Sequential Cleavage of Type I Procollagen by Procollagen N-Proteinase. An Intermediate Containing an Uncleaved Proα1(I) Chain†

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ABSTRACT: The conversion of type I procollagen to type I collagen was studied by cleaving the protein with partially purified type I procollagen N-proteinase from chick embryos. Examination of the reaction products after incubation for varying times at 30 °C indicated that, during the initial stages of the reaction, proα1(I) and proα2(I) chains were cleaved at about the same rate. As a result, all the proα2(I) chains were converted to pCα2(I) chains well before all the proα1 chains were cleaved. When the reaction products were examined by gel electrophoresis without reduction of interchain disulfide bonds, a distinct band of an intermediate was detected. The same intermediate was seen when the reaction was carried out at 35, 37, and 40 °C. The data established that over two-thirds of the type I procollagen was converted to the intermediate and that this intermediate was then slowly converted to the final product of pCcollagen. The kinetics for the reaction, however, did not fit a simple model for precursor–product relationship among substrate, intermediate, and product. Examination of the reaction products with a two-step gel procedure demonstrated that the intermediate consisted of three polypeptide chains in which the N propeptide was cleaved from one proα1 chain and one proα2(I) chain but the N propeptide was still present on one of the proα1(I) chains. In further experiments it was demonstrated that a similar intermediate was seen when a homotrimer of proα1(I) chains was partially cleaved by the enzyme. No intermediate, however, was detected when the enzyme was used to cleave type II procollagen, a homotrimer of proα1(II) chains. If the partially cleaved intermediate of type I procollagen is generated during fibril assembly in vivo, it may help to determine the structure of the collagen fibrils found in tissues.

The conversion of type I procollagen to type I collagen requires cleavage of the protein by two separate endoproteinases (Prockop et al., 1979; Bornstein & Sage, 1980). A procollagen N-proteinase cleaves the three polypeptide chains in the N-terminal region of the protein to release the N propeptides (Kohn et al., 1974; Tuderman et al., 1978; Layman, 1981). A separate C-proteinase cleaves the three polypeptide chains in the C-terminal region to release the C propeptides (Goldberg, et al., 1975; Kessler & Goldberg, 1978; Duksin et al., 1978; Leung et al., 1979; Nijieha et al., 1982).

The N-proteinase was identified in several different connective tissues, and it was purified from the tendons of chick embryos (Tuderman & Prockop, 1982). More recently, the enzyme was extensively purified from homogenates of whole chick embryos (Berger et al., 1983; Tanzawa et al., 1985). The enzyme from both tendons and homogenates of chick embryos was similar in that it cleaved the N propeptides from type I and type II procollagen but it did not cleave either type III or type IV procollagen. It was a neutral metalloproteinase which was inhibited by metal chelators, and it required calcium for optimal activity. Also, the enzyme had the unusual property of requiring procollagen in a native conformation to serve as a substrate. As judged by gel filtration chromatography, the enzyme was a large protein with an apparent molecular weight of about 320000 (Tuderman et al., 1978; Tanzawa et al., 1985). A separate procollagen N-proteinase that cleaves type III procollagen has been purified from cultures of calf fibroblasts (Nusgens et al., 1980) and smooth muscle cells (Halila & Peltonen, 1984).

Procollagen C-proteinase has been studied less extensively than the N-proteinase, but several reports demonstrated that distinct, partially cleaved intermediates were readily detected after partial cleavage of type I procollagen with the C-proteinase. The observations of Davidson et al. (1977) and Morris et al. (1979) suggested that the first step in the cleavage of procollagen by the C-proteinase was random cleavage of a proα1(I) or proα2(I) chain, and the last step was cleavage of the proα1(I) chain. Njieha et al. (1982) also observed sequential cleavage of type I procollagen by C-proteinase, but their results suggested that the last step in the reaction was cleavage of the proα2(I) chain. We have here examined the question of whether distinct intermediates can be detected during the cleavage of type I procollagen by N-proteinase.

