Formation of Enzyme-Substrate Complexes with Protocollagen Proline Hydroxylase and Large Polypeptide Substrates*

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SUMMARY

Cartilage from chick embryos was incubated with 14C-proline and puromycin in order to prepare puromycin peptides which were substrates for the synthesis of 14C-hydroxyproline with protocollagen proline hydroxylase. Stable enzyme-substrate complexes were recovered when the substrate was mixed with the enzyme and the mixtures were examined by gel filtration.

Although ascorbate, α-ketoglutarate, and ferrous iron are required for catalytic activity, formation of the enzyme-substrate complexes did not require addition of the cofactors or cosubstrates to dialyzed enzyme. Formation of the complexes was reduced but not prevented by EDTA in concentrations which inhibited catalytic activity.

Under the conditions used, the enzyme in the isolated complexes was readily saturated with substrate, but it was difficult to saturate the substrate with enzyme. With high concentrations of enzyme relative to substrate, the initial velocity for the reaction was greater than the initial velocity observed with the isolated enzyme-substrate complexes, suggesting that 1 molecule of polypeptide substrate could bind more than 1 molecule of enzyme.

The enzyme-substrate complexes were in equilibrium with free enzyme and substrate, since addition of a competing polypeptide produced a dissociation of the initial complexes and a rapid inhibition of the hydroxylation of the substrate. Most of the substrate was still recovered in the enzyme-substrate complexes under conditions in which the over-all concentration of substrate was about 100 pm polypeptide. Since the enzyme appeared to be saturated with substrate under these conditions, the results suggested that the dissociation constant for the complexes expressed in terms of the molar concentration of substrate polypeptides was probably less than 100 pM. The affinity of the enzyme for substrate decreased after partial hydroxylation of the substrate. After one-sixth of the available 14C-proline in the substrate was converted to 14C-hydroxyproline, it was no longer possible to demonstrate enzyme-substrate complexes in the gel filtration column.

Previous kinetic measurements demonstrated that the enzyme has a higher affinity for large polypeptides than for small ones of the same structure, and that a single encounter between enzyme and a large polypeptide probably involves a large segment of the polypeptide. The present results indicate that multiple encounters between enzyme and substrate are necessary to complete the hydroxylation of polypeptide substrates with molecular weights of up to 100,000. Since the affinity of the enzyme for the substrate decreases after partial hydroxylation of the substrate, the introduction of the final few hydroxyl groups into large polypeptide substrates apparently does not proceed as readily as the introduction of the first few hydroxyl groups.

The hydroxyproline in collagen is synthesized by the hydroxylation of proline in a large proline-rich and lysine-rich polypeptide precursor of collagen called protocollagen (for review, see Reference 1). Protocollagen proline hydroxylase has been partially purified from several sources (2–5), and it has been shown to require as cofactors or cosubstrates O2, Fe2+, α-ketoglutarate, and ascorbate (1). The enzyme has been studied by using as substrates synthetic polypeptides with the structure (Gly–X–Pro)n in which “X” is proline or alanine (1, 2, 6–8), denatured cuticle collagen from Ascaris lumbricoides (9), or proline-labeled protocollagen synthesized in vitro by isolated connective tissues (1). Kinetic studies with these substrates have indicated that large polypeptides are better substrates for the synthesis of hydroxyproline than short polypeptides. When the data were expressed either as concentration of prolyl residues or as concentration of polypeptide, the $K_m$ values for synthetic substrates decreased with increasing size of the polypeptide over a relatively broad range (1, 6–8), and the $K_m$ values observed with synthetic polypeptides with molecular weights of up to 10,000 were considerably greater than the $K_m$ values for larger biologically synthesized substrates (8–10). The $K_m$ value observed with denatured cuticle collagen from Ascaris with a molecular weight of about 60,000 was about 200 nm polypeptide (9), and the $K_m$ value observed for proline-labeled protocollagen with a molecular weight of about 100,000 was about 10 nm poly-

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peptide (10), indicating that the enzyme had a high affinity for these large substrates. The high affinity of protocollagen proline hydroxylase for large polypeptide substrates probably explains the early observation (11) that particular fractions of cartilage homogenates which contained protocollagen also contained significant amounts of the hydroxylase.

