STUDIES ON PROTOCOLLAGEN: IDENTIFICATION OF A PRECURSOR OF PROTO α1

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SUMMARY. In the presence of α,α-dipyridyl chick calvaria accumulate a hydroxyproline and hydroxylysine deficient collagen, protocollagen. In addition to components with the properties of proto α1 and α2 chains, a component was identified that has the properties of nonhydroxylated α1 from a precursor of collagen and is therefore designated protopro α1.

Recently we reported that an unusual form of collagen is secreted by fibroblasts in culture (1). In contrast to the typical collagen molecule, this protein is soluble under physiological conditions and contains components of higher molecular weight than α chains. Following incubation with a protease it had the properties of a typical collagen. We suggested that the soluble protein is a precursor of the collagen found in fibers, that is a procollagen, and functions in transport from the cell to the site of fiber formation. Presumably, in culture its conversion to precipitable collagen is impaired. To obtain further evidence for precursors of collagen we have studied protocollagen produced by chick calvaria in vitro. This protein accumulates in cells when the enzymes that hydroxylate proline and lysine in collagen are inhibited (2,3,4).

Protocollagen extracted from tissues cultured under anaerobic conditions or in the presence of chelating agents has been studied previously (5,6,7,8, 9). In our studies protocollagen from a similar system was chromatographed under denaturing conditions and it was found to contain a component with the characteristics of a precursor of the proto α1 chains.

MATERIALS AND METHODS. Twenty calvaria from 17-day-old chick embryos were incubated in 20 ml of a modified version of Eagle's medium as previously

described (10). β-Aminopropionitrile (100 μg/ml) was added to the media to inhibit cross-linking. α,α-Dipyridyl (1.4 mM/l) was added to some samples to inhibit the hydroxylation of proline and lysine. Proline-3,4-^{3}H (250 μc/sample) was used as a label. Labeled protocollagen was extracted from the calvaria with 0.5% acetic acid.

Molecular sieve chromatography of collagen and protocollagen under denaturing conditions was carried out as described by Piez (11). Chromatography of collagen on carboxymethyl cellulose under denaturing conditions was performed as described by Piez et al. (12), except in some cases the starting buffer was 0.02M K acetate, pH 4.8 plus 1M urea. With this solvent a linear gradient from 0 to 0.1M LiCl was used. Radioactivity was assayed in a commercially obtained counting fluid (Aquasol, New England Nuclear) with a Packard Model 3375 liquid scintillation spectrometer.

Protocollagen-3,4-^{3}H was assayed using proline hydroxylase from newborn rat skin in the tritium release assay of Hutton et al. (13).

Cyanogen bromide digestion of various fractions, with added chick skin collagen or components derived from it, was carried out as described by Epstein et al. (14). The resulting peptides were fractionated on phosphocellulose (15) or CM-cellulose (14).

RESULTS. Preliminary experiments extablished that the calvaria in the presence of α,α-dipyridyl incorporated labeled proline into protein but did not synthesize labeled peptidyl hydroxyproline. To establish that the extracts of calvaria labeled with proline-3,4-^{3}H contained protocollagen, these extracts as well as various fractions obtained by subsequent chromato graphic fractionation were tested as substrates for peptidyl proline hydroxylase. In the assay used (13) the formation of hydroxyproline is accompanied by a proportional release of tritium from the protein. In the presence of an excess of peptidyl proline hydroxylase, the tritium released from the protein is proportional to the total protocollagen present. As judged by this assay, acetic acid extracted essentially all of the proto-

collagen synthesized by the calvaria (not shown).

To investigate the size and heterogeneity of the components of protocollagen, a portion of the calvaria extract was applied to a molecular sieve column under denaturing conditions. A single major radioactive peak emerged from the column in the region where the α chains of lathyritic rat skin collagen chromatographed (Fig. 1). The material in this peak was isolated free of salt and tested as a substrate for peptidyl proline hydroxylase in the tritium release assay outlined above. As judged by this assay, the major radioactive peak contained protocollagen components (not shown).

