Solid-phase Synthesis of Substrate Model Peptides and Their Hydroxylation with Collagen Lysyl Hydroxylase

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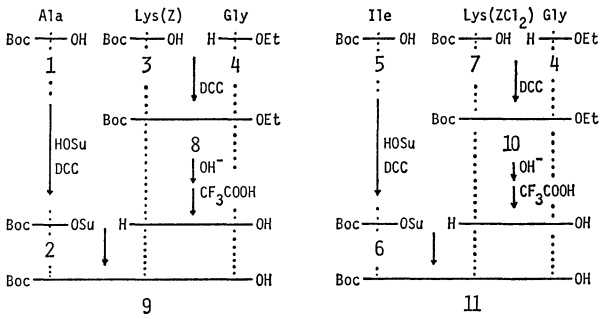
The peptides, (Ala–Lys–Gly)$_{10}$–Phe, (Ile–Lys–Gly)$_3$–Phe, and (Ile–Lys–Gly)$_5$–Phe, were synthesized by solid-phase fragment condensation method. The contaminating shorter peptides and branched chain peptides were removed by CM-cellulose column chromatography. The peptides were useful as synthetic model substrates for collagen lysyl hydroxylase. The –Ile–Lys–Gly– units in (Ile–Lys–Gly)$_5$–Phe showed a higher affinity to the enzyme than those in (Ile–Lys–Gly)$_3$–Phe. A strong substrate inhibition was observed with highly basic peptide, (Ala–Lys–Gly)$_{10}$–Phe.

Hydroxylysine and hydroxyproline are synthesized *in vivo* by enzymic hydroxylation of lysyl and prolyl residues in the peptide chain of procollagen.[1] The proline hydroxylation involves an oxygenation mechanism: The atmospheric ^{18}O is incorporated into hydroxyl group of collagen hydroxyproline.[2–4] On the other hand, hydroxylysine isolated from the chick embryos incubated in ^{18}O-enriched atmosphere did not contain ^{18}O.[4] There are two possibilities to explain the absence of ^{18}O in hydroxylysine: (1) Hydroxylation of lysyl residues involves a non-oxygenation mechanism, (2) The oxygen atom incorporated into hydroxyl group through an oxygenation mechanism is equilibrated with water oxygen by an enzymic process.

More recently the purified enzymes, lysyl hydroxylase and prolyl hydroxylase, were shown to require the same cosubstrates, namely, O_2, α-oxoglutarate, Fe^{2+} and ascorbic acid with similar K_m values.[5–7] During the enzymic hydroxylation, α-oxoglutarate is decarboxylated into CO_2 and succinate stoichiometrically to the formation of hydroxylysine or hydroxyproline.[8,9] The kinetic studies elucidated the same reaction scheme for the two hydroxylases involving an ordered binding of substrates to enzyme and an ordered release of products.[10,11] These results strongly suggest an oxygenation mechanism for lysine hydroxylation and support the second possibility mentioned above; *i.e.* an oxygenetic hydroxylation of lysyl residues is followed *in vivo* by an enzymic equilibration of hydroxyl group with water. The lysine hydroxylation will be studied separately from the oxygen equilibration and the oxygen source of hydroxyl group will be demonstrated for the first time if a synthetic substrate is subjected to the action of purified lysyl hydroxylase *in vitro*. Since the minimum sequence requirement for substrate is known as X–Lys–Gly[12] tripeptide,[9] the present paper describes the syntheses of substrate model peptides, (X–Lys–Gly)$_n$–Phe (X= Ala or Ile) and their enzymic hydroxylation with purified lysyl hydroxylase.

Experimental

Protected Tripeptides. The protected tripeptides, Boc–Ala–Lys(Z)–Gly[12] (**9**) and Boc–Ile–Lys(ZCl_2)–Gly (**11**), were synthesized by the solution method of peptide synthesis as shown in Scheme 1. The yields, melting points, and elemental analyses are shown in Table 1.



