Effect of Polymer Size on the Inhibition of Protocollagen Proline Hydroxylase by Polyproline II*

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DARWIN J. PROCKOP‡ and KARI I. KIVIRIKKO§

From the Departments of Medicine and Biochemistry, University of Pennsylvania, and the Philadelphia General Hospital, Philadelphia, Pennsylvania 19104.

**SUMMARY**

Fractations of poly-L-proline Form II ranging in molecular weight from 1,600 to 21,000 were compared in their ability to serve as competitive inhibitors for the synthesis of hydroxyproline by protocollagen proline hydroxylase. The degree of inhibition of the enzyme increased with the size of the polymer, and the $K_i$ values decreased progressively with molecular weight. The results indicated that poly-L-proline polymers with 150 to 200 residues have higher affinities for the inhibitor site or sites on the enzyme than smaller poly-L-proline fractions with the same structure in solution. The data could not be explained solely on the basis that large fractions of poly-L-proline act as multifunctional reactants with cooperative or noncooperative binding of several enzyme molecules per polypeptide. Accordingly, it appears that interaction between the enzyme and poly-L-proline involves some unusual feature such as an inhibitor site large enough to accommodate 150 to 200 prolyl residues or lateral movement of the enzyme along the polypeptide.

The enzyme protocollagen proline hydroxylase synthesizes the hydroxyproline in collagen by the hydroxylation of proline which has been incorporated into protocollagen, the large proline-rich (1-5) and lysine-rich (6) polypeptide precursor of collagen (for review see Reference 7). Studies with synthetic polypeptides (8-11) showed that the synthesis of hydroxyproline by the enzyme required a polypeptide substrate of relatively large size, but the nature of the size requirement has not been clearly established. Protocollagen proline hydroxylase does not catalyze the hydroxylation of proline in polyproline† but the enzyme is competitively inhibited by large molecular weight polymers of polyproline in the Form II conformation (12, 13). In the present report the effect of polypeptide size on the interactions with protocollagen proline hydroxylase was examined by preparing polyproline II fractions of different molecular weights and comparing their ability to inhibit the enzyme.

**MATERIALS AND METHODS**

Preparations of Polyproline—N-Carboxy-L-proline anhydride was prepared by the method of Randall (14). In order to obtain polyproline polymers of the largest size, the N-carboxy-L-proline anhydride was polymerized in pyridine without initiator. The average molecular weight of these preparations varied from 16,000 to 20,000. In order to obtain polymers of smaller size, the N-carboxy-L-proline was polymerized in acetonitrile with NaOCH₃ as the initiator. The ratio of initiator to carboxyanhydride was varied from 1:200 to 1:20. All the preparations were converted to the Form II conformation by treatment with formic acid at room temperature for 24 hours.

The molecular weight distributions of polymer preparations were relatively broad, and therefore the polymers were fractionated by gel filtration in a manner similar to that used previously to fractionate polymers of (Pro-Gly-Pro)ₖ (15). Sephacryl G-50 was used for preparations with average molecular weights less than 10,000, and Sephadex G-75 used for preparations with higher average molecular weights. The column eluates were divided into four or five fractions of about equal size, and the average molecular weight of each fraction was measured in an analytical ultracentrifuge by the midpoint technique of Yphantis (15). In addition to the polyproline II fractions obtained with these procedures, several unfractionated polymers were used. One unfractionated polymer with an average molecular weight of 4,000 was prepared for previous studies on protocollagen proline

‡ To whom requests for reprints should be sent at Philadelphia General Hospital.
§ Present address, Department of Medical Chemistry, University of Helsinki, and Children's Hospital, University of Helsinki, Helsinki, Finland.

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† Polyproline refers to polymers of poly-L-proline in the water-soluble Form II conformation (see Reference 12).
hydroxylase (13). An unfraccionated polymer with an average molecular weight of 21,000 was purchased from Mann. The average molecular weights of both these preparations were measured with an analytical centrifuge, and gel filtration indicated the molecular weight distribution in each was relatively broad. Two additional polyproline II preparations with average molecular weights of about 12,000 and 15,000 were kindly supplied by Dr. D. Wasserman, Research Division, Ethicon, Inc.

