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Enzymes Converting Procollagens to Collagens

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Conversion from procollagen to collagen is a specific process that is a requirement for proper alignment of collagen molecules to form functional fibers. This process is catalyzed by at least three structurally and functionally distinct enzymes cleaving collagen types I–III. The cleavage processes possibly taking place in the more recently discovered collagen types are not known to any extent at this time.

Two amino-terminal proteinases, one cleaving type I and type II procollagens and the other cleaving type III procollagen, have been purified close to homogeneity, and the more unspecific activity of carboxy-terminal proteinase has been isolated from several tissues. In our experimental model, however, cleavage of the carboxy-terminal propeptides of types I and III procollagen is differently affected by lysine. This suggests the presence of at least two distinct enzymes for the removal of carboxyl-terminal propeptides.

The regulation of the reaction process from procollagen to collagen is not well known at present. The importance of the phenomenon in terms of fibril formation, however, is demonstrated by several elegant studies in vitro; and certain genetic disorders in which this process is defective demonstrate the significance in vivo. Moreover, the factors shown to effect the cleavage process may be potentially beneficial in the treatment of the pathological processes with abnormal collagen accumulation such as fibrosis.

In this paper we briefly review the current knowledge of the converting enzymes, including some very recent findings of our laboratory as well as the evidence presented for the biological significance of the conversion process.

Key words: procollagen conversion, amino-terminal proteinases, carboxy-terminal proteinases

COLLAGEN: STRUCTURE AND BIOSYNTHESIS

Collagens are a genetically and structurally closely related family of connective tissue proteins, which are widely distributed in the tissues. They have a multiplicity of functions; perhaps the most central one is to give structural integrity and tensile strength to the organs [for reviews, see 1–3]. At least ten genetically distinct collagens have been reported so far [4–9]. All known mammalian collagen molecules have a common basic structure: the collagen molecule consists of three polypeptide chains

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(α-chains), which are twisted around each other into a triple-helix conformation [1-2]. In some collagen types the triple-helix is interrupted by globular domains. The helical conformation is due to an exceptional primary structure of collagen α-chains, which are basically built up to a repeating triplet of Gly-x-y, where the y site is often occupied by a proline residue, and the x site by either a proline or a hydroxyproline residue. There are other exceptional structural features in collagen molecules, such as the presence of hydroxylysyl and glycosylated hydroxylysyl residues. These unique structural features confer upon the collagen molecules certain particular properties such as the ability of collagen molecules types I–III to form fibers spontaneously. Other, so-called non-fibrillar collagen types also spontaneously form organized structures, such as the network structure formed by type IV collagen [2]. All collagen molecules and fibers are remarkably resistant to non-specific proteinases.

Initially, the α-chains of collagen are synthesized as high molecular weight precursor forms called preprocollagen α-chains. They contain a short transient hydrophobic amino-terminal, the so-called signal sequence typical to the secretory proteins, which is removed cotranslationally, while the molecule enters the cisternae of the rough endoplasmic reticulum [10–14]. In addition proα-chains still contain large extensions at both the amino- and the carboxy-terminal ends. In the rough endoplasmic reticulum cisternae the proα-chains are further processed co- and post-translationally: Certain proline and lysine residues are hydroxylated by specific, separate enzymes, and hydroxylysine residues are glycosylated again by specific enzymes [15–16]. Disulfide bridges are formed intramolecularly as well as between different proα-chains to form trimeric procollagen molecules [17–20]. After an appropriate number of prolyl residues are hydroxylated, the procollagen molecule assumes a triple-helix conformation, stable at physiological temperatures [1,21]. Furthermore, the extension peptides are glycosylated through the dolichol pathway [15].

After formation of the triple-helix, the procollagen molecules are secreted into the extracellular space via the Golgi complex [1,2]. During secretion, or immediately following it, the majority of amino- and carboxy-terminal propeptides are removed by amino- and carboxy-terminal procollagen proteinases [22–26]. The removal of the extensions of the procollagen molecules is a crucial step in the biosynthesis of collagen. Prevention or disturbance of this removal leads to perturbation of the alignment of collagen molecules and impaired fiber function [27–31]. What other possible consequences of partially prevented cleavage may be is not known at the moment. The disturbances in suggested feedback regulation of cellular collagen synthesis by the free, removed N-propeptides might effect either the quantity or the quality of the secreted collagen molecules [32,42].

In the extracellular space, collagen molecules are further modified by a copper-dependent enzyme, lysyl oxidase, to allow the collagen molecules to form covalent inter- and intra-molecular cross-links, which stabilize the fiber structure [16,33–35].

