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INHIBITION OF LYSYL HYDROXYLASE BY CATECHOL ANALOGS

J.C. MURRAY, R.H. CASSELL and S.R. PINNELL

Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, N.C. 27710 (U.S.A.)

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Summary

Catechol analogs inhibit the activity of lysyl hydroxylase (peptidyllysine, 2-oxyglutarate: oxygen 5-oxidoreductase, EC 1.14.11.4), a microsomal enzyme which catalyzes the transformation of certain lysyl residues in collagen to hydroxylysine. Chick embryo lysyl hydroxylase activity was measured by specific tritium release as tritiated water from an L-[4,5-^{3}H]lysine-labelled unhydroxylated collagen substrate prepared from chick calvaria. Catechol analogs did not bind irreversibly to either enzyme or substrate, as full activity was restored with dialysis. Addition of excess cofactor, Fe^{2+}, ascorbic acid, or α-ketoglutarate, did not affect inhibition. Kinetic analysis revealed that with respect to collagen substrate, catechol demonstrated a noncompetitive type of inhibition with a K_i of 15 μM.

Introduction

Hydroxylysine is critically important for the structural function of collagen, which provides the strength and stability for the framework of all connective tissues. Hydroxylysine residues are essential for cross-linking between collagen molecules so that unique covalent linkages between hydroxylysine side chains and their respective aldehydes can lock collagen molecules into place [1—3]. In addition, hydroxylysine is an attachment site for carbohydrate to collagen, as galactose and glucosylgalactose are *O*-glycosidically linked to hydroxyl groups of various hydroxylysine residues [4,5]. Although the effect of glycosylation of hydroxylysine on cross-link formation is not entirely understood, the isolation of collagen cross-links with carbohydrate derivatives implies some functional or regulatory relationship [6,7].

Abbreviation: Roche 4-4602, DL-serine(2,3,4-trihydroxybenzyl—hydrazide.

Hydroxylysine is formed as a post-translational modification of certain lysine residues in collagen by the action of lysyl hydroxylase (peptidyllysine, 2-oxoglutarate: oxygen 5-oxidoreductase, EC 1.14.11.4). Lysyl hydroxylase is a mixed-function oxygenase requiring O_2, Fe^{2+}, ascorbic acid and α-ketoglutarate [8—12]. Although its enzymatic mechanism is unknown, the involvement of O_2 suggests that an oxygen intermediate, such as an oxygen radical, may be involved in the hydroxylation. Since epinephrine is a known scavenger of O_2^- [13] and an inhibitor of proline hydroxylase [14], we investigated its effect on lysyl hydroxylase activity. Although the identity of the reaction intermediates remains unknown, our present investigations characterize the inhibition of hydroxylysine formation by catechol analogs.

Methods

Lysine-labelled unhydroxylated collagen substrate was prepared by incubating calvaria from 24 16-day-old chick embryos at 37°C with 1 mM, α,α'-dipyridyl, 150 μg ascorbic acid, 300 units penicillin, 300 μg streptomycin in 3.0 ml of Dulbecco's lysine-free minimal essential media (Grand Island Biological Co.). 400 μCi of L-[4,5-^{3}H]lysine (New England Nuclear, spec. act. 1 mCi/ml) were added and the culture was incubated at 37°C for 24 h in a reciprocal water-bath shaker. The calvaria were homogenized with 8 ml distilled water using a Polytron (Brinkmann) at full speed for 1 min and the homogenate was centrifuged 15 000 × *g* for 1 h. The supernatant was dialyzed against 2 l of 20 mM Tris · HCl buffer adjusted to pH 7.6 at 4°C with three changes during 18 h. The substrate preparation was placed in boiling water for 10 min to remove inherent enzyme activity. The sample was centrifuged 15 000 × *g* for 30 min and the supernatant was stored at —70°C in 2-ml aliquots. Since different unhydroxylated collagen substrate preparations incorporated varying amounts of labelled-lysine residues, each experiment was run with the same substrate preparation.