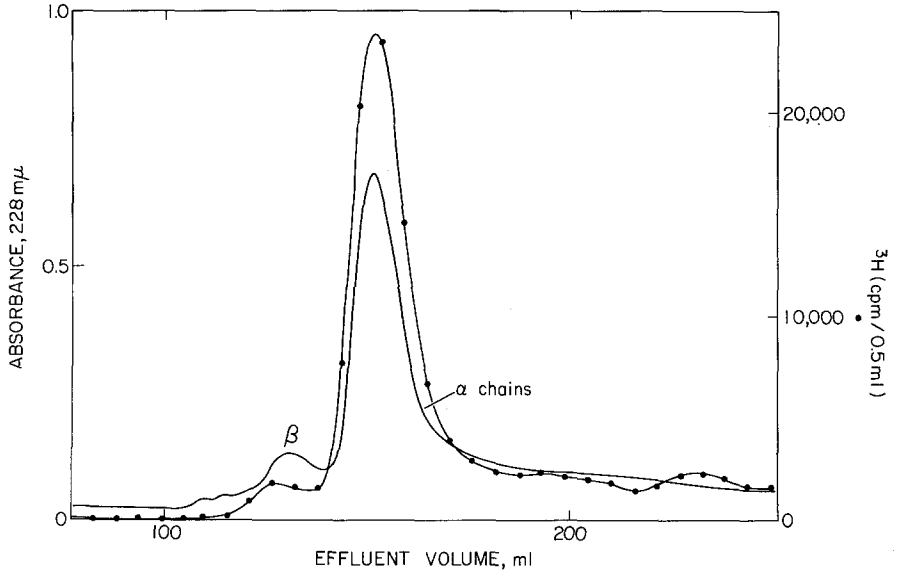

Fig. 1. Molecular sieve chromatography (Bio-Gel A 1.5 column 180 x 1.5 cm) of protocollagen isolated from chick calvaria labeled with proline-3,4-^{3}H in the presence of α,α-dipyridyl and β-aminopropionitrile (o). Denatured lathyritic rat skin collagen has been added to mark the position of the α and β components of collagen (line without points). Column was eluted at a flow rate of 7 ml/hr.

Further characterization of protocollagen was carried out by chromatographic techniques. When applied to CM-cellulose under the standard conditions used for collagen components, a large part of the radioactivity in the extract (Fig. 2) eluted at the front. Less radioactivity chromatograph-

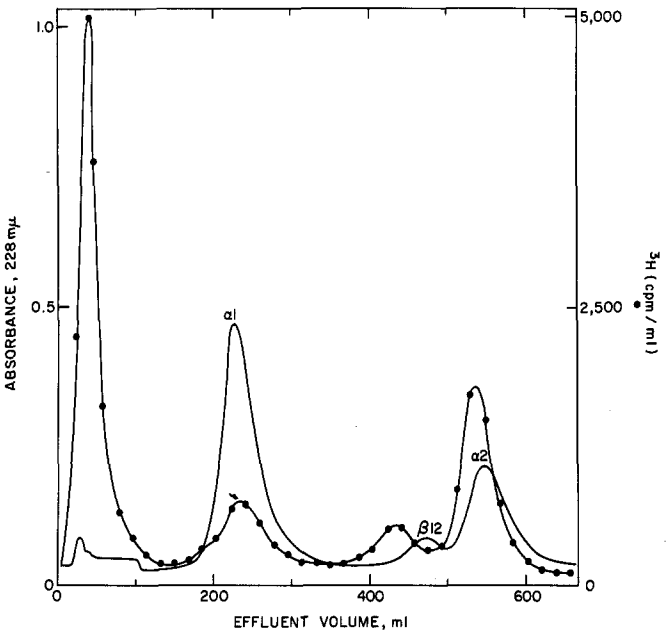

Fig. 2. CM-cellulose chromatogram of protocollagen components (o) and denatured lathyritic rat skin collagen (line without points). Elution was achieved with a linear salt gradient established with 400 ml of starting buffer (0.06M K acetate) and 400 ml limit buffer (0.06M K acetate containing 0.1N LiCl). The column was eluted at a flow rate of 200 ml/hr.

ed in the region of α1 than α2, and the ratio of α1- to α2-type material was much lower than observed with added carrier collagen. However, when the large peak at the front was tested for substrate activity with peptidyl proline hydroxylase, it was found to contain material reacting like protocollagen. When another portion of the sample was chromatographed with urea and at a lower initial buffer concentration, a radioactive peak emerged before as well as with carrier α1 and the amount of radioactivity eluting with carrier α2 was increased (Fig. 3).

