The assembly of reduced pro-α chains of type I and type II procollagen into the native triple-helical molecule was examined in vitro in the presence and absence of pure protein disulfide isomerase. The data clearly indicates that protein disulfide isomerase is able to accelerate the formation of native interchain disulfide bonds in these procollagens. It takes about 6 min after disulfide bonding before triple-helical molecules exist, while the time required to produce triple-helical type I procollagen in the presence of protein disulfide isomerase is 9.4 min and that for type II procollagen 17.2 min. These values agree with those obtained for type I and II procollagen in vivo suggesting that protein disulfide isomerase is also an enzyme catalyzing interchain disulfide bond formation in procollagen in vivo.

The formation of native disulfide bonds can proceed without any enzyme catalysis but then requires the presence of reduced and oxidized glutathione. Bonding is rather slow in such a case, however, resulting in a delay in the formation of the triple helix.

The propeptides of procollagens, the biosynthetic precursors of various collagens, contain both intrachain and interchain disulfide bonds. In type I and type II procollagen intrachain bonds are found in both the amino-terminal and the carboxyl-terminal propeptides, whereas interchain disulfides are present only between the carboxyl-terminal propeptides. In type III procollagen, both the amino-terminal and the carboxyl-terminal propeptides are linked by interchain disulfide bonds, in addition to which there are two interchain disulfide bonds at the carboxyl-terminal end of the triple-helical region (1–3).

The role of the disulfide bonds between the carboxyl-terminal propeptides in procollagens in the formation of the native triple helix has been the subject of numerous investigations (3–13). If purified collagens or procollagens are denatured in vitro, the presence of covalent cross-links such as disulfide bonds between the polypeptide chains is required for efficient refolding into a triple-helical conformation (10–13). Disulfide bond formation in the carboxyl-terminal propeptides and folding of the triple helix are closely correlated during the assembly of procollagen in the rough endoplasmic reticulum (4–9). In newly synthesized procollagen in cartilage or tendon cells triple helix formation has been assumed to occur after the formation of the disulfide bonds between the constituent polypeptides (9, 14), a theory which gains support from research into the temporal sequence of the disulfide bonds in type III procollagen during biosynthesis (6). There is also a contradictory suggestion (15), however, that initiation of folding in procollagen can take place prior to disulfide bond formation and that a noncovalent interaction could be sufficient to initiate efficient triple helix formation. The time required for triple helix formation varies markedly between cells synthesizing various collagen types, the shortest being about 8–10 min in chick embryo tendon cells synthesizing type I procollagen (1, 3, 4, 14).

The mechanism of disulfide bonding in proteins is still rather poorly understood. In vitro studies have indicated that it is a two-step process. First randomly cross-linked disulfide bonds are formed, and second these non-native disulfides are isomerized to native ones via a series of thiol-disulfide interchange reactions (16–18). The latter process seems to be the rate-limiting step. An enzyme known as protein disulfide isomerase (EC 5.3.4.1) which catalyzes such a rearrangement of disulfide bonds in vitro has been found to be widely distributed in animal tissues and is present in the microsomal fraction, where it has a wide specificity for protein disulfide substrates (18, 19). Protein disulfide isomerase activity is present in cells which actively synthesize collagen (18, 20), and its changes in the tissues of developing chick embryos (21) and between cells synthesizing collagen at markedly different rates conform with differences in prolyl 4-hydroxylase activity and the rate of collagen synthesis (21).

Forster and Freedman (22) have shown that homogeneous protein disulfide isomerase from bovine liver accelerates the in vitro reassembly of reduced type I procollagen chains into disulfide-linked trimers. The results do not suffice to establish, however, whether the trimer was triple-helical procollagen with all the correct disulfide bonds. The present paper examines the assembly of reduced pro-α chains of types I and II procollagen into the native triple-helical molecule in the presence and absence of protein disulfide isomerase, with the principal aim of studying whether the latter is an enzyme catalyzing disulfide bond formation in procollagens in vivo. A study is also made of the relationship between interchain disulfide bonding of pro-α chains and triple helix formation in the biosynthesis of procollagen.

