Sequence Position of 3-Hydroxyproline in Basement Membrane Collagen

ISOLATION OF GLYCYL-3-HYDROXYPROLYL-4-HYDROXYPROLINE FROM SWINE KIDNEY*

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SUMMARY

The position of 3-hydroxyproline was investigated in the triplet sequences of peptides released by collagenase digestion of a collagen preparation from kidney cortex. Composition of the collagen preparation indicated that it was largely or wholly of basement membrane origin. 3-Hydroxyproline was detected in only one sequence, the tripeptide, glycyl-3-hydroxyprolyl-4-hydroxyproline, which accounted for a major fraction of the total 3-hydroxyproline obtained in the peptides released by collagenase. Preliminary data, based on sequencing the peptide mixture released by collagenase treatment, suggested that, in contrast, 4-hydroxyproline occurs predominantly if not exclusively in the Y position of Gly-X-Y triplet sequences in the collagen preparation studied.

In the vertebrate collagens so far studied, both sequence analysis (1) and the substrate specificity of several vertebrate hydroxylases (2-4) lead to the generalization that 4-hydroxyproline is found only in the Y position of the typical collagen triplet, Gly-X-Y. To date, the only well established exception is the collagen of earthworm cuticle, from which the individual tripeptides, Gly-Hyp1-Ala (5) and Gly-Hyp-Ser (6), have been isolated, and in which sequence analysis of a mixture of collagenase-released peptides suggested that much, and possibly all, of the 4-hydroxyproline is in the X position (5).

In contrast, 3-hydroxyproline has been located only in the sequence Gly-3-Hyp-4-Hyp, which occurs once in the α-1 chain of mammalian skin collagen (7); it is a reasonable supposition that the same sequence also accounts for the single 3-hydroxyproline residue (per 1000 residues) of a variety of other vertebrate interstitial collagens (8).

It was of interest to examine the position of 3-hydroxyproline in basement membrane collagen, since all forms of this collagen type studied contain much higher levels of 3-hydroxyproline (9) than do any of the other collagen types (10) so far described. If the much more abundant 3-hydroxyproline of basement membrane collagen were also limited to the same sequence found in interstitial collagen, this would imply severe restrictions on the specificity of proline hydroxylation in position 3 compared with 4-hydroxylation.

Our approach was to obtain a preparation of collagen from kidney cortex by a simple, high yield procedure (11); the reported composition of this preparation indicated that it is very largely, if not entirely, basement membrane collagen (see "Discussion"), as expected from the histological appearance of mammalian kidney cortex. In any case, for the question at issue, any collagen preparation high in 3-hydroxyproline was suitable.

Collagenase digestion of such a preparation should yield largely tripeptides N-terminal in glycine (12) and the resulting peptide fraction could then be examined for the occurrence and quantity of the sequence of interest. This paper describes the isolation of Gly-3-Hyp-4-Hyp from such a peptide mixture, as well as certain technical problems encountered in sequencing this tripeptide. 3-Hydroxyproline was detected only in this peptide and its recovery represented about two-thirds of the total 3-hydroxyproline obtained in the small peptides resulting from collagenase digestion.

MATERIALS AND METHODS

Special Compounds and Peptides—trans-3-Hydroxy-L-proline was isolated as a crystalline compound from the seeds of Delonix regia, a tropical legume, by a modification of the method of Sung and Fowden (18). The reference tripeptides, Gly-3-Hyp-4-Hyp and Gly-4-Hyp-3-Hyp, were synthesized in this laboratory and will be described separately. Gly-3-Hyp-4-Hyp was prepared using 4-[14C]hydroxy-L-proline (New England Nuclear Corp.); specific activity of the labeled tripeptide was 95,000 dpm per μmol. Gly-Ser-4-Hyp and Gly-4-Hyp-Ser were obtained from Fox Chemical Co., Los Angeles, Calif., and checked as noted earlier (6); Gly-4-Hyp-Glu was purchased from the same vendor. Phenylisothiocyanate was obtained from the Pierce Chemical Co. and was used without further purification; other reagents and solvents used in sequencing were purchased as the purest grade available, liquids were redistilled before use. The PTH derivatives of glycine and 4-hydroxyproline were purchased from Pierce. These and the PTH derivative of 3-hydroxyproline were also prepared in this laboratory by the method of Edman as described by Konigsgen and Hill (14). Collagenase (CLS, Worthington Biochemical Corp.) was purified as described earlier (15) to remove possible
nonspecific proteases. Amino acid analyzer resin (DCI) and Dur-
micro Pico buffers were purchased from the Durrum Chemical Co.

