# Inhibition by Heparin of the Oxidation of Lysine in Collagen by Lysyl Oxidase†

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ABSTRACT: The generation of covalent cross-linkages in collagen is initiated by the deamination by lysyl oxidase of specific lysine residues in this connective tissue protein. Since lysyl oxidase activity is influenced by ionic ligands bound to its protein substrates, the effect of heparin, an anionic glycosaminoglycan known to bind to collagen, was explored by using collagen and elastin substrates and highly purified lysyl oxidase. Concentrations of heparin up to 1 mg $mL^{-1}$ had little effect on the enzymatic rate of oxidation if it was added prior to the addition of enzyme to a preformed fibrillar collagen substrate or to an insoluble elastin substrate. However, collagen oxidation was inhibited by 85% if this glycosaminoglycan was present at 0.4 mg $mL^{-1}$ during collagen fibril formation before addition of the enzyme. Similarly, the rate and extent of collagen fibrillogenesis in the absence of lysyl oxidase were each markedly inhibited in the presence of 0.4 mg $mL^{-1}$ heparin. Heparin also inhibited the extent of tight binding of lysyl oxidase to preformed fibrils by about 40% under conditions where enzyme activity against preformed fibrils was hardly affected. These results suggest that heparin may modulate the oxidation and thus the insolubilization of extracellular collagen fibers, possibly under conditions where elastin fiber synthesis is not affected, and that the tight binding of lysyl oxidase to collagen is not completely related to the expression of enzyme activity toward this substrate. These results also have mechanistic implications for the retarding effect of heparin on postoperative wound healing.

The oxidation of lysine residues to peptidyl aminoadipic semialdehyde at the non-triple-helical ends of collagen $\alpha$ chains is an essential posttranslational modification in the assemblage of insoluble collagen fibers. This reaction is catalyzed by lysyl oxidase (EC 1.1.4.13) and leads to the spontaneous condensation of the newly formed aldehyde residues with other vicinal aldehydes or with $\epsilon$-amino functions, thus generating the covalent cross-linkages which account for the insolubility of this connective tissue protein. Elastin is also a substrate for lysyl oxidase and thus also contains a variety of lysine-derived cross-linkages (Kagan, 1986).

Since the biosynthesis of cross-linkages is critical to the growth and repair of connective tissues, it is important to note that the expression of the activity of lysyl oxidase can be strongly influenced by factors which are either intrinsically or extrinsically related to the protein substrate. Thus, it has been shown that native, quarter (or D–) staggered fibrils represent the optimal substrate form of collagen for lysyl oxidase while collagen monomers appear not to be effective substrates (Siegel, 1974). This and related studies have led to the suggestion that the enzyme may require a binding site whose structural features are generated by the three-dimensional packing array of collagen molecules in native fibrils (Siegel, 1979; Cronlund et al., 1985). In addition to such structural requirements, the activity of lysyl oxidase is also sensitive to the ionic nature of the substrate. Thus, lysyl oxidase will oxidize a variety of cationic but not acidic globular proteins in vitro (Kagan et al., 1984), while its activity toward elastin is strongly stimulated by elastin-bound, cationic amphiphiles but completely inhibited by elastin-bound anionic amphiphiles such as fatty acids (Kagan et al., 1981).

There are several molecular species which are known to interact with collagen in the extracellular matrix and which might influence, therefore, the interaction of lysyl oxidase with collagen. Among these potential ligands, glycosaminoglycans would appear to be of particular interest since certain of these sulfated polysaccharides have been shown to alter the kinetics of collagen fibrillogenesis as well as the structural features of collagen fibrils formed in their presence in vitro (Mathews & Decker, 1968; Toole, 1969; Snowden & Swann, 1980; Lilja & Barrach, 1981, 1983; Koda & Bernfeld, 1984). In the present study, we explore the effect of heparin on the oxidation of collagen fibrils by lysyl oxidase. This glycosaminoglycan was chosen because of its presence in mammalian tissues and by virtue of the fact that it is commonly administered as an anticoagulant under postoperative conditions in which wound healing and thus collagen deposition are expected to occur.

