Studies on Protocollagen Lysine Hydroxylase. Hydroxylation of Synthetic Peptides and the Stoichiometric Decarboxylation of α-Ketoglutarate*

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ABSTRACT: A series of synthetic peptides was prepared and used to study protocollagen lysine hydroxylase, the enzyme which synthesizes the hydroxylysine in collagen. The enzyme was purified 150- to 250-fold from chick embryo extracts and it was completely free of protocollagen proline hydroxylase activity. Several synthetic peptides competitively prevented the hydroxylation of lysine-labeled [^{14}C]protocollagen by protocollagen lysine hydroxylase. When the peptides were examined directly as substrates for the enzyme, the synthesis of measurable amounts of hydroxylysine was observed for the first time. The synthesis of hydroxylysine was accompanied by the conversion of α-ketoglutarate to CO_2 and succinate, indicating that the enzyme belongs to the new class of oxygenases which decarboxylate α-ketoglutarate during substrate hydroxylation. The release of [^{14}C]CO_2 from [1-$^{14}C_1$]α-ketoglutarate was equimolar with the synthesis of hydroxylysine and therefore the amount of [^{14}C]CO_2 released could be used as a simple assay for the enzymic reaction. Three peptides were synthesized which had amino acid sequences comparable to amino acid sequences around glycosylated hydroxylysine in collagen. The peptide "L-I" had the sequence Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly, "L-II" the sequence Ala-Arg-Gly-Met-Lys-Gly-His-Arg-Gly-(Pro-Pro-Gly)$_4$, and "L-III" the sequence (Pro-Pro-Gly)$_4$-Ala-Arg-Gly-Met-Lys-Gly-His-Arg-Gly-(Pro-Pro-Gly)$_4$. When the peptides were used as substrates for protocollagen lysine hydroxylase, the V_{max} for all three peptides was the same. The K_m for L-I was twice the K_m for L-II but there was no difference in the K_m for L-II and L-III. In contrast, when L-II and L-III were compared as substrates for protocollagen proline hydroxylase, the K_m for L-II was three times the K_m for L-III. Free lysine and the tripeptide Lys-Gly-Pro did not serve as substrates for the synthesis of hydroxylysine. A very slow rate of hydroxylysine synthesis was observed with the tripeptide Ile-Lys-Gly, whereas the rate observed with the hexapeptide (Ile-Lys-Gly)$_2$ was over ten times greater. Also, lysine^8-vasopressin with the terminal sequence -Cys-Pro-Lys-Gly-NH_2 was a substrate for the synthesis of hydroxylysine. However, the V_{max} and K_m values for (Ile-Lys-Gly)$_2$ and lysine^8-vasopressin indicated that they were poorer substrates than L-I, L-II, or L-III. Reduced and carboxymethylated collagen from the cuticle of *Ascaris lumbricoides* and peptides derived from this collagen did not interact with the enzyme, even though the collagen contains about 40 residues of lysine/1000 residues and no hydroxylysine. The data obtained with the various peptides indicated that both the amino acid sequence around lysine and the length of the chain are critical determinants in the synthesis of hydroxylysine by protocollagen lysine hydroxylase.

The hydroxylysine in collagen is synthesized by the hydroxylation of lysine in a polypeptide precursor of collagen called protocollagen (for review, see Grant and Prockop, 1972). The hydroxyproline in collagen is synthesized by the hydroxylation of proline in the same polypeptide precursor and the presence of protocollagen proline hydroxylase has now been demonstrated in a number of animal tissues. Protocollagen proline hydroxylase does not hydroxylate free proline, but it hydroxylates polymers of the structure (X-Pro-Gly)$_n$ in which "X" is proline, alanine, or a variety of other amino acids but not glycine (Prockop *et al.*, 1967; Kivirikko and Prockop, 1967c; Nordwig *et al.*, 1967; Hutton *et al.*, 1968; Kivirikko *et al.*, 1969; Suzuki and Koyama, 1969; Kikuchi *et al.*, 1969). The enzymatic reaction requires O_2, Fe^{2+}, α-ketoglutarate, and a reducing agent which can be ascorbate (Grant and Prockop, 1971). The synthesis of hydroxyproline is accompanied by a stoichiometric decarboxylation of α-ketoglutarate (Rhoads and Udenfriend, 1968), and during the reaction one atom of O_2 is incorporated into the hydroxyproline while the other is incorporated into succinate (Cardinale *et al.*, 1971).

Initial studies on the biosynthesis of the hydroxylysine in collagen indicated that the hydroxylation of lysine required the same cofactors or cosubstrates as the hydroxylation of proline (Prockop *et al.*, 1966; Kivirikko and Prockop, 1967b; Hausmann, 1967; Hurych and Nordwig, 1967), and partially purified preparations of protocollagen proline hydroxylase were able to hydroxylate both proline and lysine in protocollagen (Kivirikko and Prockop, 1967b). It was originally suggested (Kivirikko and Prockop, 1967b) that both hydroxylations might be performed by the same enzyme. Subsequently, however, highly purified preparations of protocollagen proline hydroxylase were shown not to contain any lysine hydroxylase activity (Halme *et al.*, 1970; Miller, 1971) and experiments with competing polypeptides have indicated that the enzymatic site for the hydroxylation of lysine in protocollagen is separate from the enzymatic site for the hydroxylation of proline (Weinstein *et al.*, 1969).

We have recently purified protocollagen lysine hydroxylase which is free of any proline hydroxylase activity (Kivirikko and Prockop, 1972).[1] With this enzymatic preparation we have

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[1] In the course of the preparation of this manuscript Miller (1971)

examined the ability of several synthetic and natural peptides to serve as substrates for the synthesis of hydroxylysine and we have demonstrated that the synthesis of hydroxylysine involves a stoichiometric decarboxylation of α-ketoglutarate to succinate and carbon dioxide.

