Denatured Collagen from the Cuticle of *Ascaris lumbricoides* as a Substrate for Protocollagen Proline Hydroxylase*

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DAISABURO FUJIMOTO† AND DARWIN J. PROCKOP‡

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

SUMMARY

Recent studies have shown that the hydroxyproline and hydroxyllysine found in the collagen of vertebrates are synthesized by the hydroxylation of proline and lysine, which have been incorporated into a large polypeptide precursor of collagen called protocollagen. Because the collagen isolated from the cuticle of *Ascaris lumbricoides* contains less hydroxyproline and hydroxyllysine than any other known collagen, it was of interest to determine whether this collagen could serve as a substrate for the synthesis of hydroxyproline by protocollagen proline hydroxylase from a vertebrate source.

Essentially no synthesis of hydroxyproline was observed when the hydroxylase from chick embryos was incubated with native cuticle collagen solubilized by either 0.5 M NaCl extraction or pepsin treatment. Both of these large aggregate forms of the collagen, however, served as substrates for hydroxyproline synthesis after they were denatured by heating at 100° for 10 min. A subunit form of the collagen with a lower specific optical rotation than the large aggregate preparations served as a substrate without prior heat treatment, but the rate of hydroxyproline synthesis was increased by boiling the preparation. Comparison of the relative substrate activities and specific optical rotations of various forms of the cuticle collagen indicated an inverse relationship between the rate of hydroxyproline synthesis and the apparent degree of helicity.

Previous reports by others have suggested that the subunit structure of collagen from *Ascaris* cuticle may involve a helical conformation different from that found in other collagens, and it is possible that an unusual conformation of the polypeptide chain or chains of the native cuticle collagen may explain its inability to serve as a substrate for protocollagen proline hydroxylase.

The collagen isolated from the cuticle of *Ascaris lumbricoides* is similar to collagen from other sources in that it has a similar x-ray diffraction pattern (1), physical parameters which suggest a helical rodlike structure (2), a high content of glycine and imino acids (2, 3), and sensitivity to hydrolysis by a specific bacterial collagenase (2, 4). Cuticle collagen from *Ascaris*, however, differs from vertebrate collagens in several important respects. The collagen extracted with dilute NaCl solutions has a molecular weight of about 900,000 (2) instead of the 300,000 molecular weight of NaCl-solubilized tropocollagen from vertebrates, and the molecular forms obtained in this manner appear to consist of subunits of about 60,000 held together by disulfide linkages (5, 6). The subunit is probably a single polypeptide folded back upon itself to form a collagen-type triple helix (5, 6). Another prominent feature of cuticle collagen from *Ascaris* is that it contains less hydroxyproline and hydroxyllysine and more proline than any other known collagen (2, 3).

Recent studies have indicated that the hydroxyproline and hydroxyllysine found in the collagen from vertebrates are synthesized by the hydroxylation of proline and lysine which have been incorporated into a large polypeptide precursor of collagen called protocollagen (for review see Reference 7). Since cuticle collagen from *Ascaris* is similar to protocollagen from vertebrates in several respects, it was of interest to determine whether it could serve as a substrate for protocollagen proline hydroxylase from a vertebrate source. The results indicate that the cuticle collagen is a substrate for the synthesis of hydroxyproline after it is denatured, but that the native conformation of the cuticle collagen prevents interaction with protocollagen proline hydroxylase.

METHODS

Preparation of Collagens—Live *Ascaris lumbricoides* from the intestines of pigs were obtained from local slaughterhouses either in Sendai, Japan, or in Philadelphia, Pennsylvania. The
cuticles were removed after freezing and thawing of the worms, and the detached cuticles were reincised with scissors. The samples were ground in a mortar and pestle in the cold, and they were then homogenized in 0.5 M NaCl in a Teflon and glass homogenizer. The NaCl-solubilized collagen was obtained by extracting the sample three times with 0.5 M NaCl in the cold with shaking for 24 hours (2). The second and third 0.5 M NaCl extracts were precipitated with ammonium sulfate at 20% saturation to obtain the purified soluble collagen (2). In order to obtain the subunit form of the collagen, the residue remaining after the NaCl extraction was reduced with mercaptoethanol and carboxymethylated with monoiodoacetetic acid as described by McBride and Harrington (5). In order to obtain a large aggregate form of the collagen in better yield, the residue remaining after NaCl extraction was solubilized by treatment with 1 mg per ml of pepsin (crystalline; Worthington Biochemical Corporation, Freehold, New Jersey) in 0.01 M HCl at 4°C for 24 hours (8). The sample was then centrifuged at 15,000 x g for 20 min, and the supernatant fraction was precipitated with ammonium sulfate at 20% saturation after neutralizing with dilute NaOH. The collagen samples were either lyophilized or dialyzed against 0.5 M KCl and stored at 4°C.

