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Hydroxylation of (Pro-Pro-Gly)$_5$ and (Pro-Pro-Gly)$_{10}$ by Prolyl Hydroxylase. Evidence for an Asymmetric Active Site in the Enzyme†

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ABSTRACT: Previous studies with ^{14}C-labeled synthetic peptides demonstrated that prolyl hydroxylase, which synthesizes the hydroxyproline in collagen, preferentially hydroxylates the fourth triplet from the NH-terminal end of the peptide (Pro-Pro-Gly)$_5$. In the experiments reported here, the prolyl hydroxylase reaction was investigated further by preparing chemically modified derivatives of (Pro-Pro-Gly)$_5$ and by synthesizing ^{14}C-labeled preparations of (Pro-Pro-Gly)$_{10}$. Essentially, the same k_{cat} value was found for the hydroxylation of (Pro-Pro-Gly)$_5$, *N*-acetyl-(Pro-Pro-Gly)$_5$, (Pro-Pro-Gly)$_5$ methyl ester, (Pro-Pro-Gly)$_{10}$, and for larger polypeptide substrates of the enzyme. It appeared therefore that preferential hydroxylation of specific triplets in peptides of the structure (Pro-Pro-Gly)$_n$ cannot be explained by differences in the kinetic constants for individual triplets. Hydroxylation of ^{14}C-labeled preparations of (Pro-Pro-Gly)$_{10}$ demonstrated that the ninth triplet was preferentially hydroxylated over any other triplet. The results were best explained by the hypothesis that prolyl hydroxylase has an asymmetric active site in which binding subsites are located adjacent to but not symmetrical with the catalytic subsite.

Prolyl hydroxylase synthesizes the hydroxyproline in collagen by hydroxylating prolyl residues in nascent or completed chains of the precursor known as procollagen.[1] Hydroxylation of prolyl residues in procollagen is essential for the protein to fold into a triple-helical conformation at 37 °C (Berg and Prockop, 1973a; Rosenbloom et al., 1973) and folding into the triple-helical conformation is apparently necessary for procollagen to be secreted from fibroblasts at a normal rate (for review, see Prockop et al., 1976).

Collagen consists of three polypeptide chains folded into a triple helix and each chain has 330 repeating -Gly-X-Y- tripeptide sequences in which 100 of the Y positions are hydroxyproline (for review, see Traub and Piez, 1971). Therefore, one of the interesting features of the reaction catalyzed by prolyl hydroxylase is that the enzyme must hydroxylate about 100 prolyl residues in each of the three polypeptide chains of procollagen. Studies with natural substrates and with synthetic substrates have shown that the enzyme hydroxylates prolyl residues in the Y position of -X-Y-Gly- sequences in which the X position is proline, alanine, arginine, leucine, glutamate, or, apparently, a variety of other amino acids (for reviews, see Cardinale and Udenfriend, 1974; Prockop et al., 1976; Fietzek and Kühn, 1976). It appears therefore that in hydroxylating the polypeptides of procollagen prolyl hydroxylase interacts with multiple equivalent sites and in this sense it is comparable to other enzymes which operate on polymeric substrates, such as nucleic acid polymerases (Sherman and Gefter, 1976; McClure and Jovin, 1975) and methylases (for review, see Kerr and Borek, 1973).

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[1] Procollagen is the precursor form of collagen which has been isolated from a variety of embryonic tissues; it differs from collagen in that it has peptide extensions on the NH_2- and COOH-terminal ends of each of the three polypeptide chains. Type I procollagen from embryonic tendon cells (Olsen et al., 1976) and several other systems (Tanzer et al., 1974; Fessler et al., 1975; Byers et al., 1975) has been shown to consist of three such pro-α chains linked by interchain disulfide bonds among the COOH-terminal extensions.

We have attempted to examine the question of how prolyl hydroxylase interacts with substrates containing multiple hydroxylatable residues by examining homogeneous peptides of the general structure $(Pro\text{-}Pro\text{-}Gly)_n$. Previously, four preparations of $(Pro\text{-}Pro\text{-}Gly)_5$ were synthesized in which different triplets were labeled in the Y position with [^{14}C]-proline. By assaying [^{14}C]hydroxyproline in the product, it was established that the fourth triplet from the NH-terminal end was more readily hydroxylated than any other triplet in $(Pro\text{-}Pro\text{-}Gly)_5$ (Kivirikko et al., 1971). We have here extended these studies with [^{14}C]proline-labeled $(Pro\text{-}Pro\text{-}Gly)_5$, and we have also synthesized and examined [^{14}C]proline-labeled preparations of the larger peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$.

Materials and Methods

Preparation of Ac-(Pro-Pro-Gly)$_5$. Four preparations of [^{14}C]proline-labeled $(Pro\text{-}Pro\text{-}Gly)_5$ which were synthesized previously (Kivirikko et al., 1971) were converted to *N*-acetyl derivatives. The peptides were reacted with 5 molar equivalents of the *N*-hydroxysuccinimide ester of acetic acid in 50% aqueous pyridine at 22 °C for 2 or 3 days. The product was purified with a 0.9 × 15 cm column of DEAE[2]-Sephadex which was equilibrated with 0.05 M acetic acid and eluted with a gradient prepared from 50 mL of 0.05 M acetic acid and 50 mL of 0.5 M acetic acid. The fractions containing the second peak were lyophilized and the product was dried over P_2O_5 in vacuo. The purity of the product was checked by high-voltage paper electrophoresis using a buffer of 0.05 M sodium phosphate, pH 9.5. The paper was cut into sequential strips and the strips were counted in a liquid scintillation counter.