Scheme 1. Syntheses of Boc-Ala-Lys(Z)-Gly (**9**) and Boc-Ile-Lys(ZCl_2)-Gly (**11**).

Aoc–Phe–Resin.[12] Aoc–Phe–resin (0.3 mmol of Phe per g of resin) was prepared from Aoc–Phe (5 mmol) and chloromethylated copoly(styrene – 2%divinylbenzene)(Cl : 1 mmol per g resin, Protein Research Foundation, Osaka, Japan) (10 g) by the method of Loffet.[19] The unreacted chloromethyl sites were acetylated by the same method using acetic acid (20 mmol).

*Solid-phase Syntheses. (Ala–Lys–Gly)$_{10}$–Phe (**12**)*: The Aoc–Phe–resin (2 g, 0.6 mmol of Phe) was placed in a 20 ml reaction vessel and converted into Boc–(Ala–Lys(Z)–Gly)$_{10}$–Phe–resin by manual additions of **9** in ten cyclic steps as follows; (1) deprotection for 60 min with 33% (by vol) trifluoroacetic acid in dichloromethane (15 ml), (2) washing for 5 min with dichloromethane (20 ml × 3) and DMF[12] (20 ml × 2), (3) neutralization for 5 min with 5% (by vol) triethylamine in DMF (20 ml), (4) washing for 5 min with DMF (20 ml × 2) and dichloromethane (20 ml × 3), (5) condensation for 20 h with **9** (0.65 g, 1.28 mmol) and DCC[12] (0.28 g, 1.28 mmol) in dichloromethane (20 ml) and (6) washing for 5 min with dichloromethane (20 ml × 5). After ten cycles, the resin was dried (4.25 g, 100%). An aliquot (0.85 g) of the peptidyl resin was treated with anhydrous HF (15 ml) and anisole (1 ml) for 60 min at 0 °C and dried.[20] The peptide was extracted into 1% (by vol) acetic acid (10 ml × 1, 45 min, and 2.5 ml × 3, 15 min each) and the extract passed through a column (1 cm × 3 cm) of Amberlite IRA-400 (acetate form). The eluate was washed with ethyl acetate (10 ml) and freeze-dried to afford (Ala–Lys–Gly)$_{10}$–Phe (**12**) (244 mg, 77%).

*(Ile–Lys–Gly)$_3$–Phe (**13**) and (Ile–Lys–Gly)$_5$–Phe (**14**)*: Aoc–Phe–resin (2 g, 0.6 mmol of Phe) was subjected to the condensation cycles with **11** (0.8 g, 1.29 mmol) instead of **9** as described above except that THF[12]–dichloromethane (1:4 by vol) mixture was used as a solvent for the condensation reactions. After three cycles, about a half of the peptidyl resin was removed and dried to give Boc–(Ile–Lys(ZCl_2)–Gly)$_3$–Phe–resin (1.4 g, approx. 94%). The rest

Table 1. Synthesis of amino acid derivatives and peptides

	Compound	Yield %	Mp θ_m/°C	Elemental analysis (%)			Ref.
				C	H	N	
1	(DCHA[12] salt)	62	147—148	C. 64.8 F. 64.5	10.3 10.3	7.6 7.5	13
2		87	161—163	C. 50.3 F. 50.3	6.3 6.4	9.8 9.6	14
3	(DCHA salt)	65[a]	112—113	C. 66.3 F. 66.5	9.2 9.3	7.5 7.7	15, 16, 17
4	(Hydrochloride)	95	144—145	C. 34.4 F. 34.1	7.2 7.6	10.0 10.0	18
5	(DCHA salt)	93	126—126.5	C. 67.0 F. 66.8	10.8 10.8	6.8 6.9	17
6		Approx. 100	Oil				14
7		Approx. 100[a]	Oil				16, 17
8		72	68—68.5	C. 59.3 F. 59.6	7.6 7.8	9.0 9.2	
9		83	77—80	C. 56.2 F. 56.7	7.2 7.1	10.8 11.0	
10		87	94.5—95.5	C. 51.7 F. 51.9	6.2 6.4	7.9 7.7	
11		76	97—97.5	C. 52.3 F. 52.0	6.5 6.5	9.0 8.9	

a) Yield for *t*-butyloxycarbonylation.