## Materials and Methods

*Reagents.* L-[4,5-$^3$H]Lysine (2.6 Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA. Heparin used in these studies was purchased from Sigma Chemical Co., St. Louis, MO, as the product purified from porcine intestinal mucosa and had a specific activity of 140 USP units/mg.

*Preparation of Enzyme and Substrates.* Lysyl oxidase was isolated from bovine aorta as a copurified mixture of the four ionic variants of this enzyme, as previously described (Williams & Kagan, 1985). The product appeared as a single band at a molecular weight of 32 000 by sodium dodecyl sulfate gel electrophoresis (Laemmli, 1970). Neutral salt-soluble type I collagen was purified to homogeneity from fetal calf skin and characterized as previously described (Cronlund et al., 1985). Tritiated collagen was prepared by a published method (Siegel, 1974) from calvaria which had been removed from 16-day-old chick embryos and pulsed in organ culture with L-[4,5-$^3$H]lysine as described (Sullivan & Kagan, 1982). Fifty pairs of lyophilized, tritiated calvaria were rehydrated in 30 mL of 1 M NaCl/50 mM tris(hydroxymethyl)aminoethane (Tris), pH 7.6, for 90 min at 4 °C. The calvaria were then homogenized by two 30-s high-speed bursts of a Polytron

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homogenizer (Brinkmann Instruments) in this buffer; the suspension was stirred for 90 min and then centrifuged in a Beckman SS 34 rotor for 40 min at 15000 rpm. The supernatant was collected, and the collagen in the supernatant was precipitated by adding solid NaCl to a final concentration of 20% (w/v) and then stirring the mixture for 1 h at 4 °C followed by incubation without stirring at 4 °C for 24 h. The precipitate was isolated by centrifugation at 48000*g*, and the pellet was dissolved in 0.15 M NaCl/0.16 M potassium phosphate, to a final collagen concentration of 0.6 mg $mL^{-1}$, the latter determined by amino acid analysis for hydroxyproline content in an acid hydrolysate of an aliquot of the collagen solution. The solution was thoroughly dialyzed against this NaCl/phosphate buffer until the dialyzable radioactivity was at background levels. The collagen solution was stored at 4 °C for use as a substrate for lysyl oxidase assays. A tritiated, insoluble elastin substrate was prepared from aortas which had been excised from 16-day-old chick embryos and then pulsed as previously described (Sullivan & Kagan, 1972). Unlabeled, salt-soluble collagen was isolated from 16-day-old chick embryo calvaria which had not been pulse-labeled, following the procedure described for the isolation of tritiated calvarial collagen. All soluble collagen preparations were analyzed for purity by sodium dodecyl sulfate gel electrophoresis and by amino acid analysis, and each appeared to be a pure preparation of type I collagen.

*Enzyme Assays.* Assays against elastin contained 125000 cpm of the [$^3$H]elastin substrate in suspension in a final volume of 0.75 mL of 0.05 M sodium borate/0.15 M NaCl, pH 8.0. Assays against collagen contained 300000 cpm of the $^3$H-labeled collagen in a final volume of 1.0 mL of 0.016 M potassium phosphate/0.15 M NaCl, pH 7.6. Reconstituted fibril substrate forms of collagen were formed by preincubation of the soluble, tritiated collagen in this buffer and in the presence or absence of heparin at 37 °C for 60 min. Assays against the elastin or collagen subsrates were initiated by the addition of 2–4 μg of purified lysyl oxidase which had been dialyzed for 18 h against the specific buffer used in each assay to remove urea remaining from the purification of the enzyme. Complete assay mixtures using the various elastin or collagen substrates and containing or lacking heparin, as indicated, were incubated at 37 °C for 2 h with shaking. Reactions were stopped by rapidly freezing the assays in dry ice/acetone. Tritiated water formed by enzyme action was isolated by distillation of the thawed reaction mixtures in vacuo. Radioactivity in 0.5-mL aliquots of the distillates was quantified by liquid scintillation spectrometry. The activities of lysyl oxidase given in this report are the averages of duplicate or triplicate assays which did not vary by more than ±7% from their average values. Each result described was reproducible in two or more separate experiments. All assays were corrected for tritium released from the elastin or collagen substrates incubated in the absence of added enzyme and in the absence or presence of the various concentrations of heparin used in these studies.