Preparation of (Pro-Pro-Gly)$_5$-OCH$_3$. To prepare the *O*-methyl esters, the four ^{14}C-labeled preparations of $(Pro\text{-}Pro\text{-}Gly)_5$ were reacted in ether with diazomethane which was freshly prepared from nitrosomethylurea and potassium hydroxide. Excess diazomethane was removed by evaporation at 40 °C and then the ether was evaporated at reduced pressure. The residue was dissolved in a small amount of water and chromatographed on a 0.5 × 5 cm column of DEAE-Sephadex (Pharmacia; A-25 hydroxide form) which was equilibrated and eluted with water. Because the ester was not anionic, it passed directly through the column and was recovered in the first 5 mL of eluent. One-tenth milliliter of 1 M acetic acid was added, and the product was lyophilized and dried over P_2O_5 in vacuo. Amino acid analysis of the product indicated that the Pro/Gly ratio was 2:1. The purity of the peptide was also checked by paper electrophoresis using a buffer of 0.05 M sodium phosphate at pH 9.5 as described above.

Preparation of ^{14}C-Labeled (Pro-Pro-Gly)$_{10}$. [^{14}C]Proline-labeled polypeptides with the structure $(Pro\text{-}Pro\text{-}Gly)_{10}$ were synthesized by coupling ten separate tripeptide units of *tert*-amyloxycarbonyl-Pro-Pro-Gly with a specific modification of the Merrifield technique for solid-phase synthesis of peptides (Sakakibara et al., 1968). The ^{14}C label was introduced by using *tert*-amyloxycarbonyl-Pro-[^{14}C]Pro-Gly (Kivirikko et al., 1971) at specific coupling steps so that nine separate peptides, each labeled in a different triplet, were obtained. In order to synthesize peptides of the highest possible specific activities, the label was introduced by reacting first the undiluted, N-blocked tripeptide containing [^{14}C]proline with the peptide chain attached to the resin. This coupling reaction was then completed by using excess unlabeled blocked tripeptide to ensure complete addition of a triplet at each elongation step. Finally, the peptides were removed from the resin with anhydrous HF (Sakakibara et al., 1967) and they were desalted on an Amberlite IR-45 column followed by dialysis against distilled water for 24 h at 10 °C. The samples were lyophilized and dried at 80 °C over P_2O_5 in vacuo for 5 h. The specific activities of the peptides were estimated by measuring the protein concentration by optical absorbance and using an extinction coefficient of $A_{230\ nm}^{1\%}$ of 32.5 (Berg et al., 1970). ^{14}C content was assayed in a liquid scintillation counter with an efficiency of about 80%.

Preparation of Prolyl Hydroxylase and Assay of Enzymatic Activity. The enzyme was purified from 13-day-old chick embryos with a procedure involving ammonium sulfate fractionation, affinity column chromatography, and gel filtration (Berg and Prockop, 1973b, 1976). The isolated enzyme was homogeneous by polyacrylamide gel electrophoresis and had a specific activity of 4200 units per mg of protein. The value for specific activity was higher than that reported previously, because the extinction coefficient was measured directly (see below) instead of using an approximate value (Berg and Prockop, 1973b).

The enzyme activity was assayed under standard conditions with an α-keto[1-^{14}C]glutarate assay as described previously, except that 250 μg/mL of $(Pro\text{-}Gly\text{-}Pro)_n$ with an average molecular weight of 2300 (Miles Laboratories) was used as substrate instead of reduced and carboxymethylated cuticle collagen from *Ascaris lumbricoides*, variety suis (Berg and Prockop, 1973b). One unit of enzyme activity was defined as the amount of enzyme required to synthesize 1 μg of hydroxyproline in 1 h at 37 °C in a reaction volume of 4 mL and under conditions where the cofactors and cosubstrates were saturating (Kivirikko and Prockop, 1967a,b) and the peptidyl proline substrate was 250 μg/mL of $(Pro\text{-}Gly\text{-}Pro)_n$. The other components in the reaction mixture were 0.05 mM $FeSO_4$, 0.5 mM α-ketoglutarate (Calbiochem), 2 mM ascorbic acid (Fisher), 0.1 mg/mL catalase (Calbiochem), 2 mg/mL bovine serum albumin (Sigma), 0.1 mM dithiothreitol (Calbiochem), and 50 mM Tris-HCl buffer adjusted to pH 7.5 at 25 °C. Because of small decreases of specific activity with storage of the enzyme, especially with repeated freezing and thawing, the enzyme was reassayed in each experiment and all values for k_{cat} were corrected accordingly (see below).

