Formation of Interchain Disulfide Bonds and Helical Structure during Biosynthesis of Procollagen by Embryonic Tendon Cells†

J. David Schofield,‡ Jouni Uitto, and Darwin J. Prockop*

ABSTRACT: Interchain disulfide bonding in the procollagen synthesized by tendon cells from chick embryos was examined by gel filtration and polyacrylamide gel electrophoresis of reduced and nonreduced forms of the proteins in dodecyl sulfate. The results indicated that the major part of both the newly synthesized intracellular procollagen and the secreted procollagen consisted of pro-α chains linked by disulfide bonds. In addition to procollagen in which the three pro-α chains were linked by disulfide bonds the medium from the cells also contained smaller amounts of procollagen-like protein in which the collagen portion was still triple helical but the interchain disulfide bonds were missing. The observations suggested that the conversion of procollagen to collagen can occur by several discrete steps, the first of which may remove regions close to the NH_2-terminal ends containing the interchain bonds. Experiments in which the cells were pulse labeled for 4 min with [^{14}C]proline and then the label chased demonstrated that at the end of the pulse-labeling period pro-α chains were recovered which did not have interchain bonds and which were not helical as tested by limited proteolytic digestion. Interchain bonds and triple-helical structure appeared in the molecule at about the same time, and a chase period greater than the synthesis time for pro-α chains was required to obtain maximal values for interchain bonds and helical structure. In further experiments the cells were incubated with the iron chelator α,α'-dipyridyl so that prolyl and lysyl hydroxylases were inhibited. The intracellular pro-α chains were linked by disulfide bonds even though the absence of hydroxyproline in the polypeptides prevented them from becoming triple helical at 37°. The results suggested that formation of interchain disulfide bonds may play an important role in the formation of the helical structure of collagen.

Collagen is synthesized as a precursor, called procollagen, which is larger than the collagen molecule in that each of the three polypeptide chains contains additional amino acid sequences at its NH_2-terminal end (for recent review, see Schofield and Prockop, 1973). One of the functions suggested for the NH_2-terminal extensions is that they may facilitate correct association and alignment of the three polypeptide chains and thereby promote formation of the triple-helical structure of collagen. This function is a critical one since even under optimal conditions *in vitro* the formation of the collagen triple helix from isolated α chains is slow and incomplete (Beier and Engel, 1966; Kühn, 1969; Harrington and Rao, 1970). The discovery of cysteine in procollagen (Lenaers *et al.*, 1971) was of particular importance in this regard, since it raised the possibility that the three pro-α chains were linked together by disulfide bonds which are not found in collagen itself. There has been some disagreement, however, as to whether all three of the pro-α chains in procollagen are bonded together. For example, some initial reports indicated that the pro-α_2 chain of procollagen extracted from embryonic chick calvaria (Bornstein *et al.*, 1972) or from fibroblasts in culture (Tsai and Green, 1972) contained no cysteine, while other reports indicated that about equal amounts of this amino acid were present in both pro-α_1 and pro-α_2 chains of procollagen from freshly isolated enbryonic tendon cells (Uitto *et al.*, 1972). Also, it was shown that a large fraction of the procollagen secreted by freshly isolated tendon cells and by fibroblasts in culture contained interchain disulfide bonds (Dehm *et al.*, 1972). More recent observations suggest (Monson and Bornstein, 1973a), but have not conclusively established, that these apparent discrepancies may be explained by the possibility that part of the cysteine in pro-α chains is located near the extreme NH_2-terminal ends and that these regions are easily removed by endogenous peptidases prior to or during isolation.

Matrix-free cells prepared by controlled enzymic digestion of embryonic chick tendons have been shown to synthesize and secrete procollagen for several hours when incubated in suspension (Dehm and Prockop, 1971, 1972). In this system, the procollagen secreted into the medium is not converted to collagen as it is in the intact tendons from which the cells are prepared (Jimenez *et al.*, 1971). In the present work we have used this cell system to examine the stage at which the interchain disulfide bonds are formed during the intracellular assembly of procollagen and we have attempted to relate the formation of interchain disulfide bonds to the stage at which the helical structure of collagen is formed.