Materials and Methods

Synthetic Peptides. The peptides were synthesized at the Peptide Center of the Institute for Protein Research, Osaka University, Osaka, Japan. The hexapeptide $(Ile\text{-}Lys\text{-}Gly)_2$ and the peptide L-I (Table I) were synthesized by coupling four separate tripeptide units with the general structure Aoc-X-Y-Gly[2] with minor modifications of previously published techniques (Sakakibara, 1971). The details of preparation of the tripeptide units and of the coupling reaction will be described elsewhere (K. Shudo, Y. Kishida, and S. Sakakibara, 1971, in preparation). The peptides were lyophilized from acetic acid solutions and dried at room temperature over P_2O_5 *in vacuo* overnight.

The peptide L-II was prepared by synthesizing $(Pro\text{-}Pro\text{-}Gly)_4$-resin with a specific modification of the Merrifield procedure for solid-state synthesis of peptides (Sakakibara *et al.*, 1968). Aoc-amino acids were coupled stepwise to the $(Pro\text{-}Pro\text{-}Gly)_4$-resin and the peptide was then cleaved from the resin with HF (Sakakibara, 1971). The peptide L-III was prepared in the same manner, but before the peptide was cleaved from the resin, $(Pro\text{-}Pro\text{-}Gly)_4$ was added to the H_2N-terminal end by repeated coupling with Aoc-Pro-Pro-Gly. Excess HF and neutral contaminants were removed by passing the peptides in water through an anion-exchange resin (Dowex 1-X8) and the eluate was adjusted to pH 4 with acetic acid. The samples were lyophilized and dried at room temperature over P_2O_5 *in vacuo*.

Other Materials. Lysine[8]-vasopressin with the amino acid sequence Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2 was purchased from Sigma Chemical Co. (St. Louis, Mo.). Reduced and carboxymethylated collagen from the cuticle of *Ascaris lumbricoides* (var. suis) was prepared by extracting the cuticles of the worms with 0.5 M NaCl and then treating the collagen extract with mercaptoethanol and monoiodoacetic acid as described by McBride and Harrington (1967). Peptides from the reduced and the carboxymethylated collagen were prepared by treating the collagen with cyanogen bromide according to the method of Bornstein and Piez (1966). After cleavage by cyanogen bromide the resulting peptides were diluted with water, lyophilized, redissolved in water, and relyophilized.[3]

Lysine-labeled [^{14}C]protocollagen was prepared as described previously (Kivirikko and Prockop, 1967b). Seventy-two cartilagenous tibiae from 12-day-old embryos were incubated with 1 mM α,α′-dipyridyl in modified Krebs II-A medium containing glucose, phosphate buffer, and inorganic salts (Dehm and Prockop, 1971). [^{14}C]Lysine (New England Nuclear Corp.) (60 μCi) was added and the incubation was continued for 2.5 hr. The tibiae were homogenized in 8.0 ml of distilled water in a Teflon and glass homogenizer and then centrifuged at 100,000*g* for 1 hr. The supernatant was dialyzed for 24 hr against several changes of 20 mM Tris-HCl buffer adjusted to pH 7.8 at 4°. The sample was placed in boiling water for 10 min to destroy residual enzymatic activity and it was stored frozen in aliquots of 200,000 dpm.

Sodium [5-$^{14}C_1$]α-ketoglutarate (17.5 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, and [1-$^{14}C_1$]α-ketoglutaric acid from Calbiochem. The latter compound was diluted with authentic α-ketoglutarate (Calbiochem) in order to give a final specific activity of 35,000 or 60,000 dpm per 0.1 μmole. Silicic acid chromatography (see below) of the [1-$^{14}C_1$]α-ketoglutarate indicated that 73% of ^{14}C chromatographed with authentic α-ketoglutarate and 27% was eluted with higher concentrations of *tert*-butyl alcohol; similar results were previously reported by Lindstedt and Lindstedt (1970). In the experiments in which the stoichiometric evolution of [^{14}C]CO_2 during the synthesis of hydroxylysine was studied the specific activity of the [1-$^{14}C_1$]α-ketoglutarate was corrected for the 27% impurity of the radioactive material.

Partially Purified Preparations of Protocollagen Lysine Hydroxylase and Protocollagen Proline Hydroxylase. Protocollagen lysine hydroxylase was partially purified from the 15,000*g* supernatant of 3.5 kg of 13-day-old chick embryos. Purification steps included fractionation with ammonium sulfate and calcium phosphate gel, two successive chromatographies on DEAE-cellulose, and gel filtration on 8% Agarose (Bio-Gel A-1.5 m, Bio-Rad). The final preparations were completely free of any proline hydroxylase activity and the final specific activities were 150–250 times the specific activity of the original 15,000*g* supernatant. The details of the purification procedure will be published elsewhere (Kivirikko and Prockop, 1972).

Protocollagen proline hydroxylase was prepared as described by Halme *et al.* (1970) through the first step of chromatography on DEAE-cellulose. The final preparation had a small amount of lysine hydroxylase activity but this activity was not sufficient to give more than a 1% error in assaying the [^{14}C]carbon dioxide released from [^{14}C]α-ketoglutarate in the experiments on the hydroxylation of proline in synthetic peptides which contain both proline and lysine.