Determination of the Molar Extinction Coefficient for Prolyl Hydroxylase. The molar extinction coefficient and optical absorbance of prolyl hydroxylase were determined by dialyzing approximately 0.6 mg of purified enzyme in a volume of 1 mL against 0.2 M NaCl and 0.01 M Tris-HCl buffer, pH 7.8, at 4 °C, in order to remove the glycine present in the buffer used to isolate the enzyme. The absorbance was measured in a 1-cm light path at 230 and 280 nm, and the same solution was then hydrolyzed with 6 N HCl for 16 h under N_2 with 0.08 M β-mercaptoethanol (Houston, 1971). The hydrolysate was evaporated to dryness, redissolved in 1 mL of 0.2 N sodium citrate buffer, pH 2.2, and an aliquot was taken for determination of its concentration by amino acid analysis. Two separate preparations of enzyme were used for this measurement and four determinations of concentration were made for each preparation. To check these procedures, the extinction coefficient of bovine serum albumin was also determined and the values were within the range of those reported previously (Kirschenbaum, 1971).

Hydroxylation of Synthetic Peptides. The peptides $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ were studied as substrates for the enzyme by incubating them in a concentration of 500

[2] Abbreviations: k_{cat}, catalytic constant of V_m/E; DEAE, diethylaminoethyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

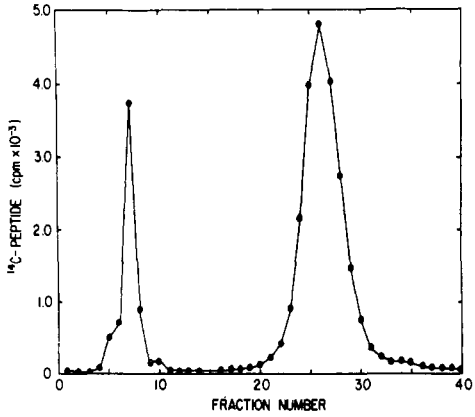

FIGURE 1: Purification of Ac-(Pro-Pro-Gly)$_5$ labeled in the first triplet with [^{14}C]proline. The preparation and purification of Ac-(Pro-Pro-Gly)$_5$ are described under Materials and Methods. The labeled peptide was applied to a DEAE-Sephadex column and eluted with an acetic acid gradient (see Materials and Methods). The fraction size was 1.15 mL and the total recovery of counts from the column was 91%.

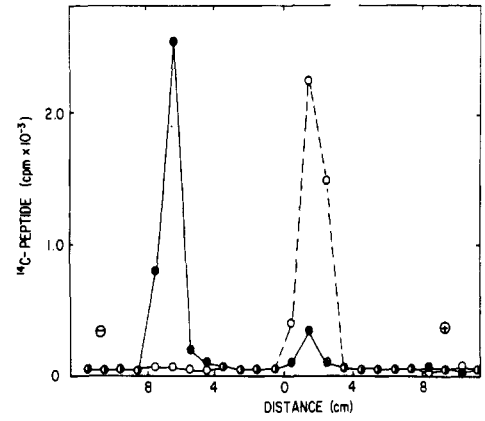

FIGURE 2: High-voltage paper electrophoresis of the peak fractions 7 and 26 from the DEAE-Sephadex column in Figure 1. The sample was applied on the center line of a strip of paper 10-cm wide. The samples were electrophoresed for 2.5 h at 2000 V. The paper was cut in 1-cm strips and each strip was counted in a scintillation counter. Control experiments demonstrated that the peak on the left comigrated with (Pro-Pro-Gly)$_5$. Symbols: fraction number 7 (●); fraction number 26 (○); anode (⊕); cathode (⊖).

μg/mL of (Pro-Pro-Gly)$_5$ or 200 μg/mL of (Pro-Pro-Gly)$_{10}$ with 1 to 14 units/mL enzyme under the conditions described above. Each ^{14}C-labeled peptide was reacted separately with enzyme and the peptides were heat denatured at 100 °C for 10 min just before hydroxylation, except in the case of the methyl esters which were heated only to 37 °C to avoid possible hydrolysis. The final incubation volume was 2 mL for the hydroxylation of the (Pro-Pro-Gly)$_5$ and 8 mL for (Pro-Pro-Gly)$_{10}$. The reaction temperature was 37 °C, and the incubation time was varied from 60 to 180 min, depending on whether initial velocities or various degrees of hydroxylation were required. The reaction was stopped by adding an equal volume of concentrated HCl, and the samples were hydrolyzed overnight at 120 °C. The hydrolysates were evaporated to dryness on a steam bath and the residues were then dissolved in distilled water. One aliquot of each sample was assayed for total amount of hydroxyproline (Kivirikko et al., 1967) and another aliquot was assayed for ^{14}C-labeled hydroxyproline (Juva and Prockop, 1966). Assay of the amount of hydroxyproline served as a check on the overall reaction in each vessel, and assay of [^{14}C]hydroxyproline indicated the hydroxylation of each specific triplet labeled with [^{14}C]proline. The observed values of dpm of [^{14}C]hydroxyproline were converted to micromoles on the basis of the specific activities of the synthetic polytripeptides. (Pro-Pro-Gly)$_5$ and (Pro-Pro-Gly)$_{10}$ labeled in the fifth and tenth triplets, respectively, were not synthesized, but it was possible to determine the amount of hydroxyproline synthesized in the fifth and tenth triplets by subtracting the amounts of hydroxyproline synthesized in the ^{14}C-labeled triplets, from the total hydroxyproline synthesized in each sample.