Kidney Collagen—Kidneys were obtained from freshly killed
swine at a local slaughterhouse or from Pel-Freez Bio-Animals,
Inc. (Rogers, Ark.) as frozen material. Collagen was purified from
swine kidney cortex according to the method of Fujimoto (11),
the essential steps being solubilization of tissue with pepsin, re-
moval of non-collagen protein by extraction with sodium hy-
droxide, and repeated salt precipitation of the collagen. Collagen
so prepared was dialyzed against 0.1 M acetic acid and stored at
−15° after lyophilization.

Amino Acid Analysis—Collagen samples and peptides were
hydrolyzed in constant-boiling HCl (1 ml per 100 mg of sample)
in evacuated, sealed ampules, at 100° for 18 to 24 hours. After hy-
drolysis, samples were evaporated to dryness in a 60° bath under a
N₂ stream and were dissolved in 0.01 M HCl for separation on the
analyzer column. Amino acids were determined with the single
column system using Technicon components. The column (0.6 ×
60 cm) was operated at 52° and a flow rate of 0.58 ml per min. To
permit separation of 3-hydroxyproline, 4-hydroxyproline, and
hydroxylysine, the Durrum Pico Buffer System II was modified
as follows. A fourth buffer (A′, pH 2.95), prepared by the addition
of HCl to Pico Buffer A, was used as the first eluant, just preceding
Pico Buffer A. In addition, Pico Buffer C was modified by reducing
the NaCl concentration to 35 g per liter and raising the pH to 4.6
with NaOH. Times for buffer changes were 45, 105, and 165 min;
this four-buffer system constituted the standard method. Trans-3-
Hydroxyproline was eluted at 60 min, trans-4-hydroxyproline at
80 min, and other amino acids were eluted in the same order as in
the original Pico Buffer System II.

Sequence Methods—Sequencing of a mixture of small peptides
separated from collagenase digests of collagen was carried out by
the subtractive Edman procedure (14), extracting the 5-thiolicino-
none derivatives with ethyl acetate.

Sequence determination of the isolated tripeptide described be-
low was carried out by two different methods. One was that of
Peterson et al. (16), involving the use of trifluoroacetic acid for the
cleavage step; the second was a modification of this procedure in
which 3 N HCl was used in place of trifluoroacetic acid. Attempts to
regenerate each amino acid from its PTH derivative, after extract-
tion into butyl acetate, were carried out by heating samples of 0.5
μmol of the PTH derivative with 50 μl of HI in a sealed evacuated
ampule for 24 hours at 125° in an autoclave. Dansyl derivatives of
glycine, 4-hydroxyproline, and 3-hydroxyproline were prepared by
the procedure of Gray (17).

RESULTS

Composition of Collagen—The amino acid composition of purified kidney collagen is shown in Table I, together with the composition of the peptide fraction solubilized by collagenase
digestion (see below). The analyses are in general agreement with those of Fujimoto (11), but include a value for 3-hydroxy-
proline, an amino acid which he did not report. It is notable
that the amino acid composition of this preparation closely
resembles that of basement membrane collagen from isolated
glomeruli (18, 19), and differs from vertebrate interstitial collagens
in the relatively high values for 3-hydroxyproline, 4-hydroxy-
proline, hydroxylysine, and branched-chain amino acids, and in the
relatively low values for proline and alanine.

