Biochem. J. (1983) **213**, 507–512
Printed in Great Britain

The source of oxygen in the reaction catalysed by collagen lysyl hydroxylase

Yasuo KIKUCHI, Yoshihiro SUZUKI and Nobuo TAMIYA
Department of Chemistry, Tohoku University, Aobayama, Sendai, Miyagi 980, Japan

(*Received 14 February 1983/Accepted 28 March 1983*)

The synthetic peptides (Pro-Pro-Gly)$_5$ and (Ile-Lys-Gly)$_5$-Phe were hydroxylated with collagen prolyl hydroxylase and lysyl hydroxylase in an $^{18}O_2$ atmosphere. The oxygen atoms in the hydroxy groups of hydroxyproline and hydroxylysine were 87% and 6.5% respectively derived from the atmospheric $^{18}O_2$. The results are consistent with those reported previously for proline hydroxylation *in vivo* [Fujimoto & Tamiya (1962) *Biochem. J.* **84**, 333–335; Prockop, Kaplan & Udenfriend (1962) *Biochem. Biophys. Res. Commun.* **9**, 192–196; Fujimoto & Tamiya (1963) *Biochem. Biophys. Res. Commun.* **10**, 498–501; Prockop, Kaplan & Udenfriend (1963) *Arch. Biochem. Biophys.* **101**, 499–503] and *in vitro* [Cardinale, Rhoads & Udenfriend (1971) *Biochem. Biophys. Res. Commun.* **43**, 537–543] and for lysine hydroxylation *in vivo* [Fujimoto & Tamiya (1963) *Biochem. Biophys. Res. Commun.* **10**, 498–501]. In view of the similarities of these two oxygenase-type hydroxylation reactions the participation of intermediates is proposed, the oxygen atoms of which are exchangeable with those of water. The atmospheric oxygen atoms incorporated into the intermediate must be equilibrated with water oxygen atoms in the slower lysyl hydroxylase reaction.

In the biosynthesis of collagen, proline and lysine are converted into hydroxyproline and hydroxylysine by enzymic hydroxylations (Stetten & Schoenheimer, 1944; Stetten, 1949; Sinex & Van Slyke, 1955; Piez & Links, 1957). An oxygenase mechanism was proposed for the proline hydroxylation on the basis of the ^{18}O incorporation in hydroxyproline synthesized in chick embryos under an ^{18}O-enriched atmosphere (Fujimoto & Tamiya, 1962, 1963; Prockop *et al.*, 1962, 1963). On the other hand, hydroxy-lysine from the same embryos did not contain ^{18}O (Fujimoto & Tamiya, 1963). Two explanations were suggested for the mechanism of lysine hydroxylation: (1) that lysine is hydroxylated through a non-oxygenase mechanism; (2) that the oxygen atom incorporated through an oxygenase mechanism is exchanged with water oxygen by a dehydratase reaction *in vivo*.

In subsequent studies it became clear that the amino acids are hydroxylated as residues in the polypeptide chain (Udenfriend, 1966; Prockop *et al.*, 1966). The enzymes prolyl 4-hydroxylase (prolyl-glycyl-peptide,2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) and lysyl hydroxylase (peptidyl-lysine,2-oxoglutarate:oxygen 5-oxidoreductase, EC 1.14.11.4) were purified (Tuderman *et al.*, 1975; Turpeenniemi *et al.*, 1977). They require molecular oxygen, 2-oxoglutarate, Fe^{2+} ions and ascorbic acid, with similar K_m values (Kivirikko & Prockop, 1972; Tuderman *et al.*, 1977; Puistola *et al.*, 1980*a*). They decarboxylate 2-oxoglutarate stoicheiometrically with the formation of the hydroxylated amino acids (Rhoads & Udenfriend, 1968; Kivirikko *et al.*, 1972). The incorporation of atmospheric oxygen atoms into hydroxyproline and succinate was demonstrated with partially purified prolyl hydroxylase (Cardinale *et al.*, 1971). These results strongly suggest an oxygenase mechanism for lysine hydroxylation, although the oxygen source of the hydroxy group of hydroxylysine has not been demonstrated with purified lysyl hydroxylase.