*Collagen Fibrillogenesis.* The kinetics of fibrillogenesis were followed by monitoring the development of turbidity accompanying fibril formation in temperature-controlled cuvettes at 310 nm in a Perkin-Elmer Model 570 spectrophotometer, according to a published procedure (Helseth & Veis, 1981). The collagen used in these fibrillogenesis studies was that isolated from chick calvaria as described and was added to initiate fibrillogenesis from a freshly dialyzed stock solution (0.6 mg $mL^{-1}$) in 0.15 M NaCl/16 mM potassium phosphate, pH 7.6. When used in these studies, heparin was added from stock solutions of this glycosaminoglycan in this buffer. Aliquots (867 μL) of the NaCl/phosphate buffer containing or lacking the specified concentrations of heparin were preequilibrated in the cuvette in the spectrophotometer at 8 °C. The sample chamber was constantly flushed with nitrogen to prevent fogging of the cuvette. An aliquot (133 μL) of the collagen stock solution was then added to the reaction mixture and the temperature maintained at 8 °C for an additional 5 min. The temperature of water circulating through the water-jacketed cuvette chamber was then jumped to bring the temperature of the contents of the cuvette to 37 °C by switching a three-way valve channeling water to the cuvette chamber from one circulating water bath set at 8 °C to another set to maintain the reaction mixture at 37 °C. Separate monitoring of the cuvette contents under these conditions indicated that equilibration to the higher temperature occurred within 1 min after switching to the second water bath. The turbidity–time plots shown are recorded from the moment of switching to the higher temperature.

*Enzyme Binding Studies.* The determination of the effect of heparin on the formation of sedimentable enzyme–collagen complexes generally followed a previously described enzyme binding assay method (Cronlund et al., 1985). Preparations of nonlabeled type I collagen purified from calf skin or chick embryo calvaria as described were used as binding matrices as specified in the text. Prior studies had shown that lysyl oxidase binds tightly both to chick embryo calvarial collagen (Siegel, 1974) and to calf skin type I collagen (Cronlund et al., 1985). Incubation mixtures (0.75 mL final volume), composed on ice, were buffered by 30 mM potassium phosphate, pH 7.6, and contained 100 μg of type I collagen added as a 0.1-mL aliquot from a stock solution in 5 mM acetic acid. Varied amounts of heparin were added from 30 mM potassium phosphate stock solutions to the mixtures either before or after collagen fibril formation was complete. Fibril formation was achieved by incubating the mixtures at 37 °C for 1 h. Lysyl oxidase was dialyzed against the potassium phosphate buffer, and then 4.2 μg of enzyme was added to each binding assay mixture after fibril formation and after the addition of heparin to the collagen suspensions. The suspensions were mixed, incubated at 37 °C for 15 min, and then sedimented by centrifugation on a Beckman Microfuge for 5 min. Aliquots (500 μL) of the supernatants were then mixed with the [$^3$H]elastin substrate (125000 cpm) in 0.25 mL of 0.05 M sodium borate, pH 7.6, and incubated for 2 h at 37 °C to assay for the enzyme remaining unbound in the supernatants obtained from the collagen suspensions. Assays were corrected for possible effects of free collagen and/or heparin in the supernatants as well as for nonenzymatic tritium release with appropriate control assays.