The collagen content of the substrate was determined using a modified method of Peterkofsky and Diegelmann [15]. The same procedure was used with 25 units of Form III collagenase (Advance Biofacturers) per assay. The percent collagen present in a portion of analyzed substrate was determined on the basis that chick embryo type I collagen contains 3.4% lysine and hydroxylysine residues [16], while noncollagen proteins contain 6.5% lysine and no hydroxylysine [17]. The formula of Diegelmann and Peterkofsky [18] was modified to account for the fact that collagen contains 52% fewer lysine residues than other proteins.

$$\% \text{ collagen} = \frac{\text{dpm in collagenase digest}}{(\text{dpm in residue} \times 0.52) + (\text{dpm in collagenase digest})} \times 100$$

The preparation of lysyl hydroxylase was modified from the procedure of Kivirikko and Prockop [19]. Lysyl hydroxylase was prepared using 100 g of 16-day-old chick embryos that had been decapitated and eviscerated. For each gram of tissue, 1.0 ml of 0.2 M NaCl, 0.1 M glycine, 50 μM 1,4-dithiothreitol (Cyclo Chemical), 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C was added. The mixture was homogenized in a blender (Oster Corporation) at full

speed for 30 s and then by Polytron at full speed for 1 min. This homogenate was made 0.1% in Triton X-100 and stirred for 2 h at 4°C and then heated to 37°C for 15 min. The homogenate was centrifuged at 500 × *g* for 10 min and then the supernatant was centrifuged for 100 000 × *g* for 1 h. The supernatant was removed and solid $(NH_4)_2SO_4$ (Allied Chemical Co.) was slowly stirred into solution until a final concentration of 17% saturation. The solution was stirred for 1 h and was centrifuged at 15 000 × *g* for 20 min. The supernatant was removed and solid $(NH_4)_2SO_4$ was slowly stirred into solution to a final concentration of 45% saturation. The solution was stirred for 1 h and centrifuged at 15 000 × *g* for 20 min. The pellet was dissolved in 0.15 M NaCl, 0.1 M glycine, 50 μM 1,4-dithiothreitol and 20 mM Tris · HCl buffer adjusted to pH 7.4 at 4°C. The enzyme preparation in a volume of 50 ml was dialyzed against 4 l of the same buffer with three changes during 18 h. The sample was diluted to a protein concentration of 30 mg/ml and was centrifuged at 15 000 × *g* for 30 min. The supernatant was stored at −70°C. Protein concentration was determined by the spectrophotometric method of Layne [20]. Purification was approximately two fold when compared to the original extract.

Lysyl hydroxylase activity was measured by a tritium release assay [21]. The incubation mixture contained in a volume of 1.5 ml, L-[4,5-^{3}H]lysine collagen substrate (6.0×10^5 dpm), partially purified lysyl hydroxylase 0.5—1.5 mg, 50 mM Tris · HCl pH 7.8, 0.5 mM α-ketoglutarate, 0.05 mM $FeSO_4$, 0.5 mM ascorbic acid, 0.1 mM 1,4-dithiothreitol, 2.25 mg bovine serum albumin and 0.15 mg catalase (Calbiochem). The reaction was initiated by addition of substrate, and samples were incubated at 37°C for 90 min. The reaction was terminated by addition of 0.1 ml of 50% trichloroacetic acid. Tritiated water was collected by vacuum distillation. 1.0 ml of the distilled sample was mixed with 10 ml of Aquasol (New England Nuclear) for counting in a Packard Tri-Carb liquid scintillation counter. The counting efficiency was 23%.

Solutions of inhibitor $1.5 \cdot 10^{-3}$ M (catechol, ephedrine, L-epinephrine bitartrate, L-norepinephrine bitartrate, phenylalanine, pyrogallol, L-tyrosine, (Sigma Chemical Co.); phenylephrine · HCl, (Winthrop Lab.); dopamine · HCl, (Arnar Stone); adrenochrome (Calbiochem); DL-serine-(2,3,4-trihydroxybenzyl)-hydrazide (Roche 4-4602) (Hoffman-LaRoche)) were prepared with distilled H_2O and 0.1 ml of inhibitor solution was added to the assay system after addition of all other components. Other concentrations of inhibitors were obtained by diluting the stock solutions of inhibitors and adding 0.1 ml of the diluted inhibitor solution to the assay system after addition of all other components.

To determine whether catechol binds irreversibly to either partially purified lysyl hydroxylase or the unhydroxylated collagen substrate, the following experiment was designed. Catechol ($2 \cdot 10^{-4}$ M) was preincubated with either enzyme preparation (15 mg) or collagen substrate (3.0×10^7 dpm). The volume of each incubation mix was 1.6 ml and the solutions were agitated for 90 min in a 37°C water bath. An aliquot (0.3 ml) was taken from each mixture for assay. The concentration of catechol during pre-incubation was $2 \cdot 10^{-4}$ M, so that the assay concentration was $4 \cdot 10^{-5}$ M. The remainder of the incubation solutions were dialyzed at 4°C against 4 l of 0.15 M NaCl, 20 mM Tris · HCl pH 7.4 with five changes over 48 h. Aliquots (0.3 ml) were taken for

assay. Controls for these experiments were incubation mixtures without catechol before and after dialysis.