Because of their chromatographic behaviour on molecular sieve and CM cellulose columns and their activity as substrates for proline hydroxylase, the proteins eluting with carrier α1 and α2 are presumed to be proto α1 and proto α2. The possibility that the material preceding proto α1 was a precursor of proto α1 was investigated using a pulse-chase approach.

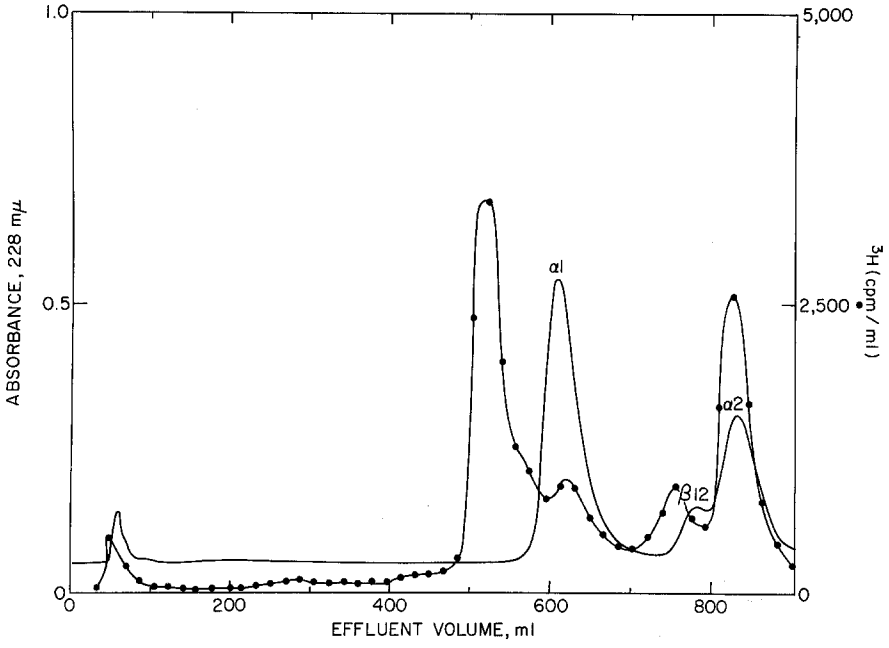

Fig. 3. CM-cellulose chromatogram of protocollagen components (o) and denatured lathyritic rat skin collagen (line without points). The sample was identical to the one described in Figure 2. In this experiment, elution was achieved with a linear salt gradient established by 400 ml of starting buffer (1 M urea, 0.02 M K Acetate, pH 4.8) and 400 ml of limit buffer (1M urea, 0.02M K acetate containing 0.1 N LiCl).

Duplicate flasks of calvaria were labeled for three hours in the presence of α,α-dipyridyl. At the end of this period, one sample was removed and the protocollagen was extracted from the tissue as described above (Pulse). The media in the other flask was replaced with non-radioactive media and the incubation continued for an additional 3 hours. Protocollagen was also prepared from these calvaria (Chase). The radioactive profile of these samples are shown in Fig. 4. In the Pulse sample the proto α1 and presumed precursor peaks were about the same size. After the chase period there was a marked reduction in the size of the precursor peak and a corresponding increase in the proto α1 peak as would be expected for a precursor-product relationship.