Assay Procedures. The hydroxylations with protocollagen lysine hydroxylase under "standard conditions" were carried out with synthetic substrates. The substrates in the amounts indicated below were incubated in a final volume of 1.0 ml containing 60–80 μg/ml of enzyme protein, 0.05 mM $FeSO_4$, 0.1 mM [1-$^{14}C_1$]α-ketoglutarate (35,000 dpm in initial experiments and 60,000 dpm in subsequent ones), 0.5 mM ascorbic acid, 0.1 mg/ml of catalase (Calbiochem), 0.1 mM dithiothreitol (Calbiochem), 2 mg/ml of bovine serum albumin (Sigma), and 50 mM Tris-HCl buffer adjusted to pH 7.8 at 25° (Kivirikko and Prockop, 1972). All the polypeptide substrates were heated to 100° for 10 min and cooled to 0° just before addition to the incubation system. The samples were incubated at 37° for 40 min and the [^{14}C]CO_2 was collected onto filter papers as described by Rhoads and Udenfriend (1968) except that the reaction was stopped by injecting 1 ml of 1 M potassium phosphate (pH 5.0). After injection of the phosphate buffer, the sealed tubes were shaken vertically in a mechanical shaker for 30 min, and the filter papers were assayed in a solvent system for liquid scintillation counting containing methyl Cellosolve and toluene (Prockop and Ebert, 1963). All values for disintegrations per minute of [^{14}C]CO_2 released were corrected for the release of [^{14}C]CO_2 observed with blank samples that did not contain substrate. In most experiments these blank values varied from 50 to 100 dpm.

also reported the separation of protocollagen lysine hydroxylase activity from protocollagen proline hydroxylase activity.

[2] Abbreviation used is: Aoc, *tert*-amyloxycarbonyl.

[3] The reduced and carboxymethylated collagen from *Ascaris* cuticle and the cyanogen bromide peptides from this collagen were kindly prepared for us by Richard A. Berg. He also measured the K_m values for the cyanogen bromide peptides as substrates for protocollagen proline hydroxylase.

TABLE I: Known Amino Acid Sequences around Glycosylated Hydroxylysine in Collagen and the Sequences of Our Synthetic Peptides.

Sequence	Code Name	Source
Naturally occurring:		
-Leu-Hyp-Gly-Met-*Hyl*-Gly-His-Arg-Gly-Phe-Ser-Gly-		α_1-CB4-5 rat skin[a]
-Gly-Met-*Hyl*-Gly-His-Arg-		Human skin, guinea pig skin, carp swim bladder[b]
-Gly-Phe-*Hyl*-Gly-Ile-Arg-		Human skin[b]
-Gly-Ile-*Hyl*-Gly-His-Arg-		Carp swim bladder[b]
Synthetic peptides:		
Ala-Arg-Gly-Ile-*Lys*-Gly-Ile-Arg-Gly-Phe-Ser-Gly	L-I	
Ala-Arg-Gly-Met-*Lys*-Gly-His-Arg-Gly-(Pro-Pro-Gly)$_4$	L-II	
(Pro-Pro-Gly)$_4$-Ala-Arg-Gly-Met-*Lys*-Gly-His-Arg-Gly-(Pro-Pro-Gly)$_4$	L-III	

[a] Butler (1970). [b] Morgan *et al.* (1970).

In experiments in which the quantitative synthesis of hydroxylysine was measured, the reaction conditions were the same as for the standard conditions except that larger amounts of enzyme were used (see Table III), the incubation was carried out for 80 min, and the reaction was stopped by the addition of 0.5 ml of 0.1 N HCl. After the filter papers used for trapping the [^{14}C]CO_2 were removed, 1.5 ml of concentrated HCl was added and the samples were hydrolyzed at 120° overnight. In order to check the recoveries through the further assay steps for hydroxylysine, 25,000 dpm of [^{14}C]hydroxylysine (New England Nuclear Corp.) were added to the hydrolysates. The samples were evaporated to dryness on a steam bath and were desalted on 1 × 4 cm columns of cation-exchange resin (AG-WX8, 200–400 mesh, H form; Calbiochem). The columns were washed with 40 ml of 0.5 N HCl and 3 ml of 4 N HCl. The samples were then eluted with 20 ml of 4 N HCl and evaporated to dryness on a steam bath. Hydroxylysine was further purified by chromatographing the samples in 3-cm streaks on thin-layer plates (Blumenkrantz and Prockop, 1971) and a specific chemical assay for hydroxylysine was then carried out (Blumenkrantz and Prockop, 1971). The volumes used for the assay were one-half of those originally recommended. Recovery of [^{14}C]hydroxylysine was checked by taking 0.1 ml of the final organic phase obtained in the chemical assay and counting it in a liquid scintillation counter. The dpm obtained were compared with the values observed when 25,000 dpm of [^{14}C]-hydroxylysine were added directly to the hydroxylysine standards which were not taken through the desalting and thin-layer chromatographic steps. The ^{14}C assays indicated that the recoveries of hydroxylysine in the desalting and chromatographic steps were over 90% in all samples, and the values for quantitative synthesis of hydroxylysine were corrected for the small variations in the recovery among the samples.

In the experiments where the conversion of [^{14}C]α-ketoglutarate to [^{14}C]succinate was followed, the incubation with enzyme was carried out under standard conditions except that 900 μg of enzyme protein was used, the concentration of α-ketoglutarate was 0.05 mM, and the sample contained 1.92 × 10^6 dpm [5-$^{14}C_1$]α-ketoglutarate. The enzymatic reaction was stopped by adding 2.5 M sulfuric acid to give a final concentration of 0.25 M. Carrier succinic acid (50 mg; 0.43 mmole) and α-ketoglutaric acid (30 mg; 0.21 mmole) were added. A 0.2-ml aliquot of the sample was then chromatographed on a column of silicic acid with 0.25 M sulfuric acid as the stationary phase and benzene with 10% *tert*-butyl alcohol as the mobile phase. The columns were prepared and the chromatography was carried out according to the procedure of Prior Ferraz and Relvas (1965) as modified by Lindstedt and Lindstedt (1970).