Digestion of Collagen by Collagenase—Samples of purified
collagen were denatured by heating at 55° for 30 min in Tris-
HCl (0.05 M in Tris, pH 7.6) containing 0.005 M CaCl₂ approxi-
mately 1.7 ml of this solution were used per mg of collagen. The
collagen solution was cooled to 37° and collagenase was added in a solution of 1 mg of enzyme per ml of Tris-CaCl₂
buffer. Approximately 1 mg of collagenase was added per 5 mg
of collagen. The digestion mixture was incubated for 90 min at
37°. Three volumes of absolute ethanol were added and the
mixture was chilled in an ice bath for 30 min before centrifuga-

Table I
Composition of kidney cortex collagen and collagenase-released peptides

| Composition of initial collagen | Collagenase released peptides |
|--------------------------------|-----------------------------|
| Gly 208                       | 324                         |
| Ala 57                        | 66                          |
| 1/2-Cys 7                     | N.D.                        |
| Val 24                        | 20                          |
| Met 12                        | 7                           |
| Ile 19                        | 18                          |
| Leu 45                        | 45                          |
| Tyr 6                         | 7                           |
| Phe 33                        | 20                          |
| Hyl 35                        | 35                          |
| Lys 13                        | 12                          |
| His 7                         | 10                          |
| Arg 39                        | 38                          |
| 3-Hyp 16                      | 20                          |
| 4-Hyp 121                     | 116                         |
| Asp 55                        | 47                          |
| Thr 20                        | 20                          |
| Ser 36                        | 42                          |
| Glu 60                        | 70                          |
| Pro 82                        | 79                          |
| 3-HYP 16                      | 20                          |
| 4-HYP 116                     | 116                         |
| Asp 55                        | 47                          |
| Thr 20                        | 20                          |
| Ser 36                        | 42                          |
| Glu 60                        | 70                          |
| Pro 82                        | 79                          |

* N.D., not detected.

At 18,000 × g for 15 min. The precipitate was washed with
30 ml of absolute ethanol and was then discarded since it con-
tained only negligible 4-hydroxyproline as determined colori-
metrically (20) on hydrolysates. Analysis of the total peptide
fraction (ethanol solution plus wash) is given in Table I. Its
composition corresponded closely to the initial collagen prepara-
tion, although the higher value for glycine suggests the presence
of some noncollagen protein in the initial preparation.

Sequence Data on Mixed Peptides—Attempts to obtain gross
information on the distribution of 3-hydroxyproline and 4-hy-
droxyproline were based on sequencing trials of the unfrac-
tionated peptide mixture obtained as described above. In con-
trast to earlier studies by this technique, both of vertebrate
collagen (21, 22) and earthworm cuticle collagen (5), unam-
biguous data were not obtained for the kidney collagen peptide
preparation. In three trials, the subtractive Edman procedure
resulted in an average loss of 46% of the initial residues at the
first step; as expected, most of the glycine was lost at this step,
but, unexpectedly, essentially all the hydroxylysine was also
lost. This prevented unequivocal assignment of either 3-hy-
droxyproline or 4-hydroxyproline to the X or the Y positions
since 40% of the initial 3-hydroxyproline and 34% of the initial
4-hydroxyproline were also lost at the first step. However, in the
subsequent two subtractive steps, 80% of the remaining 3-hy-
droxyproline was lost in Step 2, while 87% of the remaining
4-hydroxyproline was lost in Step 3.

Ion Exchange Chromatography of Small Peptide Fraction—To
examine the distribution of 3-hydroxyproline in individual pep-
tides, a sample of collagen (230 mg) was digested and treated
with collagenase exactly as described above. The ethanol-soluble

