Lysyl oxidase is secreted from fibrogenic cells as a 50-kDa proenzyme that is proteolytically processed to the mature enzyme in the extracellular space. To characterize the secreted proteinase activity, a truncated, recombinant form of lysyl oxidase was prepared as a proteinase substrate containing the sequence of the propeptide cleavage region. The processing proteinase activity secreted by cultured fibrogenic cells resists inhibitors of serine or aspartyl proteinases as well as tissue inhibitor of matrix metalloproteinases-2 (MMP-2) but is completely inhibited by metal ion chelators. Known metalloproteinases were tested for their activity toward this substrate. Carboxyl-terminal procollagen proteinase (C-proteinase), MMP-2, and conditioned fibrogenic cell culture medium cleave the lysyl oxidase substrate to the size of the mature enzyme. The NH₂-terminal sequence generated by arterial smooth muscle conditioned medium and the C-proteinase but not by MMP-2, i.e. Asp-Asp-Pro-Tyr, was identical to that previously identified in mature lysyl oxidase isolated from connective tissue. The C-proteinase activity against the model substrate was inhibited by a synthetic oligopeptide mimic of the cleavage sequence (Ac-Met-Val-Gly-Asp-Asp-Pro-Tyr-Asn-amide), whereas this peptide also inhibited the generation of lysyl oxidase activity in the medium of fetal rat lung fibroblasts in culture. In toto, these results identify a secreted metalloproteinase activity participating in the activation of prolyl oxidase, identify inhibitors of the processing activity, and implicate procollagen C-proteinase in this role.

Lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13) is a copper-dependent amine oxidase that oxidatively deaminates the ε-amino group of specific peptidyl lysine and hydroxylysine residues of collagen and of lysine in elastin. The resultant peptidyl aldehydes can spontaneously condense with other vicinal peptidyl aldehydes or with unreacted ε-amino groups to form inter- and intramolecular cross-linkages stabilizing the fibrous forms of these connective tissue structural proteins. Evidence has been presented that the inhibition of lysyl oxidase action toward collagen molecules results in the accumulation and ultimate proteolytic degradation of soluble collagen monomers, thus preventing the formation of insoluble collagen fibers. The participation of this enzyme is critical, therefore, to the development and repair of structurally sound connective tissues as in the respiratory, cardiovascular, and skeletal systems of the body.

Recent studies of the pathway by which lysyl oxidase is produced in arterial smooth muscle cells revealed that the protein is translated as a 46-kDa preproenzyme. Following signal peptide cleavage, the proenzyme undergoes N-glycosylation apparently within the propeptide region, and the resulting 50-kDa proenzyme is then secreted into the extracellular space. The secreted proenzyme is then proteolytically converted to the functional catalyst derived from the COOH-terminal sequence of the proprotein. The molecular mass of the mature, functional catalyst, as isolated from various connective tissues, ranges from 28 to 32 kDa. The involvement of a proenzyme processing protease in the biosynthetic pathway of lysyl oxidase is of particular interest, because this may represent a previously unexpected extracellular mechanism for the regulation of the production of this cross-linking enzyme. In turn, such a protease could profoundly influence the rate and/or extent of connective tissue fiber formation. The nature of this proteolytic activity is explored in the present study.
oncogene (6). The resulting RS485 cell line was then stably cotransfected with the lysyl oxidase expression vector pSV40poly(A)COD and pSV2neoi to yield the S4 cell line that secretes the 50-kDa lysyl oxidase precursor but does not process it to the mature enzyme (3). These cells were maintained in DMEM containing 4 glitler glucose, 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 1 mg/ml insulin, and 0.1 mM nonessential amino acids. They were pulse-labeled for 3 h with 50 μCi/ml [35S]methionine in the same medium lacking [35S]methionine and fetal bovine serum. The conditioned medium containing the 50-kDa glycosylated prostyl oxidase was then used as a substrate for assay of prostyl oxidase processing pro- tease activity of conditioned smooth muscle cell medium. Following incubation of the conditioned medium containing [35S]methionine labeled secreted proteinase, 20% of the conditioned medium as the source of proteinase activity, the reaction mixture was immunoprecipitated with rabbit anti-lysyl oxidase, and the immunoprecipitates were analyzed by SDS-PAGE (7) and autoradiography, as described (3). Prostyl oxidase was also biosynthetically labeled and prepared from RFL6 cells by these pulse labeling and immunoprecipitation procedures described for the S4 cells. Unlike the S4 cells, which secrete but do not process prostyl oxidase, the RFL6 cells both secrete and proteolytically process the 50-kDa precursor to the 30-kDa mature enzyme. These cells accumulate readily assayed levels of lysyl oxidase activity in the medium to a much greater extent than the smooth muscle cell cultures, thus facilitating the assessment of proenzyme activation. The RFL6 cells were used to assess the effect of an arachidonic acid inhibitor on proenzyme processing during enzyme biosynthesis.

