In Vitro Enzymatic Hydroxylation of Prolyl Residues in the \( \alpha 1\)-CB2 Fragment of Rat Collagen*

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SUMMARY

Two 36-amino acid-containing peptides (\( \alpha 1\)-CB2), isolated from rat skin and tendon collagen by cleavage of the \( \alpha 1\) chain with CNBr, were subjected to enzymatic hydroxylation with collagen proline hydroxylase. The peptides differed initially only in that the ratio of hydroxyproline to proline was considerably lower in the tendon peptide, although the imino acid content of the two peptides was the same. In vitro hydroxylation produced a 75% increase in the hydroxyproline content of the tendon peptide but only an 8% increase in hydroxyproline in the shin peptide. These findings provide direct confirmation of the suggestion, made in an earlier study, that intracellular factors other than the sequences of collagen chains in shin and tendon account for the differences in the degree of hydroxylation of susceptible prolyl residues. Intact collagen preparations from various sources were similarly shown to be underhydroxylated.

The distribution of newly formed hydroxyproline in \( \alpha 1\)-CB2 was investigated by analysis of chymotryptic fragments. Hydroxylation was largely limited to one of these fragments as predicted if hydroxylation occurred only at position 3 of the collagen triplet (Gly-X-Y), i.e., at prolyl residues followed by glycine. This finding is in agreement with sequence analyses of vertebrate collagens and with enzymatic studies using synthetic peptides.

Experimental Procedure

Materials—\( \alpha \)-Ketoglutarate-\( 1^{14} \text{C} \) was obtained from Nuclear-Chicago Corporation and New England Nuclear Corporation. This material was diluted with unlabeled \( \alpha \)-ketoglutarate to different specific activities ranging from 0.2 to 2 \( \mu \)Ci per \( \mu \)mole. \( \alpha \)-Chymotrypsin (3 times crystallized) was purchased from Worthington Biochemical Corporation. Collagen preparations from rat skin, carp swim bladder, dogfish skin, rabbit skin, chick skin, and guinea pig skin were kindly provided by Dr. Karl Piez, National Institute of Dental Research.

Methods—Rat tail tendon collagen and the \( \alpha 1\)-CB2 peptides from rat skin and tail tendon collagens were prepared as previously described (3).

Enzymatic hydroxylation was performed with collagen proline hydroxylase prepared from newborn rat skin (4). Enzyme purified through the DEAE-Sephadex step (approximately 7% pure) was used in all experiments. The standard hydroxylation system contained the following components in a volume of 1 ml:

1. Collagen (0.1 mg)
2. Enzyme (10 units)
3. \( \alpha \)-Ketoglutarate (100 mM)
4. 

\( \alpha \)-Ketoglutarate-\( 1^{14} \text{C} \) (100 \( \mu \)Ci)

Hydroxyproline in collagen is formed by enzymatic hydroxylation of certain prolyl residues in peptide linkage (see Reference 2 for review). This conversion is incomplete at individual sites as demonstrated by amino acid sequence analyses of collagen. In studies on a 36-amino acid containing peptide (\( \alpha 1\)-CB2) produced by CNBr cleavage of the \( \alpha 1\) chain, Bornstein (3) observed that a mixture of proline and hydroxyproline was obtained whenever an imino acid occurred in position 3 of the collagen triplet (Gly-X-Y). Hydroxylation of these residues was reproducibly incomplete and was not influenced by factors such as the age of the animal or iron deficiency. However, the proportions of the two imino acids varied as a function of the tissue from which the collagen was derived. Rat skin \( \alpha 1\)-CB2 contained substantially more hydroxyproline than rat tail tendon \( \alpha 1\)-CB2 although the primary structures of the two peptides were otherwise identical. Data from that study, summarized in Table 1, showed that the increased hydroxyproline content of skin \( \alpha 1\)-CB2 resulted from an increased degree of hydroxylation of certain prolyl residues in the peptide. These findings suggested that factors other than the primary structure of collagen limited the extent of hydroxylation in tendon.

The present study was undertaken to investigate the phenomenon of incomplete hydroxylation using partially purified collagen proline hydroxylase to further hydroxylate collagen substrates. By examining the hydroxylation of \( \alpha 1\)-CB2, a collagen peptide of known amino acid sequence, it was also possible to confirm the substrate specificity of proline hydroxylase suggested by studies with synthetic peptides.

