Identification of a Substrate Site for Liver Transglutaminase on the Aminopropeptide of Type III Collagen*

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The aminopropeptide of type III collagen incorporates [3H]putrescine in the presence of liver transglutaminase, and the change in incorporation with concentration indicates one binding site on each of the Mr = 15,000 subunits of the peptide. At low concentrations the incorporation was comparable to that of dimethyl casein and much greater than actin or fibrinogen. Cleavage and Edman degradation of the aminopropeptide identified the major putrescine-binding site as glutamine in position 14. The surrounding amino acid sequence (Leu-Gly-Gln-Ser) shows homology with some synthetic peptide substrates of transglutaminase.

Transglutaminases (EC 2.3.2.13) act on peptide-bound glutamine residues, catalyzing the formation of an isopeptide bond with lysine residues. Although a wide variety of amines such as putrescine or cadaverine may act as acceptor substrates in this reaction, only a limited number of glutamine residues in certain proteins will act as donor substrates (1, 2). Studies with several synthetic peptides which were designed according to donor sequences of β-casein demonstrated that certain amino acid residues adjacent to glutamine are important for enzyme recognition (3, 4). Transglutaminases have a wide distribution in cells and body fluids, but their physiological function has so far been well established only in the cross-linking of fibrin clot during hemostasis, the production of the cornified envelope during terminal differentiation in keratinocytes (1, 2). In addition to these extracellular events there is evidence for several intracellular functions of transglutaminases (5).

A possible role for transglutaminases in the metabolism of connective tissue is suggested by wound healing studies (6–8), the formation of cross-links between collagen α-chains (9), and the cross-linking of fibronectin to specific sites in collagen types I and III (10, 11). The amino acid sequence around the glutamine donor of fibronectin is known (12) but differs substantially from those of synthetic substrates (3, 4). We have recently obtained evidence for a possible transglutaminase-dependent cross-linking of the aminopropeptide of type III collagen during wound healing (8). The amino acid sequence of the aminopropeptide (13) shows in position 12–15 a segment similar to that of a synthetic transglutaminase substrate (3). In the present study, we demonstrate that glutamine 14 is the major site for incorporation of [3H]putrescine in the aminopropeptide III in the presence of liver transglutaminase.

EXPERIMENTAL PROCEDURES

Materials—Bovine and ovine aminopropeptide Col 1–3 (III) of type III collagen, the Col 1 fragment of types I and III procollagen (13, 14), and the globular domain NC1 of type IV collagen (15) were obtained from collagenase digests as described previously. Pepsin-solubilized rat skin collagen (mixture of types I and III) was purchased from Dr. H. Kleinman (National Institute for Dental Research) and was used in heat-denatured (60 °C, 1 h) form to produce α-chains. 131I-Labeled aminopropeptide Col 1–3 (III) was obtained from a radioimmunoassay kit (Behringwerke, Marburg, Federal Republic of Germany). Actin, fibrinogen, ovalbumin, and bovine serum albumin were obtained from Sigma, Hammarsten casein was from The British Drug House. Dimethyl casein (16) and guinea pig liver transglutaminase (17) were prepared by the cited methods. [4,14]H]Putrescine dihydrochloride was from Amersham Corp.

Methods—For analytical experiments 0.2 μCi of [3H]putrescine in 0.1 ml of transglutaminase buffer (0.1 M Tris/HCl, pH 8.3, containing 5 mM CaCl2 and 1 mM dithiothreitol) was mixed in a 1.5-ml Eppendorf centrifuge tube with 40 μg of each potential substrate in 0.2 ml of transglutaminase buffer. 10 μl (about 5 μg of protein) of a 20-fold dilution of the liver transglutaminase preparation were added, and the mixture was incubated at 37 °C for 90 min. An enzyme blank containing all reagents except the substrate was run with each set of tubes. After the incubation, 10 μl of bovine serum albumin in water (0.5 g/100 ml) were added as a carrier followed by 90 μl of 50% trichloroacetic acid. After incubation overnight at 4 °C, precipitates were collected by centrifugation and then washed twice with 1.0 ml of 10% trichloroacetic acid, once with 1 ml of ethanol/ether (1:1) and once with 1 ml of ether. The dry precipitates were dissolved in 50 μl of 1% sodium dodecyl sulfate by boiling for 2 min and used in 25-μl portions for counting after mixing with ACS scintillant (Amersham) and for electrophoresis after mixing with 25 μl of 50% glycerol in electrophoresis buffer containing 0.01% bromphenol blue. For quantitative comparisons the radioactive enzyme blank was subtracted from the radioactivity incorporated into each substrate. The mean ± S.E. for 10 enzyme blanks was 365 ± 22 cpm. Discontinuous polyacrylamide gels (4%/6%) were prepared and run by the procedure of Neville (18). After electrophoresis the gels were fixed in isopropanol/acetic acid/water (25:10:65), treated with Autofluor (National Diagnostics, Somerville, NJ), dried, and subjected to fluorography with Kodak X-Omat paper for 3 days.