Expression, Purification, Activation, and Assay of Metalloproteinases—MMP-2 and TIMP-2 were expressed in a recombinant vaccinia virus mammalian cell expression system (8). The construction of recombinin plasmid pt7FEMC-1 containing the baculovirus F7 promoter by cassette positioned between thymidine kinase sequences for homologous recombination has been described previously (9, 10). The cloning of human MMP-2 or TIMP-2 cDNAs into pt7FEMC-1, preparation of recombinant vaccinia viruses containing either MMP-2 or TIMP-2 plus, and purification of recombinant proteins were performed as described (9). Specific activity of the MMP-2 used here (Kapp = 500 nM, Vmax = 1 μmol/mg/min) was measured spectrophotometrically with a synthetic thiopeptide substrate (acetyl-Pro-Leu-Gly-S-S-Leu-Leu-Gly-OC2H5), where S-Leu is l-mercaptoethylcaptoprilic acid; Ref. 11) in the presence of 5, 5’-dithiobis (2-nitrobenzoic acid), as described for human skin collagenase (11) and for human fibroblast stromelysin (9).

The recombinant COOH-terminally truncated form of human stromelysin (MMP-3) was expressed in Escherichia coli cell expression system. PGEMEX-1 vector (Promega) containing the truncated version of the protein coding region of MMP-3 (obtained from Dr. Hennig Birkedal-Hansen at the University of Alabama, Birmingham) was transformed into E. coli strain BL21 and recombinant protein purified according to Refs. 12, 14, and 16. MMP-9 (92-kDa gelatinase) was expressed in mammalian p98 cells according to a method described earlier (17, 18) and the stable cell line expressing proMMP-9 was obtained from Dr. G. Goldberg (Washington University School of Medicine, St. Louis, MO). The expression system and purification of the recombinant protein has been described (15).

The PS128 stable cell line expressing proMMP-1 (interstitial collagena) (15) was obtained from Dr. G. Goldberg (Washington University School of Medicine, St. Louis, MO). The expression system and purification of the recombinant enzyme is as described (15).

All matrix metalloproteinases were expressed and purified as proenzymes. ProMMPs were activated by incubation with 1 μM 4-aminophenylmercuric acetate following which excess 4-aminophenylmercuric acetate was removed by gel-filtration. Activities of MMP-3, MMP-9, and MMP-1 were assayed according to a method developed by Dr. G. Goldberg (17) with 72-kDa gelatin-Sepharose 4B (Pharmacia Biotech Inc.) column equilibrated with the same buffer. Unbound protein was completely eluted with the equilibrating buffer, and the column was then eluted with 7.5% MeSO in this buffer. The fractions containing protein were pooled and dialyzed overnight against 40 mM Tris, 200 mM NaCl, 10 mM CaCl2, pH 7.5.

Assay of Lysyl Oxidase Activity—Aliquots of conditioned medium of RFL-6 cells were assayed for lysyl oxidase activity against a tritium-labeled recombinant human type I procollagen substrate prepared and labeled with [4,5-3H]-l-lysine (100 Ci/mmol), as described (20). Assays containing 420,000 cpm of tritiated substrate and conditioned medium were incubated at 37 °C for 1 h in a 1:1000 dilution of growth factor deficient RFL6 cell culture and were then immunoprecipitated with rabbit anti-lysyl oxidase in 0.5% BSA with 50 μM succinylated bovine, 150 mM NaCl, pH 8.2, in the presence or the absence of 50 μM β-aminopropionitrile and were incubated for 2 h at 37 °C. Enzyme activities were expressed as β-aminopropionitrile-inhibitable cpm [3H]H2O formed per 2 h per 106 cells.