Materials and Methods

Preparation of Procollagen N-Proteinase. Procollagen N-proteinase was prepared from homogenates of whole chick embryos with the procedure recently described elsewhere (Berger et al., 1983; Tanzawa et al., 1985). In brief, 12 dozen 13-day-old chick embryos (about 850 g wet weight) were homogenized in a low-salt buffer in which the enzyme was not soluble. The pellet obtained by centrifuging the homogenate at 15000*g* for 30 min was then extracted with buffer containing 2.0 M KCl and 0.1% Brij 35, and the activity in the extract was purified over 2000-fold with a three-step procedure involving chromatography on columns of DEAE-cellulose, concanavalin A–agarose, and heparin–agarose. The enzyme eluted from the heparin–agarose column was stored frozen at −70 °C for up to 3 months.

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Preparation of Procollagen Substrates. Radioactively labeled type I procollagen was prepared from the medium of freshly isolated chick embryo fibroblasts incubated with a ^{14}C-labeled mixture of amino acids (Dehm & Prockop, 1972). The type I procollagen was purified from the medium by chromatography on DEAE-cellulose (Hoffman et al., 1976).

Type II procollagen was prepared from chick embryo chondrocytes with minor modifications of previously published procedures (Curran & Prockop, 1982). Sternal cartilages from 17-day-old chick embryos were digested with trypsin and collagenase, and the washed chondrocytes were incubated at a concentration of 7.5×10^6 cells/mL in Krebs' medium II supplemented with 4 μg/mL sodium ascorbate, 6 μg/mL β-aminopropionitrile, 2 μCi/mL ^{14}C-labeled amino acid mixture, and 10% fetal calf serum at 37 °C for 2 h. The medium proteins were precipitated with 176 mg/mL ammonium sulfate, and the pellet was dissolved in and dialyzed against 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)[1] buffer, pH 7.5 at 25 °C, containing 2 M urea (Ultra-pure; Bethesda Research Labs) and 1 mM EDTA. The dialyzed procollagen was applied to a 1.5×10 cm column of DEAE-cellulose. The column was eluted with a 200-mL linear salt gradient of 0–0.2 M NaCl at 4 °C with a flow rate of 80 mL/h. The peak fractions were pooled and dialyzed against 0.1 M Tris-HCl buffer, pH 7.4 at 4 °C, containing 0.4 M NaCl. The procollagen was precipitated with 176 mg/mL ammonium sulfate and extracted into 0.1 M Tris-HCl buffer, pH 7.4 at 4 °C, containing 0.4 M NaCl. The type II procollagen was stored at −20 °C for 1 week.

The type I procollagen consisting of trimers of proα1(I) chains was prepared from the medium of skin fibroblasts from a patient with a variant of osteogenesis imperfecta (Deak et al., 1983). The fibroblasts were grown to confluency under standard conditions. They were then incubated for 24 h with 1 μCi/mL of a mixture of ^{14}C-labeled amino acids (New England Nuclear), and the procollagen from the medium was then purified by chromatography on two successive columns of DEAE-cellulose (Deak et al., 1983).

Enzymic Reaction with Procollagen N-Proteinase. Reaction of the procollagen substrates with procollagen N-proteinase was carried out essentially as described previously (Tuderman et al., 1978; Tuderman & Prockop, 1982). For reaction with type I procollagen, the assay was performed in a volume of 100 μL which contained 10 μL of [^{14}C]procollagen (about 40 000 cpm and 4 μg) in 0.4 M NaCl andd 0.1 M Tris-HCl buffer (pH 7.4 at 4 °C), 10 μL of 50 mM $CaCl_2$ in assay buffer, 70 μL of assay buffer, and 10 μL of enzyme preparation. The assay buffer consisted of 0.15 M NaCl, 0.02% NaN_3, and 50 mM Tris-HCl adjusted to pH 7.4 at 30 °C or at the temperatures indicated. One unit of enzyme activity was defined as the amount that cleaved about 5 μg of procollagen to the intermediate or to pCcollagen in 1 h at 30 °C under the conditions employed here. Because the substrates were more dilute, the conditions were modified somewhat for the reactions with type II procollagen and the procollagen consisting of proα1(I) trimers. About 40 μL of type II [^{14}C]procollagen (about 4000 cpm) was used in a reaction mixture in which the amount of assay buffer was reduced to 40 μL. In the case of the proα1(I) trimers, the substrate was first dialyzed against assay buffer containing 5.5 mM $CaCl_2$. For the reaction, 90 μL of the [^{14}C]procollagen (about 1200 cpm and 0.5 μg) was incubated directly with 10 μL of enzyme preparation.