The effect of excess Fe^{2+}, ascorbic acid and α-ketoglutarate was investigated by adding 2.5- and 5-fold excess cofactor concentration to the complete assay system before addition of catechol ($5 \cdot 10^{-5}$ M).

Kinetic analysis was performed with a modified assay system without catalase, bovine serum albumin, and 1,4-dithiothreitol. The modified assay system contained 0.7 mg lysyl hydroxylase and 1.8×10^7 dpm/ml unhydroxylated collagen substrate. The system without dithiothreitol, catalase and albumin gave 83% of whole-system activity. The assay was incubated at 37°C for 60 min. All lines were plotted according to the least-squares method [22]. Analysis of the unhydroxylated collagen substrate preparation by the collagenase-digestion method revealed that 69% of the protein was collagen. In an assay with 0.066 ml collagen substrate, approximately 2.0×10^5 dpm or ($1.8 \times 10^7 \times 0.066 \times 0.69 \times \frac{1}{4}$) dpm of lysine residues were available for hydroxylation. Based on the premise that seven of a total 34 lysine residues are hydroxylated [16], the assay with 0.066 ml substrate released 2900 Δ dpm which indicated the maximum extent (1.5%) of hydroxylation.

Results

Hydroxylysine formation was linear with protein content up to 2.0 mg and with time through 90 min. The difference in dpm between the whole system and the whole system without α-ketoglutarate was designated the enzyme activity. Since α-ketoglutarate is a cosubstrate for this reaction, and dpm for the assay system without α-ketoglutarate remain constant throughout 5 h, the assay system without α-ketoglutarate was designated the background control due to the specificity of α-ketoglutarate for lysyl hydroxylase activity.

The addition of catechol analogs inhibited the formation of hydroxylysine. 50% inhibition of hydroxylysine formation was obtained (Fig. 1) with $3 \cdot 10^{-5}$ M catechol, $2 \cdot 10^{-5}$ M dopamine, $6 \cdot 10^{-5}$ M epinephrine, and $1 \cdot 10^{-5}$ M Roche 4-4602. Several related compounds were investigated in an attempt to identify the structural requirement for inhibition of lysyl hydroxylase. The catechol moiety appeared to be necessary. The inhibitory effect was related to the number of hydroxyl groups present on the benzene ring (Table I). Trihydroxybenzene derivatives such as pyrogallol and Roche 4-4602 were the most potent inhibitors, with 98.0% and 98.9% inhibition respectively at $1 \cdot 10^{-4}$ M. 3,4-Dihydroxybenzene derivatives such as epinephrine and catechol inhibited 80.4% and 75.5% respectively at $1 \cdot 10^{-4}$ M. Monohydroxybenzene derivatives such as tyrosine and phenylephrine inhibited 17.0% and 19.0% respectively at $1 \cdot 10^{-4}$ M. The percent inhibition declined as the number of hydroxyl groups on the benzene ring decreased from three to two to one.

After preincubation of catechol with either substrate or enzyme, dialysis effectively restored complete activity. Before dialysis, aliquots from a preincubation mixture of lysyl hydroxylase and catechol ($2 \cdot 10^{-4}$ M) demonstrated 45% of control activity. Following dialysis, aliquots from these mixtures demonstrated nearly the same activity (97%) as that of aliquots from mixtures containing no inhibitor. When a similar experiment was carried out preincubat-

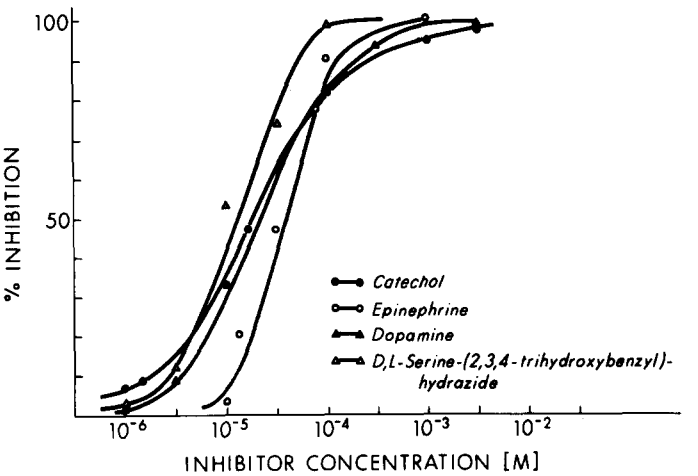

Fig. 1. Inhibition of lysyl hydroxylase activity by varying concentration of catechol (●), dopamine (▲), epinephrine (○), and Roche 4-4602 (△). Assays were performed as described in Methods.

ing the unhydroxylated collagen substrate with catechol ($2 \cdot 10^{-4}$ M), dialysis increased activity from 35% to 94%.

