Ovostatin: A Novel Proteinase Inhibitor from Chicken Egg White

II. MECHANISM OF INHIBITION STUDIED WITH COLLAGENASE AND THERMOLYSIN

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The inhibition mechanism of ovostatin was studied using rabbit synovial collagenase and thermolysin. When enzymes were complexed with ovostatin, only the proteolytic activity towards high molecular weight substrates was inhibited. Activity towards low molecular weight substrates was partially modified; the catalytic activity of collagenase bound to ovostatin was inhibited by only 40% towards 2,4-dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-d-Arg and that of thermolysin bound to ovostatin was activated about 2.6-fold towards benzoyloxy carbonyl-Gly-Leu-NH₂ and benzoyloxy carbonyl-Gly-Phe-NH₂. Collagenase-ovostatin complexes failed to react with anti-(collagenase) antibody. Saturation of ovostatin with thermolysin prevented the subsequent binding of collagenase. Ovostatin-proteinase complexes ran faster than free ovostatin on 5% polyacrylamide gel electrophoresis. Complexing ovostatin with either collagenase or thermolysin resulted in the cleavage of the quarter-subunit of ovostatin (Mₐ = 165,000) into two fragments with Mₚ = 88,000 and 78,000. On the other hand, when the inhibitory capacity of ovostatin was tested with trypsin, chymotrypsin, and papain, only partial inhibition of their proteolytic activities was observed towards azocasein. Stronger inhibition was noted when Azocoll was a substrate, however. Analyses of ovostatin-proenzyme complexes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the quarter-subunit of ovostatin was cleaved into several fragments by those enzymes. These results led us to propose that ovostatin inhibits metalloproteinases in preference to proteinases of other classes in a manner similar to α₂-macroglobulin; hydrolysis of a peptide bond by a proteinase in the susceptible region of the ovostatin polypeptide chain triggers a conformational change in the ovostatin molecule and the enzyme becomes bound to ovostatin in such a way that the proteinase is sterically hindered from access to large protein substrates and yet is accessible to small synthetic substrates. A kinetic study of collagenase binding to ovostatin gave the value of k₄/Kₐ = 6.3 × 10⁸ M⁻¹ min⁻¹. The results indicate that ovostatin is equally as good a substrate for collagenase as type I collagens.

A proteinase inhibitor which binds to mammalian tissue collagenase and thermolysin stoichiometrically (1:1 molar ratio with thermolysin) was found and purified from chicken egg white (1). The inhibitor protein was named "ovostatin" (1).

Ovostatin with Mₑ = 780,000 is a tetramer of identical subunits (Mₒ = 195,000) linked in pairs by disulfide bonds and assembled noncovalently (1). Although the molecular weight and the quaternary structure of ovostatin are very similar to those of α₂M in plasma (2, 3), the two proteins are different from one another in a number of aspects: 1) proteinase inhibitory capacity of ovostatin is insensitive to methamphetamine treatment whereas the inhibitory ability of chicken α₂M is destroyed by methamphetamine; 2) the two proteins are immunologically distinct; and 3) CNBr treatment of ovostatin and chicken α₂M generates different maps. However, NH₂-terminal sequence analyses indicate that the two proteins are highly homologous with each other (1).

In this report, we investigate the mechanism by which ovostatin inhibits the proteolytic activities of collagenase and thermolysin. Its capacity to inhibit other proteinases has been also studied. The results have led us to propose that ovostatin inhibits metalloproteinases more effectively than proteinases of other classes and that the inhibitory mechanism is very similar to that of α₂M, as proposed by Barrett and Starkey (4). The proteinase hydrolyzes a peptide bond at a specific locus within an ovostatin polypeptide chain and triggers a conformational change in ovostatin that causes it to bind to the enzyme molecule in such a way that the active site of the enzyme remains free to hydrolyze low molecular weight substrates but is restricted from reactions with large protein substrates. Kinetic studies of the binding of collagenase to ovostatin indicate that ovostatin serves as good substrate for mammalian collagenase.