In the present study the synthetic substrates (Pro-Pro-Gly)$_5$ (Sakakibara *et al.*, 1968; Kikuchi *et al.*, 1969) and (Ile-Lys-Gly)$_5$-Phe (Kikuchi & Tamiya, 1982) were hydroxylated with purified prolyl 4-hydroxylase and lysyl hydroxylase in a mixture under an $^{18}O_2$ atmosphere, and the incorporation of ^{18}O into hydroxyproline and hydroxylysine was examined.

Materials and methods

Hydroxylysine

Hydroxylysine hydrochloride (mixture of DL-hydroxylysine and allo-DL-hydroxylysine) was synthesized by the method of Van Zyl *et al.* (1951) in an

overall yield of 35% (Found: C, 35.96; H, 7.65; N, 14.46. Calc. for $C_6H_{15}ClN_2O_3$: C, 36.27; H, 7.61; N, 14.10%).

Synthetic substrates

The peptides (Pro-Pro-Gly)$_5$ and (Ile-Lys-Gly)$_5$-Phe were synthesized by a solid-phase fragment-condensation method (Sakakibara *et al.*, 1968; Kikuchi & Tamiya, 1982).

Collagen prolyl 4-hydroxylase

Collagen propyl 4-hydroxylase was extracted from 13-day chick embryos (200g wet wt.) and purified by $(NH_4)_2SO_4$ fractionation and affinity chromatography on a poly-(L-proline)–agarose column (1.5cm × 10cm) (Tuderman *et al.*, 1975). Poly-(L-proline) was synthesized by the *N*-carboxy-anhydride method (Oya *et al.*, 1970) and fractionated on a Sephadex G-50 column. The fraction of lower $K_{av.}$ (0–0.2) was coupled to Sepharose 4B (Pharmacia) by the CNBr method (Cuatrecasas, 1970), and the fraction of higher $K_{av.}$ (0.4–0.5) was used to elute the enzyme from the affinity column: $K_{av.} = (V_e - V_0)/(V_t - V_0)$, where $K_{av.}$, V_0, V_t and V_e represent available distribution coefficient, void volume, column volume and elution volume respectively. The enzyme fraction was gel-filtered on a Bio-Gel A-1.5m column (1.8cm × 100cm) and concentrated to 4ml by ultrafiltration with an Amicon PM-30 membrane. The solution contained 1.7mg of protein/ml. A sample (containing 45μg of protein) of the preparation formed 12μg of hydroxyproline during 2h incubation at 37°C in a mixture (1.5ml) containing (Pro-Pro-Gly)$_5$ (0.16mg) and essential co-substrates described below.

Collagen lysyl hydroxylase

Collagen lysyl hydroxylase was extracted from 13-day chick embryos (675g wet wt.) and purified by $(NH_4)_2SO_4$ fractionation and affinity chromatography on a concanavalin A–Sepharose 4B column (2.2cm × 18cm) as described by Turpeenniemi *et al.* (1977) except that the buffer solution did not contain $MnCl_2$. The enzyme fraction containing α-methyl D-mannoside and ethylene glycol was repeatedly diluted with the buffer solution and concentrated by ultrafiltration on a PM-30 membrane. The solution (22ml) contained 38mg of protein. Further purification was not attempted because of rapid loss of enzymic activity.