Materials and Methods

Materials. Unless otherwise indicated, these were purchased from the same suppliers as previously (Dehm and Prockop, 1971, 1972).

Isolation and Incubation of Freshly Isolated Cells from Tendon. Cells were isolated from leg tendons of 17-day-old chick embryos by controlled digestion with trypsin and purified bacterial collagenase as described previously (Dehm

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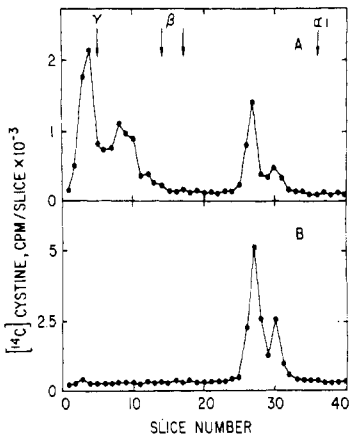

FIGURE 1: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of procollagen secreted by tendon cells. Tendon cells, 2.6×10^7, were incubated for 120 min with 0.8 μCi of [^{14}C]cystine in 3.5 ml of modified Krebs medium. The medium was dialyzed against 0.4 M NaCl and 0.1 M Tris-HCl buffer (pH 7.4) and centrifuged at 20,000g for 30 min. The medium was concentrated by adding ammonium sulfate and then prepared for electrophoresis in sodium dodecyl sulfate as described in Methods. The electrophoretic mobilities of γ, β, and α_1 chains of collagen in the same electrophoresis run were as indicated. α_2 chains were recovered in slice 40: (A) one half of the sample which was electrophoresed without reduction; (B) the other half of the sample which was reduced with 1% mercaptoethanol prior to electrophoresis.

and Prockop, 1972). In most experiments 1.4–2.0 $\times$ 10^7 tendon cells were obtained from a single embryo. The cells were incubated in modified Krebs medium without addition of fetal calf serum under the conditions described below, and then either the cells were separated from the medium by centrifugation at 1200g for 12 min at room temperature or the entire incubation system was taken for further processing.

Gel Filtration and Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. For gel filtration or polyacrylamide gel electrophoresis, the samples were denatured and reduced with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol in 0.1 M sodium phosphate (pH 7.4) (Jimenez *et al.*, 1971). In some initial experiments (see below), the reactions were carried out at 37° for 4 hr. In subsequent experiments the samples were first heated to 100° for 3 min to inactivate endogenous proteases which appeared to be present in some samples and then the reaction was allowed to proceed at 37° for 3 hr. In experiments in which the intracellular [^{14}C]protein was examined without reduction, the cells were homogenized at 4° in 50 mM iodoacetamide (Fisher Scientific Co.) to prevent disulfide exchange before treatment with sodium dodecyl sulfate.

In most instances the samples were prepared for gel filtration or polyacrylamide gel electrophoresis as described above without concentration. In occasional experiments, however, the procollagen secreted into the medium was concentrated before the treatment with sodium dodecyl sulfate. The medium was dialyzed for 24 hr at 4° against 0.4 M NaCl and 0.1 M Tris-HCl buffer (pH 7.4), centrifuged at 20,000g for 30 min to remove insoluble material, and concentrated by adding 176 mg of ammonium sulfate (Baker Chemical Co.)/ml. The precipitate was then treated with sodium dodecyl sulfate and 2-mercaptoethanol as described above.

Gel filtration on 6% agarose (Bio-Gel A-5m; 200–400 mesh; Bio-Rad) in the presence of sodium dodecyl sulfate was carried out as described previously (Jimenez *et al.*, 1971). Polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (1969) with 5% polyacrylamide gels; in some experiments the amount of cross-linking reagent was reduced to one-half. ^{14}C in the polyacrylamide gels was assayed by cutting the gels into 1-mm slices with a Mickle gel slicer (Brinkmann Instrument Co.). [^{14}C]Protein in the slices was eluted by adding 0.4 ml of a solution consisting of Protosol (New England Nuclear Corp.)–distilled water–toluene (9:1:10, v/v) (Zaitlin and Hariharasubramarian, 1970). The samples were incubated at 60° for 15 hr and subsequently cooled at −20° for 2 hr. Then 15 ml of a solvent for liquid scintillation counting was added and the samples were stored in the dark overnight before counting.

