Influence of Sequence and Charge on the Specificity of Lysyl Oxidase toward Protein and Synthetic Peptide Substrates*

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Lysyl oxidase initiates the covalent cross-linking of elastin and collagen by oxidizing lysine residues in these proteins to \( \alpha \)-aminoadipic-\( \delta \)-semialdehyde. Sequences surrounding susceptible lysines in elastin are considerably different from those in collagen and yet the same enzyme can oxidize both substrates. Possible bases of the specificity have been explored assaying for \( \text{H}_2\text{O}_2 \) release accompanying the oxidation of synthetic peptide and protein substrates. Rates of oxidation of random co-polymers were maximal with (Ala, Lys), and decreased in the order (Val, Lys)\( _n \) > (Leu, Lys)\( _n \) > (Lys)\( _n \) > (Phe, Lys)\( _n \) > (Tyr, Lys)\( _n \). The ordered polymer (Ala-Lys-Glu)\( _n \) was oxidized at only 3% of the rate of (Ala, Lys)\( _n \), implying inhibition by peptidyl glutamate. Consistent with this conclusion, kinetic analyses using ordered oligopeptides revealed that, relative to Ala-Ala-Lys-Ala-Ala, \( K_m \) is increased 3.3-fold for lysine in Ala-Ala-Lys-Glu-Ala-Ala, 2.5-fold in Ala-Ala-Lys-Arg-Ala-Ala, and 1.8-fold in Ala-Ala-Glu-Lys-Ala-Ala. Tyrosine C-terminal to lysine in such peptides also increases \( K_m \) 5-fold. In addition, lysyl oxidase oxidized lysine in various proteins with basic isoelectric points and was much less active against various acidic proteins. Lysyl oxidase was inactive against native bovine serum albumin but effectively oxidized albumin if albumin carboxyl functions were first amidated by chemical modification. These results suggest that peptides bind to lysyl oxidase in a preferred directional sense and indicate that net anionic character as well as the specific position of anionic residues in substrates can selectively effect substrate potential. Implications of these results for the oxidation of elastin and collagen are discussed.

Since the primary and higher ordered structures of elastin and collagen are quite different from each other, it is not at all clear as to what the chemical or structural determinants may be which direct the expression of catalytic activity of lysyl oxidase toward these connective tissue proteins in vitro. It does appear, however, that collagen is preferentially oxidized as an insoluble, native fibrillar structure (6) and that the oxidation of elastin-like synthetic polypeptides is enhanced if the peptides are in the cosedimented state (4). Thus, a hydrophobic, ordered polymeric structure may enhance the reactivity of protein substrates. Similarly, the oxidation of lysine in insoluble elastin is enhanced by cationic amphiphilic ligands and inhibited by anionic amphiphilic ligands prebound to the elastin substrate (7), suggesting that the net positive charge of the substrate also may be a specificity determinant. It is consistent in this regard that H1 histone, a highly basic protein, can be oxidized by lysyl oxidase in vitro (5).

In the present study, we have explored the substrate potential of synthetic lysine-containing oligo- or polypeptides of random or ordered sequence and the effect of negatively charged dicarboxylic amino acid residues in these peptides on their ability to be oxidized by lysyl oxidase. The results reveal that glutamate at different positions relative to lysine within a polypeptide differentially affects the substrate potential. These results are considered in the light of the known sequence and charge features of cross-linking sites in elastin and collagen.