Enzymic hydroxylation

The reaction mixture (total 70ml) contained (Pro-Pro-Gly)$_5$ (13mg), (Ile-Lys-Gly)$_5$-Phe (8.4mg), prolyl 4-hydroxylase (5.1mg of protein), lysyl hydroxylase (38mg of protein), 0.5mM-2-oxoglutarate, 0.05mM-$FeSO_4$, 2mM-ascorbic acid, 0.1mM-dithiothreitol, bovine serum albumin (150mg), catalase (0.8mg) and 50mM-Tris/HCl buffer, pH7.8, at 25°C. The solution containing all the components except enzyme preparations was placed in a round-bottomed flask (volume 370ml) with a side arm and a stop-cock and was degassed at 0.4kPa (3mmHg) for 5min under ice cooling. After N_2 gas at 100kPa (1atm) was introduced into the flask, the prolyl 4-hydroxylase solution (3ml) and the lysyl hydroxylase solution (22ml) were added to the mixture. The N_2 gas was evacuated off and $^{18}O_2$ gas (90atom% ^{18}O; Merck, Sharp and Dohme, Montreal, Quebec, Canada) at 20kPa (0.2atm) was introduced into the flask. The mixture was incubated at 37°C for 2h in the closed flask and freeze-dried.

Recovery of hydroxylated peptides

The freeze-dried residue of the reaction mixture was dissolved in 0.1M-acetic acid (6ml) and gel-filtered on a Sephadex G-50 (fine grade) column (Fig. 1). The peptide-containing fraction was pooled as shown in Fig. 1 and freeze-dried (16.4mg, 77% recovery by wt.). The amino acid composition of the hydrolysate of a sample (125μg) of the material is shown in Table 1.

Isolation of hydroxyproline

The hydroxylated peptide mixture (5mg) was hydrolysed with 6M-HCl (0.5ml) at 105°C for 24h, and amino acids in the hydrolysate were adsorbed on an Amberlite IR-120 (H^+ form) column (0.5cm × 0.5cm). Acidic and neutral amino acids were eluted from the column with 1M-pyridine and dried *in vacuo*. The residue was dissolved in 0.2M-sodium citrate buffer, pH2.2 (1ml), and chromatographed on the column of a JLC-8AH amino acid analyser (JEOL Co., Tokyo, Japan). The hydroxyproline-containing part of the eluate was collected and passed through an Amberlite IR-120

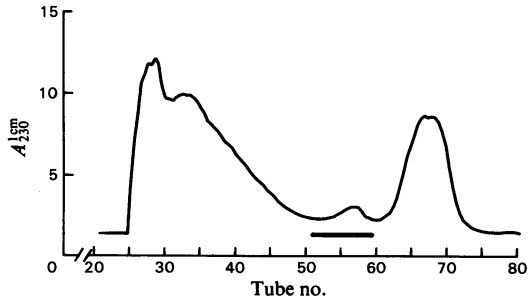


Fig. 1. *Gel filtration of the reaction mixture containing the hydroxylated peptides*

A column (2.6cm × 90cm) of Sephadex G-50 (fine grade) was eluted with 0.1M-acetic acid, and 6.3ml fractions were collected. The bar shows the fraction pooled.

Table 1. *Amino acid composition of the hydroxylated peptide mixture*

The peptide mixture was hydrolysed with 6M-HCl (0.2ml) at 105°C for 24h. Amino acids in the hydrolysate were analysed with a JLC-10D amino acid analyser (JEOL Co.).

Amino acid	Composition (nmol/125 μg)
Hyl	3.0
Lys	48.3
His	12.1
Hyp	33.9
Asp	33.8
Thr	19.6
Ser	16.2
Glu	47.1
Pro	209.2
Gly	181.3
Ala	23.2
Val	15.1
Met	3.1
Ile	27.2
Tyr	7.2
Phe	16.6

(H^+ form) column (0.8cm × 8cm). Hydroxyproline was eluted with 1M-pyridine and dried *in vacuo*. The residue was dissolved in water (1ml) and passed through a Dowex 1-X8 (H^+ form) column (0.5cm × 2cm) to remove contaminating aspartic acid. Hydroxyproline in the eluate was adsorbed on an Amberlite IR-120 (H^+ form) column (0.5cm × 0.5cm), eluted with 1M-pyridine and dried *in vacuo*. The recovery of hydroxyproline was 71 μg (39% yield).