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The precipitate was removed by centrifugation at 68,000 x g. After standing at 0° for 30 min the mixture was centrifuged at 0° for 30 min at 68,000 x g. The precipitate was removed by the addition of trichloroacetic acid to a final concentration of 5%. After standing at 0° for 30 min the mixture was centrifuged at 0° for 30 min at 68,000 x g. The precipitate was reisolated as follows. The reaction was terminated by the addition of trichloroacetic acid, and the wash after centrifugation was combined with the first supernatant fraction. The solution was extracted with 1 volume of diethyl ether to remove trichloroacetic acid and then concentrated by lyophilization. In a preliminary experiment the recovery of al-CB2 subjected to this fractionation was 83%. The partially purified peptide was chromatographed on a column of Sephadex G-50 (2 x 95 cm; Pharmacia) equilibrated with 0.2 M NaHCO₃, pH 7.9, and eluted under gravity at a flow rate of 50 ml per hour. The effluent was monitored at 230 nm with a Gilford recording spectrophotometer equipped with a flow cell. The absorbance peak corresponding in position of elution to a control preparation of al-CB2 was pooled and the peptide separated from buffer salts by lyophilization.

Control and hydroxylase preparations of al-CB2 were treated with a-chymotrypsin at 23° for 4 hours at a substrate to enzyme ratio of 50:1 (by weight) in 0.2 M NaHCO₃, pH 7.8, containing 1 mM CaCl₂. The two chymotryptic fragments were separated by Dowex 1-X2 chromatography by means of a previously described system (6).

Amino acid analyses were performed on a Beckman 120 C analyzer modified for accelerated single column gradient elution (7) and equipped with a range card for high sensitivity. For each determination 10 to 20 nmole of peptide were used. Peptides were hydrolyzed under reduced pressure in doubly distilled constant boiling HCl at 110° for 24 hours. A correction of 12% was applied for hydrolytic loss of serine. Values presented for glutamic acid include glutamine.

**RESULTS**

The experiment summarized in Table II demonstrates that al-CB2 from rat tail tendon can serve as a substrate for collagen proline hydroxylase. Increasing amounts of enzyme were used in the hydroxylating system in an attempt to attain maximal hydroxylation. The production of ¹⁴CO₂ from a-ketoglutarate-¹⁴C as previously described system (6).

- Data of Bornstein (3). al-CB2 comprises residues 16 to 51 from the NH₂-terminus of the al chain of rat skin collagen.
- Hse, homoserine.
- * Values in these columns were measured directly.
- * Determined by amino acid analysis.

**Table II**

<table>
<thead>
<tr>
<th>Enzyme concentration (mg/ml)</th>
<th>¹⁴CO₂ Assay</th>
<th>Pyrrole assay</th>
<th>Total hydroxyproline</th>
<th>New hydroxyproline</th>
<th>Total hydroxyproline</th>
<th>New hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>29.1</td>
<td>0</td>
<td>31.0</td>
<td>0</td>
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<tr>
<td>0.1</td>
<td>10.9</td>
<td>39.0</td>
<td>40.0</td>
<td>10.0</td>
<td>50.0</td>
<td>10.0</td>
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<tr>
<td>0.3</td>
<td>14.9</td>
<td>44.0</td>
<td>57.9</td>
<td>14.5</td>
<td>72.4</td>
<td>14.5</td>
</tr>
<tr>
<td>0.6</td>
<td>16.0</td>
<td>45.1</td>
<td>61.1</td>
<td>17.0</td>
<td>78.1</td>
<td>17.0</td>
</tr>
</tbody>
</table>

* 11.7 nmole of al-CB2 were used. Incubation was for 1 hour. Specific activity of a-ketoglutarate-¹⁴C was 1.8 μCi per pmole.
* Values in these columns were measured directly.
* The first value in this column was obtained by amino acid analysis of the unreacted peptide, and subsequent values for total hydroxyproline were derived by adding to this the amount of newly formed hydroxyproline.