For the pulse-chase experiments in which the resistance of the [^{14}C]protein to proteolysis was tested, the total sample (cells plus medium) was added to a one-tenth volume of 0.5 M iodoacetamide. The sample was then immediately homogenized and an aliquot of the homogenate was mixed with a one-tenth volume of a stock solution containing 3.3 mg of α-chymotrypsin (Worthington Biochemical Corp., 45 units/mg) per ml of 0.4 M NaCl and 0.1 M Tris-HCl (pH 7.4). The homogenization was carried out with the sample cooled to 4° and the enzymic digestion was at 15° for 6 hr. The digestion was begun 2 min after the sample was removed from the incubator and about 1 min after the cells were homogenized. The reaction was stopped by adding 100 μg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone (Sigma Chemical Co.) and incubating at 4° for 60 min. Treatment with dodecyl sulfate and 2-mercaptoethanol was then carried out as described prior to gel filtration.

Assays. ^{14}C in the gel filtration chromatograms was assayed in a liquid scintillation counter as described previously (Jimenez *et al.*, 1971). [^{14}C]Hydroxyproline in the column fractions and in other samples was assayed with a specific radiochemical procedure (Juva and Prockop, 1966).

Results

Interchain Disulfide Bonds among the Pro-α Chains of Secreted Tendon Procollagen. It was previously reported that when matrix-free tendon cells were incubated with [^{14}C]proline, about 85% of the nondialyzable ^{14}C secreted into the medium by cells consisted of polypeptides similar to pro-α chains (Dehm *et al.*, 1972). Of the pro-α chains recovered from the medium, 40–60% were linked by interchain disulfide bonds as measured by the elution profiles obtained by gel filtration in sodium dodecyl sulfate with and without reduction. Similar results were obtained here except that a higher proportion of the pro-α chains secreted in the medium were found to be linked by interchain disulfide bonds; the values ranged from 70 to 80% in nine separate experiments. The reason for more interchain bonding in these experiments was not apparent.

Disulfide bonding among the pro-α chains of the secreted procollagen was studied further by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figure 1). If the medium [^{14}C]protein was not reduced, 43–48% of the ^{14}C was recovered in a band with a slightly less mobility than γ chains of collagen, and 22–31% was recovered in a band with slightly less mobility than β chains of collagen. After reduction, essentially all the ^{14}C was recovered in polypeptides similar to pro-α_1 and pro-α_2 chains.

The presence of some polypeptides similar to pro-α chains but not disulfide linked suggested (see Discussion) that some of the procollagen recovered from the medium was in a partially degraded form. To establish that this apparent degradation was not an artifact produced by the method of cell preparation, intact tendons from which the cells were

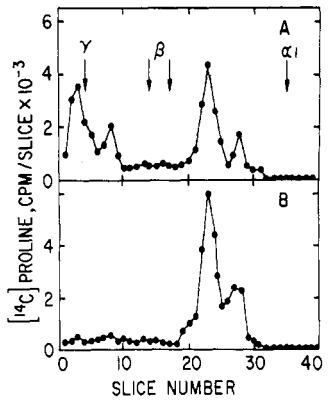

FIGURE 2: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of medium from intact tendons. Leg tendons from 17-day-old chick embryos were incubated for 120 min in 9 ml of modified Krebs medium with 10% fetal calf serum, 50 μg of β-aminopropionitrile fumarate (General Biochemical)/ml, and 10 μCi of [^{14}C]-proline. The sample was centrifuged at 1200g for 10 min and the supernatant was taken for treatment with sodium dodecyl sulfate as described in Methods. The electrophoresis run was comparable to Figure 1 but standards of α_1 and α_2 chains were recovered in slices 35 and 38, respectively: (A) sample which was electrophoresed without reduction with mercaptoethanol; (B) sample reduced with 2% mercaptoethanol prior to electrophoresis.