In the present experiments the affinity of protocollagen proline hydroxylase for large protocollagen-like substrates was shown to be great enough to permit examination of enzyme-substrate complexes by gel filtration.

**Materials and Methods**

**Preparation of Puromycin Peptides**—Forty tibiae which consisted primarily of cartilage were removed from 10-day-old chick embryos by microscopic dissection, and the tissue was incubated in a simple medium of glucose and inorganic salts at 37°C as described previously (12). Puromycin hydrochloride (Nutritional Biochemicals), 40 μg per ml, was added to the medium, and 20 min later 100 μCi of 14C-proline (New England Nuclor), 197 μCi per μmole, was added. The incubation was continued for a total of 4 hours, and the tissues were homogenized in 4 ml of distilled water. The homogenate was dialyzed against running tap water overnight, and then against 1 liter of 0.05 M KCl, 0.1 M NaCl, 0.1 M glycine, and 0.01 M Tris-HCl buffer adjusted to pH 7.8 at 4°C (3). The dialyzed sample was then placed in boiling water for 5 min, and it was diluted with the buffer used for dialysis to a final volume of 8 ml. The sample was centrifuged at 100,000 × g for 1 hour, and the supernatant solution containing puromycin peptides was stored frozen for up to 3 weeks in aliquots of 0.5 ml. The yield of 14C-labeled puromycin peptides varied from 0.34 to 1.2 × 10^6 dpm 14C with four separate preparations. Experiments in which the peptides were labeled with U-14C-puromycin of known specific activity instead of 14C-proline (see below) indicated that the preparations contained 230 to 830 pmoles of puromycin-substituted polypeptides. The variations in yield are probably explained by slight variations in the 80 to 94% inhibition of amino acid incorporation obtained with the same concentration of puromycin in different experiments (12), and by variations in the rates of protein synthesis in cartilage taken from different batches of chick embryos of the same chronological age. Except as noted, the four preparations of 14C-proline-labeled puromycin peptides gave similar results in all the gel filtration and kinetic experiments.

14C-Proline-labeled proteocollagen was prepared and stored under conditions identical to those for the puromycin peptides except that the puromycin in the incubation system was replaced by 1 mM α,α'-dipyridyl (2, 10).

**Incubation of Substrates with Protocollagen Proline Hydroxylase**—Protocollagen proline hydroxylase from chick embryo was purified through the calcium phosphate gel step (7) as recently modified by Hahne and Kivirikko (9). The enzyme was assayed with 125 μg per ml of the polytripeptide (Pro-Gly-Pro), with an average molecular weight of about 3,000. One unit of enzymatic activity was defined (9) as the amount of enzyme required to synthesize 1 μg of hydroxyproline in 1 hour under standard conditions (see below). The specific activity of the enzyme preparations was more than 50 times the specific activity of the crude extract from chick embryos, and the various enzyme preparations used here had specific activities of 5 to 10 units per mg of enzyme protein.

The enzymatic reaction with the hydroxylase from chick embryos was carried out in a final volume of 8 ml which contained from 2 to 140 units of enzyme activity; 10,000 to 50,000 dpm of 14C-proline-labeled substrate; 0.04 mM FeSO₄; 0.5 mM α-ketoglutarate; 2 mM ascorbic acid (Fisher Scientific Company, Pittsburgh, Pennsylvania); 0.05 mg per ml of catalase (Calbiochem); and 50 mM Tris-HCl buffer adjusted to pH 7.8 at 25°C (7). After the samples were incubated at 37°C with shaking for the times indicated, the reaction was stopped by adding 8 ml of concentrated HCl. The samples were hydrolyzed overnight in sealed tubes at 120°C, and the hydrolysates were evaporated to dryness in a rotary evaporator under vacuum. The residues were dissolved in 4.0 ml of water, and the samples were assayed for 14C-hydroxyproline with a specific chemical procedure (13) and for total 14C content (13).