of the resin was subjected to two more cycles with **11** (0.4 g, 0.65 mmol) and dried to give Boc–(Ile–Lys(ZCl_2)–Gly)$_5$–Phe–resin (1.7 g, approx. 96%). The peptidyl resin preparations were treated with anhydrous HF as above and the peptides extracted into 2% (by vol) acetic acid were passed through an Amberlite IRA-400 (acetate form) column as above and freeze-dried (324 mg and 360 mg, respectively).

Fractionation of the Peptide Preparations. The preparations of **12**, **13**, and **14** were fractionated on a CM-cellulose[12] column by linear gradient elutions of NaCl concentration. Fractions were pooled as shown in Fig. 1. The peptides in the fractions were desalted by gel filtrations on a Sephadex G-10 column (1.9 cm × 100 cm) equilibrated with 5 mM HCl and freeze-dried.

Collagen Lysyl Hydroxylase. The enzyme was extracted from 13-day-old chick embryos (312 g) and purified by ammonium sulfate fractionation and affinity chromatography on a column (1.5 cm × 12 cm) of concanavaline-A-Sepharose 4B (Pharmacia) as described by Turpeennien *et al.*[21] except that the buffer solution used in the affinity chromatography did not contain $MnCl_2$. The protein concentration of the enzyme solution (12 ml) was 1.2 mg/ml.

Assay Procedures. The enzymic hydroxylation of lysyl residues in substrate model peptides was assayed by the stoichiometric release of $^{14}CO_2$ from [1-^{14}C]-α-oxoglutarate as described by Rhoads and Udenfriend[8] and Kivirikko *et al.*[9] The incubation was carried out at 37 °C and $^{14}CO_2$ released was captured and counted with liquid scintillation counter as described by Ichiyama *et al.*[22]

Results

Synthetic Peptides. The preparations of **12**, **13**, and **14** were fractionated by CM-cellulose column chromatography (Figs. 1-a, b, and c). The yields and amino acid compositions are shown in Table 2. The peptides in the fraction of A-10, B-2, and C-4, namely (Ala–Lys–Gly)$_{10}$–Phe (**12**), (Ile–Lys–Gly)$_3$–Phe (**13**), and (Ile–Lys–Gly)$_5$–Phe (**14**), were used as substrates in the following enzymic reactions.

Enzymic Reactions. The time course of the reaction is shown in Fig. 2. The double reciprocal plots of the radioactivity of $^{14}CO_2$ released during 60 min incubations against the substrate concentration are shown in Fig. 3. A linear relationship was observed with **13** over the whole range of concentration examined. With **14**, a substrate inhibition was observed, however, at higher concentration than 0.5 mM of –Ile–Lys–Gly– unit. The apparent values of K_m and V_{max} were estimated by extrapolation of the linear part obtained in the lower concentration range, where the inhibition was not so significant. The values for **12** could not be obtained because of its strong substrate inhibition.

Discussion

The lysyl and prolyl residues at Y-position of –X–Y–Gly– tripeptide units in procollagen can be hydroxylated by the hydroxylases specific to them, respectively. In the present work, the peptides, (X–Lys–Gly)$_n$–Phe, were synthesized and subjected to the action of collagen lysyl hydroxylase. The syntheses were carried out by successive condensations of protected tripeptide units to phenylalanine linked to an insoluble supporting resin. Because of the incomplete reaction in the deprotection and/or condensation steps, the products were mixtures of peptides with varying number of repeating units. The peptides were separated from one another by CM-cellulose column chromatography making use of varying lysine content (Figs. 1-a, b, and c). The main component of A-10 (Fig. 1-a) was characterized as the designed peptide (Ala–Lys–Gly)$_{10}$–Phe (**12**) by amino acid composition (Table 2). Most of the other components, A-1 to A-9, were shorter peptides. The relatively low phen-