The optical rotation of the polyproline polymers was measured in a Bendix Series 1100 automatic polarimeter.

Preparation of Protocollagen Proline Hydroxylase from Chick Embryos—The enzyme was prepared from 12-day-old chick embryos with the procedures described previously (8, 9). From 200 to 400 chick embryos were used, and the enzyme was purified through the calcium phosphate gel step. The enzyme was assayed with 125 μg per ml of an unfraccionated preparation of (Pro-Gly-Pro)₆. The (Pro-Gly-Pro)₆ preparation was synthesized (15) for us by Dr. James Hayden and Dr. Albert W. Burgstahler, from the Department of Chemistry, University of Kansas. Its average molecular weight was about 3,000, and its Kₘ value was slightly greater than the Kₘ value obtained previously with fractionated preparations of (Pro-Gly-Pro)₆ ranging in average molecular weights from 4,000 to 15,000 (8, 9).

One unit of enzymatic activity was defined as the amount of enzyme required to synthesize 1 μg of hydroxyproline in 1 hour with 125 μg per ml (Pro-Gly-Pro)₆ as substrate. As indicated previously (8, 9) the specific activity of the enzyme preparations obtained by purification through the calcium phosphate gel step were over 50 times the specific activity of the crude extracts of chick embryos, and the various preparations employed here contained from 5 to 10 units per mg of enzyme protein.

Enzymatic Reaction and Assay Procedures—The enzyme reaction was carried out in a final volume of 8 ml which contained 13 to 43 units of enzyme preparation; 0.04 mM FeSO₄; 0.5 mM α-ketoglutarate; 2 mM ascorbic acid (Fisher); 0.05 mg per ml catalase (Calbiochem); and 50 mM Tris-HCl buffer, adjusted to pH 7.8 at 25° (8, 9). The optical rotation of the polyproline polymers was measured with the polymers dissolved in water at a concentration of 1 mg per ml. They all had an [α]₂₅° value of −500, indicating that they were in a stable Form II conformation (12).

Enzymic Inhibition with Various Polyproline II Fractions—Previous studies on protocollagen proline hydroxylase (13) suggested that the enzyme was more effectively inhibited by a polyproline II preparation with an average molecular weight of 9,300 which was prepared by the same techniques for other purposes indicated that over 98% of the polymer was accounted for by fractions which ranged in molecular weight from 7,700 to 11,700 Daltons.

The optical rotation of all the polyproline polymers used here was measured with the polymers dissolved in water at a concentration of 1 mg per ml. They all had an [α]₂₅° value of −500, indicating that they were in a stable Form II conformation (12).

RESULTS

Molecular Weights of Polyproline Fractions—The procedures described above provided seven fractions of polyproline II which had weight-average molecular weights of 1,600, 2,400, 3,400, 6,100, 10,100, 13,400, and 19,100 Daltons. In order to examine the distribution of molecular weights in the separate fractions, the fraction with an average molecular weight of 1,600 was rechromatographed on a Sephadex G-50 column. The column eluate was collected in 20 fractions, and polymer content was assayed with 125 pg per ml of polyproline II as substrate and with 6.3 pg per ml of polyproline II samples of different molecular weight (Fig. 2). Under these conditions, the fractionated polyproline with a molecular weight of 1,600 inhibited the reaction only about 6%, and the larger polymers inhibited the reaction about 70%.

With the type of polymerization procedures used here, the distribution of molecular weights is generally narrower for larger fractions, and therefore the molecular weight distribution for the other six fractions was probably less than shown in Fig. 1. For example, measurements similar to those shown in Fig. 1 on a fractionated polyproline with an average molecular weight of 9,300 which was prepared by the same techniques for other purposes indicated that over 98% of the polymer was accounted for by fractions which ranged in molecular weight from 7,700 to 11,700 Daltons.