After incubation at 30 °C or the temperatures indicated below, the reaction was stopped by adding 25 μL of 500 mM EDTA in assay buffer and 14 μL of 20% $NaDodSO_4$ and then heating at 100 °C for 3 min.

Polyacrylamide Gel Electrophoresis. For examination of the reaction products by gel electrophoresis, an equal volume (139 μL) of electrophoresis sample buffer containing 2% $NaDodSO_4$, 10% glycerol, and 0.001% bromophenol blue in 0.25 M Tris-HCl buffer (pH 6.8 at room temperature) was added to the sample. To reduce the protein prior to electrophoresis, 2-mercaptoethanol was added to a final concentration of 5%, and the sample was heated again at 100 °C for 3 min.

To examine the enzymic reaction products with reduced disulfide bonds, the samples were electrophoresed on polyacrylamide slabs of 1.5 mm thickness with a stacking gel of 4.5% polyacrylamide and a separating gel of 6% polyacrylamide. To examine reaction products with intact disulfide bonds, electrophoresis was carried out under similar conditions with a stacking gel of 3.5% polyacrylamide and a separating gel of 4% polyacrylamide. Urea in a concentration of 0.5 M was added to the buffers used to prepare the gels and to the running buffer.

To examine the chain composition of the unreduced reaction products, the samples were first separated by electrophoresis in a 4% polyacrylamide gel as described below. The gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid to locate the bands of protein. The gel was destained in 15% methanol and 7.5% acetic acid. Gel slices containing the protein were then stirred in electrophoresis sample buffer containing 5% 2-mercaptoethanol at room temperature for 4 h. The samples were heated to 100 °C for 3 min, and the slices were placed in the sample wells of a 6% polyacrylamide gel as described above. Electrophoresis in the second gel was then carried out under the usual conditions.

Fluorograms were prepared as described previously (Tuderman et al., 1978; Tuderman & Prockop, 1982). The fluorograms of the gels were scanned on a Joyce-Loebl densitometer.

Results

Relative Rates of Cleavage of Proα1(I) and Proα2(I) Chains. The first evidence for sequential cleavage of the N propeptides was obtained by examining the relative rates at which proα chains were converted to pCα chains after short periods of incubation with procollagen N-proteinase. As indicated in Figure 1, visual inspection of fluorograms suggested that pCα1(I) and pCα2(I) chains were generated in approximately equimolar amounts at early stages of the reaction. With longer incubation times, the ratio of pCα1(I) to pCα2(I) chains became 2:1.

As indicated in Figure 2, densitometry of the fluorograms further supported the impression that proα1(I) and proα2(I) chains were cleaved at about the same rate during the initial stages of the reaction. As a result, all the proα2(I) chains were converted to pCα2(I) chains well before all the proα1 chains were cleaved.

Detection of an Intermediate in the Conversion of Procollagen to pCcollagen. In further experiments, the reaction products obtained after digestion of type I procollagen for

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; pNcollagen, intermediate in the conversion of procollagen to collagen that contains the N propeptides but not the C propeptides; pCcollagen, intermediate in the conversion of procollagen to collagen that contains the C propeptides but not the N propeptides; proγ chains, trimers consisting of three disulfide-linked proα chains; pCγ chains, trimers consisting of three disulfide-linked pCα chains; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

