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THE STRUCTURE AND ASSEMBLY OF PROCOLLAGEN – A REVIEW

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INTRODUCTION

In the three years since experimental evidence for a biosynthetic precursor of collagen, procollagen*, was first obtained, substantial progress has been made in the characterization of the precursor (1, 2). It is somewhat surprising that the existence of procollagen was not appreciated sooner. A number of earlier studies had, in fact, identified hydroxyproline-containing tissue fractions which behaved anomalously in terms of their solubility and precipitability (3–5). In addition, different forms of collagen, characterized by additional nontriple helical sequences (telopeptides), were postulated to play a role extracellularly in the molecular packing and crosslinking of collagen (6–8). However, at that time, insufficient information regarding the nature of the collagen monomer and limitations of available techniques precluded attempts to define differences in soluble collagen fractions.

The characterization of the collagen monomer with respect to chain composition and amino acid sequence (for reviews see 9, 10) provided an adequate chemical definition of the soluble precursor of the collagen fiber. An understanding of the molecular properties of the collagen monomer led, in turn, to a greater appreciation of the need for a different form of the protein during intracellular biosynthesis and transcellular movement of the molecule (11).

*The term procollagen has occasionally been used to describe a soluble precursor of the collagen fiber. For this purpose the term has been replaced by the descriptive terms soluble collagen or collagen monomer, or by tropocollagen. In this review, the use of procollagen will be reserved to describe a precursor form of the functional collagen molecule in the sense that the terms procarboxypeptidase and proinsulin are used. This seems preferable to the use of alternative designations such as "transport form" since the prefix "pro" is widely accepted in the biological literature as indicative of a biosynthetic precursor.

While the ability of soluble collagen to aggregate spontaneously and form native fibrils under physiological conditions is a useful property in the extracellular matrix, this is clearly not desirable within the cell. Presumably some means exists to inhibit fibrogenesis intracellularly. The ability of collagen chains to achieve a triple helical conformation within the cell also required explanation. A number of studies, including the biosynthetic experiments of Vuust and Piez (12, 13), indicated that helix formation occurred rapidly. However, when experiments were performed with isolated chains in vitro, renaturation was observed to be a slow process which proceeded most efficiently under conditions far removed from those expected intracellularly (14–16). These considerations, therefore, indirectly supported the possibility that supplemental information, in the form of additional amino acid sequences, might serve to assure the proper alignment of chains during triple helix formation and to prevent intracellular fibrogenesis. It was also postulated that the properties unique to procollagen might facilitate the processes of intracellular movement and secretion of collagen.

Clearcut experimental evidence for the existence of a higher molecular weight biosynthetic precursor of collagen has come from several sources: 1) studies of the synthesis and structure of the hydroxyproline-containing material secreted into the medium by cultured fibroblasts (17–23); 2) studies of the synthesis and secretion of procollagen by freshly isolated embryonic chick cells obtained from tendon, cartilage, or lens capsule (24–29); 3) an investigation of a heritable disorder of cattle and sheep (dermatosparaxis), in which an enzymatic defect in the conversion of procollagen to collagen was found (30–35); an analogous defect has been identified as a form of the Ehlers-Danlos syndrome in man (36); 4) studies of procollagen synthesis and structure by cranial bone explants (13, 37–42); and 5) isolation of derivatives of procollagen from tissues of normal animals (43–46).

DISCUSSION

The Structure of Procollagen

Studies of procollagen synthesis by cranial bone initially utilized acetic acid extracts of tissue labeled in vitro with a radioactive amino acid (37–40). These studies provided a useful preliminary characterization of the amino acid sequence unique to the precursor proα1 chain. They indicated that the additional sequences could not, on the basis of amino acid composition, achieve the triple helical structure characteristic of collagen and that the amino acids cystine and tryptophan, absent from the majority of vertebrate collagens, were present in these regions. Extraction at acidic pH inhibited a prominent neutral pH proteolytic activity responsible for the conversion of procollagen to collagen (47–49). However, the use of acetic acid promoted limited proteolysis by other enzymes, presumably released during homogenization of the tissue, and therefore led to partial degradation of the collagen precursor.