EXPERIMENTAL PROCEDURES

Materials—Random co-polymers of lysine were prepared by polymerizing the N-carboxyanhydride of \( \alpha \)-benzoyloxy carbonyl-L-lysine with the N-carboxyanhydride of the second amino acid in a 1:1 (v/v) mixture of benzene:methylene chloride (8). Polymerizations were initiated by addition of triethylamine at a monomer:initiator ratio of 100:1 and the reactions were continued for 24 h to completion of the reaction. Polymers were separated from the mixture by precipitation in an excess of diethyl ether; the precipitates were washed with ethanol and dried in vacuo. The polymers were dissolved in benzene and the \( \epsilon \)-amino groups were deblocked by saturating the benzene solutions with anhydrous HCl. The deblocked polymers were isolated by filtration, washed with ether and ethanol, and dried in vacuo. Completion of the deblocking reaction was verified by infrared and ultraviolet spectroscopy. Amino acid analyses of acid hydrolysates of the deblocked peptides were consistent with the input ratios of the N-carboxyanhydrides used in their syntheses. The ordered co-polymer (Ala-Lys-Glu), was a gift of Dr. H. J. Goren of The University of Calgary, Alberta, Canada. Each of these polypeptides was resolved into 10,000–20,000 Da mass fractions by chromatography through Bio-Gel P-60 (Bio-Rad, Richmond, CA) in 0.5% acetic acid. Fractions of this size were pooled and lyophilized. Dipeptides of lysine were obtained from Serva Fine Biochemicals, Inc., Garden City Park, NY. Apocytochrome \( c \) was prepared from commercially obtained cytchrome \( c \) by the method of Bonciel et al. (9). All other reagents were of the highest quality obtainable.

Enzyme Purification and Assay—Lysyl oxidase was isolated from bovine aorta as a co-purified mixture of the four variants of this protein...
enzyme, as described (10, 11). The specific activity of the enzyme preparation used here was 650,000 cpm of \(^{3}H\) released/mg of protein in 2 h at 37°C from 125,000 cpm of a \(^{3}H\)-labeled insoluble aortic elastin substrate. The tritiated substrate was prepared from chick embryo aortas which had been pulsed in vitro with t-[-\(^{3}H\)]lysine by methods previously described (11). Assays of enzymatic activity toward peptide or synthetic substrates employed a continuous fluorescence assay for \(H_{2}O_{2}\) release stoichiometrically accompanying l-lysyl oxidase-dependent aldehyde formation (2, 3). Unless otherwise indicated, these assays were performed under conditions previously shown to be optimal for the expression of l-lysyl oxidase activity toward synthetic substrates (3), i.e. 50 mM sodium borate, 1.2 M urea, 0.7 mM sodium homovanillate, 20 \(\mu\)g ml\(^{-1}\) horseradish peroxidase, pH 8.2, at 55°C in a final volume of 2 ml. Assays were initiated by the addition of 2 \(\mu\)g of l-lysyl oxidase.

**Solid-phase Peptide Synthesis—**Lysine-containing oligopeptides were synthesized by solid-phase procedures (12), as described (15). A manual solid-phase peptide synthesis apparatus, resins, and amino acids containing protected functional groups used in these syntheses were each obtained from Peninsula Laboratories, Inc., Belmont, CA. One gram of 1% divinylbenzene resin substituted with 0.5 to 0.7 meq of t-Boc-L-alanine was suspended and washed in 30 ml of methylene chloride in the synthesizer reaction vessel. The t-Boc-Ala-O-resin was then suspended in 25% trifluoroacetic acid in methylene chloride for 30 min at room temperature to remove the \(\alpha\)-Boc protecting group. After deprotection, the resin was washed and neutralized with 10% triethylamine in methylene chloride and then washed with methylene chloride. The next amino acid was added as the t-Boc derivative together with the diisopropylethylamine-benzyl coupling agent, each added at a 3-fold molar excess over deprotected \(\alpha\)-amino functions on the resin. The extent of deprotection and of coupling were monitored by the colorimetric ninhydrin-based procedure of Sarin et al. (14). If necessary, the coupling reaction was repeated for an additional hour. The coupling cycle was repeated until the desired sequence was obtained. Lysine was introduced in these syntheses as the \(\alpha\)-Boc-L-β-phenylvalerylcarboxyl derivative, glutamate and tyrosine as the \(\alpha\)-Boc-N\(^{\text{d}}\)-tosyl derivative. Peptides were removed from the resin by bubbling anhydrous \(\text{HBr}\) through a suspension of the peptide resin in trifluoroacetic acid for 1.5 h at room temperature. Arginine-containing peptides were removed from the resin and remaining protecting groups were removed by boron trifluoroacetic acid. The filtrate and washings were combined and dried under nitrogen. The dried peptide was dissolved in 1% acetic acid and extracted several times with ethyl acetate. The aqueous phase was separated and freeze dried. Synthetic peptides were freed of minor contaminants by chromatography in 1% acetic acid on a 2 \(\times\) 45 cm column of Bio-Gel P-2 resin. The peptide product was identified and assessed for purity by thin layer chromatography of the column fractions on silica gel plates developed in a solvent of 1-butanolacetic acidwater, 4:1:1, and visualized by spraying the plates with 1% ninhydrin and heating at 90°C. The amino acid compositions of the peptides were established by amino acid analyses of samples hydrolyzed in 6 N HCl at 108°C for 22 h in tubes sealed in tinfoil. The peptides used in these studies were homogeneous as evidenced by thin layer chromatography and had amino acid compositions in which the individual amino acid contents did not vary from the theoretical values by more than ±7%.