The 14C-proline-labeled protocollagen was prepared by incubating cartilaginous tissue from 10-day-old chick embryos with 14C-proline and α, α'-dipyrindyl as described previously (9). After the incubation, the tissues were homogenized with water and the homogenates were centrifuged at 100,000 x g for 1 hour. The 100,000 x g supernatant fraction was dialyzed in the cold against multiple changes of 1 M KCl-0.02 M Tris-HCl, pH 7.4, for 24 hours. The preparation was boiled for 5 min, divided into aliquots of about 50,000 dpm each, and stored frozen. As indicated previously (9), each sample contained approximately 0.5 μg of proline-labeled protocollagen.

Preparation of Protocollagen Proline Hydroxylase from Chick Embryos—The enzyme was prepared from 12-day-old chick embryos with 14C-proline and α, α'-dipyrindyl as described previously (9). After the incubation, the tissues were homogenized with water and the homogenates were centrifuged at 100,000 x g for 1 hour. The 100,000 x g supernatant fraction was dialyzed in the cold against multiple changes of 1 M KCl-0.02 M Tris-HCl, pH 7.4, for 24 hours. The preparation was boiled for 5 min, divided into aliquots of about 50,000 dpm each, and stored frozen. As indicated previously (9), each sample contained approximately 0.5 μg of proline-labeled protocollagen.

Enzymatic Reaction and Assay Procedures—With Ascaris collagen or (Pro-Gly-Pro)n as substrate, the enzyme reaction was carried out in a final volume of 8 ml which contained 17 to 58 units of enzyme preparation, 0.01 M FeSO4, 0.5 M α-ketoglutarate, 2 mM ascorbic acid (Fisher), 0.05 mg per ml of catalase (Calbiochem), and 50 mM Tris-HCl buffer adjusted to pH 7.8 at 25°C (12).

After the samples were incubated at 25°C or 37°C with shaking for the times indicated, the reaction was stopped with the addition of 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 vacuum. The residues were dissolved in 5.0 ml of water, and the hydroxyproline content was assayed by a specific chemical procedure in duplicate 2.0 ml aliquots (12). In the experiments with the 14C-proline-labeled protocollagen, the hydrolyzed samples were assayed for 14C-hydroxyproline with a specific chemical procedure (13).

Measurements of Specific Optical Rotation—The optical rotation at 589 nm of the collagen preparations was measured in a Bendix Series 1100 automatic polarimeter at 23-25°C. The collagen content of the solutions was measured either by peptide absorbance at 225 nm or by quantitative assay of hydroxyproline (12). The collagen content was calculated on the basis of 2.9 g of hydroxyproline per 100 g (2).

RESULTS

Native and Denatured Cuticle Collagen as a Substrate or Inhibitor—Previous reports (2, 5, 6) indicated that the collagen extracted from Ascaris cuticle with 0.5 M NaCl has a molecular weight of about 900,000, and that the RCM preparations consist of subunits with molecular weights of about 60,000. The collagen solubilized by pepsin treatment has not been as well characterized, but the viscosity and specific optical rotation (see below) of the preparations is comparable to that of NaCl-solubilized collagen, and treatment with mercaptoethanol reduces the viscosity to the same values observed with RCM collagen. Because the yields of pepsin-solubilized collagen (12) were over 20 times greater than the yields of NaCl-solubilized collagen, and because these preparations had similar substrate activity (see below), the pepsin-solubilized collagen was used as a large aggregate form of the collagen in most of the experiments described here.

