Evidence for Structural Similarities in the Multiple Forms of Aortic and Cartilage Lysyl Oxidase and a Catalytically Quiescent Aortic Protein*

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Lysyl oxidase purified from urea extracts of various connective tissues resolves into multiple catalytically functional species upon chromatography on DEAE-cellulose in 6 M urea. The four enzyme species of bovine aorta retain their original chromatographic behavior on DEAE with time of storage and after purification to homogeneity by gel exclusion chromatography. The peptide maps of each aortic enzyme partially digested by *Staphylococcus aureus* V8 protease are very similar to each other, as are the peptide maps of complete trypsic digests of each enzyme. Such similarity also exists between the peptide maps of the aortic enzyme and the urea-extractable lysyl oxidase of bovine cartilage, as well as with the peptide maps of a catalytically quiescent protein resolved from the aortic enzyme by gel exclusion chromatography. The substrate activity profiles of the multiple aortic enzyme species are also extremely similar. Although the origin of the enzyme multiplicity remains to be established, there is evident structural and catalytic similarities between the enzyme forms.

Lysyl oxidase initiates covalent cross-linkage biosynthesis in collagen and elastin by oxidatively deaminating lysine residues within these proteins. This enzyme has been purified from urea extracts (1-5) or neutral detergent extracts (6) of a number of connective tissues and has been resolved into two (1, 2) or four (3-5) catalytically active enzyme forms upon ion exchange chromatography on DEAE-cellulose. Although there is evidence that each of the four urea-extractable chick cartilage enzyme species is active against collagen and elastin substrates (4), rigorous attempts have not been made to further characterize the nature of this enzyme multiplicity. The essential role played by lysyl oxidase in the maturation of collagen and elastin to their functional states warrants exploration of the nature and possible biological significance of the heterogeneity of this enzyme.

In the present investigation, comparisons have been made between structural features, shown by peptide-mapping procedures, and the substrate specificities of the individual forms of this enzyme purified from bovine aorta. Furthermore, the aortic enzyme species have been compared to the enzyme species of bovine cartilage and to an apparently related protein co-purifying with the aortic enzyme.

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MATERIALS AND METHODS

L-[4,5-3H]Lysine (60 Ci/mmol) was obtained from New England Nuclear. Horseradish peroxidase (195 units/mg), homovanillic acid, 1,5-diaminopentane, α-N-acetyl-t-lysine methyl ester HCl, dansyl chloride, and protein standards for gel electrophoresis were purchased from Sigma. DEAE-cellulose was obtained from Whatman. Sephacryl S-200 and Sepharose CL-4B were from Pharmacia Fine Chemical Co., Piscataway, NJ. Reagents for gel electrophoresis were products of Bio-Rad. L-1-Tosylamido-2-phenylchloromethyl ketone (TAME), 1.0 M NaCl, 0.1 M sodium phosphate, 0.016 M potassium phosphate, pH 7.7, and homovanillic acid (80 units/mg) and *Staphylococcus aureus* V8 protease (618 units/mg) were purchased from Worthington. All other chemicals were of the highest grade commercially available.

**Enzyme Purification**—Lysyl oxidase was purified from bovine aorta by the method previously described (3).

Bovine aortic cartilage was obtained fresh from the slaughterhouse, dissected free of adhering fascia, and powdered in liquid nitrogen to facilitate extraction. Lysyl oxidase activity was extracted with buffered urea solutions and partially purified by passage of the urea extract through a gelatin affinity column, applying these procedures as described for the purification of the aortic enzyme (3).

