Biosynthesis of Collagen Crosslinks: Increased Activity of Purified Lysyl Oxidase with Reconstituted Collagen Fibrils

(collagen crosslinking)

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ABSTRACT  Lysyl oxidase catalyzes the formation of crosslinking aldehydes in collagen and elastin. This report demonstrates that the enzyme has high activity with collagen precipitated as native fibrils, an apparent \( K_a \) of 0.95 \( \mu \)M, and low activity toward either soluble forms such as denatured collagen, isolated \( \alpha \) chain, or isolated \( \alpha_1\)-CB1 peptide, or precipitated collagen fibrils after pepsin treatment. These results indicate that the biosynthesis of the aldehyde crosslink intermediate probably occurs primarily after the onset of fibril formation in vivo. Biosynthesis of aldehydes and subsequent crosslinks may be related to the rate of fibril formation as well as to the concentration of lysyl oxidase in vivo.

Many of the properties of connective tissue and bone, such as high tensile strength and ability to support adjacent organs, are acquired as a result of the intermolecular crosslinking of collagen and elastin (1, 2). The initial reaction in the crosslinking of both collagen and elastin is the formation of the \( \epsilon \) aldehydes, allysine, and hydroxyallysine, from certain lysyl and hydroxylysyl residues (Fig. 1) (3, 4). Aldehyde formation is catalyzed by the enzyme lysyl oxidase (5, 6). Subsequent aldol condensation or Schiff base crosslinking reactions apparently proceed without additional enzymatic reactions (7, 8).

Lysyl oxidase was initially detected in extracts of embryonic chick epiphysal cartilage with elastin substrates prepared from 16-day chick embryo aortas (5) labeled with \( \left[ \delta^{3H} \right] \)lysine in organ culture in the presence of the lathyrone \( \beta \)-aminopropionitrile (BAPN) to inhibit aldehyde formation. The insoluble residue of the labeled aortas after homogenizing was then used as a substrate. Formation of aldehydes at the 6 position of lysyl- or hydroxylysyl residues of the \( \left[ \delta^{3H} \right] \)lysine-labeled substrate resulted in release of tritium from that position and formation of tritium water. The tritium water was isolated by distillation of individual samples. The radioactivity observed occurred with formation of lysyl- or hydroxylysyl-derived aldehydes measured by amino-acid analysis (5). When equivalent amounts of lysyl oxidase were assayed with \( \left[ \delta^{3H} \right] \)lysine elastin and collagen substrates, much higher activity was measured with the elastin substrate (6). However, formation of allysine and crosslinked components did occur after incubation of collagen with preparations of lysyl oxidase (6, 9).

The crosslinking of collagen in vitro involves very specific lysyl and hydroxylysyl residues (10, 11) and the adjacent amino-acid sequences are quite dissimilar to those reported in elastin adjacent to crosslinks (12). Since lysyl oxidase has no activity with free lysine (5) or low-molecular-weight amines (5, 13), higher enzymatic activity with elastin with enzyme prepared from cartilage, a tissue rich in collagen, seem paradoxical. This paper reports further study of the activity of purified lysyl oxidase with collagen substrates. The physical state of the substrate is a principal factor determining enzyme activity. Lysyl oxidase has high activity for collagen precipitated from solution as fibrils. This observation has made it possible to develop an assay with collagen substrates that is more sensitive than the conventional elastin assay and to define the point in the biosynthesis of the mature collagen fibril at which synthesis of crosslinking aldehydes occurs.

MATERIALS AND METHODS

Preparation of Substrates. Elastin substrates were prepared from chick embryo labeled with \( \left[ L-\delta^{3H} \right] \)lysine in organ culture as previously described (5, 14).

For preparation of collagen substrates, 18 pairs of chick calvaria piaeral bones from 17-day-old chick embryos were incubated at \( 37^\circ \) in 50-ml Erlmeneyer flasks with 10 ml of Eagle’s minimal essential medium without lysine and supplemented with 4 mg of proline, 5 mg of glycine, 5 mg of BAPN, 5 mg of ascorbic acid, and 20,000 U of penicillin G per 100 ml of medium (6). After preincubation for 30 min to reduce the free lysine pool, the medium was changed, 200 \( \mu \)Ci of \( \left[ L-\delta^{3H} \right] \)lysine was added to each flask, and the flasks were gassed with 1 min with a 5% \( \mathrm{CO}_2 \)-air mixture. After incubation for 24 hr, the calvaria from four flasks were pooled and homogenized in 40 ml of 1 M NaCl, 0.05 M Tris-HCl, pH 7.4, at \( 4^\circ \) for 1 hr in a ground-glass homogenizer and then centrifuged at \( 20,000 \times g \) for 10 min. The collagen in the supernatant was precipitated by the addition of solid NaCl to 20% concentration. The precipitate was collected by centrifugation, resus-

Fig. 1. Formation of crosslinking aldehydes in collagen by lysyl oxidase.