Isolation of hydroxylysine

The hydroxylated peptide mixture (10mg) was dissolved in 1M-pyridine (1.5ml) and applied to a CM-52 CM-cellulose (Whatman) column (0.8cm × 1cm) equilibrated with 1M-pyridine. After the column had been washed with 1M-pyridine, the basic peptide was eluted with 3M-NH_3. The basic peptide, i.e. hydroxylated (Ile-Lys-Gly)$_5$-Phe, was hydrolysed with 6M-HCl (1.2ml) at 105°C for 24h, and the amino acids in the hydrolysate were adsorbed on an Amberlite IR-120 (H^+ form) column (0.5cm × 1cm). After the neutral amino acids had been eluted with 1M-pyridine, hydroxylysine and lysine were eluted with 3M-NH_3 and dried *in vacuo*. The residue was dissolved in 1M-HCl (0.2ml) and chromatographed on an Amberlite IR-120 column (0.5cm × 3cm) in 1.2M-HCl. Hydroxylysine and lysine were eluted at 8–12ml and 16–25ml respectively. Hydroxylysine was adsorbed on an Amberlite IR-120 column (0.5cm × 0.5cm), eluted with 3M-NH_3 and dried *in vacuo*. The purification was repeated again. The yield of hydroxylysine was 23 μg (62%).

^{18}O content in hydroxyproline

Hydroxyproline (45 μg) isolated as above was heated in bis(trimethylsilyl)trifluoroacetamide (Nakarai Chemicals, Kyoto, Japan) (0.2ml) and acetonitrile (0.2ml) at 135°C for 15min (Gehrke & Leimer, 1970). The resulting tris(trimethylsilyl) derivative was subjected to g.l.c.–mass spectrometry. The JGC-20K gas chromatograph (JEOL Co.) was equipped with a 3% SE-30 column (0.6cm × 100cm) and operated with helium (3.6kg/cm^2) as carrier gas. The temperature was set at 100°C and increased at a rate of 15°C/min. The JMS-D100S mass spectrometer equipped with JMA-2000S data-analysis system (JEOL Co.) was operated at 25eV of bombarding electron energy (Fig. 2).

^{18}O content in hydroxylysine

Hydroxylysine (21 μg) isolated as above was dissolved in methanol (10 μl), and a sample (1 μl) was subjected to field-desorption mass spectrometry (Beckey, 1969) with a JMS-D300 mass spectrometer equipped with MS-FD03 field-desorption ion source and JMA-2000S data-analysis system (JEOL Co.). The emitter current was set at 0mA and increased at a rate of 3mA/min (Fig. 3).

Results

The amino acid composition of the peptides recovered from the enzymic reaction mixture (Table 1) shows that 5.8% of the peptidyl lysine residues and 24.5% of the susceptible peptidyl proline residues were hydroxylated during the incubation. As shown in Fig. 1, the peptide peak overlapped with the tailing part of the protein peak. This is probably the reason for the presence of various amino acids in the hydrolysate.

Fig. 2 shows the mass spectrum of the tris-(trimethylsilyl) derivative of the enzymically formed hydroxyproline. The peak at m/z 232 corresponds to the fragment ion containing one ^{18}O (Scheme 1). As the fragment ion has only one oxygen atom, the ^{18}O contained is assigned to the oxygen in the hydroxy group of hydroxyproline. The derivative of authentic hydroxyproline gave its corresponding fragment-ion peak at m/z 230 (not shown). When the relative intensities of the peaks at m/z 230 and 232 (25.4 and 100 respectively) and ^{18}O content of the oxygen gas (90atom%) are considered, the incorporation yield of atmospheric oxygen into hydroxyproline is calculated to be 87%.