The addition of excess cofactors Fe^{2+}, ascorbic acid and α-ketoglutarate did not affect the inhibition of lysyl hydroxylase by catechol. With lysyl hydroxylase activity 63% inhibited by $5 \cdot 10^{-5}$ M catechol, the separate addition of each cofactor, resulting in 2.5 mM α-ketoglutarate, 0.25 mM $FeSO_4$ or 2.5 mM ascorbic acid, did not affect the inhibition by catechol more than 3%. When both 0.25 mM $FeSO_4$ and 2.5 mM ascorbic acid were added to the same $5 \cdot 10^{-5}$ M catechol-inhibited system, lysyl hydroxylase activity was 9% greater than the same system without excess cofactors.

Kinetic analysis (Fig. 2) revealed a noncompetitive type of inhibition by catechol with respect to varying collagen substrate concentrations. The reciprocal plots were straight lines converging to intersection to the left of the $1/V$ axis and just below or near the $1/S$ axis. Both the slope and y-intercept of these

TABLE I

REDUCTION OF LYSYL HYDROXYLASE ACTIVITY

The reaction mixture was prepared as described in the text with the inhibitor being the final addition to the system. % reduction was calculated with respect to the control assay mixture without inhibitor.

Compound (10^{-4} M)	% Reduction
Roche-4-4602	98.9
Pyrogallol	98.0
Epinephrine	80.4
Norepinephrine	76.7
Catechol	75.5
Phenylephrine	19.0
Tyrosine	17.0
Adrenochrome	13.0
Phenylalanine	10.5
Ephedrine	10.0

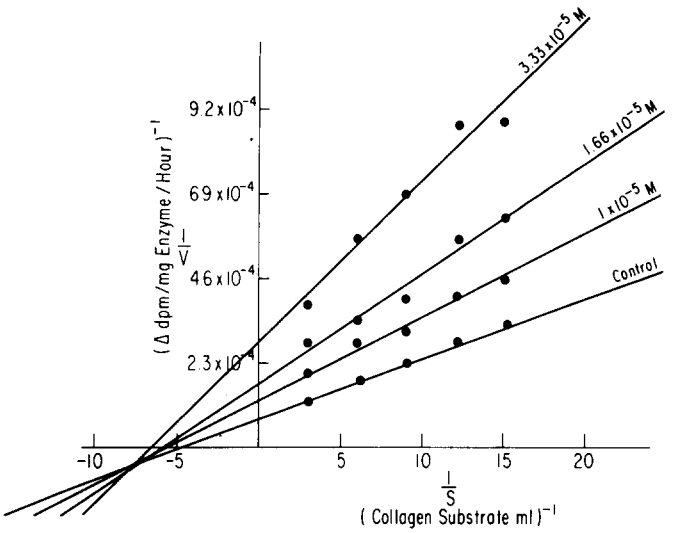

Fig. 2. Double reciprocal plots of initial rate collagen lysyl hydroxylation and catchol concentration. The modified assay system is described in Methods.

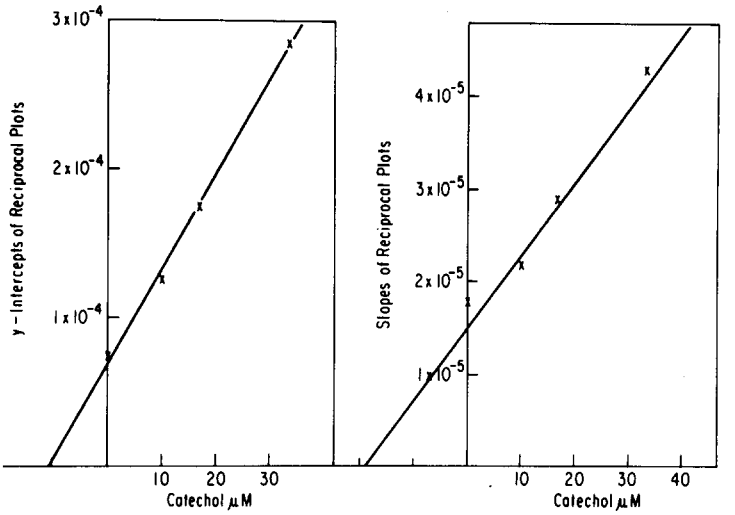

Fig. 3. Replots of *y*-intercepts and slopes from double reciprocal plots versus catechol concentration.

reciprocal lines varied with inhibitor concentration as shown by the replots (Fig. 3). The linearity of the replots implied that, if catechol does bind to the enzyme, it does not bind to the same enzyme form which combines with collagen substrate [23]. The K_i calculated from these replots was 15 μM.