**Gel Filtration**—Gel filtration was carried out with columns (1.5 × 24 cm) of polyacrylamide gel (Bio-Gel P-300, Calbiochem) or of cross-linked dextran (Sephadex G-200, Pharmacia). Initial experiments were carried out with the polyacrylamide column, but subsequently it was found that the results were essentially the same and that flow rates were better with the dextran column. The column was equilibrated and eluted at 4°C with the same buffer as was used for the dialysis of the puromycin peptides (above). Fractions of 1.2 to 1.4 ml were collected, and 0.1- to 0.4-ml aliquots were assayed for 14C or 3H with 20 ml of the solvent system for liquid scintillation counting as described previously (13). The excluded volume of the columns corresponded to the first 8 or 9 fractions, and the total volume of the columns was recovered in 33 to 35 fractions.

**Results**

**Gel Filtration of Puromycin Peptides**—Previous experiments demonstrated that after embryonic cartilage was incubated with puromycin and 14C-proline, extracts of the tissue contained small amounts of nondialyzable polypeptides which were rich in 14C-proline (12) and which served as substrates for the synthesis of 14C-hydroxyproline (14). Since puromycin inhibits protein synthesis by randomly terminating the growth of nascent polypeptides on ribosomes (15), the puromycin peptides in these preparations were heterogeneous in size (12, 16). Also, since collagen synthesis accounts for only about half of the 14C-proline incorporated into protein by the embryonic cartilage (10, 16), about half of the 14C in the preparations of puromycin peptides was in polypeptide intermediates for the synthesis of proteins other than collagen. The puromycin peptides were used here as substrates because they were of high specific activity, they were larger than available synthetic polypeptides, and they could be examined more readily by gel filtration (12, 16) than protocollagen from the same tissue. Over 95% of the 14C in the puromycin peptides was accounted for by 14C-proline and a small amount of 14C-hydroxyproline. Experiments in which the puromycin peptides were maximally hydroxylated with protocollagen hydroxylase (see below) or digested with bacterial collagenase (16) indicated that 50 to 76% of the 14C in the preparations was in protocollagen-like peptides.

Gel filtration of the puromycin peptides on a Bio-Gel P-300...
were obtained when the Peak I fractions were incubated at 37°C for 1 hour before rechromatography, and when the columns were kept at 37°C instead of 4°C. The results suggested that the enzyme-substrate complexes were bound the 14C-labeled peptides in the preparation of puromycin peptides. Protocollagen proline hydroxylase, 9.4 units, was mixed with 600,000 dpm of 14C-labeled puromycin peptides, and the mixture in a final volume of 0.9 ml was chromatographed under the same conditions used in Fig. 1. 

Further evidence that the 14C-labeled puromycin peptides recovered in the exclusion volume (Peak I in Fig. 2) were in enzyme-substrate complexes was obtained by incubating the various column fractions with the cofactors required for the synthesis of 14C-hydroxyproline (Table I). The puromycin peptides contained only small amounts of 14C-hydroxyproline, and 14C-hydroxyproline accounted for 2 to 8% of the total 14C in various preparations. Incubation of the puromycin peptides by themselves or with the required cofactors did not give synthesis of 14C-hydroxyproline. Incubation of the puromycin peptides with excess protocollagen proline hydroxylation and the required cofactors increased the ratio of 14C-hydroxyproline to total 14C to 20 to 25%. As discussed elsewhere (16), these values for the ratio of 14C-hydroxyproline to total 14C indicate a complete hydroxylation of the appropriate 14C-prolyl residues in the protocollagen-like peptides which are found in crude extracts of the cartilage. Assay of the fractions eluted from the gel filtration column indicated that the ratio of 14C-hydroxyproline to total 14C in the peptides in the exclusion volume (Peak I in Fig. 2) and in the retarded peak (Peak II) was about the same as the ratio of 14C-hydroxyproline to total 14C in the initial mixture of the puromycin peptides (Column 2 in Table I).

