Biochemical characterization of the native tissue form of type X collagen from embryonic chick sternal cartilage and identification of a chymotrypsin-sensitive site within its triple-helical domain

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We isolated and characterized the intact native tissue form of type X collagen from the presumptive calcification region of lathyritic chick-embryo sterna and from organ cultures incubated in the presence of β -aminopropionitrile (β APN). The administration of β -APN *in vivo* greatly increased the solubility of type X collagen and allowed the extraction of quantitative amounts of these molecules under non-denaturing non-proteolytic conditions. Biosynthetic studies *in vitro* showed that the addition of β APN during labelling resulted in a 4-fold increase in the extractability of the newly synthesized type X collagen. Biochemical characterization of the intact type X collagen extracted from the tissues or biosynthesized in the organ cultures showed that type X collagen is composed of 59000- M_r chains that do not undergo conversion into shorter polypeptides. Despite the marked solubilization of type X collagen upon administration of β APN, a substantial proportion remained tissue-bound and could only be extracted by employing proteolytic digestion followed by disulphide bond reduction. These findings indicate that type X collagen in the tissues is stabilized by at least two different mechanisms, one involving β APN-sensitive cross-links and the second through interactions with disulphide-bonded proteins. Limited proteolytic digestion with chymotrypsin of tissues containing 1.0 M-NaCl-insoluble type X collagen resulted in its complete solubilization. The majority of type X collagen molecules extracted with chymotrypsin were approx. 10% shorter than those obtained after limited pepsin digestion (M_r 40000 versus M_r 45000) and showed the selective loss of a single CNBr-cleavage peptide. These findings indicate the existence of chymotrypsin-sensitive sites within the triple-helical domain of the molecules.

INTRODUCTION

Type X collagen is a short-chain collagen found almost exclusively in cartilaginous tissues undergoing endochondral ossification (reviewed in Schmid & Linsenmayer, 1987). The role of type X collagen is not known at the present time, although because of its topographic and temporal segregation to tissues undergoing osteogenesis, it is speculated that it may be of fundamental importance in the process of endochondral bone formation (Jimenez *et al.*, 1986). Biosynthetic studies using pulse-chase experiments in chick tibiotarsus explants (Kielty *et al.*, 1985) suggested that type X collagen is first synthesized as a procollagen molecule containing three identical 59000- M_r chains, which are converted into 49000- M_r polypeptides in a time-dependent manner. Similar findings were obtained by Ayad *et al.* (1987) in bovine growth-plate cartilage. However, another report suggested that type X collagen molecules are proteolytically processed to an even smaller species composed of 30000- M_r polypeptide chains (Capasso *et al.*, 1984). A definitive characterization of the tissue form of type X collagen has not been possible, because these molecules can only be extracted by limited proteolytic digestion or under denaturing conditions.

The purpose of the present study was to characterize intact native type X collagen molecules from embryonic chick sternum. To this end, collagens from the sterna of lathyritic chick embryos and the newly synthesized collagens from β -aminopropionitrile (β APN)-treated organ cultures of embryonic chick sterna were extracted under non-proteolytic conditions and the intact type X collagen molecules were characterized with regard to their molecular size, extracellular processing and presence of

chymotrypsin-sensitive imperfections within their triple-helical domains.

METHODS

Organ cultures of embryonic chick sterna

Sterna from 20-day-old White Leghorn chick embryos were removed and the presumptive calcification cartilage was separated, finely minced and incubated for 24 h in tissue-culture medium (N.C.T.C. B5 MEM/Earle's BSS) containing 10% (v/v) fetal-calf serum, 100 μ g of ascorbic acid/ml and 10 μ Ci of [14 C]proline (275 mCi/mmol; Amersham International)/ml as described in detail previously (Jimenez *et al.*, 1986; Reginato *et al.*, 1986). At 20 min before labelling, β APN (100 μ g/ml) was added to the culture medium of experimental samples. Control samples did not receive β APN to resemble more closely normal cartilage matrix biosynthesis. After labelling, the minced cartilage was homogenized in 1.0 M-NaCl/proteinase inhibitor buffer as described previously (Jimenez *et al.*, 1986). The solubilized material was removed by centrifugation at 1400 g for 60 min and the insoluble tissue was sequentially extracted with acetic acid, acetic acid/pepsin, DTT buffer (twice) and acetic acid/pepsin as described previously (Remington *et al.*, 1983, 1984). Each extraction was carried out for 48 h at 4 °C. Following centrifugation at 1400 g for 60 min, the supernatants were removed and after extensive dialysis against 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, portions were taken for determination of their hydroxy[14 C]proline content by the method of Juva & Prockop (1966).

