IDENTIFICATION AND PARTIAL CHARACTERISATION OF THE NON-COLLAGENOUS AMINO- AND CARBOXYL-TERMINAL EXTENSION PEPTIDES OF CARTILAGE PROCOLLAGEN

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Summary: Procollagen secreted by embryonic chick cartilage cells has been partially purified by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography. Analyses of the products of digestion by human rheumatoid synovial collagenase and bacterial collagenase indicated the presence of non-collagenous peptide sequences at the N- and C-termini. Both regions were found to incorporate [^{35}S]cystine but inter-chain disulphide bonds were restricted to a C-terminal location. Electrophoretic analysis gave apparent molecular weights of 13000 and 36000 daltons for the respective N- and C-terminal extensions.

Introduction. Recent studies have established that cartilagenous tissues contain a genetically-distinct collagen with the chain composition $[\alpha 1(II)]_3$ which is synthesised in a precursor form having non-collagenous peptide extensions which have been tentatively assigned to an N-terminal location (1-3). It is now clear, however, that attempts to characterise procollagens have frequently been hampered by their susceptibility to limited non-specific proteolysis but where appropriate precautions have been taken it has been possible to demonstrate that type I procollagen comprises pro-α chains having non-collagenous peptide sequences of approx. 20 000 and 35 000 daltons at the N- and C-termini respectively (4-7). In this paper we present data demonstrating that type II procollagen similarly contains N- and C-terminal extensions.

Experimental. Cells isolated from the sterna of 17-day old chick embryos (1,3) were incubated at a concentration of approx. 10^7 cells/ml

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of modified Krebs medium containing 20% (v/v) dialysed foetal calf serum. Incubations were conducted for 3h at 37°C in the presence of [5-^{3}H]proline (10 Ci/mmol) and [^{35}S]cystine (49 Ci/mmol). The cells and medium were then separated by centrifugation at 1200g for 10 min and three proteinase inhibitors were immediately added to the medium to final concentrations of 1.7 mM-phenyl-methyl-sulphonyl fluoride (PMSF), 10 mM-N-ethylmaleimide (NEM) and 25 mM-EDTA. All subsequent steps were conducted at 4°C and preliminary purification of the labelled procollagen was achieved by precipitation with $(NH_4)_2SO_4$ at 30% saturation. The precipitate was collected by centrifugation at 18000 g for 60 min and redissolved in 0.1M-Tris-HCl buffer, pH 7.4, containing 0.4M-NaCl, 1.7 mM-PMSF, 10 mM-NEM and 25 mM-EDTA. Undissolved material was removed by centrifugation, the procollagen reprecipitated with $(NH_4)_2SO_4$ and then dissolved in 50 mM-Tris-HCl buffer (pH 8.3 at 22°C) containing 2M-urea, 4 mM-EDTA and 0.1% Triton X-100 and dialysed overnight against this buffer. After a second dialysis against buffer from which EDTA was omitted the sample was applied to a DEAE-cellulose column (1.6 x 10cm) and eluted with a 0 - 0.3M -NaCl gradient (6). Fractions (1.35 ml) were collected and an aliquot assayed for radioactivity in a Packard 2450 Tri-Carb Spectrometer using a toluene-based scintillation fluid.

Studies to determine the size and location of the extension peptides of procollagen were carried out using bacterial collagenase and a highly purified preparation of human rheumatoid synovial collagenase (8). The bacterial enzyme (Worthington Biochemicals Ltd., CLSPA grade) was further purified (9) and incubations were carried out for 90 min at 37°C in 0.05M-Tris-HCl buffer, pH 7.6, containing 5mM-$CaCl_2$ and 2.5mM-NEM. Samples were heated at 90°C for 2 min in the presence of 1% (w/v) sodium dodecylsulphate (SDS) containing either 1% (v/v) 2-mercaptoethanol or 0.1M-iodoacetamide and subsequently analysed by SDS-gel electrophoresis (10) or gel filtration on 8% agarose (Biogel A-1.5m, 200-400 mesh) eluted with 0.02M-Tris-HCl buffer, pH 7.4, containing 0.1% SDS and 0.02% sodium azide (3). Incubations with the human collagenase were carried out in 20 mM-Tris-HCl buffer, pH 8.0, containing 0.17M-NaCl and 10 mM-$CaCl_2$ at 25°C for 24h, and the products of digestion analysed as above.

