

Ultrastructural Immunolocalization of Lysyl Oxidase in Vascular Connective Tissue

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Abstract. The localization of lysyl oxidase was examined in calf and rat aortic connective tissue at the ultrastructural level using polyclonal chicken anti-lysyl oxidase and gold conjugated rabbit anti-chicken immunoglobulin G to identify immunoreactive sites. Electron microscopy of calf aortic specimens revealed discrete gold deposits at the interface between extracellular bundles of amorphous elastin and the microfibrils circumferentially surrounding these bundles. The antibody did not react with microfibrils which were distant from the interface with elastin. There was negligible deposition of gold within the bundles of amorphous elastin and those few deposits seen at these sites appeared to be associated with strands of microfibrils. Lysyl oxidase was similarly

localized in newborn rat aorta at the interface between microfibrils and nascent elastin fibers. Gold deposits were not seen in association with extracellular collagen fibers even after collagen-associated proteoglycans had been degraded by chondroitinase ABC. However, the antibody did recognize collagen-bound lysyl oxidase in collagen fibers prepared from purified collagen to which the enzyme had been added in vitro. No reaction product was seen if the anti-lysyl oxidase was preadsorbed with purified lysyl oxidase illustrating the specificity of the antibody probe. The present results are consistent with a model of elastogenesis predicting the radial growth of the elastin fiber by the deposition and crosslinking of tropoelastin units at the fiber-microfibril interface.

LYSYL oxidase plays a pivotal role in the biosynthesis of collagen and elastin by oxidizing peptidyl lysine to peptidyl α -amino adipic- δ -semialdehyde, the precursor to the covalent crosslinkages in these connective tissue proteins. The lysine-derived crosslinkages that evolve from this aldehyde stabilize the fibrous structures of elastin and collagen, thus providing anchoring points important to the tensile and/or elastic properties exhibited by these proteins (10, 21).

It appears likely that lysyl oxidase functions in vivo in the extracellular space and, indeed, the enzyme is secreted into the growth medium by cultured fibroblasts (13, 24) and smooth muscle cells (8). Little is known about the localization of lysyl oxidase in either the intracellular or extracellular compartments, however. Siegel et al. (22) had demonstrated at the level of the light microscope that the enzyme appears to be associated with extracellular fibers of collagen in fibrotic liver, using fluorescently labeled anti-lysyl oxidase as a probe. Its localization has not been probed at the ultrastructural level, however, nor has it been examined in an elastin-rich tissue. In the present report, we describe the localization of lysyl oxidase in the extracellular matrices of bovine calf aorta and in newborn and adult rat aorta using ultrastructural immunocytochemical methods and a polyclonal antibody to bovine aortic lysyl oxidase that we have recently described (2).

Materials and Methods

Preparation and Assay of Enzyme

Lysyl oxidase was isolated from 4-M urea extracts of bovine aorta by a modification (27) of the published procedure (11). The modified procedure substitutes chromatography through Cibacron Blue-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) for the N,N' -DEAE cellulose step originally described, thus providing a method which co-purifies the four ionic variants of lysyl oxidase normally resolved by DEAE cellulose chromatography. Prior studies had established that the peptide maps of proteolytic digests of each species were remarkably similar as were the molecular masses of each variant (32,000 D) and the substrate and inhibitor profiles of each (25). Because the four species display such structural and catalytic similarities, use of the co-purified mixture for certain immunological, catalytic, and physical studies seems warranted especially since co-purification provides larger quantities of enzyme.

Preparation of Antibody

Lysyl oxidase purified as described appeared homogeneous by SDS PAGE performed according to Laemmli (12). The enzyme was freed of possible contaminants by excising the band corresponding to lysyl oxidase from the acrylamide gel for use as the antigen. A narrow strip of the gel slab was cut out and separately stained with Coomassie Brilliant Blue and used as a guide for excising the section of the unstained gel corresponding to the electrophoretic migration position of lysyl oxidase at 32,000 D. This gel-purified antigen was then used to raise antibodies in chickens as described (2). Animals were bled at bi-weekly intervals and serum samples were screened for anti-lysyl oxidase activity by a modification of the dot blot procedure of Hawkes et al. (9). The co-purified 32,000-D lysyl oxidase antigen

(5 µg/dot) was immobilized on nitrocellulose, the blot was then incubated with dilutions of the immune serum and then with rabbit anti-chicken antibody coupled to horseradish peroxidase (Miles-Yeda, Inc., Elkhart, IN). The blot was thoroughly washed as described by Hawkes et al. (12) before and after incubations with the first and second antibody, respectively. Immobilized immune complexes were visualized by incubation of the blot with the peroxidase substrate, 4-chloronaphthol, to develop visible color. Freshly obtained immune serum developed a titer of 1:16,000 by this technique. Preimmune chicken serum yielded negative reactions in this procedure, as did controls from which primary antibody or the lysyl oxidase antigen had been omitted.

Immunoprecipitation of ¹²⁵I-Labeled Antigen

Purified bovine aortic lysyl oxidase was iodinated with Na¹²⁵I in the presence of 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril (Iodogen; Pierce Chemical Co., Rockford, IL) as described (7) and then incubated at 26°C with a 1:30 dilution of chicken anti-lysyl oxidase for 90 min. Immune complexes were precipitated by further incubation with rabbit anti-chicken IgG (Miles Laboratories, Inc., Elkhart, IN) for 120 min. The precipitate was isolated by centrifugation through a cushion of 1 M sucrose at 4300 g for 7 min, resuspended in 50 mM Tris, (pH 7.6) 1 M NaCl, 1% sodium deoxycholate, and 1% Triton X-100 and reisolated by centrifugation. The pellet was dissolved and heated at 100°C for 2 min in gel electrophoresis sample buffer containing 6 M urea, 62.5 mM Tris (pH 6.8), 4% 2-mercaptoethanol, 3% SDS, and 2% bromophenol blue, and electrophoresed in 10% crosslinked polyacrylamide slab gels, according to Laemmli (12).