The NaCl-solubilized, pepsin-solubilized, and RCM cuticle collagen were incubated with purified protocollagen proline hydroxylase from chick embryo (Table I). Essentially no synthesis of hydroxyproline was observed with untreated NaCl-solubilized or untreated pepsin-solubilized cuticle collagens. Both of these collagen preparations, however, served as substrates for the synthesis of hydroxyproline when they were heated at 100°C for 10 min, and they were incubated with the hydroxylation system immediately after cooling to 37°C. Some synthesis of hydroxyproline was seen with native RCM cuticle collagen, but the amount of hydroxyproline synthesized increased when the preparation was boiled before incubation with the enzymatic system. As indicated, the comparison of the native and denatured forms of RCM collagen was made by incubating the system at 25°C, inasmuch as the Tm value for this collagen is 32°C in contrast to the Tm of 51°C for NaCl-solubilized cuticle collagen (6).

Hydroxylation of Denatured Subunits of Cuticle Collagen—For determination of the extent to which proline in Ascaris collagen could be hydroxylated by the chick embryo enzyme, denatured RCM collagen was incubated with an excess of enzyme for 0 to 120 min (Table II). The hydroxyproline content of the collagen was increased approximately 4-fold. On the basis of a value of 19 residues per 1000 for Ascaris collagen (2), the hydroxyproline

1 The abbreviation used is: RCM collagen, subunits obtained by reduction and carboxymethylation of the insoluble cuticle collagen.
content increased to a final value of about 80 residues per 1000. Addition of fresh enzyme and cofactors to the enzymatic system after incubation for 120 min did not produce an additional increase in hydroxyproline.

In order to examine the kinetics of the reaction, the time course for the hydroxylation of denatured RCM collagen was studied (Fig. 1). With a given concentration of the collagen substrate, the reaction was linear until the hydroxyproline content was increased to about 2.5 times the initial level. Under the conditions described in Fig. 2, the reaction was linear for about 30 min with 3.7 μg per ml of denatured RCM collagen, and it was linear for about 90 min with 59 μg per ml of denatured RCM.

**Table I**
Various forms of cuticle collagen from Ascaris as substrates for protocollagen proline hydroxylase

In Experiment 1, 140 μg of NaCl-solubilized, pepsin-solubilized, or RCM collagen were incubated with 21 units of protocollagen proline hydroxylase at 37° for 40 min. In Experiment 2, 280 μg of each collagen were incubated with 48 units of enzyme at 37° for 60 min. In Experiment 3, 140 μg of RCM collagen were incubated with 58 units of enzyme at 25° for 120 min. Incubation conditions were as described in the text. Boiled samples were heated to 100° for 10 min and then cooled to 25° immediately before adding to the incubation system.

<table>
<thead>
<tr>
<th>Collagen substrate</th>
<th>Hydroxyproline</th>
<th>Before incubation</th>
<th>After incubation</th>
<th>Net synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg</td>
<td>μg</td>
<td>μg</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl-solubilized</td>
<td>4.4</td>
<td>5.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Pepsin-solubilized</td>
<td>5.6</td>
<td>5.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>RCM, boiled</td>
<td>14.4</td>
<td>13.0</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl-solubilized, boiled</td>
<td>7.8</td>
<td>20.6</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>Pepsin-solubilized, boiled</td>
<td>10.8</td>
<td>22.8</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>RCM, boiled</td>
<td>8.3</td>
<td>32.2</td>
<td>23.9</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCM</td>
<td>3.9</td>
<td>6.2</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>RCM, boiled</td>
<td>4.2</td>
<td>12.9</td>
<td>8.7</td>
<td></td>
</tr>
</tbody>
</table>

**Table II**
Maximal hydroxylation of denatured RCM collagen

Denatured RCM collagen (114 μg) was incubated with 50 units of protocollagen proline hydroxylase at 37° for the times indicated. After incubation of Sample D for 120 min, another 50 units of enzyme together with 4 μmoles of α-ketoglutarate, 16 μmoles of ascorbic acid, and 0.32 μmole of FeSO₄ were added, and the incubation was continued for an additional 60 min.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation time</th>
<th>Hydroxyproline</th>
<th>Residues of hydroxyproline per 1000 residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>2.78</td>
<td>19</td>
</tr>
<tr>
<td>B</td>
<td>60</td>
<td>11.8</td>
<td>81</td>
</tr>
<tr>
<td>C</td>
<td>120</td>
<td>11.2</td>
<td>77</td>
</tr>
<tr>
<td>D</td>
<td>180</td>
<td>11.6</td>
<td>80</td>
</tr>
</tbody>
</table>

* Calculated value based on assumption of 19 residues of hydroxyproline per 1000 residues of amino acids in cuticle collagen from Ascaris (2).