1 Durrum Pico Buffer System Instruction Manual, Durrum
Chemical Corp., 1971, Palo Alto, Calif.
peptides were then subjected to further fractionation as follows. The ethanol solution and wash were combined, taken to dryness, and dissolved in 16 ml of water; 4-ml aliquots were passed through a polyacrylamide column (Bio-Gel P-2, 100 to 200 mesh, Bio-Rad Laboratories) to remove salt and to fractionate the peptides roughly by size. The column (2.6 × 100 cm, previously calibrated with [U-14C]proline, H2O, several di- and tripeptides, and bradykinin) was eluted with ammonium propionate buffer (0.03 M, pH 4.5) at a flow rate of 1 ml per min at room temperature. Peptides were detected by their absorption at 230 nm. Four separate runs were needed to fractionate the entire sample; corresponding volumes from each run, representing a "small peptide" fraction (eluting in the range between bradykinin and [U-14C]proline), were pooled from each of the runs. This fraction contained most of the 3-hydroxyproline eluted from the column (Table II). Only small quantities of 3-hydroxyproline were measured in hydrolyzed samples from fractions emerging from the column both before and after the small peptide fraction. It should be noted that only the final fractions, which were high in salt, were not analyzed for their 3-hydroxyproline content. Fractions from the P-2 column, which were analyzed, accounted for 70% of the initial 3-hydroxyproline in the original collagen preparation (Table II). A similar proportion of the 4-hydroxyproline and glycine (not shown in Table II) was similarly recovered. Analyses of the residue remaining after collagenase digestion indicated that only negligible 3-hydroxyproline (less than 5% of that in the initial collagen sample) remained with the insoluble material, as was also true for 4-hydroxyproline.

The mixture of small peptides was then fractionated on the amino acid analyzer column, as described for amino acid separation, except that a larger column (0.9 × 60 cm, flow rate 1.12 ml per min) was used. A stream-splitting arrangement diverted 90% of the analyzer column output to a fraction collector while 10% passed through the autoanalyzer-recorder system. The recorder peaks and the collected samples could then be correlated, and desired fractions pooled, desalted (Bio-Gel P-2, as above), hydrolyzed, and analyzed to determine amino acid composition. Because of the limited capacity of the analyzer column, the "small peptide" fraction was divided into several portions, each representing about 65 mg of collagen. Results of each of several runs were identical. One such chromatogram is shown in Fig. 1. Because we were interested primarily in the occurrence of 3-hydroxyproline and 4-hydroxyproline, hydrolysates of each set of pooled fractions (corresponding to the major peaks shown in Fig. 1) were not generally subjected to complete amino acid analysis; instead the analyzer run was terminated after the emergence of glycine. Peak B was of special interest because it alone contained 3-hydroxyproline; it was therefore analyzed completely with the results shown in the legend to Fig. 1. As noted there, the major components of Peak B were glycine, 3-hydroxyproline, and 4-hydroxyproline, but other amino acids were also present.

**Separation of Pure Peptides from Peak B**—Of the 13 peaks analyzed (Fig. 1), only Peak B contained 3-hydroxyproline and represented two-thirds of the 3-hydroxyproline present in the small peptide fraction (Table II). It was therefore of interest to separate the mixture of peptides in Peak B into pure components. A lower pH (pH 2.6) for the first buffer was found to separate completely Peaks B and C, and to partly resolve Peak B into a main peak and shoulder (Fig. 2). Several aliquots of the small peptide fraction were therefore run on the amino acid analyzer column using buffer at the lower pH (prepared by addition of HCl to Pico Buffer A) with the results shown in Fig. 2. Fractions corresponding to the first portion of Peak B (B1, Fig. 2), the second portion of Peak B (B2, Fig. 2), and Peak C were separately pooled, desalted (Bio-Gel P-2, as above), and analyzed. After acid hydrolysis, fractions representing B1 contained glycine, 4-hydroxyproline, 3-hydroxyproline, and glutamic acid in the respective ratios 1:1.02:0.71:0.29; Peak B1 was therefore consistent with two tripeptides containing Gly(3-Hyp, 4-Hyp) and Gly(Glu, 4-Hyp) in the ratio of about 7:3. Fractions corresponding to B2 were desalted, lyophilized, redissolved in a small volume of water, applied in a streak to Whatman No. 3MM paper (previously water-washed), and subjected to descending paper chromatography. The paper was developed for 24 hours at room temperature with n-butyl alcohol-acetic acid-water (4:1:5). Ninhydrin-stained guide strips indicated two well separated bands; one (B1a) moved 11.5 cm from the origin and stained yellow, the other (B1b) moved 8 cm from the origin and stained purple. Elution of corresponding strips of the paper yielded peptides which had the composition shown in Table III.
TABLE III
Composition of peptides isolated from collagenase digest of basement membrane collagen