EXPERIMENTAL PROCEDURES

Materials—[2,3,4,5-3H]Proline (110 Ci/mmol) was obtained from Amer sham International (Amersham, Bucks, Great Britain). Fertilized eggs of White Leghorn chickens were purchased from Sipikarja hoitajien Liitto r.v. (Hameenlinna, Finland). Trypsin (grade TRTCPK) was obtained from Worthington, and α-chymotrypsin (type II) and soybean trypsin inhibitor were from Sigma.

Preparation of Protein Disulfide Isomerase—Protein disulfide isomerase was purified from whole chick embryos as described elsewhere (23, 24). The purification procedure contained two ammonium sulfate frac tionations, CM-Sephadex C-50 cation exchange chromatography, followed by DEAE-Sephacel anion exchange chromatography, and gel filtration on Sephacryl S-300. The enzyme was pure as judged by
FIG. 1. Autoradiophotogram of isolated radiolabeled type I procollagen. A, isolated type I procollagen was subjected to slab polyacrylamide gel electrophoresis in sodium dodecyl sulfate in the absence (lane I) and presence (lane II) of dithiothreitol. B, scanning profiles of lanes I and II indicating the presence of type I procollagen and pC-collagen in lane I and that of pro-α chains and pC-α chains in lane II.

SDS polyacrylamide gel electrophoresis.

Preparation of Radioactively Labeled Procollagen—Tendons and sterna were dissected from 17-day chick embryos and the tendon and cartilage cells isolated by trypsin and collagenase digestion (25). From 0.8 to 1.0 × 10^7 cells from 120-160 embryos were used for the experiments. These were suspended in 30 ml of Krebs medium containing 2% fetal calf serum and incubated at 37 °C for 4 h in the presence of 500 μCi of [3H]proline. 3.0 ml of Krebs medium containing a protease inhibitor mixture was then added to give final concentrations of 1.0 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, and 25 mM disodium EDTA. The cells were removed by centrifugation at 1200 × g for 10 min. The medium procollagen was precipitated by adding 176 mg/ml ammonium sulfate (30% saturation) and stirring the solution overnight. The protein precipitate was centrifuged at 1200 × g for 30 min and dissolved in 10 ml of 0.4 M NaCl and 0.1 M Tris-HCl buffer, pH 7.4, at 4 °C and stirred at 4 °C for 4 h. The dissolved material was centrifuged at 10,000 × g for 30 min, and the supernatant was then dialyzed against the NaCl-Tris-HCl buffer to give type I or type II procollagen (2.5-4.5 × 10^6 dpm/ml), specific activity 33 × 10^6 dpm/mg.

Reduction of Type I and Type II Procollagen to Pro-α Chains—Type I and type II procollagen were reduced for 20 min at 37 °C in the presence of 10 mM dithiothreitol (22). Dithiothreitol was removed from the solution by centrifugation in Sephadex G-25 columns equilibrated with 0.1 M acetic acid (26). Aliquots containing reduced pro-α chains were lyophilized and stored at -70 °C.

