Type-III procollagen assembly in semi-intact cells: chain association, nucleation and triple-helix folding do not require formation of inter-chain disulphide bonds but triple-helix nucleation does require hydroxylation

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Procollagen assembly is initiated within the endoplasmic reticulum by three α -chains associating via their C-propeptides (C-terminal propeptides). To study the requirements for the association of procollagen monomers at synthesis we have reconstituted the initial stages in the folding, assembly and modification of procollagen using semi-permeabilized cells. By translating a type-III procollagen 'mini-gene' which lacks part of the triple-helical domain, we demonstrate that these cells efficiently carry out the assembly of hydroxylated, triple-helical, procollagen trimers and allow the identification of specific disulphide-bonded intermediates in the folding pathway. Mutant chains, which lack the ability to form inter-chain disulphide bonds within the C-propeptide, were still able to assemble within this system. Furthermore, characterization of the trimeric molecules formed

suggested that inter-chain disulphide bonds had formed within the C-telopeptide (C-terminal telopeptide). However, when hydroxylation of prolyl and lysyl residues was inhibited no inter-chain disulphide bonds were formed in the C-telopeptide, indicating that hydroxylation is required for the initial nucleation of the triple-helical domain. Mutant chains which lacked the ability to form inter-chain disulphide bonds within the C-propeptide or the C-telopeptide could still assemble to form trimeric triple-helical molecules linked by inter-chain disulphide bonds within the N-propeptide (N-terminal propeptide). These results indicate that inter-chain disulphide bond formation within the C-propeptide or the C-telopeptide is not required for chain association and triple-helix formation.

INTRODUCTION

The folding pathway of procollagen has been described as a series of steps punctuated by the formation of disulphide bonds [1]. Initially the N-terminal and C-terminal propeptides (N- and C-propeptides) fold and form intra-chain disulphide bonds. The C-propeptides then associate and form inter-chain disulphide-bonded trimers. This association both ensures the correct registration of the three chains allowing triple-helix formation to proceed in a C- to N- direction [2] and regulates assembly such that the constituent chains associate in a collagen type-specific manner [3]. Inter-chain disulphide bonds form between the individual C-propeptide chains prior to triple-helix formation, leading to the suggestion that these covalent linkages may be required to stabilize the association between the individual chains [4–6]. For type-III procollagen, inter-chain disulphide bonds are also formed within the C-terminal telopeptide (C-telopeptide), a short non-collagenous sequence linking the triple-helical domain with the C-propeptide [7]. The C-telopeptide has been postulated to have a role both in C-propeptide assembly and in nucleating triple-helix formation [8]. Inter-chain disulphide bonds also form within the N-propeptide [9] but this does not occur, however, until folding of the triple helix is complete, indicating that they form after association of the chains via their C-propeptides [10]. The C-propeptide, therefore, plays an essential role in the assembly of procollagen ensuring selective association of three α -chains with the C-telopeptide forming a nucleation point for triple-helix propagation.

We have previously described the cell-free translation, translocation and trimerization of a type-III procollagen 'mini-gene' in a system consisting of a rabbit reticulocyte lysate supplemented with dog pancreatic microsomes [11]. This shortened molecule

[$\alpha 1(\text{III})\Delta 1$] encodes the N-propeptide, including the N-terminal signal sequence, a truncated triple-helical domain, and the C-propeptide. We investigated the role of inter-chain disulphide bond formation within the C-propeptide in the initial interaction of the α -chains and showed that the molecular mechanisms underlying chain recognition do not rest solely with the ability to form these bonds. Although these molecules were able to trimerize in this system they were unable to form triple helices as the microsomal membranes are unable to hydroxylate proline and lysine residues, a modification which is essential for stable triple-helix formation [12,13]. To study the association of hydroxylated pro- α -chains we have used a procedure to selectively permeabilize the plasma membrane of cells grown in culture, which releases the cytosol while retaining a functionally and morphologically intact endoplasmic reticulum (ER) [14,15]. These semi-permeabilized cells (SP-cells) can then be included instead of microsomal membranes in a conventional cell-free translation system. For this study we chose a cell-line (HT-1080) which contains procollagen-processing enzymes including prolyl 4-hydroxylase to enable the complete reconstitution of the folding pathway of procollagen at synthesis [16]. Microsomal membranes isolated from this cell-line have been shown previously to assemble and hydroxylate type-X collagen synthesized *in vitro* [17].