**Assay of Enzyme Activity**—The insoluble elastin substrate was prepared from chick embryo aorta which had been previously labeled in organ culture with L-[4,5-3H]lysine as previously described (7, 8). Collagen substrate for lysyl oxidase was prepared from the calvariae of 17-day-old chick embryos. The calvariae were pulsed with L-[4,5-3H]lysine in vitro and the labeled calvarial collagen subsequently purified according to the method of Siegel (9). The elastin substrate was treated with bacterial collagenase to remove contaminating collagen previously shown present (4, 10). Bacterial collagenase (Advanced Biofactures, Lynbrook, NY) used for this purpose was further purified before use by chromatography on Sephacryl S-200 in 50 mM Tris buffer, pH 7.6, 5 mM CaCl₂.

Lysyl oxidase activity was measured both with the collagen and elastin substrates by the tritium release method of Pinnell and Martin (7). Samples of enzyme were dialyzed at 4°C against 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.8, to remove urea and then incubated with 250,000-cpm aliquots of either substrate in 0.75 ml of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.8, buffer. The collagen substrate was preincubated for 60 min at 37°C to promote fibril formation before addition of the enzyme (9). One unit of enzyme activity is defined as that quantity releasing 1 cpm of distillable tritium as 3H2O/ml of assay mixture in 2 h at 37°C.

Enzyme activity against nonpeptidyl amine substrates was determined by the peroxidase-coupled fluorometric assay described by Trackman et al. (11), using an assay buffer of 50 mM sodium borate, pH 8.2, in 1.2 M urea at 55°C. All reported lysyl oxidase activity against elastin, collagen, or nonpeptidyl amines was fully inhibited by 0.2 mM β-aminopropionitrile present in the assay.

**DEAE-cellulose Chromatography**—Samples of lysyl oxidase to be chromatographed or rechromatographed on DEAE were dialyzed when necessary against 6 M urea, 0.016 M potassium phosphate, pH 7.7, and applied to a DEAE-cellulose column (1 X 11 cm) which had been previously equilibrated in this buffer. Elution was achieved with a 360-ml linear salt gradient between 0 and 0.4 M NaCl in 6 M urea, 0.016 M potassium phosphate, pH 7.7. Fractions (2 ml) were analyzed for enzyme activity with the insoluble elastin substrate and the gradient measured with a YSI Model 31 conductivity bridge.

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electro-
Comparison of Lysyl Oxidase Species

RESULTS

DEAE-cellulose Chromatography

The activity profile characteristic of bovine aortic lysyl oxidase eluted from DEAE-cellulose is shown in Fig. 1 (bottom). Four distinct peaks of enzyme activity are evident with a shallow salt gradient in 6 M urea, with peak maxima appearing at conductivities equivalent to 0.02, 0.05, 0.08, and 0.11 M NaCl, respectively. As previously reported (3), subsequent chromatography of each of these enzymes on Sephacryl S-200 eliminates contaminating proteins. Polyacrylamide gel electrophoresis in SDS of enzyme peaks purified by this additional step demonstrates that each appears homogeneous and each has a molecular weight of 32,000 ± 800 under reducing conditions (Fig. 2). Since both the DEAE and Sephacryl S-200 chromatographic steps are performed in 6 M urea, protein-protein interactions are unlikely to account for the resolution of the multiple species of enzyme. Nevertheless, this possibility was tested, as was the possibility of time-dependent equilibration between the resolved species in vitro. Peak II of the DEAE-profile of Fig. 1 (bottom) was pooled dialyzed, and rechromatographed on DEAE-cellulose. As shown (Fig. 3A), this peak elutes again at the original gradient position equivalent to 0.05 M NaCl. As noted, a contaminant of Peak I is resolved by this treatment. The Peak II enzyme elutes from DEAE at

FIG. 1. DEAE-cellulose chromatography of bovine aortic (bottom) and bovine cartilage (top) lysyl oxidase. ---, \( A_{\text{max}} \) enzyme activity; . . . , conductivity.

