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KINETIC PATTERNS OF PROTOCOLLAGEN HYDROXYLASE AND FURTHER STUDIES ON THE POLYPEPTIDE SUBSTRATE

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SUMMARY

The kinetics of the protocollagen hydroxylase reaction were examined by the technique of measuring the initial velocities as a function of the concentration of one reactant with a series of fixed concentrations of a second reactant, and with a constant concentration of the remaining reactants. From the data a rate equation was developed for the interaction of the enzyme with ascorbate, α-ketoglutarate, oxygen, and polypeptide substrate. The kinetic data were consistent with a scheme involving consecutive bimolecular additions of ascorbate and α-ketoglutarate followed by the random order addition of oxygen and polypeptide substrate, but other kinetic schemes were not excluded.

Studies with a purified enzyme system indicated that α-ketoglutarate was not consumed in stoichiometric amounts in the reaction.

Further studies on the polypeptide substrate supported earlier indications that the enzyme can hydroxylate polypeptides without triple helical structure. When proline-labeled [^{14}C]protocollagen was boiled and quenched, the synthesis of [^{14}C]-hydroxyproline was 74% of the control value. Also, the polypeptide (Gly–L-Ala–L-Pro)$_n$ which apparently does not have triple helical structure in solution was found to serve as a substrate for the synthesis of hydroxyproline by the enzyme. The K_m value and maximum velocity for (Gly–L-Ala–L-Pro)$_n$ were slightly lower than for (L-Pro–Gly–L-Pro)$_n$ under the same conditions.

INTRODUCTION

The enzyme protocollagen hydroxylase[1,2] synthesizes hydroxyproline by the hydroxylation of proline in protocollagen, the large proline-rich[3–7] and lysine-rich[1,8–10] polypeptide precursor of collagen. Free proline, proline in tripeptides, or proline in

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poly-L-proline does not serve as a substrate for the enzyme[11], but the enzyme hydroxylates proline in polytripeptides of the structure $(Pro–Gly–Pro)_n$ (refs. 1, 2, 11, 12). The same enzyme may be involved in the synthesis of the hydroxylysine in collagen[1], and the hydroxylation of both proline[3,5,13–16] and lysine[1,8–10,17] in protocollagen requires atmospheric oxygen, ferrous iron, α-ketoglutarate, and ascorbate. In the present paper an attempt was made to examine the kinetic patterns of the enzyme, and to examine further the requirements for the polypeptide substrate.

MATERIALS AND METHODS

Materials

The polytripeptide with the structure $(L\text{-Pro–Gly–L-Pro})_n$ was purchased from the Yeda Research and Development Co., Rehovoth, Israel. The polymer was prepared and fractionated as prescribed previously[2,18] and it had an average molecular weight by ultracentrifugation of about 4000. The polytripeptide with the structure $(Gly–L\text{-Ala–L-Pro})_n$ was also purchased from the Yeda Research and Development Co. The polytripeptide was fractionated in the same manner as the $(L\text{-Pro–Gly–L-Pro})_n$, and it had an average molecular weight of about 8000. Proline-labeled [^{14}C]-protocollagen was prepared from chick embryo cartilage as described previously[1,3]. Sodium 2-keto[5-^{14}C]glutarate, specific activity 9.33 mC/mmole, was purchased from Nuclear Chicago Corp. Poly-L-proline, Form II, with a molecular weight of about 15 000 was a gift from Dr. D. WASSERMAN, Research Division, Ethicon, Inc.