EXPERIMENTAL PROCEDURES

Materials—Ovostatin was purified as described by Nagase et al. (1). The molar concentration of ovostatin was determined by using Mₑ = 780,000 (1). Rabbit synovial collagenase and sheep anti-(rabbit fibroblast collagenase) F(ab')₂, coupled to agarose were prepared as described previously (5). Cysteine proteinase affinity label, Ep-475 (6) was kindly provided by Dr. K. Hanada, Taisho Pharmaceutical Co., Omiya, Japan. Chymotrypsin (bovine, type I-S), papain (type

1 Mₑ is used for the weight-average molecular weight determined by equilibrium ultracentrifugation.

2 The abbreviations used are: α₂M, α₂-macroglobulin; F(ab')₂, di-valent antigen binding fragment of immunoglobulin G; SDS, sodium dodecyl sulfate; Dip N, diisopropyl phosphorofluoridate; Z, benzoxycarbonyl; DNP, 2,4-dinitrophenyl; Boc, t-butyloxycarbonyl-; NMec, 4-methyl-7-coumarylamine; Ep-475, L-trans-epoxysuccinylleucylamido-(3-methyl)butane; E-64, L-trans-epoxysuccinylleucylamido(4-guanidino)butane.
Ill), Dip-F, Brij 35, 4-nitrophenyl acetate, and Z-Gly-Phe-NH₂ were from Sigma Chemical. D-P-Arc-Pro-Arg-NMe₄, DNP-Pro-Gly-Gly-Ile-Ala-Gly-Gly-Arg (DNP-peptide), and Z-Gly-Leu-NH₂ were from Peninsula Laboratories. Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) was from Pierce and ¹²⁵I (specific activity, 16.4 mCi/µg) from Amersham. 5'-Dithio-bis (2-nitrobenzoic acid) was from Calbiochem. Other chemicals were described in the preceding paper (1).

Enzymic Assays—Collagenolytic-NMec, DNP-Pro-Gly-Gly-Ile-Ala-Gly-Gly-Arg (DNP-peptide), and Z-Gly-Leu-NH₂ were prepared against reconstituted ²³⁴C-acetylated collagen fibers (7). One unit of collagenase digests 1 µg of collagen/min at 37 °C. Collagenase activity and its inhibition by ovostatin were also assayed by viscometry at 27 °C using pepsin-treated collagen (8) as substrate in the presence of 50 mM L-arginine to prevent premature formation (8). Collagenase activity was measured in Cannon-Fenske viscometers (Cannon Instrument Co.). Collagenase activity was expressed as percent of initial specific viscosity (ν₀). The reaction was terminated after 180 min by adding 2.0 ml of 5% (v/v) acetic acid, and the fluorescence read within a linear range of fluorescamine assay. The fluorescence of the resulting solution was measured in a Hitachi MPF-2A fluorescence spectrophotometer using an excitation wavelength at 390 nm and an emission wavelength at 490 nm.

Activity against azocasein was measured as described previously (10). Thermolysin, trypsin, and chymotrypsin activities were measured in 0.1 M Tris/HCl buffer, pH 7.8, containing 10 mM Ca²⁺. Activity of papain was measured in 0.1 M potassium phosphate buffer, pH 6.4, containing 5 mM cysteine and 5 mM EDTA. Enzymic activity was measured by estimating the number of a-amino groups liberated after hydrolysis of the substrate by fluorescamine (11). To each sample (2.5 ml) 0.5 ml of fluorescamine (0.15 mg/ml in 0.1 M boric acid/NaOH buffer, pH 8.2, containing 0.1% Brij 35) was added. Assay tubes were rotated at 23 °C for an appropriate time. After brief centrifugation, the supernatant was aspirated and the fluorescence of the fluorescamine and proteinase activity was measured within this range. For papain assay, 0.1 M potassium phosphate buffer, pH 6.4, containing 5 mM cysteine, 5 mM EDTA and 0.05% Brij 35 was used.

Hydrolysis of Synthetic Substrates—Z-Gly-Leu-NH₂ and Z-Gly-Phe-NH₂ were used as substrates for thermolysin. Stock solutions of the substrates (50 mM in dimethyl sulfoxide, stored at -20 °C) were diluted 25-fold with 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM Ca²⁺ and 0.15 M NaCl was incubated with 50 µl of enzyme solution in the same buffer in a 3-ml conical centrifuge tube with a stopper at 37 °C for 2 h. The reaction was stopped by adding 0.2 ml of 1 M HCl. The digestion products were extracted with 1.4 ml of ethyl acetate, 1-butanol (10:1.5, v/v) by Vortex mixing for 7.5 min, containing 10 mM Ca²⁺ and 0.15 M NaCl was incubated with 50 µl of enzyme solution in the same buffer in a 3-ml conical centrifuge tube with a stopper at 37 °C for 2 h. The reaction was stopped by adding 2.0 ml of 5% (v/v) acetic acid, and the fluorescence read within a linear range of fluorescamine assay. The fluorescence of the resulting solution was measured in a Hitachi MPF-2A fluorescence spectrophotometer using an excitation wavelength at 390 nm and an emission wavelength at 490 nm, which was additive with simultaneous mixing on a Vortex mixer. Where necessary, the sample was diluted severalfold with 0.1 M sodium phosphate buffer, pH 8.5, containing 20 mM EDTA to read within a linear range of fluorescamine assay. The fluorescence of the reaction solution was measured in a Hitachi MPP-2A fluorescence spectrophotometer using an excitation wavelength at 390 nm and an emission wavelength at 475 nm. Glycine (10–50 mmol) was used as a standard to estimate the amount of substrate hydrolyzed. For blanks, thermolysin was incubated with substrates in the presence of 33 mM EDTA with or without appropriate amounts of ovostatin.