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gated further. In order to avoid the occasional variability introduced by aging of the enzyme, all the experiments reported here were carried out with enzyme preparations which had been stored less than 4 weeks and which retained 85% or more of their initial activity.

The effect of polypeptide size was investigated further by measuring the $K_i$ values of different polyproline II samples. The $K_i$ value for the unfractionated polymer with an average molecular weight of 4,000 was 4.0 and 6.4 µg per ml at two levels of inhibitor (Fig. 3). The $K_i$ value for the fractionated polymer with an average molecular weight of 6,100 was about 3.7 µg per ml; the $K_i$ value for the fractionated polymer with an average molecular weight of 10,100 was about 2.1 µg per ml; and the $K_i$ value for the fractionated polymer with an average molecular weight of 13,400 was about 1.1 µg per ml (Fig. 4). A $K_i$ value of 0.2 µg per ml was previously obtained (8) for an unfractionated polyproline polymer with an average molecular weight of 15,000, and a value of 0.4 µg per ml was obtained with the same preparations in the experiments reported here. The $K_i$ value for the unfractionated polymer with an average molecular weight of 21,000 was 0.18 and 0.19 µg per ml at two levels of inhibitor (not shown).

The $K_i$ values for the polyproline polymers decreased with increasing size when the values were expressed on the basis of micrograms per ml (Table I), or the equivalent basis of molar concentration of prolyl residues (Fig. 5). Also, there was a

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TABLE I

<table>
<thead>
<tr>
<th>Polyproline polymer</th>
<th>Observed $K_i$ value</th>
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<tr>
<td>mol/ml</td>
<td>µg/ml</td>
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<tr>
<td>4,000</td>
<td>4.9, 6.4</td>
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<tr>
<td>6,100</td>
<td>3.7</td>
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<tr>
<td>10,100</td>
<td>2.1</td>
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<tr>
<td>13,400</td>
<td>1.1</td>
</tr>
<tr>
<td>15,000*</td>
<td>0.30, 0.40</td>
</tr>
<tr>
<td>21,000*</td>
<td>0.18, 0.19</td>
</tr>
</tbody>
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* Unfractionated polymers.
Inhibitors, whereas oligopeptides and polymers with the sequence Gly-Pro-Ala were both poor substrates and weak inhibitors, whereas oligopeptides and polymers with the sequence Gly-Pro-Gly (11) or Pro-Pro-Pro were effective competitive inhibitors for the enzyme (8-11). The results indicated that at least 6 residues were required for significant synthesis of hydroxyproline, but conflicting results were reported as to the optimal size of the polypeptide substrates. We observed (8, 9) that $K_m$ values (expressed as micrograms per ml) for polymers with the repeating sequence Gly-Pro-Pro or Gly-Ala-Pro decreased with increasing size when oligopeptides or polymers with 0 to about 50 residues were compared, but there was no further decrease in $K_m$ values when polymers with about 50 residues were compared with polymers of the same sequence with 100 or 150 residues. Hutton et al. (10) reported results with tritiated (Pro-Gly-Pro)$_n$ that were similar, but they found that one fraction with about 100 residues (molecular weight of about 8000) had a smaller $K_m$ value than two fractions with about 50 residues. The discrepancy may be explained by instability of the tritiated polymers (10) or by structural differences in solution among different preparations of apparently similar polymers. Sakakibara et al. (17) reported that homologous fractions of (Pro-Pro-Gly)$_n$ prepared by a Merrifield-type synthesis are less soluble and have much sharper melting curves than Engel et al. (15) observed with comparable fractions of (Pro-Gly-Pro)$_n$ prepared by a polymerization procedure. The use of polyproline to study the effect of polymer size on binding of polypeptides to procollagen hydroxylase offers a method for circumventing some of these problems, since larger molecular weight preparations are available, the polymers are water soluble in the Form II conformation, and the conformation of the polymers is independent of size over a broad range (see Reference 12).