Abbreviations used: β APN, β -aminopropionitrile; DTT, dithiothreitol.

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Gel electrophoresis

The labelled proteins were examined by SDS/PAGE in 8%-polyacrylamide slab gels under reducing and non-reducing conditions as described previously (Jimenez *et al.*, 1986). After electrophoresis, the gels were processed for fluorography with En³Hance (New England Nuclear) and exposed to X-Omat AR film (Eastman Kodak). Two-dimensional CNBr-cleavage peptide mapping was performed as described previously (Reginato *et al.*, 1986).

Quantitative analysis of the relative proportions of extracted collagens from control and β APN-treated organ cultures

In order to examine the relative proportion of type X collagen solubilized with each of the extractants, samples from each extract were electrophoresed on SDS/8%-polyacrylamide gels under reducing and non-reducing conditions before and after limited pepsin digestion, and submitted to fluorography. The fluorographs were scanned in a linear-drive densitometer at 540 nm and the areas under each peak quantified employing a planimeter. The densitometric analysis was carried out in slightly underexposed fluorographs to assure linearity between the calculated areas and the absolute amount of radioactivity in each band as described in previous papers (Jimenez *et al.*, 1986; Reginato *et al.*, 1986).

Extraction of intact type X collagen from normal and lathyritic embryonic chick sternum

To characterize the form of type X collagen found *in vivo*, 10 dozen 17-day-old embryonated chicken eggs were rendered lathyritic by injection of 100 μ l of a sterile solution containing 150 μ g of β APN/ml in 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, into their chorioallantoic membranes. At day 20, their sterna were removed and the presumptive calcification region cartilage was extracted with 1.0 M-NaCl/proteinase inhibitor buffer. The solubilized proteins were examined by SDS/PAGE in polyacrylamide slab gels followed by staining with Coomassie Brilliant Blue (Bio-Rad-250).

Pulse-chase labelling of embryonic chick sternum organ cultures

Presumptive calcification cartilage from 20-day-old chick sterna was pulse-labelled with 50 μ Ci of [¹⁴C]proline/ml in tissue-culture medium containing 10% (v/v) fetal-calf serum and 100 μ g of ascorbic acid/ml. After 1 h the medium was removed, the tissues were rinsed and portions of cartilage were distributed into 15 separate culture tubes containing fresh medium and 100 mM unlabelled proline to dilute the pool of labelled proline. At different intervals following the pulse, triplicate cultures were harvested, the media were removed and the tissues were separately homogenized. The newly synthesized collagens present in the tissues at each period of chase were solubilized with the 1.0 M-NaCl/proteinase inhibitor buffer.

Extractability of newly synthesized type X collagen with chymotrypsin

Sterna from 20-day-old embryos were removed and the presumptive calcification region was labelled with [¹⁴C]proline as described above. The labelled collagens remaining in the tissues after extraction of soluble molecules with 1 M-NaCl/proteinase inhibitor buffer were extracted for 24 h with 1 mg of chymotrypsin/ml in 0.15 M-NaCl/50 mM-Tris at 4 °C followed by extraction with DTT buffer as indicated above. After extensive dialysis, the extracts were analysed by SDS/PAGE and two-dimensional CNBr-cleavage peptide mapping as indicated above.