Hydrolysis of DNP-peptide by collagenase was measured spectrophotometrically by a modification of the procedure of Masui et al. (12). A 50-µl portion of 1 mM DNP-peptide in 50 mM Tris/HCl, pH 7.5, containing 10 mM Ca²⁺ and 0.15 M NaCl was incubated with 50 µl of enzyme solution in the same buffer in a 3-ml conical centrifuge tube with a stopper at 37 °C for 2 h. The reaction was stopped by adding 0.2 ml of 1 M HCl. The digestion products were extracted with 1.4 ml of ethyl acetate, 1-butanol (10:1.5, v/v) by Vortex mixing for at least 30 s followed by brief centrifugation. The absorption of the extract was measured at 365 nm in a 1-ml cuvette with a 1-cm pathlength. Hydrolysis of Boc-Val-Pro-Arg-NMe₄ by trypsin was measured as described by Nagase and Barrett (13). The reaction mixture (20 µl) in 0.1 M Tris/HCl, pH 7.8, containing 10 mM Ca²⁺ was terminated by adding 2.0 ml of 5% (v/v) acetic acid, and the fluorescence read with a Hitachi MPP-2A fluorescence spectrophotometer using an excitation wavelength at 380 nm and an emission wavelength at 490 nm (14).

Active Site Titration—The molar concentrations of trypsin solution and chymotrypsin solution were determined by titration with 4-nitrophenyl-4′-guanidino benzoate as described by Chase and Shaw (15), and with 4-nitrophenyl acetate as described by Bender et al. (16). The molar concentration of papain was determined using Ep-475 (6) instead of Ep-64 essentially as described by Barrett et al. (17). Papain solutions (50 µl; approximately 5 µM in 0.1 mM potassium phosphate buffer, pH 6.4, containing 5 mM cysteine and 5 mM EDTA) were mixed with 50 µl of 1–10 µM Ep-475 and incubated at 23 °C for 30 min. Residual activity against azocasein was then measured, and the results were plotted against inhibitor concentration. The intercept of a straight line through the points and the abscissa (concentration of Ep-475) was used to determine the molar concentration of the enzyme solution.

¹²⁵I-labelling of Proteins—Purified collagenase, thermolysin, trypsin, and papain were labeled with ¹²⁵I by the iodogen method as described by Praker and Speck (18).

Binding Rate Assay of Collagenase and Thermolysin to Ovostatin—¹²⁵I-labeled collagenase and ¹²⁵I-labeled thermolysin were incubated with a 2–10 molar excess of ovostatin at 25 °C in 50 mM Tris/HCl buffer, pH 7.8, containing 0.15 M NaCl and 5 mM Ca²⁺. To terminate the reaction of ¹²⁵I-labeled collagenase to bind to ovostatin, a 25-µl portion was removed periodically and mixed immediately with 25 µl of electrophoresis sample buffer containing 50 mM 1,10-phenanthroline and 10% (v/v) ethanol. To terminate the ¹²⁵I-thermolysin reaction, electrophoresis sample buffer containing 0.2% bovine serum albumin, thermolysin (600 µg/ml), and 50 mM 1,10-phenanthroline and 10% (v/v) ethanol was added. Samples were run on 5% polyacrylamide gels. Proteins were stained with Coomassie brilliant blue R-250, dried, and exposed to Kodak X-Omat (XRP-1) film. The film was developed with a Kodak RPX-Omat processor. Ovostatin bands that had incorporated ¹²⁵I-labeled enzymes were excised from the gel and counted for incorporated radioactivity in a Beckman γ counter.