Initial studies of the material synthesized and secreted by cultured or freshly isolated cells used either reducing agents or limited cleavage with pepsin, procedures

which also altered the structure of the biosynthetic precursor. More systematic studies of the procollagen secreted by cultured cells indicated that a substantial fraction of the protein contained interchain disulfide bonds (19–21, 25). When embryonic chick cranial bones were extracted either at neutral pH in the presence of a number of enzyme inhibitors (41) or by rapid extraction in acetic acid (42), essentially all the recently synthesized procollagen was shown to exist as a high molecular weight product linked by interchain disulfide bonds (Fig. 1). Goldberg and Sherr (23) arrived at a similar conclusion in regard to the procollagen synthesized by cultured human fibroblasts. Nondisulfide-linked chains identified in earlier studies presumably arose by limited proteolysis either during culture of the tissue or during subsequent processing of the protein. Similarly, the shorter nondisulfide-linked chains identified in the skin of dermatosparactic animals (30) apparently represent partial degradation products of native procollagen. In this case, limited proteolysis may occur at least in part in vivo since these components persist even when precautions are taken to limit proteolysis during extraction (C. M. Lapière, personal communication).

The general concept which emerges from these studies is that the biosynthetic precursor of collagen is assembled from chains higher in molecular weight than α chains, consisting, in the case of the predominant collagen synthesized in skin, bone, and tendon, of two different types of chains, proα1 and proα2 (Fig. 2). Subsequently, interchain disulfide bonds form between the additional sequences in the precursor chains. One published study suggested that the collagen precursor may be synthesized as a single continuous polypeptide chain (50). Other investigators have not obtained evidence for a precursor resistant to reduction by sulfhydryl compounds; the postulate of a single chain precursor also contradicts biosynthetic evidence for separate initiation points for synthesis of proα1 and proα2 chains (13).

If the additional sequences in procollagen facilitate triple helix formation, their location at the amino terminal ends of α chains would seem necessary. Electron microscopic examination of segment-long spacing (SLS) aggregates of procollagen from a number of sources has indeed demonstrated the existence of a poorly banded region at the amino terminal end of the molecule (Fig. 3) (25, 28, 31, 45). The additional sequences are covalently linked to α chains since SLS aggregates with amino terminal extensions could be formed by renaturation of dissociated chains obtained from dermatosparactic procollagen (31). The unusual (for collagen) conformation and composition of this amino terminal region of the protein is reflected in the ability of enzymes such as chymotrypsin and pepsin to selectively cleave this region, provided the triple helical conformation of the main body of the molecule remains intact (17–19, 23, 25, 31, 37, 41, 48, 51).

The large size and presence of disulfide bonds in procollagen have largely precluded accurate determinations of the molecular weight of the protein by calibrated SDS molecular sieve chromatography or acrylamide gel electrophoresis. Molecular weight determination by band sedimentation analysis (42) may possess certain advantages but resolution by this method is limited. Estimates of the molecular weight of constituent proα chains can be made by acrylamide gel electrophoresis of the reduced protein in the presence of SDS. Interpretation of these experiments remains complicated by the fact that proα chains consist of chemically different regions. Studies have shown that collagen chains

