INHIBITION BY COLLAGEN CHAINS OF LYSYL HYDROXYLASE OF CHICK EMBRYO AND OF WI-38 HUMAN LUNG FIBROBLASTS

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Received March 16,1976

SUMMARY

Lysyl hydroxylase from chick embryos was strongly inhibited by heat-denatured collagens from various vertebrate sources, and by separated α chains and β components of rat tail tendon collagen. The kinetics exhibited with this enzyme when heat-denatured calf or rabbit skin collagens was used showed a mixed type of inhibition. On the other hand, a preparation of homologous heat-denatured 4,5-^{3}H-L-lysine-labeled collagen, in itself an extremely poor substrate for the hydroxylase, showed non-competitive inhibition with a K_i of about 8-9 μM. Finally, the lysyl hydroxylase preparations from WI-38 fetal human lung fibroblasts and from transformed WI-38 cells (WI-38 VA 13) were also inhibited by heat-denatured collagens or, where tested, by separated collagen chains.

INTRODUCTION

Lysyl hydroxylase, also called protocollagen lysyl hydroxylase or collagen lysyl hydroxylase is a dioxygenase that catalyzes the hydroxylation of lysine residues in pro-α chains of collagen as they are synthesized on polyribosomes (for reviews on the hydroxylation of certain prolyl and lysyl residues already incorporated in peptide linkages, see refs. 1-4). The enzyme requires α-ketoglutarate as a cosubstrate and ferrous ion as an activator; it also has a dependence on ascorbate or another reducing substance (5,6). It can hydroxylate lysine residues in an experimentally produced "precursor" of collagen chains called "protocollagen", a material identical with the chains of collagen except that neither its proline or hydroxylysine residues have been hydroxylated. The enzyme also can hydroxylate lysine residues in synthetic polypeptides containing sequences of X-Lys-Gly (7). Because the cofactor requirements of collagen lysyl and prolyl hydroxylases are identical, at one time the two activities were presumed due to a single enzyme (5),

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but indeed selective inhibition studies are able to distinguish the two. Thus, a preparation containing both activities is inhibited in its action against ^{14}C-proline residues in protocollagen by poly-L-proline, $(gly\text{-}pro\text{-}pro)_n$, or reduced carboxymethylated collagen of Ascaris (RCM-Ascaris collagen) (8). These materials do not inhibit collagen lysyl hydroxylase activity in the same preparation. Thus the two activities are due to separate enzymes, and in fact have been separated from one another by column chromatography (6, 9, 10).

In a previous communication (11) we reported that prolyl hydroxylase is inhibited by heat-denatured collagens and by separated α-chains of collagen. In the present paper evidence is provided that lysyl hydroxylase is inhibited similarly, and the nature of the inhibition is described.

MATERIALS AND METHODS

4,5-^{3}H-L-Lysine and 3,4-^{3}H-L-proline were obtained from the New England Nuclear Corp. Ichthyocol (carp swim bladder collagen), and the skin collagens of calf, guinea pig and rabbit were gifts of Dr. S. Takahashi. Pure α1 and α2 chains, as well as β_{11} components, of rat tail tendon collagen were supplied kindly by Dr. O.O. Blumenfeld. RCM-Ascaris collagen was purchased from Applied Science Laboratories, Inc. Purified collagenase of Clostridium histolyticum (4 units per mg) was obtained from Boehringer Mannheim-Gmbh.

The RCM-Ascaris collagen was coupled to Sepharose by a procedure based on that described by Berg and Prockop (12). The collagen-Sepharose was shown to have a ratio of Asp:Gly:Pro identical with the ratio found in the uncoupled RCM-Ascaris collagen. The amount of glycine in bound form in the coupled material was 3 μmoles per ml of packed Sepharose used.

Enzyme preparations: Partially purified chick embryo lysyl hydroxylase was prepared according to the method of Kivirikko and Prockop (6). First, a fraction precipitating between 17-45% saturation with ammonium sulfate was prepared. That material was redissolved at 4° in a buffer consisting of 0.15 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris-HCl at pH 7.4 (hereafter referred to as the "working buffer"), and exhaustively dialyzed against the same buffer. When assayed, this material had both hydroxylase activities, but was especially rich in prolyl hydroxylase. To minimize the complications of competition for the protocollagen substrate in which both the prolyl and lysyl residues are essentially unhydroxylated, some of the kinetic experiments required that the level of prolyl hydroxylase activity be reduced. Accordingly the enzyme preparation was subjected to differential affinity chromatography as follows.