Western Blot Analyses—Protein samples were resolved by electro-phoresis on 10% SDS-polyacrylamide gels. The proteins were transferred to Hybond-ECL (Amersham Corp.) nitrocellulose membrane by electroblotting. The membrane was treated with 5% skim milk as the blocking reagent in Tris-buffered saline-Tween (TBS-T; Amersham Corp.) for 2 h at room temperature and then incubated with rabbit anti-lysyl oxidase in 0.5% blocking reagent in TBS-T for 1 h. After washes in TBS-T, the blots were incubated with a 1:1000 dilution of goat anti-rabbit second antibody coupled to horseradish peroxidase. Bands were visualized with an enhanced chemiluminescent reagent (Amersham Corp.) followed by exposure to NEF-496 autoradiography film (DuPont NEN).

NH2-terminal Sequencing—NH2-terminal sequences were obtained on protein bands that were isolated from SDS gel electrophorograms and then transferred to nitrocellulose membranes. The membrane-bound samples were analyzed on an ABI 476A protein sequencing system utilizing Edman chemistry at the Boston University School of Medicine DNA/Protein Core Facility.

Construction of the Fusion Plasmid—The full-length rat lysyl oxidase cDNA clone as a Sall fragment in a Bluescript plasmid, pBS/SCOD, as described previously (21). The Sall site was then digested with BglII and pBS/SCOD was digested with a mixture of XhoI and BssHII, and the resulting 4067- and 500-base pair fragments were gel purified. Two additional BssHII sites flanking the lysyl oxidase cDNA in pBS/SCOD proved to be resistant to BssHII digestion. The 500-base pair fragment was cut with BglII and ligated to the 4067-base pair fragment combined with a mixture of the 8-mer (AGTCTAGA) and the 15-mer (CGCGCTTAGACTGCG) in the BglII site and BssHII sites to create an XhoI site. The ligation mixture was then cut with BssHII and transferred into DH5α competent E. coli cells. DNA was isolated from the resulting colonies and screened by double digestion with XhoI and SalI endonucleases. The correct product was identified (17) with an additional fragment on the gel, 0.4 kb larger than the expected size. The correct plasmid was then digested with BglII (New England Biolabs, Beverly, MA) containing DNA encoding the maltose-binding protein whose terminal sequence contains a factor Xa cleavage site. The resulting plasmid, pMALc-232, was cloned in the DH5α host cells. The cells were grown in LB medium (Sigma) containing 2 g liter-1 glucose and 100 μg ml-1 ampicillin at room temperature until an OD600 of 0.4. The cells were then induced with 0.4 mM isopropyl-β-D-thiogalactoside (0.3 mM), incubated overnight, and harvested by centrifugation at 4000 × g for 20 min, and the pellet was resuspended in 20 mM Tris, 200 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 10 mM 2-mercaptoethanol, pH 7.4, quick frozen at −70 °C, thawed, and lysed by sonication. After centrifugation, the supernatant was passed through an amylose-linked Sepharose affinity column and eluted with a maltose-containing buffer. The eluates were concentrated and electrophoresed on 10% SDS-PAGE gels.

The expected 76-kDa fusion protein (based on the molecular masses...
of the 43-kDa maltose-binding protein, 0.7 kDa derived from the intervening residues encoded by the cDNA sequence linking the two proteins, and the truncated 32-kDa recombinant lysyl oxidase protein (Fig. 3) was identified (band FP, Fig. 1). Close inspection of this band indicated that it represented two closely spaced bands at 76 ± 1 kDa, possibly reflecting the tendency of products expressed in this E. coli system to undergo some degree of proteolysis (New England BioLabs manual). A second band appearing at 65 kDa appeared to be a nonspecific, cross-reacting protein because preincubation of the polyclonal antibody with pure bovine aorta lysyl oxidase did not reduce its appearance in Western blots. In contrast to the fusion protein at 76 ± 1 kDa, this cross-reacting band was not affected by any of the protease treatments used in this study, and its presence did not influence the conclusions of this study. Moreover, attempts at purifying the antibody fraction from the polyclonal rabbit serum did not eliminate the reactivity with this band. The 76-kDa fusion protein preparation was cleaved by factor Xa to produce a 43- and a 33-kDa protein, indicating that the factor Xa Arg-Ile cleavage site within the intervening sequence of the fusion protein is accessible to this protease (not shown).