For the preparative experiments, liver transglutaminase (0.2 ml) was rechromatographed in transglutaminase buffer on a small (0.6 × 15 cm) Sephadex G-50 column, and the material eluting at Vs was collected. 0.5 ml of this preparation was mixed with 4 mg of Col 1 (III) in 1 ml of transglutaminase buffer and 5 μl (5 μCi) of [3H]putrescine. After incubation for 5 h at 37 °C the mixture was dialyzed against 0.15 M NaCl in 50 mM Tris/HCl, pH 7.5, and chromato- graphed on a Sephadex G-50 column (1.4 × 30 cm). The radioactive protein in the single peak which was eluted after Vs was isolated.
After complete reduction and S-carboxymethylation this material was subjected to cleavage with trypsin and thermolysin, and the digests were separated as described before (13, 14). Edman degradation in a Beckman Sequencer followed standard procedures (19) and digests were separated as described before (13, 14). Edman degradation was subjected to cleavage with trypsin and thermolysin, and the peptide was analyzed by varying the concentration of the enzyme (1, 11) shows that the aminopropeptides are indeed good substrates for liver transglutaminase. Other connective tissue proteins such as the aminopropeptide Col 1(I) of type I collagen, Col(I) and Col(II) chains, and the globular domain NC1 of type IV collagen were found to be poor substrates. Incorporation of putrescine into the aminopropeptide Col 1–3(III) showed some dependence on conformation, since a 5-fold decrease was observed after complete reduction of disulfide bonds. Degradation of Col 1–3(III) to the fragment Col 1(I), however, did not change the incorporation; this demonstrates that the globular domain of the aminopropeptide contains essentially all of the putrescine labeling sites.

Electrophoretic analysis of the putrescine-labeled aminopropeptide showed that most of the label is in a band co-migrating with an authentic sample of [125I]-labeled aminopropeptide (Fig. 1, lanes 1 and 2). A distinct radioactive band was also observed at the top of the running gel; this did not correspond to any protein band when examined by Coomassie Blue staining. This band presumably arose due to covalent cross-linking of the aminopropeptide. Similar bands at the top of the stacking and running gels were also found in the labeling of dimethyl casein (Fig. 1, lane 4).

The number of putrescine-labeling sites in the aminopropeptide was analyzed by varying the concentration of the reagent. A straight line derived from the double-reciprocal plot (Fig. 2) intercepts the vertical axis at a value for 1/P of 0.36, corresponding to 2.78 nmol. This indicates binding of about 1 mol of putrescine in each identical Mr = 15,000 subunit of the aminopropeptide. High performance liquid chromatography of an exhaustive proteolytic digest of the labeled peptide (8) showed that 58% of the label occurred as γ-glutamyl putrescine.

The precise position of the putrescine acceptor site was determined by cleaving the reduced and alkylated sheep peptide Col 1(III) with trypsin. This, as shown before for calf Col 1(III) (13), allowed the separation on Sephadex G-50 of a 65-residue peptide T1 with 8 glutamine residues from the

### Table I

| Protein                                      | Radioactivity incorporated<sup>a</sup> | 10<sup>6</sup> cpm/20 μg | cpm/nmol |
|----------------------------------------------|----------------------------------------|--------------------------|----------|
| **Aminopropeptides**                        |                                        |                          |          |
| Sheep Col 1–3(III)                           | 20.9 ± 4.3                             | 27.9<sup>b</sup>         |          |
| Sheep Col 1(III)                             | 18.4 ± 1.5                             | 24.5                     |          |
| Sheep Col 1–3(III)<sup>c</sup>              | 4.6                                    | 6.1                      |          |
| Calf Col 1–3(III)                            | 51.4                                   | 68.5<sup>b</sup>         |          |
| Sheep Col 1(I)                              | 1.2                                    | 1.6                      |          |
| **Collagens**                                |                                        |                          |          |
| α1(I), α1(III) chains                        | 0.2                                    | 0.04                     |          |
| NC1 domain, collagen IV                      | 1.8                                    | 1.3<sup>b</sup>          |          |
| **Other proteins**                           |                                        |                          |          |
| Dimethyl casein                              | 64.1 ± 4.2                              | 85.4                     |          |
| Casein                                       | 6.7 ± 1.6                              | 8.9                      |          |
| Fibrinogen                                   | 1.4                                    | 0.5<sup>b</sup>          |          |
| Actin                                        | 1.7                                    | 0.8                      |          |
| Serum albumin                                | 0.2                                    | 0.07                     |          |
| Ovalbumin                                    | 0.02                                   | 0.01                     |          |

<sup>a</sup> Average values (n = 3 or 6) obtained in the presence of 0.043 μM [3H]putrescine. Error limits (± S.E.) are given for n = 6.