Both anti-lysyl oxidase serum and preimmune serum were partially purified before use as ultrastructural probes. Each serum was dialyzed against 0.02 M Tris HCl (pH 8.0) and applied to columns (1 × 1.5 cm) of DEAE-Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA). The IgG components of the sera passed through this column and were completely eluted by washing with 0.02 M Tris HCl (pH 8.0). Anti-lysyl oxidase activity was recovered in the IgG eluate as determined by the dot/blot titration method.

Generation of Enzyme-Collagen Complexes In Vitro

Type I collagen was isolated from fetal calf skin by an adaptation of the method of Monson and Bornstein (15) as previously described (6). The acid extraction step was avoided to preserve the N- and C-terminal telopeptides intact. Collagen types in the neutral salt extract were separated by precipitation with specific concentrations of NaCl. The type I product was chromatographed over DEAE cellulose under conditions that should resolve type I tropocollagen from procollagen species and from acidic proteoglycans. The purity of the product as type I tropocollagen was established by amino acid analysis and by SDS PAGE, each of which indicated the absence of procollagens in this preparation. The product was found to be free of contamination by type III collagen by the delayed reduction gel electrophoresis method of Sykes et al. (26). Fibrils of type I collagen were prepared by adding 50 µl of a 5 mM acetic acid solution of collagen (0.57 mg ml⁻¹) to 1.5 ml polyethylene tubes on ice and diluting to 0.5 ml with 5 mM acetic acid. To this was added 0.5 ml of a buffer composed of 30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, 30 mM Na₂HPO₄, and 135 mM NaCl (pH 7.5). Fibrils were formed by incubating this mixture at 26°C for 3 h. Lysyl oxidase in 6 M urea was dialyzed against 5 mM potassium phosphate (pH 7.5) before use in these studies. Aliquots of 50 µl containing 2 µg of the dialyzed enzyme were added to tubes containing the preformed collagen fibrils, the mixtures were incubated at 37°C for 10 min and centrifuged at 10,000 g for 1.5 min. The supernatants were removed and the pellets were gently suspended in the fibril-forming buffer and reisolated by centrifugation to remove unbound enzyme. The pellet of the enzyme-collagen complex was then fixed by treatment with 4% paraformaldehyde for 2 h on ice and processed as above for electron microscopy.

Immunohistochemistry

Aortas of 2-wk-old calves, newborn rat pups and adult Sprague Dawley rats were freshly obtained and random pieces of aortic tissue were finely minced and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at 0°C. The tissue was washed four times in this phosphate buffer in the absence of fixative and then processed into Lowicryl K4M using the low temperature embedding method of Carlemalm et al. (3). The embedded samples were polymerized by exposure to ultraviolet light and thin (60–70 nm) sections were cut and mounted on collodion-coated nickel grids. A two-step indirect immunogold procedure (16) was used to localize antigen in the

tissue specimens reacting with chicken anti-lysyl oxidase. Mounted thin sections were etched in 10% H₂O₂, washed in distilled deionized water, and incubated in a 1:30 dilution of normal goat serum for 30 min. The grids were transferred directly into chicken anti-lysyl oxidase diluted 1:1, 1:10, or undiluted and incubated in a moist chamber for 1–24 h at 4°C. The sections were then washed four times in a buffer of 0.1 M Tris and 0.1% BSA (pH 7.2), and incubated for 1 h with a 1:6 dilution of rabbit anti-chicken IgG conjugated with 10-nm particles of colloidal gold (E. Y. Laboratories, Inc., San Mateo, CA). The grids were extensively washed in filtered Tris-BSA buffer, rinsed twice in distilled deionized water, air-dried, and stained with uranyl acetate and lead citrate. Control sections were incubated with preimmune chicken serum, chicken anti-lysyl oxidase preincubated with excess purified lysyl oxidase before addition to tissue sections or gold-conjugated rabbit anti-chicken IgG in the absence of anti-lysyl oxidase.

After immunogold labeling of lysyl oxidase, some grids were stained with a 1% palladium chloride solution for 5–15 min, rinsed thoroughly, and counterstained with uranyl acetate and lead citrate before observation in the electron microscope (14).

Enzyme Digestion

To account for the possibility that proteoglycans might mask the immunoreactivity of lysyl oxidase associated with collagen fibers, freshly isolated calf aorta was finely minced and incubated with 1 U ml⁻¹ of chondroitinase ABC (Miles Laboratories, Inc.) in 0.1 M Tris buffer (pH 8.0) containing 0.5% BSA for 1 h at 37°C (20). Control specimens were incubated under these conditions but in the absence of chondroitinase ABC. After incubation, the tissue preparations were each divided into two aliquots, one of which was fixed and embedded for immunolabeling as described above, while the other was stained with 1% ruthenium red in McIlvaines buffer, (pH 5.6) for 30 min at room temperature. The tissue was then fixed overnight at 4°C in a solution of 3% glutaraldehyde and 1% ruthenium red in McIlvaines buffer. The next day, the tissue was quickly rinsed four times with buffer containing ruthenium red, postfixed for 2 h in 1% osmium tetroxide, and then dehydrated through ascending concentrations of acetone and embedded routinely in Epon for electron microscopy.