Berg and Prockop (12) had prepared an RCM-Ascaris collagen-Agarose column to which prolyl hydroxylase presumably was bound selectively, and were able to elute the enzyme with $(pro\text{-}gly\text{-}pro)_n$. The enzyme was free of lysyl hydroxylase activity. By a variation of these procedures we were able to obtain a lysyl hydroxylase preparation with a significantly reduced level of prolyl hydroxylase activity. For this purpose, the crude dialyzed ammonium sulfate fraction was diluted with the working buffer to a protein concentration of 8.4 mg per ml, and the solution clarified by centrifugation at 30,000 x g for 15 minutes. The supernate was then passed through a series of 4 columns connected one with another in a vertical arrangement; each column contained 1 ml of packed RCM-Ascaris collagen-Sepharose previously equilibrated with buffer. Elution was performed

with working buffer at a flow rate of 0.25 - 0.30 ml per min and fractions of 1 ml each were collected. The first 4 fractions to emerge were discarded, and the succeeding fractions assayed for both prolyl and lysyl hydroxylase. Less than 4% of the initial prolyl hydroxylase activity was eluted whereas up to 31% of the lysyl hydroxylase activity was recovered. This resulted in a considerable enrichment of lysyl hydroxylase relative to prolyl hydroxylase activity.

Crude cell free extracts of WI-38 and transformed WI-38 VA 13 subline 2RA fibroblasts were obtained using growth, detaching, washing, harvesting and ultrasonic disrupting procedures previously described (11).

Preparation of 4,5-^{3}H-L-lysine-labeled protocollagen and collagen: Lysine-labeled protocollagen was prepared by incubating tibias from 11-day old chick embryos with 4,5-^{3}H-L-lysine (54 mCi/μmole) in the presence of α,α'-dipyridyl and isolated using a procedure (13) as slightly modified in a previous study (11) for the preparation of 3,4-^{3}H-proline-labeled protocollagen. After incubation, the homogenized tibia suspension was centrifuged at 100,000 x g for 45 minutes, the supernate dialyzed against 20 mM Tris-HCl at pH 7.6 and autoclaved for 20 minutes at 120° C. Insoluble material was removed by centrifugation and the supernate redialyzed against the same buffer. Preparations of protocollagen varying in specific activity from 2.7 to 6.6 x 10^6 cpm per mg were obtained routinely.

Using the same procedure for incubation and processing of tibia, except that α,α'-dipyridyl was not added to inhibit hydroxylation, and instead additions were made to stimulate it (0.5 mM L-ascorbate, 0.1 mM α-ketoglutarate and 0.1 mM Fe $(NH_4)_2(SO_4)_2$), lysine-labeled collagen was prepared and autoclaved. This preparation, as distinct from "denatured" protocollagen, is referred to as "fully hydroxylated" denatured collagen, and prepared thus with radioactive lysine, had a specific activity of 5.1 - 5.8 x 10^6 cpm per mg.

Samples of the ^{3}H-labeled protocollagen and collagen preparations were digested with collagenase and fractionated on a Dowex 50 WX2 or Type P chromobeads ion exchange (Technicon chemicals, S.A.) column to determine characteristic collagen or protocollagen tripeptides. Using a modification of a published procedure (14), a kinetic study was made of the release by collagenase of radioactivity into an acid medium (5% trichloroacetic acid + 0.25% tannic acid). From the results obtained, respective preparations were estimated to be greater than 60% of protocollagen or collagen.

Assay of lysyl hydroxylase activity: Hydroxylation of the labeled lysine residues in autoclaved protocollagen was measured by the detritiation method of Miller (15). The detritiation method was used for assay of prolyl hydroxylase with an appropriate substrate labeled in its proline residues (16, 11).