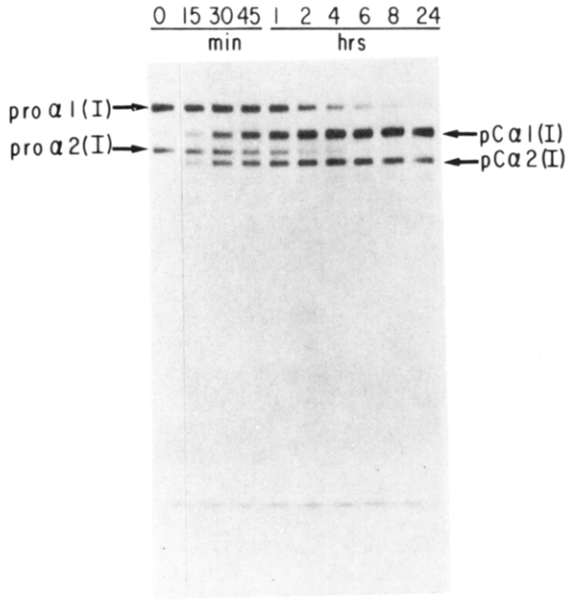

FIGURE 1: Conversion of type I procollagen to pCcollagen by procollagen N-proteinase. Type I procollagen from chick embryo fibroblasts was incubated with about 0.6 units of procollagen N-proteinase at 30 °C under conditions described in the text. The reaction products were then reduced and examined by electrophoresis in a 6% polyacrylamide gel. A fluorogram of the gel is shown.

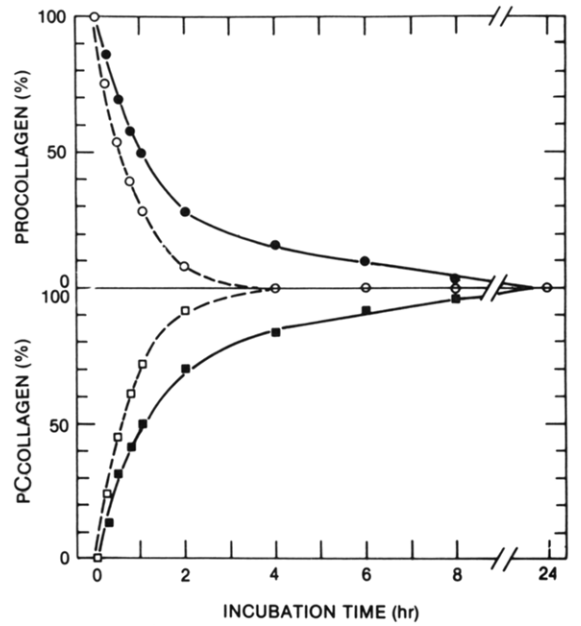

FIGURE 2: Time course for the conversion of procollagen to pCcollagen by procollagen N-proteinase. Data were obtained by densitometry of fluorograms similar to that shown in Figure 1. (●) Proα1(I); (○) proα2(I); (■) pCα1(I); (□) pCα2(I).

varying times were examined by gel electrophoresis without reduction of the interchain disulfide bonds that link the C-terminal propeptides. The samples were separated in a 4% polyacrylamide gel which made it possible to detect both proγ chains and pCγ chains. As indicated in Figure 3, the reaction products obtained after short periods of incubation with procollagen N-proteinase contained not only proγ chains and pCγ chains but also a third band with an intermediate rate of migration. The intermediate band was present after reaction of the substrate with the N-proteinase for 15 min. Densitometry of the fluorograms (Figure 4) indicated that, after reaction for about 45 min, the intermediate band accounted for 55–70% of the reaction products. With longer reaction

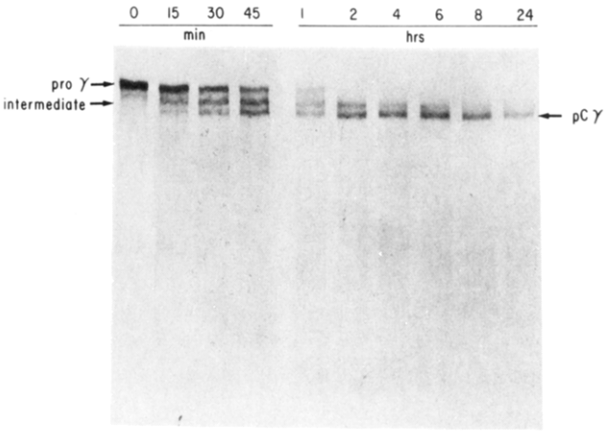

FIGURE 3: Demonstration of an intermediate in the cleavage of procollagen by procollagen N-proteinase. Conditions were similar to those in Figure 1 except that the reaction products were examined without reduction in gels of 4% polyacrylamide.