Purification of protocollagen hydroxylase

The enzyme was prepared with slight modifications of the previously published procedures[1,2,11]. A total of 400 12-day-old chick embryos were used as the source of enzyme, and the $(NH_4)_2SO_4$ fraction (30–65% satn.) of the chick embryo extract was prepared as described previously[2]. The calcium phosphate gel step was modified in the following manner. The dialyzed $(NH_4)_2SO_4$ fraction was diluted with 0.05 M KCl to a protein concentration of 15 mg/ml, and a suspension of calcium phosphate gel (Calbiochem), 12 mg solid per ml, was added slowly. After 5.5 ml of gel per 1000 mg protein was added, the suspension was stirred for 12 min, and it was centrifuged for 10 min at 15 000 $\times$ g. The same amount of gel was added to the supernatant fraction, and a second pellet was obtained. This step was repeated until 5 separate pellets were obtained. Each of the 5 pellets was eluted in two steps with potassium phosphate buffer. The first elution was with 0.02 M potassium phosphate (pH 7.0) and the second elution was with 0.15 M potassium phosphate (pH 7.3). In each case 3 ml of buffer were used per ml of gel suspension used to obtain the pellet. Each elution was carried out by thoroughly dispersing the gel and stirring it for 30 min in the eluting buffer, and then centrifuging the suspension at 15 000 $\times$ g for 10 min. The eluates were precipitated with solid $(NH_4)_2SO_4$ to 48% satn., and after centrifugation, the pellets were dissolved in a small volume of 0.1 M KCl and 0.02 M Tris–HCl buffer (pH 7.2). The fractions were then dialyzed and frozen in 5-ml aliquots as described earlier[2]. Enzyme fractions with the highest specific activity were usually obtained in the 0.15 M eluate of the third and fourth, or the fourth and fifth pellets.

Enzymatic reaction and assay procedures

The enzymatic reaction under standard conditions was carried out in a final volume of 8 ml which contained 0.2–0.3 mg/ml enzyme protein purified through the calcium phosphate step; 125 μg/ml $(Pro–Gly–Pro)_n$; 0.05 mM $FeSO_4$; 0.5 mM α-ketoglutarate; 2 mM ascorbic acid (Fisher Sci.); 0.05 mg/ml catalase (Calbiochem); and 0.05 M Tris–HCl buffer, adjusted to pH 7.8 at 25° (ref. 2). The samples were incubated at 37° with shaking for 1 h, and the reaction was stopped by the addition of 8 ml conc. HCl. The samples were hydrolyzed overnight in sealed tubes at 120°, and the hydrolysates were evaporated to dryness *in vacuo*. The residues were dissolved in 5.0 ml of water, and the hydroxyproline content was assayed by a specific chemical procedure[19] in duplicate 2.0-ml aliquots. [^{14}C]Hydroxyproline was assayed by oxidation to [^{14}C]pyrrole (ref. 20).

In experiments where α-ketoglutarate was assayed, the reaction was stopped by the addition of 12 ml of 11% trichloroacetic acid, the samples were filtered, and the α-ketoglutarate was assayed in triplicate on 6.0-ml aliquots by the procedure of BARAK, BECKENHAUSER AND QUAIFE[21].

The protein content of enzyme preparations was measured by peptide absorbance at 225 mμ using serum albumin as a standard, and by ninhydrin assays of protein hydrolysates.

RESULTS

Modified purification of enzyme

The enzyme was previously reported[1] to be purified 600–1200-fold when the specific activity was measured with proline-labeled [^{14}C]protocollagen as a substrate. Studies with $(Pro–Gly–Pro)_n$ as substrate indicated, however, that the [^{14}C]protocollagen substrate tended to overestimate the specific activity of the most purified preparation of enzyme, and that the overall purification obtained was probably 400–600-fold. Because of the relatively large losses of enzyme in the purification procedures, and because the most purified preparations lost their activity rapidly, attempts were made to improve the first two steps in the purification procedure to give relatively large amounts of stable enzyme. With the modifications described here, the enzyme obtained after the calcium phosphate gel step synthesized 13–18 μg hydroxyproline per mg enzyme protein per h when 250 μg/ml $(Pro–Gly–Pro)_n$ was used as substrate. The same preparation synthesized 9–13 μg hydroxyproline per mg enzyme protein per h when 125 μg/ml polymer was used as substrate.