The field-desorption mass spectrum of the enzymically formed hydroxylysine is shown in Fig. 3. As the relative molecular mass of hydroxylysine is 162, the peak at m/z 163 is assigned to the pseudomolecular ion, $(M+1)^+$, of hydroxylysine containing no ^{18}O. The $(M+1)^+$ ion of ^{18}O-containing hydroxylysine should appear at m/z 165. In

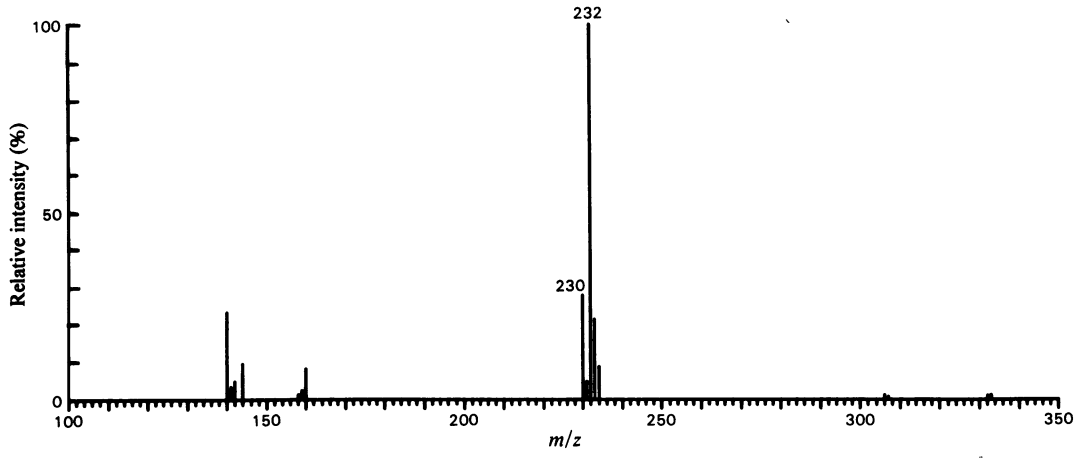


Fig. 2. *Mass spectrum of the tris(trimethylsilyl) derivative of hydroxyproline formed enzymically in the presence of molecular* $^{18}O_2$

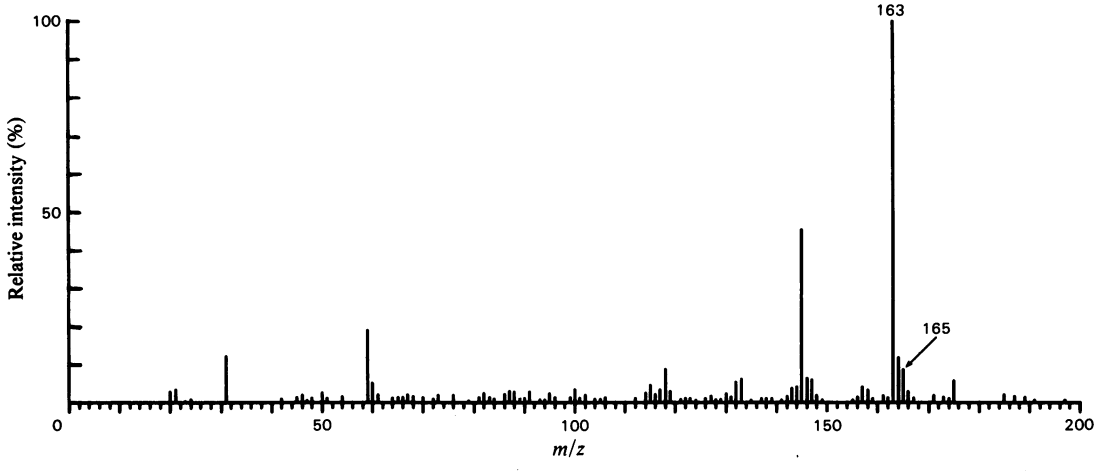
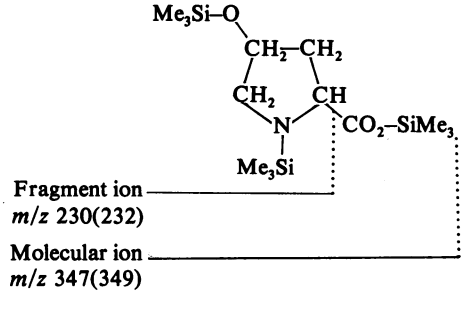


Fig. 3. *Field-desorption mass spectrum of hydroxylysine formed enzymically in the presence of molecular* $^{18}O_2$



Scheme 1. *Expected fragmentation of tris(trimethylsilyl)hydroxyproline*
The *m/z* values of ^{18}O-containing ions are shown in parentheses.