Results. Previous studies have demonstrated that cartilage cells secrete type II procollagen (1,3) and provided suitable precautions are taken the procollagen can be recovered from the medium as a disulphide-linked trimer of pro-α1(II) chains each having a molecular weight of approx. 140 000 daltons (7). These studies have also shown that when cartilage cells are incubated with radioactive proline, procollagen is the major labelled species found in the medium. Thus, when cells were incubated with [^{3}H]proline and [^{35}S]cystine and the medium proteins analysed by DEAE-cellulose chromatography following $(NH_4)_2SO_4$ precipitation, the elution position of procollagen was identifiable as the major ^{3}H-containing peak eluting at a NaCl concentration of 0.10M

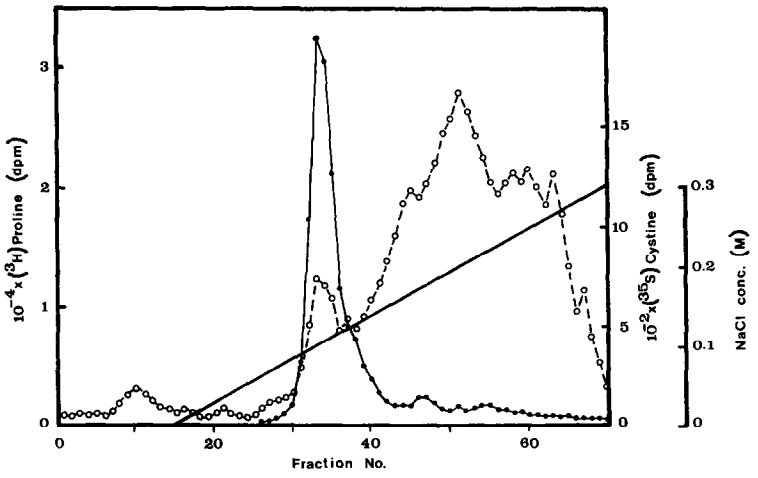

Fig. 1. DEAE-Cellulose chromatography of partially purified cartilage procollagen.
Proteins secreted during a 3h incubation with [^{3}H]proline and [^{35}S]-cystine were precipitated in the presence of protease inhibitors by the addition of $(NH_4)_2SO_4$ to 30 per cent saturation and then analysed by DEAE-cellulose chromatography.
●——●, elution of [^{3}H]proline; o - - - o, elution of [^{35}S]cystine.

(Fig. 1). Coincident with this [^{3}H]procollagen was a peak of [^{35}S]-cystine (Fig. 1) and when the peak (fractions 32-35) was analysed by gel electrophoresis after reduction with mercaptoethanol the ^{35}S and ^{3}H labels were found to migrate in a single peak having a mobility identical to that of pro-α1(II) chains (Fig. 2a).

The fractions recovered from the DEAE-cellulose column were digested with either human rheumatoid synovial collagenase or bacterial collagenase after dialysis against the appropriate enzyme buffer. When cartilage procollagen was digested with the human enzyme and the products analysed by gel electrophoresis under non-reducing conditions two major components, each containing [^{3}H]proline and [^{35}S]cystine, were observed to migrate in positions in the region of pro-α chains and α-chains respectively (Fig. 2b). Following reduction, the band in the α region [designated pro-α1(II)$_A$] was unchanged but the higher molecular weight component was absent and a new peak [designated pro-α1(II)$_B$] was observed