FIG. 2. SDS-polyacrylamide gel electrophoresis of purified aortic lysyl oxidase Peaks I-IV. Positions of standard proteins and their molecular weights are identified. 68K, for example, represents \( M_r = 68,000 \).
Comparison of Lysyl Oxidase Species

Peptide Maps of Partial Proteolytic Digests

Peptide mapping procedures were used to approximate the degree of structural similarities or differences which might exist among the four aortic enzyme forms. Lysyl oxidase (Peak III, 12 μg) was digested with varying amounts of *S. aureus* V8 protease for 30 min, and the resulting peptides were resolved on an 18% SDS-polyacrylamide gel as described under "Materials and Methods." Densitometric scans of the gels (Fig. 4) reveal that the enzyme is digested by this protease and that 2-4 μg of protease produce an array of peptides appropriate for comparative peptide maps. Linear peptide maps were prepared from the four purified enzyme species partially digested with 2.5 μg of *S. aureus* V8 protease (Fig. 5). The profiles are clearly very similar. Furthermore, the correspondingly sized peptides of the different digests are produced in similar ratios from the four enzyme forms (Table I). Peptide map profiles of V8 protease digests of aldolase (Mr = 45,000) and carbonic anhydrase (Mr = 29,000) were considerably different from those of the lysyl oxidase species and from each other (data not shown), confirming the expected uniqueness of peptide map profiles of digests of structurally different proteins.

Peptide Maps of Complete Proteolytic Digests

To further test the possibility that there may be structural differences between the four species, each was digested to

**NH₂ Termini**

NH₂-terminal amino acid analyses were performed on each of the four purified enzyme peaks after reduction and alkylation. α-Dansyl-aspartic acid was the only α-dansyl-amino acid detected in each case. ε-N-Dansyl-lysine and O-dansyl-tyrosine were also present in the acid hydrolysates of each peak. Thus, each peak terminates with NH₂-terminal aspartic acid or its amide. These results also support the conclusion that each preparation consists of a single polypeptide chain.

**FIG. 3.** Recchromatography of aortic lysyl oxidase Peak II on DEAE-cellulose. A, recchromatography of Peak II taken from the experiment of Fig. 1 (bottom). B, recchromatography of Peak II after its further purification on Sephacryl S-200. C, recchromatography of fractions 19-28 of chromatographic profile shown in B of this figure. ——, A₄₅₀; △—△, enzyme activity; ——, conductivity.

this position even after its further purification on Sephacryl S-200 (Fig. 3B). The enzyme activity fractions of the experiment shown in Fig. 3B were pooled and chromatographed again on DEAE and again the enzyme eluted at the same position in the gradient (Fig. 3C). The enzyme had been in buffered 6 M urea for 15 days between the first and last chromatography on DEAE-cellulose. Thus, these results indicate that the enzyme forms do not equilibrate with each other in vitro significantly. The constancy of elution behavior on DEAE before and after purification on Sephacryl S-200 indicates that protein-protein interactions are unlikely bases of the separation originally achieved on DEAE-cellulose. The same results were obtained with Peaks I and III (data not shown). Peak IV was not tested in this fashion due to the limited quantity of this enzyme form available.

Treatment of unresolved mixtures of the four enzyme species or of the individually purified enzyme species with alkaline phosphatase or *Clostridium perfringens* neuraminidase prior to chromatography on DEAE-cellulose did not change the elution characteristics of the individual enzymes, nor did preincubation of the species with 10 mM 2-mercaptoethanol at 4 °C for 4 h (data not shown). These results argue against the possibility that the heterogeneity stems from varying degrees of covalent modification of a parent species by sialic acid or by inorganic phosphate and more tentatively argue against the concept that the heterogeneity reflects the presence of disulfide isomers of the proteins.