Results

Reactivity of Antibody with Lysyl Oxidase

SDS gel electrophoresis of the purified preparation of bovine aortic lysyl oxidase used as antigen in the present study demonstrated that it consisted of an apparently homogeneous protein species of 32,000 D (Fig. 1 A). The reactivity of the chicken anti-lysyl oxidase against this protein was demonstrated by immunoprecipitation of a preparation of purified lysyl oxidase that had been radioactively labeled with ¹²⁵I (Fig. 1 B). Studies in progress have demonstrated that the antibody also precipitates considerably larger isotopically labeled proteins obtained from the cell matrix of calf aortic smooth muscle cells pulsed with radioactive amino acids in culture (1). It is assumed that these proteins are precursors of lysyl oxidase or are otherwise related to the 32,000-D product since the immunoreactivity with these species is competitively prevented by preincubation of the chicken anti-lysyl oxidase with the unlabeled, purified 32,000-D species (1). The antibody is unreactive against peptides solubilized by hot oxalic acid extraction of bovine ligament elastin, is not reactive toward bovine fibronectin, and does not precipitate products which are susceptible to bacterial collagenase from cultured calf smooth muscle cells (data not shown). The assumption that the antibody is specific for forms of lysyl oxidase in cellular and tissue specimens is supported by the competing effect of the purified antigen.

Immunocytochemistry

Bundles of amorphous elastin in a section of calf aorta are shown in Fig. 2, A–C, with microfibrils most evident at the

periphery of the bundles. Gold deposits signifying the presence of lysyl oxidase are also present at the periphery of the elastin bundle in association with microfibrils. Microfibrils that are not at the elastin interface do not bind the antibody. The distribution of lysyl oxidase is continuous around the bundles. Fig. 3, *A* and *B*, represent presumed earlier stages of elastin formation in newborn rat aorta where microfibrils predominate over amorphous elastin. In addition to apparent complexes of lysyl oxidase with microfibrils at the periphery of the developing elastin bundle (Fig. 3 *A*), there are linear deposits of lysyl oxidase running through the bundle in association with thin strands of microfibrils. Fig. 3 *B* shows a bed of microfibrils with extremely small areas of amorphous elastin. Lysyl oxidase appears in linear, seemingly periodic deposits within the microfibrillar bed. This figure gives the impression of a microfibril-lysyl oxidase template within which linear bands of elastin will ultimately develop.

Adult rat aorta, reacted with anti-lysyl oxidase and gold-labeled second antibody (Fig. 4), reveals a thin ring of microfibrils delineating amorphous elastin. Microfibrils are fewer in number and much less dense than in the newborn aorta. Gold labeling of the elastin-microfibril complex is considerably less than in developing elastin, suggesting that much less lysyl oxidase is available for binding by the antibody.

Gold deposits are not seen in control specimens prepared using chicken anti-lysyl oxidase that had been preadsorbed by excess purified bovine aortic lysyl oxidase before incubation with fixed tissue (Fig. 5), thus illustrating the specificity of the gold labeling patterns seen in this study. Completely negative results were also obtained with preimmune chicken serum or if the chicken anti-lysyl oxidase but not the gold-labeled second antibody was omitted from the immunolocalization protocol described in Materials and Methods. The gold deposits seen in bovine calf, rat pup, and adult rat aortae thus appear to localize epitopes found in purified 32,000-D bovine aortic lysyl oxidase.

Gold deposits are not seen on the longitudinal or transverse sections of collagen fibrils in the aortic sections examined (see Fig. 2), nor were gold deposits clearly visible within cell structures in the process of secretion or transport to the extracellular microfibrillar loci. The apparent lack of gold deposits on collagen fibers was somewhat surprising since prior studies have shown that purified lysyl oxidase binds to native fibers of type I collagen *in vitro* (6). Gold deposits were also not seen on collagen fibers of tissue specimens that had been preincubated with chondroitinase ABC to remove proteoglycans associated with collagen before incubation with the first and second antibodies (data not shown). To further investigate this result, complexes of purified lysyl oxidase with native fibers of type I collagen were generated *in vitro* as described and examined by the anti-lysyl oxidase immunolocalization technique. As shown (Fig. 6) gold deposits are seen in association with these collagen fibers. Collagen fibers to which the enzyme had not been added *in vitro* did not exhibit gold deposits.

Discussion

The present report identifies immune-reactive product in association with microfibrillar material found at the periphery of elastic fibers in the extracellular space. The absence of