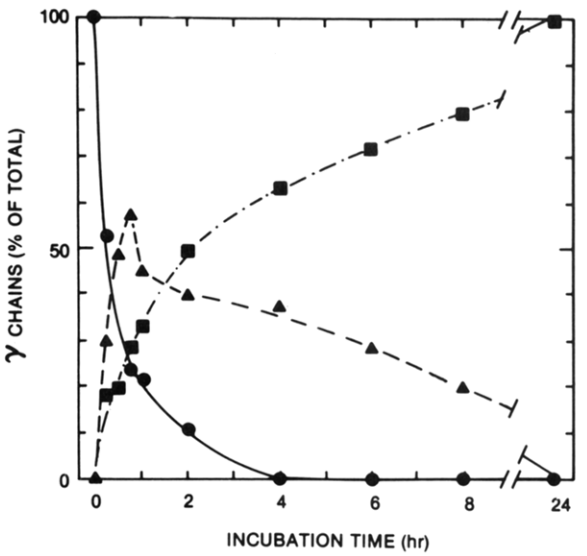

FIGURE 4: Time course for the conversion of procollagen to pCcollagen. Data were generated by densitometry of fluorograms similar to that shown in Figure 3. (●) Procollagen; (▲) intermediate; (■) pCcollagen.

times, the intermediate bands disappeared, apparently by complete conversion to pCγ chains. The results suggested therefore a precursor–product relationship between the proγ chains of procollagen, the intermediate, and the final product of pCγ chains.

The standard condition for cleavage of procollagen by N-proteinase involved incubation of the reaction mixture at 30 °C (Figures 1–4). To establish whether or not the same intermediate was observed at higher temperatures, the experiments were repeated at 35, 37, and 40 °C. An intermediate with the same mobility was seen at all the temperatures examined. The rate of conversion was somewhat faster at the higher temperatures, but a distinct intermediate band was readily identified (Figure 5).

To examine further the kinetics of the reaction, a simple model of the form

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

where A is the uncleaved substrate, B is the intermediate, C is the final product, and k_1 and k_2 are first-order rate constants (Fersht, 1977) was curve fitted to the data. The rate constant k_1 was estimated to be about 2.0 by curve fitting the equation

$$A(t) = e^{-k_1 t}$$

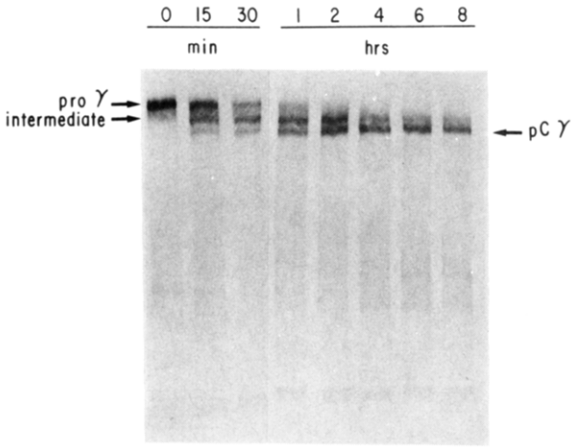

FIGURE 5: Intermediate in the cleavage of procollagen by N-proteinase at 40 °C. Conditions as in Figure 3 except that the enzymic reaction was carried out at 40 °C instead of 30 °C.

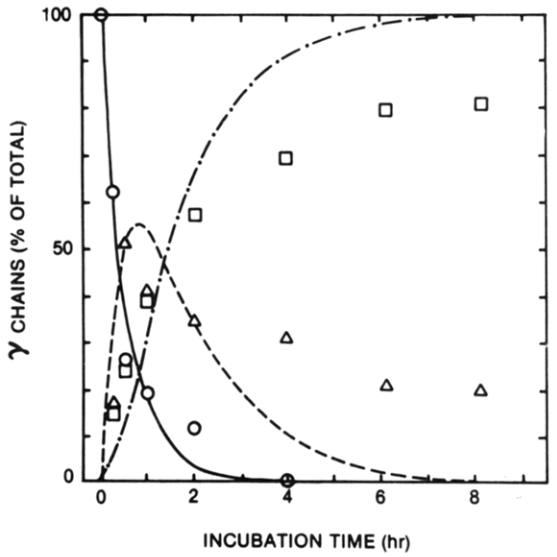

FIGURE 6: Conversion of procollagen to pCcollagen by procollagen N-proteinase. Data were obtained by densitometry of gels similar to those shown in Figures 3 and 5 except that the reaction was carried out at 35 °C. The curves are theoretical curves drawn according to the equations presented in the text. (○) Procollagen; (△) intermediate; (□) pCcollagen.