## RESULTS

*Effect of Heparin on Lysyl Oxidase Activity.* The activity of lysyl oxidase toward collagen substrates is strongly favored if the collagen molecules are allowed to assemble into quarter-staggered native fibrils by thermal induction in neutral salt buffers prior to the addition of the enzyme. Indeed, there is a lag in tritium release if lysyl oxidase is added to collagen molecules in solution prior to fibril formation, this delay in enzyme activity reflecting the time-dependent assemblage of the collagen into quarter-staggered fibrils (Seigel, 1974). Thus, the effect of the presence of heparin on the oxidation of lysine in the tritiated collagen substrate was assessed under two different conditions. In one case, the collagen substrate was preincubated at 37 °C for 1 h in assay buffer to allow maximum fibril formation before the addition of heparin and

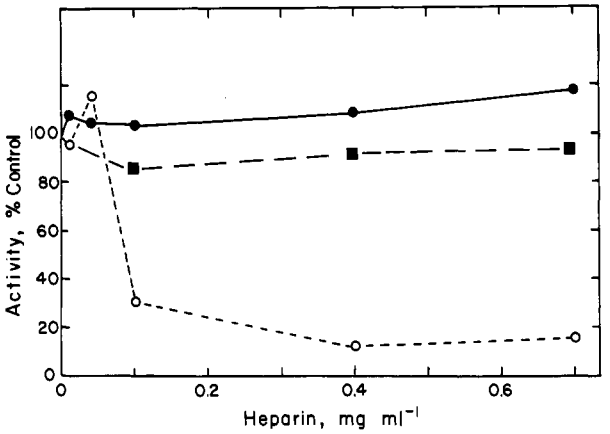


FIGURE 1: Effect of heparin on the oxidation of collagen and elastin by lysyl oxidase. (●) Heparin added to preformed collagen fibrils; (○) heparin added before and present during collagen fibrillogenesis; (■) oxidation of insoluble elastin substrate. In each case, assays were begun by the addition of enzyme.

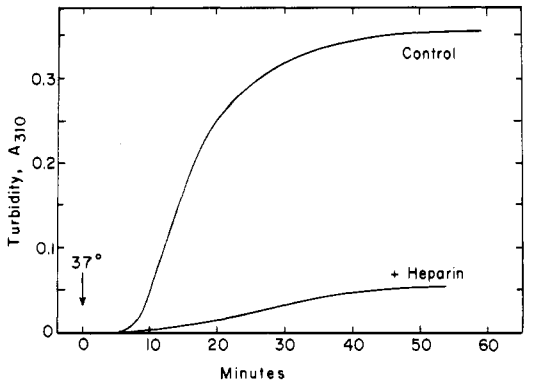


FIGURE 2: Effect of heparin (0.4 mg $mL^{-1}$) on collagen fibrillogenesis. See Materials and Methods for details.

enzyme. Alternatively, heparin was added to a solution of collagen molecules at 4 °C in assay buffer, and the mixture was then incubated at 37 °C for 1 h prior to the addition of enzyme. As shown (Figure 1) heparin has little effect on the initial rate of enzyme-catalyzed tritium release from collagen fibrils preformed in its absence. In contrast, heparin strongly inhibits collagen oxidation if the glycosaminoglycan is added at the concentrations indicated prior to the 1-h gelation period. In both cases, the catalytic reaction was initiated by the addition of lysyl oxidase at the end of the 1-h preincubation at 37 °C. Visual inspection of the tubes indicated that the presence of heparin during the preincubation of the collagen substrate at 37 °C appeared to reduce the mass of fibrils which formed.

As also shown in Figure 1, the presence of heparin in assays for the oxidation of the insoluble [$^3$H]elastin substrate by lysyl oxidase only marginally affects the enzyme-catalyzed rate of tritium release, with inhibition ranging from 14% to 6% over concentrations of heparin ranging from 0.1 to 1 mg $mL^{-1}$.

*Effect of Heparin on Collagen Fibrillogenesis.* The effect of heparin on collagen fibrillogenesis was assessed by following the development of turbidity at 310 nm resulting from the aggregation of chick calvaria type I collagen molecules into fibrils in vitro, as described under Materials and Methods. As shown in Figure 2, a sigmoidal turbidity vs time curve results as fibrillogenesis proceeds upon the switching of the temperature of the cuvette contents from 8 to 37 °C. The presence of 0.4 mg $mL^{-1}$ heparin in the collagen solution prior to and during gelation strongly inhibited the development of collagen fibrils. Thus, the lag time, estimated by extrapolation of the linear phase of the fibrillogenesis curve to the abscissa, is increased from 8 min in the absence of heparin to 12 min in its presence. Moreover, the maximum turbidity that is reached in the presence of heparin is reduced by approximately 85%.