Reoxidation of Pro-α Chains to Procollagen—Reduced pro-α chains (1.25-2.25 × 10^6 dpm) were reoxidized at 37 °C in 200 μl of 1.0 mM EDTA, 0.1 M Tris-HCl buffer, pH 7.5, containing reduced glutathione (GSH) and oxidized glutathione (GSSG) (22). Two aliquots of 20 μl were removed from the reoxidation mixture after various intervals of 0 min to 6 h. One aliquot was immediately added to 100 μl of SDS-PAGE sample buffer consisting of 2% (w/v) sodium dodecyl sulfate, 15% (v/v) glycerol, 0.001% (w/v) bromphenol blue, and 0.1 M iodoacetate in 62.5 mM Tris-HCl buffer, pH 6.8, preheated to 80 °C. The sample was maintained at 80 °C for a further 10 min and then stored at -20 °C. The other aliquot was added to 15 μl of 0.1 M Tris-HCl buffer, pH 7.5, at 37 °C containing trypsin (100 μg/ml) and α-chymotrypsin (250 μg/ml) and incubated at 37 °C for 5 min to digest non-triple-helical material (9). The digestion was stopped by adding 5 μl of 0.1 M Tris-HCl buffer containing soybean trypsin inhibitor (5.5 mg/ml). After that 80 μl of SDS-PAGE sample buffer with iodoacetate was added. The samples were stored at -20 °C until used.

Reoxidation in the presence of protein disulfide isomerase was performed in the same way except that 20 μl of pure protein disulfide isomerase (5 ng) was added to the reoxidation mixture.

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed under nonreducing conditions using 5% acrylamide as the separating gel (27). The gels were impregnated with 2.5-diphenyloxazole for fluorography (28), dried under a vacuum, and exposed to Kodak X-Omat film at -70 °C for 3-14 days.

Quantification of Protein Bands in Slab Gels—Procollagen, collagen, and pro-α chains detected on the film were quantified using a densitometer connected to a Spectra-Physics SP 4100 computing integrator.

RESULTS

Isolated Procollagens—Types I and II procollagen were labeled biosynthetically by incubating chick embryo tendon and cartilage cells in the presence of [3H]proline. Although the procollagens were isolated in the presence of proteinase inhibitors (1.0 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 25 mM EDTA), large portions of both types were processed to pC-collagen, i.e. procollagen without the amino-terminal propeptide. The amount of intact procollagen in the type I (Fig. 1) and type II procollagen preparations was 40 and 60%, respectively. Treatment with 10 mM dithio-
threitol reduced the procollagen and pC-collagen to pro-α chains and pC-α chains, respectively. The radioactivity found in pro-α1(I) band in SDS-slab gel electrophoresis was 2.2 times that in the pro-α2(I) band, and a similar radioactivity ratio was found when the pC-α1(I) band was compared with the pC-α2(I) band.

**Reoxidation of Type I Pro-α Chains to Triple-helical Pro-collagen**—The rate of reoxidation of pro-α chains was monitored by measuring the disappearance of pro-α1(I) and pro-α2(I) chains upon SDS-polyacrylamide gel electrophoresis after various times. Polymeric material corresponding in molecular weight to trimeric pro-α chains was noticed on the gel, indicating the presence of covalent linkages between pro-α chains.

The rate of triple helix formation was monitored by following the appearance of α1(I) and α2(I) chains upon SDS-polyacrylamide gel electrophoresis after trypsin and chymotrypsin digestion of samples withdrawn at various points in time.

The reoxidation of pro-α chains was achieved with an oxidizing system containing GSH and GSSG. Several concentrations of this system were used (Table I, Fig. 2). The half-time for the disappearance of the pro-α1(I) and pro-α2(I) chains in the presence of 10 μM GSH, 1.0 μM GSSG was 20.6 min, whereas when 1.0 mM GSH, 0.1 mM GSSG was used this was shortened to 11.2 min. Further increases in the GSH/GSSG concentration led to longer half-times for disulfide bonding, 20 mM GSH, 2.0 mM GSSG giving a value of 21.1 min. When a highly oxidizing system of 5.0 mM GSSG was used, the value was 10.9 min (Table I). The disappearance of both pro-α1(I) and pro-α2(I) chains obeyed the same kinetics, giving similar half-times to the corresponding pro-α chains (not shown).