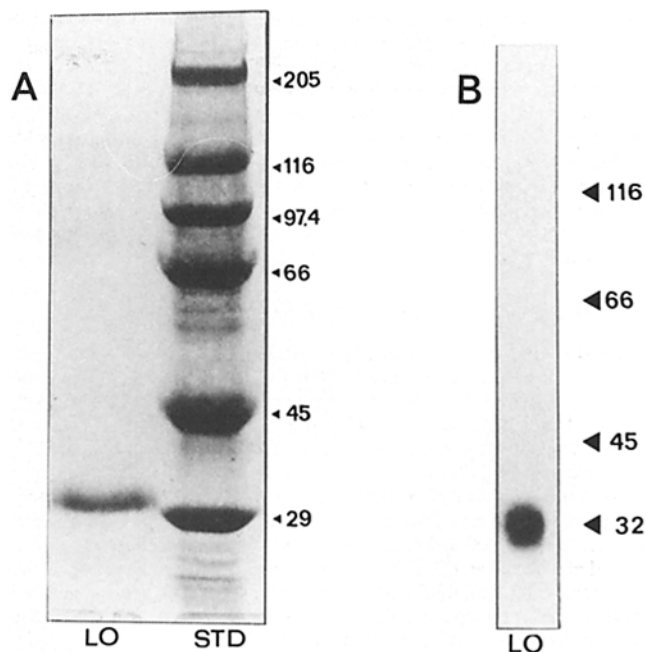


Figure 1. (A) SDS PAGE of lysyl oxidase (left lane) purified from bovine aorta. The molecular mass of the purified lysyl oxidase is 32,000 D. Standard proteins of the molecular masses ($\times 10^{-3}$) indicated are in the right lane. (B) SDS PAGE of the immunoprecipitated ^{125}I -labeled derivative of the purified lysyl oxidase shown in A. [^{125}I]Lysyl oxidase was precipitated from solution by incubation with chicken anti-lysyl oxidase and then with rabbit anti-chicken IgG. The immunoprecipitate was dissolved and heated at 100°C in the electrophoresis sample buffer before electrophoretic resolution on the gel. The radioactive band was visualized by autoradiography.

gold deposit in specimens treated with preimmune serum, in specimens treated with immune serum that had been pretreated with the 32,000-D aortic lysyl oxidase antigen that had been purified to apparent homogeneity, and in those specimens in which the primary antibody was omitted argues in favor of the conclusion that the discrete gold deposits identify lysyl oxidase protein at these sites. The complete inhibition of the deposition of gold by preincubation of anti-lysyl oxidase with purified lysyl oxidase is consistent with the conclusion that the antibody both recognizes