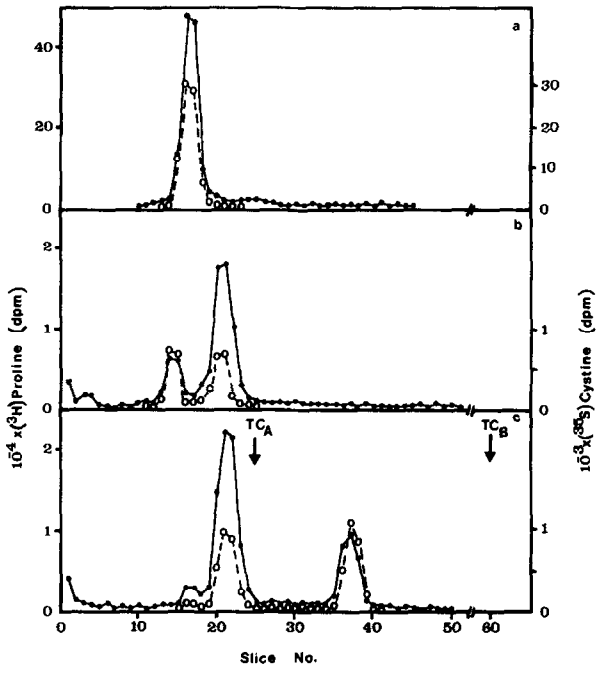

Fig. 2. Electrophoresis of dual-labelled cartilage procollagen treated with human rheumatoid synovial collagenase on 7.5% polyacrylamide gels.
(a) Procollagen denatured in SDS and reduced with mercaptoethanol.
(b) Procollagen denatured in SDS and alkylated with iodoacetamide after digestion with human collagenase.
(c) Procollagen denatured in SDS and reduced with mercaptoethanol after digestion with human collagenase. The migration positions of TC_A and TC_B fragment of α1(II) chains are indicated.
●——●, [^{3}H]proline; o- - - o, [^{35}S]cystine.

in a position between markers of TC_A and TC_B fragments derived from cartilage collagen (Fig. 2c). Because collagenous proteins behave anomalously on SDS-gel electrophoresis and as these components are hybrids of collagenous and non-collagenous sequences no definitive molecular weights could be assigned by this technique. However, when the molecular weights of pro-α1(II)$_A$ and pro-α1(II)$_B$ were determined by SDS-agarose gel filtration values of 90000 and 62000 daltons respectively were obtained (Fig. 3).

In order to evaluate the molecular size of the non-collagenous

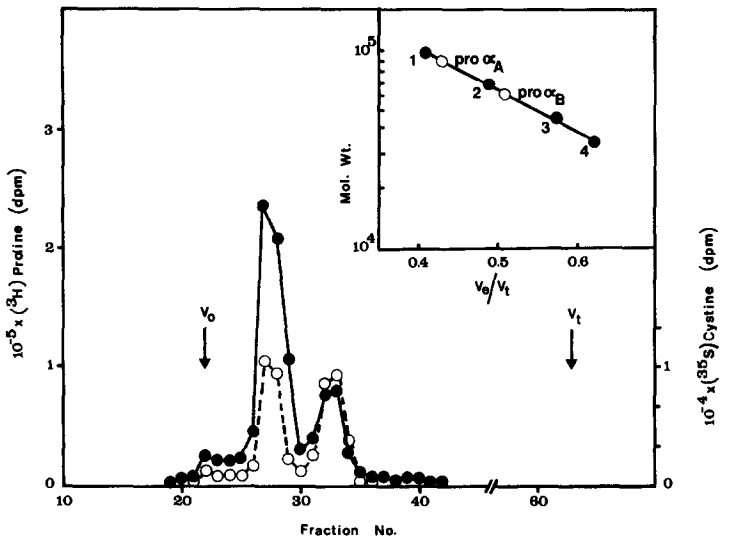

Fig. 3. Gel filtration on SDS-agarose of dual-labelled cartilage procollagen after digestion with human rheumatoid synovial collagenase. Procollagen was incubated with the human enzyme for 24h at 25°C and the sample was then denatured in SDS and reduced with mercaptoethanol. Analysis was carried out on Bio-Gel A-1.5m (column size 90 x 1.6 cm) and fractions (2.8 ml) collected and assayed for radioactivity. The void volume (V_o) and total volume (V_t) of the column are indicated. ●——●, elution of [^{3}H]proline; o- - -o, elution of [^{35}S]cystine. INSET: Calibration of SDS-agarose column using standards 1, collagen α-chains; 2, bovine serum albumin; 3, ovalbumin; 4, pepsin.

sequences in cartilage procollagen, purified preparations of dual-labelled precursor were digested with bacterial collagenase. Analysis by gel electrophoresis under reducing conditions revealed two [^{35}S]cystine-labelled peaks having molecular weights of 36000 and 13000 daltons (Fig. 4). The distribution of [^{3}H]proline-labelled peptides (not shown) was very similar giving a peak coincident with the larger [^{35}S]cystine-labelled component but the region corresponding to molecular weights of 5-15000 daltons was more complex presumably because of incomplete digestion of the α-chain fragments. It should also be noted that electrophoresis under non-reducing conditions revealed a dual-labelled peak of approx. 100000 daltons which was found to represent a disulphide-linked trimer of the larger molecular weight extension (Fig. 4).