to the data. The rate constant k_2 was then estimated by curve fitting the equations

$$B(t) = \frac{-k_1}{k_1 - k_2}(e^{-k_1 t} - e^{-k_2 t})$$

$$C(t) = \frac{k_2}{k_1 - k_2}(e^{-k_1 t}) - \frac{k_1}{k_1 - k_2}(e^{-k_2 t}) + 1$$

to the data. The best value for k_2 was 0.75. As indicated in Figure 6, the experimental values provided a reasonably close fit to the theoretical curves for values up to 2 h. Beyond 2 h, the conversion of the intermediate was too slow to fit this simple model. These observations were not explained, in any simple way, by inactivation of the enzyme during the reaction, since in separate experiments using pNcollagen as a substrate the reaction was found to be linear for 12 h (data not shown).

Chain Composition of the Intermediate in the Cleavage of Type I Procollagen by N-Proteinase. To determine the chain composition of the intermediate, the reaction products were examined in a two-step gel procedure. After partial cleavage of type I procollagen, the reaction products were first separated without reduction in a 4% polyacrylamide gel. The protein

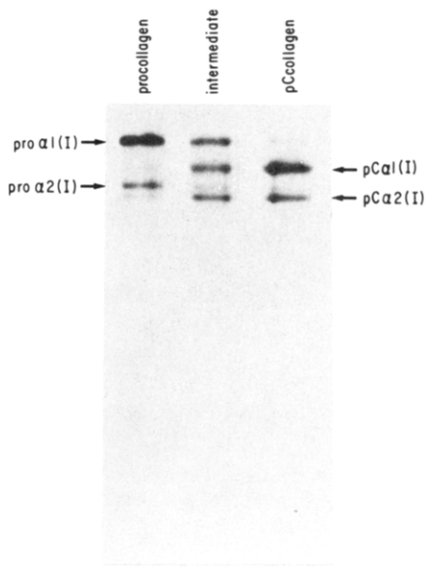

FIGURE 7: Intermediate in the cleavage of type I procollagen by procollagen N-proteinase. Type I procollagen from chick embryo fibroblasts was incubated with 0.6 unit of procollagen N-proteinase for 45 min at 30 °C. The reaction products were then separated in a 4% polyacrylamide gel without reduction as shown in Figure 3. Slices containing procollagen, the intermediate, and pCcollagen were then reduced and separated by electrophoresis in a second gel of 6% polyacrylamide.

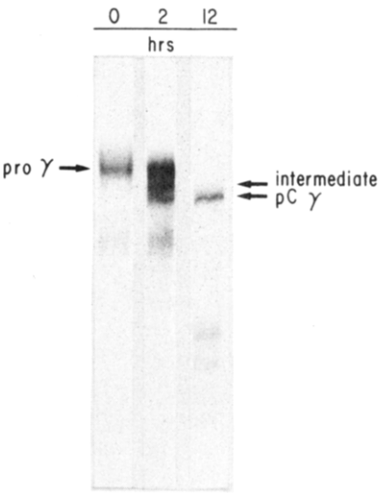

FIGURE 8: Intermediate in the cleavage of a homotrimer of proα1(I) chains by procollagen N-proteinase. A homotrimer of proα1(I) chains was isolated from the medium of fibroblasts of a variant of osteogenesis imperfecta and incubated with 0.6 unit of N-proteinase at 30 °C as described in the text. The reaction products were then separated without reduction by electrophoresis in a 4% polyacrylamide gel. A distinct band of the intermediate was more apparent on the original fluorogram than in the photograph presented here.

bands were located by staining of the gel, and then gel slices containing the protein bands were reduced and electrophoresed in a second gel. As indicated in Figure 7, proα1(I) and proα2(I) chains in a stoichiometry of about 2:1 were recovered from the initial substrate of type I procollagen. Also, after complete cleavage of the procollagen, pCα1(I) and pCα2(I) chains were recovered in a stoichiometry of about 2:1. From the band of intermediate protein, three types of chains were recovered. These consisted of a pCα1(I) chain, a pCα2(I) chain, and a proα1(I) chain in a stoichiometry of about 1:1:1.

