Further Hydroxylation of Lysyl Residues in Collagen by Protocollagen Lysyl Hydroxylase *in Vitro*†

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ABSTRACT: Additional hydroxylation of lysyl residues in collagen was studied by incubation of several collagens *in vitro* with partially purified protocollagen lysyl hydroxylase. All collagens tested served as substrates for further hydroxylation if they were thermally denatured before reaction with the enzyme. With large amounts of the enzyme the degree of hydroxylation of lysyl residues in calf skin collagen increased from about 20 to over 40%. In experiments with isolated α1 and α2 chains, additional hydroxylysine was synthesized to a greater extent with α1 chains than with α2 chains. However, the initial hydroxylysine content in α1 chains is only about half of that in α2 chains and, therefore, the final hydroxylysine content of the α2 chains was larger than that of the α1. The peptide α1-CB8-HA1 obtained by hydroxylamine cleavage of a specific cyanogen bromide peptide from the α1 chain of rat skin collagen was also tested as a substrate. The 99 amino acids in the peptide were previously sequenced and the level of hydroxylation of the two lysyl residues in the peptide was found to be only about 10%. The peptide served as a substrate for further hydroxylation, but lysyl residues in this peptide were less susceptible to hydroxylation than lysyl residues in whole collagen. The difference may be attributable to the amino acid sequences adjacent to the two lysyl residues in the peptide. In contrast to the results obtained with denatured collagens, the degree of hydroxylation of native collagen was very low, and most or all of the hydroxylation observed with native collagen can be explained by the presence of small amounts of denatured collagen. The results suggest, therefore, that the formation of a triple-helical structure may be one of the critical factors limiting the degree of hydroxylation of lysyl residues during collagen biosynthesis.

Hydroxylysine and hydroxyproline in collagen are synthesized by hydroxylation of certain lysyl and prolyl residues in a polypeptide precursor of collagen known as protocollagen (for reviews, see Grant and Prockop, 1972, and Kivirikko, 1973). These hydroxylations are catalyzed by two separate enzymes, prolyl hydroxylase (Halme *et al.*, 1970; Rhoads and Udenfriend, 1970; Berg and Prockop, 1973a) and lysyl hydroxylase (R. L. Miller, 1971; Kivirikko and Prockop, 1972; Popenoe and Aronson, 1972), and both reactions require molecular oxygen, ferrous iron, α-ketoglutarate, and a reducing agent which can be ascorbate (Grant and Prockop, 1972; Kivirikko, 1973). The prolyl hydroxylase specifically hydroxylates prolyl residues in the "Y position" of repeating -X-Y-Gly- sequences of collagen and synthetic peptides. The lysyl hydroxylase also appears to be specific for lysyl residues in such Y positions. However, the hydroxylation of both lysyl residues (*e.g.*, Butler, 1968, 1972; Bornstein, 1969; Balian *et al.*, 1971, 1972; Wendt *et al.*, 1972a) and prolyl residues (*e.g.*, Bornstein, 1967; Butler and Ponds, 1971; Balian *et al.*, 1971, 1972; Wendt *et al.*, 1972b; Fietzek *et al.*, 1972a,b) was found to be incomplete in that collagen in a single tissue contains some molecules in which a given amino acid position is occupied by a hydroxylysyl or hydroxyprolyl residue and other molecules in which the same position is occupied by a lysyl or prolyl residue. It was also found that it is possible to increase the average level of hydroxylation of prolyl residues in several collagens by about 5–15% by incubation *in vitro* with prolyl hydroxylase and an increase of about 75% was observed with a specific peptide derived from tendon collagen (Rhoads *et al.*, 1971).

In the present study, the level of hydroxylation of lysyl residues was investigated by incubating collagens with lysyl hydroxylase and by assaying for additional hydroxylation. Such studies were of special interest, because amino acid sequences of the α chains of collagen indicated that the level of hydroxylation of several lysyl residues is considerably lower than the level of hydroxylation of prolyl residues (above references). Furthermore, it is known that the level of hydroxylation of lysyl residues in certain collagens changes with age (Miller *et al.*, 1967; Barnes *et al.*, 1971a,b), and that the hydroxylysine content of collagen from various tissues varies widely (E. J. Miller, 1971; Kefalides, 1971). Further information about the hydroxylation of specific lysyl residues was obtained by hydroxylation of a peptide of known amino acid sequence prepared from the α1 chain of rat skin collagen.