<sup>b</sup> Expressed per subunit.

<sup>c</sup> After total reduction and alkylaion of disulfide bonds.

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**Fig. 1.** Polyacrylamide gel electrophoresis of proteins labeled with [3H]putrescine by liver transglutaminase. Lane 1, sheep aminopropeptide Col 1–3 (III); lane 2, [125I]-labeled aminopropeptide (for reference); lane 3, bovine serum albumin; lane 4, dimethyl casein.

**Fig. 2.** Double reciprocal plot of putrescine binding to sheep aminopropeptide Col 1–3(III) in the presence of liver transglutaminase, P, putrescine bound (nmol); S, total putrescine concentration (mM). 40 μg of peptide (0.89 nmol) in 0.3 ml transglutaminase buffer containing 4 μCi [3H]putrescine, 10 μl of 20-fold-diluted enzyme, and 0.0175–3.5 mM unlabeled putrescine were incubated for 2 h at 37 °C and then processed as described under "Experimental Procedures."
blocked N-terminal 19-residue peptide T2 containing 2 glutamine residues. The smaller peptide contained about 95% of the label and showed an amino acid composition identical to that of the T2 calf peptide (Fig. 3). Further cleavage of peptide T2 with thermolysin and passage over Dowex 50X2 allowed the isolation of the tripeptide pyrrolidone carboxylic acid-Gln-Glu which, due to a blocked N terminus did not bind to the column. 90% of the radioactivity was in the bound fraction. On elution, the major radioactive peak contained two peptides corresponding to positions 4–11 and 12–16 of the amino acid sequence shown in Fig. 3, as demonstrated by Edman degradation. Since the label is mainly present as γ-glutamyl putrescine, it is clear that glutamine in position 14 is the major putrescine acceptor site of the aminopropeptide.

**DISCUSSION**

Our data clearly demonstrate strong incorporation of putrescine into the aminopropeptide of type III collagen. This was distinctly greater than that for actin, fibrinogen, or unmodified casein, each of which is known to be a substrate for liver transglutaminase (20, 21). Incorporation is basically restricted to a single glutamine (position 14) within each subunit of the aminopropeptide and this is located in a sequence (Leu-Gly-Gln-Ser) showing a degree of homology to the sequence of a synthetic transglutaminase substrate (Fig. 3). However, other features of the surrounding sequence differ substantially; these include an alanine in the aminopropeptide position 18 which causes a considerable decrease in activity when placed into the synthetic peptide substrate (3). This could indicate the influence of conformational features in enzyme recognition, particularly since the glutamine is located within a disulphide loop of the aminopropeptide (13) and reduction of disulfide bonds causes a considerable decrease in incorporation. Conformational differences may also explain the differences in labeling of sheep and calf aminopropeptides (Table I) which otherwise are identical in the amino acid sequences around the glutamine donor sites.

Putrescine labeling was rather low for other potential substrates of the extracellular matrix such as α-chains of fibrillating collagens, the globular domain NC1 of basement membrane collagen type IV, and the aminopropeptide of type I collagen. The latter observation is very likely due to the absence of a Leu-Gly-Gln-Ser sequence in the aminopropeptide of type I collagen (13, 14) (Fig. 3).

Previous wound healing studies (8) have demonstrated that much of the putrescine label is found in a small protein which showed the same chromatographic properties as authentic aminopropeptide of type III collagen. Other labeled components were of larger size but could be degraded to similar smaller material by bacterial collagenase. Together with the present data using authentic aminopropeptide of collagen type III, this evidence indicates a preferential transglutaminase-catalyzed cross-linking of these structures during connective tissue repair. This may include cross-linking between the subunits of the aminopropeptide (see Fig. 1) as well as cross-linking to other matrix components (8, 22). The actual enzyme responsible remains to be identified but it is probably a net factor XIIIa, the transglutaminase from blood, which does not catalyze the binding of putrescine to the aminopropeptide.

Whether transglutaminase-catalyzed modification of the aminopropeptide is involved in biological processes other than tissue remodeling remains an open question. It is of interest in this context that most, if not all, type III collagen fibrils retain on their surface the aminopropeptide which is considered to prevent further fibril growth (23). It is possible that covalent cross-linking of the aminopropeptide contributes to its high metabolic stability. Other cross-links may include those to fibronectin (10, 11), as indicated by a close correspondence between the distribution of the two structural components, as shown by electron microscopy (24).

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