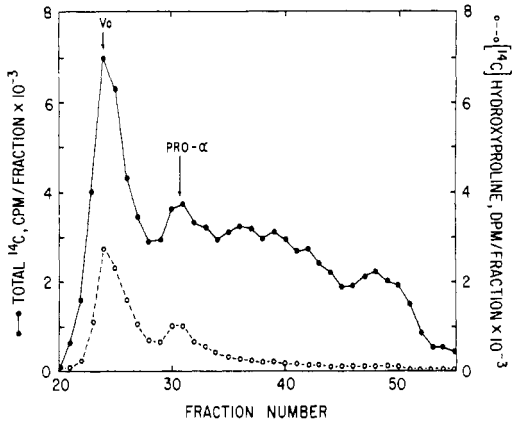

FIGURE 3: Gel filtration in sodium dodecyl sulfate of unreduced intracellular proteins labeled under steady-state conditions. Tendon cells, 1.3 $\times$ 10^8, were incubated with 10 μCi of [^{14}C]proline in 17 ml of modified Krebs medium for 2 hr. The cells were recovered by centrifugation, and then homogenized at 4° in 50 mM iodoacetamide, 1% sodium dodecyl sulfate, and 0.1 M sodium phosphate (pH 7.4). The sample was then prepared for gel filtration as described in text. The V_0 (void volume of gel filtration columns) and the elution position of pro-α chains are indicated. The V_t (total volume of gel filtration columns) was in fraction 72.

obtained were incubated with [^{14}C]proline and the medium from the incubation system was examined. As indicated (Figure 2), part of the [^{14}C]protein recovered from the medium consisted of pro-α chains which were disulfide linked and part consisted of similar polypeptides which did not contain interchain disulfide bonds.

In further experiments reported elsewhere (Schofield *et al.*, 1974; Dehm *et al.*, 1974), it was shown that the interchain disulfide bonds in tendon procollagen were formed among the three NH_2-terminal extensions. After digestion of the procollagen with purified bacterial collagenase, a trimer was obtained which reacted with specific antibodies to procollagen and which was shown to consist of two kinds of monomers in a ratio of about 2:1.

Interchain Disulfide Bonds in Intracellular Tendon Procollagen. Tendon cells were incubated for 2 hr with [^{14}C]proline so that the steady-state labeling of the intracellular proteins was achieved. Cells were homogenized and the [^{14}C]-protein in the homogenate was then examined by gel filtration in sodium dodecyl sulfate with and without reduction. Control experiments indicated that over 90% of the nondialyzable ^{14}C in the cells was solubilized by the treatment with dodecyl sulfate and recovered from the agarose column.

When the intracellular [^{14}C]protein was reduced with mercaptoethanol, most of the intracellular [^{14}C]hydroxyproline was recovered in polypeptides which were about the same size as pro-α chains.[1] When the intracellular [^{14}C]protein was examined without reduction, about 60% of the [^{14}C]hydroxyproline eluted in a molecular form larger than pro-α chains (Figure 3). The observations indicated therefore that the cells contained procollagen in which the pro-α chains were linked by disulfide bonds.

Stage at Which Interchain Disulfide Bonds Form in Tendon Cells. To determine the stage at which interchain bonds were formed in procollagen, a series of pulse-label and chase experiments were carried out with the tendon cells. In most of the experiments the cells were pulse labeled with [^{14}C]proline for 4 min, *i.e.*, about two-thirds of a synthesis time (Vuust and Piez, 1972; Miller *et al.*, 1973), and then the label was chased by adding 100 μg/ml of [^{12}C]proline. The chase with [^{12}C]proline was effective in that in the experiment shown the total [^{14}C]protein in the cells at the end of the pulse-labeling period was 1.21 $\times$ 10^6 cpm; it increased by 33% during the first 3 min of the chase period but was constant thereafter.