Materials and Methods

Preparation of Collagen Substrates and of α1-CB8-HA1. The 0.5 M sodium chloride soluble collagen was prepared from the skin of normal rats, and 0.1 M acetic acid soluble collagen from the skin of normal rats, lathyritic rats, normal calves, and from the tail tendon of normal rats as described by Steven and Jackson (1967). The α1 and α2 chains were prepared from collagen extracted with 1 M NaCl from the skin of lathyritic rats (Bornstein and Piez, 1966), and the peptide α1-CB8-HA1 was prepared from this collagen as reported previously (Balian *et al.*, 1971).

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Partially Purified Lysyl Hydroxylase. Thirteen-day-old chick embryos (2.5 kg) were homogenized and lysyl hydroxylase was partially purified from the 15,000*g* supernatant (Kivirikko and Prockop, 1972). The final preparations were free of prolyl hydroxylase activity, and the final specific activities of the lysyl hydroxylase were about 100–200 times the specific activity of the original 15,000*g* supernatant.

Other Materials. The peptide L-I was synthesized at the Peptide Center of the Institute for Protein Research, Osaka University, Osaka, Japan. It has the amino acid sequence: Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly, and its properties as a substrate for lysyl hydroxylase have been reported previously (Kivirikko *et al.*, 1972a).

[1-^{14}C]α-Ketoglutaric acid was purchased from Calbiochem, and was diluted with authentic α-ketoglutarate (Calbiochem) to give a final specific activity of 60,000 dpm/0.1 μmol. As reported previously, 30% of the ^{14}C in the preparation of [1-^{14}C]α-ketoglutarate was in other compounds (Lindstedt and Lindstedt, 1970; Kivirikko *et al.*, 1972a), and the specific activity of the final α-ketoglutarate preparation was corrected accordingly.

Assay Procedures. The reaction with lysyl hydroxylase under "standard conditions" was carried out in a final volume of 1.0 ml containing 40–60 μg of enzyme preparation, 0.05 mM $FeSO_4$, 0.1 mM [1-^{14}C]α-ketoglutarate (60,000 dpm), 0.5 mM ascorbic acid, 0.1 mg of catalase (Calbiochem), 0.1 mM dithiothreitol (Calbiochem), 2 mg of bovine serum albumin (Sigma), and 50 mM Tris-HCl buffer adjusted to pH 7.8 at 25° (Kivirikko and Prockop, 1972). The collagen preparations and the peptides 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 $^{14}CO_2$ 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 counted in a scintillant containing Methyl Cellosolve and toluene (Prockop and Ebert, 1963). All values for disintegrations per minute of $^{14}CO_2$ released were corrected for the release of $^{14}CO_2$ observed with blank samples that did not contain substrate. In most experiments these blank values varied from 50 to 200 dpm.

In experiments in which the synthesis of hydroxylysine was quantitated by amino acid analysis the reaction conditions were the same except that larger amounts of the enzyme were used, and the incubation was carried out for 80 min. After the filter papers were removed, four samples each containing 100 μg of collagen were pooled. A control sample was prepared with 400 μg of collagen and other components present in the test sample. The samples were dialyzed at 4° for 16 hr against several changes of 0.02 M ammonium bicarbonate (pH 7.6), containing 0.1 mM calcium chloride. After dialysis the samples were incubated at 37° for 4 hr with 100 μg of purified bacterial collagenase (Sigma), and dialyzed for 16 hr against 50 ml of distilled water in sealed tubes with shaking. The solution outside the dialysis bag was evaporated to dryness, and the residue was hydrolyzed in 3 ml of 6 M HCl for 18 hr at 120°. The samples were evaporated to dryness and dissolved in 3 ml of buffer used for amino acid analysis, and the analysis was carried out using a Jeol JLC-5AH automatic amino acid analyzer. The final values for hydroxylysine were corrected for small losses during the processing of the samples on the basis of initial and final values for hydroxyproline content of the samples.