RESULTS

Effects of β APN on the extractability of newly synthesized collagens

Addition of β APN to the organ cultures resulted in a marked increase in the solubility of newly synthesized collagens. The soluble pool comprising collagens extracted with 1.0 M-NaCl/proteinase inhibitor buffer and 0.5 M-acetic acid represented less than 13% of the newly synthesized collagens in the control cultures, whereas it represented greater than 60% in the cultures treated *in vitro* with β APN. The samples obtained by repeated pepsin extractions contained a very small proportion of newly synthesized collagen in both cases since only 6.9% and 2.7% of the total collagen were present in the control and β APN-treated cultures respectively. Treatment of the remaining tissue with DTT buffer under non-denaturing conditions resulted in marked solubilization of the pepsin-insoluble collagen from control (77.1%) and β APN-treated (85.0%) tissues.

Characterization of newly synthesized collagens from control and β APN-treated organ cultures

As shown in Fig. 1, the electrophoretic patterns under reducing conditions of all extracts from control and β APN-treated cultures were qualitatively similar. The 1.0 M-NaCl extracts (lanes 1 and 4) showed a prominent band corresponding to α 1(II) collagen chains and other bands migrating above and below it. Two of the bands migrating above correspond to intact type XI collagen chains. The major band observed below α 1(II) collagen chains migrated with an apparent M_r of 59000 and corresponded to α 1(X) collagen polypeptides. A marked increase in the proportion of α 1(X) collagen chains present in the 1.0 M-NaCl extract from β APN-treated organ cultures (lane 4) relative to the control cultures (lane 1) was found. The electrophoretic pattern of the labelled proteins solubilized with pepsin showed faint bands corresponding to α 1(II)_p and to 45000- M_r α 1(X)_p collagen chains in the control and in the β APN-treated cultures (lanes 2 and 5).

Fig. 1. SDS/PAGE fluorograph of newly synthesized collagens from control organ cultures and organ cultures treated *in vitro* with β APN from the presumptive calcification cartilage of 17-day-old chick embryo sterna

The tissues were labelled and extracted as described in the Methods section. Lanes 1–3, control organ cultures: lane 1, 1.0 M-NaCl extract; lane 2, pepsin extract; lane 3, DTT extract. Lanes 4–6, β APN-treated organ cultures: lane 4, 1.0 M-NaCl extract; lane 5, pepsin extract; lane 6, DTT extract.

Table 1. Relative proportion of type II and type X collagens in extracts from control and β APN-treated chick sternal presumptive calcification cartilage organ cultures

Aliquots from each of the extracts were examined by SDS/PAGE under reducing conditions after limited proteolytic digestion with pepsin as described in the Methods section. Following fluorography, the relative proportions of types II and X collagens were calculated from densitometric analysis of the fluorographs. The values are the averages of values obtained with three separate samples. The pepsin extracts were not included in this analysis because of the very low amounts of labelled collagens.

Extractant	Treatment	Proportion of each collagen chain (% of total)		Ratio of type X/type II
		Type II	Type X	
1.0 M-NaCl+0.5 M-acetic acid	Control	64.8	8.5	0.13
	β APN	56.8	30.3	0.53
DTT	Control	51.3	46.2	0.90
	β APN	54.7	40.4	0.74

Samples from the DTT extracts from control (lane 3) and β APN-treated cultures (lane 6) showed bands corresponding to $\alpha 1(\text{II})_p$ collagen chains, a small proportion of pepsin-cleaved type XI collagen chains and a prominent 45000- M_r band of pepsin-cleaved type X collagen $\alpha 1(\text{X})_p$. The final residues from control and β APN-treated tissue contained peptides that migrated with or near the tracking front of the gel.

Relative proportions of type II and type X collagens in extracts from control and β APN-treated organ cultures

Table 1 shows the relative proportions of type II and type X collagen present in the 1.0 M-NaCl-soluble plus 0.5 M-acetic acid-soluble pool and in the DTT extracts from control and β APN-treated organ cultures. The pepsin extracts were not included in this analysis as they contained very small amounts of collagen. The soluble pool from tissues treated with β APN contained a lower relative proportion of type II collagen chains than untreated tissues (56.8% versus 64.8%). No difference in the relative proportion of these chains was found in the DTT extracts from control or β APN-treated tissues (51.3% versus 54.7%). In contrast, the relative proportion of type X collagen chains found in the soluble pool was almost 4 times higher in the β APN-treated cultures than in the control samples (30.3% versus 8.5%). Using the relative proportion of type X to type II collagen chains in each extract as an index of solubilization, a 4-fold higher ratio of type X/type II collagen was found in the soluble pool of β APN-treated organ cultures (0.53 versus 0.13), indicating a more profound effect of β APN on the solubility of type X collagen than on the solubility of type II collagen.