In this report we have reconstituted the efficient folding and assembly of a type-III procollagen 'mini-gene' and shown that we can follow the formation of specific disulphide-bonded intermediates formed during the folding and assembly of procollagen. We have used this system to investigate the role of inter-chain disulphide bond formation on procollagen assembly. The results demonstrate that inter-chain disulphide bond formation within the C-propeptide or within the telopeptide is not a

prerequisite for triple-helix formation. We also show that the nucleation of the triple-helical domain prior to helix propagation can be prevented by the inclusion of inhibitors of proline and lysine hydroxylation, suggesting a role for this modification in the association of the three chains at the C-telopeptide.

MATERIALS AND METHODS

Reagents

Restriction enzymes, T7 RNA polymerase, and nucleotides were obtained from Boehringer (Lewes, East Sussex, U.K.). Nuclease-treated rabbit reticulocyte lysate (Flexi-lysate), amino acids minus methionine, RNasin ribonuclease inhibitor, were purchased from Promega (Southampton, U.K.). L-[³⁵S]Methionine was purchased from Amersham International (Amersham, Bucks., U.K.). HT-1080 human fibrosarcoma cells (A.T.C.C. CCL121) were from the American Type Culture Collection (Rockville, MD, U.S.A.). Tissue culture reagents were purchased from Gibco Life Technologies Ltd. (Glasgow, Scotland, U.K.). Digitonin was obtained from Calbiochem (Nottingham, U.K.). SP-cells were prepared as described [15]. Bacterial collagenase form III was from Advance Biofacture (Lynbrook, NJ, U.S.A.). All other reagents were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Construction of recombinant plasmids

Construction of the procollagen type III 'mini-gene' $\alpha 1(\text{III})\Delta 1$ has been described previously [11]. Site-directed mutagenesis was performed essentially as described by Kunkel et al. [18], except that 1 unit of T7 DNA polymerase (USB, Amersham, U.K.) was used in the extension reaction.

Transcription *in vitro*

Transcription reactions were carried out as described by Gurevich et al. [19]. Recombinant $\alpha 1(\text{III})\Delta 1$ and cysteine mutants (10 μg) were linearized with *Hind*III and transcribed using T3 RNA polymerase. Reactions were incubated at 37 °C for 4 h, followed by phenol/chloroform extraction and ethanol precipitation. RNA was resuspended in 50 μl of RNase-free water containing 1 mM dithiothreitol (DTT) and 40 units of RNasin.

Translation *in vitro*

RNA was translated using a rabbit reticulocyte lysate (Flexi-Lysate) at 30 °C in the absence of exogenous DTT. The translation reaction (25 μl) contained 17.5 μl of reticulocyte lysate, 0.5 μl of 1 mM amino acids (minus methionine), 0.4 μl of 100 mM KCl, 0.5 μl of ascorbic acid (0.5 $\mu\text{g}/\text{ml}$), 15 μCi of L-[³⁵S]methionine, 1 μl of transcribed RNA and approx. 10^5 SP-cells. After translation, *N*-ethylmaleimide (NEM) was added to a final concentration of 25 mM. SP-cells were isolated by centrifugation in a microfuge at 10000 *g* for 5 min. The pellet was resuspended in an appropriate buffer for subsequent enzymic digestion or for immunoprecipitation.

Antibody preparation and immunoprecipitation

An antibody was raised to a peptide consisting of the last 133 amino acids of human type-III procollagen. The antigen was prepared by first isolating a 1300 bp *Nco*I/*Hind*III fragment from $\alpha 1(\text{III})$. This fragment was end-filled using the Klenow fragment of DNA polymerase and subcloned into the *Sma*I site of the *Escherichia coli* expression plasmid pET3ap [20]. BL21(DE3) cells were transformed with the resulting recombinant plasmid pET-III-*Nco*. A 50 ml culture was grown to an

A_{590} of 0.5 and expression induced with isopropyl thiogalactoside (400 mM) for a further 3 h. Cells were harvested, washed with 50 mM Tris/HCl, pH 8, containing 2 mM EDTA and resuspended in 4 ml of lysis buffer (50 mM Tris/HCl, pH 8, 2 mM EDTA, 1% Triton X-100) plus 100 $\mu\text{g}/\text{ml}$ lysozyme and incubated at 30 °C for 30 min. The lysed cells were passed several times through a 21 gauge needle to shear the DNA and centrifuged at 12000 *g* for 15 min at 4 °C. The pelleted material, which contained inclusion bodies, was solubilized in 2 ml of 7 M urea prior to preparative electrophoresis. One-fifth of the total volume of solubilized inclusion bodies was added to an equal volume of SDS/PAGE buffer and loaded across a 15% polyacrylamide gel. Narrow slices were cut from the ends and middle of the gel and stained in 5% (v/v) methanol/7.5% (v/v) acetic acid/20% (v/v) ethanol/0.25% (w/v) Coomassie Blue to visualize proteins. The stained strips were then lined up with the unstained gel and the appropriate band was excised. The protein was electroeluted and dialysed overnight against PBS prior to use as an antigen.