TABLE I: Further Hydroxylation by Lysyl Hydroxylase of Denatured Collagens Assayed by the Release of $^{14}CO_2$ from [1-^{14}C]α-Ketoglutarate.[a]

Expt	Substrate	Substrate Concn (μg/ml)	$^{14}CO_2$ Released (dpm)
1	Acid-soluble skin collagen from lathyritic rat	300	890
	Synthetic peptide L-I	500	2,160
2	NaCl-soluble skin collagen from normal rat	300	890
	Acid-soluble skin collagen from normal rat	100	750
		300	1,190
	Acid-soluble tendon collagen from normal rat	100	760
		300	1,020
	Synthetic peptide L-I	500	2,440
3	Synthetic peptide $(Gly\text{-}Pro\text{-}Pro)_n$ mol wt 6600	250	0
	Synthetic peptide L-I	500	10,660[b]

[a] Collagen substrates were prepared and the incubations with lysyl hydroxylase were carried out as described in Methods. All the substrates were denatured by heating at 100° for 10 min. [b] The amount of enzyme in expt 3 was four times that in expt 1 and 2.

All counting of ^{14}C was performed in a Wallac liquid scintillation spectrometer with an efficiency of 85% and a background of 25 cpm.

Results

Demonstration of in Vitro Hydroxylation of Lysyl Residues in Denatured Collagens. In order to study *in vitro* hydroxylation of lysyl residues, denatured collagens from various sources were incubated with lysyl hydroxylase, and the release of $^{14}CO_2$ from [1-^{14}C]α-ketoglutarate was assayed. Previous studies had demonstrated that the synthesis of hydroxylysine is accompanied by a stoichiometric decarboxylation of α-ketoglutarate, and therefore the evolution of $^{14}CO_2$ could be used to follow the enzymic reaction (Kivirikko *et al.*, 1972a). It had also been demonstrated that synthetic peptides having amino acid sequences comparable to amino acid sequences adjacent to glycosylated hydroxylysine in collagen serve as substrates for the enzymic synthesis of hydroxylysine (Kivirikko *et al.*, 1972a). In the present study the peptide L-I, which has the amino acid sequence Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly was used as a standard substrate.

The results indicate that acid-soluble collagen from the skin of lathyritic rats, acid-soluble collagen from the skin and tail tendon of normal rats, and salt-soluble collagen from the skin of normal rats all served as substrates for further hydroxylation of lysyl residues (Table I). There were no marked differences in the degree of additional hydroxylation of these collagens, and there also were no differences in the degree of hydroxylation of acid-soluble collagens from rat skin and calf skin (compare expt 2 in Table I to expt 1 and 2 in Table III). To verify that none of the $^{14}CO_2$ released was due to the hydroxylation of prolyl residues, a large amount of the enzyme was incubated with the peptide $(Gly\text{-}Pro\text{-}Pro)_n$, which serves as a substrate for the hydroxylation by prolyl hydroxylase

TABLE II: Effect of Thermal Denaturation on the Further Hydroxylation of Two Preparations of Acid-Soluble Skin Collagen from Calf.[a]

Expt	Temp (°C)	Substrate	Substrate Concn (μg/ml)	$^{14}CO_2$ Released (dpm)
1	37	Preparation I		
		Native[b]	300	150
		Denatured	300	1130
		Preparation II		
		Native	300	120
		Denatured	300	960
2	30	Preparation I		
		Native[b]	100	<30[c]
		Denatured	100	480

[a] The preparations of collagen were denatured by heating at 100° for 10 min, and the incubations with lysyl hydroxylase were carried out at 37 or 30° as described in Methods. [b] When preparation I was incubated for 4 hr at 30 or 20° with trypsin, 4.5 or 1.5% of the hydroxyproline was recovered in dialyzable peptides as described in text. [c] The values observed with duplicate samples were 25 and 26 dpm.

TABLE III: Comparison of Rat or Calf Skin Collagen and the Fragment α1-CB8-HA1 of Rat Skin Collagen as Substrates for Further Hydroxylation by Lysyl Hydroxylase.[a]

Expt	Substrate	Substrate Concn (μg/ml)	$^{14}CO_2$ Released (dpm)
1	α1-CB8-HA1	200	130
		500	370
	Acid-soluble rat skin collagen	100	640
		300	910
	Synthetic peptide L-I	500	2040
2	α1-CB8-HA1	300	320
		1000	410
	Acid-soluble calf skin collagen	100	720
		300	1130
	Synthetic peptide L-I	500	2080

[a] The collagen substrates and the hydroxylamine fragment HA1 of the cyanogen bromide peptide CB8 of α1 chain of rat skin collagen were prepared as described in Methods. The substrates were denatured, and the incubations with lysyl hydroxylase were carried out as described in Methods.