As an additional index of the efficacy of the solid-phase method employed, the sequence of one of these peptide products, \(\text{H}_{2}\text{N-Ala-Ala-Lys-Ala-Ala} \text{COOH}\), was analyzed on a Beckman Automated Peptide Sequencer and was found to have the desired sequence.

**Oxidation of peptidyllysine by l-lysyl oxidase**

Each substrate was assayed at a concentration of 0.5 mg ml\(^{-1}\) in the fluorometric assay for peroxide release. Ratios in parentheses refer to input ratios of carboxyamidine derivatives of amino acids used in the synthesis of random polypeptides. All other polypeptides listed are ordered sequence polymers, as indicated in each case.

| Substrate | Initial | Relative \(n\)nmol H\(^{2}\)O
t 10 min\(^{-1}\) | velocity |
<table>
<thead>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{Ala-Lys)}), (2:1)</td>
<td>0.69</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>(\text{Val-Lys)}), (1:1)</td>
<td>0.39</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>(\text{Lys)})</td>
<td>0.27</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>(\text{Leu-Lys)}), (1:1)</td>
<td>0.23</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>(\text{Phe-Lys)}), (1:1)</td>
<td>0.19</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>(\text{Tyr-Lys)}), (1:1)</td>
<td>0.11</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>(\text{Tyr-Lys)}), (3:1)</td>
<td>0.13</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>(\text{Ala-Lys-Glu})</td>
<td>0.02</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>(\text{Gly-Lys-Pro-Gly})</td>
<td>0.30</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>(\text{Gly-Lys-Glu-Val-Pro})</td>
<td>0.37</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>(\text{Lys-Tyr)})</td>
<td>0.11</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>(\text{Tyr-Lys)})</td>
<td>0.11</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>(\text{Lys-Glu} \text{(or) Glu-Lys)})</td>
<td>0.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

The abbreviation used is: t-Boc, \(\text{t-butyloxycarbonyl-}\).
Substrate Specificity of Lysyl Oxidase