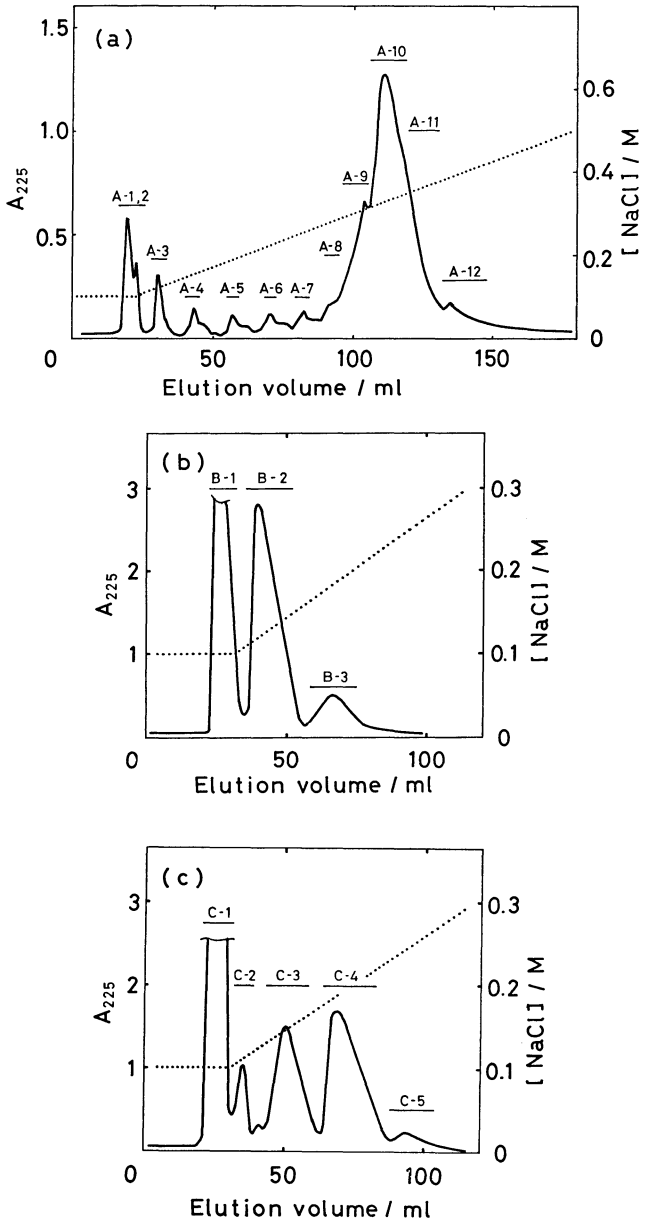


Fig. 1. CM-cellulose column chromatography of peptides prepared by fragment condensation in solid-phase method.
Crude preparations of (a) $(Ala–Lys–Gly)_{10}$–Phe (**12**), 185 mg; (b) $(Ile–Lys–Gly)_3$–Phe (**13**), 195 mg; and (c) $(Ile–Lys–Gly)_5$–Phe (**14**), 203 mg, were dissolved in 0.05 M NaCl (20 ml) and applied on a CM-cellulose column (2.6 cm × 50 cm) equilibrated with 0.1 M NaCl. The column was eluted with 0.1 M NaCl (70 ml) and with 0.1 M to 0.6 M NaCl (linear gradient, total 2000 ml) at 150 ml/h. Fractions of 15 ml were collected. —: Absorbance at 225 nm, ……: concentration of NaCl.