**Fig. 1.** Time course for the hydroxylation of denatured RCM collagen. Boiled RCM collagen, 140 μg, was incubated with 20 units of protocollagen proline hydroxylase at 37° for the times indicated. Other conditions were as described in text.

**Fig. 2.** Effect of substrate concentration on initial velocity for the hydroxylation of denatured RCM collagen. The enzymatic system contained 17 units of enzyme, and the incubations were performed at 37°. In order to obtain initial velocities with low concentrations of substrate, the system was incubated for 20 and 20 min, and the initial velocity was calculated as the mean of the two observed values (see text and Fig. 1). In order to obtain initial velocities with high concentrations of substrate, the system was incubated for 60 and 90 min, and the initial velocity was calculated as the mean of the two observed values.
collagen. Incubating varying amounts of RCM collagen with the enzymatic system gave Michaelis-Menten kinetics for the reaction (Fig. 2), and double reciprocal plots were linear. The apparent $K_m$ value for the substrate was about $10 \mu g$ per ml. A $K_m$ value of $110$ to $125 \mu g$ per ml was observed under the same conditions with the preparation of (Pro-Gly-Pro)$_n$. (12). The maximal velocity for the denatured RCM collagen was about $15 \mu g$ of hydroxyproline per hour, and under the same conditions a maximal velocity of about $29 \mu g$ per hour was observed with the preparation of (Pro-Gly-Pro)$_n$.

In separate experiments (not shown), it was found that no hydroxyproline was synthesized with denatured RCM collagen as the substrate of $\alpha$-ketoglutarate, ascorbic acid, or ferrous iron hydroxyproline was synthesized with denatured RCM collagen.

**Relationship between Helical Conformation and Substrate Activity**—As reported by Harrington and his collaborators, and the $[\alpha]_D^{25}$ of NaCl-solubilized cuticle collagen is about $-420^\circ$ and comparable to that of native collagen from vertebrates (2), and the specific optical rotation of RCM subunits is about $75%$ the value of the NaCl-solubilized collagen (5, 6). Heat denaturation of the two preparations at $75^\circ$ for 30 min reduces the optical rotation to the same final value. After cooling, the specific optical rotation of denatured NaCl-solubilized collagen returns to $90%$ of the initial value in 1 hour, but RCM collagen renatures at a much slower rate.

In order to relate the conformation of the different collagen preparations to their substrate activity, the rate of hydroxylation of native and boiled pepsin-solubilized collagen and of native and boiled RCM collagen were compared. After the pepsin-solubilized collagen was boiled for 10 min, the $[\alpha]_D^{25}$ decreased from $-432^\circ$ to less than $-250^\circ$ (Fig. 3). At room temperature, the $[\alpha]_D^{25}$ returned to a plateau value of $-320^\circ$ in 30 min. Native RCM collagen had an initial $[\alpha]_D^{25}$ value of $-316^\circ$, and after the collagen had boiled for 10 min this value decreased to $-200^\circ$. After storage of the collagen at $4^\circ$ for 4 weeks, the $[\alpha]_D^{25}$ returned to $-318^\circ$ (Table III).

In order to compare the conformation of the various collagen preparations, the native pepsin-solubilized collagen was assumed to have $100%$ of the initial helicity of the collagen, and the denatured RCM collagen had $0%$ helix. The relative rate of hydroxylation was calculated on the basis that the rate of hydroxylation of denatured RCM collagen at either $25^\circ$ or $37^\circ$ was $100%$.

**TABLE III**

<table>
<thead>
<tr>
<th>Collagen substrate</th>
<th>$-[\alpha]_D^{25}$</th>
<th>Rate of hydroxyproline synthesis</th>
<th>$Relative helicity$</th>
<th>Relative rate of hydroxyproline synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCN, denatured</td>
<td>209</td>
<td>7.8</td>
<td>68.5</td>
<td>100</td>
</tr>
<tr>
<td>RCN, untreated</td>
<td>316</td>
<td>3.7</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td>RCN, renatured</td>
<td>318</td>
<td>3.7</td>
<td>51</td>
<td>47</td>
</tr>
<tr>
<td>Pepsin-solubilized, renatured</td>
<td>326</td>
<td>30.4</td>
<td>54</td>
<td>44</td>
</tr>
<tr>
<td>Pepsin-solubilized, untreated</td>
<td>432</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