Fig. 3 the relative intensity of the peak at *m/z* 165 is 8.6. In the mass spectrum of authentic hydroxylysine it should be 0.8 according to the natural abundance of isotopes, and it was 2.9 in the observed spectrum (not shown). When the relative intensities of the peaks at *m/z* 163 and 165 and the ^{18}O content (90 atom %) are taken into account, the incorporation of atmospheric oxygen into the hydroxy group of hydroxylysine is calculated to be 6.5%.

The following observation excludes the possibility that the hydroxy oxygen of hydroxylysine was exchanged with water oxygen during the experimental procedure; the fragment ion II (Scheme 2) appeared at *m/z* 243 in the mass spectrum (Fig. 4)

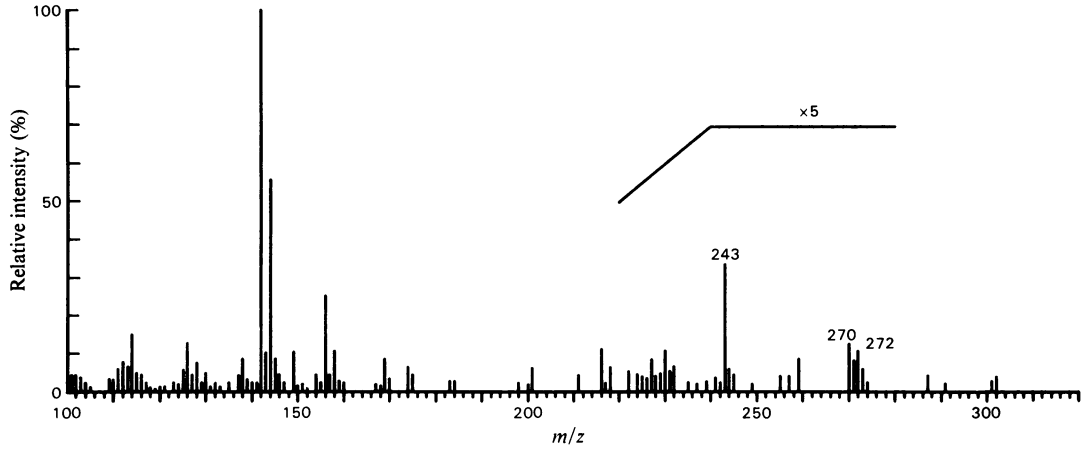
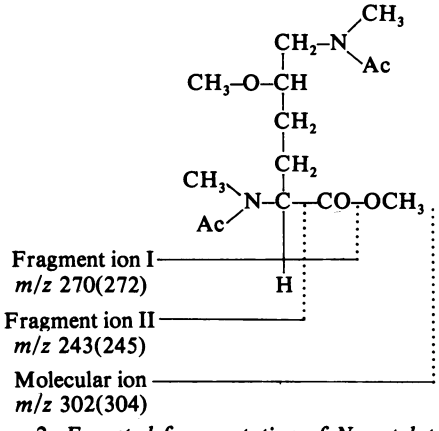
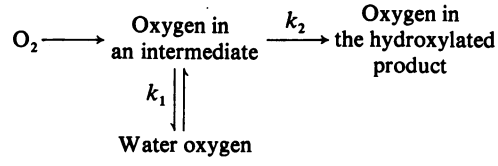


Fig. 4. *Mass spectrum of N-acetylated and permethylated hydroxylysine*
Hydroxylysine (1 mg) was heated in 6 M-HCl (0.5 ml) containing $H_2{}^{18}O$ (73 atoms % ^{18}O) at 105°C for 120 h. The *N*-acetylated and permethylated derivate was prepared by the method of Morris *et al.* (1971). The M-52 mass spectrometer (Hitachi Co., Tokyo, Japan) was set at 80°C of direct-point temperature and operated at 25 eV of bombarding electron energy.