For determining K_m and k_{cat} values for peptides such as (Pro-Pro-Gly)$_5$, Ac-(Pro-Pro-Gly)$_5$, (Pro-Pro-Gly)$_5$-OCH$_3$, and (Pro-Pro-Gly)$_{10}$, various concentrations of the peptides were incubated with enzyme in a volume of 1 mL under the conditions outlined above. The synthesis of product was followed by determining the amount of $^{14}CO_2$ released from α-keto[1-^{14}C]glutarate after a 20-min incubation. Typically, the substrates were dissolved in H_2O at a concentration of approximately 2 mg/mL and the pH was adjusted to 7. All substrate concentrations were determined by amino acid analysis. The substrates were diluted and final concentrations ranged from 23 to 760 μM for determination of the K_m of (Pro-Pro-Gly)$_5$ and its derivatives. The substrate concentration ranged from 8 to 90 μM for the K_m determination of (Pro-Pro-Gly)$_{10}$. The data were analyzed by plotting the results in Lineweaver-Burk plots. Values of k_{cat}[2] were obtained by calculating the units of enzyme activity producing a given V_m and dividing the V_m by the actual amount of enzyme present during the reaction. The amount of enzyme present was estimated from the specific activity of 4200 units/mg for purified enzyme. The reaction time was varied for the lowest and highest substrate concentrations to ensure that initial velocities were being measured during each experiment.

Results

Effect of Modification of NH- and COOH-Terminal Groups on the Asymmetric Hydroxylation of (Pro-Pro-Gly)$_5$. As discussed previously (Kivirikko et al., 1971), there are several possible explanations for the observations that the fourth triplet from the NH-terminal end in ^{14}C-labeled (Pro-Pro-Gly)$_5$ was more readily hydroxylated than any other triplet in the peptide. One possible explanation is that the negative charge at the COOH-terminal end, or the positive charge at the NH-terminal end, determines which triplet is initially hydroxylated. To explore this possibility, derivatives of (Pro-Pro-Gly)$_5$ were prepared in which either the NH-terminal end or the COOH-terminal end was chemically blocked.

The *N*-acetyl derivative was synthesized as discussed under Methods and purified by chromatography on DEAE-Sephadex. Because the derivative was more anionic than (Pro-Pro-Gly)$_5$, it bound more tightly to the column and was eluted with an acetic acid gradient (Figure 1). The product appearing in the second peak was found to be free of (Pro-Pro-Gly)$_5$, the starting material, when examined by paper electrophoresis at pH 9.5 (Figure 2). The *O*-methyl ester was also purified by chromatography on DEAE-Sephadex and the peptide passed directly through the column, whereas the unblocked peptide remained bound. The product had a mobility distinct from (Pro-Pro-Gly)$_5$ and appeared homogeneous when examined by paper electrophoresis at pH 9.5 (not shown).

To examine the effect of end-group modification on hydroxylation of (Pro-Pro-Gly)$_5$, ^{14}C-labeled derivatives were hydroxylated with prolyl hydroxylase under conditions in which 1.9 to 3.8% of the available -X-Pro-Gly- sequences were

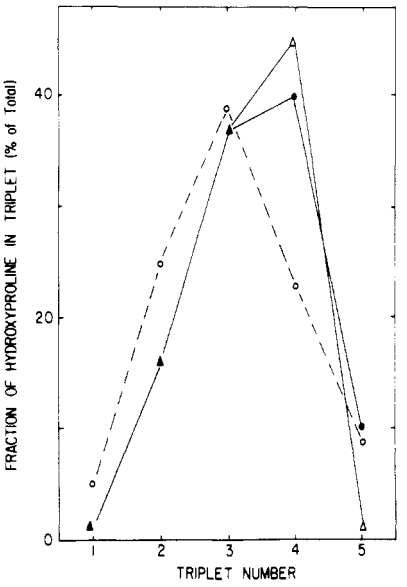

FIGURE 3: Fraction of hydroxyproline in individual triplets of (Pro-Pro-Gly)$_5$, Ac-(Pro-Pro-Gly)$_5$, and (Pro-Pro-Gly)$_5$-OMe. The fraction of hydroxyproline in each triplet is the amount of hydroxyproline found in the triplet divided by the amount of hydroxyproline found in the total peptide. The procedures for determining the amounts of hydroxyproline in each triplet are given in Materials and Methods. The overall extents of hydroxylation of the peptide are 2.3% of maximal for (Pro-Pro-Gly)$_5$, 1.41% of maximal for Ac-(Pro-Pro-Gly)$_5$, and 3.8% of maximal for (Pro-Pro-Gly)$_5$-OMe. Symbols: (Pro-Pro-Gly)$_5$ (●); Ac-(Pro-Pro-Gly)$_5$ (○); and (Pro-Pro-Gly)-OMe (△).