Peptides Bla and Blb were separated from Peak Bl (Fig. 2) by paper chromatography as described in the text. Peptide C was obtained from the amino acid analyzer column (Fig. 2). All figures are molar equivalents, taking glycine as 1.

| Peptide | Glycine | 3-Hydroxyproline | 4-Hydroxyproline | Glutamic acid | Serine |
|---------|---------|------------------|------------------|---------------|--------|
| Bla     | 1.00    | 1.02             | 0.96             | 0             | 0      |
| Blb     | 1.00    | 0                | 1.00             | 1.15          | 0.97   |
| C       | 1.00    | 0                | 0.91             | 1.00          | 0.97   |

Table III also shows the composition of fractions corresponding to Peak C.

**Sequence of Peptide Bla**—In preparation for sequencing the isolated Peptide Bla, trials were first carried out on synthetic Gly-3-Hyp-4-Hyp, which had been prepared with tritiated 4-hydroxyproline (see "Materials and Methods"). Sequencing by the dansyl-Edman technique (22) verified the presence of NH₂-terminal glycine, but was unreliable in sequencing the last two residues since it was not possible to achieve a satisfactory separation of dansyl derivatives of 3-hydroxyproline and 4-hydroxyproline even with the use of some 25 solvents using both thin layer and polyamide sheets.

Sequencing trials utilizing the synthetic peptide were then carried out by the subtractive Edman method, with the use of trifluoroacetic acid. This technique was disappointing in that, after the removal of glycine, approximately two-thirds of the residual dipetide was recovered as the diketopiperazine of 3-Hyp-4-Hyp. This was determined by fractionating the aqueous residue (following Edman step 1) on the analyzer column; approximately two-thirds of the radioactivity applied to the column emerged without retardation, while the remaining one-third was eluted as a ninhydrin-positive peak at 135 min. Each radioactive peak, on hydrolysis, gave only equimolar 3-hydroxyproline and 4-hydroxyproline, supporting the conclusion that the first peak was cyclic (3-Hyp-4-Hyp) while the retarded peak was the linear dipetide (3-Hyp-4-Hyp).

An additional complication in determining the sequence of this peptide appeared on H₁ hydrolysis of the extracted PTH amino acids obtained at successive steps. By this procedure, the model peptide gave glycine, as expected, after hydrolysis of the first PTH amino acid. Hydrolysis of what should have been PTH-3-hydroxyproline, however, yielded no 3-hydroxyproline but two unidentified peaks, each with a high A₆₇₀/A₄₄₀ ratio (typical of a primary amino acid); one peak eluted in the position of glycine. Hydrolysis of the PTH derivative prepared from free 3-hydroxyproline also yielded two components one of which eluted from the analyzer column in the position of glycine. In contrast, hydrolysis of PTH-4-hydroxyproline under the same conditions yielded equimolar hydroxyproline and allohydroxyproline, the total representing the quantity of free amino acid expected.

Definitive sequence data were obtained, however, by means of the subtractive Edman procedure, when aqueous 3 N HCl was substituted for trifluoroacetic acid. The results, shown in Table IV, indicated the sequence of the naturally occurring peptide to be Gly-3-Hyp-4-Hyp.

As an additional proof of this sequence, Peptide Bla was cochromatographed with a mixture of the two model tripeptides, Gly-4-Hyp-3-Hyp and Gly-3-Hyp-4-(G-³H)Hyp. The two synthetic peptides were well separated on the analyzer column, eluting with the standard buffer system, and appeared at 106 and 118 min, respectively. Peptide Bla coeluted with the radioactive peak of the latter peptide (Fig. 3).

The sequence of Blb was not determined. The amino acid composition (Table II) suggests the possibilities, Gly-4-Hyp-Glu, Gly-4-Hyp-Glu, or one of the two corresponding tripeptides with glutamic acid in place of glutamate. Only the model tripeptide, Gly-4-Hyp-Glu, was available to us for comparison. When this was chromatographed (descending chromatography, 17 hours, Whatman No. 3MM paper, solvent: n-butyl alcohol-acetic acid-water, 5:1:4) the Rₐ of peptide Blb was found to be 0.02 while that of Gly-4-Hyp-Glu was 0.05, indicating their difference.