When the hydroxylations were carried out using lysine labeled [^{14}C]protocollagen as the substrate, 200,000 dpm of the substrate were incubated in a final volume of 2 ml containing 1–2 μg/ml of enzyme protein, 0.05 mM $FeSO_4$, 0.5 mM α-ketoglutarate, 0.5 mM ascorbic acid, 0.1 mg/ml of catalase, 0.1 mM dithiothreitol, 1.5 mg/ml of bovine serum albumin, and 50 mM Tris-HCl buffer adjusted to pH 7.8 at 25° (Kivirikko and Prockop, 1972). The samples were incubated at 37° for 30 min and the reaction was stopped by adding 20 ml of cold acetone (Weinstein *et al.*, 1969). After being allowed to stand in the cold for 30 min, the samples were centrifuged at about 3000*g* for 30 min and the supernatants were removed with suction. The pellets were dried by gently blowing nitrogen into the tubes and were suspended in 1 ml of distilled water followed by 6 ml of 0.3 M citrate–phosphate buffer (pH 6.4). [^{14}C]Hydroxylysine in the sample was assayed with a specific chemical procedure (Blumenkrantz and Prockop, 1969) using one-half the volumes originally suggested.

In experiments in which polypeptides were tested as substrates for protocollagen proline hydroxylase, the enzymatic reaction was carried out in a final volume of 1.0 ml, which contained 40 μg of the preparation of proline hydroxylase, 0.05 mM $FeSO_4$, 0.1 mM [1-$^{14}C_1$]α-ketoglutarate (35,000 dpm), 2 mM ascorbic acid, 0.2 mg of catalase, 0.1 mM dithiothreitol, 2 mg of bovine serum albumin, and 50 mM Tris-HCl buffer adjusted to pH 7.8 at 25° (see Halme *et al.*, 1970). The samples were incubated at 37° for 20 min and the [^{14}C]CO_2 was trapped and counted as described above.

All counting of ^{14}C was performed in an Intertechnique liquid scintillation spectrometer with an efficiency of 89% and a background of 25 cpm.

Results

Design and Synthesis of Peptides L-I, L-II, and L-III. Previous experiments indicated that free lysine did not serve as a substrate for protocollagen lysine hydroxylase, an observation which suggested that lysine had to be in a peptide structure in order to react with the enzyme. Synthetic peptides that might

TABLE II: Effect of the Peptides L-II and L-III on the Hydroxylation of [^{14}C]Lysine-Labeled Protocollagen by Protocollagen Lysine Hydroxylase.[a]

Addn (mg/ml)	[^{14}C]Hydroxylysine Synthesized (dpm)	% Control
None[b]	5850	
	6650	
	6920	
L-II (0.1)	4400	68
L-II (1.0)	1100	15
L-III (0.1)	3920	61
L-III (1.0)	1370	21

[a] The hydroxylation of [^{14}C]lysine-labeled protocollagen was carried out as described in Methods. [b] Control samples.

TABLE III: Quantitative Relationship between the Synthesis of Hydroxylysine and the Evolution of Carbon Dioxide.[a]

Expt	Substrate (mg)	[^{14}C]CO_2 Released[b] (nmoles)	Hydroxylysine Synthesized (nmoles)	[^{14}C]CO_2: Hydroxylysine
1	L-I (0.4)	46	47	0.98
2	L-II (0.6)	34	38	0.89

[a] Incubations were carried out as described in Methods. The amount of enzyme protein was 0.9 mg in expt 1, and 0.7 mg in expt 2. In order to obtain relatively large amounts of hydroxylysine for quantitative assay, large amounts of enzyme and long incubation times were used. [b] The nmoles of CO_2 released were calculated from the observed dpm of [^{14}C]CO_2 trapped and the specific activity of the [1-$^{14}C_1$]α-ketoglutarate. Values are corrected for negative controls which contained no substrate. The observed counts per minute for the negative controls were 10.7% (expt 1) and 8.2% (expt 2) of the observed counts per minute in the samples containing substrate.

serve as substrates for the synthesis of hydroxylysine were designed by examining known sequences in collagen in which glycosylated hydroxylysine occurs (Table I) (Butler, 1970; Morgan *et al.*, 1970). Unglycosylated hydroxylysine is known to occur in other sequences in collagen but in several of these sequences the position occupied by hydroxylysine is not fully hydroxylated and contains significant amounts of lysine (Butler, 1968, 1970; Miller *et al.*, 1969). Although the data are incomplete, it seems reasonable to assume that the positions in the polypeptide chains that are both hydroxylated and glycosylated would be most readily hydroxylated. In the four sequences known to contain glycosylated hydroxylysine as well as in several additional sequences that contain unglycosylated hydroxylysine, the hydroxylysine is always in the position immediately preceding glycine. In the four known sequences containing glycosylated hydroxylysine the amino acid preceding hydroxylysine is either methionine, phenylalanine, or isoleucine. The "X" position in the "X-Y-Gly" triplet following the glycosylated hydroxylysine is either histidine or isoleucine. The "Y" position in the triplet following the glycosylated hydroxylysine is arginine. Accordingly, the synthetic polytripeptides were designed so that the "X" position in the triplet containing lysine was either isoleucine or methionine, the "X" position in the following triplet was either histidine or isoleucine, and the "Y" position in the same triplet was arginine. In order to study the effect of chain length, L-II was synthesized with four triplets of Pro-Pro-Gly at the HOOC-terminal end and L-III with four triplets of Pro-Pro-Gly at both the H_2N- and HOOC-terminal ends. As indicated, the sequence of L-I is not directly comparable to the sequences of L-II and L-III.