Examination of the intracellular [^{14}C]protein indicated that at the end of the pulse-labeling period, about 67% of the [^{14}C]hydroxyproline was in polypeptides of about the same size as pro-α chains (Figure 4). The elution profile was essentially the same with or without reduction, indicating that little if any of the [^{14}C]hydroxyproline-containing polypeptides were disulfide linked. After a chase period of 3 min, about 25% of the [^{14}C]hydroxyproline in the system eluted as polypeptides larger than pro-α chains when the protein was not reduced with mercaptoethanol. Proportionately more of the [^{14}C]hydroxyproline was recovered in such larger molecular weight components as the chase period was increased to 6 and 9 min (Figure 4).

In the same experiments the relative amount of collagen polypeptides which were in a helical conformation was tested by incubating the intracellular [^{14}C]protein with α-chymotrypsin. As indicated by previous reports (Drake *et al.*, 1966; Layman *et al.*, 1971; Jimenez *et al.*, 1971; Uitto and Prockop, 1974a,b) the amount of [^{14}C]protein in a collagen type of triple helix can be estimated by determining the per cent of the initial [^{14}C]hydroxyproline which undergoes limited digestion and which is recovered as α chains after α-chymotrypsin treatment under appropriate conditions. At the end of the pulse-labeling period, less than 10% of the [^{14}C]hydroxyproline in the system was in polypeptides resistant to α-chymotrypsin digestion. After chase periods of 3, 6, and 9

[1] Although precursor polypeptide chains larger than pro-α chains have been reported in studies with cultured fibroblasts (Church *et al.*, 1973), no evidence for such larger polypeptides was found in the tendon cells used here even though it was possible to carry out appropriate pulse-label and chase experiments and it was possible to examine the size of over 90% of the intracellular [^{14}C]protein. The observations on the larger precursor polypeptides (Church *et al.*, 1973) are difficult to relate to the data presented here, since one of the standards used to estimate molecular size by Church *et al.* was pro-α chain-like polypeptides from the skin of cattle with dermatosparaxis; these polypeptides may be partially degraded forms which are intermediates between complete pro-α chains and α chains (see Schofield and Prockop, 1973).

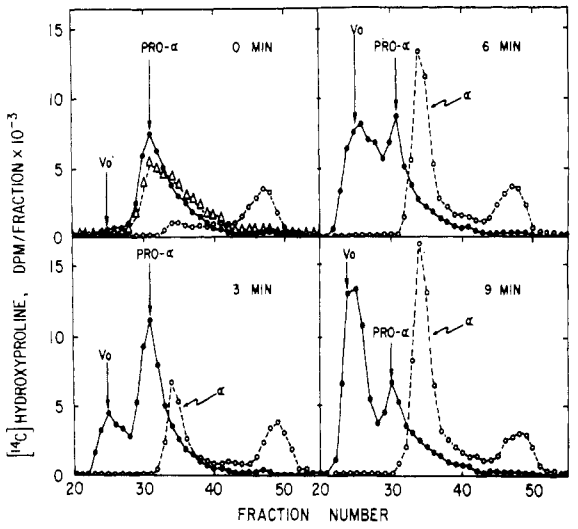

FIGURE 4: Pulse-chase experiments with tendon cells. Tendon cells, 1.2×10^9, were incubated with 100 μCi of [^{14}C]proline in 30 ml of modified Krebs medium for pulse-labeling period of 4 min. The label was then chased by adding 1 ml of Krebs medium containing 3.1 mg of [^{12}C]proline. Aliquots of 6 ml were removed after chase periods of 0, 3, 6, and 9 min. The samples were immediately homogenized with 0.6 ml of 0.5 M iodoacetamide at 4°. One half of the sample was then taken for gel filtration in sodium dodecyl sulfate with or without reduction and the other half was taken for α-chymotrypsin digestion prior to gel filtration as described in Methods. The chromatographic conditions were similar to those in Figure 3. The elution position of the pro-α chains and α chains were determined by assaying [^{14}C]hydroxyproline in the column fractions. Symbols: elution of [^{14}C]hydroxyproline in samples chromatographed without reduction or proteolytic digestion (●—●); elution of [^{14}C]hydroxyproline in sample reduced with mercaptoethanol but not subjected to proteolysis (△- - - - -△); elution of [^{14}C]hydroxyproline in samples digested with α-chymotrypsin (O- - - - - -O).