GENERAL FEATURES IN THE CONVERSION OF PROCOLLAGEN TO COLLAGEN

Several lines of evidence suggest that the conversion of procollagen to collagen requires specific proteinases, and unspecific tissue proteinases are clearly not involved in this process in any significant extent. First, the sequence data of released propep-

tides and tissue collagens show that cleavage always takes place in the constant sequences [36–41]. Second, both amino- and carboxy-terminal trimeric extensions can be found in tissue and cell cultures after the conversion, and the free amino-terminal extension can be demonstrated in serum as well [66-68]. Thus the removal occurs en bloc, this type of cleavage providing very special requirements for the proteinases. This high specificity of the converting enzymes is probably required both for the proper alignment of molecules to form proper collagen fibers and for the specific regulation of the conversion process.

The converting enzymes characterized to any extent so far share certain characteristics [43–48,50]: They are neutral proteinases and require Ca^{+2} for maximal activity. The amino-terminal proteinases are not significantly inhibited either by serum or by several common proteinase inhibitors [43–48]. The carboxy-terminal proteolytic activity seems to be partially or totally inhibited by serum but is also resistant to several inhibitors of proteolysis [50]. Further, at least the amino-terminal proteinases are glycoproteins, and all the reported converting enzymes have exceptionally high molecular weights when compared to other proteolytic enzymes. All the converting enzymes seem to be inhibited by certain divalent cations such as zinc, since this cation inhibits conversion in tissue cultures [52].

In the type I procollagen molecule, the N-terminal propeptides are cleaved prior to the C-terminal propeptides [22,25,49,56]; and in the type III procollagen, the N-terminal propeptides are removed very slowly, and partially processed type III pN-collagen [26] molecules can be found in many tissues [29,30,46]. In type II procollagen no preferential order has been observed in the removal of N- and C-terminal propeptides [28,55]. However, there does not appear to be an obligatory order for the removal of any of the two propeptides from the procollagen molecule in the formation of the collagen fiber.

The removal of the C-terminal propeptides is necessary for fibril formation, whereas molecules with intact terminal N-propeptides can still form fibrils, but they are smaller in diameter than the fibers formed from fully processed collagen molecules [31]. Thin fibrils of both type I and type III pN-collagen are mostly found during embryonic development [30,54]; those formed from type III pN-collagen have been demonstrated to be present in small amounts also in adult tissues [29]. Thus removal of N-propeptides may have an important function in the control of fibril growth. High amounts of unprocessed pN-collagen can be found in tissues in certain genetic disorders of connective tissue (see below).

It has been previously suggested that the removal of C-propeptide of both type I and III procollagens is inhibited by arginine in chick embryo blood vessel cultures [24,26]. Thus it was suggested that the same proteolytic enzyme is responsible for the removal of C-propeptide in both of these collagen types. We have, however, recently studied the effect of lysine on the conversion of procollagen to collagen in aortic smooth muscle cell cultures. In these studies no inhibition of the removal of the carboxy-terminal extension of type III procollagen was observed, while the conversion of type I pC-collagen to collagen was clearly prevented [Halila, Ryhänen, and Peltonen, submitted for publication]. Thus we assume that two different proteolytic enzymes are needed to convert type I and type III pC-collagen to collagen. In favor of this assumption is also the clear difference in the cleavage sites of these two molecules. Type I pC-collagen is cleaved at the Ala-Asp-bond in both the $\alpha 1$(I) and the $\alpha 2$(I) chains, while type III pC collagen is cleaved at the Arg-Asp-bond.

SPECIFIC ENZYMES RESPONSIBLE FOR THE CLEAVAGE

The amino-terminal propeptides of both type I and type II procollagen are cleaved by the same enzyme (type I N-proteinase), which has a molecular weight (MW) of 260,000 as estimated by gel filtration. This enzyme has been purified to homogeneity from chick embryo tendons and also from calf tendons [43,44,47]. The enzyme is an endopeptidase requiring Ca-ions as cofactors; the cleavage site for this enzyme is Pro-Gln [36] in proα(I) and Ala-Gln [37] in proα2(I) chain. For maximal activity, the enzyme requires a substrate with triple-helical conformation. The peptides analogous to the cleavage site in the proα1(I) chain have been synthesized, and they effectively inhibited cleavage by the enzyme [61]. The binding of enzyme activity to concanavalin A suggests the presence of certain carbohydrate moieties. EDTA inhibits the enzyme activity, whereas other inhibitors of proteolytic enzymes, such as N-ethylmaleimide (NEM) and phenylmethyl sulfonylfluoride (PMSF), have no effect [43]. A polyclonal antiserum has been developed that is capable of precipitation and partial inhibition of the enzyme activity [44]. The localization of the enzyme, its possible subunit structure or amino acid content, and its sequence are currently unknown.