**FIG. 4.** Linear peptide maps of partial digests of Peak III aortic lysyl oxidase using different amounts of *S. aureus* V8 protease (SV8). The migration position of the protease is indicated (SV8). There was no evidence for autodigestion products of the protease. Intact lysyl oxidase migrates just to the left of the intact protease.
comparison with trypsin and the peptides produced were resolved by the two-dimensional thin layer peptide-mapping procedure described. A composite sketch traced from such peptide maps is shown in Fig. 6. The amino acid compositions of the enzyme species (3) predict that 25-28 tryptic peptides are expected/mol of subunit for each of the four enzyme peaks. The numbers of peptides found were in good agreement with theory, i.e. 25, 26, 26, and 24 for Peaks I, II, III, and IV, respectively. Although there are a few peptides which are uniquely or partially specific for the different enzyme forms, 21 of the peptides produced are common to all four digests, while there are 26 peptides common to Peaks II and III.

Therefore, both partial and complete peptide mapping procedures reveal that considerable similarity exists between the enzyme forms. However, these results do point toward limited differences among the enzyme forms.

Substrate Specificity

The possibility that the different enzyme species may have different substrate specificities was tested using purified, tritium-labeled collagen and elastin substrates, as well as nonpeptidyl amine substrates, previously shown to be oxidized by lysyl oxidase (11). As shown in Table II, each of the isolated species oxidizes each of the substrates tested. The profiles of activity toward this series of substrates are quite similar for each of the enzyme species. Thus, the enzymes appear to be quite similar catalytically as well as structurally.

Comparison of Aortic Lysyl Oxidase with Related Enzymes and Proteins

Cartilage Lysyl Oxidase—Urea-soluble bovine knee cartilage lysyl oxidase was extracted and partially purified as described under "Materials and Methods." The DEAE-cellulose elution profile of the cartilage enzyme (Fig. 1, top) con-

![Fig. 5. Peptide maps of V8 protease digest of aortic lysyl oxidase Peaks I-IV.](image)

**TABLE I**

Distribution of peptides generated by S. aureus V8 protease

The data are presented as the per cent total Coomassie blue-stained protein as determined from densitometry of the scans in Fig. 5.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>M,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16,900</td>
<td>40</td>
<td>36</td>
<td>28</td>
<td>41</td>
</tr>
<tr>
<td>15,250</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>11,580</td>
<td>17</td>
<td>15</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>11,040</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>10,000</td>
<td>28</td>
<td>36</td>
<td>40</td>
<td>32</td>
</tr>
</tbody>
</table>

![Fig. 6. Two-dimensional peptide map (composite) of complete tryptic digest of aortic lysyl oxidase Peaks I-IV. Solid black areas represent peptides common to all four peaks. Cross-hatched areas are found only in those peaks specifically designated.](image)

**TABLE II**

Substrate specificity of the isolated enzyme peaks

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme activities*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme peak 1:1:1 mixture of 4 peaks</td>
</tr>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Collagen</td>
<td>616±42</td>
</tr>
<tr>
<td>Elastin</td>
<td>1211±73</td>
</tr>
<tr>
<td>Ratio: (collagen/elastin)</td>
<td>2</td>
</tr>
<tr>
<td>1,5-Diaminopentane (D)</td>
<td>0.69±0.02</td>
</tr>
<tr>
<td>n-Butylamine (B)</td>
<td>0.17±0.04</td>
</tr>
<tr>
<td>α-N-Acetyl-l-lysine-O-methyl ester (L)</td>
<td>0.27±0.01</td>
</tr>
<tr>
<td>Ratio: (D/L)</td>
<td>2.6</td>
</tr>
<tr>
<td>(D/B)</td>
<td>4.1</td>
</tr>
<tr>
<td>(L/B)</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*Activities are presented as initial rates of tritium release from the elastin or collagen substrate (counts/min^3H released/2 h/µg) or of H_2O_2 formation from the amine substrates (nanomoles of H_2O_2/min/µg).
tains four prominent peaks of enzyme activity, eluting at conductivities approximating those of Peaks I, II, III, and IV of the bovine aortic enzyme (Fig. 1, bottom). Peak II of the cartilage enzyme was pooled and analyzed by SDS-gel electrophoresis. This enzyme preparation contained a major protein band with a molecular weight of 32,000 ± 800, coinciding with the position of highly purified bovine aortic Peak II enzyme (Fig. 7). The bovine aortic and cartilage enzymes were compared by the linear peptide mapping procedure. In view of the limited amount of cartilage enzyme available, the multiple forms of this enzyme were co-purified by passage of the initial urea extract through gelatin-Sepharose as described, followed by SDS-gel electrophoresis of the 6 M urea effluent (the enzymatically active fraction) of this affinity column. The prominent band at $M_w = 32,000$, containing the unresolved cartilage enzyme species seen in the DEAE-cellulose elution profile, was excised from the gel, and a linear peptide map was prepared from the V8 protease digest of this isolated protein band. The purified, unresolved mixture of the aortic enzyme species was prepared in identical fashion, and peptide maps of lysyl oxidase of these two enzyme preparations were compared (Fig. 8). As shown, there is considerable similarity between the profiles of the peptides produced from the two enzyme sources.