Abbreviations: allysine, \( \alpha \)-aminoadipic-\( \delta \)-semialdehyde; hydroxyallysine, \( \alpha \)-aminoadipic-\( \delta \)-hydroxy, \( \delta \)-semialdehyde; BAPN, \( \beta \)-aminopropionitrile; PBS, solution of 0.1 M NaH_2PO_4, 0.15 M NaCl, pH 7.8.
Enzymatic Collagen Crosslinking in Fibrils

**Results**

Previous studies of lysyl oxidase have been limited by lack of an efficient purification method (5, 6, 9). When impure enzyme fractions were added to labeled collagen substrate and incubated for 3 hr, approximately 50 cpm of net tritium release was observed compared to more than 4000 for elastin substrates (Table 1).

This difference in enzyme activity for the two substrates could not be attributed solely to the higher specific activity of...
the elastin substrate labeled with (4, 5) [H]lysine, since the specific activity of the lysyl residues in the elastin substrate was only four times greater than the specific activity of the hydroxylysyl and lysyl residues in the collagen substrate. Previously, higher activity with elastin substrates was thought to reflect the greater number of lysyl residues in elastin that formed crosslinks (5). However, when I tested highly purified preparations with collagen substrates, I observed a marked increase in enzyme activity (Table 1). This activity with collagen substrates was inhibited by mixing small amounts of the initial enzyme extract with the purified enzyme, although this caused little reduction in activity with the elastin substrate. An explanation for the difference between substrates was suggested by a lag period of approximately 1 hr before aldehyde formation began in incubations with pure enzyme (Fig. 2). In contrast, the reaction begins immediately with elastin substrates (5, 14). A lag of similar duration is observed during the heat-induced precipitation of collagen from solution as fibrils (21). Electron microscopy of the collagen substrate after 1 hr of incubation confirmed that native collagen fibrils were present then, but not prior to incubation. Observations of the solution during 1 hr of incubation both visually and by Klett colorimetry showed an increasing opacity of the solution that began at 30–30 min of incubation and plateaued by 40–50 min, as has been previously reported to occur during fibril precipitation (21). The amount of collagen that precipitated was studied by measuring the radioactivity in the supernatant and precipitate after incubation at 37° for 1 hr and comparing it to initial radioactivity in the supernate. These results (not shown) indicated that 85–70% of the radioactivity was precipitated from solution during incubation. However, when crude enzyme was incubated with the collagen substrate for 3 hr and then centrifuged at 30,000 x g for 30 min at 37°, all of the radioactivity remained in the supernatant. Amino-acid analysis of the precipitate after precipitation confirmed that it was essentially pure collagen with one-third glycyl residues and 106 hydroxyprolyl residues per 1000 amino acids (16). When the precipitate was separated from the supernatant after initial heat precipitation, more than 98% of the observed enzyme activity was for precipitated collagen fibrils. In subsequent experiments, the substrate was precipitated at 37° for 60 min to allow fibril formation to occur before addition of enzyme fractions. When the substrate was precipitated for 1 hr, enzyme activity observed with the initial extract and partially purified preparations was higher than the activity with pure enzyme without precipitation (Table 1, third column). Furthermore, inhibition no longer occurred when impure and pure enzymes were mixed. Duplicate samples gave values within 2%. To determine the nature of the enzyme kinetics with the fibrillar substrates, various amounts of substrate were pre-incubated for 1 hr and then incubated for 1 hr with 5 μg of purified enzyme. A Lineweaver–Burk plot of the data obtained (Fig. 3) shows a typical linear relationship with apparent K_m of 0.95 μM calculated on the basis of a molecular weight for monomeric collagen of 285,000 (1). To test whether the collagen and elastin assays were measuring the same or different enzyme molecules, fractions collected from the DEAE-cellulose column were assayed with both substrates (Fig. 4). No difference in the elution of enzyme activity was observed. These results suggest that the same enzyme is active on both substrates. To obtain collagen in fibrillar and soluble form at comparable temperatures, 0.05 M arginine was used to inhibit fibril formation (21). When arginine was added to the assay tube before fibril formation, collagen fibril formation was prevented.
and little enzyme activity was observed (Table 2). However, 0.05 M arginine did not affect activity if it was added to the reaction mixture after fibril formation. Arginine also had no effect on activity when assayed with elastin substrates (not shown).