For immunoprecipitation the cell pellet was resuspended in 100 μl of immunoprecipitation buffer [50 mM Tris/HCl buffer, pH 7.5, containing 1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA] with 0.5% (w/v) SDS and translation products denatured by heating at 95 °C for 5 min. The denatured products were then diluted with 1 ml of immunoprecipitation buffer and immunoprecipitated with 1 μl of antiserum followed by absorption on to Protein A-Sepharose.

Pepsin digestion

Isolated SP-cells were resuspended in 0.5 M acetic acid in 1% (v/v) Triton X-100 and incubated with pepsin (100 $\mu\text{g}/\text{ml}$) for 2 h at 20 °C or for 16 h at 4 °C. Digestions were stopped by neutralization with 1 M Tris base and proteins precipitated with ethanol at a final concentration of 27% (v/v).

Chymotrypsin/trypsin digestion

Precipitated protein from pepsin digests were resuspended in 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 10 mM EDTA and 1% (v/v) Triton X-100. Chymotrypsin and trypsin were added to a final concentration of 250 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ respectively and samples incubated at room temperature for 2 min. The digestion was stopped by the addition of soy bean trypsin inhibitor to a final concentration of 500 $\mu\text{g}/\text{ml}$ and 5 vols. of boiling SDS/PAGE sample buffer and samples were boiled for 3 min.

Heat denaturation

Pepsin-treated samples were resuspended in 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 10 mM EDTA and 1% (v/v) Triton X-100, and 10 μl aliquots were placed in a DNA thermal cycler. A stepwise temperature gradient was set up from 35 to 44 °C with the temperature being held for 2 min at 1 °C intervals. For a given temperature at the end of 2 min the sample was assayed at room temperature with chymotrypsin/trypsin for 1 min, as described above.

Post-translational hydroxylation

Translations were carried out in the presence of pro $\alpha 1(\text{III})\Delta 1$ mRNA and α, α' -dipyridyl (0.5 mM) for 90 min and the translation terminated by the addition of cycloheximide to a final concentration of 0.5 mM. Iron in the form of FeSO₄ was then added to a final concentration of 5 mM to reverse the inhibition of hydroxylation by α, α' -dipyridyl. Samples were taken at various

times and disulphide bond formation stopped by the addition of NEM (25 mM). The samples were then separated by SDS/PAGE.

PAGE

Samples were prepared for electrophoresis by mixing with 5 vol. of SDS/PAGE loading buffer [0.0625 M Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and Bromophenol Blue] in the presence or absence of 50 mM DTT and boiled for 5 min. After electrophoresis, gels were processed for autoradiography and exposed to Kodak X-Omat AR film, or images quantified by phosphorimage analysis.

RESULTS

Synthesis, translocation and disulphide bond formation of procollagen in SP-cells

We have previously shown that SP-cells can efficiently translocate, glycosylate and assemble a variety of secretory and membrane-bound proteins [15]. To determine whether they were capable of processing type-III procollagen we translated pro- $\alpha 1(\text{III})\Delta 1$ RNA in a rabbit reticulocyte lysate in the presence of HT-1080 SP-cells. Ascorbic acid was included in the translation reaction to optimize the activity of prolyl 4-hydroxylase (the inclusion of this reagent did not alter the redox conditions sufficiently to affect disulphide bond formation). Translation was

carried out for various time periods and terminated by the addition of 25 mM NEM to prevent further formation or rearrangement of disulphide bonds. Cells were isolated from the translation mixture to remove untranslocated protein, lysed and the type-III procollagen immunoprecipitated with a polyclonal antibody that recognizes the C-propeptide. At early time points and when the samples were reduced prior to electrophoresis, a single translation product was seen which was progressively converted into a slower-migrating species (Figure 1, upper panel, lanes 1–6). When a similar time course was carried out in the presence of α, α' -dipyridyl (an inhibitor of prolyl 4-hydroxylase), a single translation product was seen at early time points with a slight broadening of the band at later time points (Figure 1, upper panel, lanes 7–12). The slower-migrating product synthesized in the absence of α, α' -dipyridyl was not synthesized in the presence of this inhibitor, indicating that this alteration in mobility is due to hydroxylation of procollagen, a modification that is known to affect electrophoretic mobility [21,22]. The broadening of the band seen in the presence of α, α' -dipyridyl could be due to either incomplete inhibition of hydroxylation or to other post-translational modifications such as glycosylation.