min, the amount of [^{14}C]hydroxyproline in resistant polypeptides gradually increased to about 60%.

The results from the pulse-chase experiments were further examined by comparing the per cent of [^{14}C]hydroxyproline

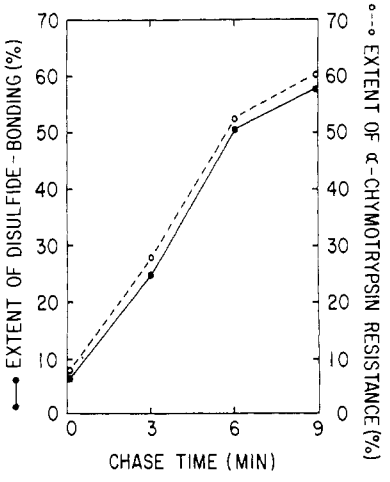

FIGURE 5: Extent of disulfide bonding and resistance to digestion by α-chymotrypsin in pulse-chase experiment with tendon cells. The values are from the experiment shown in Figure 5. The extent of disulfide bonding was calculated as the ratio of [^{14}C]hydroxyproline which eluted in the V_0 of the gel filtration column to the total [^{14}C]-hydroxyproline in the chromatogram. The extent of resistance to α-chymotrypsin digestion was calculated as the ratio of [^{14}C]hydroxyproline recovered in the α chain peak after digestion to the total nondialyzable [^{14}C]hydroxyproline in the sample before the proteolytic digestion (Uitto and Prockop, 1974a).

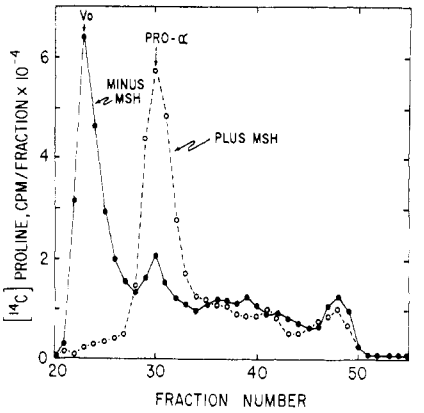

FIGURE 6: Demonstration of interchain disulfide bonding in protocollagen from tendon cells. Tendon cells, 2.5×10^8, were incubated in 34 ml of modified Krebs medium containing 20 μCi of [^{14}C]-proline and 0.3 mM α,α'-dipyridyl. The samples were incubated for 2 hr and then adjusted to 1% sodium dodecyl sulfate and 50 mM iodoacetamide by addition of stock solutions. The samples were immediately heated to 100° for 3 min and incubated at 37° for 3 hr prior to gel filtration as in Figure 4. Symbols: sample not reduced with mercaptoethanol (●—●); sample reduced with 1% mercaptoethanol (O- - - -O).

recovered in disulfide-linked material larger than pro-α chains, and the amount of [^{14}C]hydroxyproline in polypeptides resistant to α-chymotrypsin (Figure 5). The results indicated a close correlation between these two parameters, suggesting that the formation of the interchain disulfide bonds and the formation of helical structure of collagen occurred at about the same stage during the intracellular biosynthesis.