To study whether factors other than the physical state of the collagen molecule were important for enzyme activity, activity for various substrates derived from [6-3H]lysine collagen was studied. As might be expected, significant enzyme activity was only observed with precipitated collagen fibrils. Soluble substrates such as denatured collagen or isolated \( \alpha \) chains had low activity. Purified isolated \( \alpha 1 \)-CB1, the amino-terminal cyanogen bromide peptide from the collagen \( \alpha 1 \) chain, with a single lysyl residue previously shown to be converted to allysine in vivo when part of the collagen molecule (6, 10), was also inactive as a soluble peptide. After incubation with lysyl oxidase, the peptide was recovered, rechromatographed on phosphocellulose (18), and analyzed by amino-acid analysis (18). Both techniques indicated that the peptide had not been altered or degraded during incubation. These experiments confirmed that soluble proteins were inactive substrates. Pepsin-treated collagen was then used as a substrate to test whether any insoluble lysyl residue might be an active substrate. In initial experiments with pepsin-treated collagen in PBS, only 5–10% of the radioactivity precipitated after 60-min of incubation. However, 65% of the radioactivity precipitated when the experiment was repeated in 0.3 M sodium phosphate (22). Lysyl oxidase activity was not particularly affected by this difference in ionic strength as measured with the elastin substrate; however, neither crude nor highly purified enzyme was active with the precipitated pepsin substrate in either PBS or 0.3 M sodium phosphate. When the normal collagen substrate was assayed in the presence of 130 \( \mu \)g/ml of pepsin-treated collagen, some inhibition of enzyme activity was observed (Table 3). This inhibition was linear with increasing pepsin treated collagen concentration and had an apparent inhibition constant of approximately 0.90 \( \mu \)M. This probably indicates that the enzyme binds to normal and pepsin-treated collagen to a similar degree. Finally, to determine whether small peptides might become substrates by either interaction with collagen fibrils in solution or by absorption to fibrils, isolated [6-3H]lysine \( \alpha 1 \)-CB1 (final concentration 10 \( \mu \)g/ml) was added to unlabelled pepsin-treated collagen in 0.3 M sodium phosphate and incubated with purified lysyl oxidase. \( \alpha 1 \)-CB1 was chosen as the soluble peptide in this experiment, since it forms the amino-terminal portion of the collagen molecule before cyanogen bromide cleavage (18), is a substrate for lysyl oxidase when part of the native collagen molecule, (6) and is known to interact with other collagen molecules during fibril formation (23). However, there was no enzyme activity and no indication that the peptide had been altered during incubation with purified lysyl oxidase.

**TABLE 3.** Substrate specificity of lysyl oxidase

<table>
<thead>
<tr>
<th>Substrate labeled with [6-3H]lysine</th>
<th>Net (^{3}H) release (cpm/200,000 substrate cpm)( \dagger )</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native collagen fibrils</td>
<td>1470</td>
<td>100</td>
</tr>
<tr>
<td>Denatured collagen( \dagger )</td>
<td>131</td>
<td>8.9</td>
</tr>
<tr>
<td>Isolated ( \alpha ) chains</td>
<td>68.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Isolated ( \alpha 2 ) chains</td>
<td>76.3</td>
<td>5.2</td>
</tr>
<tr>
<td>Isolated ( \alpha 1)-CB1( \dagger )</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pepsin-treated collagen( \dagger )</td>
<td>6.3</td>
<td>0.4</td>
</tr>
<tr>
<td>( \alpha 1)-CB1 with unlabeled pepsin-treated collagen( \dagger )</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Native collagen fibrils with unlabeled pepsin-treated collagen( \dagger )</td>
<td>944</td>
<td>64.3</td>
</tr>
</tbody>
</table>

\( \dagger \) Incubations were for 3 hrs at 37\( ^\circ \). Substrates were dissolved in PBS buffer. Total volume of the incubation mixture in each assay tube was 1.5 ml. One-milliliter aliquots were assayed. Lysyl oxidase was added at 3 \( \mu \)g per tube.

\( \dagger \) \(^{3}H\) release without added enzyme varied from approximately 150–200 cpm/500,000 cpm native or denatured collagen to 40–70 cpm with isolated \( \alpha \) chains, pepsin-treated collagen, or \( \alpha 1\)-CB1.