Table I

**Sequence of al-CB2 and degree of hydroxylation at individual positions**

- Gly-Ala-Hyp-Gly-Pro-Gln-Gly-Phe-Gln-Gly-Pro-Hyp-15
- Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Ala-Ser-Gly-Pro-Hse-4OH 25
- Total 4.9 (4.9) d
- 3.1 (3.0) d

Hydroxyproline content

<table>
<thead>
<tr>
<th>Position</th>
<th>Hydroxyproline contenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Tendon</td>
</tr>
</tbody>
</table>

It was identical with the skin peptide. An experiment was carried out in which both peptides were subjected to hydroxylation under the same conditions (Table III). The hydroxyproline content of tendon al-CB2 increased approximately 75% compared to only 8% for the skin peptide. In both cases the hydroxyproline content approached, but did not exceed, the theoretical maximum of 6 residues per peptide, based on the proposed specificity of proline hydroxylase (9-15).

Evidence that proline hydroxylase acts in vitro on only those prolyl residues followed by glycine (i.e. prolyl residues occupying position 3 of the collagen triplet) has come solely from studies with synthetic collagen-like peptides. In order to determine the specificity of proline hydroxylase on a natural collagen substrate...
Hydroxylation of \( \alpha \)-CB2 Vol. 246, No. 13

same increase would be observed in Cl as in C2 since the two random hydroxylation, on the other hand, approximately the in vitro hydroxylation is limited to position 3, one would expect chymotryptic peptides contain equal numbers of prolyl residues as C2, although both fragments contain 3 imino acid residues 1-18 and 19-36, were not separated from Cl and C2 by

and to rule out the possibility of random hydroxylation (i.e. at both positions 2 and 3) the distribution of newly formed hydroxyproline in \( \alpha \)-CB2 was examined.

Chymotrypsin cleaves \( \alpha \)-CB2 into two fragments, C1 (residues 1 to 20) and C2 (residues 21 to 36) (3). As shown in Table I, C1 from tendon \( \alpha \)-CB2 contains 3 to 4 times as much hydroxyproline as C2, although both fragments contain 3 imino acid residues in position 3 of the collagen triplet. Consequently, if \textit{in vitro} hydroxylation is limited to position 3, one would expect to find substantially more hydroxyproline formed in C2. With random hydroxylation, on the other hand, approximately the same increase would be observed in C1 as in C2 since the two chymotryptic peptides contain equal numbers of prolyl residues (4.6 in C1 and 4.3 in C2).

In Table IV the data for proline and hydroxyproline are tabulated as degree of hydroxylation (hydroxyproline per total imino acid). Hydroxylation in C1 increased from 31 to 38%, approach-
Degree of hydroxylation of prolyl residues in rat tail tendon α1-CB2 before and after hydroxylation with proline hydroxylase

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of hydroxylation</th>
<th>C1 (1 to 20)</th>
<th>C2 (21 to 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After hydroxylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theoretical maximum*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Assuming that hydroxylation takes place only at prolyl residues followed by glycyl residues. In C1 there are three such prolyl residues in a total of seven and in C2, three in a total of five.

TABLE VI

Further hydroxylation by proline hydroxylase of denatured collagen from various sources

<table>
<thead>
<tr>
<th>Source</th>
<th>New hydroxyproline</th>
<th>Increase</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmole/50 μg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat tail tendon</td>
<td></td>
<td>5.6</td>
<td>12.0</td>
</tr>
<tr>
<td>Rat tail tendond*</td>
<td></td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Experiment 2*</td>
<td></td>
<td>8.3</td>
<td>15.5</td>
</tr>
<tr>
<td>Calf skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 3*</td>
<td></td>
<td>5.4</td>
<td>11.6</td>
</tr>
<tr>
<td>Rat skin</td>
<td></td>
<td>4.5</td>
<td>9.5</td>
</tr>
<tr>
<td>Carp swim bladder</td>
<td></td>
<td>4.8</td>
<td>12.3</td>
</tr>
<tr>
<td>Dogfish skin</td>
<td></td>
<td>2.9</td>
<td>9.4</td>
</tr>
<tr>
<td>Rabbit skin</td>
<td></td>
<td>3.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Chick skin</td>
<td></td>
<td>2.9</td>
<td>5.3</td>
</tr>
<tr>
<td>Guinea pig skin</td>
<td></td>
<td>2.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Enzyme concentration, 0.25 mg per ml; collagen concentration, 25 μg per ml; incubation period, 1 hour; specific activity of α-ketoglutarate-1-14C, 0.2 μCi per μmole.
\* Not denatured before hydroxylation.

