Maturation of Collagenous Tissue: Specific In Vivo Proteolytic Cleavage of Only α1(I) Chains.

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Summary: A partially cleaved α1(I) chain, α1χ, has been isolated from earlier synthesized or older (acid-extracted) guinea pig skin collagen. The α1χ component is shown to be absent from the newly synthesized (neutral salt-extracted) collagen. This degradation is a result of specific in vivo proteolytic sission of α1(I) chain since the soluble collagen has no corresponding product from the α2 chain. The in vivo proteolytic cleavage is believed to result from processes related to natural physiological maturation of collagenous tissue.

A number of fractions of soluble collagen may be extracted from skin: a) newly synthesized (salt extractable); and b) earlier synthesized or older collagen (successive dilute acid extractable). Several investigators have reported chromatographic heterogeneity of chains from these soluble collagens. Three detectable α chains were reported in soluble collagen of rat skin, calf skin (1-3) and chick bone (4). The α1(I) chain was reported to be separated into an aldehyde [$\alpha 1(I)^{ald}$][3] and a lysine [$\alpha 1(I)^{lys}$] form from soluble rat skin collagen when chromatographic conditions were favorable (5). In the latter report, separation of the two forms of the α2 chain also occurred under the same conditions. However, it is obvious from the data presented that the separation in the latter report was not consistent.

Another α1(I) component, α1', was reported to exist in the newly synthesized (neutral-salt-soluble) and the earlier synthesized or older (acid-soluble) collagen from 3-week old chick skin (7). It was separated from the acid soluble extract and α1(I)' was identical in all respects with α1(I) chain except that it lacked only the 19 residue sequence of the α1(I) chain that is represented by α1(I)CB0 and α1(I)CB1 (7). In addition the relative proportions of α1(I)' to α1(I) increased

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[3]Aldehyde form of α1(I) chain - Lysine residue in N-terminal non-helical region is converted to a residue of α-amino-adipic-δ-semialdehyde by lysyl oxidase.

with successive acid extractions. On gel electrophoresis by the method of Nagai (7), α1(I)' migrated with the α1(I) chain (6).

We wish to report here the chromatographic separation of at least five α components from acid-soluble guinea pig skin collagen and the isolation of the three α1(I) components $\alpha 1_a$, $\alpha 1_b$ and $\alpha 1\chi$, the first two being the aldehyde and lysine form of an α1(I) chain respectively. The $\alpha 1\chi$ component is shown not to be present in the neutral-salt extract. It arises from specific in vivo cleavage of an α1(I) chain in the normal process of physiological maturation.

Methods and Materials

Shaven minced skins from 200 g male guinea pigs were extracted twice with ice cold 0.4 M NaCl, 0.05M Tris-HCl at pH 7.4 and three successive times with ice cold 1% acetic acid. All the neutral salt extractions were performed in the presence of inhibitors of both neutral and acidic proteolytic enzymes. The concentration of the enzyme inhibitors were 0.001M benzamidine, 0.001M phenymethylsulfonyl fluoride, 0.1mM iodoacetamide, and 0.02M EDTA for neutral salt extraction; and 0.001M phenylmethylsulfonyl fluoride, 0.005M benzamidine and 0.1mM iodoacetamide for the acid extractions. The two neutral salt soluble extracts were combined but the three successive 1% acetic acid soluble extracts were kept separate. The collagen was purified by two salt precipitations and two reconstitutions as fibrils with resolution in 0.5% acetic acid after each precipitation and reconstitution.

The collagen chains were separated and isolated by slight modification of the CM-52 cellulose chromatography method described earlier (8). The collagen chains were also chromatographed on Bio-Gel A 5M agarose columns in 1M $CaCl_2$, 0.05M Tris-HCl at pH 7.5 as also described (13).

Gel electrophoresis in sodium dodecylsulfate (9) and in 6M urea in a modified Sakai and Gross method (10) without spacer or sample gels was used to monitor the gelatin chains. The gels were stained with Comassie Blue and the quantity of each band was estimated by planimetry of the profile obtained using a gel scanner on the Gilford Model 2400 spectrophotometer.

Amino acid composition of the isolated α1(I) components was determined in triplicate using an automatic amino acid analyzer after hydrolysis in vacuo under N_2 with 3N tosyl acid at 110°C for 48 hrs (11). Quantitative determination of one nanomole of amino acid was possible.