Characterization of the native form of type X collagen

To characterize the authentic form of type X collagen found *in vivo*, cartilage from the presumptive calcification region of sterna from lathyritic chick embryos was homogenized and extracted in 1.0 M-NaCl buffer containing proteinase inhibitors. The solubilized proteins were examined by electrophoretic analysis and staining with Coomassie Brilliant Blue. The results are shown in Fig. 2. Two major bands were observed, one corresponding to $\alpha 1(\text{II})$ collagen chains and the other to intact type X collagen chains, which migrated with an apparent M_r of 59000 (lane 1). Limited pepsin digestion of a sample from this extract resulted in the conversion of the 59000- M_r chain into the expected 45000- M_r pepsin-resistant chains of type X collagen (lane 2). The pepsin-resistant band migrating just above the 45000- M_r pepsin-digested type X collagen was not characterized further.

Pulse-chase labelling of embryonic chick sternum

The processing of type X collagen was studied by employing pulse-chase labelling of the organ cultures of the presumptive calcification region of the sternum (Fig. 3a). The pulse-chase experiment showed that type X collagen did not undergo proteolytic processing into any lower- M_r form other than the 59000- M_r species even after an extended chase period of 24 h.

In certain experiments, we observed the spontaneous conversion of the 59000- M_r species into a discrete component of lower M_r during prolonged incubation or storage of the 1.0 M-NaCl extracts at 4 °C. As illustrated in Fig. 3(b), the spontaneous cleavage of the 59000- M_r form of type X collagen (lane 1) into a smaller component migrating with an apparent M_r of 49000 (lane 2) was observed. Proteolytic digestion with pepsin of 1.0 M-NaCl extracts containing the 59000- M_r and 49000- M_r molecules resulted in the conversion of these two molecular species into molecules corresponding to the 45000- M_r pepsin-resistant form of type X collagen (lane 3).

Extractability of newly synthesized type X collagen with chymotrypsin

The patterns of Fig. 4 show the electrophoretic analysis of the chymotrypsin-solubilized collagens from presumptive calcifi-

Fig. 2. SDS/PAGE of the 1.0 M-NaCl extract from 20-day-old lathyritic chick-embryo presumptive calcification sternal cartilage stained with Coomassie Brilliant Blue

The tissues were removed from chick embryos that had been injected with 15 μ g of β APN into their chorioallantoic membranes 3 days before they were killed. Lane 1, 1.0 M-NaCl extract; lane 2, same as lane 1 after pepsin digestion.

Fig. 3. SDS/PAGE fluorograph of 1.0 M-NaCl extracts of presumptive calcification cartilage organ cultures (a) at various intervals of a pulse-chase experiment and (b) after prolonged storage at 4 °C

(a) Tissues from 20-day-old chick-embryo sterna were pulse-labelled for 1 h with [14 C]proline in the presence of β APN, and after labelling they were chased with fresh medium containing 100 mM unlabelled proline for 0–24 h as described in the Methods section. Separate samples were harvested at the indicated times during the chase period, homogenized and extracted with 1.0 M-NaCl buffer. Lane 1, 0 h; lane 2, 0.5 h; lane 3, 1 h; lane 4, 2 h; lane 5, 24 h. (b) Lane 1, intact 1.0 M-NaCl extract; lane 2, same as lane 1 after prolonged storage; lane 3, same as lane 2 after limited pepsin digestion.