Since there was no recovery of enzymic activity after forming the ovostatin-enzyme complex, the anticipated reaction scheme for formation of irreversible ovostatin-enzyme complex (EI*) is described as the following:

\[ E + \frac{1}{k_1} \rightleftarrows E {I} \rightleftarrows E {I}^* \]

where \( K_i = \frac{k_{-1}}{k_1} \)

\[ K_i \]

\[ \frac{1}{k_{-1}} = K_i = \frac{k_1}{k_{-1}} \]

\[ \frac{1}{k_{-1}} = K_i = \frac{k_1}{k_{-1}} \]

Results

Binding of Collagenase to Ovostatin—Progressive binding of rabbit synovial collagenease to ovostatin was demonstrated by viscometric assay. A severalfold excess of ovostatin was incubated with collagenase at 27 °C for 0, 15, or 30 min before addition of collagen substrate. When the enzyme and ovostatin were added to collagen at time 0, the binding of collagenase to ovostatin was slow: inhibition was observed only after 30 min (Fig. 1A). However, when the enzyme and ovostatin were incubated without collagen, binding of collagenase to ovostatin occurred more quickly: by 30 min of incubation, almost all of the collagenase was bound to ovostatin.

Viscometric measurements of each sample were terminated after 180 min by adding EDTA (20 mM, final), and the
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Fig. 1. Viscometric analysis of progressive inhibition of collagenase by ovostatin. A, rabbit synovial collagenase (0.95 unit) was incubated with 348 μg of ovostatin in 0.1 M Tris/HCl buffer, pH 7.8, containing 20 mM Ca2+ and 0.3 M NaCl (total, 0.72 ml) at 27 °C for 15 min (■), and 30 min (□). Then, after adding 120 μl of 0.5 M arginine and 360 μl of collagen (2 mg/ml) to the mixture, 1 ml of the total mixture was subjected to viscometric assay at 27 °C. For the control experiment, 0.95 unit of collagenase was used without ovostatin (■). For 0 min of incubation (C), ovostatin, collagen, and the buffer were mixed first at 27 °C, and then 0.95 unit of collagen was added to the mixture just before the viscosity changes were monitored. Collagen and ovostatin alone are shown (■). B, after the measurement of viscosity changes of each experimental solution over the period of 180 min, the reaction was stopped by adding 20 mM EDTA at the final concentration, and a portion was run on SDS-gel electrophoresis (7.5% total acrylamide). Lane 1, collagen alone incubated at 27 °C for 180 min; lane 2, collagen and collagenase; lanes 3–5, collagenase and ovostatin incubated for 0, 15, and, 30 min prior to the addition of collagen, respectively; lane 6, collagen incubated with ovostatin. The arrow indicates the quarter-subunits of ovostatin. αA, αB, and βA are the cleavage products of α and β components.

reaction products were analyzed on SDS-gel electrophoresis (Fig. 1B). The amount of specific reaction products of collagen (αA, αB, and βA) produced by collagenase were progressively reduced by prolonged incubation of collagenase with ovostatin.

Binding of collagenase was studied further with 125I-labeled collagenase. Ovostatin (0.45 μM) was incubated with 125I-labeled collagenase (60 units) at 25 °C, and a portion of the mixture was removed periodically and mixed immediately with 25 mM 1,10-phenanthroline to stop the reaction. 125I-labeled collagenase bound to ovostatin was analyzed by measuring the radioactivity incorporated into the ovostatin band after running the samples on 5% polyacrylamide gels (Fig. 2). This technique was used after it was found that collagenase inactivated by chelating agents was unable to bind to ovostatin.

The progressive binding of collagenase to ovostatin with time in accordance with Equation 4 was obtained (Fig. 2, inset).

\[
\ln \frac{E1^* - E1^*}{E1^*} = -k_{app} \cdot t
\]  

(4)

The initial enzyme concentration was measured as \(E1^*\), the total \(E1^*\) formed at infinite time. \(E1^*\) is the amount of \(E1^*\) formed at time \(t\). The values of \(k_{app}\) obtained with various concentrations of ovostatin in this manner were then plotted according to Equation 3 (Fig. 3). The straight line was drawn according to the median values of \(K_i\) and \(k_2\) obtained by the method of Eisenenthal and Cornish-Bowden (20). The dissociation constant, \(K_i\), and the first order rate constant for the irreversible formation of ovostatin-collagenase complex, \(k_2\), were \(5.7 \times 10^{-7}\) M and 0.36 min\(^{-1}\) (\(k_2/K_i = 6.3 \times 10^4\) M\(^{-1}\) min\(^{-1}\)).