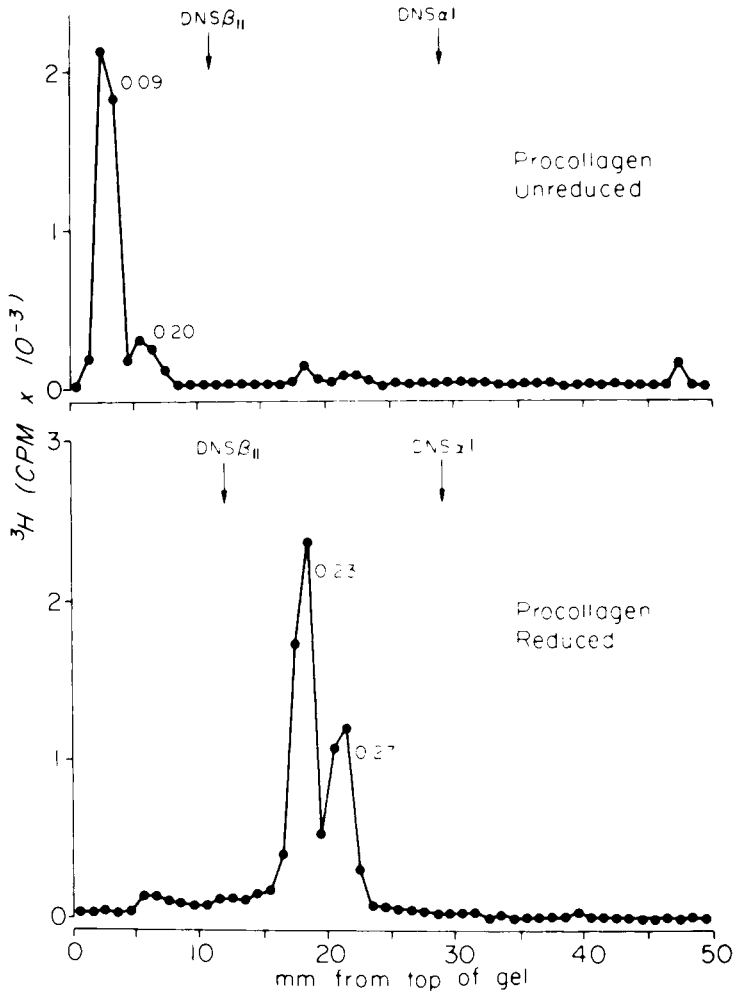

Fig. 1. Sodium dodecyl sulfate acrylamide gel electrophoresis of ^{3}H-proline-labeled procollagen extracted from embryonic chick cranial bone at neutral pH in the presence of enzyme inhibitors (41). Top, gel run in the absence of reducing agents and in the presence of 0.1 M iodoacetamide. Bottom, gel run in the presence of 2% 2-mercaptoethanol. The arrows indicate the position of migration of dansylated α and β collagen components used as internal markers. The numbers indicate migration rates (Rfs) relative to dansylated α1–CB7. Under these conditions the Rfs of acid-extracted proα1 and proα2 chains are 0.28 and 0.33, respectively.

migrate more slowly on acrylamide gels than other proteins of equivalent weight (40, 52), presumably because these chains bind less SDS per unit mass.

Proα chains obtained from human fibroblast procollagen (20) and chick cranial bone procollagen (41) migrate similarly on SDS acrylamide gel columns relative to marker α and β collagen components. Based on collagen chain standards, a molecular weight of approxi-