The results obtained here indicated that polyproline polymers with 150 to 200 residues have higher affinities for the inhibitor site or sites on the enzyme than smaller polyproline fractions with the same structure in solution. The degree of inhibition of the enzyme increased with the size of the polyproline with fractions ranging in molecular weight from 1,000 to over 10,000, and there was a progressive decrease in $K_i$ values with the size of the polymer in all the fractions examined when the values were expressed either in molar concentration of polyproline or in molar concentration of polymer. The $K_i$ values appeared to approach a limiting value with polymers of 150 to 200 residues, but accurate comparisons could not be made because the (Pro-Gly-Pro)$_n$ substrate with a molecular weight of about 3,000 had a much higher $K_m$ value than the $K_i$ values observed with the larger fractions of polyproline.

A recent re-evaluation (12) of the physical data available on polyproline II indicated that the molecule probably does not have a completely rigid helical structure in solution as was previously assumed. The high optical rotation and other properties, however, indicated that polyproline II contains a high degree of structure in solution, and that the structure is an extended one without any association between individual chains. Also, it is clear that the structure does not vary with size in polymers which contain more than 6 or 7 residues. Accordingly, the higher affinity of protocollagen hydroxylase for the large polyproline polymers is difficult to explain, and at least three schemes should probably be considered. Scheme A, the large polymers might be multifunctional reactants, and separate short segments of the large polyproline polymers might bind several enzyme molecules; Scheme B, the inhibitor site on the enzyme might be an extensive one, and the large polymers might occupy more of the inhibitor site; and Scheme C, the enzyme might move laterally along polypeptide substrates or inhibitors, and because of their greater length, the larger polyproline polymers might have significantly smaller turnover numbers.

The data presented here are difficult to account for solely on the basis that polyproline polymers are multifunctional reactants (Scheme A). If the binding of several enzyme molecules were noncooperative, the $K_i$ values should be about the same for...
large polymers and for small ones when the values are expressed on the basis of molar concentration of prolyl residues. In fact, a slight increase in $K_i$ values might be seen in the range where the polymer was of insufficient size to accommodate an integer number of enzyme molecules. The decrease in $K_i$ values with size could be explained by cooperative binding of several enzyme molecules, but this possibility is inconsistent with the kinetics of the reaction. If there were cooperative binding to the inhibitor, it is unlikely that double reciprocal plots of initial velocity and substrate concentration would be linear, or that they would indicate a purely competitive type of inhibition. Also, the $K_i$ values observed with cooperative binding would probably vary with enzyme or inhibitor concentration, but such effects could not be shown.

The available data do not distinguish between the other two schemes. When examined by gel filtration (18, 19), the enzyme appeared near the exclusion peak of a Sephadex G-200 column. It seems unlikely that the enzyme has an inhibitor site large enough to have relatively intimate contact with 150 to 200 prolyl residues in polyproline, but this possibility has not been completely excluded. The possibility that the enzyme moves laterally along polypeptide substrates or inhibitors is suggested primarily by analogy with polymerases which use DNA or RNA templates. Preliminary results of single turnover experiments with the polypeptide substrates support this suggestion, but it is clearly more difficult to apply the scheme to polyproline than to polypeptide substrates where the hydroxylation of successive prolyl residues could be assumed to impart lateral motion to the enzyme. The data obtained with polyproline would be consistent with a mechanism involving lateral motion if it were assumed that the enzyme could attach at any point on the polyproline, but it could only fall off at the ends of the polymer. One variant on this type of mechanism (Scheme C) is that an enzyme molecule once bound to a polyproline molecule might re-bind to the same polyproline molecule, because the diffusion rate of the large polyproline molecules is relatively small and as a result the local concentration of polyproline might be much greater than the average concentration in the solution as a whole. This possibility, however, is not supported by the fact that the observed $K_i$ values were not sensitive to changes in the concentration of enzyme of polyproline over the ranges which could be examined. Also, the differences between the local and average concentrations would have to be very large in order to explain the 100- to 150-fold differences observed in $K_i$ values (in molar concentration of polymer) between the polyproline fractions with molecular weight of 4,000 and 15,000 to 21,000.

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