Kinetic studies on the reaction

The kinetics of the reaction were examined by the technique of measuring the initial velocities as a function of the concentration of one reactant with a series of fixed concentrations of a second reactant, and with a constant concentration of the remaining reactants. The substrates for the protocollagen hydroxylase reaction are atmospheric O_2, ascorbate, the polypeptide in which proline is hydroxylated, and possibly α-ketoglutarate. Fe^{2+}, which is also involved in the reaction, and which is loosely bound to the enzyme[1], was maintained at constant and saturating concentrations in these studies.

The K_m values under the standard conditions of the assay for ascorbate, α-

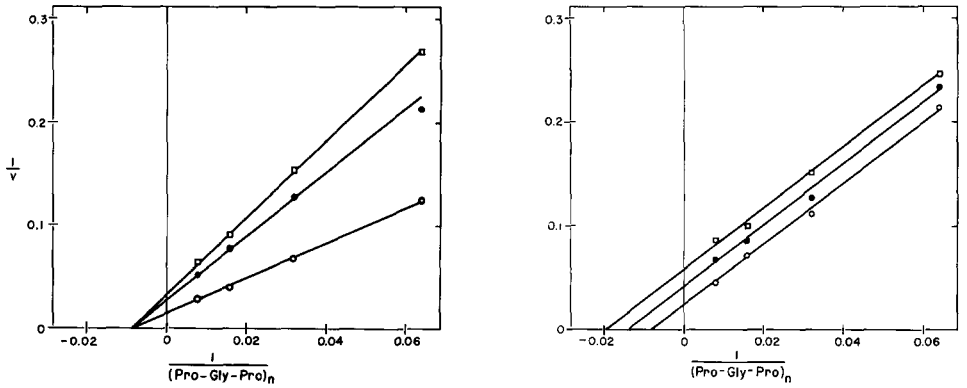

Fig. 1. Double reciprocal plots of initial velocity as a function of (Pro–Gly–Pro)$_n$ concentration at three constant concentrations of O_2. The concentrations of O_2 used were air (○); 4 vol.% O_2 (●); 3 vol.% O_2 (□). The velocities were expressed as μg hydroxyproline synthesized in 60 min, and the (Pro–Gly–Pro)$_n$ concentrations as μg/ml. Other reactants were as described in text for the enzymatic reaction under standard conditions.

Fig. 2. Double reciprocal plots of initial velocity as a function of (Pro–Gly–Pro)$_n$ concentration at three constant concentrations of ascorbate. The concentrations of ascorbate were 2 mM (○); 0.4 mM (●); 0.3 mM (□). Other conditions as in Fig. 1.

ketoglutarate, Fe^{2+}, and (Pro–Gly–Pro)$_n$ have been reported previously[2]. The K_m value for ascorbate varied from 2 to $4 \cdot 10^{-4}$ M in different experiments, but the other K_m values were relatively constant. The K_m value for atmospheric O_2 was estimated for the first time with purified enzyme, and it was found to be 6% under the conditions employed here. Because of variations in the specific activity of different enzyme preparations, all plots shown here represent data obtained in a single experiment. In each case results similar to those shown in the figures were obtained in at least two additional experiments.