Binding of Thermolysin to Ovostatin—A rapid binding of thermolysin was observed: all of 125I-labeled thermolysin (5 μg/ml) bound to ovostatin (300 μg/ml) within 20 s at 25 °C, pH 8.0. Accurate kinetic constants for the formation of the ovostatin-thermolysin complex could not be determined by the method used for collagenase binding even after considerable dilution of the enzyme and ovostatin.

SDS-Polyacrylamide Gel Electrophoresis of Ovostatin-Collagen and Ovostatin-Thermolysin Complexes—Various amounts of rabbit synovial collagenase or thermolysin were complexed with ovostatin and the complexes were analyzed by SDS-gel electrophoresis with reduction. Both enzymes cleaved the quarter-subunit of ovostatin and generated specific fragments with \(M_r = 85,000\) and \(M_r = 78,000\) (Fig. 4). Both enzymes presumably cleave peptide bonds at the same locus of the ovostatin polypeptide chain. The amount of proteolytic fragments generated correlated with the amount
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Ovostatin (1). In order to examine whether or not the inhibition of those enzymes is due to the binding of inhibitor to the active sites of the enzymes, low molecular weight synthetic substrates were tested. When collagenase was complexed with ovostatin, activity against DNP-peptide was inhibited about 40%. The degree of inhibition did not change with increasing ratios of the enzyme to ovostatin in the assay where sufficient amounts of ovostatin were present to bind to all collagenase.

On the other hand, when thermolysin was saturated with ovostatin, the enzymic activity towards Z-Gly-Leu-NH$_2$ and Z-Gly-Phe-NH$_2$ (at pH 8.2) increased about 2.6-fold (Fig. 5). Extrapolation of the linear segment of the curve in Fig. 5 shows the maximal activation to occur at 1:1 molar ratio of ovostatin to thermolysin. The activation phenomenon was further examined by double reciprocal plots of the hydrolysis rate versus the substrate concentration in the presence or absence of 2.5-molar excess of ovostatin. The result showed that the $k_{\text{cat}}$ value of thermolysin against Z-Gly-Leu-NH$_2$ increased from 5.8 s$^{-1}$ (without ovostatin) to 16.2 s$^{-1}$ (with ovostatin) but the $K_m$ value (3.45 mM) was unchanged. Similar activation of thermolysin bound to ovostatin was observed at pH 6.7.

These data indicate that ovostatin does not block the active site of collagenase or thermolysin. Thus, inhibition of proteolytic activities of these metalloproteinases is considered to be due to steric hindrance caused by the conformationally altered ovostatin molecule formed by hydrolysis of a peptide bond.

Immunoreactivity of Ovostatin-Collagenase Complex with Anti-(collagenase) Antibody—If the inhibition of proteolytic activity of these enzymes were due to steric hindrance by the macromolecule, we anticipated that the ovostatin-enzyme complex-activities of collagenase and thermolysin towards collagen and azocasein, respectively, were inhibited by ovostatin (1). Longer incubation (at least 24 h at 23°C) of the ovostatin-enzyme complex did not change the amount of fragments produced, suggesting the complex is quite stable. No cleavage occurred with inactive enzymes.

Hydrolysis of Synthetic Substrates by Ovostatin-Enzyme Complexes—Activities of collagenase and thermolysin towards collagen and azocasein, respectively, were inhibited by o.
complex would fail to bind to antibody raised against the enzyme. We chose collagenase with $M_\text{r} = 45,000$ (22) rather than thermolysin ($M_\text{r} = 36,000$); the larger enzyme may be more exposed towards the surface of the inhibitor-enzyme complex and hence more available for antibody binding.

125I-labeled collagenase or that complexed with ovostatin was passed through an anti-(collagenase) F(ab')2-agarose column. The 125I-labeled collagenase-ovostatin complex failed to bind to anti-(collagenase) F(ab')2-agarose, whereas the majority of free 125I-labeled collagenase bound readily (Fig. 6), and could be eluted from the column with 8 M urea. (Recovery of the radioactivity from the column was about 60%.) These data suggest that the majority of antigenic determinants on the enzyme were hindered from access to antibody.