Incubation of the Peak I fractions with the required cofactors resulted in the synthesis of 14C-hydroxyproline (Table I and Fig. 4). Addition of fresh enzyme to the Peak I fractions increased the rate of the reaction (see below), and there was a slight increase in the final amount of 14C-hydroxyproline synthesized (Table I). The maximal values for 14C-hydroxyproline to total 14C obtained with Peak I fractions ranged from 34 to 38%. Calculations based on the proline and hydroxyproline content of pure vertebrate collagens indicated that the maximal theoretical value for this ratio after hydroxylation of a pure polypeptide precursor of collagen is 41 to 46% (16). The results indicated, therefore, that the enzyme in Peak I specifically bound the 14C-labeled peptides in the preparation of puromycin peptides which were substrates for protocollagen proline hydroxylation.

Incubation of the Peak II fraction with the required cofactors did not give any significant synthesis of 14C-hydroxyproline.
The same conditions. The results therefore indicated that peak proline to total 14C to 11 to 17% in various experiments. This reduced the amount of 14C recovered in peak I to about one-third of the amount obtained with a control sample (see Fig. 2). The presence of 0.1 mM EDTA completely inhibited the enzymatic hydroxylation of the puromycin peptides.

Effect of Cosubstrates and Cofactors in Formation of Enzyme-Substrate Complexes—Since the enzyme-substrate complexes were recovered from the gel filtration column without addition of α-ketoglutarate, FeSO₄, or ascorbate (Fig. 2), it was apparent that addition of these cofactors or cosubstrates was not essential for the binding of substrate polypeptides to the enzyme.

Treatment of the enzyme with EDTA under conditions which completely inhibited the reaction reduced but did not prevent the formation of enzyme-substrate complexes. EDTA in a final concentration of 0.1 mM was added to enzyme, and then puromycin peptides were added under the same conditions as those used in Fig. 2. The sample was then chromatographed on a Sephadex G-200 column which was eluted with buffer containing 0.1 mM sodium-EDTA. The presence of 0.1 mM EDTA reduced the amount of 14C recovered in peak I to about one-third of the amount obtained with a control sample (see Fig. 2). The presence of 0.1 mM EDTA completely inhibited the enzymatic hydroxylation of the puromycin peptides.

Saturation of Enzyme with Substrate and Saturation of Substrate with Enzyme—Further experiments indicated that under the conditions used above the enzyme was essentially saturated with substrate polypeptides. In the experiment shown in Fig. 9, about 1.08 × 10⁶ dpm of 14C were recovered in peak I from a sample which contained 9.4 units of enzyme and 6 × 10⁶ dpm of puromycin peptides. When the experiment was repeated with twice the amount of the same preparation of puromycin peptides, about 1.02 × 10⁶ dpm were recovered in peak I (not shown).

Kinetic measurements indicated, however, that the substrate polypeptides in peak I were not saturated with enzyme. When the reaction was carried out with enzyme-substrate complexes which were isolated from a mixture containing excess substrate, the reaction was linear for about 15 min (lower curve, Fig. 4). The increase in the initial velocity of the reaction observed when the ratio of enzyme to substrate was increased by adding an excess of enzyme to unfractionated puromycin peptides, there was a marked increase in the initial velocity of the reaction (upper curve, Fig. 4). The increase in the initial velocity of the reaction observed when the ratio of enzyme to substrate was greater than the ratio in the isolated enzyme-substrate complexes suggested that 1 mole of substrate can bind more than 1 mole of enzyme.