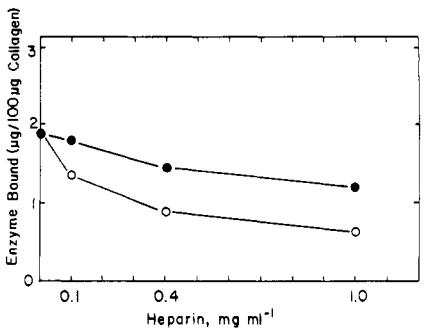


FIGURE 3: Effect of heparin on the binding of lysyl oxidase to fibrillar calf skin type I collagen. (●) Heparin added to preformed collagen fibrils; (○) heparin present during collagen fibril formation.

*Effect of Heparin on the Binding of Lysyl Oxidase to Collagen.* Lysyl oxidase can bind to the triple-helical portion of collagen in reconstituted fibrils as a sedimentable complex with a dissociation constant of 0.32 µM (Cronlund et al., 1985). Such binding has been suggested to be essential to the expression of enzyme activity toward the fibrillar collagen substrate. It was thus of interest to examine the effect of heparin on the formation of this bound complex. As shown (Figure 3), increasing amounts of heparin inhibit the binding of lysyl oxidase to fibrils of calf skin type I collagen generated in the presence or absence of heparin. The total binding seen in the absence of heparin is reduced by about 40% at 1 mg $mL^{-1}$ heparin in samples in which heparin was added after fibril formation while binding is reduced by approximately 68% at 1 mg $mL^{-1}$ heparin when heparin was present during fibril formation. The dissociation constant for the collagen–enzyme complex formed in the absence of heparin calculated from the data of Figure 3 was 0.5 µM, in reasonable agreement with the value of 0.32 µM previously determined (Cronlund et al., 1985). The apparent $K_d$ was increased to 2.8 µM for the enzyme complex of fibrils formed in the presence of 1 mg $mL^{-1}$ heparin and to 1.1 µM if heparin was added after fibril formation. Comparing the results seen Figures 2 and 3, it is also of interest to note that there is significant binding of enzyme to the reduced mass of fibrils generated in the presence of heparin. The results of such binding studies were essentially the same as those shown in Figure 3 if chick calvaria type I collagen was used as the binding matrix (not shown).

## Discussion

There has been continued interest in the influence of glycosaminoglycans and proteoglycans on the metabolism and structure of extracellular macromolecules (Toole, 1981; Poole, 1986). Evidence for the interaction of collagen with proteoglycans (Koda & Bernfeld, 1984; Vogel et al., 1984; Smith et al., 1985; Oegema et al., 1975; Toole & Lowther, 1968; Junqueira et al., 1980) and/or glycosaminoglycans (Mathews & Decker, 1968; Snowden & Swann, 1980; Lilja & Barrach, 1981, 1983; Keller et al., 1986) has been obtained either from direct binding studies or from observations that these polymers can influence the rate of formation and/or the morphology of collagen fibrils which form in their presence.

In the present study, it has been observed that heparin can markedly inhibit the ability of lysyl oxidase to oxidize lysine in collagen fibrils formed in the presence of this glycosaminoglycan. In contrast, little if any effect on the rate of oxidation of the collagen substrate resulted if heparin was