When the samples were separated without prior reduction higher-molecular-mass products were visualized (Figure 1, lower panel). At early time points only the monomeric protein was seen but this quickly disappeared with the concomitant appearance of higher-molecular-mass products. In the absence of α, α' -dipyridyl the higher-molecular-mass products appeared as a major band with a molecular mass corresponding to a trimer (Figure 1, lower panel, lanes 2–6). However, in the presence of the inhibitor, two major products were observed with molecular masses corresponding to a dimer and trimer. The trimer synthesized in the absence of α, α' -dipyridyl had a faster mobility than the trimer synthesized in the presence of inhibitor (Figure 1, lower panel, compare lanes 6 and 12).

To investigate the basis for this difference in mobility of the trimer we carried out the following experiment. Pro $\alpha 1(\text{III})\Delta 1$ chains were synthesized for 90 min in the presence of α, α' -dipyridyl and the translation terminated by the addition of cycloheximide. Iron in the form of FeSO_4 was then added to reverse the inhibition of hydroxylation by α, α' -dipyridyl. Samples were taken at various times and disulphide bond formation stopped by the addition of NEM (25 mM). The samples were then separated by SDS/PAGE under reducing or non-reducing conditions (Figure 2). When the samples were reduced prior to electrophoresis no hydroxylated product could be seen after synthesis in the presence of α, α' -dipyridyl (Figure 2, lane 1). However, post-translational hydroxylation of the translation product was observed after addition of iron as judged by the appearance of a slower-migrating band (Figure 2, lanes 2–4). The appearance of this product coincided with the appearance of a faster-migrating trimer when the samples were separated without prior reduction (Figure 2, lanes 5–8). The intensity of the slower-migrating trimer and the dimer decreased with time illustrating a precursor-product relationship. It has been demonstrated previously that the hydrodynamic volume of type-III procollagen trimers decreases when disulphide bonds are present at both ends of the triple helix, i.e. within both the C- and N-propeptides [1,10]. Thus, it seems reasonable to suggest that the slower-migrating trimer seen when procollagen is unhydroxylated corresponds to molecules which are inter-chain disulphide bonded at one end (the C-propeptide) whereas the faster-migrating trimer seen when procollagen is hydroxylated corresponds to molecules which are linked at both ends (see Figure 9).

To investigate whether the procollagen synthesized in our system had formed a correctly aligned triple helix we determined

Figure 1 Autoradiograph of a time course of disulphide bond formation in procollagen in the presence and absence of α, α' -dipyridyl

RNA coding for type-III procollagen 'mini-gene' [pro $\alpha 1(\text{III})\Delta 1$] was translated for various lengths of time as indicated in a reticulocyte lysate in the absence of added DTT. Translations were carried out in the presence of SP-cells either in the absence (lanes 1–6) or presence (lanes 7–12) of α, α' -dipyridyl. Products of translation were treated with NEM (20 mM), immunoprecipitated with antibodies specific for type-III procollagen and separated without (lower panel) or with (upper panel) prior reduction with DTT (50 mM). Products were separated by SDS/PAGE through a 7.5% gel and visualized by autoradiography.

Figure 2 Autoradiograph of translation products synthesized in the presence of α,α' -dipyridyl after post-translational addition of iron

Translation of $\alpha\alpha 1(\text{III})\Delta 1$ RNA was carried out for 90 min in the presence of SP-cells and α,α' -dipyridyl. Cycloheximide was added to a final concentration of 0.5 mM to inhibit translation and iron was added in the form of FeSO_4 (5 mM) and reactions allowed to continue for various lengths of time at 30 °C prior to the addition of NEM (20 mM). Products were immunoprecipitated and separated by SDS/PAGE through a 7.5% gel under either reducing (lanes 1–4) or non-reducing (lanes 5–8) conditions.