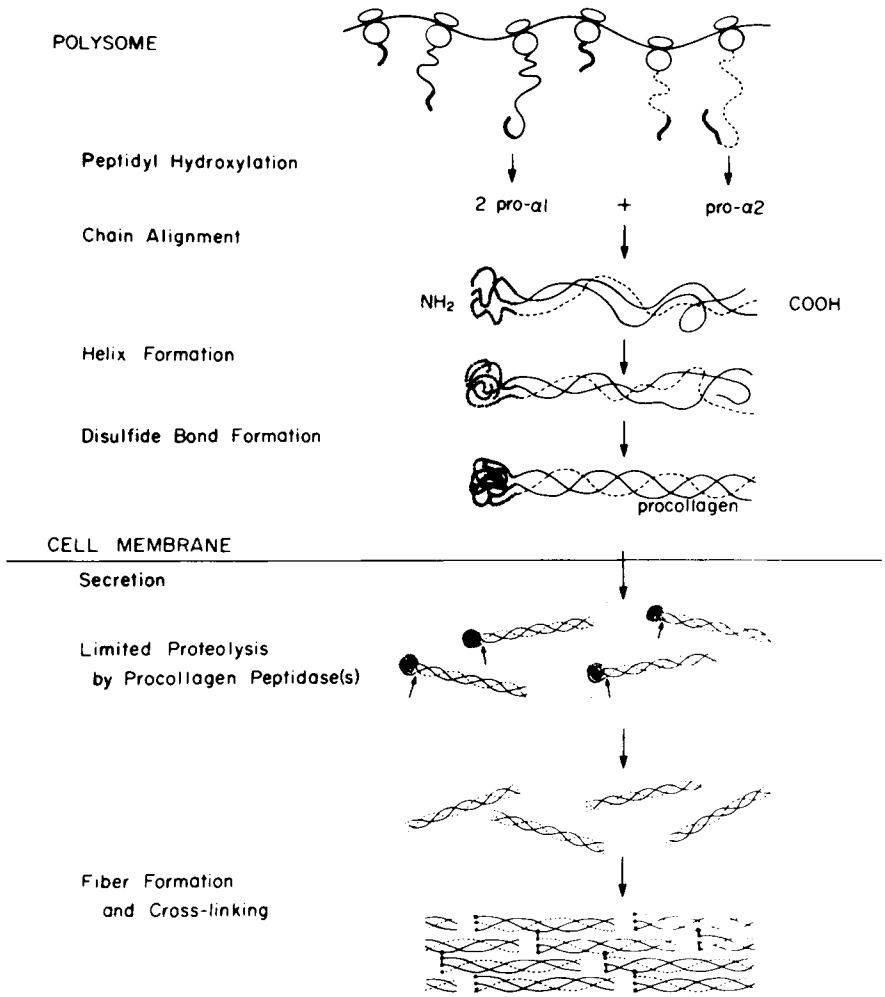

Fig. 2. Scheme depicting the biosynthesis of procollagen, conversion to collagen, and extracellular fibrogenesis. It is likely that alignment and association of the three chains and helix formation occur as a concerted process, at least in part prior to release of chains from ribosomes. Disulfide bond formation appears to take place largely following completion of chain synthesis. Reproduced with permission from (2).

mately 135,000 was calculated for the proα chain of chick bone procollagen (unpublished data). Proα chains obtained from procollagen after acid extraction of calvaria consistently migrate more rapidly with an estimated molecular weight of 115,000 to 120,000 (38) indicating that acid extraction leads to limited proteolysis of the collagen precursor. However, the uncertainty inherent in such estimates is underscored by the observation that