The peptide L-I was homogeneous by thin-layer chromatography, and by paper electrophoresis at pH 3.6 in 0.2 M acetate–pyridine buffer, at pH 4.8 in 0.2 M acetate–pyridine buffer, and in 0.2 M pyridine. *Anal.* Calcd for L-I·4AcOH·3H_2O: C, 48.42; H, 7.54; N, 17.59. Found: C, 48.33; H, 7.55; N, 17.13.

Paper electrophoresis of L-II and L-III at pH 4.8 in 0.2 M acetate–pyridine buffer indicated a small amount of heterogeneity in the peptides. The calculated amino acid analysis for L-III was Pro 16, Gly 11, Ala 1, Met 1, His 1, Lys 1, and Arg 2. Amino acid analysis indicated that about 80% of the dry weight was accounted for by amino acids and after applying this correction the found values were Pro 15.7, Gly 11.0, Ala 0.69, Met 0.66, His 0.90, Lys 0.72, and Arg 1.82. The observed values for L-II showed a similar agreement with calculated values.

Synthetic Peptides as Competing Substrates for the Hydroxylation of Lysine-Labeled [^{14}C]*Protocollagen.* In order to determine whether the synthetic peptides interacted with protocollagen lysine hydroxylase, preparations of the enzyme were incubated with [^{14}C]lysine-labeled protocollagen and the synthesis of [^{14}C]hydroxylysine was assayed with a specific chemical procedure (Table II). Addition of the synthetic peptides L-II and L-III decreased the synthesis of [^{14}C]hydroxylysine, indicating that the peptides were either inhibitors of the enzyme or competitive substrates for the hydroxylase.

Quantitative Synthesis of Hydroxylysine and Stoichiometric Evolution of CO_2. In order to establish that the synthetic peptides were substrates, L-I and L-II were incubated with relatively large amounts of protocollagen lysine hydroxylase. Hydroxylysine was partially purified from the reaction mixture with two chromatographic steps and then assayed with a specific chemical procedure. The results indicated that there was quantitative synthesis of hydroxylysine with both peptides and that up to 14% of the lysine in the peptide was converted to hydroxylysine under the conditions of the experiment (Table III).

In the same experiments the stoichiometry between the synthesis of hydroxylysine and the decarboxylation of α-ketoglutarate was examined. The amount of CO_2 released was calculated from the observed dpm of [^{14}C]CO_2 and the specific activity of the [1-$^{14}C_1$]α-ketoglutarate used in the experiments. The ratio of [^{14}C]CO_2 released to the amount of hydroxylysine synthesized was 0.98 and 0.89, indicating that the release of CO_2 was equimolar with the synthesis of hydroxylysine.

Demonstration that Succinate Is a Product of the Enzymic Reaction. In order to determine whether α-ketoglutarate is decarboxylated to succinate in the enzymic reaction, [5-$^{14}C_1$]-α-ketoglutarate was incubated with the enzyme. Large amounts of enzyme and a low concentration of α-ketoglutarate were employed so that a large fraction of the [5-$^{14}C_1$]α-ketoglutarate was converted to product. Chromatography of the reaction

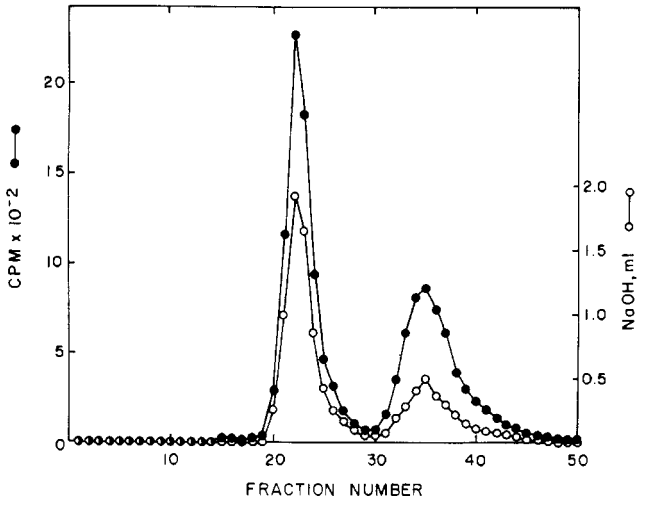

FIGURE 1: Conversion of [5-$^{14}C_1$]α-ketoglutarate to [^{14}C]succinate during the synthesis of hydroxylysine. The enzyme reaction was carried out as described in the Materials and Methods section. The sample was chromatographed on a 2.5 × 7 cm column of silicic acid with 0.25 M sulfuric acid as the stationary phase and benzene with 10% *tert*-butyl alcohol as the mobile phase (Prior Ferraz and Relvas, 1965; Lindstedt and Lindstedt, 1970). Fractions of 4.7 ml were collected. The scale on the left (●) indicates the cpm observed in 0.2-ml aliquots of the fractions. The scale on the right (○) indicates amount of 0.025 M NaOH required to titrate the succinic acid and α-ketoglutaric acid carriers in 4.5 ml of the fractions. The first peak of ^{14}C corresponds to the chromatographic position of the succinic acid carrier and the second peak corresponds to the chromatographic position of the α-ketoglutaric acid carrier.

products indicated two peaks of ^{14}C, one corresponding to the chromatographic position of the carrier α-ketoglutaric acid and the other to the chromatographic position of the carrier succinic acid (Figure 1). The recovery of the carrier α-ketoglutaric acid and succinic acid was over 95%. The results indicated that over half of the [5-$^{14}C_1$]α-ketoglutarate was converted to [^{14}C]succinate under the conditions of the experiment.