its sensitivity to proteolysis with pepsin, chymotrypsin and trypsin, an assay that has been shown previously to be specific for triple-helical collagen [23]. Preliminary experiments (results not shown) revealed that digestion of the translation products with a combination of chymotrypsin and trypsin yielded a product which was difficult to separate from undigested C-propeptide, which has been shown to be partially resistant to digestion with these proteases [23]. However, if we first treated the translation products to digestion with pepsin prior to chymotrypsin/trypsin treatment the C-propeptide was digested leaving a protease-resistant fragment corresponding to correctly folded triple-helical domain (Figure 3A). Translation products synthesized in the presence of α,α' -dipyridyl were completely degraded by this treatment (results not shown). To confirm that the protease-resistant fragment was the triple-helical domain we carried out a heat denaturation experiment which demonstrated that the fragment was completely degraded when the temperature was raised above 41 °C (Figure 3, lanes 9–11). The protease-resistant fragment remaining at each temperature was quantified by phosphorimage analysis to determine the melting temperature (T_m) which was calculated to be approx. 39.5 °C (Figure 3B). This relatively high T_m compared with the T_m of fragments produced by digestion with interstitial collagenases (36 °C) probably reflects the higher percentage of hydroxyproline residues in the type-III 'mini-gene' (15% compared with 13%). This demonstrates that the procollagen synthesized within our system was hydroxylated and folds to form a correctly aligned triple helix.

Role of inter-chain disulphide bond formation in triple-helix formation

Inter-chain disulphide bonds can form within the C-propeptide, the C-telopeptide and the N-propeptide of type-III procollagen. We had shown previously that point mutations changing in-

Figure 3 Autoradiograph illustrating the heat denaturation of 'mini-gene' collagen triple helix

(A) Translation of $\alpha\alpha 1(\text{III})\Delta 1$ RNA was carried out for 90 min in the presence of SP-cells. SP-cells were isolated by centrifugation, solubilized and either analysed directly by SDS/PAGE (lane 1) or treated with pepsin (100 $\mu\text{g}/\text{ml}$) and precipitated with ethanol (27%, v/v). Precipitates were resuspended in chymotrypsin/trypsin digest buffer and heated to a set temperature as indicated prior to being digested with a combination of chymotrypsin (250 $\mu\text{g}/\text{ml}$) and trypsin (100 $\mu\text{g}/\text{ml}$) (lanes 2–11). The samples were analysed by SDS/PAGE through a 12.5% polyacrylamide gel. Gels were fixed, dried and radiolabelled proteins visualized by autoradiography or band intensities quantified by phosphorimage analysis. (B) The resulting band intensities were plotted versus temperature to form a heat denaturation curve. The temperature (T_m) of 50% degradation is indicated and is approx. 39.5 °C.

dividual cysteine residues within the C-propeptide thought to be involved in inter-chain disulphide bond formation to serine abolished trimer formation [11]. These results were obtained using a cell-free system supplemented with dog pancreas microsomes, which do not hydroxylate procollagen. To determine the effect of hydroxylation on inter-chain disulphide bond formation within the C-telopeptide and the N-propeptide we translated these Cys-mutants in the presence of SP-cells. We initially concentrated on one mutant (cys2) which has the second of the eight cysteines within the C-propeptide mutated (Figure 4). This mutant had been shown previously to be able to form intra-chain but not inter-chain disulphide bonds [11]. A time course of translation was carried out (Figure 5) either in the absence (upper panel) or presence (lower panel) of α,α' -dipyridyl. In the absence of α,α' -dipyridyl a single translation product was observed at early time points, when the samples were reduced prior to electrophoresis, which was converted into a slower-migrating product (Figure 5, upper panel, lanes 1–6). When the

Figure 4 Schematic representation of the procollagen chain encoded by pro α 1(III) Δ 1 recombinant minigene

This recombinant encodes the N-propeptide, including the N-terminal signal sequence, a truncated triple-helical domain, and the C-propeptide. The numbers refer to the number of amino acids in each domain. The numbers in parentheses indicate the size of the full-length triple-helical region. The position of the cysteine residues within the coding region are indicated with a line and numbered within the C-propeptide. The amino acid sequence of the last part of the triple-helical domain and the C-telopeptide is included.

Figure 5 Autoradiograph of a time course of disulphide bond formation of cys2 mutant

RNA coding for cys2 mutant was translated for various lengths of time in a reticulocyte lysate either in the absence (upper panel) or presence (lower panel) of α,α' -dipyridyl. Products of translation were treated with NEM (20 mM), immunoprecipitated with antibodies specific for type-III procollagen and separated by SDS/PAGE through a 7.5% gel without (lanes 7–12) or with (lanes 1–6) prior reduction with DTT (50 mM). Immunoprecipitated proteins were visualized by autoradiography.