Purified protein disulfide isomerase (5 ng of enzyme protein) accelerated the reoxidation of the pro-α chains, a half-time of 3.7 min for pro-α1(I) and pro-α2(I) chains being observed with 10 μM GSH, 1.0 μM GSSG or 1.0 mM GSH, 0.1 mM GSSG (Fig. 2, Table I). pC-chains gave similar half-times in the presence of protein disulfide isomerase. The reoxidations of both pro-α and pC chains again obeyed the same kinetics (Fig. 2).

The reoxidized pro-α chains formed a triple-helical molecule, but there was a lag period of 6 min between the disappearance of the pro-α chains and the appearance of the triple-helical conformation (Fig. 3A), the latter obeying sigmoid-like kinetics (Fig. 3A) and giving half-times of 17.1-26.0 min in the absence of protein disulfide isomerase and 9.4 min in its presence. As seen in Fig. 3B, the disappearance of free pro-α1(I) and free pro-α2(I) chains is described by a first order process, just as the appearance of triple-helical procollagen after a 6-min lag period can be also described by a first-order process. This implies that the process occurring in the lag period takes place according to zero order kinetics.

**Formation of the Triple-helical Molecule from Pro-α(II) Chains**—The half-time for the disappearance of pro-α(II) chains in the presence of 10 μM GSH, 1.0 μM GSSG as the oxidative system was found to be about 90 min, as was that observed with a 10 mM GSH, 1.0 mM GSSG system. In the presence of protein disulfide isomerase the kinetics gave a half-time of 11.6 min with both concentrations of the GSH/GSSG system (Fig. 4), and a similar result was found with the pC-α(II) chains (not shown). The appearance of triple-helical procollagen obeyed sigmoid-like kinetics, giving a half-time of 17.2 min (Fig. 5) in the presence of protein disulfide isomerase.

**Comparison of Interchain Disulfide Bond and Triple Helix Formation**

**Table I**

| Oxidizing system* | t_{1/2} of free pro-α chain formation | t_{1/2} of triple helix formation | Lag time between pro-α and triple helix formation |
|-------------------|-------------------------------------|---------------------------------|-----------------------------------------------|
|                   | Without PDI | With PDI | Without PDI | With PDI | Without PDI | With PDI |
| GSH/GSSG          |            |          |            |          |            |          |
| Type I procollagen|            |          |            |          |            |          |
| 10 μM, 1.0 μM     | 20.6       | 3.7      | 26.0       | 9.4      | 5.4-5.7    |          |
| 1.0 mM, 0.1 mM    | 11.2       | 3.7      | 17.1       | 9.4      | 5.7-5.9    |          |
| 20 mM, 2.0 mM     | 21.1       | 6.1      | ND*        | ND       | ND         |          |
| 2.0 mM, 2.0 mM    | 10.7       | 3.7      | ND         | ND       | ND         |          |
| 0.0 mM, 5.0 mM    | 10.9       | ND       | ND         | ND       | ND         |          |
| Type II procollagen|          |          |            |          |            |          |
| 10 μM, 1.0 μM     | 88.2       | 11.6     | ND         | 17.1     | 5.5        |          |
| 1.0 mM, 0.1 mM    | 80.1       | 11.6     | ND         | 17.3     | 5.7        |          |

*Reoxidation of pro-α chains of types I and II procollagen into triple-helical procollagen was studied using various concentrations of reduced (GSH) and oxidized (GSSG) glutathione as an oxidizing system in the reaction mixture. 5 ng of pure protein disulfide isomerase (PDI) was used in the reaction.

*ND, not determined.
Protein Disulfide Isomerase

FIG. 3. Kinetics of reoxidation of pro-α chains of type I procollagen into triple-helical conformation in the presence of protein disulfide isomerase. A, pro-α chains were incubated with protein disulfide isomerase in the presence of 1.0 mM GSH, 0.1 mM GSSG, and the disappearance of pro-α chains (O) and appearance of trypsin/chymotrypsin-resistant α chains (●) were monitored at various intervals on SDS-PAGE. Similar results were obtained in five separate experiments. B, the data from A presented on a semi-logarithmic scale. O, disappearance of pro-α chains; ●, disappearance of trypsin/chymotrypsin sensitive α chains (100% - value from A at each time point).