added to the preformed collagen fibrils prior to the addition of enzyme. The maximum inhibition of collagen oxidation was seen when 0.4 mg $mL^{-1}$ heparin was present during fibril formation. This concentration of heparin also markedly inhibited the rate and extent of collagen fibril formation. Since the reconstituted quarter-staggered fibril is a considerably more effective substrate for lysyl oxidase than native collagen monomers (Siegel, 1974), it is likely that the inhibition of collagen oxidation is largely due to the effect of heparin on the extent of formation and possibly the morphology of collagen fibrils. In this regard, it is of interest that the width of collagen fibrils grown in the presence of heparin or other highly sulfated glycosaminoglycans exceeds that of fibrils generated in the absence of these glycosaminoglycans (Wood, 1960; Keech, 1961; Lilja & Barrach, 1983) while glycosaminoglycans of this category have also been observed to induce both native and segment-long-spacing (SLS) fibrillar structures (Lilja & Barrach, 1981). Thus, it is possible that heparin may alter the steric relationship between collagen monomers within the fibrils formed in its presence in a fashion that alters the expression of the catalytic action of lysyl oxidase. It should be noted, however, that although the rate of oxidation is reduced to approximately 15% of control with fibrils grown in the presence of heparin, this level approximates the amount of fibrils accumulating in the presence of heparin relative to fibrils grown in its absence (see Figure 2). This suggests that such fibrils may be effective substrates for lysyl oxidase and that the inhibitory effect of heparin on collagen oxidation is likely to be related primarily to the degree of its ability to inhibit collagen fibrillogenesis.

The negligible effect of heparin on the oxidation of fibrils generated in its absence indicates that heparin bound to collagen under these conditions does not compete against catalytically productive interactions of lysyl oxidase with this substrate. While there is evidence for (Koda & Bernfeld, 1984) and against (Oegema et al., 1975; Toole & Lowther, 1968) the binding of proteoglycans and/or glycosaminoglycans to preexistent collagen fibrils in vitro, heparin is known to bind to a high-affinity site in the triple-helical region of type I collagen monomers between residues 755 and 933 of the $\alpha 1$ chains (Keller et al., 1986). The sedimentable binding of lysyl oxidase to collagen fibrils also occurs in the triple-helical region of collagen and may involve residues 925–932 since this sequence is conserved in the $\alpha$ chains of type I, II, and III collagens, aligns opposite to the oxidizable, N-terminal telopeptide lysine of an adjacent tropocollagen unit within native fibrils, and, consistent with the charge preference of lysyl oxidase, is highly cationic (Seigel, 1976; Cronlund et al., 1985). It is not certain, however, that such tightly bound complexes of lysyl oxidase are identical with catalytically productive enzyme–substrate complexes. Indeed, as seen in this study, addition of heparin to preformed fibrils reduces sedimentable binding of enzyme by as much as 40% although there is no significant reduction in the rate of enzyme catalysis by corresponding concentrations of heparin. Thus, at least this fraction of tightly bound enzyme appears to be unrelated to catalytic events, pointing toward more than one mode of tight binding of the enzyme to this connective tissue protein.

In preliminary studies on the specificity of the effects noted here, we have observed that 0.4 mg $mL^{-1}$ concentrations of hyaluronate or chondroitin sulfate reduce the initial rate of collagen oxidation by 36% ± 3% and 38% ± 3%, respectively, regardless of whether the glycosaminoglycan is added either before or after fibril formation. These results point toward the conclusion that the binding of certain glycosaminoglycans other than heparin to preexistent native collagen fibrils may modulate the biosynthesis of cross-linkages in this connective tissue protein by interference with the expression of lysyl oxidase activity.

Although heparin is an anionic agent and anionic ligands of elastin can suppress the oxidation of this substrate by lysyl oxidase (Kagan et al., 1981), heparin had little effect on elastin oxidation as noted in the present study. This is likely due to the fact that heparin is hydrophilic since prior studies indicated that anionic amphiphiles must be sufficiently hydrophobic to bind to elastin to inhibit its oxidation (Kagan et al., 1981). The present results do raise the possibility, however, that heparin might direct the specificity of lysyl oxidase toward elastin at the exclusion of collagen by virtue of its inhibition of collagen oxidation. It would be of considerable interest if similar mechanisms were to contribute to the differential contents of cross-linked fibers of elastin or collagen found in various tissues in vivo. Although not explored in the present study, it is possible that specific glycosaminoglycans might influence elastin fibrillogenesis by altering the rate or extent of accretion of tropopelastin units to the nascent elastin fibril before lysine oxidation occurs. Indeed, dermatan and heparan sulfates have been found to be associated with $\beta$-aminopropionitrile-induced lathyritic elastin in chick embryo aorta (Pasquali-Ronchetti et al., 1983).