Molar Amounts of Substrate Polypeptides in Enzyme-Substrate Complexes—Since puromycin inhibits protein synthesis by being

### Table I

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* Assays for 14C-hydroxyproline were made on samples containing at least 15,000 dpm of 14C, and the observed counts per min for the 14C-pyrrole obtained from 14C-hydroxyproline in the assay were at least 10 times the background of 9 cpmp for the counting system. Results are expressed as the ratio of 100 × dpm of 14C-hydroxyproline to total 14C in sample.

### Figure 4

Synthesis of 14C-hydroxyproline with isolated enzyme-substrate complexes, and with excess enzyme and unfractionated substrate. In one experiment, the peak I fractions from a chromatograph similar to that shown in Fig. 2 were pooled and divided into seven equal fractions, each containing 19,000 dpm of 14C. The fractions were then incubated at 37°C with 2 mM ascorbic acid, 0.5 mM α-ketoglutarate, and 0.04 mM FeSO₄ for the times indicated. In the second experiment 31,000 dpm of unfractionated puromycin peptides were incubated with 117 units protocollagen proline hydroxylase under the same conditions. ○ --- ○, 14C-hydroxyproline synthesized with isolated peak I fractions; ⋅⋅⋅⋅⋅⋅⋅, 14C-hydroxyproline synthesized when unfractionated puromycin peptides were incubated with excess protocollagen proline hydroxylase. Values indicate amount of 14C-hydroxyproline (14C-HYPRO) synthesized.

### Figure 5

Kinetic measurements indicated, however, that the substrate polypeptides in peak I were not saturated with enzyme. When the reaction was carried out with enzyme-substrate complexes which were isolated from a mixture containing excess substrate, the reaction was linear for about 15 min (lower curve, Fig. 4). When the ratio of enzyme to substrate was increased by adding an excess of enzyme to unfractionated puromycin peptides, there was a marked increase in the initial velocity of the reaction (upper curve, Fig. 4). The increase in the initial velocity of the reaction observed when the ratio of enzyme to substrate was greater than the ratio in the isolated enzyme-substrate complexes suggested that 1 mole of substrate can bind more than 1 mole of enzyme.

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in incorporated into the COOH-terminal end of nascent polypeptides (15), it was possible to estimate the molar amounts of peptide in the enzyme-substrate complexes by preparing puromycin peptides which were labeled with $^3$H-puromycin of known specific activity. Puromycin peptides were prepared as described above except that 200 $\mu$Ci of $^3$H-puromycin (O-methyl-$^3$H, New England Nuclear), $2.4 \times 10^4$ dpm per mole, was used as the source of radioactive label instead of $^{14}$C-proline. The total yield of labeled nondialyzable peptides was $2.0 \times 10^4$ dpm of H. On the assumption that a single molecule of $^3$H-puromycin had been incorporated per polypeptide chain, the results indicated that the yield of puromycin-substituted polypeptides was about 830 pmol. The yield of $^{14}$C-proline-labeled puromycin peptides in the same experiment was $1.2 \times 10^5$ dpm.

Ten units of enzyme were mixed with a saturating amount (see above) of the $^3$H-puromycin peptides ($1.9 \times 10^4$ dpm or 79 pmol), and the mixture was chromatographed under the same conditions as the experiment shown in Fig. 2. A total of $1.0 \times 10^4$ dpm of $^3$H was recovered in the Peak I fractions, indicating that about 4.2 pmol of $^3$H-puromycin-labeled peptides were recovered in the enzyme-substrate complexes formed with 10 units of enzyme. A total of $2.0 \times 10^4$ dpm of $^{14}$C was recovered in the Peak I fractions when the substrate consisted of puromycin peptides labeled with $^{14}$C-proline prepared under identical conditions in this experiment. Accordingly, the specific activity of the substrate labeled with $^{14}$C-proline was about $4.8 \times 10^4$ dpm per pmol. Based on this value, the amount of substrate polypeptide bound to enzyme with the three other preparations of $^{14}$C-proline-labeled puromycin peptides ranged from 3.4 to 23 pmol of polypeptide per 10 units of enzyme, and the mean value was 9.0 pmol. Adjusted for the total volume of the Peak I fractions, the mean concentration of substrate in these fractions was about $1.4 \times 10^{-13}$ M per polypeptide. Rechromatography of the Peak I fractions on a second gel column (Figs. 3 and 6) involved a 7-fold greater dilution, and therefore the mean concentration of substrate in the exclusion volume from the second column was about $2 \times 10^{-19}$ M per polypeptide. Because of the technical limitations imposed by the specific activity of the polypeptides used for these experiments, it was not possible to examine higher dilutions of the enzyme-substrate complexes.