Results and Discussion

The gel electrophoresis run in 6M urea of the salt-soluble (gel 1) and the second acid-soluble (gel 2) extracts of the purified collagen preparations are shown in figure 1. The band labeled $\alpha 1\chi$ is also present in the first acid-soluble extract although a smaller amount is present. Note that the $\alpha 1\chi$ band is absent

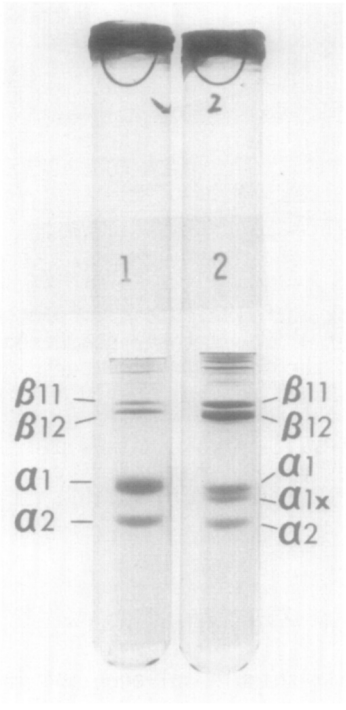

Figure 1: Polyacrylamide gel electrophoresis in 6M urea of purified collagen extracts prepared in the presence of neutral and acidic protease inhibitors. The running gel concentration was 6.8% with a bisacrylamide to acrylamide ratio of 1 to 37.5. No sample and spacer gels were used. Constant current at 1.5 milliamp per tube (I.D. 5 mm) for 3 hrs.
Gel 1 - Neutral-salt-souble guinea pig skin Type I collagen with all the Type III removed.
Gel 2 - Second 1% acetic-acid-soluble guinea pig skin collagen.

from the salt-soluble extracts (gel 1). On gel electrophoresis in sodium dodecyl-sulfate no separation of $\alpha 1\chi$ from $\alpha 1$ occurs in the acid-soluble collagen and both salt-soluble and acid-soluble collagen look qualitatively identical to gel 1. Only these variations were noted: there is quantitatively more α in the salt-soluble, and the mobility of the chains are different in both systems of gel electrophoresis, as would be expected. It was found that the proportion of the $\alpha 1\chi$ band increased with each acid-soluble extract. The three successive 1% acetic acid extracts contained 3.5%, 31% and 38% of $\alpha 1\chi$ of the total amount of $\alpha 1$ chain in the acid-soluble collagen, respectively, while the salt-soluble collagen possessed none.

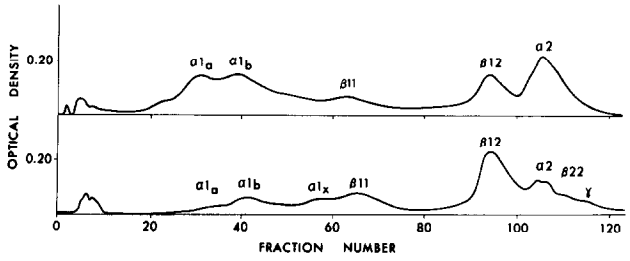

Figure 2: CM52 - carboxymethylcellulose chromatography on 2.5 x 12 cm jacketed column maintained at 41°C. A three-chambered varigrad was used with 600 ml of starting buffer in the first two chambers and 600 ml of limit buffer in the last. Flow rate was 90 ml per hr. and 10 ml fractions were collected. The column was monitored at 230 mμ with a Gilford 2400 spectrophotometer.
Upper pattern - Neutral-salt-soluble guinea pig skin Type I collagen with all the Type III removed.
Lower pattern - Second 1% acetic-acid-soluble guinea pig skin collagen.

The elution profiles from CM-52 cellulose chromatography appear in figure 2. The upper curve represents salt-soluble collagen and indicates that α1(I) has two forms: $\alpha 1_a$ and $\alpha 1_b$ and is in accordance with what has been observed earlier (5). A precursor-product relationship between $\alpha 1(I)^{lys}$ and $\alpha 1(I)^{ald}$ had also been demonstrated earlier (5). The same type of result is seen to occur with the α2 peak which also contains some γ component as revealed by gel electrophoresis. This observation was also seen previously when it was reported that the broadened heterogeneous α2 peak contains $\alpha 2^{lys}$ and $\alpha 2^{ald}$ (5). The lower (Fig. 2) pattern represents the profile of the second acid-soluble extract. The amounts of the $\alpha 1_a$, $\alpha 1_b$ and α2 peaks are less and the last exhibits some resolution of the $\alpha 2^{lys}$ and $\alpha 2^{ald}$ chains. A new peak appears on the leading edge of the β11 component labeled $\alpha 1\chi$. It also appears in the first acid-soluble extract but is more exaggerated in the second and third acid-soluble extracts.

$\alpha 1_a$, $\alpha 1_b$ and $\alpha 1\chi$ were isolated by rechromatography and gel filtration on a calibrated column of Bio-Gel A 5M agarose. No differences in molecular weight were detectable and all three were calculated as 93,000 daltons. As judged by gel electrophoresis in sodium dodecylsulfate and 6M urea, column chromatography and gel filtration, all three isolated chains were apparently homogeneous.