(Kivirikko and Prockop, 1967; Kivirikko *et al.*, 1972b). No release of $^{14}CO_2$ was observed with this substrate (Table I, expt 3).

Comparison of Native and Denatured Collagen as Substrates for Further Hydroxylation. The above experiments were all carried out with denatured collagens. To study whether there was any difference between the rates of hydroxylation of native and denatured collagen, two native preparations of acid-soluble calf skin collagen were reacted with the enzyme. When the experiment was carried out at 37° and with 300 μg/ml of collagen, the release of $^{14}CO_2$ with the native collagen preparations was only about 13% of that observed with the denatured collagens (Table II).

Because it seemed possible that native collagen preparations may contain a small fraction of denatured collagen either initially or after the incubation at 37°, the experiment was repeated with a lower collagen concentration and at a lower temperature. The release of $^{14}CO_2$ observed at 30° with 100 μg/ml of native collagen was less than 6.3% of that observed with the same concentration of denatured collagen (Table II).

The question of whether native collagen preparations contained a small fraction of denatured collagen was studied further by trypsin treatment. In these experiments, 10 mg of collagen preparation I (Table II) was incubated with 1 mg of trypsin in 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM calcium chloride for 4 hr at 30 or 20°, and then the sample was dialyzed for 16 hr at 4° against distilled water. The solution outside the dialysis bag was evaporated to dryness, hydrolyzed with 6 M HCl for 16 hr at 120°, and assayed (Kivirikko *et al.*, 1967) for hydroxyproline. The results indicated that after treatment with trypsin at 30° 4.5% of the hydroxyproline in native collagen was recovered in dialyzable peptides, and after treatment at 20° 1.5%. Accordingly it seems that the native collagen preparations contained some denatured collagen, and at least part of the small release of $^{14}CO_2$ observed with native collagen was due to the hydroxylation of lysyl residues in denatured collagen. It appears therefore that although lysyl residues in denatured collagen can readily be hydroxylated further there is very little, if any, additional hydroxylation of lysyl residues in native collagen.

Hydroxylation of Lysyl Residues in the Fragment α1-CB8-HA1 of Rat Skin Collagen. In order to examine the hydroxylation of specific lysyl residues, the peptide α1-CB8-HA1 was studied as a substrate for hydroxylation by lysyl hydroxylase after thermal denaturation. The peptide contains 99 amino acid residues, and its amino acid sequence has been determined (Balian *et al.*, 1971). The peptide has two lysyl residues, both of them in the sequence -Gly-Ala-Lys-Gly-, and the level of *in vivo* hydroxylation of these two residues is only about 10% (Balian *et al.*, 1971). The peptide was found to serve as a substrate for the *in vitro* hydroxylation of lysyl residues (Table III). However, the degree of hydroxylysine synthesis was substantially lower than that observed with comparable concentrations of collagen. Because the molecular weight of the peptide was only about one-tenth of that of a collagen chain, the difference in hydroxylysine synthesis between the peptide and collagen was even greater when the concentrations of substrates were expressed on a molar basis.

Quantitative Relationship between the Synthesis of Hydroxylysine and the Evolution of Carbon Dioxide in the Further Hydroxylation of Collagen. Experiments were carried out to study whether the stoichiometry between the synthesis of hydroxylysine and the release of $^{14}CO_2$ from [1-^{14}C]α-ketoglutarate, which was previously observed in the hydroxylation of synthetic peptides (Kivirikko *et al.*, 1972a), also holds for the further hydroxylation of collagen. Because the net synthesis of hydroxylysine had to be assayed as the difference between the initial hydroxylysine content and the value after the hydroxylation, experimental conditions were designed to give a large increase in hydroxylysine in order to minimize the analytical error. This was achieved by using relatively small amounts of collagen and large amounts of the enzyme (Table IV). It was not possible to assay the small amounts of hydroxylysine directly in the incubation mixture, which contained the enzyme, bovine serum albumin, catalase, and several other substances. Therefore, the collagen was examined by digestion with collagenase followed by assay for hydroxy-

TABLE IV: Relationship between the Synthesis of Hydroxylysine and the Evolution of Carbon Dioxide in the Further Hydroxylation of Lysyl Residues in Collagen.[a]

