 μ g of α_2 M and assayed for 4h; $\blacktriangle$, 5 μ l samples preincubated with 180 μ g of α_2 M and assayed for 20h; $\blacksquare$, 20 μ l samples not preincubated, but assayed directly for various periods. These amounts of collagenase were chosen so that the maximum lysis was always within the linear range of the assay. The activities are expressed as a percentage of those of controls without preincubation.

but this increased to 11 h in the presence of collagen. In other experiments, in which suboptimal (2 mM) concentrations of Ca^{2+} were present during the incubation in the presence of collagen, the half-time for inhibition increased to over 30 h. The single exponential progress of the inhibition suggests that only one collagenase was present.

Collagenase of human granulocytes

An extract of granules from human polymorphonuclear leucocytes was prepared, and shown to have activity against collagen in solution and against reconstituted collagen fibrils. The granule extract was mixed with normal human plasma (3 or 33% final concentration) or purified $\alpha_2\text{M}$ (giving final concentrations equivalent to 3, 33 or 167% plasma). A duplicate set of tubes also contained 1 mM-Dip-F to inactivate any serine proteinase that might otherwise have interfered by degrading the collagenase (Lazarus *et al.*, 1972) and also by competing with collagenase for binding by $\alpha_2\text{M}$. The mixtures were left at 4°C overnight, and were then assayed for activity against collagen in solution and reconstituted collagen fibrils. At all concentrations of plasma or purified $\alpha_2\text{M}$ there was complete inhibition, whereas control mixtures containing no plasma protein retained full activity. In other experiments when human plasma was added at the start of the collagenase assay, without preincubation, very little inhibition was detected. Lazarus *et al.* (1968) found that the collagenase of polymorphonuclear leucocytes was not inhibited by serum, but their experiments were made without preincubation of the enzyme with serum, and without the use of an inhibitor of serine proteinases. By analogy with the results obtained with collagenase of human synovial fluid, it now seems likely that no inhibition was detected in these earlier experiments because the collagenase- $\alpha_2\text{M}$ reaction is rather slow, especially in the presence of collagen.

Binding and inhibition of non-mammalian metal proteinases by $\alpha_2\text{M}$

As part of an attempt to establish whether metal proteinases with specificities different from that of mammalian collagenases interact similarly with $\alpha_2\text{M}$, the Zn^{2+} -requiring collagenases of *Crotalus atrox* venom and *Clostridium histolyticum* and the Zn^{2+} - and Ca^{2+} -containing proteinase (thermolysin) from *Bacillus thermoproteolyticus* were used.

Proteinase of Crotalus atrox venom. The activity of the *Crotalus* venom (5 $\mu\text{g}/\text{ml}$) against Azocoll (see the Materials and Methods section) was 95% inhibited by

EDTA (10 mM) and 90% inhibited by 1,10-phenanthroline (0.5 mM); it was therefore largely attributable to the zinc proteinase of this venom (Mella *et al.*, 1967), which seems to have collagenolytic activity (Simpson, 1972).

Reaction mixtures (4 ml) containing 10, 100, 300 and 600 μg of $\alpha_2\text{M}$ were prepared, without preincubation of the enzyme (20 μg) with $\alpha_2\text{M}$. Inhibition produced by these amounts of $\alpha_2\text{M}$ was 13, 45, 74 and 94% respectively.

Clostridial collagenase. A mixture of clostridial collagenase (10 μg) with $\alpha_2\text{M}$ (625 μg) in 150 μl of 100 mM-Tris-HCl buffer, pH 7.6, containing 5 mM- CaCl_2 was left at 4°C for 30 min. The activity of the enzyme was not decreased in assays with Pz-Pro-Leu-Gly-Pro-D-Arg as substrate, compared with a control without $\alpha_2\text{M}$. $\alpha_2\text{M}$ (625 μg) decreased by 63%, 59% or 52% the activity of clostridial collagenase (10, 50 or 100 ng) against reconstituted collagen fibrils, without preincubation, in a 20 h assay.

In a further experiment, a mixture of $\alpha_2\text{M}$ (6.25 mg) with an excess of clostridial collagenase (1.75 mg) was analysed by gel filtration. The mixture in 0.5 ml of 50 mM-Tris-HCl buffer, pH 7.5, containing 5 mM- CaCl_2 and 200 mM-NaCl was first left for 30 min at 4°C; it was then run on a column (1.5 cm $\times$ 28 cm, 50 cm^3) of Sephadex G-200 in the same buffer, and 1 ml fractions were collected at 4 ml/h.