Incubation of the affinity purified fusion protein (Fig. 1, lane 1) with conditioned smooth muscle cell medium resulted in the proteolytic conversion of the 76-kDa fusion protein into 30- and 46-kDa fragments (Fig. 1, lanes 2 and 3, respectively). This reaction can be assessed with this substrate simply by Coomasie Blue staining of protein bands resolved by SDS-PAGE without the need for isotopic labeling of the precursor, because of the relative purity and greater quantities of the protease substrate (not shown). The 46-kDa fragment reacted with a polyclonal antibody specific for the maltose-binding protein (MBP, lane 3) but did not react with polyclonal anti-lysyl oxidase as observed by Western blotting (lane 2). The 30-kDa band reacted with anti-lysyl oxidase but not with the anti-maltose-binding serum (LO, lane 2).

Peptide Inhibitors—The thiol-based peptide inhibitor of the MMP family, HS-CH2-CH(CH2-CH(CH3)2)-CO-Phe-Ala-NH2, and its S-acetylated derivative were purchased from Peptides International (Louisville, KY). The acetyl-Met-Val-Gly-Asp-Asp-Pro-Tyr-Asn-amide peptide was synthesized by solid phase peptide chemistry and purified to homogeneity by high pressure liquid chromatography. The NH2- and COOH-terminal residues of this peptide were derivatized by acetylation and amidation, respectively, by QCB, Inc.

RESULTS

Processing Activity in Conditioned Smooth Muscle Cell Medium—As previously demonstrated, the S4 cell line secretes the 50-kDa precursor form of lysyl oxidase but does not process it to the smaller, mature enzyme. Consistent with the latent nature of the proenzyme, lysyl oxidase activity is not detectable in the conditioned S4 cell medium (3). Thus, these cells were used to obtain an isotopically labeled form of the proenzyme for the assay of the proenzyme processing protease activity secreted into the medium by cultured neonatal aortic smooth muscle cells. S4 cells were pulse-labeled with [35S]methionine; the conditioned medium was decanted and incubated with the conditioned medium obtained from neonatal rat aorta smooth muscle cells cultured for 18 h in serum-free DMEM. Following 3 h of incubation at 37 °C, the reaction mixtures were immunoprecipitated with polyclonal antilylsyl oxidase. As shown (Fig. 2, lane 1), the [35S]-labeled 50-kDa band corresponds to the precursor species of lysyl oxidase (3). Incubation with the native, cell-free conditioned medium of the SMC cultures results in the conversion of the 50-kDa species to a product migrating as a 30-kDa species, consistent with the mature enzyme (lane 2). Preincubation of the antibody with excess lysyl oxidase purified from bovine aorta prevents the appearance of both the 30- and 50-kDa species in the immunoprecipitates of the reaction mixtures, indicating their immunological identity with lysyl oxidase (not shown). Prior heat inactivation (100 °C for 10 min) of the conditioned SMC medium prevents the conversion of the 50-kDa species to the 30-kDa species (lane 3). The presence of PMSF (lane 4) or aprotonin (lane 5), inhibitors of serine proteases, does not inhibit this conversion. In contrast, the presence of 1 mM EDTA in the incubation mixture prevents the cleavage (lane 6).

In the course of these studies, it became apparent that the availability of larger quantities of a more conveniently prepared substrate for the assay of the SMC protease was desirable. For that purpose, a recombinant 76-kDa protein was expressed in E. coli consisting of maltose-binding protein fused to that portion of the lysyl oxidase proenzyme suspected to contain the proprotein proteolytic processing site contiguous with the remaining COOH-terminal sequence representing the mature portion of lysyl oxidase (Fig. 1; also see Fig. 8 and "Discussion"). The presence of the maltose-binding domain within this fusion protein permitted the facile purification of this product from the bacterial lysate by affinity chromatography (see "Materials and Methods"). As shown in Fig. 3, the cleavage of the fusion protein by SMC medium was not prevented by inhibitors of serine (PMSF and aprotinin), thiol (leupeptin), or acid proteases (pepsatin) but was inhibited by EDTA, EGTA, and prior heat inactivation of the SMC medium, consistent with the results presented in Fig. 2. It is important to note that Western blot analysis of the conditioned smooth muscle cell medium alone did not reveal the presence of lysyl oxidase protein bands, consistent with our repeated observations that lysyl oxidase produced by these cells in culture associates predominantly with the insoluble cell layer/matrix fraction. The results presented at Figs. 2 and 3 indicate that full-length or truncated recombinant precursor forms of lysyl oxidase prepared in mammalian or bacterial cells, respectively, can be cleaved to the same apparent size by the SMC-conditioned medium and that the conversion of the proprotein to the mature enzyme species is catalyzed by a heat-labile secreted metalloprotease.