Table 1

Amino Acid Compositions of α1 Chains

Isolated From Soluble Guinea Pig Skin Collagen.

	Res/1000 total residues[a]				
	[α1(I)^{ald}]	[α1(I)^{lys}]			
	$\alpha 1_a$	$\alpha 1_b$	$\alpha 1\chi$	α1(I)[d]	α1(I)[e]
4-Hyp	90	86	84	96	99
Asp	47	48	45	45	46
Thr[b]	18	17	16	16	16
Ser[b]	41	40	40	43	44
Glu	79	81	81	78	78
Pro	115	108	114	125	129
Gly	331	331	344	333	342
Ala	132	128	124	119	122
Val	16	16	16	16	15
Met[c]	6.5	6.5	3.8	6.5	6.5
Ile	8.5	8.1	8.5	8	7.9
Leu	20	20	20	20	20
Tyr	3.0	2.3	0	3.7	3.8
Phe	12	12	11	12	12.2
OHlys	5.9	5.9	5.2	5.1	5.1
Lys	29	31	31	29	29
His	2.1	2.3	2.1	2.0	2.0
Arg	52	52	50	49	49

a Actual value listed for less than 10 residues
b Not corrected for destruction on hydrolysis
c Methionine plus methionine sulfoxide
d Recalculated for res/1000 residues from ref. 12
e Ref. 12 - Composition of the α1 chain

The amino acid compositions of $\alpha 1_a$, $\alpha 1_b$ and $\alpha 1\chi$ are presented in Table 1.

Note that tyrosine is completely absent from $\alpha 1\chi$ and there is less methionine. When four times the amount of $\alpha 1\chi$ as of $\alpha 1_a$ or $\alpha 1_b$ was subjected to amino acid analyses, tyrosine was still not present, although only 0.2 residue would have been detected. The amino acid composition of the $\alpha 1_a$ and $\alpha 1_b$ are in good agreement with values reported for $\alpha 1$(I) by others (12).

The amino acid composition of $\alpha 1\chi$, except for the lack of tyrosine and less methionine, clearly identifies it as an $\alpha 1$(I) chain. The absence of tyrosine suggests that cleavage of some N- and C-terminal non-helical residues have occurred since two tyrosines exist in $\alpha 1$(I)CB1 and one is in $\alpha 1$(I)CB(7,6) of soluble guinea pig skin collagen (12). A partially cleaved $\alpha 1$(I) chain, $\alpha 1'$, has been reported earlier from chick skin collagen as having only the $\alpha 1$(I)CB0 and $\alpha 1$(I)CB1 peptides missing (6). The data in the latter report suggest that the partially cleaved $\alpha 1$(I) chain, $\alpha 1'$, also appears in the newly synthesized (salt-extracted) as well as the earlier or older (acid-extracted) collagen (6). The absence of tyrosine from $\alpha 1'$ could not be ruled out because of cross-contamination with $\alpha 1(I)^{ald}$ and $\alpha 1(I)^{lys}$. The latter paper (6) could not determine whether the presence of the cleaved $\alpha 1$(I) chain, $\alpha 1'$, was due to: a) *in vivo* proteolytic removal of the N-terminal non-helical region of the molecule, or b) cleavage *in vitro* during extraction and purification by nonspecific proteolytic enzymes.

In this report the extractions and purifications were carried out in the presence of neutral and acidic proteolytic enzyme inhibitors. The partially cleaved $\alpha 1$(I) chain, $\alpha 1\chi$, does not appear to be present in the newly synthesized (salt-extracted) collagen but only appears in the earlier synthesized or older (acid-extracted) collagen. In view of these facts we would like to propose that $\alpha 1\chi$ results from *in vivo* proteolytic cleavage of collagen at the N- and C-terminal portions of the molecule and that this is related to the natural physiological maturation of the tissue. Furthermore no partially cleaved $\alpha 2$ chain was evident on gel electrophoresis in 6M urea and CM-cellulose chromatography and therefore this *in vivo* mechanism is specific only for $\alpha 1$(I) chains.

Since a precursor-product relationship has already been established for $\alpha 1_b$ [$\alpha 1(I)^{lys}$] and $\alpha 1_a$ [$\alpha 1(I)^{ald}$], respectively (5), and $\alpha 1\chi$ is derived from $\alpha 1(I)$ chains, we would like to suggest that $\alpha 1_a$ [$\alpha 1(I)^{ald}$] and $\alpha 1\chi$ have a precursor-product relationship, respectively, in in vivo maturation.

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