As is shown in Fig. 4, analysis of the effluent fractions revealed two peaks of activity against the synthetic substrate Pz-Pro-Leu-Gly-Pro-D-Arg, the first being correlated with the peak of $\alpha_2\text{M}$ eluted at the void volume (39% of the bed volume) whereas the second appeared at about 60% of the bed volume (the elution volume of the single peak obtained when 1.1 mg of clostridial collagenase was run alone). The material forming the first peak was considered to be purified $\alpha_2\text{M}$ -clostridial collagenase complex, and it was tested for activity against the synthetic substrate and collagen fibrils. Enzyme from the lower-molecular-weight peak of activity was taken as a control. The $\alpha_2\text{M}$ -collagenase complex separated from free enzyme was able to solubilize the radioactive collagen fibrils completely, but the activity was only 3% of that of an amount of enzyme from the control peak having identical activity against the synthetic peptide.

It was concluded from these experiments that $\alpha_2\text{M}$ binds clostridial collagenase forming a complex fully active against a substrate of low molecular weight, but markedly inhibited in its action on collagen.

Rate of interaction with $\alpha_2\text{M}$. Clostridial collagenase (0.2 μg in 100 μl of Tris- Ca^{2+} buffer containing 1 mg of bovine serum albumin/ml) was incubated at 25°C with 190 μg of $\alpha_2\text{M}$ in a total volume of 150 μl . At various times up to 16 h, 100 μg of trypsin was added to mixtures prepared as above, followed 5 min

Fig. 4. Binding of clostridial collagenase by α_2 M

A mixture of clostridial collagenase with α_2 M (—), or the collagenase alone (----), was run on a column of Sephadex G-200 and the distributions of enzymic activity and α_2 M (horizontal bar) were determined. Enzymic activity was measured with a synthetic substrate of low molecular weight (see the text for details).

later by Tos-LysCH₂Cl to give 0.40 μ mol in a final volume of 0.40 ml. The samples were assayed for activity against collagen fibrils and the results were compared with those for controls incubated without α_2 M. In two experiments made in this way the half-time for loss of activity in the presence of α_2 M was 1.4 (range 1.2–1.6) h. The addition of excess of trypsin was adopted for stopping the collagenase– α_2 M reaction because this enzyme is known to saturate α_2 M almost instantaneously (Lebreton de Vonne *et al.*, 1971). The trypsin was then selectively inactivated with Tos-LysCH₂Cl so that it should not destroy the collagenase or otherwise affect the collagenase assay.

Thermolysin. Thermolysin (100 μ g) was incubated at 37°C for 30 min with 3.75 mg of α_2 M in 1.25 ml of Tris–Ca²⁺ buffer containing 200 mM–NaCl. The mixture was run on a column (1.5 cm $\times$ 28 cm, 50 cm³) of Sephadex G-200 in the same buffer, and assays with Pz-Pro-Leu-Gly-Pro-D-Arg were made on the effluent fractions. It was found that over 95% of the enzymic activity was eluted at the void volume (38% of bed volume), the remainder being eluted at 69% of bed volume.

The effect of α_2 M (100 μ g) on the activity of thermolysin (2 μ g) against azocasein was tested. An assay started immediately after the enzyme and α_2 M had been mixed showed 89% inhibition, and this value

was not increased by a preincubation (150 min) of the enzyme with α_2 M.

Demonstration of proteolytic cleavage of α_2 M by metal proteinases

It has been shown by Harpel (1973) that the interaction of several serine proteinases with α_2 M involves a proteolytic attack on the α_2 M. The quarter subunits of α_2 M, of molecular weight about 185 000, were shown to be cleaved, apparently near their mid-point, to yield products of about 85 000 molecular weight.

To determine whether the metal proteinases attack α_2 M in the same way, α_2 M was incubated with a range of concentrations of trypsin, thermolysin and rabbit synovial-cell collagenase. Reaction mixtures (50 μ l) contained 25 μ l of α_2 M solution (0.32 mg/ml) in 100 mM–NaCl, and 25 μ l of enzyme (1, 2, 5, 10, 20 or 50 μ g/ml, or units/ml for synovial-cell collagenase) in 50 mM–Tris–HCl buffer, pH 7.6, containing 10 mM–CaCl₂. After 30 min incubation at 40°C, 50 μ l of a solution containing sodium dodecyl sulphate (20 mg/ml), glycerol (50% v/v), 2-mercapto-ethanol (1%, v/v), Bromophenol Blue (100 μ g/ml) and Tris and Bicine (each 10 mM) was introduced and the tubes were stood in a beaker of boiling water for 5 min. Samples (50 μ l) were then run in electrophoresis in polyacrylamide gels (see the Materials and Methods section).

Some representative gels from these experiments are shown in Plate 2. The results with trypsin confirmed those of Harpel (1973). The results obtained with thermolysin and synovial-cell collagenase were very similar to those with trypsin; again a marked accumulation of the characteristic products during the course of the degradation was detected, but in enzyme excess these were further degraded. The exact molar ratios of α_2 M to the metal proteinases in the incubation mixtures could not be determined, but partial conversion of quarter subunits into the smaller fragments was seen in mixtures in which α_2 M was certainly in excess.