For the assay of lysyl hydroxylase the reaction mixture contained the following in a final volume of 0.5 ml: Tris-HCl, 24 μmoles at pH 8.0; Fe $(NH_4)_2(SO_4)_2$, 0.25 μmole; L-ascorbate, 0.5 μmole; α-ketoglutarate, 0.25 μmole; dithiothreitol, 0.05 μmole; bovine serum albumin, 1 mg; crystalline catalase (20 mg per ml), 10 μl; and 200 μl of a solution of autoclaved lysine-labeled protocollagen of specific activity as will be indicated. Reaction was initiated by addition of the preparation containing lysyl hydroxylase, and the mixture incubated for 60 minutes at 30° C. Reaction was terminated by addition of 10 μmoles of α,α'-dipyridyl-HCl. The assay mixture was then transferred to the side-arm of a Thunberg tube and the water collected by sublimation under high vacuum. Radioactivity was measured with 54% efficiency in a scintillation counting spectrometer and corrected for non-enzymatic release of ^3H_2O from the substrate. Using a saturating concentration of substrate, the enzymatic activity was linear with time for about 2 hours, and proportional to the concentration of enzyme when protein concentrations varied between 0.4 - 1.2 mg.

Preparation of heat denatured collagen solutions: Except for ichthyocol that was gelatinized at 50°C, all other collagen preparations in solution (1 mg/ml) were autoclaved at 120° C for 15 minutes. Insoluble material was removed by centrifugation, and the protein concentration in the supernate determined according to Lowry et al. (17). The amounts of collagens are expressed as equivalents of bovine serum albumin although, on a weight basis, collagens yield only about 50% of color given by albumin.

RESULTS AND DISCUSSION

Results summarized in Table I show that heat-denatured collagens of carp swim bladder and from the skins of several vertebrate species, as well as separated α chains and β components of rat tail tendon collagen, strongly inhibited the lysyl hydroxylase of a preparation obtained from chick embryos. RCM-Ascaris collagen, however, had no such inhibitory effect, as had already been demonstrated by Weinstein et al. (8). Ascaris collagen is known to have no hydroxylysine residues, and presumably no hydroxylatable lysine residues. These inhibitory effects of denatured collagens and of collagen chains were obtained both with lysyl hydroxylase preparations of high prolyl hydroxylase activity (preparations carried only through the ammonium sulfate fractionation step) and with others relatively freed of prolyl hydroxylase activity by differential affinity chromatography.

Collagens isolated from various sources may, in their heat-denatured forms, undergo further in vitro enzymatic hydroxylation of their lysine residues (18). The observed inhibition of lysyl hydroxylase by exogenous heat-denatured collagen might then perhaps represent a dilution of radioactive substrate rather than a true inhibition. To examine this possibility, "fully hydroxylated" 4,5-^{3}H-L-lysine-labeled collagen was prepared and tested both as substrate and inhibitor for the lysyl hydroxylase of chick embryos. Table II (line 2) shows that ^{3}H-lysine-labeled homologous heat-denatured collagen could itself be only hydroxylated further in vitro to the extent of 9% of that by which the labeled denatured protocollagen could be hydroxylated (Table II, line 1). When the homologous denatured collagen was added to the incubation mixture containing denatured protocollagen, lysyl hydroxylase activity was inhibited by about 31% (line 3 of the Table). In the same table homologous denatured lysine-labeled collagen is shown to have inhibited a prolyl hydroxylase preparation by about 40%; in a previous publication we had

TABLE I

Effects of Collagens and Collagen Chains on Lysyl Hydroxylase Activity from Chick Embryos

Additions	Cpm of ^3H_2O Released	% Inhibition
None	2,038	-
Ichthyocol, 30 µg	257	87
60 µg	116	94
Calf skin collagen, 22 µg	1,175	42
44 µg	853	58
Guinea pig skin collagen, 25 µg	1,142	44
50 µg	862	58
Rabbit skin collagen, 23 µg	1,372	33
46 µg	1,019	50
RCM-Ascaris collagen, 41 µg	2,252	-
82 µg	2,285	-
RTT α_1 - chain, 22 µg	451	78
44 µg	247	88
RTT α_2 - chain, 19 µg	484	76
38 µg	307	85
RTT β_{11} - component, 17 µg	373	82

The reaction mixtures were set up as described under Materials and Methods and contained 94 µg of 4, 5-^{3}H-L-lysine protocollagen (6.3×10^6 cpm/mg) and 0.66 mg of a freshly prepared chick embryo lysyl hydroxylase preparation carried through the ammonium sulfate fractionation step.

reported a greater degree of inhibition of prolyl hydroxylase by another denatured collagen preparation labeled with radioactive proline.