Figure 6 Autoradiograph of products of translation of cysteine mutants translated in the presence or absence of α,α' -dipyridyl

RNA coding for pro α 1(III) Δ 1 or cysteine mutants was translated for 60 min in a reticulocyte lysate and SP-cells either in the absence (upper panel) or presence (lower panel) of α,α' -dipyridyl. Cells were isolated after translation, solubilized and products of translation were separated by SDS/PAGE through a 7.5% gel either under reducing (lanes 1–5) or non-reducing (lanes 6–10) conditions and visualized by autoradiography.

same samples were separated without prior reduction, inter-chain disulphide-bonded trimers were observed (Figure 5, upper panel, lanes 7–12) which had a mobility identical to wild-type trimers synthesized in the absence of α,α' -dipyridyl (Figure 6, upper panel, lanes 6 and 8). As this mutant is unable to form inter-chain disulphide bonds within the C-propeptide then this demonstrates that inter-chain disulphide bonds can form within either the C-telopeptide or the N-propeptide.

When α,α' -dipyridyl was included during translation, hydroxylation was inhibited as inferred by the absence of a slower-migrating product when the samples were reduced prior to electrophoresis (Figure 5, lower panel, lanes 7–12). At later time points there was a reduction in the amount of product immuno-

Figure 7 Autoradiograph of translation products from *cys2*, *cys2teloCS* and Δ *teloCS* mutant RNAs before and after digestion with pepsin, chymotrypsin and trypsin

Left panel: RNA coding for either *cys2* (lanes 1,2,7,8), *cys2teloCS* (lanes 3,4,9,10) or Δ *teloCS* (lanes 5,6,11,12) was translated in a reticulocyte lysate for 90 min either in the presence (lanes 2,4,6,8,10,12) or absence (lanes 1,3,5,7,9,11) of α,α' -dipyridyl. Translation products were either analysed directly (lanes 1–6) or treated with pepsin, chymotrypsin and trypsin (lanes 7–12) and separated by SDS/PAGE through a 12.5% gel under reducing conditions and visualized by autoradiography. Right panel: samples from protease digests of translation products synthesized in the absence of added α,α' -dipyridyl were also separated without prior reduction. The protease-resistant fragment from wild-type translation products is included for comparison (lane 1).

precipitated, which may reflect reduced affinity of the antibody for this product or degradation of the translation product, a process which has previously been demonstrated in SP-cells [15]. No trimers were seen when the same samples were separated without prior reduction but intra-chain disulphide bond formation was observed as judged by an increase in mobility of the monomer (Figure 5, lower panel, lanes 7–12). These results confirm those previously obtained with dog pancreas microsomes [11] and indicate that if hydroxylation is inhibited then inter-chain disulphide bond formation within the C-telopeptide or the N-propeptide does not occur.

To investigate further the role of inter-chain disulphide bond formation within the C-propeptide on triple-helix formation we assessed the ability of the other cysteine mutants which had the first, third or fourth cysteine replaced with serine to form trimers in the presence of SP-cells (Figure 6). The results demonstrate that only the wild-type, *cys2* and *cys3* were able to form inter-chain-disulphide-bonded trimers (Figure 6, upper panel, lanes 6, 8 and 9). The formation of trimers with *cys2* and *cys3* does not occur when α,α' -dipyridyl is present during translation (Figure 6, lower panel, lanes 8 and 9) but some dimerization does occur, presumably via *cys3* in the case of the *cys2* mutant or *cys2* in the case of the *cys3* mutant. Both in the presence and absence of α,α' -dipyridyl the monomer of the wild-type, *cys2* and *cys3* can form intra-chain disulphide bonds. However, the monomer of the *cys1* and *cys4* mutants, which do not form trimers, forms a more diffuse pattern when separated under non-reducing conditions (Figure 6, both panels, lanes 7 and 10). This suggests that they do not form the same pattern of intra-molecular disulphide bonds as the wild-type, *cys2* or *cys3* polypeptides.