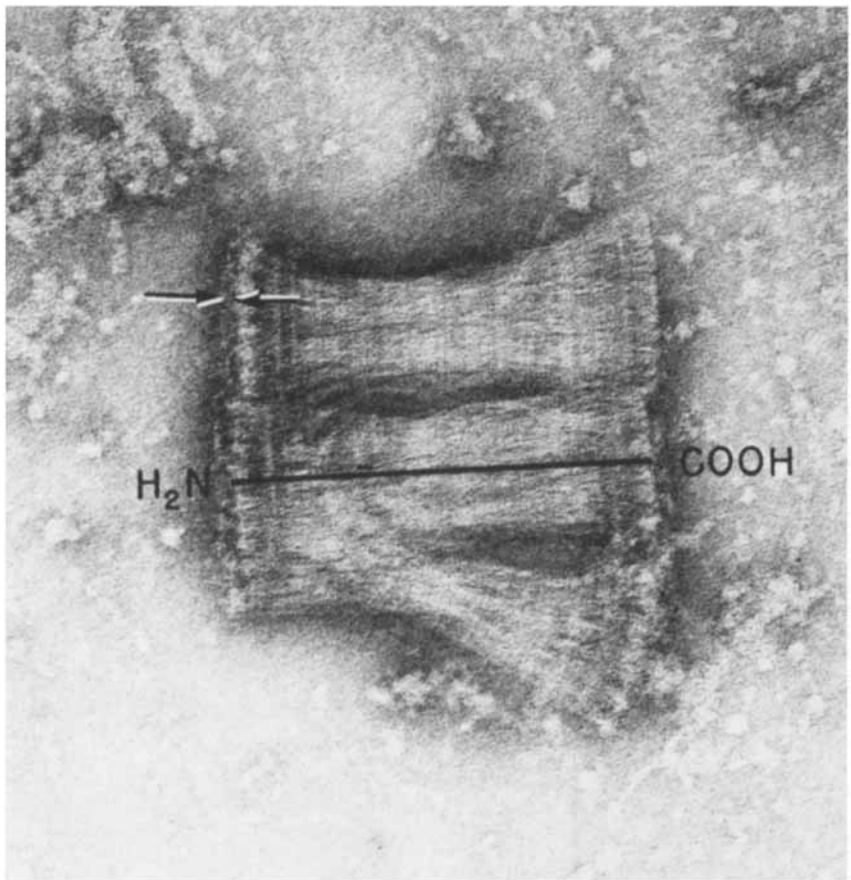

Fig. 3. Electron micrograph of segment-long spacing aggregates of procollagen extracted from the skin of a dermatosparactic lamb. The extent and orientation of an aggregate obtained from a normal animal is indicated by the horizontal line. An additional poorly banded region at the H_2 N-terminal end of the aggregate (arrows) is evident. Negative staining with 2% ammonium molybdate. Magnification 192,000 X. (The electron micrograph was kindly supplied by Dr. Bjorn Olsen, University of Oslo.)

α1 and α2 chains, which have very similar molecular weights based on peptide composition and amino acid sequence data, migrate quite differently in this system (52).

Since the existence of genetically distinct tissue-specific collagens has been clearly established (53–56) it is likely that these proteins are also secreted as higher molecular weight biosynthetic precursors. Indeed, evidence for a synthetic precursor of basement membrane collagen (29) and cartilage collagen (28, 57) has been published. Preliminary evidence for existence, in the medium of cultured skin fibroblasts, of a biosynthetic precursor of type III collagen, found in skin and blood vessels, has also been obtained (58).

Procollagen-Derived Peptides

An additional approach to the determination of the molecular weight and amino acid composition of proα chains has been the isolation and analysis of procollagen-derived peptides. Cyanogen bromide-produced and collagenase-resistant peptides (40, 59) were isolated after treatment of purified proα chains from acid-extracted procollagen. These peptides were shown to have molecular weights of approximately 20,000 and 15,000, respectively. The amino acid compositions of these peptides agreed well with the difference in amino acid composition between acid-extracted proα1 and α1 chains and indicated that the majority of the sequence unique to the truncated precursor chain was present in these peptide derivatives. Since the starting material, acid-extracted procollagen, was in itself a derivative of the native precursor (41), these fragments represented only a fraction of the additional sequence in this precursor chain. Furthmayr et al. (60) used a similar approach to isolate a collagenase-resistant fragment from the p-α1 chain of dermatosparactic procollagen. This peptide had a molecular weight similar to the cyanogen bromide peptide isolated from the acid-extracted proα1 chain of chick bone procollagen (40), but the amino acid composition was substantially different. Analyses of a peptide excised from dermatosparactic procollagen by procollagen peptidase (49) were in good agreement with those of Furthmayr et al. (60).

Sherr et al. (61) have also employed bacterial collagenase digestion of procollagen secreted into the medium by cultured human diploid fibroblasts to isolate a collagenase-resistant fragment. This fragment retained the predominant antigenic determinants of the intact precursor molecule and was shown to contain interchain disulfide bonds. The molecular weight of the peptide varied with the method of determination but the best data indicated a molecular weight of 75,000 to 80,000 for the disulfide-bonded fragment and 25,000 for the reduced and alkylated material. The amino acid analysis of this fragment again differed substantially from that obtained with chick bone and bovine skin procollagen-derived peptides and differences also existed in comparison with compositions deduced from amino acid analyses of chick tendon proα chains eluted from SDS acrylamide gel electrophoresis columns (27).