Clostridial collagenase (Worthington) was treated with iodoacetate and Dip-F, and then subjected to gel filtration as described by Peterkofsky & Diegelmann (1971) to remove traces of contaminating proteolytic activity. The characteristic degradation of α_2 M was produced by this enzyme only with difficulty: a solution having an E_{280} of 0.50 was mixed in equal volumes with the α_2 M solution as described above, and incubated at 40°C for 30, 60 or 120 min. Proteins from the enzyme sample were seen in the stained electrophoresis gels as two zones of mobility intermediate between the quarter subunits and the characteristic products, which appeared clearly at 60 and 120 min. The relatively slow cleavage

of the quarter subunits of α_2M by clostridial collagenase at high concentration is consistent with the slowness of binding and inhibition. The action of both synovial-cell collagenase and clostridial collagenase was almost completely prevented by inclusion of 1mM-1,10-phenanthroline in the reaction mixtures, as was to be expected for zinc-dependent enzymes.

Discussion

Barrett & Starkey (1973) suggested that all endopeptidases react with α_2M in essentially the same way, although there were few data about the metal proteinases. This idea was in conflict with reports from several other laboratories which indicated that collagenases were not inhibited by serum proteins. Our present results reconcile these conflicting points of view, and show that the interaction of α_2M with metal proteinases, including collagenase, is fundamentally the same as that with other endopeptidases.

The existence of metal endopeptidases in animals and man has only recently been recognized, and as yet none of these enzymes has been characterized in sufficient detail to show unequivocally which metal ions are involved in their activity. The bacterial metal proteinases typically contain Zn^{2+} , and usually Ca^{2+} (Seifter & Harper, 1971; Colman *et al.*, 1972), and the inhibition of rabbit fibroblast collagenase by 1,10-phenanthroline and EDTA (Werb & Burleigh, 1974) suggests that it may require the same cations. The sensitivity of the specific collagenase of tadpole tail (Nagai *et al.*, 1966) to chelating agents, like that of some snake venom proteinases (Mella *et al.*, 1967; Simpson, 1972), indicates that these enzymes, too, are metal proteinases.

The collagenase obtained from rabbit synovial cells in tissue culture is known to have the characteristics of a 'specific' collagenase, that is to say it cleaves the triple helix of native collagen at a point three-quarters of the way from the *N*-terminus, giving characteristic products at 24°C (Werb *et al.*, 1973; Werb & Burleigh, 1974). This enzyme was used in much of the present work both because of the likely biological importance of specific collagenases (Evanson *et al.*, 1968) and because the apparent strict specificity of the enzyme seemed likely to pose a critical test of the hypothesis that α_2M reacts with essentially all endopeptidases.

The finding that the synovial-cell collagenase was inhibited by normal human serum is consistent with several previous reports about other specific collagenases (Evanson *et al.*, 1968; Eisen *et al.*, 1970a; Dresden *et al.*, 1972; Sakamoto *et al.*, 1972a). Our failure to find any indication of an inhibitory role for any serum protein other than α_2M contrasts with the results of Eisen *et al.* (1971) who concluded

that α_1 -trypsin inhibitor is inhibitory for human skin collagenase. On the contrary, our results are consistent with those of Donoff *et al.* (1971) who found that serum deficient in α_1 -trypsin inhibitor was not less inhibitory than normal serum for a collagenase from wounded rabbit skin, and Harris *et al.* (1969) who could not decrease the inhibitory capacity of serum for rheumatoid synovial collagenase by a heat treatment that would have destroyed α_1 -trypsin inhibitor.

Like Abe & Nagai (1972a,b), we were unable to show dissociation of collagenase from α_2M by gel filtration, and conclude with them that the binding is exceedingly tight, or irreversible. It therefore seems that some other inhibitor of much lower affinity was responsible for the inhibition in tissue extracts, reversible by gel filtration, reported by Bauer *et al.* (1971), Eisen *et al.* (1971), Fullmer *et al.* (1972) and Sakamoto *et al.* (1972b). The existence of such an inhibitor was suggested by Abe & Nagai (1973).

Our finding that the inhibitory capacity of α_2M for collagenase can be blocked by first allowing the inhibitor to react with cathepsin B1 or trypsin explains the observation by Harris *et al.* (1969) that trypsin, but not hyaluronidase, will neutralize the inhibitory activity of serum and synovial fluid. Sakamoto *et al.* (1972a) found that α -chymotrypsin, elastase and papain, as well as trypsin, could saturate the inhibitor in horse serum; these are all proteinases known to be bound by α_2M (Barrett & Starkey, 1973).