Scheme 2. *Expected fragmentation of N-acetylated and permethylated hydroxylysine*
The *m/z* values of ^{18}O-containing ions are shown in parentheses.



Scheme 3. *Reaction scheme involving an oxygen exchange*

of the *N*-acetylated and permethylated derivative of hydroxylysine that had been heated in 6 M-HCl containing $H_2{}^{18}O$ previously. The presence of fragment ion I at *m/z* 270 and 272 indicates that the oxygen in the carboxy group was equilibrated with water oxygen (^{18}O). Hydroxylysine kept in 3 M-NH_3 containing $H_2{}^{18}O$ at room temperature for 2 h gave a similar result (not shown).

Discussion

Two reaction mechanisms proposed for proline hydroxylation (Lindblad *et al.*, 1969; Cardinale *et al.*, 1971; Hamilton, 1971) can explain the incorporation of atmospheric oxygen into hydroxyproline and succinate (Cardinale *et al.*, 1971). In the present study, 87% of the hydroxy group of hydroxyproline was derived from molecular oxygen ($^{18}O_2$). On the other hand, the oxygen in the hydroxy group of hydroxylysine was mostly from water. These results confirm the observation made on lysine hydroxylation *in vivo* (Fujimoto & Tamiya, 1963) but are inconsistent with the mechanisms proposed for the proline hydroxylation. It is also evident, however, that molecular oxygen is essential for the two hydroxylase reactions, and that these two reactions are similar to each other in many apsects, including kinetic parameters (Myllylä *et al.*, 1977; Puistola *et al.*, 1980*b*). To explain all these observations, we propose the participation of intermediates, the oxygen atoms of which are exchangeable with those of water. Although nothing can be suggested on the mechanism of the oxygen exchange, it took place efficiently in the hydroxylation of lysine *in vivo* and in the reaction with the partially purified enzyme *in vitro*. It is unlikely that

the lysyl hydroxylase preparation used in the present study contained an enzyme that catalyses the exchange of the hydroxy oxygen of hydroxylysine with that of water through dehydration and hydration. The intermediates that contain an exchangeable oxygen are probably involved in both lysyl and prolyl hydroxylations.

The low incorporation yield of atmospheric oxygen into the hydroxy group was reported also in the hydroxylation of 4-methyl-2-oxovalerate (Sabourin & Bieber, 1982) and of *p*-hydroxyphenylpyruvate (Lindblad *et al.*, 1970). In these cases, the substrates contain 2-oxo acid groups to be decarboxylated during the reactions and the enzymes require molecular oxygen, Fe^{2+} ions and ascorbic acid. The oxygen exchange through similar intermediates may be common in the reactions of this particular class of hydroxylases (Scheme 3). The extent of the oxygen incorporation from the atmosphere depends on the k_1/k_2 ratio. In lysine hydroxylation, the oxygen equilibration is much faster than the hydroxylation ($k_1 \gg k_2$), and in proline hydroxylation the ratio is reversed ($k_1 \ll k_2$). In the hydroxylation of 4-methyl-2-oxovalerate and *p*-hydroxyphenylpyruvate, k_1 and k_2 are presumed to be of comparable magnitude.

We thank Professor Masanao Oya and Professor Ryoichi Katagai of Gunma University for practical suggestions in the synthesis of poly-(L-proline). We are also grateful to Professor Hideki Sakurai, Dr. Yasuhiro Nakadaira and Mr. Akihiro Nakamura of this department for g.l.c.–mass spectrometry, to Mr. Hiroshi Kameyama of the technology department for field-desorption mass spectrometry, and to Mr. Hisaku Abe of this laboratory for amino acid analysis.