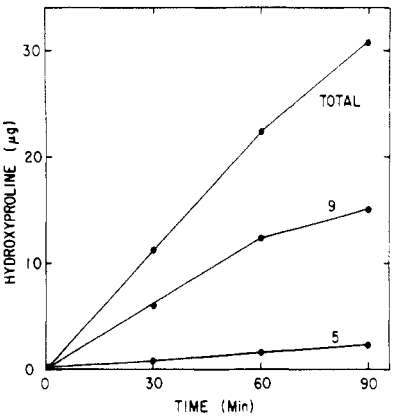

FIGURE 4: Time course for the synthesis of hydroxyproline in the fifth and ninth triplet and the overall synthesis of hydroxyproline in the total peptide of (Pro-Pro-Gly)$_{10}$. The conditions are described under Materials and Methods; the concentration of substrate was 200 μg/mL, the volume was 8 mL, and the temperature during hydroxylation was 37 °C. The amount of enzyme was 15.2 units for the experiment in which the ninth triplet was labeled and 56 units for the experiment in which the fifth triplet was labeled. The values for the total amount of hydroxyproline synthesized when the fifth triplet was labeled were normalized to the values obtained when the ninth triplet was labeled. Symbols: Total, hydroxyproline in the total peptide; 9, hydroxyproline in ninth triplet; 5, hydroxyproline in the fifth triplet.

converted to -X-Hyp-Gly-. The results showed that hydroxyproline in the *O*-methyl ester had the same distribution as in the unmodified peptide, and that the fourth triplet was hydroxylated more readily than any other triplet (Figure 3). With the *N*-acetyl derivative, however, the third triplet was hydroxylated more readily than any other. Also, there appeared to be a somewhat greater hydroxylation of the first and second triplets in the modified peptide than in (Pro-Pro-Gly)$_5$.

K_m Values and Catalytic Constants for the Hydroxylation of Peptide Substrates. In order to determine the k_{cat} values for the hydroxylation of -X-Pro-Gly- sequences in various peptides, the molar extinction coefficient for prolyl hydroxylase was determined as described under Materials and Methods and it was found to be $\epsilon_{280} = 0.36 \times 10^6$ M^{-1} cm^{-1} and $\epsilon_{230} = 1.77 \times 10^6$ M^{-1} cm^{-1}. The optical absorbances were $A_{280\,nm}{}^{1\%} = 15.7$ and $A_{230\,nm}{}^{1\%} = 77.3$. Using these values of the extinction coefficient, the specific activity of purified enzyme was 4200 units/mg instead of 2700 units/mg as reported previously (Berg and Prockop, 1973b).

Hydroxylation of the peptides with prolyl hydroxylase demonstrated that the modified peptides Ac-(Pro-Pro-Gly)$_5$ and (Pro-Pro-Gly)$_5$-OMe had the same K_m for the reaction as the unmodified peptide (Table I). Also, the k_{cat} values for the modified peptides were essentially the same as for the unmodified peptides. As noted previously, the K_m for (Pro-Pro-Gly)$_5$ was considerably greater than the K_m for (Pro-Pro-Gly)$_{10}$. Also as indicated, the K_m for all the synthetic peptides was about four orders of magnitude larger than the K_m for the natural substrate with a molecular weight of about 100 000 per chain. However, the k_{cat} values for the synthetic peptides were essentially the same as that of the natural substrate.

Preparation of ^{14}C-Labeled (Pro-Pro-Gly)$_{10}$. Nine preparations of [^{14}C]proline-labeled (Pro-Pro-Gly)$_{10}$ were synthesized with the procedures previously described (Kivirikko et al., 1971). The specific activities of the nine peptides varied from 0.73 to 1.01 × 10^5 dpm/μmol. The variations in the specific activities were probably accounted for by the small differences in the completeness with which the blocked radioactively-labeled tripeptide was linked to the peptide on the resin before the reaction was completed with the addition of unlabeled, blocked tripeptide (see Materials and Methods).

TABLE I: Kinetic Constants for the Hydroxylation of -X-Pro-Gly- Sequences in Various Peptides.

Peptide	K_m (μM peptide)	k_{cat} (s^{-1})
(Pro-Pro-Gly)$_5$	300	5.3
(Pro-Pro-Gly)$_5$-OMe	300	4.7
Ac-(Pro-Pro-Gly)$_5$	280	3.3
(Pro-Pro-Gly)$_{10}$	30	3.6
Protocollagen	0.002	4.0,[a] 6.0[b]

[a] Value reported by Berg and Prockop (1973c). [b] Value reported by Tuderman et al. (1975).

Time Course for the Hydroxylation of the Fifth and Ninth Triplet in (Pro-Pro-Gly)$_{10}$. Prolyl hydroxylase was incubated with 200 μg/mL of two different preparations of (Pro-Pro-Gly)$_{10}$, one labeled in the ninth triplet and the other labeled in the fifth triplet. The results indicated that, under the conditions of the experiment, the overall hydroxylation of the peptide was linear for up to 60 min and then the rate decreased slightly between 60 and 90 min (Figure 4). Assays for [^{14}C]-hydroxyproline indicated that more of the hydroxyproline synthesized was in the ninth triplet than in the fifth triplet.