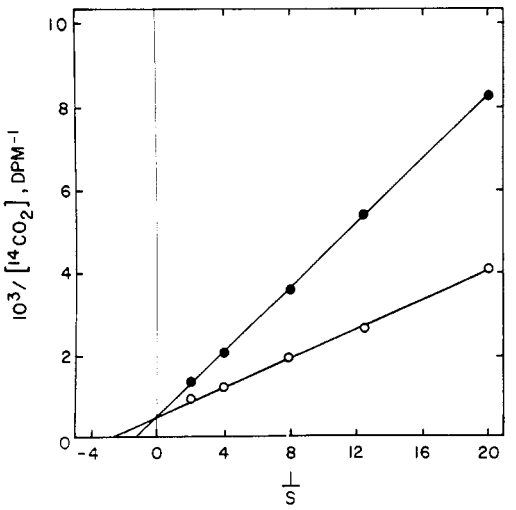

FIGURE 2: Double-reciprocal plots of substrate concentration and the initial velocity for the release of [^{14}C]CO_2 from [1-$^{14}C_1$]α-ketoglutarate. The concentration of the peptide substrate is expressed in milligrams per milliliter. Reaction in which L-II was used as the substrate (○); reaction in which L-III was used as the substrate (●).

TABLE IV: Comparison of L-I, L-II, and L-III as Substrates for the Synthesis of Hydroxylysine by Protocollagen Lysine Hydroxylase.[a]

Substrate	No. of Amino Acid Residues	Rel V_{max}	K_m	
			mg/ml	mM
L-I	12	100	0.5	0.4
L-II	21	100	0.4	0.2
L-III	33	100	0.7	0.2

[a] Protocollagen lysine hydroxylase was incubated with the various substrates under standard conditions as described in Methods. Values shown are the mean of at least three experiments. The enzyme was free of any proline hydroxylase activity.

A Rapid Assay for Protocollagen Lysine Hydroxylase. Although net synthesis of hydroxylysine was observed with the synthetic peptide substrates (above), large amounts of enzyme were required and the assay procedures were tedious. Accordingly, a rapid assay for the enzyme was developed based on the stoichiometric release of [^{14}C]CO_2 from [1-$^{14}C_1$]α-ketoglutarate. The K_m values were measured for Fe^{2+}, α-ketoglutarate, and ascorbate (Kivirikko and Prockop, 1972), and then saturating concentrations of Fe^{2+} and ascorbate were employed in the assay. The concentration of α-ketoglutarate was only twice the K_m concentration because higher concentrations of [1-$^{14}C_1$]α-ketoglutarate produced higher blank values of [^{14}C]CO_2 and higher concentrations of carrier α-ketoglutarate reduced the sensitivity of the assay. It was previously shown that the activity observed with protocollagen proline hydroxylase was increased if catalase (Kivirikko and Prockop, 1967c), dithiothreitol, and bovine serum albumin (Rhoads *et al.*, 1967) were added to the incubation system. Similar effects were observed with protocollagen lysine hydroxylase (Kivirikko and Prockop, 1972). Therefore optimal concentrations of these substances were employed.

Under the conditions of the assay the production of [^{14}C]-CO_2 during the synthesis of hydroxylysine was linear with incubation periods of up to 60 min and it was proportional to the amount of enzyme added. In experiments in which the substrate concentration was varied it was found that double-reciprocal plots of initial velocity and substrate concentration were linear (Figure 2). The assay was found to be reproducible and it was possible to assay about 40 samples within 2 hr. With the assay various substrates for the enzyme could be compared and the purification of the enzyme could be followed (Kivirikko and Prockop, 1972).

Comparison of L-I, L-II, and L-III as Substrates for Protocollagen Lysine Hydroxylase. In order to examine some of the structural requirements for peptide substrates of the enzyme, the V_{max} and K_m were compared for L-I, L-II, and L-III. The results indicated that there was no significant difference in the V_{max} obtained with L-I, L-II, or L-III (Table IV). When the K_m values were compared on the basis of the molar concentration of the polytripeptides, the value for L-II was about one-half the value for the shorter polytripeptide L-I. Since however the sequences around lysine were not the same in L-I and L-II, it was not clear whether the difference in K_m

TABLE V: Comparison of L-II and L-III as Substrates for the Synthesis of Hydroxyproline by Protocollagen Proline Hydroxylase.[a]

Substrate	No. of Amino Acid Residues	Rel V_{max}	K_m mg/ml	K_m mM
L-II	21	100	0.2	0.1
L-III	33	100	0.1	0.03

[a] Partially purified protocollagen proline hydroxylase was incubated with the substrates as indicated in the Methods. The values are the mean of two experiments. The enzyme contained a small amount of lysine hydroxylase activity but this was not sufficient to give more than a 1% error in assaying the hydroxylation of proline by the evolution of $[^{14}C]CO_2$ from [1-$^{14}C_1$]α-ketoglutarate.

could be attributed to the difference in chain length or in amino acid sequence. There was no significant difference in K_m values between L-II and L-III, an observation which indicated either that increasing chain length beyond seven triplets has no effect or that the chain must be extended with triplets other than Pro-Pro-Gly in order to further improve interaction with protocollagen lysine hydroxylase.

Because L-II and L-III contained Pro-Pro-Gly triplets, they were also examined as substrates for protocollagen proline hydroxylase (Table V). The K_m for L-III was about one-third the value for L-II when expressed in terms of the molar concentration of polytripeptide. It may be noted that the K_m values for these peptides with the proline hydroxylase were lower than the values with the lysine hydroxylase (compare Tables IV and V).