Formation in Type I and II Procollagens—As indicated in this paper, the formation of trimeric pro-γ chains is possible in an oxidizing milieu without enzyme catalysis. An increase in the concentration of the glutathione-oxidizing system to millimolar level admitted reduced the time required for formation of the pro-γ chain in the case of type I procollagen, but even in this case interchain disulfide bonding and triple helix formation was considerably slower than that found in the presence of protein disulfide isomerase. Interchain disulfide bonding of pro-α chains was accelerated 4–8-fold for type I procollagen and about 8-fold for type II procollagen when the pro-α chains were oxidized in the presence of protein disulfide isomerase (Table I). It was noticed that interchain disulfide bonding occurred much faster in type I procollagen than in type II.

The trimeric pro-γ chain was converted to a triple-helical conformation, as shown by the resistance of the molecule to trypsin and chymotrypsin. Protein disulfide isomerase does not seem to have any effect on the process converting the trimeric pro-γ chain to a triple-helical conformation, as the lag time of about 6 min between the pro-γ chain and the triple-helical conformation was observed both with and without the enzyme. The lag-time was the same in types I and II procollagen. Pro-α chains of type I procollagen formed a triple helix in 9.4 min in the presence of protein disulfide isomerase and pro-α chains of type II procollagen in 17.2 min.

DISCUSSION

This paper describes the effect of protein disulfide isomerase on the formation of interchain disulfide bonds in types I and II procollagen. The data clearly indicates that this enzyme is able to accelerate the formation of native interchain disulfide bonds in these procollagens. Disulfide bonding precedes triple helix formation, a period of about 6 min being needed before the triple-helical molecules exist, probably due to cis/trans isomerization of peptide bonds in the triple helix folding. This latter is reported to be the rate-limiting step in triple helix propagation (12, 29). Measurements by nuclear magnetic

FIG. 4. Kinetics of interchain disulfide bonding in type II procollagen (semilog scale). The rate of disulfide bond formation was monitored by measuring the disappearance of pro-α chains on SDS-PAGE after various periods of reoxidation with protein disulfide isomerase and 1.0 mM GSH, 0.1 mM GSSG (●), without protein disulfide isomerase but with 1.0 mM GSH, 0.1 mM GSSG (●), and without protein disulfide isomerase or any oxidizing system (A). Similar results were obtained in four separate experiments.

FIG. 5. Kinetics of reoxidation of pro-α chains of type II procollagen into a triple-helical conformation in the presence of protein disulfide isomerase. Pro-α chains were incubated with protein disulfide isomerase in the presence of 1.0 mM GSH, 0.1 mM GSSG. The disappearance of pro-α chains (●) and the appearance of trypsin/chymotrypsin-resistant α chains (●) were monitored on SDS-PAGE at various time intervals. Similar results were obtained in four separate experiments.
resonance spectroscopy have shown that 16 ± 2% of the X-Pro and 8 ± 2% X-Hyp bonds are in the cis configuration in unfolded collagens (30), which means that about 30 bonds/α chain have to be isomerized prior to folding of the triple helix. The growth of the triple helix is thought to proceed from a single nucleus at the carboxyl-terminal end in a zipper-like fashion at a constant rate, this giving rise to zero-order kinetics (12), as also found in this study. The time required to produce triple-helical type I procollagen in vitro in the presence of protein disulfide isomerase was 9.4 min, the corresponding value for type II procollagen being 17.2 min. These in vitro values fit very well with those obtained for type I and type II procollagen in cellulo (1, 3, 4, 14, 15), suggesting that protein disulfide isomerase is also an enzyme catalyzing interchain disulfide bond formation in vivo.