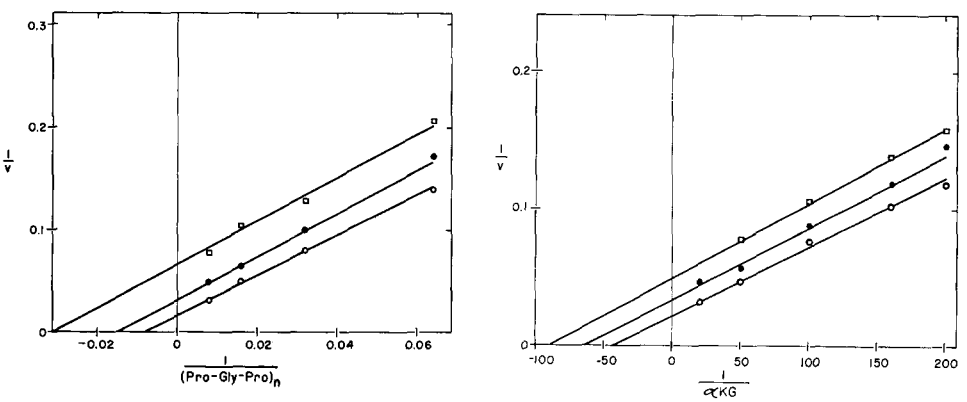

Fig. 3. Double reciprocal plots of initial velocity as a function of (Pro–Gly–Pro)$_n$ concentration at three constant concentrations of α-ketoglutarate. The concentrations of α-ketoglutarate were 0.5 mM (○); 0.02 mM (●); 0.01 mM (□). Other conditions as in Fig. 1.

Fig. 4. Double reciprocal plots of initial velocity as a function of α-ketoglutarate concentration at three constant concentrations of ascorbate. The concentrations of ascorbate were 2 mM (○); 0.2 mM (●); 0.1 mM (□). The concentration of (Pro–Gly–Pro)$_n$ was 125 μg/ml, and other conditions were as in Fig. 1. αKG = α-ketoglutarate.

Lines intersecting on the abscissa were obtained in double reciprocal plots of initial velocity and concentration of $(Pro–Gly–Pro)_n$ when three concentrations of O_2 were used for the incubation (Fig. 1). By contrast, parallel lines were obtained in the double reciprocal plots of initial velocity and concentration of $(Pro–Gly–Pro)_n$ when three different levels of ascorbate were used (Fig. 2). Parallel lines in double reciprocal plots of initial velocity and concentration of $(Pro–Gly–Pro)_n$ were also obtained when three different concentrations of α-ketoglutarate were examined (Fig. 3). In addition, parallel lines were obtained with reciprocal plots of initial velocity and concentration of α-ketoglutarate when three different concentrations of ascorbate were used (Fig. 4).

Studies on the possible consumption of α-ketoglutarate in the reaction

α-Ketoglutarate is apparently a specific requirement for the reaction[2,16], and no synthesis of hydroxyproline is obtained in the absence of α-ketoglutarate[11,16]. It was earlier reported that α-ketoglutarate disappeared during the protocollagen hydroxylase reaction with relatively crude preparations of the enzyme[16]. Omission of ascorbate or Fe^{2+} inhibited the hydroxylation, however, without changing the rate of disappearance of α-ketoglutarate in the crude preparations, and it was suggested[16] that transamination was a major pathway for metabolism of α-ketoglutarate in the crude enzyme preparations. The question of consumption of α-ketoglutarate in the reaction was re-examined with a more purified preparation of enzyme and with $(Pro–Gly–Pro)_n$ as the polypeptide substrate. Because relatively small amounts of

TABLE I

STUDIES ON THE POSSIBLE CONSUMPTION OF α-KETOGLUTARATE IN THE PROTOCOLLAGEN HYDROXYLASE REACTION

$(Pro–Gly–Pro)_n$, 125 μg/ml, was incubated with protocollagen hydroxylase under the standard conditions as described in text, except that the α-ketoglutarate concentration was 0.02 mM. Samples were prepared in quadruplicate, and after incubation for 1 h, two tubes of each sample were taken for the α-ketoglutarate assay, and two tubes for the hydroxyproline assay. Assays of the duplicate tubes agreed within ± 5 mμmoles for α-ketoglutarate, and within ± 2 mμmoles for hydroxyproline. Poly-L-proline II, 2.5 μg/ml, was added to the sample indicated before the incubation.