Expt	Sample	Amino Acid Analysis: Hyl Found (nmol)	Amino Acid Analysis: Hyl Synthesized (nmol)	$^{14}CO_2$ Assay: $^{14}CO_2$ Released (dpm)	$^{14}CO_2$ Assay: Hyl Synthesized (nmoles)
1	Control	6.98			
	Control	7.04			
	Hydroxylated	14.60	7.59	4520	7.53
2	Control	6.93			
	Hydroxylated	15.06	8.13	5170	8.61

[a] The substrates were denatured and the incubations were carried out for 80 min as four separate samples, each containing 100 μg of calf skin collagen, and 500 μg (expt 1) or 600 μg (expt 2) of enzyme. The specific activity of the enzyme used in expt 2 was about twice that used in expt 1. After the hydroxylation, the four samples were pooled, and the assay was continued as described in Methods. The values dpm of $^{14}CO_2$ released are mean values of the four separate samples. The nanomoles of hydroxylysine synthesis obtained by $^{14}CO_2$ assay were calculated from the observed disintegrations of $^{14}CO_2$ trapped and the specific activity of the [1-^{14}C]α-ketoglutarate.

lysine in the dialyzable peptides as described in Methods. The results of the experiments indicated that there was a large increase in the hydroxylysine content after the incubation with lysyl hydroxylase, and that the increase in hydroxylysine was equimolar with the evolution of $^{14}CO_2$ (Table IV).

Extent of Further Hydroxylation of Lysyl Residues in Collagen and in α1-CB8-HA1. Maximal hydroxylation of lysyl residues was studied in experiments in which varying amounts of lysyl hydroxylase were added to the incubation system containing a constant amount of collagen or the peptide α1-CB8-HA1 (Table V). The synthesis of hydroxylysine was assayed by the evolution of $^{14}CO_2$ from [1-^{14}C]α-ketoglutarate, and the disintegrations per minute of $^{14}CO_2$ were converted to nanomoles of hydroxylysine on the basis of equimolar synthesis of hydroxylysine and decarboxylation of α-ketoglutarate. Up to 44% of the lysyl residues in calf skin collagen were hydroxylated. In contrast, only about 18% of lysyl residues was hydroxylated in the peptide α1-CB8-HA1 (Table V).

It was not possible to demonstrate an exact limit in the extent of hydroxylation, but the increments were small with more than about 270 μg of enzyme and with longer incubation times (Table V, expt 3), suggesting that the highest values observed were near maximal.

Further Hydroxylation of Lysyl Residues in Separate α1 and α2 Chains of Collagen. Because the α2 chains of mammalian collagens have about twice the hydroxylysine content of the α1 chains (see Eastoe, 1967), experiments were carried out with isolated α1 and α2 chains of skin collagen from lathyritic rats. The results indicated that the synthesis of additional hydroxylysine with small amounts of the enzyme was about the same with both chains, whereas with large amounts of enzyme somewhat less hydroxylation was observed with α2 chains (Figure 1, lower curves). However, when the results were

TABLE V: Extent of Further Hydroxylation by Lysyl Hydroxylase of Denatured Acid-Soluble Calf Skin Collagen and of the Fragment α1-CB8-HA1 of Rat Skin Collagen.[a]

Expt	Substrate	Enzyme (μg)	$^{14}CO_2$ Released (dpm)	Hyl Synthesized (nmol)	% Lys Hydroxylated (% of Lys + Hyl Residues)
1	α1-CB8-HA1	0	0	0	10[b]
		45	330	0.55	12.5
		90	580	0.97	14.4
		180	850	1.42	16.4
		270	1040	1.73	17.8
		360	1080	1.80	18.1
	Calf skin collagen	0	0	0	19.7
		45	2080	3.47	29.6
		90	3050	5.08	34.1
		180	3960	6.60	38.4
		270	4230	7.05	39.7
		360	4170	6.95	39.4
2	Calf skin collagen	700	4740	7.90	42.1
3	Calf skin collagen	700[c]	5030	8.38	43.5
4	Calf skin collagen	600[d]	5170	8.61	44.1