To determine whether a correctly aligned triple helix had formed with the *cys2* mutant we digested the translation products synthesized in the presence and absence of α,α' -dipyridyl with a combination of pepsin, chymotrypsin and trypsin (Figure 7). A protease-resistant fragment was obtained when translations were

Figure 8 Autoradiograph of products of translation from C-telopeptide mutants

RNA coding for either wild-type (lanes 1 and 4), *cys2teloCS* mutant (lanes 2 and 5) or Δ *teloCS* (lane 3 and 6) mutant were translated in a reticulocyte lysate for 60 min in the presence of SP-cells either in the absence (lanes 1–3) or presence (lanes 4–6) of α,α' -dipyridyl. Products of translation were immunoprecipitated with antibodies specific for type-III procollagen and separated by SDS/PAGE through a 7.5% gel under non-reducing conditions and visualized by autoradiography.

carried out in the absence of α,α' -dipyridyl but not when this inhibitor was present (Figure 7, left panel, lanes 7 and 8). When the same samples were separated without prior reduction higher-molecular-mass trimers were seen for both the wild-type and *cys2* translation products (Figure 7, right panel, lanes 1 and 2). This demonstrates that inter-chain disulphide bond formation occurred within the C-telopeptide of the wild-type and *cys2* mutant. Significantly, this shows that for the inter-chain disulphide bonds to form within the C-telopeptide the triple-helical domain must be hydroxylated as these covalent links do not form when the *cys2* mutant is translated in the presence of α,α' -dipyridyl.

The critical experiments were to ascertain whether this disulphide bond was required to ensure propagation of the triple helix. To do this we created a double mutation which converted the two adjacent cysteine residues within the C-telopeptide to serine (Δ *teloCS*). We also mutated these residues in the *cys2* mutant, so creating a procollagen chain which could not form inter-chain disulphide bonds within the C-propeptide or within the C-telopeptide (*cys2teloCS*). This mutant could still potentially form an inter-chain disulphide bond within the N-propeptide but only if the procollagen chain assembled correctly and if the triple helix was formed. The telopeptide mutants were translated in the presence of SP-cells either in the presence or absence of α,α' -dipyridyl and were either analysed by sensitivity to proteolysis (Figure 7) or by electrophoresis under non-reducing conditions (Figure 8). When the translation products from the *cys2teloCS* mutant were separated without prior reduction an inter-chain disulphide-bonded trimer was formed but only when α,α' -dipyridyl was not present during translation (Figure 8, lanes 2 and 5). These trimers had a slower mobility than the wild-type trimer (lane 1), indicative of them being joined at one end of the

trimer (see Figure 9). No dimers were formed either in the presence or absence of α,α' -dipyridyl. This mutant was also able to form a correctly aligned triple helix as judged by the appearance of a protease-resistant fragment after digestion when translations were carried out in the absence of α,α' -dipyridyl but not in its presence (Figure 7, left panel, lanes 9 and 10). No trimer was seen when this fragment was separated without prior reduction, which was expected as the C-telopeptide cysteines have been mutated (Figure 7, right panel, lane 3). These results indicate that in the absence of α,α' -dipyridyl this mutant folds correctly and forms a triple helix which allows the formation of an inter-chain disulphide bond within the N-propeptide. An important conclusion from these experiments was that the formation of inter-chain disulphide bonds within the C-telopeptide is not required for nucleation of the triple helix. When the translation products from the $\Delta 1$ teloCS mutant were separated without prior reduction inter-chain disulphide bonds were formed even when α,α' -dipyridyl was present during translation (Figure 8, lanes 3 and 6). The trimers formed in the absence of α,α' -dipyridyl did not migrate at the same position as the wild-type trimers (Figure 8, lane 3) but ran as a doublet. This doublet could arise from differences in the extent of inter-chain disulphide bond formation within the C-propeptide (see Figure 9). Dimers were also formed which migrated with a slightly different mobility than the dimers formed when the wild-type chains were translated in the presence of α,α' -dipyridyl (Figure 8, lane 4). Thus, the $\Delta 1$ teloCS mutant can still form inter-chain disulphide bonds within the C-propeptide, giving rise to dimers and trimers when translated in the absence or presence of α,α' -dipyridyl. In the absence of inhibitor, the mutant chains are able to become inter-chain disulphide-bonded at the N-propeptide, giving rise to a trimer doublet. The fact that the dimer and trimer do not migrate at the same position as the wild-type dimers and trimers suggests that formation of the C-telopeptide disulphide causes the differences in mobilities of the inter-chain disulphide-bonded oligomers. This mutant was also shown to form a correctly aligned triple-helical domain as judged by the appearance of a protease-resistant fragment (Figure 7, left panel, lanes 11 and 12) which, as expected, did not migrate as a trimer when separated without prior reduction (Figure 7, right panel, lane 4).