**Competition between Thermolysin and Collagenase to Bind to Ovostatin**—If the mechanism of ovostatin involves irreversible binding to the enzyme and restriction of its access to large substrates as a result of the conformational changes triggered following proteolysis, it would be expected that if ovostatin is saturated with one enzyme it should no longer inhibit another enzyme that is catalytically different. Since stoichiometric inhibition was observed only by thermolysin and collagenase (stoichiometric inhibition was not observed with serine and cysteine proteinases tested, see below), these enzymes were used in these studies. Thermolysin (0.55 $\mu$M) was reacted at various molar ratios of ovostatin. A portion of each mixture was then tested for residual activity against azocasein, and another portion was tested for the capacity to inhibit collagenolysis. Fig. 7 indicates that an excess of thermolysin completely abolished the ability of ovostatin to inhibit collagenase added subsequently. Inhibition of collagenase activity was observed only after complete inhibition of thermolysin was attained by ovostatin, at which time free ovostatin would be expected to increase.

**Changes in Electrophoretic Mobility of Ovostatin by Reacting with Proteinases**—Upon reaction with proteinases or with NH$_2$ salt, the increase in mobility of human $\alpha_2$M both in rate and pore-limit electrophoresis was noted (23-25). This is due to the conformational changes in $\alpha_2$M molecule that allow it to pass through sieving gels more readily (25). Upon reacting with proteinases, similar changes in electrophoretic mobility of ovostatin were noted. It was found that treatment of ovostatin with thermolysin (Fig. 8) or collagenase (data not shown) increased the mobility of ovostatin on 5% polyacrylamide gel. However, unlike human $\alpha_2$M, the treatment of ovostatin with CH$_3$NH$_2$ did not change the mobility of the molecule on rate electrophoresis (1). Chicken $\alpha_2$M, which ran

![Fig. 5. Activation of thermolysin towards synthetic substrates by ovostatin.](image)

![Fig. 6. Absence of immunoreactivity of collagenase-ovostatin complexes with anti-(collagenase) antibody.](image)

![Fig. 7. Competition between thermolysin and collagenase to bind ovostatin.](image)
faster than ovostatin, increased its mobility slightly upon reaction with thermolysin (Fig. 8).

Lack of Covalent Linking of Proteinases to Ovostatin—It has been reported that when \( \alpha_2M \) reacts with proteinases some of the enzymes become covalently linked to \( \alpha_2M \) (28–30). Since there are structural similarities between \( \alpha_2M \) and ovostatin, we examined whether or not ovostatin forms a covalent bond with proteinases. \( ^{125}\text{I}-\)Collagenase and \( ^{125}\text{I}-\)thermolysin were reacted with ovostatin and the complexes were run on SDS-gel electrophoresis under reducing conditions. \( ^{125}\text{I}-\)labeled enzymes were localized by autoradiography. As shown in Fig. 9, none of the proteinase molecules became covalently bound to ovostatin. Determination of thiol groups of free ovostatin (2.4 pmol) using 5,5′-dithio-bis-(2-nitrobenzoic acid) both in the presence and in the absence of 2% SDS (31) indicated that there are no thiol groups in ovostatin. No thiol groups were liberated even after reacting with a 2-molar excess of thermolysin or trypsin. These results and the observation that the inhibitory activity of ovostatin on proteinases was not altered by the CH\(_3\)NH\(_3\) treatment (1) indicated that the ovostatin molecule does not contain a thiol-ester bond.

**Fig. 8.** Rate gel electrophoresis of free ovostatin and an ovostatin-enzyme complex. Ovostatin and chicken \( \alpha_2M \) were reacted with the equimolar thermolysin for 10 min at 23 °C, pH 7.8. The samples were run on 5% polyacrylamide gel electrophoresis. Lane 1, ovostatin; lane 2, ovostatin-thermolysin complex; lane 3, chicken \( \alpha_2M \); and lane 4, chicken \( \alpha_2M \)-thermolysin complex.

**Fig. 9.** Autoradiography of \( ^{125}\text{I}-\)labeled proteinases-ovostatin complex. Ovostatin (15 \( \mu \)g in 25 \( \mu \)l of 50 mM Tris/HCl buffer, containing 8 mM Ca\(^{2+}\)) was reacted with 0.2 unit of \( ^{125}\text{I}-\)collagenase (ovostatin excess) or with a 0.5 molar ratio of \( ^{125}\text{I}-\)thermolysin in an equal volume of the same buffer. The mixtures were incubated at 23 °C for 1 h and the reaction was terminated by adding 20 mM EDTA (final concentration). The samples were then subjected to SDS-gel electrophoresis (7.5% total acrylamide) under reducing conditions as described in the legend to Fig. 4. The gel was stained for proteins, dried, and autoradiographed. Lane 1, autoradiograph of \( ^{125}\text{I}-\)collagenase-ovostatin complex. The major doublet with \( M_r \), 45,000 and 49,000 is trypsin-activated collagenase (22) and the minor band with \( M_r \), 57,000 is procollagenase (22). Lane 2, autoradiograph of \( ^{125}\text{I}-\)thermolysin-ovostatin complex. I, II, and III indicate the positions where the quarter-subunit of ovostatin and proteolytic fragments of ovostatin run, respectively. Molecular weight standards are as in Fig. 4. 94K represents \( M_r \), 94,000, for example.