Demonstration that Formation of the Helical Structure Is Not Essential for Formation of the Interchain Disulfide Bonds. When tendon cells are incubated with the appropriate concentration of the iron chelator α,α'-dipyridyl, the cells synthesize and accumulate [^{14}C]protocollagen, the nonhydroxylated collagen precursor similar to procollagen except that it contains no hydroxyproline or hydroxylysine and is correspondingly rich in proline and lysine (Juva *et al.*, 1966; Berg and Prockop, 1973b). Recent data have demonstrated that the protocollagen which accumulates under these conditions is nonhelical at 37° (Berg and Prockop, 1973a,b; Jimenez *et al.*, 1973; Uitto and Prockop, 1973, 1974a). Cells were incubated with [^{14}C]proline and 0.3 mM α,α'-dipyridyl so that they synthesized and accumulated [^{14}C]protocollagen. To insure that the [^{14}C]protocollagen did not become helical during its extraction from the cells, the cells were suspended in 50 mM iodoacetamide and 1% sodium dodecyl sulfate at 37° immediately after the incubation and then were heated at 100° for 3 min. Most of the [^{14}C]protocollagen was disulfide bonded (Figure 6) even though the protein had been prevented from forming the triple-helical structure either in the cells or during its subsequent processing.

Discussion

Most of the available data suggest that the intrachain and interchain disulfide bonds found in some proteins are not of major importance in facilitating the formation of the correct structures during biosynthesis but that the disulfide bonds simply stabilize the correct three-dimensional structures of these proteins after they have formed (for recent reviews, see Anfinsen, 1972, and Wetlaufer and Ristow, 1973). This conclusion is in part based on the observation that many proteins with complex tertiary and quaternary structures do not contain disulfide bonds. Also, it has been shown that following

denaturation *in vitro*, several proteins without disulfide bonds fold into the correct tertiary and quaternary structures in a few min and therefore at rates which appear rapid enough to account for their synthesis time in cells. Ribonuclease and other proteins which contain intrachain disulfide bonds tend to refold into the correct structure at relatively slow rates when disulfide bonds are reduced and then allowed to reoxidize. This observation, however, has been explained by formation of inappropriate disulfide bonds from improper pairing of the half-cysteine residues during the reoxidation *in vitro*. The discovery of a "microsomal" enzyme which catalyzes interchange of disulfide bonds is thought to explain why such inappropriate intrachain disulfide bonds do not delay formation of the correct structure of proteins *in vivo* (Anfinsen, 1972). In the case of immunoglobulins, the best studied example of proteins comprised of subunits held together by interchain disulfide bonds, it has been shown that dissociated L and H chains of IgG can reassociate *in vitro* under conditions in which formation of interchain bonds is prevented (Olins and Edelman, 1964; Björk and Tanford, 1971). The resulting tetramers have the same physical properties and much of the biological activity of the native molecule. In further support of the conclusion that the role of intrachain and interchain disulfide bonds is simply to stabilize correct protein structures, it has been pointed out that such bonds are not present in most intracellular proteins and that they are found primarily in proteins which are synthesized for "export" into extracellular spaces where they are subject to a fluctuating environment (Anfinsen, 1972). Although these arguments and conclusions have been generally accepted, it should be noted that kinetic data on the folding of most proteins are incomplete and they have not rigorously excluded the possibility that during the biosynthesis of at least some proteins either intrachain or interchain bonds are necessary for the formation of the correct structure at a rate which is biologically adequate.

The conclusion that disulfide bonds simply serve to stabilize protein structure cannot be applied, without considerable qualification, to the special case of procollagen and collagen. The portion of the procollagen molecule containing the interchain disulfide bonds is removed either as the molecule is secreted or shortly thereafter (see Schofield and Prockop, 1973). Although a small amount of procollagen or partially degraded procollagen is present in the extracellular fiber (Veis *et al.*, 1973), collagen accounts for the bulk of the protein and therefore it is extremely unlikely that disulfide bonds contribute to the impressive tensile strength of such fibers. A further consideration is that collagen differs from other proteins in that all the hydrogen bonds necessary for helical structure are interchain (for review, see Traub and Piez, 1971) and therefore the individual polypeptides cannot assume secondary or tertiary structure without chain association. This circumstance suggests that disulfide bonds may play a critical role in the formation of the helical structure of collagen.