The procollagen preparations used here study contained both procollagen and pC-collagen and it was possible to measure the disappearance of free pro-α chains and pC-α chains on the slab gel. It was noticed that both pro-α(I) chains and pro-α2(I) chains obeyed the same kinetics and had the same half-time for their disappearance. Furthermore, it was found that pC-α1(I) and pC-α2(I) chains obeyed the same kinetics as pro-α1(I) and pro-α2(I) chains, indicating that the association of pro-α chains starts in the carboxyl-terminal part of the procollagen molecule. Our data are in good agreement with the earlier findings (6-12, 29), showing that the association and interchain disulfide bonding of carboxyl-terminal propeptides of procollagen precedes the formation of a triple helix in the collagenous domain of the molecule. The conversion of pro-α chains to fully disulfide-linked trimers is thought on the basis of data from pulse-chase experiments (8, 31) to occur through intermediates, in which two pro-α chains are linked by interchain disulfide bridges, although no accumulation of dimers of pro-α chains (β chains) could be detected during the association of pro-α chains in the present study. Whether this discrepancy is due to a difference in the sensitivity of the assays or to a difference in the experimental conditions used in the various studies remains to be solved.

The results obtained in this study indicate that the formation of native disulfide bonds can proceed without any enzyme catalysis in the presence of an oxidizing system consisting of reduced and oxidized glutathione. In such a case the process is rather slow, however. Great kinetic barriers are encountered in the formation of trimeric pro-γ chains, resulting in a delay in the formation of the triple helix. These data further support the hypothesis that disulfide bond formation is an essential event for triple helix formation (10, 11, 29).

Kao et al. (31) have also studied the formation of disulfide bonds in procollagen using pulse-chase labeling and found that the formation of disulfide bonds, measured in terms of the disappearance of free radiolabeled pro-α chains, had a half-time of 10 and 20 min for procollagen of types I and II, respectively. However, taking into account the fact that the amount of radioactively labeled procollagen was increasing during the chase period the values obtained for free pro-α chains at various chase times would seem to be too high and thus give excessively high values for the half-time for disulfide bonding. If the data of Kao et al. (31) are recalculated taking this into account, a half-time of about 4 min is found for the disulfide bonding of type I procollagen, a value which fits perfectly with that obtained in vitro here in the presence of protein disulfide isomerase.

Disulfide bonding seems to be a slower process in type II procollagen than in type I, both in vivo (1, 3, 4, 14, 15) and in this in vitro study. As indicated here and in our earlier work on cells synthesizing different collagen types (20), the marked differences in the rates of formation of the interchain disulfide bonds between the various procollagen types are not due to corresponding differences in protein disulfide isomerase activity. It, therefore, seems likely that they are due to differences in structure between the procollagens. There is a high homology of primary sequences between the type I and II procollagen carboxyl-terminal propeptides (32-34) and the site of cysteine residues is well conserved, so that it seems surprising to find that the rate of disulfide pairing is not similar in the two types. There are some differences in structure between the carboxyl-terminal propeptides of the two types, however, which may explain the marked difference in disulfide bonding.

The carboxyl-terminal propeptide of the pro-α1(I) and pro-α1(II) chains contains 8 cysteine residues, whereas the pro-α2(I) chain has only 7 (32-34), an odd number which thus offers a free thiol group for the rearrangements of disulfides. This may be the reason for the more rapid formation of interchain disulfides in type I procollagen than in type II, which has an even number of cysteine residues. It would also explain the fact that a type I homotrimer consisting of three pro-α1(I) chains, and thus having an even number of cysteine residues in the carboxyl-terminal propeptide of the molecule, involves a higher degree of post-translational modifications than normal type I procollagen (35). This is probably due to the increased half-time for disulfide bonding in the homotrimer and thus increased time for triple helix folding, which allows more time for post-translational modifications to occur (see Refs. 1 and 3).

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