The elution patterns obtained when the Peak I fractions were rechromatographed (Figs. 3 and 6) indicated that more than 75% of the substrate was in enzyme-substrate complexes, and therefore the mean concentration of free substrate was less than 25% of the total or less than $0.5 \times 10^{-10}$ M per polypeptide. It was not possible to extract the molar concentrations of enzyme, but the enzyme appeared to be saturated with substrate under the conditions of these experiments (see above), and therefore it is unlikely that the concentration of free enzyme was greater than the concentration of free substrate. On the assumption that the concentrations of free substrate and free enzyme were $0.5 \times 10^{-10}$ M or less, the results suggested that the dissociation constant expressed on the basis of the molar concentration of substrate was no greater than $[0.5 \times 10^{-10}]^2/[1.5 \times 10^{-10}] = 2 \times 10^{-14}$ M.

**Dissociation of Enzyme-Substrate Complexes after Complete or Partial Hydroxylation of Substrate Polypeptides**—Further chromatographic experiments indicated that the enzyme had considerably less affinity for the puromycin peptides after they were either completely or partially hydroxylated. Puromycin peptides were incubated with protocollagen hydroxylase and optimal concentrations of cofactors so that essentially all the substrate $^{14}$C-prolyl residues were converted to $^{14}$C-hydroxyprolyl residues and no a-ketoglutarate. Another third of the sample was incubated in $37^\circ$ for 1 hour with 2 mM ascorbic acid, 0.04 mM FeSO$_4$, and 0.001 mM $\alpha$-ketoglutarate so that about one-sixth of the available substrate $^{14}$C-proline was converted to $^{14}$C-hydroxyproline (see text). $\bullet$—$\bullet$, sample incubated without $\alpha$-ketoglutarate; $\odot$—$\odot$, sample incubated with limiting amount of $\alpha$-ketoglutarate.

**Fig. 5 (left).** Gel filtration of puromycin peptides after complete hydroxylation with protocollagen proline hydroxylase. $^{14}$C-Proline-labeled puromycin peptides, $1.2 \times 10^4$ dpm, were incubated at $37^\circ$ for 1 hour with 28 units of protocollagen proline hydroxylase and the standard concentrations of the required cofactors. About $5.4 \times 10^4$ dpm of the reaction mixture were then chromatographed under the same conditions used in Figs. 1 and 2. $\bullet$—$\bullet$, $^{14}$C in fractions; $\odot$—$\odot$, $^{14}$C-hydroxyproline in fractions.

**Fig. 6 (center).** Rechromatography of Peak I fractions after partial hydroxylation of the substrate in the enzyme-substrate complexes. A mixture of $3.6 \times 10^4$ dpm of puromycin peptides and 12 units of protocollagen proline hydroxylase was chromatographed under the same conditions as were used in Fig. 2. The Peak I fractions were pooled, and one-third of the sample was incubated at $37^\circ$ for 1 hour with 2 mM ascorbic acid, 0.04 mM FeSO$_4$, and no $\alpha$-ketoglutarate. Another third of the sample was incubated at $37^\circ$ for 1 hour with 2 mM ascorbic acid, 0.04 mM FeSO$_4$, and 0.001 mM $\alpha$-ketoglutarate so that about one-sixth of the available substrate $^{14}$C-proline was converted to $^{14}$C-hydroxyproline (see text). $\bullet$—$\bullet$, sample incubated without $\alpha$-ketoglutarate; $\odot$—$\odot$, sample incubated with limiting amount of $\alpha$-ketoglutarate.