Because of the difficulty of isolating sufficient amounts of the intact protein, the number of intrachain and interchain disulfide bonds in procollagen has not been definitively determined and the currently available data do not exclude the possibility that procollagens from some sources may be devoid of interchain bonds (see Schofield and Prockop, 1973). However, in the case of the tendon cells employed here, the most complete form of the molecule contains interchain disulfide bonds among the three pro-α chains. As previously reported with cultured fibroblasts (Goldberg and Scherr, 1973), both the intracellular and secreted procollagen contained interchain bonds. Some degradation of the molecule was seen with the tendon cells in that the medium contained some polypeptides which were triple helical and similar in size to pro-α chains but which were not linked by interchain bonds. Similar polypeptides were recovered from the medium of intact tendon and therefore the results support previous indications (Uitto *et al.*, 1972; Goldberg and Scherr, 1973; Monson and Bornstein, 1973a) that the conversion of procollagen to collagen can occur by several discrete steps, the first of which may remove regions close to the NH_2-terminal end containing the interchain bonds. However, the data do not resolve the important question whether this stepwise conversion is quantitatively more important *in vivo* than the one-step conversion which has been suggested on the basis of observations in cattle with dermatosparaxis (Lapière *et al.*, 1971).

The pulse-label and chase experiments reported here demonstrated that interchain disulfide bonding occurs at a relatively late stage during the biosynthesis of tendon procollagen. After a pulse period of 4 min, [^{14}C]hydroxyproline-containing polypeptides of the same size as pro-α chains were labeled but essentially none of these peptides or of shorter peptides were disulfide linked. It was only after a chase period of 9–12 min that most of the intracellular pro-α chains were linked by interchain bonds. Since the synthesis time for pro-α chains has been shown to be about 6 min for two different interstitial collagens (Vuust and Piez, 1972; Miller *et al.*, 1973), the formation of the interchain bonds apparently occurs after assembly of amino acids into pro-α chains is completed and perhaps after the pro-α chains are released from ribosomes.

In the pulse-chase experiments the polypeptides became triple helical, as judged by resistance to proteolysis, at about the same time as the interchain bonds formed. It might be noted that the test of helicity which was employed was the recovery of [^{14}C]hydroxyproline in α chains after digestion with α-chymotrypsin. This test would not have detected peptides which were smaller than α chains and triple helical. Since, however, the pro-α chains present at the end of pulse-labeling period were nonhelical, it seems unlikely that any shorter peptides were helical. Although the data demonstrated that interchain bonding and formation of the triple helix occurred at about the same time, it was not possible in these experiments to determine whether one of these events preceded the other. However, the experiments in which the cells were incubated so that they synthesized and accumulated protocollagen demonstrated that the interchain bonding could occur without the pro-α chains becoming triple helical. The protocollagen which accumulates in tendon cells during inhibition of the proline and lysine hydroxylases in the presence of the iron chelator α,α'-dipyridyl or under anaerobic conditions is converted to triple-helical procollagen if inhibition of the hydroxylases is reversed (Juva *et al.*, 1966; Uitto and Prockop, 1973, 1974a). It is clear therefore that under these experimental conditions, and presumably in circumstances where connective tissues are subject to intermittent anoxia *in vivo*, synthesis of the interchain disulfide bonds precedes formation of the triple helix.

The observations presented here as well as recent observations with lens cells synthesizing basement membrane collagen (Grant *et al.*, 1973) suggest the hypothesis that formation of interchain disulfide bonds may play an important role in the biosynthesis of procollagen. The synthesis of such bonds may in fact be essential for the triple helix to form at an appropriately rapid rate *in vivo*.

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Addendum

Following the submission of this manuscript, three manuscripts from other laboratories have appeared which have provided further evidence for the conclusion that the three pro-α chains of procollagen are linked by interchain disulfide bonds (Sherr *et al.*, 1973; Fessler *et al.*, 1973; Monson and Bornstein, 1973b).

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