Distribution of Hydroxyproline in (Pro-Pro-Gly)$_{10}$ with Varying Degrees of Hydroxylation of the Peptide. To determine the distribution of hydroxyproline in the various triplets of (Pro-Pro-Gly)$_{10}$, the nine ^{14}C-labeled preparations of (Pro-Pro-Gly)$_{10}$ were hydroxylated with prolyl hydroxylase. The times of hydroxylation and the amounts of enzyme were varied to give different extents of hydroxylation in four experiments so that between 1.3 and 23.4% of the total Y-position

TABLE II: Fraction of Hydroxyproline in Individual Triplets with Varying Extents of Hydroxylation of (Pro-Pro-Gly)$_{10}$.

	Fraction of Hydroxyproline in Triplet (% of Total)[a]			
	Experiment No.			
Triplet	1	2	3	4
1st	0	0	0	0
2nd	2.4	2.5	2.5	3.7
3rd	4.5	5.9	5.1	6.7
4th	5.4	7.2	5.9	9.3
5th	6.0	6.7	7.5	8.2
6th	5.8	6.7	6.1	8.6
7th	5.3	6.3	7.1	9.2
8th	16.2	19.2	16.0	16.6
9th	51	48	44	27
10th	0	0	5.8	10.6

[a] The fraction of hydroxyproline in each triplet is calculated as the amount of hydroxyproline in the triplet divided by the amount of hydroxyproline in the total peptide. Extent of hydroxylation of the total peptide was 1.3% of maximal in Expt 1, 3.4% of maximal in Expt 2, 10.7% of maximal in Expt 3, and 23.4% of maximal in Expt 4.

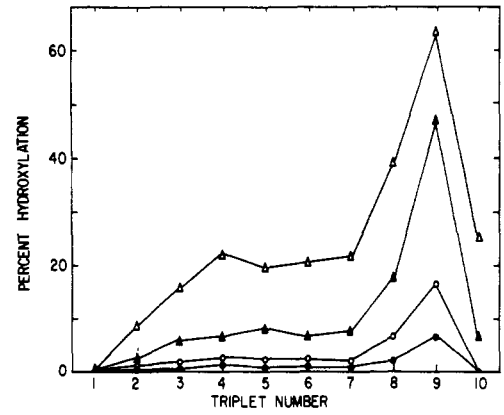

FIGURE 5: Hydroxylation of individual triplets for different overall extents of hydroxylation of (Pro-Pro-Gly)$_{10}$. The hydroxylation of a given triplet is expressed as the amount of hydroxyproline found in a given triplet divided by the amount of hydroxyproline which would have been found in the triplet if it were maximally hydroxylated. The procedures for determining the amounts of hydroxyproline in each triplet are given under Materials and Methods. The amount of prolyl hydroxylase and time of incubation were adjusted so that the extent of hydroxylation of the total peptide was 1.3% (●); 3.4% (○); 10.7% (▲); and 23.4% (△).

prolyl residues in the peptide were converted to hydroxyproline. When the overall hydroxylation was 23.4%, about 27% of the hydroxyproline was found in the ninth triplet (Table II) and over 60% of the Y-position prolyl residues in the ninth triplet were hydroxylated (Figure 5). With lower extents of overall hydroxylation, the degree of hydroxylation of all the triplets decreased, but a larger fraction of the hydroxyproline synthesized was found in the ninth triplet. The fraction of hydroxyproline in the eighth triplet remained relatively constant with variations in the overall extent of hydroxylation (Table II).

Calculations on Helix Formation during the Hydroxylation of (Pro-Pro-Gly)$_{10}$. Since the triple-helical conformation of protocollagen prevents the hydroxylation of this substrate, and since (Pro-Pro-Gly)$_{10}$ and (Pro-Hyp-Gly)$_{10}$ form triple-helical structures in aqueous solution (see Sakakibara et al., 1968; Berg et al., 1970; Sutoh and Noda, 1974a,b; Prockop et al., 1976; Engel et al., 1977), it was possible that some of the observations made here were explained by (Pro-Pro-Gly)$_{10}$ becoming triple helical during the enzymatic reaction and therefore unavailable for further hydroxylation. However, calculations based on published data indicated that little if any of the peptide could have become triple helical under the conditions employed here. For relatively short peptides like (Pro-Pro-Gly)$_{10}$ and (Pro-Hyp-Gly)$_{10}$, the T_m is concentration dependent according to eq 5 from Engel et al. (1977). From the measured values of $\Delta H°$ and $\Delta S°$ (Engel et al., 1977), it was calculated that at a concentration of 200 μg/mL the T_m of (Pro-Pro-Gly)$_{10}$ is 9 °C. Therefore, none of the substrate could have folded into a stable triple-helical conformation during the enzymatic hydroxylation performed at 37 °C. Similar calculations based on the measured $\Delta H°$ and $\Delta S°$ values for (Pro-Hyp-Gly)$_{10}$ (Engel et al., 1977), indicated that at a concentration of 200 μg/mL the T_m of this peptide is 46 °C. Therefore, at equilibrium fully hydroxylated (Pro-Pro-Gly)$_{10}$ would be triple helical. However, the kinetics of helix formation are also concentration dependent and the initial rate of helix formation is equal to $3kc^2$, where k is the forward rate constant and c is the initial concentration of single-stranded peptide (Sutoh and Noda, 1974a). The rate constant, k, has not yet been measured for (Pro-Hyp-Gly)$_{10}$, but Sutoh and Noda (1974a) found k to be about 1000 s^{-1} M^{-2} at 25 °C for (Pro-Pro-Gly)$_{10}$. On the likely assumption that the value of k is about the same for (Pro-Hyp-Gly)$_{10}$, it was calculated that nonhelical (Pro-Hyp-Gly)$_{10}$ at a concentration of 200 μg/mL and at 37 °C folds into a triple-helical conformation at a rate of only 2%/h. Since little if any fully hydroxylated (Pro-Pro-Gly)$_{10}$ was present in the experiments reported here and since the formation of triple helix is concentration dependent, it is unlikely that appreciable amounts of the peptides became triple helical under the conditions used here for hydroxylation by prolyl hydroxylase.