FIG. 1. Reciprocal plots of initial rates of oxidation of ordered oligopeptides. Ordinates: reciprocal of nanomoles of H\textsubscript{2}O\textsubscript{2} released min\textsuperscript{-1}. Each of the plotted lines represents linear regression analysis. The correlation coefficients for each of the four plots in the upper half are 0.92, 0.97, 0.94, and 0.96, from left to right, respectively; in the lower half, the values are 0.93, 0.99, and 0.94, from left to right, respectively.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_m$ (M x 10\textsuperscript{-4})</th>
<th>$V_{max}$ (nml H\textsubscript{2}O\textsubscript{2} 10 min\textsuperscript{-1})</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Ala\textsubscript{2}-Lys-Ala\textsubscript{2}</td>
<td>8.2</td>
<td>0.43</td>
<td>0.053</td>
</tr>
<tr>
<td>II. Ala\textsubscript{2}-Lys-Ala\textsubscript{2}-Lys-Ala\textsubscript{2}</td>
<td>6.8</td>
<td>0.66</td>
<td>0.097</td>
</tr>
<tr>
<td>III. Ala\textsubscript{2}-Lys-Ala\textsubscript{2}-Arg-Ala\textsubscript{2}</td>
<td>29.6</td>
<td>0.97</td>
<td>0.032</td>
</tr>
<tr>
<td>IV. Ala\textsubscript{2}-Lys-Arg-Ala\textsubscript{2}</td>
<td>20</td>
<td>0.93</td>
<td>0.047</td>
</tr>
<tr>
<td>V. Ala\textsubscript{2}-Lys-Glu-Ala\textsubscript{2}</td>
<td>75.9</td>
<td>0.47</td>
<td>0.006</td>
</tr>
<tr>
<td>VI. Ala\textsubscript{2}-Glu-Lys-Ala\textsubscript{2}</td>
<td>14.4</td>
<td>0.21</td>
<td>0.014</td>
</tr>
<tr>
<td>VII. Ala\textsubscript{2}-Lys-Tyr-Ala\textsubscript{2}</td>
<td>41</td>
<td>0.79</td>
<td>0.019</td>
</tr>
</tbody>
</table>

The $V_{max}$ but increases the $K_m$ 3.6-fold, thus decreasing the $V_{max}/K_m$ ratio. If arginine is introduced peptide-bonded to the lysine carbonyl (Peptide IV), the kinetic constants are similar to those of Peptide III, suggesting that the difference in peptide length between Peptide I and Peptides II, III, and IV is not the prominent factor in changing the kinetic constants but rather that the introduction of the second positively charged residue generally increases $V_{max}$ and, in the case of the arginine substitutions, may increase the $K_m$ as well. The substitution of glutamate C-terminal to lysine (Peptide V) markedly increases the $K_m$ to a value 3.3-fold over that of Peptide I, while exerting little effect on the $V_{max}$ value. This substrate has the lowest $V_{max}/K_m$ value of those tested and thus is the least effective substrate by this criterion. In contrast, substitution of glutamate to the a-amino group of lysine (Peptide VI) increases the $K_m$ only by 1.8-fold over that of Peptide I, while decreasing the $V_{max}$ to one-half of that of Peptide I. Substitution with tyrosine bonded to the carbonyl of lysine (Peptide VII) increases the $K_m$ 5-fold while increasing $V_{max}$ to a lesser degree, thus decreasing the $V_{max}/K_m$ ratio of Peptide I by 64%. In summary, the largest effects seen relative to the kinetic constants obtained with Peptide I are the marked reductions in the $V_{max}/K_m$ ratios resulting from introduction of glutamate or tyrosine in the indicated positions in these sequences.

The suppressing effect of negatively charged peptide carboxylate functions on the activity of lysyl oxidase suggests that such an effect may extend to proteins. It was of interest, therefore, to test the possibility that the potential of a protein to be oxidized by lysyl oxidase may correlate with the isoelectric point of the potential protein substrate. The results shown in Fig. 2 generally support this hypothesis in that the proteins in this list with isoelectric points greater than 4 all serve as substrates for lysyl oxidase while those with more acidic isoelectric points display negligible or no ability to be oxidized. Although substrate potential does correlate with the
Substrate Specificity of Lysyl Oxidase

overall acidity or basicity of this group of proteins, there is marked variation in the initial rates of oxidation obtained with those basic proteins which are substrates but otherwise have similar pI values. This likely reflects differences in the concentration and/or accessibility of surface lysine residues among this group of proteins. Although bovine serum albumin is not a substrate for lysyl oxidase, it is particularly noteworthy that this protein becomes an effective substrate if its carboxylate functions are first covalently modified to neutral amide functions by treatment of the native protein with ammonia and a water-soluble carbodiimide, as described under “Experimental Procedures.” The generation of substrate potential in albumin accompanies the change in pI from 4.7 to 11.4 resulting from amidation of its carboxylate functions (Fig. 2).