Cleavage of a Homotrimer of Proα1(I) Chains and of Type II Procollagen. In further experiments, we examined the question of whether sequential cleavage of the N propeptides also occurred when procollagen N-proteinase was incubated

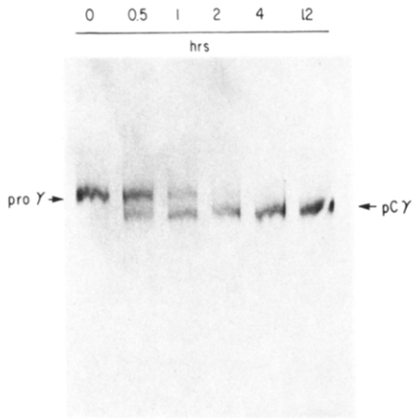

FIGURE 9: Cleavage of type II procollagen by procollagen N-proteinase. Type II procollagen was isolated from the medium of chondrocytes as described in the text and then incubated with 0.6 unit of procollagen N-proteinase at 30 °C as described in text. The reaction products were separated without reduction by electrophoresis in a 4% polyacrylamide gel. No band of intermediate is apparent in the fluorograms.

with procollagens which were homotrimers. In the first series of experiments, a homotrimer of proα1(I) chains was used as a substrate. As indicated in Figure 8, an intermediate with the same mobility as the intermediate seen with type I procollagen was detected when the homotrimer was incubated with procollagen N-proteinase. In a second series of experiments, the substrate employed was type II procollagen which consists of the homotrimer proα1(II) chains. As indicated in Figure 9, no intermediate was detected in reaction products of partially cleaved type II procollagen.

Discussion

The results presented here provide information about both the kinetics and the intermediates generated as procollagen N-proteinase cleaves the three polypeptide chains of type I procollagen. Since an intermediate containing only one uncleaved proα chain was readily detected, the results demonstrated that most of the reaction proceeds by a pathway in which the third chain is cleaved more slowly than the first two. Since the intermediate contained a single uncleaved proα1(I) chain, the results also demonstrated that most of the substrate is cleaved in a sequential order in which the last chain cleaved is a proα1(I) chain.

It should be noted that the kinetics seen here are unusual for an endoproteinase, since with most such enzymes the susceptible peptide bonds in the substrate become more accessible and the rate of the reaction increases as the protein is partially cleaved and begins to unfold (Fruton, 1974). If procollagen N-proteinase conformed to this general pattern, one would expect that intermediates would be difficult to detect and that the most readily detectable intermediate would be the first cleavage product containing one cleaved and two uncleaved propeptides. It is of interest that two other exceptions to this general pattern of endoproteinases are vertebrate collagenase (Sunada & Nagai, 1983) and procollagen C-proteinase (Davidson et al., 1977; Morris et al., 1979; Njieha et al., 1982). With both vertebrate collagenase and procollagen C-proteinase, partially cleaved substrate molecules similar to the intermediate described here are readily detected in the reaction products.