Incubation system	*Incubation time (min)*	*α-Ketoglutarate (mμmoles)*		*Hydroxyproline synthesized (mμmoles)*
		Recovered	*Consumed*	
Complete	0	160	0	0
Complete – $(Pro-Gly-Pro)_n$	60	150	10	0
Complete	60	120	40	120
Complete + poly L-Pro II	60	170	−10*	80

* Assay for α-ketoglutarate gave 10 mμmoles higher value than negative control.

product are formed in the reaction, the possible disappearance of α-ketoglutarate had to be examined with small concentrations of this reactant. No significant consumption of α-ketoglutarate was observed when the complete system was incubated without $(Pro–Gly–Pro)_n$ and 160 mμmoles α-ketoglutarate (Table I). When $(Pro–Gly–Pro)_n$ was added, 120 mμmoles hydroxyproline were synthesized and 40 mμmoles

TABLE II

HYDROXYLATION OF NATIVE AND DENATURED [^{14}C]PROTOCOLLAGEN WITH PURIFIED PROTOCOLLAGEN HYDROXYLASE

[^{14}C]Protocollagen was heated at 100° for 5 min and cooled rapidly to 0° for 3 min. The sample was then added to the standard hydroxylation system except that no $(Pro–Gly–Pro)_n$ was added. The hydroxylation system was preincubated at 37° before adding the [^{14}C]protocollagen, and the incubation was then continued for 10 min. Control sample of native [^{14}C]protocollagen was incubated similarly, but it was not heated to 100°. All samples contained 50 000 disint./min of either native or denatured [^{14}C]protocollagen.

Incubation system	*[^{14}C]Protocollagen substrate*	*[^{14}C]Hydroxyproline (disint./min)*
Complete – enzyme	Denatured	200*
Complete	Denatured	6250
Complete	Native	8500

* The [^{14}C]protocollagen substrate initially contained 200 disint./min [^{14}C]hydroxyproline.

α-ketoglutarate were consumed. In five similar experiments small amounts of α-ketoglutarate were consumed during the synthesis of hydroxyproline, but the α-ketoglutarate consumption was only one-fifth to one-half of the amount of hydroxyproline synthesized. Similar results were obtained in experiments in which disappearance of α-[^{14}C]ketoglutarate was measured by radioactive counting of the final solvent extracts in the assay procedure.

When 2.5 μg/ml of the competitive inhibitor poly-L-proline II (ref. 2) was added to the system, the synthesis of hydroxyproline was reduced from 120 to 80 mμ-moles (Table I). Under these conditions, however, there was no significant consumption of α-ketoglutarate.

Further studies on the polypeptide substrate

The question of whether the enzyme required a polypeptide substrate with a helical configuration similar to native collagen[12] was studied further by incubating

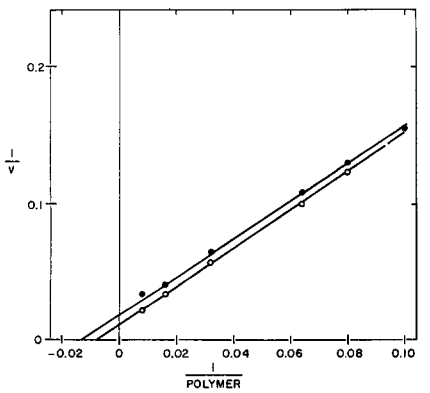

Fig. 5. Hydroxylation of $(Gly–Ala–Pro)_n$, molecular weight about 8000 (●), and $(Pro–Gly–Pro)_n$, molecular weight about 4000 (○), with protocollagen hydroxylase. The concentrations of the polypeptide substrates were expressed as μg/ml. Other conditions as described in text for the enzymatic reaction under standard conditions.

denatured protocollagen with purified protocollagen hydroxylase. Proline-labeled [^{14}C]protocollagen was heated to 100° for 5 min. The sample was then added to a complete incubation mixture at 37°, and the hydroxylation was allowed to proceed for 10 min. Under these conditions the amount of [^{14}C]hydroxyproline synthesized was 74% of that synthesized with a control sample of native proline-labeled [^{14}C]protocollagen (Table II). Because of the small amounts of [^{14}C]protocollagen available, it was not possible to make physical measurements on the amount of helical structure, but extensive studies (see ref. 22) on the renaturation of collagen suggest that no significant amount of helix should be present under the conditions used.