NH2-terminal Sequence Analysis—In an effort to define the site of cleavage of the proprotein acted upon by the smooth muscle cell protease activity, advantage was taken of the availability of the relatively more abundant quantities of the fusion...
protein substrate described herein. This substrate was incubated with conditioned smooth muscle cell medium and then transferred onto a polyvinylidene difluoride membrane. The 30-kDa band was visualized by Ponceau S, excised from the membrane, and subjected to automated microsequencing analysis. The sequence so obtained was Asp-Asp-Pro-Tyr-Asn.

The profile of inhibitor sensitivity and the cleavage site specificity of the SMC medium proteolytic activity pointed toward candidate extracellular metalloproteinases that may participate in the extracellular maturation of prolysyl oxidase. Among possible candidates, procollagen C-proteinase has been purified from chick calvaria and conditioned medium of cultures of mouse fibroblasts and of organ cultures of chick embryo tendons and sterna (18, 22). This metalloproteinase normally processes procollagen, cleaving the pro-α1(1) and pro-α2(1) chains at an Asn-Ala-Asp bond and pro-α1(111) at a Gly-Asp bond to release the corresponding COOH-terminal extension peptides (18, 22).

This enzyme operates at nearly neutral pH, is Ca²⁺-dependent, and may require a second metal in addition to Ca²⁺. It is inhibited by metal chelators but not by inhibitors of serine or cysteine proteinases. Additional consideration was given to 72-kDa gelatinase (type IV collagenase; matrix metalloproteinase-2), which is secreted by mammalian cells of various tissue origins (23, 24) and has been reported to be the major secreted metalloproteinase constitutively expressed by human vascular smooth muscle cells (25, 26). In this regard, analyses in the present study of the conditioned medium of neonatal rat aorta SMC medium by gelatin zymography revealed a single gelatinolytic activity that appeared to be 72-kDa MMP-2 both by molecular mass and by its sensitivity to antibody specific for MMP-2 (data not shown). Other matrix metalloproteinases, including MMP-9 (92-kDa gelatinase) and stromelysin are synthesized and secreted upon stimulation of human vascular SMC by specific cytokines (26).

Thus, the following enzymes were screened for their potential to cleave the fusion protein substrate: procollagen C-proteinase, purified from the medium of organ cultures of chick embryo tendons; MMP-2, partially purified from neonatal rat SMC conditioned medium; and purified, recombinant human MMP-2; purified recombinant 92-kDa gelatinase, stromelysin, and MMP-3 (interstitial matrix metalloproteinase), each in their catalytically active forms. As shown (Fig. 4), among these, only MMP-2 and procollagen C-proteinase cleaved the fusion protein substrate to yield a 30-kDa product the apparent size of which was indistinguishable from that of the product resulting from cleavage by conditioned SMC medium. As noted in the legend of Fig. 4, approximately equal amounts of activity units of each of the various metalloproteinases were individually incubated with the fusion protein, with the exception that a lesser quantity of the C-proteinase was employed. The results indicate that the C-proteinase is the most effective of the proteinases employed in this assay. When incubated with 10-fold larger quantities of each of these metalloproteinases, the fusion protein substrate was again selectively cleaved to yield proportionately larger quantities of the 30-kDa product by the C-proteinase and by MMP-2, whereas the 92-kDa gelatinase and interstitial collagenase nonspecifically degraded fusion protein to a variety of products of different sizes (not shown).

To further characterize the processing proteinase activity, the effects of TIMP-2, a naturally occurring protein inhibitor of the MMP family (27), and the effects of a thiol-based peptide, HS-CH₂-C(CH₂-C(H₃)₂)-CO-Phe-Ala-NH₂, a potent inhibitor for the group of Zn²⁺-dependent matrix metalloproteinases (28), were assessed. An S-acetylated derivative of the thiol peptide is inactive toward these enzymes and serves as a control for the effect of the noninhibitory components of the peptide.