A number of explanations may be advanced to account for these differences in amino acid composition. Substantial species-specific differences may exist, although this would not be expected if these peptide regions play a specific and critical role in chain alignment, association, and initiation of helix formation. Possibly, different regions of the sequences unique to the precursor chain have been analyzed in the several cited studies. In the study of Sherr et al. (61) the presence of a sequence derived from the proα2 chain, which is probably different in composition from that of proα1, may contribute to differences in the overall composition. Finally, rigid criteria for purity were not always applied in these preliminary studies.

In recent experiments (62) a collagenase-resistant disulfide-linked peptide has been isolated from procollagen extracted from chick cranial bone. The fragment appeared to be similar in its electrophoretic behavior to that isolated by Sherr et al. (61) from procollagen in the medium of cultured fibroblasts. A similar fragment was identified in the medium of cultured cranial bone (62). This fragment was also disulfide-linked and retained

the antigenic determinants predominating in the intact precursor molecule. Analysis of the molecular weight of the collagenase-resistant fragment, after reduction and alkylation and electrophoresis on 10% SDS acrylamide gels, indicated a molecular weight of approximately 40,000. This value must be considered preliminary since the possibility of carbohydrate in the precursor region (60) may modify the behavior of the fragment on acrylamide gel. The data, however, indicate that the molecular weight of the precursor chain is at least 135,000 and perhaps greater if collagenase susceptible linkages exist within sequences unique to the precursor chain (38).

Immunologic Studies

Antibodies to determinants specific to the biosynthetic precursor have been obtained by injection of rabbits with the proα1 chain of acid-extracted chick procollagen (59), dermatosparactic procollagen (63), procollagen obtained from cultured human fibroblasts (64) and from cultured embryonic chick tendon fibroblasts (65). Antibodies to dermatosparactic bovine procollagen were used in indirect immunofluorescence tests to show that immunoreactive material existed in several connective tissues of the normal animal. These studies suggest that derivatives of procollagen, or perhaps the released peptides, persist in these tissues for substantial periods of time (63). Antibodies to procollagen, labeled with ferritin, were used by Olsen and Prockop (66) and Nist et al. (67) to demonstrate the presence of procollagen in the cisternae of the rough endoplasmic reticulum and in several components of the Golgi complex in tendon and corneal fibroblasts. These studies provide strong evidence for the participation of the Golgi in the pathway of secretion of the procollagen molecule.

Assembly of Procollagen

It has been assumed that the additional sequences in procollagen play a role in chain alignment, association, and initiation of helix formation. Experiments designed to examine this question are limited by the difficulty in obtaining a sufficient quantity of unmodified procollagen to yield measurable renaturation of triple helical protein. Veis et al. (45) have reported that a procollagen fraction, obtained from mature bovine skin, regained a triple helical conformation more rapidly than bovine collagen. Procollagen preparations such as that obtained by Veis et al. (45) as well as dermatosparactic procollagen and acid-extracted procollagen are not entirely suitable for such experiments since these proteins lack the entire complement of additional sequences and might therefore not be expected to fold correctly. Indeed, experiments with acid-extracted procollagen (68; von der Mark and Bornstein, unpublished) indicate that the rate of renaturation of acid-extracted procollagen is no greater than that of collagen.