and is specific for lysyl oxidase. The fact that gold is not deposited on all microfibrils but is only deposited on those at the interface with elastin indicates that the antibody is not directed against microfibrils themselves or against a component common to all microfibrils. Moreover, the distribution of gold deposits is not consistent with that expected of fibronectin since collagen fibers in tissue specimens were not reactive with anti-lysyl oxidase in the present study but would be expected to react with antibodies directed against fibronectin (28).

The presence of lysyl oxidase in association with microfibrillar material surrounding elastin fibers is of particular interest. These results suggest that elastogenesis is characterized by accumulation of microfibrillar structures adjacent to the surface of the fibrogenic cell; lysyl oxidase associates with these microfibrils, after which elastin fibrils appear within the microfibril-lysyl oxidase aggregates. The microfibrillar material remains in association with the periphery of the elastin component but is not evident within the elastin fiber which remains structurally amorphous. A model for the

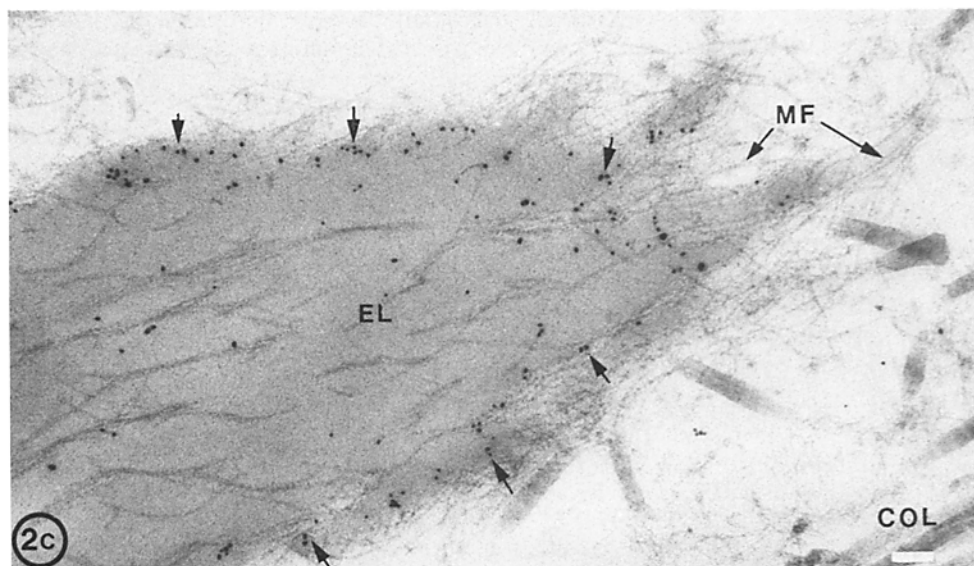
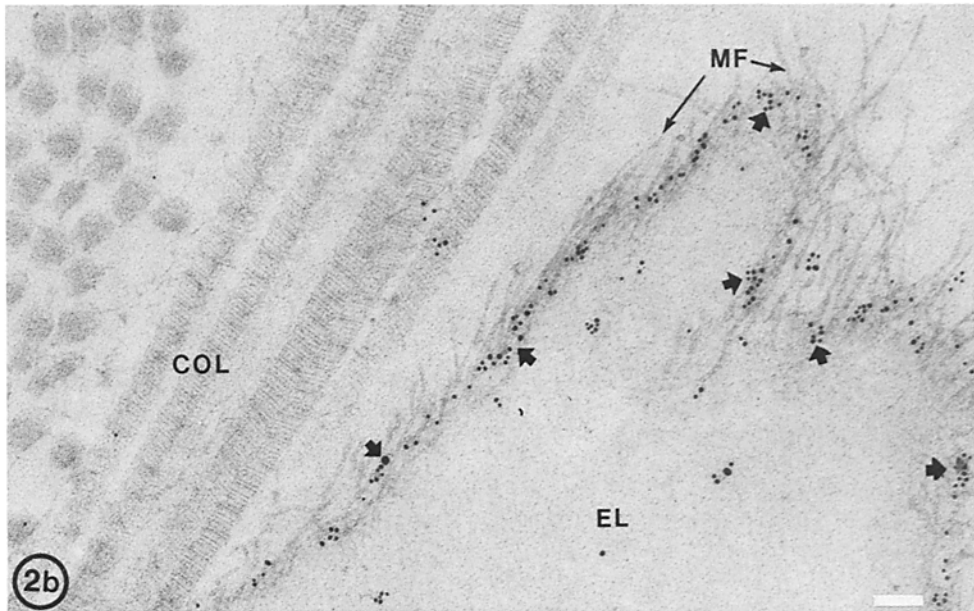
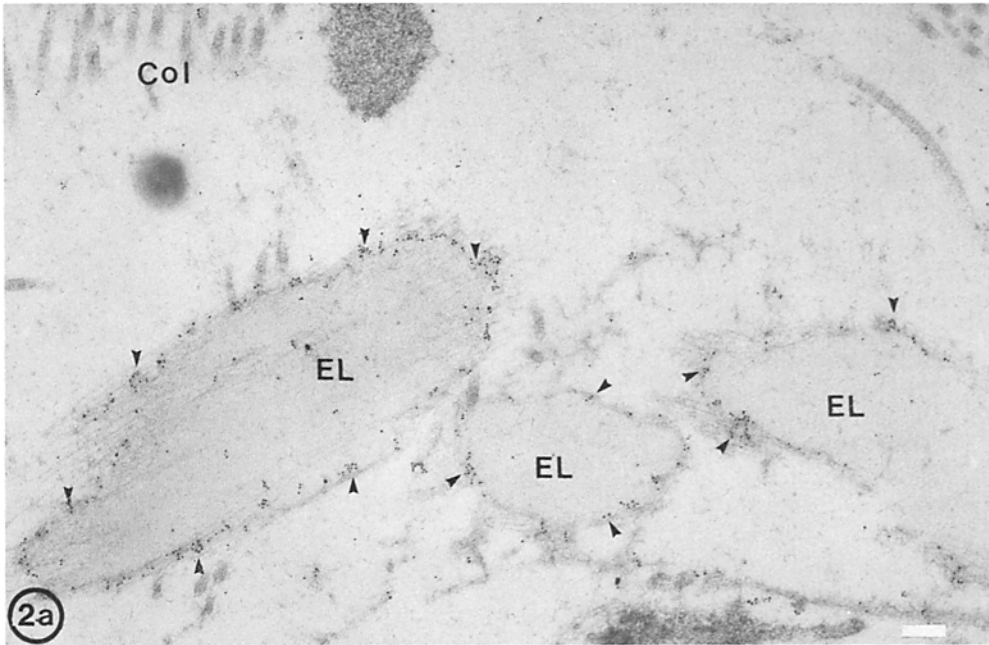