There have been several reports which indicate that the administration of heparin suppresses wound healing and scarring in burned, wounded, or surgically treated tissues (Pilcher & Barker, 1970; Stinchfield et al., 1956; Ohwiler et al., 1959; Frisbie, 1986; Rosenberg, 1977). This activity has been largely attributed to the anticoagulant effect of heparin resulting from its enhancement of the inhibition of proteases of the clotting cascade (Rosenberg, 1977; Tollefsen, 1984) which in turn might also account for the suppression by heparin of smooth muscle cell proliferation in tissue injury (Clowes & Karnovsky, 1973). While the concentrations of heparin resulting in the inhibition of collagen oxidation in vitro range from 0.1 to 1 mg $mL^{-1}$, it should be noted that heparin doses of up to 10 000 USP units per day may be administered to prevent postoperative deep venous thrombosis, while as much as 45 000 USP units per day have been administered for treatment of established thrombosis (Zimmerman & Plow, 1983). Since it is possible that cumulative binding effects may occur in vivo, the present results thus raise the possibility that such high doses of administered heparin may inhibit wound healing and reduce scar tissue formation at least partly by inhibition of the lysyl oxidase catalyzed oxidation of lysine in collagen, thereby preventing cross-linking of newly secreted collagen molecules into insoluble fibers.

**Registry No.** Lysyl oxidase, 9059-25-0; heparin, 9005-49-6.

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# Complete Amino Acid Sequence of Ovine Salivary Carbonic Anhydrase†

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ABSTRACT: The primary structure of the secreted carbonic anhydrase from ovine salivary glands has been determined by automated Edman sequence analysis of peptides generated by cyanogen bromide and tryptic cleavage of the protein and *Staphylococcus aureus* V8 protease, trypsin, and α-chymotrypsin subdigests of the large cyanogen bromide peptides. The enzyme is a single polypeptide chain comprising 307 amino acids and contains two apparent sites of carbohydrate attachment at Asn-50 and Asn-239. The protein contains two half-cystine residues at 25 and 207 which appear to form an intramolecular disulfide bond. Salivary carbonic anhydrase shows 33% sequence identity with the ovine cytoplasmic carbonic anhydrase II enzyme, with residues involved in the active site highly conserved. Compared to the cytoplasmic carbonic anhydrases, the secreted enzyme has a carboxyl-terminal extension of 45 amino acids. This is the first report of the complete amino acid sequence of a secreted carbonic anhydrase (CA VI).

Several different isozymes of carbonic anhydrase (CA)[1] (carbonate dehydratase, EC 4.2.1.1) have been reported from mammalian sources. The best characterized are the cytoplasmic isozymes termed CA I, CA II, and CA III (Tashian et al., 1983). Another type of isozyme has been isolated from bovine lung membranes (Whitney & Briggle, 1982) and human kidney membranes (Wistrand, 1984). These enzymes appear to be intrinsic membrane proteins and have been called CA IV. A fifth isozyme (CA V) has been isolated from the mitochondria of guinea pig hepatocytes (Dodgson et al., 1980) and its amino-terminal amino acid sequence determined (Hewett-Emmett et al., 1987). In 1979, we described an unusual carbonic anhydrase from the ovine parotid gland and saliva (Fernley et al., 1979) which does not seem to fit into any of these categories. Subsequently, this enzyme has been purified and characterized more thoroughly (Fernley et al., 1984, 1988). The equivalent enzyme has been purified also from rat saliva (Feldstein & Silverman, 1984) and more recently from human saliva (Murakami & Sly, 1987). These enzymes have apparent subunit molecular masses of 42 000–

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[1] Abbreviations: TFA, trifluoroacetic acid; CA, carbonic anhydrase; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin; Tris·HCl, tris(hydroxymethyl)-aminomethane hydrochloride; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid.

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