Discussion

Lysyl hydroxylase catalyzes the unique post-transitional modification of certain lysyl residues in collagen to hydroxylysine. Since hydroxylysine par-

ticipates directly in intermolecular cross-linking necessary for the structural stability of connective tissue, the regulation of hydroxylysine formation would be expected to influence the integrity of synthesized collagen. Previous investigations have shown that hydroxylysine formation may be inhibited by metal chelators such as α,α'-dipyridyl or anaerobiosis [24]. The toxicity of these conditions limits their pharmacological usefulness [25]. We are proposing an alternative approach to inhibit hydroxylysine formation and subsequently to modify collagen metabolism.

The requirements for hydroxylysine formation are Fe^{2+}, O_2, α-ketoglutarate and a reducing agent such as ascorbic acid. In studies of prolyl hydroxylase, which shares similar requirements, properties and intracellular locations with lysine hydroxylase, the oxygen of the hydroxyl group of hydroxyproline has been shown to be derived from atmospheric oxygen and not from water by the use of $^{18}O_2$ [26,27]. Although no studies with $^{18}O_2$ and lysine hydroxylation have been reported, the similarities between lysyl and prolyl hydroxylases imply that the oxygen of the hydroxyl group in hydroxylysine may have a similar origin. It might be expected that these reactions would involve an oxygen intermediate which is not tightly bound to the enzymes and that it would be possible to inhibit the reactions by trapping this oxygen intermediate. We felt that a good candidate for such an oxygen intermediate was the superoxide radical anion, which might be removed by such compounds as epinephrine [13] and nitro blue tetrazolium [28], as well as by superoxide dismutase. Epinephrine and nitro blue tetrazolium have previously been shown to inhibit prolyl hydroxylase [14]. We have shown that epinephrine and other catechol analogs, as well as nitro blue tetrazolium, inhibit lysyl hydroxylase [29]. However, the evidence for superoxide as an intermediate in the reactions remains circumstantial, since attempts by ourselves * and others [30] to demonstrate inhibition of lysyl or prolyl hydroxylase by superoxide dismutase have been unsuccessful.

Although the mechanism of inhibition of lysyl hydroxylase by catechol analogs is not entirely clear, several characteristics have been observed. Of the catechol analogs which inhibited lysyl hydroxylase activity, the critical structural feature appeared to be the number of hydroxyl groups on the benzene ring. For some unknown reason the inhibitory efficacy of catechol analogs increased with increasing hydroxyl groups on the benzene ring, so that two hydroxyl groups were necessary for at least 75% inhibition at $1 \cdot 10^{-4}$ M. Since the catechol moiety appeared crucial for inhibition of lysyl hydroxylase, further investigations of the inhibition were conducted with catechol. Catechol did not irreversibly modify unhydroxylated collagen substrate or lysyl hydroxylase, since dialysis effectively removed the inhibition. The apparent availability of cofactors or cosubstrate was not diminished by catechol, since the addition of excess cofactors did not affect the inhibition of lysyl hydroxylase. Preliminary kinetic analysis revealed a linear noncompetitive type of inhibition for catechol with a K_i of 15 μM. Catechol apparently did not affect collagen substrate binding to lysyl hydroxylase, and the degree of inhibition of hydroxy-

* Murray, J.C., Pinnell, S.R. and Cassell, R.H., (1977) manuscript in preparation

lysine formation by catechol depended upon the inhibitor concentration and K_i.

The inhibition of hydroxylysine formation potentially has clinical significance. Numerous clinical conditions, including pulmonary fibrosis, liver cirrhosis, hypertrophic scars and keloids, are characterized by excessive fibrosis and scarring. If hydroxylysine formation and subsequent intermolecular cross-linking could be controlled pharmacologically, collagen accumulation might be prevented. Since collagenase is more efficient against poorly cross-linked or uncross-linked collagen, such modified collagen would be expected to be efficiently degraded [31].

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