Bornstein and Click (unpublished) have attempted to renature procollagen, obtained in such a way as to preserve, in so far as possible, the native structure of the protein (41). Since the triple helix is resistant to pepsin, refolding of procollagen was assayed by determination of the fraction of protein that, after limited cleavage with pepsin, migrated in the position of α chains on sodium dodecyl sulfate acrylamide gel electrophoresis. A

TABLE I. Renaturation of Collagen and Procollagen

Protein[a]	Time after Denaturation (hr)[b]	Renaturation (%)[c]
Collagen	0	0
	6	0
	24	8
	48	7
Procollagen	0	0
	6	26
	24	35
	48	43

[a]0.1 mg/ml in 0.5 M acetic acid; heat denatured at 50°C for 30 min.
[b]In 0.5 M acetic acid, 15°C.
[c]Calculated as fraction of cpm migrating as α chains on acrylamide gel electrophoresis after pepsin digestion (6 hr, 15°C).

marked enhancement of renaturation of the disulfide bonded procollagen was observed in comparison with collagen (Table I). This finding was expected since the artificially cross-linked α chain trimer or γ component of collagen regains the triple helical conformation rapidly (69). However, it has not yet been possible to demonstrate efficient folding of procollagen after reduction of disulfide bonds.

Of considerable importance to an understanding of the folding of the procollagen molecule has been the recent demonstration that a normal complement of peptidyl hydroxyproline is required to confer stability to the native protein at 37°. It was shown that the melting temperature of the unhydroxylated collagen monomer is 24°C, some 15 degrees lower than the temperature at which the normally hydroxylated protein unfolds (51, 70–73) (Fig. 4). The hydroxyl group of hydroxyproline may confer stability to the triple helix by participating in intramolecular hydrogen bonds either directly (73) or through the intermediacy of a water molecule (74). These data, together with evidence indicating that hydroxyproline exists on nascent chains (75, 76), suggest that, normally, peptidyl hydroxylation occurs during polypeptide chain elongation. Very possibly, triple helix formation of the previously synthesized chains occurs prior to termination of polypeptide chain synthesis. In this manner, the attainment of a helical conformation may regulate the extent to which individual prolyl and lysyl residues are hydroxylated. Consistent with this suggestion is the observation that both prolyl (51, 77) and lysyl hydroxylases (78) fail to hydroxylate collagen chains in a triple helical conformation. It seems clear, however, that when peptidyl hydroxylation is blocked, as for example by addition of α, α'-dipyridyl, completed procollagen chains which accumulate intracellularly can be hydroxylated after removal of the enzymatic block (79, 80).

The role of disulfide bonds in the molecular assembly of procollagen is less well understood. A number of investigators (29, 42, 81) have suggested that interchain disulfide bond formation may not occur until after completion of polypeptide chain synthesis. Possibly, noncovalent interactions between the nontriple helical regions of the proα chains may be sufficient to initiate helix formation whereas disulfide bond formation may be a relatively late event in the molecular assembly process. The data of