References

Beckey, H. D. (1969) *Int. J. Mass Spectrum. Ion Phys.* **2**, 500–503

Cardinale, G. J., Rhoads, R. E. & Udenfriend, S. (1971) *Biochem. Biophys. Res. Commun.* **43**, 537–543

Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065

Fujimoto, D. & Tamiya, N. (1962) *Biochem. J.* **84**, 333–335

Fujimoto, D. & Tamiya, N. (1963) *Biochem. Biophys. Res. Commun.* **10**, 498–501

Gehrke, C. W. & Leimer, K. (1970) *J. Chromatogr.* **53**, 201–208

Hamilton, G. A. (1971) *Prog. Bioorg. Chem.* **1**, 83–157

Kikuchi, Y. & Tamiya, N. (1982) *Bull. Chem. Soc. Jpn.* **55**, 1556–1560

Kikuchi, Y., Fujimoto, D. & Tamiya, N. (1969) *Biochem. J.* **115**, 569–574

Kivirikko, K. I. & Prockop, D. J. (1972) *Biochim. Biophys. Acta* **258**, 366–379

Kivirikko, K. I., Shudo, K., Sakakibara, S. & Prockop, D. J. (1972) *Biochemistry* **11**, 122–129

Lindblad, B., Lindstedt, G., Tofft, M. & Lindstedt, S. (1969) *J. Am. Chem. Soc.* **91**, 4604–4606

Lindblad, B., Lindstedt, G. & Lindstedt, S. (1970) *J. Am. Chem. Soc.* **92**, 7446–7449

Morris, H. R., Williams, D. H. & Ambler, R. P. (1971) *Biochem. J.* **125**, 189–201

Myllylä, R., Tuderman, L. & Kivirikko, K. I. (1977) *Eur. J. Biochem.* **80**, 349—357

Oya, M., Katagai, R., Uno, K. & Iwakura, Y. (1970) *Kogyo Kagaku Zasshi* **73**, 2371–2377; cited in *Chem. Abstr.* (1971) **75**, 6397e

Piez, K. A. & Links, R. C. (1957) *J. Biol. Chem.* **229**, 101–109

Prockop, D. J., Kaplan, A. & Udenfriend, S. (1962) *Biochem. Biophys Res. Commun.* **9**, 192–196

Prockop, D. J., Kaplan, A. & Udenfriend, S. (1963) *Arch. Biochem. Biophys.* **101**, 499–503

Prockop, D. J., Weinstein, E. & Mulveny, T. (1966) *Biochem. Biophys. Res. Commun.* **22**, 124–128

Puistola, U., Turpeenniemi-Hujanen, T. M., Myllyä, R. & Kivirikko, K. I. (1980*a*) *Biochim. Biophys. Acta* **611**, 40–50

Puistola, U., Turpeenniemi-Hujanen, T. M., Myllyä, R. & Kivirikko, K. I. (1980*b*) *Biochim. Biophys. Acta* **611**, 51–60

Rhoads, R. E. & Udenfriend, S. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **60**, 1473–1478

Sabourin, P. J. & Bieber, L. L. (1982) *J. Biol. Chem.* **257**, 7468–7471

Sakakibara, S., Kishida, Y., Kikuchi, Y., Sakai, R. & Kakiuchi, K. (1968) *Bull. Chem. Soc. Jpn.* **41**, 1273

Sinex, F. M. & Van Slyke, D. D. (1955) *J. Biol. Chem.* **216**, 245–250

Stetten, M. R. (1949) *J. Biol. Chem.* **181**, 31–37

Stetten, M. R. & Schoenheimer, R. (1944) *J. Biol. Chem.* **153**, 113–132

Tuderman, L., Kuutti, E. R. & Kivirikko, K. I. (1975) *Eur. J. Biochem.* **52**, 9–16

Tuderman, L., Myllylä, R. & Kivirikko, K. I. (1977) *Eur. J. Biochem.* **80**, 341–348

Turpeenniemi, T. M., Puistola, U., Anttinen, H. & Kivirikko, K. I. (1977) *Biochim. Biophys. Acta* **483**, 215–219

Udenfriend, S. (1966) *Science* **152**, 1335–1340

Van Zyl, G., van Tamelen, E. E. & Zuidema, G. D. (1951) *J. Am. Chem. Soc.* **73**, 1765–1767