$[\alpha]_D^{25}$ values were measured as described in Fig. 3. In order to measure the rate of hydroxylation at $25^\circ$, $140 \mu g$ of RCM collagen were incubated with 58 units of enzyme for 60 or 90 min. The rate of synthesis was linear for 90 min, and the two observed values for micrograms of hydroxyproline synthesized were then used to calculate the micrograms of hydroxyproline synthesized per hour per 100 units of enzyme. For measurement of the rate of hydroxylation at $37^\circ$, $140 \mu g$ of RCM or pepsin-solubilized collagen were incubated with 21 units of enzyme for 30 or 60 min. The two observed values for micrograms of hydroxyproline synthesized were then used to calculate the micrograms of hydroxyproline synthesized per hour per 100 units of enzyme.

![Fig. 3. Specific optical rotation of pepsin-solubilized and RCM collagen. $[\alpha]_D^{25}$ values were measured with collagen concentrations of 700 $\mu g$ per ml in 0.5 M KCl. Samples were heated at 100$^\circ$ for 10 min and cooled to room temperature in a water bath. ○——○, pepsin-solubilized collagen; O——O, RCM collagen.](http://www.jbc.org/)

**DISCUSSION**

The enzyme which hydroxylates the proline in protocollagen has been found in a variety of tissues (7, 9, 14), and it has been purified from chick embryos (9, 12) and subsequently from newborn rat skin (15). The enzyme requires as cofactors or cosub-
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article atmospheric oxygen, ferrous iron, a ketoglutarate, and ascorbate (see Reference 7). The enzyme will not hydroxylate free proline or proline in tripeptides (7), and it is strongly inhibited by poly-L-proline Form II but not by poly-L-proline Form I (16). The polypeptide substrates for the enzyme used in previous studies have been biologically synthesized protocollagen with a molecular weight of 100,000 or greater (7), or polypeptides of the structure (Gly-X-Pro), in which “X” is proline (19) or alanine (10) but not glycine. The results obtained with denatured Ascaris cuticle collagen indicate a third type of substrate for the enzyme. The cofactors or co-substrates required for the hydroxylolation of proline in cuticle collagen by the chick embryo enzyme were the same as those required for the hydroxylation of vertebrate protocollagen or synthetic polypeptides. The maximal rate of hydroxyproline synthesis was less with the denatured Ascaris collagen than with (Pro-Gly-Pro), under the same conditions, but the K₉ value observed with the denatured Ascaris collagen was considerably less than the K₉ value for the preparation of (Pro-Gly-Pro),.

Incubation of denatured cuticle collagen with excess enzyme for prolonged periods of time indicated that the collagen could be hydroxylated from a hydroxyproline content of 19 residues per 1000 to a final content of about 80 residues per 1000 residues, a value comparable to the hydroxyproline content of vertebrate or cuticle collagen. The initial proline content of Ascaris cuticle collagen is 290 residues per 1000 (2); therefore, even after maximal hydroxylation of proline in cuticle collagen by the chick embryo hydroxylation, the proline content is still about 230 residues per 1000, or considerably greater than the proline content of vertebrate collagen. The initial hydroxyproline synthesis was less with the denatured Ascaris collagen than with (Pro-Gly-Pro), under the same conditions, but the K₉ value observed with the denatured Ascaris collagen was considerably less than the K₉ value for the preparation of (Pro-Gly-Pro),.

Native NaCl-solubilized and native pepsin-solubilized collagen from Ascaris cuticle collagen do not serve as substrates for the chick embryo hydroxylase. In fact, the substrate activity of various forms of pepsin-solubilized collagen and RCM collagen appeared to be inversely proportional to the relative degree of native conformation as measured by specific optical rotation. The results with Ascaris cuticle collagen are in marked contrast to results obtained with protocollagen from chick embryos (10, 20) or calf skin (11) where no marked difference in substrate activity has been observed between native and denatured preparations. The ready hydroxylation of both native and denatured protocollagen from vertebrates indicates that the triple helical structure of native forms of these collagens neither interferes with the enzymatic reaction nor is essential for it. As noted above, however, the studies with poly-L-proline suggest some conformational require-
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