**Fig. 7 (right).** Effect of a competing polypeptide substrate on the formation of enzyme-substrate complexes. Protocollagen proline hydroxylase (30 units) was mixed with 100,000 dpm of puromycin peptides, and the mixture was chromatographed under the same conditions used in Fig. 2. $\bullet$—$\bullet$, control sample; $\bigstar$—$\bigstar$, sample to which 1.4 mg per ml of denatured and carboxymethylated collagen from *Ascaris* cuticle (9) was added just prior to chromatography.
Subsequent gel filtration of the hydroxylated sample indicated that the amount of 14C-labeled peptides which appeared in the exclusion volume with the enzyme was markedly reduced (compare Fig. 5 with Fig. 2). Most of the 14C-hydroxyproline in the sample appeared in the leading edge of the elution peak (Fig. 5), indicating that the protocollagen-like peptides which served as substrates were larger than the other peptides in the substrate preparation. The small amount of 14C-labeled peptides which still appeared in the exclusion volume may indicate a small fraction of the hydroxylated peptides specifically bound to the enzyme, or nonspecific coprecipitation of some of the peptides with denatured proteins from the enzyme preparation. A small precipitation of protein without any loss in enzymatic activity was usually seen after incubating the enzyme at 37° for 1 hour.

In order to examine the effect of partial hydroxylation of the substrate, a mixture of enzyme and puromycin peptides was chromatographed on a G-200 column, and the Peak 1 fractions were incubated with optimal amounts of all the cofactors (Table I). After the partial hydroxylation of the substrate in the isolated enzyme-substrate complexes, the substrate peptides were no longer eluted in the exclusion volume of the column (Fig. 6), indicating that the enzyme had less affinity for the partially hydroxylated polypeptides than for the initial substrate polypeptides. Results similar to those shown in Fig. 6 were obtained when fresh enzyme was added to the partially hydroxylated enzyme-substrate complexes before rechromatography. The peptides which dissociated from the initial enzyme-substrate complexes eluted earlier than the major peak of peptides which were substrates for the enzyme (compare Figs. 5 and 6), probably because the initial enzyme-substrate complexes were formed with the largest peptides in the substrate preparation.

**Dissociation of Enzyme-Substrate Complexes in Presence of Competing Polypeptides**—To demonstrate that the enzyme-substrate complexes were in equilibrium with free enzyme and substrate, the enzyme was mixed with puromycin peptides under the conditions used in Fig. 2, then denatured collagen from Ascaris cuticle (9) was added to the sample before it was chromatographed. The addition of the competing substrate resulted in the release of a large part of the 14C-labeled substrate from the initial enzyme-substrate complexes (Fig. 7).

Kinetic measurements with competing polypeptides indicated that the dissociation of the initial enzyme-substrate complexes was more rapid than the hydroxylation of the substrate in the complexes (Fig. 8). Of the enzyme, 39 units were used to hydroxylate 30,000 dpm of puromycin peptides so that the rate of the reaction was considerably faster than the rate observed with the isolated enzyme-substrate complexes (*lower curve*, Fig. 4). Addition of the competitive inhibitor, poly-L-proline (17), inhibited the reaction 75%, and addition of denatured collagen from the cuticle of Ascaris inhibited the reaction about 88% (Fig. 8). The percentage inhibition was the same when the competing polypeptide was added to the enzyme first, or when it was added after the substrate so that the initial enzyme-substrate complexes had already formed.