There have been previous reports of two human collagenases not inhibited by serum, the enzyme from polymorphonuclear leucocytes (Lazarus *et al.*, 1968), and the high-molecular-weight collagenase of synovial fluid (Harris *et al.*, 1969). In contrast with these earlier results, we have found that the leucocyte collagenase and all the activity of several synovial-fluid samples was inhibited by α_2M . It seems probable that the failure of earlier workers to detect inhibition was due to the rather slow rate of interaction of α_2M with these enzymes. Slow progressive inhibition of thrombin, a serine proteinase, by α_2M was previously reported by Lanchantin *et al.* (1966). For the leucocyte collagenase the presence of less specific, more rapidly reacting leucocyte proteinases, which would tend to presaturate the α_2M , might also have prevented the inhibition.

Competition by the substrate, collagen, also was a factor in the inhibition of the synovial-fluid collagenase by α_2M , greatly increasing the half-time of inhibition. The partial inhibition (20%) observed by Harris *et al.* (1969) could be accounted for by the presence of the substrate. The results of Norman (1958) on the effect of substrate on inhibition of plasmin by serum may also be interpreted in this way (see Barrett & Starkey, 1973). Our results indicate the need to consider the importance of incubation

time and conditions in the interpretation of data on the inhibition of collagenase by α_2 M and serum.

It has been reported that the collagenolytic activity of synovial fluids can be separated into two components by gel filtration, the component of higher molecular weight being poorly inhibited by serum and that of lower molecular weight being rapidly inhibited (Harris *et al.*, 1969). In unpublished work with the synovial fluids described in the present paper, we have obtained similar results using purified α_2 M. In the absence of other evidence for the existence of two distinct collagenases in synovial fluid, and on the basis of the kinetic data presented in the present paper, an explanation for these findings would be that the higher-molecular-weight component was a macromolecular complex in which the collagenase had less reactivity with α_2 M. This possibility is supported by the finding that collagenase obtained from human synovial tissue in culture was rapidly and completely inhibited by α_2 M.

Clostridial collagenase has been found by Bannister & Evans (1973) to be inhibited by chicken serum, although Eisen *et al.* (1970b) were unable to obtain inhibition with human serum, and Sakamoto *et al.* (1972b) obtained no inhibition with horse serum. In our hands, the bacterial collagenase was slowly bound by α_2 M, and was not inhibited with respect to a pentapeptide substrate, but almost completely inhibited with collagen.

In the present work, the metal proteinase of *Crotalus atrox* venom was inhibited by α_2 M. A bacterial metal proteinase, thermolysin, was also shown to be bound and inhibited by α_2 M. Among the non-collagenolytic metal proteinases is an enzyme from rheumatoid synovial tissue in culture that has been shown to be inhibited by serum (Harris & Krane, 1972).

Barrett & Starkey (1973) argued that the simplest hypothesis that was consistent with all the experimental data required proteolytic cleavage of the macroglobulin in one particular region as the first stage of the interaction of an endopeptidase with α_2 M. While the present work was in progress Harpel (1973) published evidence that the binding of several serine proteinases involved cleavage of the α_2 M near the mid-point of one or more of the quarter subunits. We have now confirmed Harpel's (1973) results for trypsin, and shown that thermolysin, rabbit synovial-cell collagenase and clostridial collagenase attack α_2 M in a similar way. It has also been shown (A. J. Barrett, unpublished work) that cathepsin B1, a thiol proteinase, and cathepsin D, a carboxyl proteinase, cleave α_2 M in the same region; thus it is clear that α_2 M can act as a substrate for endopeptidases of all four classes. The more specific proteinases, including clostridial collagenase, presumably interact slowly with α_2 M because proteolytic cleavage is the rate-limiting step.

The interaction of α_2 M with metal proteinases is likely to be of physiological importance, since a mechanism exists for the selective removal of α_2 M-endopeptidase complexes from the circulation (Ohlsson, 1971). It has been suggested (Barrett & Starkey, 1973) that in the blood, α_2 M regulates the activity of endopeptidases mainly by clearing them from the circulation, rather than by inhibition. It seems possible that α_2 M acts in this way to ameliorate the effects of the metal proteinases of snake venoms and pathogenic bacteria. Inhibition may be more important for the collagenases of joint tissues, however. It has been shown by Abe & Nagai (1972a,b) and by the present authors (unpublished work) that the complex of collagenase with α_2 M can exist in appreciable quantities in the synovial fluid in rheumatoid arthritis. This presumably implies that neither the leucocytes nor the lining cells of the synovium have the capacity to phagocytose the complex, and in this situation the essentially complete inhibition of collagenases by α_2 M probably represents an important protection of the articular cartilage.

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