As shown (Fig. 5A), TIMP-2 inhibited processing of the fusion protein by purified recombinant MMP-2 (lanes 1 and 2) but failed to inhibit the cleavage by the conditioned SMC me-
Methionine as described under "Materials and Methods" during the biosynthesis of the enzyme by fibrogenic cells, concluding that both are specific for the same Gly-Asp bond in the mature region of prolysyl oxidase and corresponds to the NH2-terminal sequence recently identified in mature lysyl oxidase. The NH2-terminal amino acid sequences of the 30-kDa products cleaved from the FP substrate by different proteinase sources. The arrows and underlining indicate the location within the propeptide cleavage region of the NH2 termini generated by proteolysis.

![Proteolytic Activation of Prolysyl Oxidase](image)

**Proteolytic Activation of Prolysyl Oxidase**

| Protease Source | N-Terminal Sequence |
|-----------------|---------------------|
| SMC Media       | DDPYN.............  |
| C-Proteinase    | DDPYNPYN...........|
| MMP-2           | LRPPSHV............|

The NH2-terminal amino acid sequences of the 30-kDa products generated by MMP-2 and C-proteinase were determined to assess the site of cleavage in the propeptide sequence within the fusion protein substrate. As shown (Fig. 6), the NH2-terminal sequence resulting from cleavage by MMP-2 indicates that this enzyme hydrolyzes an Asn-Leu bond. Both conditioned SMC medium and the purified C-proteinase yield the same cleavage profile to the 30-kDa enzyme in cultures actively synthesizing lysyl oxidase. Although procollagen C-proteinase is the most promising candidate among those tested for the source of processing protease activity, there does not appear to be an inhibitor that is specific for that protease. In view of the ability of an oligopeptide (Tyr-Tyr-Arg-Ala-Asp-Ala) to mimic the C-proteinase cleavage site in the proc-α1(I) chain to compete with the procollagen substrate of this catalyst (6–12 mM inhibits by 45–78%) (30), a similar approach was adopted in this instance by assessing for the effect of a synthetic peptide mimicking a conserved, octameric sequence containing the GDD cleavage site in prolysyl oxidase, Ac-Met-Val-Gly-Asp-Asp-Pro-Tyr-Asn-amide, on C-proteinase activity in the cell-free assay system and on prolysyl oxidase processing activity in RFL6 cell cultures. Initial experiments established that 1 mM of this peptide inhibits procollagen C-proteinase activity against the fusion protein substrate (Fig. 7).

This peptide inhibitor, as well as the thiol and acetylated thiol peptide inhibitors, were then tested for their effect on the de novo generation of lysyl oxidase activity in cultures of RFL6 cells. RFL-6 fibroblasts were cultured to confluency in T75 flasks and pulse labeled with [35S]methionine for 2 h in the presence of 50 μM S-acetylated peptide (lane 1), MeSO carrier (lane 2), or 50 μM HS peptide (lane 3). The conditioned medium of each incubation was then immunoprecipitated with anti-lysyl oxidase as described. LO, lysyl oxidase.

**Proteolytic Activation of Prolysyl Oxidase**

**Protease Source**

| N-Terminal Sequence |
|---------------------|
| SMC Media           | DDPYN............. |
| C-Proteinase        | DDPYNPYN...........|
| MMP-2               | LRPPSHV............|

**Propeptide Cleavage Region**

..GASRRANRTASPQQPQLSINLRPPSHVDRMVGGPPYWYK..
Discussed Previous studies have provided evidence that lysyl oxidase is synthesized as a proenzyme that is secreted as a 50-kDa N-glycosylated protein. The secreted proenzyme is then proteolytically processed in the extracellular space to a functional 30-kDa product. The present study indicates that the process-lytically processed in the extracellular space to a functional glycosylated protein. The secreted proenzyme is then proteo-