Figure 2. (A) Low power view of elastic fibers from bovine calf aorta. Gold particles (*arrowheads*) demonstrate lysyl oxidase localized to the elastin-microfibril interface surrounding the amorphous elastin bundle (EL). Col, collagen. Bar, 0.2 μm . (B) Edge of elastin bundle from bovine calf aorta. Amorphous elastin (EL) is bordered by microfibrils (MF). Lysyl oxidase antibody labeled with 10-nm gold particles (*arrows*) is present at the interface of elastin and microfibrils in an irregular distribution. Not all microfibrils are associated with lysyl oxidase. Collagen (COL) appearing in longitudinal and cross section displays no lysyl oxidase. Bar, 0.1 μm . (C) Edge of elastin bundle from bovine calf aorta also stained with palladium chloride. The outline of the amorphous elastin (EL) can be more readily appreciated in this section. Lysyl oxidase antibody labeled with gold (*arrowheads*) is present at the elastin-microfibril interface. Those microfibrils (MF) away from the elastin bundle do not bind the antibody. Note occasional gold deposits on the few microfibrils seen within the amorphous elastin. Bar, 0.1 μm .

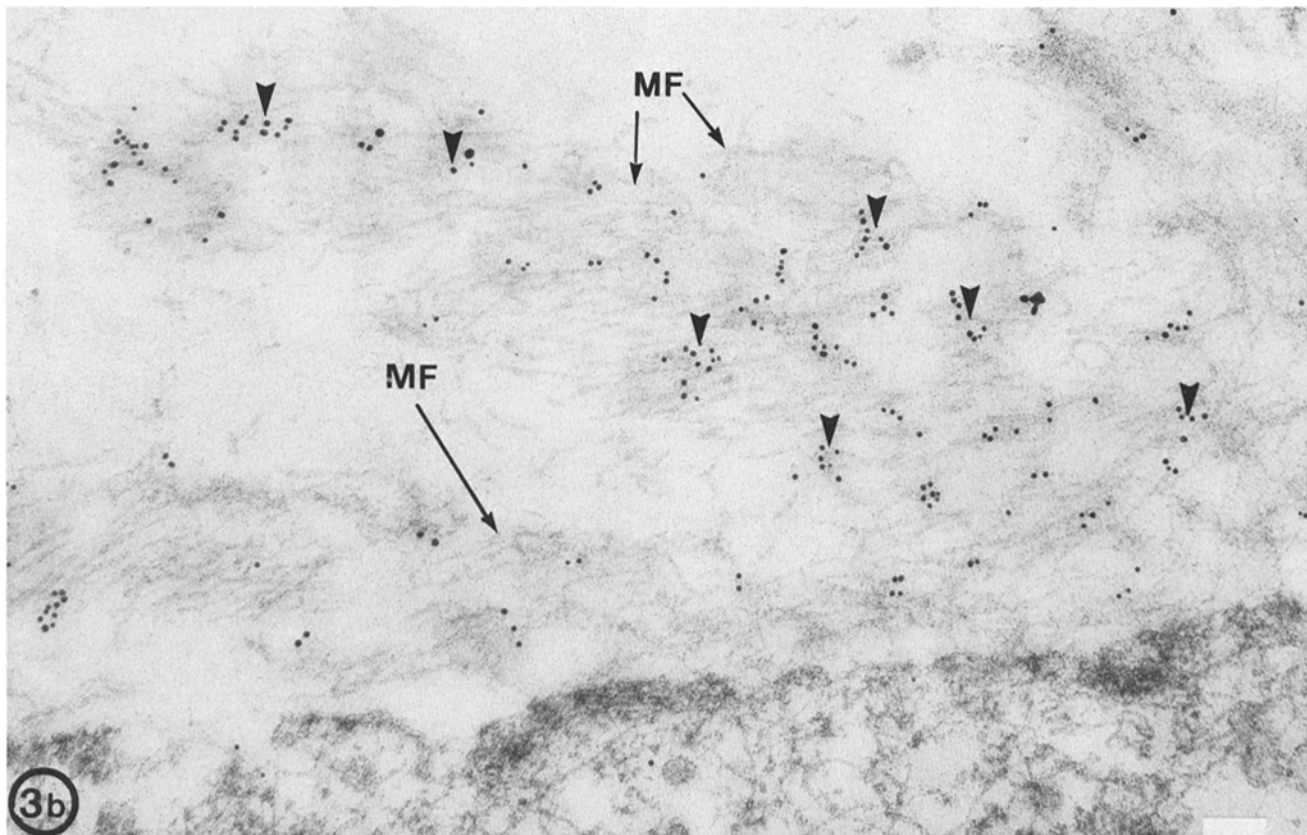
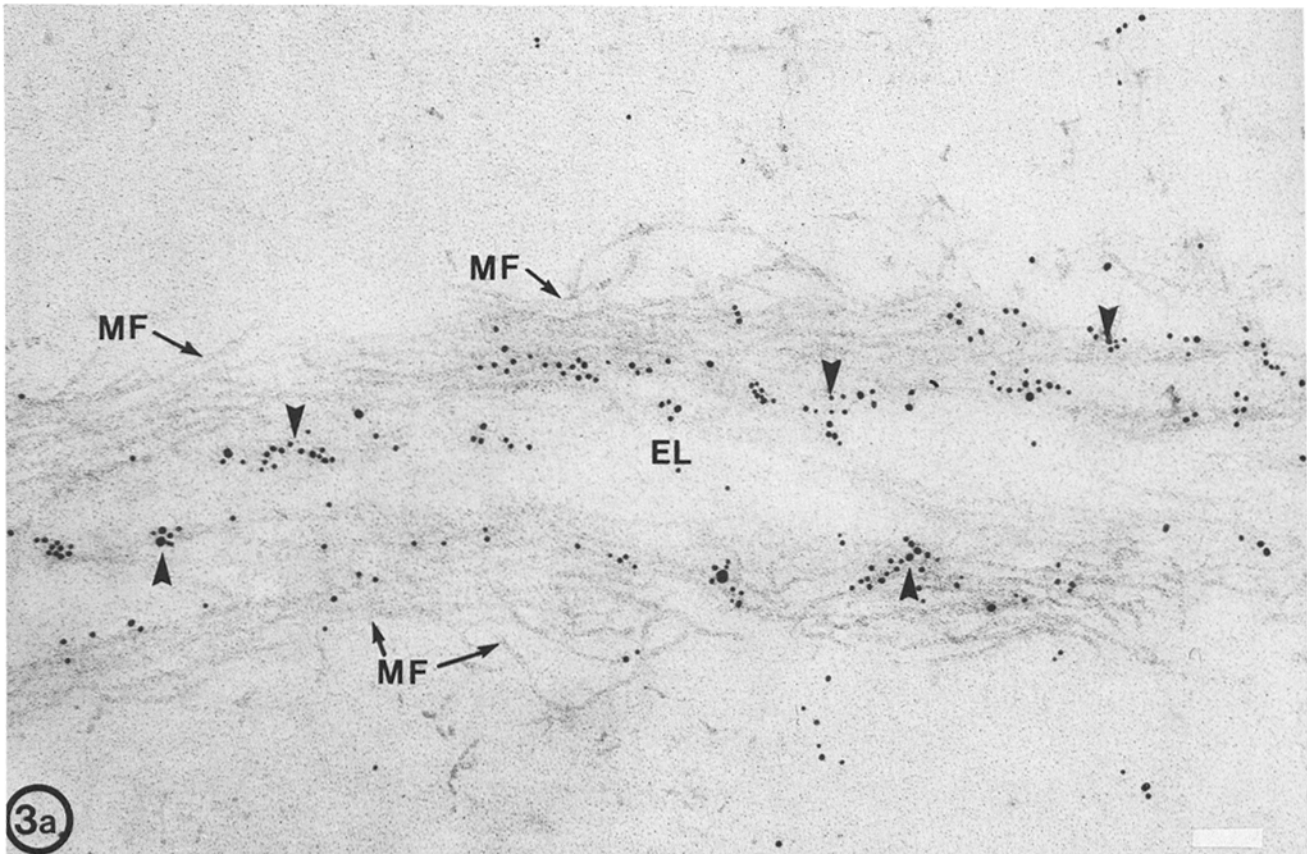
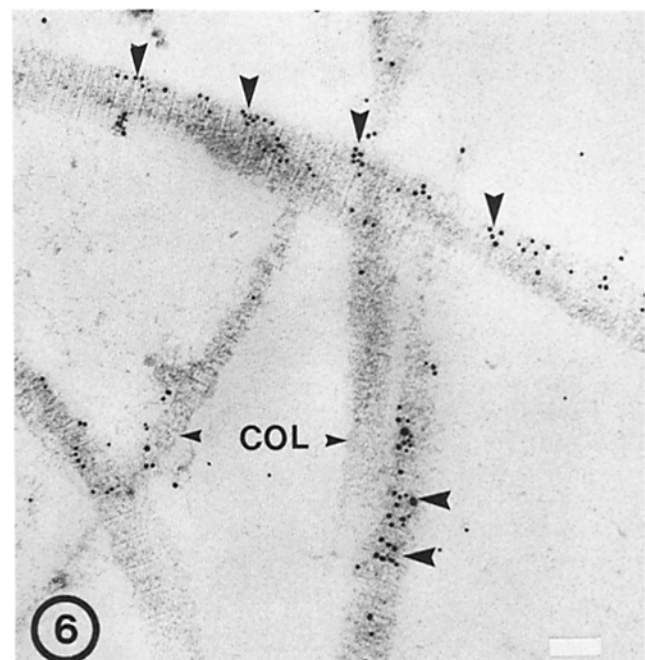
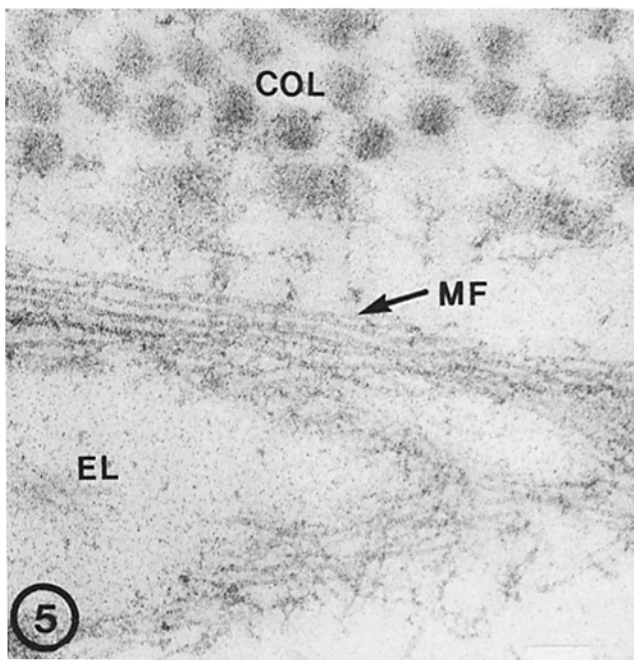
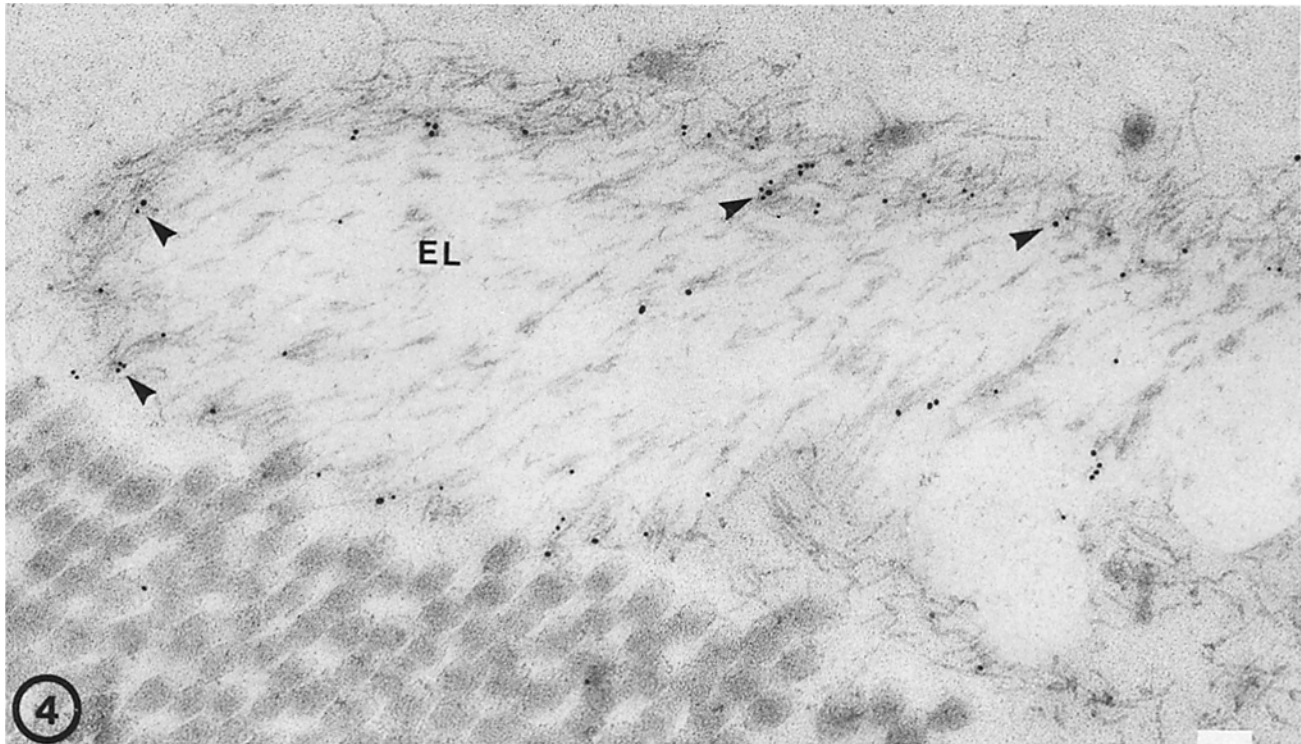