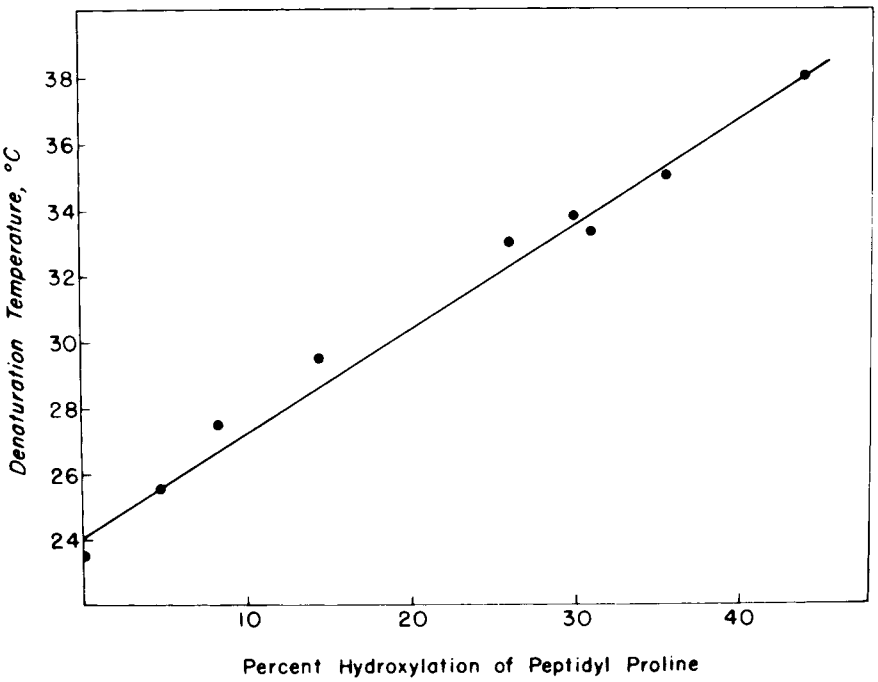

Fig. 4. Plot of the denaturation temperature (Tm) of procollagen as a function of the extent to which peptidyl proline is hydroxylated. Procollagen samples with varying hydroxyproline contents were obtained by incubating embryonic chick tendon fibroblasts with different concentrations of α, α' dipyridyl, an inhibitor of prolyl hydroxylase. Reproduced with permission from (71).

Schofield et al. (82) suggest that disulfide bonding and helix formation, as measured by resistance to proteolysis with chymotrypsin, may develop concomitantly. If this is so, the triple helix may not form until after completion of polypeptide chain synthesis, even though peptidyl hydroxylation occurs on the nascent chain. Treatment with dithiothreitol of cells which had accumulated underhydroxylated procollagen intracellularly markedly reduced the rate at which helix formation occurred (81). Whether this effect can be attributed solely to reduction of interchain disulfide bonds remains to be demonstrated.

Conversion of Procollagen to Collagen

The conversion of procollagen to collagen occurs extracellularly (1, 2, 83) but whether one or more enzymatic activity is required to effect this conversion is unclear. A neutral pH enzymatic activity, procollagen peptidase, has been shown to convert dermatosparactic and acid-extracted procollagen to collagen (47–49). The enzymatic activity from normal calf tendon was purified to homogeneity (49) and was shown to be an endopeptidase which excises the amino terminal regions of the precursor chains *en bloc* rather than in multiple fragments. However, the substrate used, dermatosparactic

procollagen, is clearly not the native collagen precursor. It will therefore be necessary to show that calf tendon procollagen peptidase acts similarly on the disulfide bonded native precursor.

Indirect evidence for the existence of multiple steps in the conversion of procollagen to collagen has come from the work of Veis and his associates (44, 45). These studies detected the presence, in the tissues of normal animals, of chains intermediate in size between those of the native precursor and the collagen monomer. If such intermediates are incorporated into soluble collagen fibers, they may play a role in the molecular packing of collagen molecules, but the argument for such a role is speculative. Present concepts of molecular packing in the collagen fiber (84) do not provide a role in fibril formation for molecules with amino terminal extensions.

SUMMARY

Collagen is synthesized as a biosynthetic precursor, procollagen, with additional sequences at the amino terminal ends of the three chains. These sequences differ in composition and conformation from the triple helical body of the molecule and are stabilized by interchain disulfide bonds.

Studies with procollagen-derived peptides obtained by collagenase digestion of the purified precursor and from the medium of cultured bone have confirmed the presence of interchain disulfide bonds in the region unique to procollagen. Preliminary determinations indicate a molecular weight of approximately 40,000 for the additional sequence in each proα chain.

Antibodies have been developed to determinants unique to procollagen. Such antibodies, labeled with ferritin, have been used to demonstrate the presence of procollagen in cisternae of the rough endoplasmic reticulum and in the Golgi complex of tendon and corneal fibroblasts.

It is likely that the additional sequences in procollagen participate in chain alignment and accelerate triple helix formation. Peptidyl hydroxylation normally occurs on nascent chains and appears to be inhibited by the triple helical conformation of the substrate. Hydroxyproline contributes to the thermal stability of the triple helix, presumably by contributing to interchain hydrogen bonds. The role of interchain disulfide bonds in the assembly of procollagen remains to be established.

The conversion of procollagen to collagen occurs extracellularly and is achieved by at least one and possibly more proteolytic steps.

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