The results of several investigations have indicated that vertebrate collagen proline hydroxylases recognize only those prolyl residues in position 3 of the collagen triplet. Kivirikko and Prockop (9) and Hutton et al. (10), using enzyme from chick embryo and labeled repeating tripeptides of the form (Pro\*-Gly-Pro)ₙ, demonstrated that hydroxylation occurred at the labeled residue. A similar conclusion was reached by Norvid and Pfab (11) with calf embryo skin enzyme, but in addition, these authors showed that (Pro-Gly-Hyp-Pro)ₙ did not yield 14C-hydroxyproline. Hutton, Kaplan, and Udenfriend (12) found that when biologically prepared hydroxyproline-deficient substrate (protocollagen) was subjected to enzymatic hydroxylation in vitro, 37% of the hydroxyproline could be recovered as Gly-Pro-Hyp. The polytripeptide (Gly-Ala-Pro)ₙ was shown to be a substrate of proline hydroxylase from chick embryo by Kivirikko, Bright, and Prockop (13), and in a more recent study from the same laboratory it was reported that (Ala-Pro-Gly)ₙ was hydroxylated more than 10 times as rapidly as (Pro-Gly-Gly-Pro)ₙ (14). In an earlier report from this laboratory (15), it was shown that proline hydroxylase from newborn rat skin catalyzed the hydroxylation of only one of three prolyl residues in bradykinin (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg), the one located in position 3. The fact that a bradykinin derivative containing 3,4-H-proline in position 2 yielded no \(^3\)H upon treatment with enzyme ruled out even low levels of hydroxylation at this position. A recent study by McGee, Rhoads, and Udenfriend (16) demonstrated that while the bradykinin molecule

1 Standard deviation.
could be hydroxylated at position 3, analogues containing either alanine or sarcosine (N-methylglycine) in place of glycine were completely inactive as substrates. All of these results are consistent with the fact that hydroxyproline has been found only in position 3 in all vertebrate collagens examined (19).

Collagen from earthworm cuticle, on the other hand, contains hydroxyproline in both positions 2 and 3 (20). Such a finding had been predicted by Rao and Harrington (21) on the basis of the thermal stability of this collagen. The difference between earthworm and vertebrate collagens can be accounted for by the specificity of earthworm proline hydroxylase. Nordwig and Pfab (11) treated \(^{14}\text{C-Pro-Gly-Pro}\) and \(^{14}\text{C-Pro-Gly-\text{Pro}}\) with earthworm hydroxylase and found \(^{14}\text{C} \) hydroxyproline produced in both cases.

Our experiments with a collagen peptide containing a variety of different amino acids confirm the substrate specificity of collagen proline hydroxylase suggested by experiments with synthetic repeating tripeptides and bradykinin. The hydroxyproline content of \(\alpha 1\)-CB2 after hydroxylation was always slightly less than the number of imino acid residues in position 3. The reaction proceeded to about 85% completion in spite of very high enzyme concentrations. This behavior suggests that the ease with which a given site is hydroxylated depends on whether neighboring sites are already hydroxylated. Juva and Prockop (22) arrived at the same conclusion by means of another line of evidence. These authors studied the formation of complexes between proline hydroxylase and large polypeptide substrates and found that the affinity of the enzyme for substrate decreased after partial hydroxylation had occurred. A complex could no longer be demonstrated after approximately one-sixth of the susceptible prolyl residues had been hydroxylated.

Our experiments with intact collagen indicate that, although susceptible prolyl residues are present, they are not accessible to the enzyme unless the collagen is thermally denatured. Kikuchi, Fujimoto, and Tamiya (23), using synthetic polymers of the type \((\text{Pro-Pro-Gly})\), \(n = 1 \) to 20, also reached the conclusion that triple helices are poor substrates compared to single chains. Similarly, in the case of \(\text{Ascaris} \) collagen, a protein abnormally low in hydroxyproline, \(\text{in vivo} \) hydroxylation cannot occur unless tertiary structure is disrupted (24, 5). Heat treatment of protocollagen, on the other hand, does not increase its substrate activity and in fact usually decreases it, as several laboratories have reported (13, 28, 27). In none of these studies, however, was it demonstrated that the protocollagen which was used existed in a triple helical conformation before denaturation. These considerations suggest that proline hydroxylase completes its function in \(\text{in vivo} \) before collagen chains achieve appreciable tertiary structure.

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