[a] The substrates were denatured and the incubations with 100 μg/ml of the substrates were carried out as described in Methods, except that the incubation time was 80 min in expt 1, 2, and 4, and 160 min in expt 3. The nanomoles of CO_2 released were calculated from the observed disintegrations of $^{14}CO_2$ trapped and the specific activity of the [1-^{14}C]α-ketoglutarate. The values were converted to nanomoles of hydroxylysine synthesized on the basis of the stoichiometric release of CO_2 during the hydroxylation of lysine (Table IV). The values in expt 1 and 3 represent single determinations, those in expt 2 and 4 means of four determinations. [b] According to various previous analyses on the peptide α1-CB8-HA1 (Balian *et al.*, 1971) about 10% of the two lysyl residues in the peptide were hydroxylated. [c] The incubation time was 160 min. [d] The specific activity of the enzyme preparation used in expt 4 was about twice that used in expt 1–3.

expressed as a sum of original hydroxylysine and additional hydroxylysine, considerably higher values were noted with α2 chains than with α1 chains (Figure 1, upper curves). The shape of the curves suggest that the values observed with 300 μg of the enzyme were approaching a limit, and thus the maximal hydroxylysine content for α2 chains appears to be higher than that for α1 chains.

Discussion

Hydroxyproline and hydroxylysine in collagen are synthesized by enzymic reactions which occur after proline and lysine are incorporated into peptide linkage. It is of interest that the hydroxyproline content of most collagens from higher organisms is relatively constant and ranges from about 90

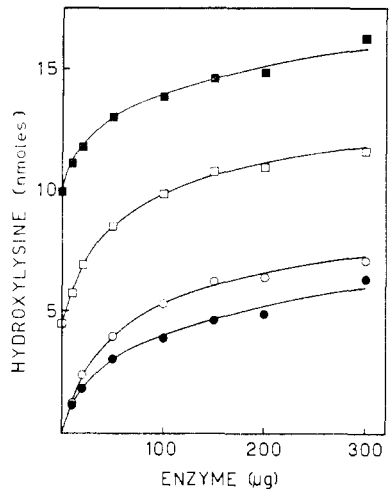

FIGURE 1: Further hydroxylation of lysyl residues in isolated $\alpha 1$ and $\alpha 2$ chains of skin collagen from lathyritic rats. The incubations with 100 μg of denatured $\alpha 1$ or $\alpha 2$ chains and varying amounts of lysyl hydroxylase were carried out as described in Methods, except that the incubation time was 80 min. The nanomoles of hydroxylysine synthesized were calculated from the observed disintegrations of $^{14}CO_2$ trapped and the specific activity of the [1-^{14}C]α-ketoglutarate (Table IV). The synthesis of additional hydroxylysine observed with $\alpha 1$ (○) and $\alpha 2$ (●) chains is shown by the lower curves. The values for the final hydroxylysine contents after hydroxylation of the $\alpha 1$ (□) and $\alpha 2$ (■) chains are shown by upper curves.

to 100 residues per polypeptide chain of about 1000 amino acid residues whereas the hydroxylysine content is more variable and ranges from about 5 to 20 residues per chain[1] (for review, see Grant and Prockop, 1972). Furthermore, the level of hydroxylation of lysyl residues in certain collagens decreases with age (Miller *et al.*, 1967; Barnes *et al.*, 1971a,b), and an increase by about 30–50% in the level of hydroxylation of lysyl residues was recently observed in rat and chick bone collagen in vitamin D deficiency (Toole *et al.*, 1972; Barnes *et al.*, 1973).

One of the explanations offered for the variable hydroxylysine contents of the collagens from different tissues has centered on the specificity of the lysyl hydroxylase (Grant and Prockop, 1972; Kivirikko *et al.*, 1972a). Although the enzyme hydroxylates lysyl residues in the Y positions of the repeating -X-Y-Gly- sequences of collagen, it has been suggested that the particular amino acids found in the X position of the same triplet or in either X or Y positions of adjacent triplets may greatly influence interaction with the enzyme. The results presented here support this suggestion in that lysyl residues in the peptide $\alpha 1$-CB8-HA1 were considerably less susceptible to further hydroxylation than average lysyl residues in skin or tendon collagen.