Table III shows that lysyl hydroxylase of WI-38 cells of two different passage numbers, of "intermediate" and "old" age in terms of this model of aging, were inhibited similarly by

TABLE II

Inhibition of Lysyl and Prolyl Hydroxylases from Chick Embryos by 4, 5-^{3}H-L-Lysine-Labeled Collagen

Substrate	Cpm of ^3H_2O Released	
	Lysyl Hydroxylase	Prolyl Hydroxylase
1) Lysine-labeled autoclaved protocollagen (6.6×10^6 cpm/mg, 160 µg)	4,619	
2) Lysine-labeled autoclaved collagen (5.8×10^6 cpm/mg, 160 µg)	432	
3) 1) plus 2)	3,494	
4) 3, 4-^{3}H-L-proline-labeled autoclaved protocollagen (15×10^6 cpm/mg, 84 µg)		5,528
5) 4) plus 2)		3,362

The reaction mixtures for lysyl hydroxylase activity were set up as described under Materials and Methods and the enzyme used (0.90 mg in each assay) was a preparation carried through the ammonium sulfate fractionation step. The preparation of prolyl hydroxylase from chick embryos and the conditions of its assay were as previously described (11). The enzyme (0.16 mg in each assay), stored in the frozen state for several months, had retained full prolyl hydroxylase activity and was free of lysyl hydroxylase activity.

heat-denatured rabbit skin collagen. In addition, transformed WI-38 cells of the line WI-38 VA 13, examined in their logarithmic phase of growth, also were inhibited with respect to lysyl hydroxylase activity by this heat-denatured collagen; and, as observed with the chick embryo enzyme (Table I), more effective inhibition occurred when separated α2 chains of rat tail tendon collagen were used. Similar inhibition of prolyl hydroxylase of the two kinds of cells by heat-denatured collagens was reported previously (11).

The K_m of protocollagen as prepared here and used as a substrate for lysyl hydroxylase of the chick embryo was approximately 1 µM (Figure I). That is of the same order of magnitude

TABLE III

Effect of Collagens and Collagen Chains on Lysyl Hydroxylase Activity of WI-38 Fibroblasts and Transformed WI-38 VA 13 Cells

Source of Crude Extracts	Additions	Cpm of ^3H_2O Released	% Inhibition
WI-38 fibroblasts, passage 21, at confluency	None	949	-
	Rabbit skin collagen, 93 µg	495	48
WI-38 fibroblasts, passage 47, at confluency		1,479	-
	Rabbit skin collagen, 93 µg	528	64
WI-38 VA 13 cells, in logarithmic phase of growth	None	525	-
	Rabbit skin collagen, 93 µg	171	67
	Rat tendon tail α_2-chain, 120 µg	29	94

Assay mixtures as described under Materials and Methods containing 128 µg of 4,5-^{3}H-L-lysine protocollagen (2.65×10^6 cpm/mg) as substrate. The amount of crude extract protein in each assay was as follows: WI-38 fibroblasts from passage 21, 330 µg; WI-38 fibroblasts from passage 47, 300 µg; WI-38 VA 13 cells, 252 µg.

as the K_m of another preparation of protocollagen used as a substrate for chick embryo prolyl hydroxylase (11). Figure I also describes the nature of the inhibition of lysyl hydroxylase by the various denatured collagens used. The homologous collagen prepared from chick embryo tibia (incidentally labeled with ^{3}H-lysine), inhibited lysyl hydroxylase non-competitively with a K_i of 8 - 9 µM (Figure IA). However, the inhibition by either denatured calf skin collagen or rabbit skin collagen was of a mixed kind as indicated by the complex kinetics shown in Figure IB. Whether this difference in the nature of inhibition is due to an intrinsic difference between the chick embryonic collagen and the skin collagens or, alternatively, to an influence stemming from possible differences in crosslinking, cannot be stated at this time. Of interest is that inhibition