Peptides were prepared from protein from the precursor α1, α1 and α2

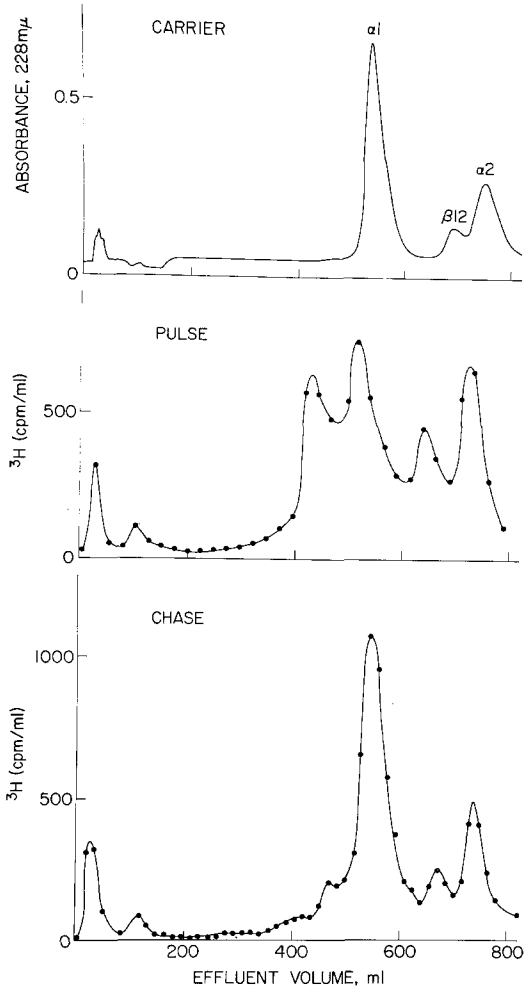

Fig. 4. CM-cellulose elution pattern of absorbance of denatured carrier rat skin collagen (top) and pattern of radioactivity of protocollagen components (middle and bottom). Protocollagen was isolated from chick calvaria following a pulse label for 3 hours with proline-3,4-^{3}H (middle) and after a subsequent incubation for 3 hours with nonradioactive medium (bottom).

peaks of protocollagen and from authentic α chains by digestion with CNBr. The radioactive profile of the peptides from proto α1 and its precursor closely followed the absorbance of CNBr-derived peptides from chick skin collagen α1 both on phosphocellulose and CM-cellulose, except that the precursor material contained a peptide present in only trace amounts in proto α1. Peptides from proto α2 cochromatographed with the peptides from

chick skin collagen α2. Details of these experiments will be published later.

DISCUSSION. Our studies indicate that two forms of the α1 chain occur in protocollagen preparations from chick bone. Lukens (9) has reported similar results previously. He postulated that these two peaks were nonhydroxylated α1 chains that differed in that a lysyl residue in one is present as an aldehyde in the other. Heterogeneity of this type in α1 and α2 chains is well known. The lysine-aldehyde conversion represents the first step in cross-linking in this protein. However, our studies support a different explanation. First, β-aminopropionitrile, a known inhibitor of the enzyme oxidizing lysyl residues in collagen (10), was added to the media in which the calvaria were incubated so that significant amounts of the aldehyde form would not be present (16). Second, radioactivity in the early peak in the pulse-chase experiment moved to the proto α1 peak. The lysine-aldehyde conversion causes movement in the opposite direction since the precursor lysine form is more basic (17). These facts support the interpretation that the peak preceding proto α1 is a precursor of proto α1. This component is then a double precursor of α1 and is designated protopro α1. Presumably, similar precursors exist for α2.

There are some differences between the precursor observed in protocollagen and the procollagen synthesized by human fibroblasts in culture. In particular, after denaturation the fibroblast protein contains components of greater molecular weight than are observed in protocollagen. The explanation for this difference is unclear.

It is possible that protopro α1 contains peptide material not present in proto α1 since CNBr digests of this chain contained a peptide present in only trace amounts in proto α1. We have also observed (unpublished observations) that the putative precursors observed in both the protocollagen and fibroblast systems are more soluble than collagen. These differences suggest that the physiological precipitation of collagen is

controlled by enzymatic modifications of the precursor at the site of fiber formation. A model of this type has been proposed by Speakman (18) who suggested that the extra peptide material on the pro $\alpha 1$ and $\alpha 2$ chains could facilitate the exact alignment of the chains in the assembly of the macromolecule. Presumably another function would be to determine the chain composition of the collagen.

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