There are at least two possible explanations for the generation of a slowly reacting intermediate during the cleavage of type I procollagen by procollagen N-proteinase. One is that the conformation of the peptide bonds around the cleavage

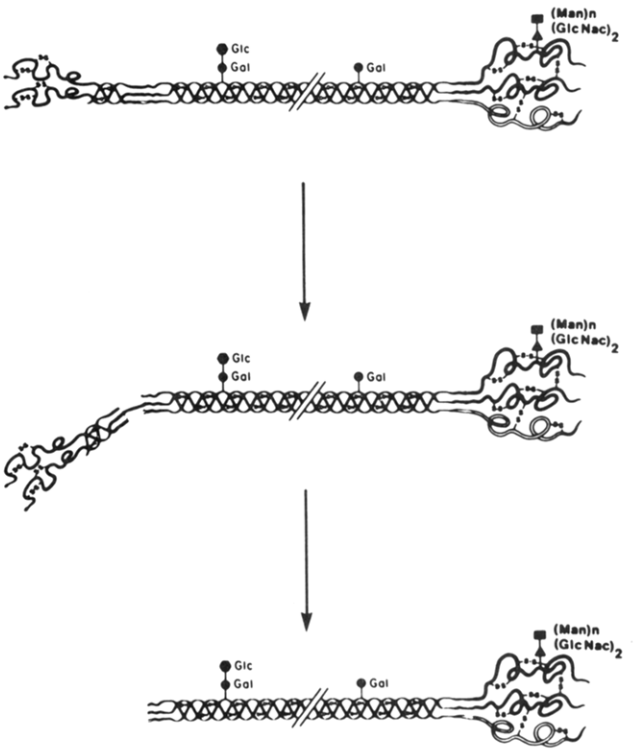

FIGURE 10: Schematic model for the stepwise cleavage of procollagen to pCcollagen by procollagen N-proteinase.

site changes markedly after the first two proα chains are cleaved (Figure 10). Procollagen N-proteinase has a highly specific requirement for a procollagen substrate with the correct, native conformation. It will not cleave thermally denatured procollagen (Tuderman et al., 1978; Tanzawa et al., 1985). Also, it will not cleave two recently identified type I procollagens in which the conformation of the N-proteinase cleavage site is altered by genetic mutations that shorten one of the three proα chains in the molecule by deleting -Gly-X-Y- sequences from the α chain domain (Williams & Prockop, 1983; Sippola et al., 1984). Therefore, it is possible that after N-proteinase cleaves the first two proα chains, the region of the protein containing the cleavage site partially unfolds and thereby makes the remaining proα chain more resistant to cleavage.

A second and alternative explanation is that the last proα chain is cleaved more slowly because it is not in an equivalent position to either the first or the second proα chain in the native conformation of the protein. Within the triple-helical structure of the collagen, the three α chains are displaced one amino acid position relative to each other along the axis of the helix [see Hulmes et al. (1980) and Bender et al. (1983)]. Therefore, it is possible that within the native conformation of type I procollagen, the second proα1(I) chain is cleaved more slowly because it is displaced more toward or away from the center of the molecule than either the proα2(I) chain or the first proα1(I) chain.

Both of these explanations are consistent with the observation that an intermediate containing one uncleaved proα chain was also detected when a homotrimer or proα1(I) chains was used as a substrate for procollagen N-proteinase. The detection of the intermediate after partial cleavage of the homotrimer of proα1(I) chains clearly demonstrates that the generation of the intermediate does not depend on any special contribution that the proα2(I) chain makes to the structure of the native protein. However, neither of the two explanations presented above can easily explain the further observation that

no intermediate was detected during the cleavage of type II procollagen. In the case of type II procollagen, it may be that because of differences in primary structure, the molecule in the region of the cleavage site remains more rigid and does not unfold as much as type I procollagen after cleavage of the first two proα chains. Alternatively, the third proα chain may be in a more accessible conformation in type II procollagen than in type I procollagen.

The data clearly established that at least two-thirds of type I procollagen is converted to an intermediate containing one uncleaved proα1(I) chain and that this intermediate is slowly converted to the final product. The kinetics of the reaction, however, did not fit a simple model for a precursor–product relationship among substrate, intermediate, and product. There are several possible explanations for this situation, such as a time-dependent conformational change in the intermediate (Figure 10), product inhibition, or an alternate pathway for some of the substrate.

Several investigators have suggested a specific role for the propeptides of procollagen in the assembly of normal collagen fibrils (Veis et al., 1973; Fleischmajer et al., 1981, 1983; Hulmes, 1983; Miyahara et al., 1984). Therefore, if the partially cleaved intermediate detected here is generated during the fibril assembly in vivo, it may help to determine the structure of the fibrils found in tissues.

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