**Fig. 10.** Inhibition of trypsin, chymotrypsin, and papain by ovostatin. Trypsin (48 pmol) and chymotrypsin (54 pmol) were incubated with ovostatin at various molar ratios in 0.1 M Tris/HCl buffer, pH 7.8, containing 10 mM Ca\(^{2+}\) at 23 °C for 10 min. For papain (21 pmol) assay, 0.1 M potassium phosphate buffer, pH 6.4, containing 5 mM cysteine and 5 mM EDTA was used. The mixtures were then assayed for 2 h at 23 °C for the residual proteolytic activities against azocasein (●–●) or Azocoll (〇–〇). Since Azocoll is not a good substrate for chymotrypsin, the activity on Azocoll is not shown.
Inhibition Mechanism of Ovostatin

Fig. 11. Cleavage of ovostatin with trypsin, chymotrypsin, and papain. Ovostatin (16.7 μg) was reacted with trypsin, chymotrypsin, and papain for 10 min at the molar ratios (enzyme/ovostatin) of 1/8 (lanes 1), 1/4 (lanes 2), 1/2 (lanes 3), 1 (lanes 4), and 2 (lanes 6), in the conditions described in Fig. 12. The reaction was terminated by 2 mM Dip-F for trypsin and chymotrypsin and by 0.1 mM Ep-475 for papain. The samples were then mixed and boiled with an equal volume of the electrophoresis sample buffer with SDS and 2-mercaptoethanol, and subjected to SDS-gel electrophoresis as in Fig. 4. O, ovostatin alone; lanes 6 contain the same amount of enzyme alone as in (lanes 5). Protein standards were as in Fig. 4.

Fig. 12. Accessibility of soybean trypsin inhibitor (Kunitz) to ovostatin-trypsin complexes. Trypsin (12 pmol) was reacted with ovostatin at various molar ratios at 23 °C for 10 min (●—●), 6 h (○—○) and 18 h (△—△). A portion of the mixtures was then mixed with 48 pmol of soybean trypsin inhibitor for 10 min at 23 °C, and assayed for amidase activity against Boc-Val-Pro-Arg-NMec. The other portion was assayed for amidase activity without the inhibitor as control.

Analyzes of ovostatin-enzyme complexes by SDS-gel electrophoresis showed that trypsin, chymotrypsin, and papain digested the quarter-subunit of ovostatin into several fragments (Fig. 11). Thus, it may be speculated that in those cases the enzymes are more exposed towards the surface of the ovostatin molecule due to multi-cleavage of the subunits which may result in less inhibitory capacity of ovostatin against these classes of proteinases.

Accessibility of soybean trypsin inhibitor (Kunitz) to trypsin of the ovostatin-trypsin complexes was also tested (Fig. 12). After reacting ovostatin with trypsin at various molar ratios for 10 min, 6 h, or 18 h at 23 °C, a 4-molar excess of soybean trypsin inhibitor was added to the mixture and the residual activity against Boc-Val-Pro-Arg-NMec was measured. There were no marked changes in amidase activity of trypsin complexed with ovostatin. At high ovostatin/trypsin molar ratios, about 70% of trypsin became resistant to soybean trypsin inhibitor. The protection of trypsin by binding to ovostatin did not change at least over 18 h at 23 °C, indicating that there was no dissociation of trypsin from the complex with prolonged incubation, even in an excess of trypsin.

DISCUSSION

The majority of protein inhibitors of proteinases react with active sites of enzymes and are capable of abolishing enzymic activities towards all substrates. An exception to those inhibitors has been described for a plasma glycoprotein, α2M. The inhibition mechanism of α2M is unique: it inhibits the great majority of endopeptidases regardless of their catalytic mechanism (4). The reaction of a proteinase and α2M is initiated by proteolytic attack of the enzyme on a particular region located near the middle of the macroglobulin polypeptide chain. The cleavage of a peptide bond triggers a conformational change in α2M which, in turn, “traps” the enzyme molecule (4). When α2M complexes with a proteinase only the proteolytic activity of the enzyme towards high molecular weight substrates is inhibited. Activity towards low molecular weight substrates and reactivity to affinity labels are retained.