Discussion

Enzymes which operate on polymeric substrates have several features which distinguish them from enzymes operating on small molecules. These distinguishing features include a relatively large active site which contains binding subsites for monomeric units of the substrate and a catalytic subsite located at a unique position within the active site (Berger and Schechter, 1970). The finding that prolyl hydroxylase selectively hydroxylated the fourth triplet of (Pro-Pro-Gly)$_5$ (Kivirikko et al., 1971) stimulated interest in studying further the interaction of prolyl hydroxylase with synthetic peptide substrates. As discussed previously (Kivirikko et al., 1971), there appear to be at least three explanations as to why, after the initial hydroxylation of (Pro-Pro-Gly)$_5$, the distribution of hydroxyproline is asymmetric, instead of being either evenly distributed or symmetrically distributed around the middle triplet: (a) electrostatic interactions between the enzyme and the charged NH- and COOH-terminal groups may produce asymmetric binding of the peptide; (b) the reaction may involve a processive mechanism in which the enzyme binds either randomly or selectively to specific triplets, and then moves laterally toward the COOH terminus as hydroxylation of the peptide proceeds, and (c) the enzyme may have an active site in which the binding subsites are asymmetrically distributed around the catalytic subsite.

The effect of electrostatic charges on the end groups was tested here with two techniques, chemical modification of the end groups of (Pro-Pro-Gly)$_5$ and extending the peptide with

additional triplets of -Pro-Pro-Gly-. No effect on the distribution of hydroxyproline was observed when the COOH-terminal residue was converted to the *O*-methyl ester. The negative charge on the COOH-terminal end, apparently therefore, has no effect in directing hydroxylation of the peptide. Chemically modifying the NH-terminal end of $(Pro\text{-}Pro\text{-}Gly)_5$ changed the distribution so that the middle triplet was preferentially hydroxylated and this observation suggested that the positive electrostatic charge contributed to the asymmetry. However, the more exaggerated asymmetry seen with initial hydroxylation of $(Pro\text{-}Pro\text{-}Gly)_{10}$ indicated that the charge on the NH-terminal group was a minor effect. $(Pro\text{-}Pro\text{-}Gly)_{10}$ can be considered as R-$(Pro\text{-}Pro\text{-}Gly)_5$, where R consists of five triplets of -Pro-Pro-Gly-. Since the active sites of enzymes or binding sites of antibodies combine with up to about seven residues (Rupley, 1967; Kabat, 1968; and Berger and Schechter, 1970), it seems unlikely that one can explain the asymmetry seen with the initial hydroxylation of $(Pro\text{-}Pro\text{-}Gly)_{10}$ by the positive charge on the NH terminus which is located 25 residues from the hydroxylated prolyl residue in the ninth triplet.

There are several reasons for considering the possibility that prolyl hydroxylase may hydroxylate polypeptides of the structure $(Pro\text{-}Pro\text{-}Gly)_{10}$ with a processive mechanism (see Grant and Prockop, 1972). In particular, previous studies with prolyl hydroxylase demonstrated that large polypeptides have a much higher affinity for the enzyme than short polypeptides containing the same amino acid sequences (Juva and Prockop, 1969; McGee et al., 1971; Kivirikko et al., 1972; Berg and Prockop, 1973c). As discussed elsewhere (see Prockop et al., 1976), it seems possible therefore that prolyl hydroxylase is similar to one DNA methylase (Drahovsky and Morris, 1971) and to DNA polymerase II (Sherman and Gefter, 1976), two enzymes which apparently operate on macromolecular substrates with processive mechanisms.