**Peak B Protein**—As noted, the aortic enzyme species may be purified to apparent homogeneity by passage of the peaks resolved on DEAE through Sephacryl S-200 in 6 M urea (3). An elution profile of the aortic DEAE-Peak III enzyme chromatographed through Sephacryl S-200 is shown in Fig. 9 (top). The appearance of three prominent protein peaks, designated A, B, and C, respectively, is characteristic of the corresponding profiles for each of the four aortic enzyme species similarly chromatographed on this column medium, as reported (3). Peak B, corresponding by its elution position to a molecular weight of approximately 60,000, was pooled, concentrated, and passed again through this column in buffered 6 M urea. The protein eluted at its original elution position (Fig. 9, bottom). This protein preparation did not catalyze the release of tritium from the tritiated elastin substrate nor did it alter the expression of enzyme activity of the C peak (active Peak III lysyl oxidase) when these two proteins were assayed together against the elastin substrate. Thus, Peak B does not appear to be an active form of lysyl oxidase nor to modify the catalytic expression of the active enzyme. Polyacrylamide gel electrophoresis in SDS of this protein (Peak B) revealed a prominent band at $M_w = 32,000$, coincident with the position of the active enzyme (Fig. 9, inset, lane C).2 Lanes A and B, also shown in

2 There is an additional less prominent band at $M_w = 24,000$ in the gels of the “B” peaks and, less so, in the sample of the Peak III enzyme. This band develops from the intact purified enzyme with storage at 4 °C in 6 M urea for extended periods of time. Peptide map analyses of the $M_w = 24,000$ band associated with the B peak or with the active enzyme peak are very similar to those of newly purified lysyl oxidase. These properties are consistent with the possibility that the $M_w = 24,000$ band may be a proteolytically derived fragment of the enzyme. Storage in 1 mM phenylmethylsulfonyl fluoride does not
the inset of Fig. 9, represent corresponding analyses of the B Peaks isolated from Sephacryl chromatography of DEAE-Peak I and of DEAE-Peak II, respectively, showing that each enzyme fraction resolved on DEAE is accompanied by this protein band as well. The protein band in the acrylamide gel corresponding to $M_r = 32,000$ was eluted and compared to the active Peak III aortic lysyl oxidase by peptide maps of limited V8 protease digests of each protein (Fig. 8). As shown, this protein also is remarkably similar to the enzyme by this criterion.

**DISCUSSION**

The present analysis of the multiple forms of bovine aortic lysyl oxidase reveals that these enzymes are remarkably similar to each other, both structurally and catalytically. The structural homology among the enzyme species as evidenced by peptide map analyses suggests that the enzyme forms may be post-translational derivatives of a common molecule. However, the alternative possibility of separate genomic origin of each species cannot be eliminated at present. Furthermore, the similarities in the substrate activity profiles observed in this study argue against but do not completely eliminate the possibility that the heterogeneity of lysyl oxidase may yet prove to have functional consequences. Since the enzyme is isolated under dissociating conditions in 4-6 M urea, the normal subunit-subunit contacts are likely disrupted and may not appropriately reform under assay conditions with possible effects on enzyme specificity. Furthermore, there may be subtle differences in specificities of the different enzyme forms for different lysine residues in collagen and in elastin yet to be detected.