The results described for ovostatin here indicate that the mechanism by which ovostatin inhibits collagenase and thermolysin is analogous to that described for α2M (4). 1) Inhibition of collagenase and thermolysin activity by ovostatin was observed when collagen and azocasein were used as their respective substrates. The activity towards low molecular weight substrates was retained. This clearly indicates that the active sites of the enzymes are unblocked. 2) When collagenase complexed with ovostatin, its immunoreactivity to a specific antibody raised in sheep was lost completely. 3) Saturations of ovostatin with thermolysin prevented subsequent binding to collagenase. 4) Both collagenase and thermolysin hydrolyzed the Mr = 165,000 polypeptide chain and produced two fragments of Mr = 88,000 and 78,000 despite the fact that the specificities of these two enzymes are quite different (32, 33). 5) The rearrangement of the quaternary structure of ovostatin upon reaction with proteinases was demonstrated by a faster mobility of the protein in 5% polyacrylamide gel rate electrophoresis. The conformational change was also supported by electron microscopy.3

Mammalian collagenases are known to hydrolyze interstitial collagen types I, II, and III (32, 34–36), although a lesser activity towards gelatin and azocasein has been reported for some collagenases (36–38). Our kinetic data obtained for collagenase binding to ovostatin demonstrated that ovostatin is an equally good substrate for collagenase. Since only an

3 G. C. Ruben, H. Nagase, and E. D. Harris, Jr., unpublished work.
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active enzyme can bind to ovostatin, it is assumed that hydrolysis of a peptide bond in the proteinase-susceptible "bait" region of ovostatin is essential for the complex formation. Thus, the $k_{cat}/K_m$ value of $6.3 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ can be compared with the $k_{cat}/K_m$ values reported for human skin collagenases towards various type I collagen: 11.1 $\times 10^6 \text{M}^{-1} \text{s}^{-1}$ (human), 7.1 $\times 10^6 \text{M}^{-1} \text{s}^{-1}$ (coll); 4.2 $\times 10^6 \text{M}^{-1} \text{s}^{-1}$ (guinea pig), and 3.6 $\times 10^6 \text{M}^{-1} \text{s}^{-1}$ (rat) (36). Competition between collagen and ovostatin for collagenase was demonstrated in Fig. 1.

The cause of the increase in activity of thermolysin complexed with ovostatin towards low molecular weight substrates is not clear. This may, however, reflect the microenvironment created by an ovostatin "trap." Although the activity towards low molecular weight substrates of a proteinase bound to $\alpha_2$M is usually normal or slightly changed (39), there have been a few reports of an enhancement of the activity (40-42). Particularly, Twumasi et al. (42) reported about a 9-fold increase of the $k_{cat}$ of leukocyte elastase without any apparent changes in the $K_m$ when succinyl-trialanine-4-nitroanilide was used as substrate.

Inhibition of trypsin, chymotrypsin (serine proteinases), and papain (cysteine proteinase) by ovostatin was somewhat different from that of the metalloproteinases mentioned above. Only partial and nonstoichiometric inhibition was found when azocasein was used as substrate. This is in contrast to the results obtained with chicken $\alpha_2$M: as shown in our preceding paper, chicken $\alpha_2$M stoichiometrically inhibits trypsin activity towards azocasein (1). However, stronger inhibition was observed when Azocoll was the substrate (Fig. 10). The discrepancy between the two results may be explained by accessibility of substrates to the ovostatin-enzyme complex. As shown by SDS-gel electrophoresis, trypsin, chymotrypsin, and papain hydrolyze ovostatin molecules into several fragments. Our recent observation with pancreatic elastase was similar to these (data not shown). Therefore, these enzymes may form less rigid-ovostatin-enzyme complexes. Furthermore, azocasein is a heat-denatured soluble protein whereas Azocoll is an insoluble particle. Therefore, the accessibility of substrates to the ovostatin-enzyme complex and the dissociation of the substrate from the enzyme may be essential for the function of $\alpha_2$M as a proteinase inhibitor.

Since ovostatin forms covalent bonds with proteinases, the following applications for it as a reagent may be proposed. 1) Purification of an active proteinase may be achieved by reacting the proteinase with ovostatin, and by subsequent isolation of the complex and the dissociation of the enzyme from the complex. 2) The molecular weight of an active proteinase may be determined by isolation of a radio-labeled proteinase bound to ovostatin either by molecular sieve or immunoprecipitation technique and by subsequent SDS-gel electrophoresis and autoradiography.

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