DISCUSSION

The present studies reveal the potential of lysyl oxidase to oxidize peptidyllysine in a variety of protein sequence environments, although certain residues vicinal to peptidyllysine markedly alter the potential for lysine oxidation. The most notable effect among the specific sequences examined in this study is the considerable increase in $K_\text{m}$ resulting from the insertion of glutamate at the carboxyl of peptidyllysine. Since there is little effect on the $V_{\text{max}}$ by this negatively charged residue, it appears that the primary influence of this substitution is to severely reduce the affinity of the neighboring peptidyllysine for the active site of the enzyme. This presumably reflects the formal negative charge of the glutamate residue, and, in that sense, these data are consistent with prior observations that lysyl oxidase activity toward an insoluble elastin substrate is considerably enhanced by elastin-bound cationic amphiphiles and is strongly inhibited by anionic, amphiphilic elastin ligands (7). The unfavorable effect of negative charges in substrates is further evidenced by the generation of substrate potential in albumin, which is otherwise not oxidized by lysyl oxidase, upon the amidation of its carboxylate functions, and by the apparent correlation of substrate potential with basic isoelectric points of proteins.

The present results also reveal, however, that substrate potential is adversely affected not only by the net anionic charge of a substrate but also by the specific sequence position of a dicarboxylic amino acid residue relative to that of the substrate lysyl residue. It seems unlikely that the marked decrease in substrate affinity by glutamate C-terminal to lysine is due primarily to ion pairing between the γ-carboxyl function and the ε-amino group to block the entrance of the lysine side chain into the active site since molecular models reveal that such ion pairing appears equally possible with either the -Lys-Glu- or -Glu-Lys- sequence. Thus, the considerably larger increase in $K_\text{m}$ induced by glutamate substituted to the lysine α-carboxyl function implies that such peptide substrates bind to the active center in a preferred directional sense. Further, it seems likely that there are multiple binding interactions between the polypeptide substrate and lysyl oxidase adjacent to and at a distance from the oxidizable lysine residue, indicated by the different effects on the kinetic parameters induced by glutamate at different peptide positions and by the increase in $V_{\text{max}}$ by the introduction of arginine to the lysine carboxyl function or at a position three residues C-terminal to lysine.

Thus, it appears that there are two levels of charge-dependent effects influencing the susceptibility of peptidyllysine to oxidation by lysyl oxidase. The effects of ligands on elastin oxidation and the correlation of substrate potential with protein charge point toward an electrostatic field effect important in the approximation of substrates with the lysyl oxidase molecule. Consistent with the observed charge relationships, the amino acid compositions (11) and migration behavior of the lysyl oxidase variants upon isoelectric focusing reveal that lysyl oxidase is an acidic protein. At the level of the active site, the sequence-specific perturbation of the $K_\text{m}$ by glutamate implicates the presence of an ionized, presumably anionic residue or residues located at or near the substrate-processing site which may more readily interact with and repel glutamate C-terminal to peptidyllysine in an appropriately bound peptide substrate. This possibility is supported by the much lower increase in $K_\text{m}$ induced by the replacement of glutamate with a cationic arginine C-terminal to lysine. The presence of a specificity-determining anionic site in the enzyme has also been inferred from analyses of the substrate specificity of lysyl oxidase for alkyl diamines of varying chain length (17) and from the specificity of the enzyme for various β-substituted ethylamine derivatives as suicide inhibitors (17). The increase induced in $V_{\text{max}}$ by substitution of the ordered oligopeptides with arginine or a second peptidyllysine suggests that vicinal positive charge may enhance product release or other components of the catalytic mechanism of lysyl oxidase.