The requirements for the polypeptide substrate were also studied with a preparation of (Gly–L-Ala–L-Pro)$_n$ with a molecular weight of 8000, in order to determine whether the enzyme can hydroxylate proline in large polypeptides with this sequence. The proline in the polypeptide was hydroxylated, and the K_m value observed was about 80 μg/ml (Fig. 5). With concentrations of (Gly–Ala–Pro)$_n$ greater than 100 μg/ml, however, double reciprocal plots began to deviate from linearity, and in several other experiments the deviation from linearity was apparent at concentrations of 60 μg/ml. In the same experiment (Fig. 5) double reciprocal plots obtained with (Pro–Gly–Pro)$_n$ were linear, and the observed K_m value was about 120 μg/ml. As indicated, the maximal velocity observed with (Gly–L-Ala–L-Pro)$_n$ was slightly lower than the maximal velocity with (Pro–Gly–Pro)$_n$.

DISCUSSION

The synthesis of hydroxyproline by protocollagen hydroxylase involves enzyme, Fe^{2+}, ascorbate, α-ketoglutarate, atmospheric O_2, and the polypeptide substrate. The kinetic data obtained here are described by the rate equation,

$$\frac{E_0}{v} = \frac{1}{k}\left(1 + \frac{AB}{[RH]\,[O_2]} + \frac{A}{[O_2]} + \frac{B}{[RH]}\right) + \frac{C}{[Asc]} + \frac{D}{[\alpha KG]},$$

where E_0 is the initial enzyme concentration; v is the initial velocity; RH is the polypeptide substrate; Asc is ascorbate; αKG is α-ketoglutarate; and k, A, B, C, and D are constants. The rate equation predicts that when the concentrations of ascorbate and α-ketoglutarate are high, the K_m value for the polypeptide substrate is independent of the concentration of O_2, and double reciprocal plots with fixed levels of O_2 will give lines intersecting on the horizontal axis (Fig. 1). When the concentrations of O_2 and α-ketoglutarate are high, the K_m value for the polypeptide will vary with the ascorbate concentration, and double reciprocal plots with fixed levels of ascorbate will give parallel lines (Fig. 2). With high concentrations of O_2 and ascorbate, the K_m value for the polypeptide will vary with the α-ketoglutarate concentration, and double reciprocal plots with fixed levels of α-ketoglutarate will give parallel lines (Fig. 3). Similarly, when the concentrations of O_2 and polypeptide are high, the K_m value for α-ketoglutarate will vary with the ascorbate concentration, and the corresponding double reciprocal plots will be parallel (Fig. 4). Also, the rate equation involves at least one first-order rate constant (k), and a finite maximal velocity will always be observed with high concentrations of reactants (Figs. 1–4).

In the case of reactions involving two substrates, a choice from among several

arbitrary kinetic schemes such as 'sequential' and 'ping-pong' is frequently made on the basis of convergence or non-convergence in initial velocity patterns together with additional data such as product inhibition and equilibrium isotope exchange (see ref. 23). However, convergence or non-convergence of initial velocity patterns depends not only on the kinetic mechanism, but also on the numerical values of the rate constants (see ref. 24). For example, the parallel initial velocity patterns obtained for the glucose oxidase reaction might suggest a ping-pong mechanism in which the first product is released from the enzyme before the second substrate combines with it. However, direct transient state kinetic measurements have not yet indicated the exact location of product release steps[25,26]. Because of the values of the rate constants in this particular case, parallel-line initial velocity patterns would be obtained whether the release of the first product precedes or follows combination of the enzyme with the second substrate. With three or more substrates participating in a reaction, the steady-state kinetic analysis becomes very complex and requires extensive investigation of initial velocity patterns.

Because of technical difficulties in studying protocollagen hydroxylase, all the possible kinetic patterns could not be obtained. It was not possible, for example, to examine variations in the concentrations of two reactants with several fixed concentrations of a third reactant. It is unlikely that the complete kinetic patterns will become available in the forseeable future, and attempts to demonstrate a reduced enzyme species using ascorbic acid oxidase[27] were unsuccessful (D. J. PROCKOP and K. I. KIVIRIKKO, unpublished data). The kinetic data described here are consistent with a relatively simple kinetic scheme involving consecutive bimolecular addition of ascorbate and α-ketoglutarate followed by the random order addition of O_2 and the polypeptide. The available data, however, do not exclude other kinetic schemes.