\( \dagger \) Denatured by heating to 60\( ^\circ \) for 15 min.

\( \dagger \) Two hundred micrograms of unlabeled pepsin-treated collagen added per incubation tube.

**Fig. 4.** DEAE-cellulose chromatography of lysyl oxidase previously partially purified by absorption to Sepharose 4-B resin that had had lathyritic rat skin collagen coupled to it (affinity resin). The enzyme was applied to a 1.3 × 10-cm DEAE column at 25° in 6 M urea, 0.05 M Tris-HCl, \( \phi H \) 7.6. Elution was with a linear 0–1.0 M NaCl gradient in a total volume of 200 ml in a solution of 6 M urea, 0.05 M Tris, \( \phi H \) 7.6. The absorbance of the effluent was continuously monitored at 280 nm; flow rate was 4.5 ml/min with 9-mL fractions collected. Fractions were dialyzed against 0.15 M NaCl, 0.1 M NaHPO\(_4\), \( \phi H \) 7.8, overnight and 1-mL aliquots were assayed with 500,000 cpm of either [6-3H]-lysyl collagen (●) or [4,5-\(^{3}H\)]lysyl elastin (□) substrates. Incubations were for 3 hr at 37\( ^\circ \).
with elastin substrates. However, activity is largely dependent on the physical state of the substrate molecule. There is little activity with proteins or peptides that remain soluble during assay, such as denatured collagen, native collagen in the presence of arginine, isolated \( \alpha \) chains, or the amino-terminal collagen peptide, \( \alpha_1 \)-CB1. However, there is high activity with precipitated collagen fibrils, elastin, or a coacervate of soluble elastin (24). Although the substrate specificity cannot be completely defined by this study, the experiment with pepsin-treated collagen fibrils indicates that enzyme activity is not high for all lysyl residues in the solid state. Specific steric relationships are probably required for activity, since activity remained low even after addition of purified \( \alpha_1 \)-CB1 to the pepsin-treated fibrils. Perhaps the purified lysyl oxidase used in this study is only one of a class of enzymes that are able to utilize lysyl or hydroxyllysyl residues in collagen or elastin as substrates. The initial extraction procedure in PBS may not have solubilized an enzyme acting primarily in helical regions. Against this possibility, however, are the observations that the purified enzyme is active with both collagen and elastin, and the increase in specific activity during purification is similar for both substrates. Furthermore, the same enzyme molecule would seem to act on both proteins, since analysis of the DEAE-cellulose chromatograph with both substrates yielded a single peak of enzyme activity.

The assay with fibrillar collagen should facilitate characterization of lysyl oxidase with respect to normal collagen crosslinking and pathologic states with postulated abnormalities of crosslinking. The assay is more rapid and sensitive than the conventional elastin assay and uses a well-defined substrate. Although the substrate is a precipitate, the linear Lineweaver-Burk plot is evidence that it follows typical enzyme kinetics over short incubation times. The high inhibition with pepsin-treated collagen implies that the binding site for the enzyme is on the helical region of the molecule although the catalytic site is in the nonhelical region of the same or a different molecule in the fibril. The affinity for the helical binding site may still be sufficiently great after catalysis occurs that enzyme displacement is slow unless other collagen molecules are immediately adjacent.

Recent studies of the biosynthesis of collagen have shown that collagen is synthesized as a large, soluble precursor, procollagen (25, 26). Presumably, following secretion, the precursor-specific region of procollagen is enzymatically removed and then self assembly into collagen fibrils occurs (27). It is of interest that the initial enzyme extracts and the partially purified enzyme preparation appeared to inhibit fibril formation in the collagen substrate described in this paper. Since a wide variety of low-molecular-weight compounds such as arginine or aspartic acid inhibit fibril formation (21), this may be a nonspecific effect. On the other hand, this study indicates that formation of lysine-derived aldehydes occurs after fibril formation has begun. It will be of interest to determine whether this reaction occurs on the outside of the growing fibril or in the interior of an assembled fibril. In either case, it seems likely that the rate of fibril formation as well as the concentration of lysyl oxidase is a significant factor regulating the biosynthesis of lysine-derived aldehydes and subsequent crosslinking in vivo. If fibril formation does regulate the rate of crosslinking, then specific inhibiting factors may be present in rapidly growing tissues, such as embryonic cartilage, to modulate the rate of fibril formation and subsequent crosslinking.

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