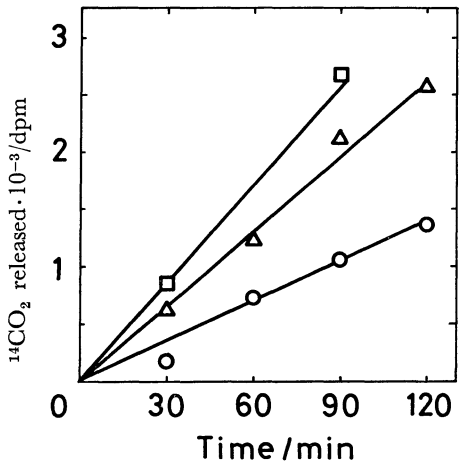


Fig. 2. Time course of the enzymic reaction.
Peptide $(Ala–Lys–Gly)_{10}$–Phe (**12**) (0.31 mg/ml) (○), $(Ile–Lys–Gly)_3$–Phe (**13**) (0.32 mg/ml) (△), or $(Ile–Lys–Gly)_5$–Phe (**14**) (0.32 mg/ml) (□) was incubated with the enzyme (0.06 mg protein/ml) and [1-^{14}C]-α-oxoglutarate (60000 dpm/ml) at 37 °C in 3 ml of reaction mixture.

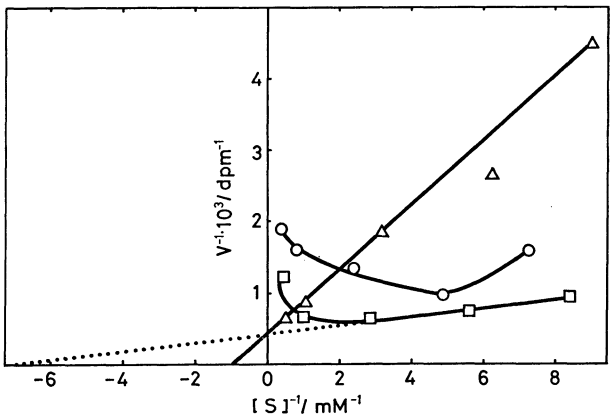


Fig. 3. Double reciprocal plot of initial rate of enzymic hydroxylation against the substrate concentration.
The initial rate is expressed as dpm of $^{14}CO_2$ released from [1-^{14}C]-α-oxoglutarate (9×10^4 dpm) during the incubation (60 min) with the enzyme (0.22 mg protein/ml) and varying amount of substrate peptide in 1 ml of the reaction mixture. Substrate concentration, [S], is expressed as mM of –X–Lys–Gly– unit. —○—: $(Ala–Lys–Gly)_{10}$–Phe (**12**), —△—: $(Ile–Lys–Gly)_3$–Phe (**13**), —□—: $(Ile–Lys–Gly)_5$–Phe (**14**).

ylalanine content in the fractions A-3 to A-7 is probably due to the contamination of peptides without phenylalanine at C-termini. A very small extent of contamination of such peptides (1 to 2% by weight) produced by random cleavage of peptide bond during the HF treatment will affect the amino acid composition. Fraction A-11 was a tailing part of A-10 and fraction A-12 contained branched-chain peptides which resulted from the deprotection of N^{ε}-group on exposures to 33% trifluoroacetic acid. Although a more acid-stable 2,4-dichlorobenzyloxycarbonyl (ZCl_2) group[16] was used for the protection of N^{ε}-group in the syntheses of **13** and **14**, the branched-chain peptides, B-3 and C-5, were produced (Figs. 1-b and c). The presence of considerable amount of shorter peptides besides $(Ile–Lys–Gly)_5$–Phe (Fig. 1-c) reflects the low reaction yield in the solid-phase fragment condensation of **11**. The large absorption values observed with peptide B-1 and C-1 are mainly due to the presence of anisole in these fractions. The