Removal of the N-propeptide of type III procollagen requires a specific endopeptidase (type III N-proteinase), clearly separate from the enzyme that cleaves type I and type II procollagens [46–48]. This enzyme does not accept type I or IV procollagen or heat-denatured type III procollagen as its substrate. It removes the amino-terminal extension from both type III pN-collagen and type III procollagen, has a molecular weight of 70,000, and requires Ca^{2+} for maximal activity. The enzyme has been partially purified from calf aortic smooth muscle cell cultures [48] and to apparent homogeneity from human placental tissue [Halila and Peltonen, submitted for publication]. Analogically to type I amino-terminal proteinase, the enzyme activity is inhibited by EDTA and EGTA, but it is not affected by serum or several proteinase inhibitors. Polyclonal antibodies produced against the enzyme protein inhibit enzyme activity effectively. All the evidence collected so far suggests a monomeric structure for the enzyme protein. The amino acid content and sequence as well as the tissue localization of this enzyme are not yet known.

The enzyme or enzymes responsible for the removal of the carboxy-terminal propeptide of procollagens is characterized to a much lesser extent. An enzyme activity cleaving carboxy-terminal extension from types I–III and V procollagen has been isolated from chick embryo membranous bones and chick, human, and murine fibroblasts cultures [24,25,49–51]. The enzyme appears to have a molecular weight of 80,000 daltons. Like the N-proteinases, it requires Ca^{2+} as a cofactor, and thus it is inhibited by metal chelators. Unlike N-proteinases, the enzyme is inhibited with serum, and it is capable of cleaving heat-denatured substrates [50]. The cleavage site in proα1(I) and proα2(I) chains is at the Ala-Asp-bond and in proα1(III) chain at the Arg-Asp-bond [39–41]. The cleavage site in type II procollagen is probably also at the Ala-Asp-bond [57].

In short-term tendon and cartilage cultures certain amino acids, polyamines, and structurally related compounds have been shown to inhibit the removal of carboxy-terminal extensions from both type I and type II procollagen [24,55,58]. Amino acids—such as β-alanine, arginine, hydroxylysine, lysine, and ornithine, but not, for example, alanine, glycine, or leucine—are effective inhibitors. To further map the structural features necessary for inhibition, we studied a number of lysine

derivates in cartilage culture. D- and L-lysine seemed to be equally effective. Other potent inhibitors were S-2-aminoethylcysteine, ϵ-aminocaproic acid, and dipeptides lysyl-valyl, lysyl-leucyl, and lysyl-aspartyl [55,58]. N_α-acetyl-L-lysine is also inhibitory, while N_ϵ-acetyl-L-lysine is not. Thus the critical structural feature of the inhibitory compound seems to be the presence of a free amino group, other than α-amino group, at the end of a hydrocarbon chain. Furthermore, the carbon chain length of the compound appears to be of some importance, lysine being a more effective inhibitor than β-alanine, an amino acid with a shorter carbon chain length. In tissue culture conditions, contrary to the results obtained by isolated and partially purified carboxy-terminal proteinase, neither the removal of the carboxy- nor that of the amino-terminal propeptide is affected by serum. This discrepancy may be due to a poor tissue penetration of serum inhibitors.

There have been reports that acidic proteinases with lower specificity may be involved in the physiological conversion of procollagen to collagen [59]. The major part of conversion, however, occurs extracellularly, in neutral pH. Moreover, this conversion seems to exhibit a remarkable specificity in several in vitro experiments. For example, the fact that the conversion of type I and II pC-collagen can be inhibited with quite specific inhibitors such as arginine, lysine, and ϵ-aminocaproic acid suggests that there are a very limited number of proteolytic enzymes capable of catalyzing the conversion.

GENETIC DISORDERS IN MAN CAUSED BY DEFECTIVE CONVERSION

The removal of both amino- and carboxy-terminal propeptides is crucial for the proper alignment of molecules to form stable and functional fibers. As the removal of the C-propeptide is an absolute requirement for fibril formation, the defect in this process would most probably be lethal. If removal of the N-propeptides from type I procollagen is inhibited, abnormally thin fibers are formed, resulting in well-characterized genetic disorders in man.

Ehlers-Danlos Syndrome Type VII

A genetic defect in type I N-proteinase-enzyme or mutation in the substrate close to the cleavage site of the proα2(I) chain leads to impaired removal of the amino-terminal propeptides in type I collagen in the Ehlers-Danlos syndrome type VII [60,62]. This biochemical defect leads to typical clinical picture including loose joints and mild stretchability and bruisability of the skin. A related animal disorder, dermatosparaxis, was already earlier demonstrated to have an analogous biochemical defect [63–65].

Osteogenesis Imperfecta (OI)

One variant of osteogenesis imperfecta with some clinical characteristics of Ehlers-Danlos syndrome, such as bilateral dislocations of the hips, has been shown to have a deletion of the proα2(I) chain. This prevents the removal of N-propeptide and results in the persistence of pN-collagen molecules in the subject's tissues. The other clinical features included blue sclerae and radiological evidence of wormian bones, findings typical to OI, but unusually for this disorder, there was no history or evidence of broken bones [69,70].

No detailed studies have been reported about possible defects in the conversion of collagen types other than type I in connective tissue disorders of man. Thus even the existence of such defects remains an open question.

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