Although the roles of ascorbate and Fe^{2+} have not been studied in detail, it is probable that ascorbate is oxidized in order to generate reduced enzyme, and that changes in the valence state of the iron bound to the enzyme are involved in this reaction. A mechanism of this type has recently been demonstrated for copper and ascorbate in the dopamine β-hydroxylase reaction[27]. It is more difficult to explain the role of α-ketoglutarate which is an absolute[11,16] and apparently specific[2,16] requirement for the synthesis of hydroxyproline. Quantitative assays of α-ketoglutarate in the system used here indicated that it is not consumed in stoichiometric amounts, but the data do not exclude cyclical consumption and regeneration of α-ketoglutarate during the reaction. The most probable role for α-ketoglutarate is that it mediates an alteration in the enzyme which is required for a subsequent combination with polypeptide substrate and O_2. It is of interest that α-ketoglutarate is also a requirement for thymine 7-hydroxylase[28] and the hydroxylation of α-butyrobetaine to carnitine[29], and that these hydroxylations involve atmospheric O_2, Fe^{2+}, and ascorbate.

The synthesis of hydroxyproline observed with $(Gly\text{–}L\text{-}Ala\text{–}L\text{-}Pro)_n$ indicates that polymers of this structure can also serve as a substrate for the enzyme. This finding is consistent with the fact that the sequence –Gly–Ala–Hyp– is relatively common in collagen[30]. The K_m value and maximal velocity for $(Gly\text{–}Ala\text{–}Pro)_n$ were slightly lower than for $(Pro\text{–}Gly\text{–}Pro)_n$ under the same conditions, and the K_m values for both polytripeptides were considerably higher than the K_m value previously obtained[1] with biologically prepared proline-labeled [^{14}C]protocollagen.

Because the conditions for the hydroxylation differed, and because of technical difficulties in estimating protocollagen content, the reported K_m value of 0.9 μg/ml for protocollagen may be subject to some inaccuracy, but it is unlikely that errors in the assays can account for the 100-fold differences in K_m values. As discussed elsewhere[2], the differences in size between the synthetic polymers and protocollagen also cannot account for discrepancy in K_m values, and therefore the reasons for the differences between the polytripeptides and protocollagen are not apparent.

Previous studies with crude preparations of the enzyme suggested that the ability of polypeptides to serve as substrates for the hydroxylation of proline may depend on the ability of the polypeptides to form a helical structure similar to that of collagen[12]. Measurement of the K_m values of $(Pro–Gly–Pro)_n$ preparations with different sizes were not, however, consistent with this possibility[2]. Experiments with crude enzyme preparations indicated that 50–100% of the substrate activity was retained in gelatinized [^{14}C]protocollagen[3,7,16], and similar results were obtained here in quenching experiments with [^{14}C]protocollagen and purified enzyme. These observations suggest that the enzyme can hydroxylate proline in non-hecilal polypeptides, and this conclusion is further supported by the synthesis of hydroxyproline with $(Gly–L\text{-}Ala–L\text{-}Pro)_n$. Physical measurements have shown that $(Gly–L\text{-}Pro–L\text{-}Ala)_n$ (ref. 31) differs from $(L\text{-}Pro–Gly–L\text{-}Pro)_n$ in that it does not form triple helical structures in aqueous solution, and preliminary observations (W. TRAUB, personal communication) indicate that the same is true of polytripeptides with the structure $(Gly–L\text{-}Ala–L\text{-}Pro)_n$.