The data obtained here do not completely exclude all possible types of processive mechanisms, but they speak against a mechanism in which adjacent triplets are hydroxylated sequentially as the enzyme moves along a polymeric substrate. If prolyl hydroxylase hydroxylated $(Pro\text{-}Pro\text{-}Gly)_{10}$ with such a processive mechanism, all of the triplets in $(Pro\text{-}Pro\text{-}Gly)_{10}$ should be hydroxylated to the same relative extent independently of the overall extent of hydroxylation. The results presented here exclude this mechanism, since it was found that one triplet, the ninth from the NH_2-terminal end of the peptide, was hydroxylated more than any other triplet when the overall extent of hydroxylation was varied from 1.3 to 23.4%.

The results here also exclude a processive mechanism involving hydroxylation of the ninth triplet with an obligatory hydroxylation of an adjacent triplet, since hydroxylation of the eighth or tenth triplets did not occur in parallel with the hydroxylation of the ninth triplet (Table II). The data do not exclude the possibility that the small amount of hydroxylation seen in the second to seventh triplets may have been introduced into the peptide through a processive mechanism in which two or more adjacent triplets are sequentially hydroxylated. This, however, seems unlikely on the basis of the observations with triplets eight, nine, and ten.

On the basis of the data presented here, the asymmetry in the hydroxylation of $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ is best explained by the enzyme having binding subsites which are asymmetrically distributed around the catalytic subsite or subsites. It seems unlikely that the asymmetry can be accounted for by the catalytic subsite displaying different reactivities with individual triplets in the peptides, since the k_{cat} was essentially the same for $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$ and for protocollagen (Berg and Prockop, 1973c). If however the active site of prolyl hydroxylase contains binding subsites which preferentially combine with one or more triplets on the NH_2-terminal side of the triplet which is hydroxylated, short peptides with repeating triplets would, as observed here and previously (Kivirikko et al., 1972), be preferentially hydroxylated at one end. A similar hypothesis has been proposed to explain the binding of synthetic peptides to papain (Berger and Schechter, 1970).

The evidence presented here for a complex, asymmetric active site in prolyl hydroxylase may help to explain several apparent anomalies in the hydroxylation of pro-α chains during the synthesis of procollagen in vivo, such as the incomplete hydroxylation of specific prolyl residues (for review, see Fietzek and Kühn, 1976). Hydroxylation in vivo may, however, be more complex because it occurs on larger polypeptides, it can occur during translation, and it is influenced by the nature of the amino acid occupying the X position of the triplet hydroxylated (see above) and the amino acids occupying the X and Y positions of adjacent triplets (for reviews see Cardinale and Udenfriend, 1974; Prockop et al., 1976).

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A Confirmation of the Phase Behavior of *Escherichia coli* Cytoplasmic Membrane Lipids by X-Ray Diffraction†

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ABSTRACT: The lipid fatty acid composition of the cytoplasmic membranes of *Escherichia coli* can be varied by growing an unsaturated fatty acid auxotroph in the presence of different fatty acid supplements. Electron spin resonance (ESR) studies of spin-label partitioning into the cytoplasmic membranes of different lipid fatty acid compositions as a function of temperature have been interpreted as indicating a broad order-to-disorder transition in the membrane lipids, the end points of the transition depending upon the fatty acid composition. We have utilized x-ray diffraction to confirm the ESR studies for three different fatty acid supplements (oleic, elaidic, and bromostearic). We found that the characteristic end-point temperatures detected by ESR were indeed the end-point temperatures of a broad order-to-disorder transition of the cytoplasmic membrane lipids. In addition, Patterson functions calculated from lamellar x-ray diffraction from partially oriented cytoplasmic membranes indicate a decrease in average membrane thickness upon fatty acid chain melting.

Upon melting or freezing, lipids in intact membranes or in extracted form undergo a broad phase transition (Luzzati, 1968; Engleman, 1970). This broad transition is defined by distinct end points and can be described using phase diagrams. Electron spin resonance (ESR)[1] has been used to define the end points of the phase transition in binary lipid mixtures (Shimshick and McConnell, 1973). A lower characteristic temperature (t_l) marks the onset of melting. Melting of the two components continues as temperature is increased until an upper characteristic temperature (t_h) is reached. Below t_l all lipid is in a frozen state; above t_h, all lipid is in a liquid state. Hence, t_l lies on the solidus curve and t_h lies on the fluidus curve of the phase diagram for the system. Over the temperature range bounded by t_l and t_h, there exists an equilibrium mixture of fluid and solid lipid phases; this equilibration requires lateral diffusion (Kornberg and McConnell, 1971; McConnell et al., 1972).

The spin-label probe Tempo was used in ESR studies of cytoplasmic membranes isolated from cells of an unsaturated fatty acid auxotroph of *E. coli* grown with single fatty acid supplements (Linden et al., 1973b). Partitioning of Tempo between hydrocarbon and aqueous phases of the membranes revealed that there were two characteristic temperatures defining the course of melting of the membrane lipids. Aqueous dispersions of lipids extracted from these membranes were examined in the same manner and yielded similar characteristic temperatures. By analogy with ESR studies of model binary lipid mixtures, these temperatures were designated as the upper and lower characteristic temperatures of the membrane lipid phase transition. This analogy is appropriate since analysis of the membrane phospholipid fatty acid composition and

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[1] Abbreviations used: Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxyl; ESR, electron spin resonance.