Previous studies (Kivirikko *et al.*, 1972a) on the substrate requirements of lysyl hydroxylase indicated that in addition to the amino acid sequences, the length of the peptide chain was a critical determinant for the reaction. The results with $\alpha 1$-CB8-HA1, however, cannot be explained by differences in chain length, because the peptide had 99 amino acid residues but was a poorer substrate than the peptide L-I, which contained only 12 amino acid residues. It seems likely therefore that either the sequence -Gly-Ala-Lys-Gly- or some of the sequences in adjacent triplets make the $\alpha 1$-CB8-HA1 peptide a particularly ineffective substrate. The differences in the hydroxylation between $\alpha 1$ and $\alpha 2$ chains of collagen are probably also attributable to differences in amino acid sequences. It might be noted that the currently available data suggest that the amino acids in adjacent sequences may have a more important effect on lysyl hydroxylase than on prolyl hydroxylase. It was previously shown that prolyl hydroxylase readily hydroxylates -Gly-Ala-Pro-Gly- sequences (Kivirikko *et al.*, 1968), and prolyl residues in the collagen from *Ascaris* cuticle (Fujimoto and Prockop, 1968). On the other hand, lysyl hydroxylase poorly hydroxylated the -Gly-Ala-Lys-Gly- sequences in $\alpha 1$-CB8-HA1 and was previously reported not to interact with *Ascaris* cuticle collagen even though it contains some 40 lysyl residues and no hydroxylysine (Kivirikko *et al.*, 1972a).

A second factor which might explain the variable hydroxylysine contents of different collagens is variability in the concentrations of substrate, enzyme, and cofactors in cells synthesizing collagen and variability in the time that the substrate is exposed to active enzyme during its intracellular processing. The results presented here clearly demonstrate that the hydroxylysine contents of collagens from tissues such as skin and tendon do not reflect the maximal extent to which lysyl hydroxylase can hydroxylate these proteins. Although it was previously shown that the hydroxyproline contents of such collagens can be increased by reaction with prolyl hydroxylase, the increases were relatively small (Rhoads *et al.*, 1971). The hydroxylysine contents of similar collagens could be doubled by a reaction with the lysyl hydroxylase, and on this basis the level of hydroxylation of lysyl residues is clearly much lower than that of prolyl residues. It has recently been found that there are large differences in the levels of lysyl hydroxylase activity in various tissues of chick embryos of the same age and large differences exist even within the same tissue from chick embryos of varying ages (L. Ryhänen and K. I. Kivirikko, in preparation). It seems reasonable to assume therefore that such differences in the activity of the lysyl hydroxylase may help to explain differences in the hydroxylysine contents of different collagens.

The results presented here suggest a third factor which might affect the hydroxylysine content of various collagens, namely, the conformation of the protein during the time it is exposed to the hydroxylase. Although lysyl residues in denatured collagen were readily hydroxylated, very little, if any, further hydroxylation of lysyl residues was observed with native collagen. This observation is consistent with previous observations on the additional hydroxylation of prolyl residues in collagen (Rhoads *et al.*, 1971) and with several recent observations with protocollagen. Sufficient quantities of triple-helical protocollagen have recently been isolated to study its thermal stability by optical rotation. The results demonstrated that the unhydroxylated protein has a T_m of 24°, a value which is about 15° lower than the T_m of collagen (Berg and Prockop, 1973b). Similar results were obtained in experiments on the resistance of protocollagen to digestion with proteolytic enzymes (Jimenez *et al.*, 1973). These observations as well as studies on the intracellular hydroxylation of protocollagen (Uitto and Prockop, 1973) suggest that hydroxylation of polypeptide chains helps to establish a helical conformation. Experiments with protocollagen have also demonstrated that when protocollagen is triple helical, it cannot be hydroxylated by prolyl hydroxylase (Berg and Prockop, 1973c). The latter observation together with the

[1] The collagen found in basement membranes differs in several important respects from the collagens in other tissues (Kefalides, 1971). Its hydroxyproline content is about 130 residues/1000 amino acid residues and its hydroxylysine content is about 40 residues/1000 amino acid residues.

results on further hydroxylation of prolyl residues in collagen (Rhoads *et al.*, 1971), and the results obtained here on further hydroxylation of lysyl residues in collagen, suggest that when newly synthesized polypeptide chains become helical within the cell, further hydroxylation of prolyl and lysyl residues ceases. Recent observations on the hydroxylation of lysyl residues in native and denatured protocollagen are consistent with this suggestion (L. Ryhänen and K. I. Kivirikko, to be published). Consequently, differences among tissues in the stage at which the polypeptides become helical may contribute to differences in the hydroxylysine contents of collagens from these tissues.

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