Table 2. Yields[a] and amino acid compositions[b] of fractionated pfptides

Fraction	Yield wt%	Amino acid composition[c]			Fraction	Yield wt%	Amino acid composition			
		Ala	Gly	Phe			Ile	Lys	Gly	Phe
A-1, 2	1.4				B-1	16.9	2.0	2.1	2.2	1
A-3	1.1	3.5	3.7	1	B-2	70.0	2.9	2.8	3.0	1
A-4	1.3	6.4	6.5	1	B-3	4.7	3.9	4.8	4.8	1
A-5	0.8	6.2	6.2	1						
A-6	1.1	8.3	8.3	1	C-1	6.3	2.1	2.2	2.1	1
A-7	1.5	9.9	10.0	1	C-2	10.3	3.3	3.2	3.4	1
A-8	2.5	7.9	8.0	1	C-3	36.9	4.1	4.0	4.3	1
A-9	13.2	9.5	9.6	1	C-4	31.8	4.8	4.9	5.2	1
A-10	43.0	10.3	10.3	1	C-5	4.5	5.4	6.0	6.2	1
A-11	12.1	10.4	10.6	1						
A-12	5.0	12.1	12.9	1						

a) Yields are based on the amount of peptides subjected to the fractionation. b) Peptides were hydrolyzed with 6 M HCl at 105 °C for 24 h in evacuated tubes and the resulting amino acid mixtures were subjected to amino acid analysis with JLC 8AH amino acid analyzer (JEOL). Amino acid compositions are expressed as molar ratios to phenylalanine content. c) The lysine contents were not determined.

Table 3. Comparison of model peptides as substrate for collagen lysyl hydroxylase

Substrate	Relative V_{max}	K_m	
		mg/ml	mM of -X-Lys-Gly-
$(Ile\text{-}Lys\text{-}Gly)_2$		1.2[a]	4[a]
$(Ile\text{-}Lys\text{-}Gly)_3$-Phe	1	0.35	1
$(Ile\text{-}Lys\text{-}Gly)_5$-Phe	1[b]	0.05[b]	0.14[b]
L-III[c]		0.7[a]	0.2[a]

a) Values reported by Kivirikko *et al.*[9] b) Values obtained by extrapolation (see Experimental). c) synthetic peptide; $(Pro\text{-}Pro\text{-}Gly)_4$-Ala-Arg-Gly-Met-Lys-Gly-His-Arg-Gly-$(Pro\text{-}Pro\text{-}Gly)_4$.[9]

amino acid compositions of B-2 and C-4 agreed with those expected for **13** and **14**, respectively.

The lysyl residues in the model substrates were hydroxylated by collagen lysyl hydroxylase. When K_m values of various synthetic substrates are compared on molar basis of -X-Lys-Gly- unit (Table 3), the longer is a peptide, the higher is the affinity per unit to the enzyme. The average K_m value (0.14 mM) per -Ile-Lys-Gly- unit in **14** is almost equal to the K_m value (0.2 mM) reported for -Met-Lys-Gly- unit in a synthetic model peptide of collagen.[9] The effect of the chain length is obvious merely on the affinity of the peptide to the enzyme. No difference was observed between the V_{max} values of **13** and **14** (Fig. 3 and Table 3).

The substrate inhibition observed for **12** over the whole range of the concentration examined here and for **14** at higher concentration may be due to a high basicity of the peptides because the positive charges per molecule increase with increasing n value of $(X\text{-}Lys\text{-}Gly)_n$-Phe. A highly basic peptide may inactivate the enzyme which is charged negatively at pH 7.4.[21]

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12) Abbreviations for amino acids are according to the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). All amino acids except for glycine are of L configuration. Other abbreviations used: Aoc, *t*-pentyloxycarbonyl; Boc, *t*-butyloxycarbonyl; CM, carboxymethyl; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DMF, *N*,*N*-dimethylformamide; HOSu, *N*-hydroxysuccinimide; Z, benzyloxycarbonyl; $Z(Cl_2)$, 2,4-dichlorobenzyloxycarbonyl.
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