Figure 3. (a) Newborn rat aorta; developing bundles of elastin. Bundles of amorphous elastin (*EL*) surrounded by extensive array of microfibrils (*MF*), some of which appear to run through the bundles. Aggregates of 10-nm gold particles localizing lysyl oxidase (*arrowheads*) appear at the interface of elastin and microfibrils. (b) Bed of microfibrils (*MF*) with little amorphous elastin (*EL*). Aggregates of lysyl oxidase (*arrowheads*) appear in a pattern that may define sites of subsequent elastin deposition. Bars, 0.1 μm .



Figures 4-6 (Fig. 4) Adult rat aorta with amorphous elastin (*EL*) and microfibrils (*MF*). Gold particles (*arrowheads*) identify sparse localization of lysyl oxidase associated with adult fibers. (Fig. 5) Bovine calf aorta; incubated with excess purified exogenous lysyl oxidase before application of anti-lysyl oxidase. Lack of gold labeling signifies specific inhibition of antibody binding to elastin (*EL*), microfibrils (*MF*), and collagen (*COL*). (Fig. 6) Isolated pellet of native type I collagen (*COL*) complexed with exogenous lysyl oxidase. Note binding of antibody (*arrowheads*) to collagen fibers. Bars, 0.1 μ m.

morphogenesis of elastin fibers has been proposed that is consistent with the present observations. This model suggests that microfibrils are first formed in the extracellular space in the pericellular environment and then may serve as a scaffolding for elastin deposition (4, 5, 17-19). Noting the continuing association of microfibrils with the developing elastin fiber, Cleary et al. (5) had speculated that the

microfibrillar material may be associated with lysyl oxidase. The present findings substantiate that possibility. Indeed, the present observation that lysyl oxidase is associated with microfibrillar deposits before and after the appearance of amorphous elastin within the area circumscribed by the microfibrils is consistent with the hypothesis that this complex plays an essential role in elastin fiber biosynthesis. The

paucity of microfibrillar material and gold deposits in the interstices of the amorphous elastin fiber is consistent with a model predicting the radial growth of the elastin fiber by the deposition and crosslinking of newly accreting tropoelastin precursors of elastin at the fiber-microfibril interface. It seems possible that the microfibrillar material may actively participate in this process by providing a matrix for the alignment of enzyme, tropoelastin and the preexistent elastin fiber, thus facilitating radial fiber growth.

The association of lysyl oxidase with only some of the microfibrils suggests selectivity of the elastogenic process. Indeed, the linearity of the localization of lysyl oxidase on microfibrils where little amorphous elastin is visualized (Fig. 3 B) further suggests that this complex may act as a template for deposition of the elastic fiber. It is also evident that lysyl oxidase remains associated with the microfibrillar components of mature elastic bundles as revealed by the micrographs of the enzyme localization in the adult rat aorta. The persistence of enzyme in the adult tissue may signal that the synthesis and/or repair of elastin fibers continues as the animals age. It is important to note, however, that the immunological marker for lysyl oxidase does not differentiate between catalytically functional and nonfunctional enzyme.

Notably, gold deposits were not found in association with extracellular collagen fibers in tissue specimens, although the enzyme was seen in association with collagen when lysyl oxidase was added to collagen fibers in vitro. In contrast with the present results, collagen fibers in the extracellular matrices of fibrotic rat liver and chick tendon were labeled in a prior study that used immunofluorescently labeled antibody directed against lysyl oxidase purified from chick cartilage (22). The purification method used for the isolation of the chick cartilage enzyme involved chromatography of urea extracts of cartilage on DEAE cellulose and on collagen-Sepharose columns. In contrast, the isolation of bovine aorta lysyl oxidase used as antigen in the present study employed DEAE cellulose, Cibacron Blue Sepharose and gel exclusion chromatographic steps (27). It has been our experience that gel exclusion chromatography in 6 M urea is required to free the enzyme product of traces of fibronectin and other high molecular weight contaminants (27). We had previously observed this also to be necessary with enzyme purified by DEAE cellulose and collagen-Sepharose (11). Because specific information was not given about the specificity of the antibody raised against the chick cartilage enzyme (22), it appears possible that the immunolocalization seen in the earlier study (22) may in part reflect a reaction of the antibody with other collagen-bound macromolecules such as fibronectin. Moreover, although collagen- or elastin-specific forms of lysyl oxidase have not been identified by assays of isolated enzymes in vitro, it also remains possible that the discrepancy between the immunolocalization results of the present study with those of Siegel et al. (22) may reflect such enzyme specificity differences between the chick cartilage and bovine aortic enzymes in vivo.

It is unlikely that collagen-associated proteoglycans prevented access of the antibody to lysyl oxidase since preincubation of tissue specimens with chondroitinase ABC did not alter the distribution of the antibody binding sites. It is possible, however, that the extracellular collagen fibers seen in the tissue specimens are fully crosslinked products from which

bound lysyl oxidase had previously dissociated. Indeed, recent studies have noted that collagen-lysyl oxidase complexes formed in vitro do slowly dissociate (6). Further, although the purified enzyme used as antigen in the present study oxidizes both collagen and elastin substrates in vitro, the possibility has not been excluded that collagen-specific forms of lysyl oxidase may exist in vivo that are not recognized by the antibody directed against the bovine aortic enzyme.

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