Peptide C (Fig. 2, Table III) was not sequenced, but was compared, in elution behavior on the analyzer column, with the two synthetic peptides Gly-Ser-4-Hyp and Gly-4-Hyp-Ser. With the standard system these eluted at 2 hours 43 min and 2 hours 23
min, respectively; peptide C coeluted with the former. On this basis the sequence Gly-Ser-4-Hyp is assigned to C.

**DISCUSSION**

Detection of 3-hydroxyproline in only a single peptide, Gly-3-Hyp-4-Hyp, coupled with the good recovery of total 3-hydroxyproline in this peptide (considering the losses expected from such a fractionation procedure) indicates that this is a predominant component. Hyp-FHyp, coupled with the good recovery of total 3-hydroxyproline in this peptide (considering the losses expected from such a sequence in the single isolated sequence by examining other collagens prepared using different methods), shows that it represents very largely basement membrane collagen. This is based on the analytical data of Table I which indicate a position occurrence of the single 3-hydroxyproline per a chain of skin collagen, presumably chiefly of basement membrane origin, and yields sequence data which agree with that found earlier for the position occurrence of the single 3-hydroxyproline per a chain of skin collagen. Our methods could have missed the occurrence of 3-hydroxyproline at very low levels in a variety of other sequences, and it will be of interest to test the possibility of unique sequence determination of the single isolated sequence by examining other collagens rich in 3-hydroxyproline.

While our collagen preparation was derived by a method which does not involve preliminary isolation of morphologically identified glomeruli or renal tubules, we can conclude with some confidence that it represents very largely basement membrane collagen, and particularly, the collagen derived from glomeruli. This is based on the analytical data of Table I which indicate a value for 3-hydroxyproline essentially equal to that reported for a purified population of isolated glomeruli (18, 19); the level of 3-hydroxyproline reported for renal tubular basement membrane is considerably lower than that for glomeruli (25). Any significant contamination of our collagen preparation by interstitial collagen should have appreciably lowered the 3-hydroxyproline value from that observed, since the latter collagen averages only about one residue of 3-hydroxyproline per thousand (8). A similar argument holds for the high hydroxylysine to lysine ratio (Table I) which, in our preparation, exceeds that of common vertebrate interstitial collagens (8) by a factor of about 10.

We should note certain difficulties encountered in our attempts to sequence the peptide Gly-3-Hyp-4-Hyp. First, we were unable to separate the dansyl derivatives of these two position isomers of hydroxyproline; comparable difficulty might be expected in chromatographic separation of the PTH derivatives. Furthermore, coincident with removal of NH2-terminal glycine by the Edman procedure, cyclization of the residual dipeptide, 3-Hyp-4-Hyp, proved troublesome, but was circumvented by using aqueous 3 N HCl in place of trifluoroacetic acid in this step.

In addition, the hydrolysis of PTH-3-Hyp led to unidentified products (one of which might have been glycine) but to no detectable 3-hydroxyproline. Such degradation of PTH-3-Hyp, as a β-hydroxy-α-amino acid, may be compared with destruction of PTH-serine or PTH-threonine on acid hydrolysis (26).

Finally, it is worth calling attention to the curious results obtained on attempting a sequence analysis of the initial peptide mixture. We have no explanation for the loss of almost 50% of the amino acid residues, including virtually all of the hydroxylysine, during the first Edman step. The unusually high content of hydroxylysine, and particularly glycosylated hydroxylysine, characteristic of basement membrane collagen (9) prompts the speculation that an unknown reaction of these residues with the Edman reagent may result in the removal of associated peptides from the aqueous phase. This would account for the large loss of varied residues in addition to the loss of all the hydroxylysine. In contrast, the expected loss of only one-fourth to one-third of the initial residues was observed at this step with peptide mixtures derived from other types of collagen (5, 21, 22) which are low in hydroxylysine.

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