Fig. 4. SDS/PAGE fluorograph of chymotrypsin and DTT extracts from organ cultures

Lane 1, 1.0 M-NaCl extract; lane 2, chymotrypsin extract; lane 3, DTT extract after chymotrypsin extraction.

cation cartilage labelled *in vitro* under control conditions (no β APN added). As described above, the initial 1.0 M-NaCl extracts contained the 59000- M_r chains of type X collagen (lane 1). Chymotrypsin was an extremely potent extractant as it solubilized more than 90% of the NaCl-insoluble labelled molecules. Electrophoretic analysis of the molecules solubilized with chymotrypsin (lane 2) showed the appearance of a prominent new band of lower M_r , [$\alpha 1(X)_{chymo}$] in addition to the 45000- M_r type X collagen chains. The M_r of this low- M_r fragment, estimated by comparison with collagenous standards, was about 40000. Subsequent extraction of the chymotrypsin-resistant fraction with DTT/NaCl showed minimal additional solubilization of type X

Fig. 5. Fluorograph of a two-dimensional CNBr-cleavage peptide map of the chymotrypsin extracts

First dimension was samples of a chymotrypsin extract from the presumptive calcification cartilage under reducing conditions. Electrophoresis in the second dimension was performed after digestion with CNBr as indicated in the Methods section. Direction of electrophoresis is indicated by large arrows. The peptides of type X collagen are shown by small arrowheads. The peptide missing from the 40000- M_r chymotrypsin fragment of type X collagen is shown by the large arrowhead.

collagen and the extract contained only type II collagen chains (lane 3).

Characterization of the low- M_r chymotrypsin-derived type X collagen polypeptide

In order to confirm that the 40 000- M_r polypeptides represented a chymotrypsin-derived type X collagen fragment and to attempt to identify the site of cleavage, two-dimensional CNBr-cleavage peptide analysis was performed. The result shown in Fig. 5 demonstrates that the 40 000- M_r fragment displayed the characteristic CNBr-cleavage peptide pattern of type X collagen. The pattern of CNBr-cleavage peptides obtained from the 40 000- M_r chymotrypsin-derived fragment was identical with that of pepsin-cleaved type X collagen except that one of the five characteristic CNBr-cleavage peptide fragments was absent (arrowhead). These findings indicate that chymotrypsin cleaved the type X collagen within its helical domain, resulting in the loss of one specific CNBr-cleavage peptide from the molecule.

DISCUSSION

The most complete information on the structure of type X collagen has been derived from the nucleotide sequence of the type X collagen gene (Ninomiya *et al.*, 1986; Lu Valle *et al.*, 1988). The deduced amino acid sequence indicates that type X collagen contains a triple-helical domain of 460 amino acid residues and a 170-residue-long non-collagenous C-terminal domain for a total molecular size of 59 000 M_r . The exact size of the molecule that is assembled in the extracellular matrix is not known, although some reports have suggested the proteolytic processing of the 59 000- M_r type X collagen species to smaller fragments (Capasso *et al.*, 1984; Kielty *et al.*, 1985; Ayad *et al.*, 1987).

The purpose of the present study was to characterize further the authentic tissue form of type X collagen from the presumptive calcification cartilage of embryonic chick sternum rendered lathyrictic by exposure *in vivo* and *in vitro* to β APN. Very small amounts of type X collagen were extracted with 1.0 M-NaCl from radiolabelled control organ cultures. However, culture in the presence of β APN resulted in 4-fold greater amounts of soluble type X collagen molecules. The remainder of type X collagen could be solubilized only after extraction with a NaCl/DTT buffer following extensive proteolytic treatment of the tissues with pepsin. Injection of β APN *in vivo* into the air sacs of 17-day-old chick embryos also caused a marked increase in the solubility of type X collagen. However, our findings that a substantial portion of newly synthesized molecules remained tissue-bound even after repeated pepsin digestion and became solubilized only after disulphide-bond reduction indicate that stabilization of type X collagen in the matrix is established by at least two different mechanisms. The first mechanism involves the formation of β APN-sensitive inter- and intra-molecular cross-links. The second mechanism, however, is less obvious. On the basis of our results, we postulate that additional tissue stabilization of type X collagen occurs through the formation of a molecular structure with a protein or proteins that are highly cross-linked by disulphide bonds. This structure appears to stabilize the type X collagen molecules in the matrix despite extensive digestion with pepsin and does not allow their solubilization unless the disulphide bonds are reduced.