The substrate specificity of procollagen C-proteinase against the model lysyl oxidase substrates found here is consistent with the known specificity of this enzyme for type I procollagen in which this enzyme cleaves an Ala-Asp bond in the pro-α(I) and pro-α2(II) chains and a Gly-Asp bond in the pro-α(I)III chain (22, 26). Cronshaw et al. (29) have previously noted the similarity between the known substrate specificity of procollagen C-proteinase and the putative cleavage site predicted from their analysis of the NH2-terminal of the isolated mature pig skin enzyme. Additional although indirect support for the role of this proteinase in prolyl oxidase processing stems from: 1) the common susceptibility of the rat fibroblast and procollagen C-proteinase processing activity to inhibition by the synthetic peptide containing the GDD cleavage sequence; 2) the common susceptibility of other proproteinases by enzymes such as furin (32). Thus far, however, evidence that cleavage may occur at these sites has not been seen. It is of interest that minor immunoreactive bands are seen in Figs. 1, 3, and 5 which this enzyme cleaves an Ala-Asp bond in the pro-α(I) and pro-α2(II) chains and a Gly-Asp bond in the pro-α(I)III chain. The cysteine 21–alanine 22 bond is a putative signal peptide cleavage site (34).

Comparison of the primary structures predicted from the cDNA sequences of human, rat, mouse, and chick prolyl oxidase reveals that the only Asp-Asp sequence occurs in the Gly-Asp-Asp-Pro-Tyr-Asn-NH2 peptide.

The possibility that other sites may also be cleaved in prolyl oxidase catalyst certainly must be considered. Notably, there are two polybasic sites in the rat proenzyme, i.e. Arg-Arg-Arg at residues 62–64 and Arg-Arg at residues 134–135. Such polybasic sequences are known to be the sites of proteolytic processing of a variety of other proproteins by enzymes such as furin (32). Thus far, however, evidence that cleavage may occur at these sites has not been seen. It is of interest that minor immunoreactive bands are seen in Figs. 1, 3, and 5, and at a molecular mass of approximately 32,000 daltons. Efforts at determining the NH2-terminal of this minor band have yet to be successful. This may reflect intermediates or secondary products generated during the proteolytic processing of prolyl oxidase that do not accumulate significantly. As noted, the 72-kDa MMP-2 is also able to cleave the substrate to apparently the same size as obtained by cleavage with the SMC medium and C-proteinase. However, NH2-terminal sequencing showed that the cleavage site in this case is at an Asn-Leu bond located 12 amino acids upstream from the Gly-Asp-Pro-Tyr-Asn-NH2 peptide. The possibility that other sites may also be cleaved in prolyl oxidase catalyst certainly must be considered.
critical participation of lysyl oxidase in the accumulation of insoluble collagen fibers. It is of further interest in this regard that cleavage of the propeptides from procollagen by the NH₂-terminal and C-proteinases is essential for the subsequent, spontaneous assembly of the processed collagen molecule into quarter-staggered, native fibrils. Moreover, as seen with type I collagen, collagen molecules must be assembled into native fibrillar aggregates to become an effective substrate for lysyl oxidase (33). Recent studies suggest that fibril formation of collagen may be necessary for lysine oxidation by permitting the intermolecular neutralization of an unfavorable anionic charge near the potentially susceptible, N-telopeptide lysine by a cationic site within the triple helical sequence of a neighboring, Ω-staggered molecule. Thus, procollagen C-proteinase may play a doubly essential role in the cross-linking process by: 1) converting procollagen to the collagen molecule that can then assemble into the quaternary fibrillar structure essential to collagen lysine oxidation and 2) by converting the latent prolyl oxidase to the fully functional catalyst capable of oxidizing lysine in the newly preassembled native fibrils, thus, in essence, generating the active forms of the collagen substrate and of the lysyl oxidase catalyst. More definitive assessment of this intriguing possibility awaits the development and/or discovery of specific chemical or macromolecular inhibitors of the activity or expression of procollagen C-proteinase.

In view of the markedly different sequences surrounding peptidyl lysine in collagen and elastin substrates (41), the possibility that different forms of lysyl oxidase may exist with specificity for one and another of these substrates has long been considered. However, there is no evidence presently available that this is the case. Indeed, purified lysyl oxidase readily oxidizes purified preparations of both elastin and collagen substrates (1) as well as elastin-like (42) and collagen-like synthetic polypeptides (41). Thus, although procollagen C-proteinase plays an essential role in the post-translational processing of procollagen, the present study raises the possibility that this proteinase may be involved in the processing of an enzyme participating in the post-translational modification of both elastin and collagen.

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Metalloproteinase Activity Secreted by Fibrogenic Cells in the Processing of Prolysyl Oxidase: POTENTIAL ROLE OF PROCOLLAGEN C-PROTEINASE
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