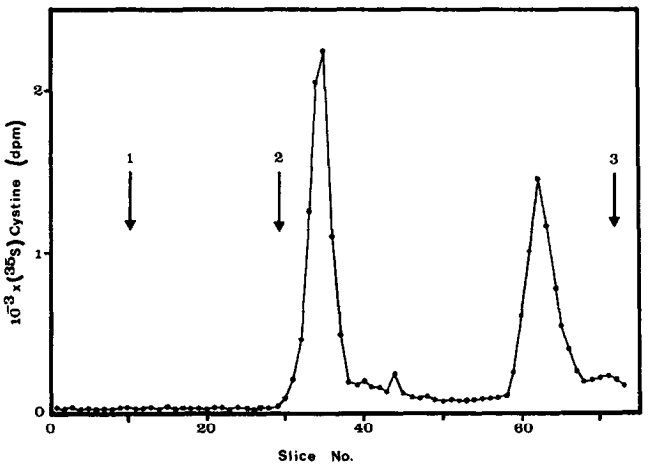

Fig. 4. SDS polyacrylamide gel electrophoresis of cartilage [^{35}S]-procollagen treated with bacterial collagenase.
Procollagen was incubated with purified bacterial collagenase for 1.5h at 37°C and the sample was then denatured in SDS and reduced with mercaptoethanol. Electrophoresis was carried out on 11% polyacrylamide gels and the molecular weights of the procollagen-derived [^{35}S]peptides estimated by reference to a calibration curve determined using several standard proteins analysed under identical conditions. The migration positions of the unreduced C-terminal peptides, ovalbumin (mol. wt. 45000 daltons), and bromophenol blue are labelled 1, 2 and 3 respectively.

Discussion: The human collagenase employed in this study has been shown to cleave type II collagen at a specific locus three quarters of the length of the molecule from the N-terminal end to yield two triple helical moieties, TC_A and TC_B, comprising α1(II) chain fragments of approx. 74000 and 21000 daltons respectively (11). When cartilage procollagen was digested with this enzyme and the products examined by gel electrophoresis under reducing conditions, two components [pro-$\alpha1(II)_A$ and pro-$\alpha1(II)_B$] were obtained, neither of which corresponded with the migration position of the α-chain fragments of TC_A and TC_B (Fig. 2c). Since the molecular weights of pro-$\alpha1(II)_A$ and pro-$\alpha1(II)_B$ were estimated to be 90000 and 62000 daltons respectively (Fig. 3) it is clear that pro-$\alpha1(II)_B$ is too small to be the product amino-terminal to the site of collagenase cleavage and must therefore represent the carboxyl-terminal fragment of cartilage

procollagen. These observations suggest that additional amino acid sequences are present at both the N- and C-termini and that the C-terminal fragment contained a larger extension than the N-terminal fragment.

Isotopic labelling studies demonstrated that both extensions incorporate [^{35}S]cystine, an amino acid not found in α1(II) chains. However, inter-chain disulphide bonds appear to be restricted to the C-terminus for only the pro-α1(II)$_B$ fragment had a decreased mobility under non-reducing conditions when it migrated in the region of pro-α1(II) chains (Fig. 2b). Analyses of the [^{35}S]cystine-labelled peptides isolated from digests of cartilage procollagen with bacterial collagenase (Fig. 4) indicated that the N- and C-terminal extensions had molecular weights of 13000 and 36000 daltons respectively. These values compare with 18-20000 and 33-37000 daltons determined for the N- and C-terminal extensions of chick cranial bone procollagen (5,6). The lower value obtained for the N-terminal extension of cartilage pro-α chains may be a consequence of either partial proteolysis during the isolation and purification procedures or the presence of bacterial collagenase-susceptible sequences in this peptide (12). Alternatively, the possibility of molecular size differences between procollagen types I and II cannot be excluded and it is noteworthy that a non-collagenous glycopeptide (mol. wt. 13200 daltons) has recently been isolated from chick cartilage procollagen (13).

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