Failure of Ascaris Cuticle Collagen and Peptides from the Collagen to Interact with Protocollagen Lysine Hydroxylase. Previous observations indicated that reduced and carboxymethylated collagen from *Ascaris* cuticle did not act as an inhibitor or competing substrate (Weinstein *et al.*, 1969) for the hydroxylation of [^{14}C]lysine-labeled protocollagen by crude preparations of protocollagen lysine hydroxylase. These observations were of interest because this collagen contains about 290 glycyl, 290 prolyl, and 40 lysyl residues per 1000 amino acids (McBride and Harrington, 1967). It contains only 16 hydroxyprolyl residues and no hydroxylysine (McBride and Harrington, 1967), and it serves as a substrate for protocollagen proline hydroxylase after the collagen is denatured (Fujimoto and Prockop, 1968). Experiments carried out here showed directly that the reduced and carboxymethylated collagen from *Ascaris* cuticle did not serve as substrate (Table VI). Also, peptides prepared from the reduced and carboxymethylated *Ascaris* collagen did not serve as substrates and did not inhibit the hydroxylation of L-I, even though these peptides were still substrates for protocollagen proline hydroxylase with K_m values of 0.01–0.04 mg/ml.[3]

Examination of Free Lysine, Lys-Gly-Pro, Ile-Lys-Gly, and (Ile-Lys-Gly)$_2$ as Substrates for Protocollagen Lysine Hydroxylase. Attempts were made to determine whether protocollagen lysine hydroxylase can hydroxylate simpler substrates than L-I, L-II, and L-III. As observed previously with crude preparations of the enzyme (Kivirikko and Prockop, 1967a), free lysine was not a substrate (Table VII). No synthesis of hydroxylysine was observed with the tripeptide Lys-Gly-Pro but a very small synthesis was consistently observed with the tripeptide Ile-Lys-Gly. The hexapeptide (Ile-Lys-Gly)$_2$ was a better substrate and under the same conditions the amount of hydroxylysine synthesized with the hexapeptide was over ten times greater than with the tripeptide. However, the amount synthesized with the hexapeptide was still less than with L-I which contained 12 amino acids.

Because of the slow rate of hydroxylation with the tripeptide Ile-Lys-Gly, the V_{max} and K_m could not be determined.

TABLE VI: Failure of Reduced and Carboxymethylated Collagen from *Ascaris* Cuticle and Peptides from the Collagen to Interact with Protocollagen Lysine Hydroxylase.[a]

Substrate (mg/ml)	Competing Peptide (mg/ml)	$[^{14}C]CO_2$ Released (dpm)	% of Control
L-I (0.4)		1230	
		1240	
RCM[b] (0.1)		0	0
		0	0
RCM-CB[c] (0.1)		0	0
		0	0
L-I (0.4)	RCM (0.1)	1310	106
		1220	99
L-I (0.4)	RCM-CB (0.1)	1230	100
		1250	101

[a] Protocollagen lysine hydroxylase was incubated with the various substrates under standard conditions as described in Methods. [b] RCM is reduced and carboxymethylated collagen from the cuticle of *Ascaris lumbricoides* with an average molecular weight of about 60,000 (McBride and Harrington, 1967). [c] RCM-CB is a mixture of peptides which was obtained by cleaving RCM with cyanogen bromide (Bornstein and Piez, 1966). The molecular weights of the peptides ranged from 2000 to 25,000.

TABLE VII: Examination of Free Lysine, Lys-Gly-Pro, Ile-Lys-Gly, and (Ile-Lys-Gly)$_2$ as Substrates for Protocollagen Lysine Hydroxylase.[a]

Expt	Substrate (mg/ml)	$[^{14}C]CO_2$ Released (dpm)	% Control
1	L-II (0.5)	890	
	Lysine (1.0)	0	0
	Lys-Gly-Pro (1.0)	0	0
2	L-I (0.5)	1550	
	Ile-Lys-Gly (0.5)	30	2
	(Ile-Lys-Gly)$_2$ (0.5)	500	32
3	L-I (0.5)	2720	
	Ile-Lys-Gly (0.5)	110	4

[a] Protocollagen lysine hydroxylase was incubated with the substrates as indicated under standard conditions in Methods.

TABLE VIII: Comparison of L-II, (Ile-Lys-Gly)$_2$, and Lysine^8-Vasopressin as Substrates for Protocollagen Lysine Hydroxylase.[a]

Substrate	Rel V_{max}	K_m mg/ml	K_m mM
L-II	100	0.4	0.2
(Ile-Lys-Gly)$_2$	50	1.2	2.0
Lysine^8-vasopressin	50	1.2	1.1

[a] Protocollagen lysine hydroxylase was incubated with the substrates under standard conditions as described in Methods.

However, such measurements could be carried out with the hexapeptide (Table VIII) and they indicated that it was a poorer substrate than L-I, L-II, or L-III (Tables IV and VIII).

Hydroxylation of Lysine^8-Vasopressin. In order to study further whether lysine in amino acid sequences other than those found in protocollagen could be hydroxylated, the lysine hydroxylase was incubated with lysine^8-vasopressin which contains the HOOC-terminal sequence -Cys-Pro-Lys-Gly-NH_2. The peptide was found to serve as a substrate for the synthesis of hydroxylysine when the reaction was assayed by the evolution of [^{14}C]CO_2 from [1-$^{14}C_1$]α-ketoglutarate. Because the structure of lysine^8-vasopressin differed so markedly from that of collagen, the reaction was also followed by quantitative assays of hydroxylysine. Under the same conditions as were used in expt 1 of Table III, 22 nmoles of hydroxylysine was synthesized. However, the rates of synthesis observed with lysine^8-vasopressin were less than with L-I, L-II, or L-III. Kinetic measurements carried out with the [^{14}C]CO_2 assay indicated that the V_{max} for lysine^8-vasopressin was only half and the K_m was considerably greater than for L-I, L-II, and L-III (Tables IV and VIII).