**DISCUSSION**

The results directly show the high affinity of protocollagen proline hydroxylase for large polypeptide substrates. The substrate used here consisted of a heterogeneous mixture of polypeptides obtained by incubating embryonic cartilage with 14C-proline and puromycin. When a sample consisting of the puromycin peptides and the enzyme was examined by gel filtration, the polypeptides in the mixture of polypeptides which were substrates for the synthesis of hydroxyproline were specifically bound to the enzyme. Since it was shown that the enzyme-substrate complexes were in equilibrium with free enzyme and substrate peptides (see below), the principal explanation for the stability of the complexes in the gel filtration columns is that their dissociation constants are considerably less than the concentration of the enzyme-substrate complexes. Most of the substrate was still recovered in the complexes when the over-all concentration of substrate was about 100 μM polypeptide, and the results suggested that the dissociation constant for the complexes expressed in terms of the molar concentration of substrate polypeptides is probably less than 100 μM. This value is lower than might be expected from previous kinetic measurements, which indicated that the Kₚ value for protocollagen and similar
polypeptides was about 10 mM polypeptide (10). It should be noted, however, that an assignment of such values to interactions of polypeptides with procollagen hydroxylase is complicated by the unusual nature of the interactions. Since the enzyme interacts with large segments of polypeptides (8, 17), and since a single large polypeptide can apparently bind more than one enzyme molecule (see below), it will probably be necessary to define the mechanism of each interaction before deciding the units in which to express the concentration of substrate. Also, it may be necessary to define explicitly the meaning of $K_m$ values for these interactions.

The high affinity of the enzyme for the polypeptide substrates did not depend on the presence of unbound ascorbate, $\alpha$-ketoglutarate, or ferric iron, since it was not necessary to add these cofactors or cosubstrates to the mixture of dialyzed enzyme and puromycin polypeptides in order to demonstrate the enzyme-substrate complexes. The formation of enzyme-substrate complexes was reduced but not prevented by addition of EDTA in concentrations which completely inhibited enzymatic activity.

The affinity of the enzyme for the polypeptide substrates decreased after the substrates were completely or partially hydroxylated. After isolated enzyme-substrate complexes were incubated with limiting amounts of the required cofactors, so that about one-sixth of the available substrate $\text{^{14}C}$-proline was hydroxylated, the partially hydroxylated substrates were no longer bound to the enzyme in the gel filtration column. This observation is consistent with the fact that the enzyme is inhibited by poly-L-proline but not by poly-L-hydroxyproline (10).

Previous kinetic measurements with polypeptides of varying size demonstrated that the enzyme interacts with large segments of polypeptide chains with molecular weights of 5,000 to 20,000 (8, 17). The results suggested either that the enzyme has an unusually large binding site, or that the enzyme moves laterally along polypeptides so that larger polymers have significantly smaller turnover numbers (17). The present observations indicate that even though the enzyme interacts with relatively large segments of polypeptide chains, multiple encounters between the enzyme and the substrate are necessary for the complete hydroxylation of large polypeptides with molecular weights of up to 100,000. This conclusion was suggested by the evidence that 1 molecule of substrate can bind more than 1 molecule of enzyme, and by the dissociation of the enzyme-substrate complexes after partial hydroxylation of the substrate in the complexes. Further evidence was provided by examining the effect of competing polypeptides on the reaction. When a polypeptide which was either a competitive inhibitor or a competing substrate was added to the enzymatic system, the initial enzyme-substrate complexes dissociated, and there was a rapid inhibition of the reaction with the initial substrate. Even in the presence of relatively large concentrations of enzyme, the dissociation of the initial enzyme-substrate complexes was more rapid than the hydroxylation of the substrate in the complexes.

The requirement for multiple encounters between enzyme and substrate and the decreased affinity of the enzyme for partially hydroxylated substrates may help to explain both the relatively long time required to obtain complete hydroxylation of procollagen in connective tissue cells (1) and the incomplete hydroxylation of specific prolyl residues in the $\alpha_1$ chain of collagen (18). Since the affinity of the enzyme for the substrate decreased after partial hydroxylation of the substrate, the results obtained here indicate that the introduction of the final few hydroxyl groups into the polypeptide substrate apparently does not proceed as readily as the introduction of the first few hydroxyl groups.

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