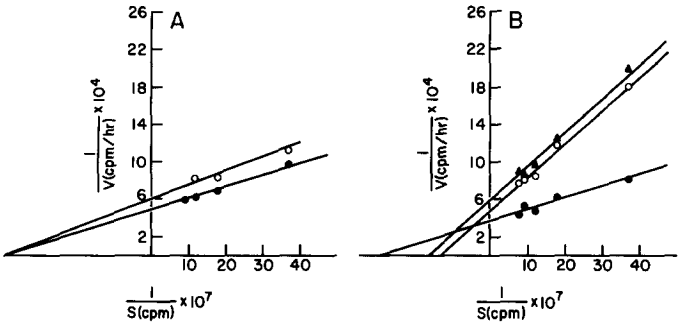

Fig. 1. Inhibition of chick embryo lysyl hydroxylase by homologous "fully hydroxylated" collagen, calf skin collagen and rabbit skin collagen. 4, 5-^{3}H-L-Lysine-labeled protocollagen with a specific activity of 6.6×10^{6} cpm was used as the substrate in all experiments. The enzyme used was a preparation eluted from an RCM-Ascaris collagen-Sepharose column, and concentrated approximately 3-fold by ultrafiltration through an Amicon PM-30 membrane. Prior to the affinity chromatography step, the preparation had a relative ratio of prolyl to lysyl hydroxylase activity of 17.3 to 1; this ratio was reduced to 2.5:1 in the eluate emerging from the column. The reaction mixtures contained 0.5 mg of enzyme protein with all other assay components as described under Materials and Methods. A: ●——●, no inhibitor; o——o, 94 µg of homologous collagen, incidentally labeled with ^{3}H-lysine (cf. Table II). Specific activity 5.1×10^{6} cpm/mg. B: ●——●, no inhibitor; o——o, 21 µg of calf skin collagen; ▲——▲, 23 µg of rabbit skin collagen.

of the lysyl hydroxylase by the denatured homologous collagen was noncompetitive, and that indeed this same collagen was a very poor substrate for further hydroxylation of lysine residues (see Table II). In general, lysyl residues of isolated and denatured collagens can be hydroxylated to a further extent in vitro (18), and this may indeed make the kinetics of inhibition more complex.

Experimentally, most tissues studied show greater prolyl hydroxylase than lysyl hydroxylase activity (see refs. 1-4 and 19). Those observations accord with results in the present study with chick embryo and WI-38 hydroxylases. Since even the lysyl hydroxylase purified by affinity chromatography contains residual prolyl hydroxylase activity, and the substrate used has both hydroxylatable proline and lysine residues, the order of hydroxylation could not be inferred from these studies. Recent evidence suggests that both prolyl and lysyl hydroxylases are associated with the rough endoplasmic reticulum of tendon and cartilage cells (20). The reported presence

in nascent procollagen peptides of hydroxyproline and also hydroxylysine (21, 22) has been extended by findings that glycosylation also appears to be initiated in the course of collagen synthesis on the ribosomes (23). In the case of nascent procollagen chains, therefore, the matter whether lysine and proline residues are hydroxylated independently or dependently is of considerable interest. At this time no information exists concerning even whether an ordered and dependent priority governs the hydroxylation of one lysine or proline residue vis a vis another.

In conclusion, the observations that both hydroxylase activities are inhibited by denatured collagens(that contain some separated α chains), and indeed by separated α chains and β components per se, may be relevant to the physiological regulation of collagen synthesis. Conceivably the accumulation of hydroxylated chains within a cell making collagen could affect the rate or perhaps turn off further collagen chain synthesis.

Acknowledgments. This work was supported by Grant NIH 1P01 HD07173. The WI-38 starter cultures (cell packs) used in these studies were obtained through contract N01-HD-4-2828, Stanford University, from the National Institute of Aging. The authors express their thanks to Dr. Sam Seifter for his interest and help in the preparation of this manuscript.

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