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REFERENCES

1 K. I. KIVIRIKKO AND D. J. PROCKOP, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 782.
2 K. I. KIVIRIKKO, AND D. J. PROCKOP, *J. Biol. Chem.*, 242 (1967) 4007.
3 D. J. PROCKOP AND K. JUVA, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 661.
4 A. A. GOTTLIEB, B. PETERKOFSKY AND S. UDENFRIEND, *J. Biol. Chem.*, 240 (1965) 3099.
5 J. HURYCH AND M. CHVAPIL, *Biochim. Biophys. Acta*, 97 (1965) 361.
6 L. N. LUKENS, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 1235.
7 K. I. KIVIRIKKO AND D. J. PROCKOP, *Biochem. J.*, 102 (1967) 432.
8 D. J. PROCKOP, E. WEINSTEIN AND T. MULVENY, *Biochem. Biophys. Res. Commun.*, 22 (1966) 124.
9 E. HAUSMANN, *Biochim. Biophys. Acta*, 133 (1967) 591.
10 J. HURYCH AND A. NORDWIG, *Biochim. Biophys. Acta*, 140 (1967) 168.
11 K. I. KIVIRIKKO AND D. J. PROCKOP, *Arch. Biochem. Biophys.*, 118 (1967) 611.
12 D. J. PROCKOP, K. JUVA AND J. ENGEL, *Z. Physiol. Chem.*, 348 (1967) 553.
13 D. FUJIMOTO AND N. TAMIYA, *Biochem. J.*, 84 (1962) 333.
14 D. J. PROCKOP, A. KAPLAN AND S. UDENFRIEND, *Arch. Biochem. Biophys.*, 101 (1963) 494.
15 J. J. HUTTON, A. L. TAPPEL AND S. UDENFRIEND, *Anal. Biochem.*, 16 (1966) 384.
16 J. J. HUTTON, A. L. TAPPEL AND S. UDENFRIEND, *Arch. Biochem. Biophys.*, 118 (1967) 231.
17 E. A. POPEONE, R. B. ARONSON AND D. D. VAN SLYKE, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 393.

18 J. ENGEL, J. KURTZ, E. KATCHALSKI AND A. BERGER, *J. Mol. Biol.*, 17 (1966) 255.
19 K. I. KIVIRIKKO, O. LAITINEN AND D. J. PROCKOP, *Anal. Biochem.*, 19 (1967) 249.
20 K. JUVA AND D. J. PROCKOP, *Anal. Biochem.*, 15 (1966) 77.
21 A. J. BARAK, H. C. BECKENHAUSER AND M. A. QUAIFE, *Gastroenterology*, 52 (1967) 655.
22 W. F. HARRINGTON AND P. H. VON HIPPEL, *Advan. Protein Chem.*, 16 (1961) 1.
23 W. W. CLELAND, *Ann. Rev. Biochem.*, 36 (1967) 77.
24 Q. H. GIBSON, *Ann. Rev. Biochem.*, 35 (1966) 435.
25 Q. H. GIBSON, B. E. P. SWOBODA AND V. MASSEY, *J. Biol. Chem.*, 239 (1964) 3927.
26 H. J. BRIGHT AND Q. H. GIBSON, *J. Biol. Chem.*, 242 (1967) 994.
27 S. FRIEDMAN AND S. KAUFMAN, *J. Biol. Chem.*, 240 (1965) 4763.
28 M. T. ABBOTT, S. LIN, E. K. SCHANDL, R. F. LEE, T. S. PARKER AND R. J. MIDGETT, *Federation Proc.*, 26 (1967) 451.
29 G. LINDSTEDT, S. LINDSTEDT, T. MIDVEDT AND M. TOFFT, *Biochem. J.*, 103 (1967) 19P.
30 K. HANNING AND A. NORDWIG, in S. FITTON-JACKSON, R. D. HARKNESS, S. M. PARTRIDGE AND G. R. TRISTRAM, *Structure and Function of Connective and Skeletal Tissue*, Butterworth, London, 1965, p. 7.
31 P. J. ORIEL AND E. R. BLOUT, *J. Am. Chem. Soc.*, 88 (1966) 2041.