Characterization of the soluble type X collagen isolated from lathyrictic tissues showed that these molecules were composed entirely of 59 000- M_r polypeptides, and no evidence that they were processed to shorter polypeptides was found by employing pulse-chase experiments. Our results therefore do not support previous reports that type X collagen is proteolytically processed

in the tissue to smaller polypeptide chains (Capasso *et al.*, 1984; Kielty *et al.*, 1985; Ayad *et al.*, 1987). However, our findings that prolonged incubation *in vitro* and storage of tissue extracts resulted in spontaneous proteolysis of the 59 000- M_r chains to yield 49 000- M_r components may explain this discrepancy.

In order to gain further understanding of the molecular structure of type X collagen within the tissue matrix, we examined the effects of chymotrypsin on its extractability. We found that, in contrast with pepsin, incubation with chymotrypsin resulted in a very effective extraction of type X collagen, this perhaps being due to its ability to produce a more widespread proteolytic cleavage of the putative protein network that contributes to stabilization of this collagen in the matrix.

Characterization of the type X collagen molecules extracted with chymotrypsin showed that a large proportion were composed of 40 000- M_r polypeptides. These findings indicated the existence of chymotrypsin-sensitive sites within the triple-helical domain of the molecule. Two-dimensional CNBr-cleavage peptide maps of the chymotrypsin-extracted samples showed the loss of a single peptide (Fig. 5, arrowhead), suggesting that chymotrypsin cleavage was not random but most probably occurred at a specific site within the molecule. In order to identify the chymotrypsin-cleavage site, the amino acid sequence deduced from the nucleotide sequence of the gene (Ninomiya *et al.*, 1986) was examined. The sequence of type X collagen shows the presence of two types of imperfections at several regions within the triple helix (Fig. 5). One type of imperfection is a Gly-Xaa-Gly sequence instead of Gly-Xaa-Yaa; the second type of imperfection contains a Gly-Xaa-Yaa-Xaa-Yaa sequence in place of two Gly-Xaa-Yaa triplets. Out of the five imperfections of the

Fig. 6. Diagram summarizing the possible proteolytic cleavage of type X collagen

(a) The arrowhead shows the location of the possible chymotrypsin-cleavage site. The arrows show the pepsin-cleavage sites at both ends of the triple-helical domain. The location of methionine residues as predicted from the nucleotide sequence (Ninomiya *et al.*, 1986) is shown. The asterisk shows the likely CNBr-cleavage peptide that is lost as a result of chymotrypsin cleavage. The dashes above the triple-helical regions denote short imperfections and the squares denote long imperfections. (b) Schematic diagram of possible chymotrypsin and mammalian collagenase cleavage of type X collagen.

first type, three have a Gly-Phe-Gly sequence and two a Gly-Ile-Gly sequence. Two out of three imperfections of the second type contain Gly-Leu or Gly-Ile, the sequence cleaved by mammalian collagenase within collagenous triple helices, and one contains a Gly-Phe-Thr sequence. The most likely cleavage site for chymotrypsin appears to be the Gly-Phe bond located in the imperfection of the second type closest to the *N*-terminus (Fig. 6, arrowhead). Cleavage at this site would yield a product of M_r approx. 40 000. The loss of a single CNBr-cleavage peptide also supports this possibility (CNBr-cleavage peptide marked with an asterisk in Fig. 6a). Other possible sites are less likely since they would yield shorter polypeptides.

From the CNBr-cleavage peptide mapping and nucleotide sequence data, a tentative model for the cleavage of type X collagen with chymotrypsin has been constructed (Fig. 6b). The diagram shows the possible cleavage sites of the 59 000- M_r form of type X collagen to the 45 000- M_r form by pepsin and to the 40 000- M_r fragment exclusively by chymotrypsin as well as the three fragments resulting from mammalian collagenase digestion (Schmid *et al.*, 1986). However, sequence studies of the chymotrypsin digestion products will be necessary to determine exactly which bonds within the $\alpha 1(X)$ collagen helix are cleaved.

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