The chemical basis of the separation on DEAE-cellulose is apparently of an ionic nature. Nonionic adsorptive interactions with the DEAE-cellulose matrix are presumably minimal or absent since the chromatography is performed in 6 M urea. Furthermore, the separation does not appear to reflect protein-protein interaction. As indicated, it does not seem that such charge differences between the species reflect covalently linked sialic acid or inorganic phosphate. Since the enzyme is extracted, stored, and purified in 6 M urea, it is possible that amino acid side chain charge in the enzyme species may be differentially carbamoylated due to cyanate derived from urea. However, if such a charge-altering modification were the basis of the heterogeneity, it would be expected that all of the forms would eventually be converted to one derivative due to continued carbamoylation with time. This does not occur, however. Furthermore, amino acid analyses of acid hydrolysates of each of the purified forms do not contain detectable levels of homocitrulline, the carbamoylated derivative of endopeptidyllysine (3). This also argues against such an artifactual source of the enzyme heterogeneity.

Although the lack of effect of neuraminidase treatment provides evidence against the presence of sialic acid, other carbohydrate moieties may be components of lysyl oxidase and contribute to the heterogeneity of enzyme forms. Tanzer and Housley (18) have recently demonstrated the association of uronic acid with a form of lysyl oxidase solubilized from bovine cartilage with neutral detergent. However, the identity of the neutral detergent form with the urea-extractable enzyme is not at all clear, although the present study reveals the marked structural similarity of the urea-extractable bovine aortic and cartilage enzymes. The aortic urea-extractable forms do not bind to concanavalin A-Sepharose (3) and, therefore, are assumed not to contain appropriately accessible mannopyranosyl or glucopyranosyl residues, consistent with the affinity of this lectin. However, recent studies in our laboratories utilizing liquid chromatographic analysis of enzyme hydrolysates do suggest the presence of as yet unidentified complex carbohydrate units in each of the aortic enzyme species. Thus, the heterogeneity may prove to reflect differential glycosidic derivatization of the enzyme. Certainly, other possibilities, including limited differences in primary sequence, variation in amide content, as well as the possibility that the forms may represent proteolytic derivatives of a common precursor, remain to be fully assessed.

Peptide maps have been used to advantage in the present study to identify a catalytically quiescent protein whose peptide profile is very similar to that of lysyl oxidase and which elutes from Sephacryl S-200 just prior to the active lysyl oxidase species. While lysyl oxidase and this protein resolve on the gel exclusion medium in 6 M urea, they have the same migration on SDS-polyacrylamide gels under reducing and denaturing conditions. These proteins may, therefore, be conformational, disulfide-linked, or aggregate-induced isomers of each other. Catalytic activity is not generated in this protein by the addition of cupric ion, a cofactor of lysyl oxidase (19, 20, nor by pyridoxalphosphate, a second putative enzyme cofactor (21). Furthermore, brief treatment of the protein with trypsin does not convert it to the corresponding functional lysyl oxidase species. Thus, the source of this protein is not clear, although it appears to be structurally related to lysyl oxidase. As proposed for the protein which is immunologically cross-reactive to prolyl hydroxylase (22), the tenable possibilities include the hypothesis that this protein may be or derive from a precursor to the enzyme or that it reflects an inactivated derivative of the catalytically active enzyme. It is of interest that this protein or its closely related species occurs in the identical elution position of the Sephacryl S-200 profiles of each of the DEAE-peaks of enzyme activity. The origin and significance of this group of proteins are subjects of continued investigation.

**REFERENCES**


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Comparison of Lysyl Oxidase Species