Examination of the known sequences of the cross-linkage regions of elastin and collagen reveals interesting relationships with the present data. Elastin is secreted from fibrogenic cells as a 72,000-Da monomeric precursor, identified as tropoelastin (18, 19). This protein contains about 48 lysines/1000 residues and, extrapolating from studies on insoluble elastin (20), contains approximately 9 nonamidated dicarboxylic residues. Thus, tropoelastin is predominantly a cationic protein which becomes less basic as its lysine residues are oxidized, properties which are consistent with the present observation that highly basic proteins are more actively oxidized by lysyl oxidase. Lysine in tropoelastin is predominantly localized in alanine-rich sequences, such as the following (21):

- Ala-Lys-Ala-Ala- (1)
- Ala-Lys-Ala-Lys-Tyr- (2)

Such sequences are consistent with the preference of lysyl oxidase for lysine in sequences containing less bulky neutral residues. Both of the lysine residues in peptide (1) can be oxidized in vivo following which intramolecular condensation yields the aldol condensation product cross-linkage (21). The sequence of peptide (2) yields the structure of the anhydridolysinonorleucine Schiff base cross-linkage in which only one of the lysine donors to this intramolecular cross-linkage is oxidized. Based on the sequences of cross-linked elastin peptides, Foster et al. (21) have suggested that the tyrosyl residue linked to the carboxyl group of the second lysine in peptide sequence 2 inhibits its oxidation by lysyl oxidase, consistent with the present finding that tyrosine in that position increases the $K_\text{m}$ 5-fold relative to Ala,-Lys-Ala, and reduces the $V_{\text{max}}/K_\text{m}$ ratio 2.8-fold. It should be noted that Meacham and Foster (22) have isolated the sequence -Gly-Ala-Glu-aminoadipic-δ-semialdehyde- from insoluble elastin of copper-deficient pig aorta. However, it is possible that the purification of the elastin protein in that study by extraction with hot alkali may have hydrolyzed specific amide functions including that sequenced as glutamate N-terminal to the oxidized lysine residue. Nevertheless, lysine more frequently occurs in glutamate-free segments of the tropoelastin sequence (19).

The sequences of the N- and C-terminal telopeptides of the

2 H. M. Kagan, unpublished observations.
a1(I) chain of collagen (23, 24) are shown below:

\[
\begin{align*}
N\text{-terminal:} & \quad pGlu-Leu-Ser-Tyr-Gly-Tyr-Asp-Glu-Lys-Ser-Thr-Gly- \\
& \quad 1^{1N} \quad 9^{9N} \\
C\text{-terminal:} & \quad \text{Pro-Pro-Gln-Gln-Glu-Lys-Ala-} \\
& \quad \text{His-Asp-Gly-Gly-Arg-Tyr-Tyr}
\end{align*}
\]

Lysine \(9^{9N}\) and lysine \(17^{1C}\) are each oxidized in vivo to \(\alpha\)-aminoacidipic-\(\delta\)-semialdehyde and, as such, each participates in cross-linkage formation (25, 26). These lysine sequences suggest an incongruity with the present data in view of the glutamate residues N-terminal to lysine in both sequences and with respect to the additional dicarboxylate aspartate residue at position \(7^{N}\). Nevertheless, it is consistent that C-terminal glutamate rather than N-terminal glutamate is most inhibitory to lysine oxidation in the ordered sequence oligopeptides. Notably, Fukae and Mechanic (27) have found evidence that lysine \(17^{1C}\) is oxidized well before lysine \(9^{9N}\) in Type I collagen in vivo. This temporal sequence of oxidation may well result from the two anionic residues immediately preceding lysine \(9^{9N}\), rendering this residue less susceptible to lysyl oxidase than lysine \(17^{1C}\). It is of interest, as has been noted previously (1), that there is near identity between the sequence to telopeptide lysine residues which are oxidized by lysyl oxidase with the extracellular matrix (1) raises additional questions about factors which may control the specificity of this connective tissue enzyme.

**REFERENCES**