Discussion

Although protocollagen proline hydroxylase has been extensively studied in recent years, little work has been carried out with protocollagen lysine hydroxylase. The principal reasons for this have been the lack of suitable substrates for the enzyme and the absence of a convenient assay for the reaction. As a result, purification of the enzyme has been difficult and only crude preparations have been available.

As a first approach to solving these problems attempts were made to design peptide substrates for the synthesis of hydroxylysine. Collagens from different tissues vary markedly in their content of hydroxylysine, and amino acid sequencing has indicated that the collagen of a single tissue can contain some molecules in which a given amino acid position is occupied by hydroxylysine and other molecules in which the same position is occupied by lysine (for review, see Grant and Prockop, 1972). All collagens have been found to contain a small but variable amount of carbohydrate in the form of galactose or glucosylgalactose in O-glycosidic linkage to hydroxylysine (Butler and Cunningham, 1966; Spiro, 1969). Although the available data are incomplete, it seems reasonable to assume that the positions in the polypeptide chain which contain glycosylated hydroxylysine are the most readily hydroxylated. Accordingly, the first peptide substrates which were synthesized for the studies reported here contained amino acid sequences comparable to known sequences in collagen in which lysine is both hydroxylated and glycosylated. All three synthetic peptides with amino acid sequences comparable to those in collagen served as substrates for the synthesis of hydroxylysine and with these substrates a quantitative synthesis of hydroxylysine was demonstrated for the first time. The synthesis of hydroxylysine was accompanied by the decarboxylation of α-ketoglutarate. The results therefore established protocollagen lysine hydroxylase as the fourth hydroxylase which decarboxylates α-ketoglutarate in the course of substrate hydroxylation (Lindstedt *et al.*, 1968; Rhoads and Udenfriend, 1968; McCroskey *et al.*, 1971). Succinate and carbon dioxide were shown to be products of the reaction and the amount of carbon dioxide released was equimolar with the amount of hydroxylysine synthesized. Although the reaction mechanism is not known in detail, the scheme involving a peroxide intermediate suggested by Lindstedt and Lindstedt (1970) for the hydroxylation of γ-butyrobetaine to carnitine is probably applicable to both protocollagen proline hydroxylase and protocollagen lysine hydroxylase.

The quantitative relationship between the synthesis of hydroxylysine and the release of [^{14}C]CO_2 from [^{14}C]α-ketoglutarate made it possible to develop a rapid and simple assay for the enzyme. With the assay kinetic experiments can be carried out and the assay has been helpful in developing a purification procedure for the enzyme (Kivirikko and Prockop, 1972). The enzyme can also be assayed by using [^{14}C]lysine-labeled protocollagen as a substrate and this assay is both more specific and more sensitive than the assay with [^{14}C]-α-ketoglutarate. However, variations between different preparations of the biologically prepared [^{14}C]protocollagen and the difficulty of preparing protocollagen make this assay less useful than the [^{14}C]α-ketoglutarate assay for most studies on protocollagen lysine hydroxylase.

Comparison of the data obtained with different peptides provided some initial information as to the critical determinants for the hydroxylation of lysine in a peptide linkage. Free lysine and the tripeptide Lys-Gly-Pro were not hydroxylated. A very slow rate of hydroxylysine synthesis was observed with the tripeptide Ile-Lys-Gly, but the rate observed with the hexapeptide (Ile-Lys-Gly)$_2$ was over ten times greater. Also, lysine^8-vasopressin with the terminal sequence -Cys-Pro-Lys-Gly-NH_2 was a substrate for the synthesis of hydroxylysine. These data suggest that a tripeptide of the structure Lys-Gly-X does not interact with the enzyme but a single triplet of X-Lys-Gly fulfills a minimum requirement for recognition by the enzyme. Also, the data indicate that extending the peptide with an additional triplet of X-Lys-Gly or with an unrelated sequence of amino acids greatly facilitates hydroxylation of the lysine.

None of these simpler peptides, however, was as readily hydroxylated as the three peptides with amino acid sequences comparable to those found in collagen. The V_{max} for all three of these peptides was the same, but the K_m for L-II and L-III was about one-half the K_m for L-I. This difference can be attributed either to differences in the amino acid sequences or to an effect of chain length, since L-I contained a total of only 4 X-Y-Gly triplets whereas L-II contained 7 and L-III contained 11 triplets. The effect of chain length was not apparent in the comparison of K_m values for L-II and L-III which had identical structures except that L-III had four additional Pro-Pro-Gly triplets at the NH_2-terminal end. The results indicated therefore that although chain length is critical with short peptides, increasing chain length beyond seven triplets has no effect, or the chain must be extended with trip-

lets other than Pro-Pro-Gly in order to further improve interaction with protocollagen lysine hydroxylase.

The observation that reduced and carboxymethylated collagen from the cuticle of *Ascaris* and peptides from this collagen do not interact with protocollagen lysine hydroxylase is surprising, since this collagen contains about 40 residues of lysine per 1000 residues (McBride and Harrington, 1967) and it is a good substrate for the synthesis of hydroxyproline by the proline hydroxylase (Fujimoto and Prockop, 1968). The failure of the collagen from *Ascaris* (Weinstein *et al.*, 1969) and the peptides derived from it to interact with lysine hydroxylase indicated that either all the lysyl residues are in the "X position" of repeating triplets of X-Y-Gly, or that the collagen contains lysyl residues in the "Y position" but in sequences which prevent them from interacting with the enzyme. It is of interest that in the recently reported sequence of α_1-CB-5 (Butler, 1970) there are two lysyl residues in the Y position which apparently are not hydroxylated *in vivo* since they do not contain hydroxylysine.

Further studies with a large variety of different peptides will be necessary to establish all the critical structural features which influence the interaction of substrates with the enzyme. The present data, however, demonstrate that both the amino acid sequence around lysine and the length of the peptide chain are critical determinants.

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