DISCUSSION

We have been able to reconstitute the translocation, disulphide bond formation and assembly of a type-III procollagen 'mini-gene' into a correctly aligned triple-helical molecule, in a system which mimics the processes as they would occur within an intact cell. We were able to identify distinct disulphide-bonded intermediates in the folding pathway by alkylating samples and separating the translation products under non-reducing conditions. This has enabled us to evaluate quickly the effect of specific mutations within the coding sequence of this protein on its folding and assembly within a functionally and morphologically intact ER. The results from this work are summarized in Figure 9. Under normal conditions, the polypeptide is translocated into the ER essentially in a monomeric state and intra-chain disulphide bonds are formed as shown by their greater mobility in comparison with the reduced protein. The formation of inter-chain disulphide bonds then occurs as shown by the appearance of higher-molecular-mass species which migrate with the molecular mass of a trimer. That a triple helix was formed was confirmed by the appearance of a protease-resistant fragment when the translation products were treated with proteases which have been shown to be highly selective, in that they will completely digest the triple-helical domain unless a correctly aligned triple

Transcript	hydroxylation inhibited	disulphide bonded products
type III procollagen 'mini-gene' proc $\alpha 1$ (III) $\Delta 1$	yes	 slow-migrating trimer dimer monomer Fig. 1
type III procollagen 'mini-gene' proc $\alpha 1$ (III) $\Delta 1$	no	 fast-migrating trimer Fig. 1
cys2-mutant	yes	 dimer monomer Fig. 6A
cys2-mutant	no	 fast-migrating trimer Fig. 6B
$\Delta 1$ teloCS mutant	yes	 slow-migrating trimer dimer Fig. 8
$\Delta 1$ teloCS mutant	no	 trimers dimer Fig. 8
cys2teloCS	yes	 monomer Fig. 8
cys2teloCS	no	 slow-migrating trimer Fig. 8

Figure 9 Summary of results indicating the different disulphide-bonded intermediates formed during the translation of type-III minigene and cysteine mutants

Hydroxylation was inhibited by the inclusion of α,α' -dipyridyl during translations. X marks the position of the cysteine to serine mutation within the procollagen molecule. Circle, N-propeptide; rod, triple-helical domain; oval, C-propeptide.

helix has formed [23]. When hydroxylation is inhibited by the addition of α,α' -dipyridyl both inter-chain disulphide-bonded dimers and trimers are seen. The trimer formed under these conditions has a slower mobility than the trimer formed in the absence of α,α' -dipyridyl. This is due to the fact that in the absence of inhibitor the triple helix folds and inter-chain disulphide bonds form within both the C-telopeptide and the N-propeptide, thus reducing the hydrodynamic volume [10]. In the presence of α,α' -dipyridyl the triple helix does not fold and inter-chain disulphide bonds are only formed within the C-propeptide. When the inhibition is reversed by the addition of excess iron post-translationally, the triple helix folds and inter-chain disulphides are formed within the C-telopeptide and N-propeptide as shown by the disappearance of the dimer and slower-migrating trimer and the appearance of a faster-migrating trimer.

By studying the folding and assembly of the cys2 mutant we were able to show that inter-chain disulphide bonds can only form within the C-telopeptide if the triple-helical domain is hydroxylated. Here inter-chain disulphide bonds do not form when hydroxylation is inhibited either by carrying out the translation in the presence of dog pancreas microsomes or in the

presence of SP-cells and α,α' -dipyridyl. This could be explained if hydroxylation was required for the initial nucleation of the triple helix, which would then allow the formation of the inter-chain disulphide bonds within the C-telopeptide. This would suggest that there is an initial association of the C-propeptides followed by a nucleation of the last part of the triple-helical domain, and that this nucleation is dependent upon proline hydroxylation. Thus, for the inter-chain disulphide bond to form within the C-telopeptide the last part of the triple-helical domain must nucleate, allowing the cysteine residues to come into close proximity and for the disulphides to form. A similar conclusion has been drawn from results obtained with type-XII collagen [24]. In this study inhibition of hydroxylation prevents inter-chain disulphide bond formation within the COL1 domain, a region thought to be involved in the initial recognition and registration of the triple helix. There are at least two possible explanations for these results. In the presence of α,α' -dipyridyl, the enzyme prolyl 4-hydroxylase may form a stable complex with the triple-helical domain and prevent the nucleation site in adjacent chains from interacting. It is known that this enzyme can form a stable complex with mutant procollagen chains which do not fold correctly [25]. We have also obtained preliminary cross-linking data which demonstrate that prolyl 4-hydroxylase forms a stable complex with unhydroxylated procollagen chains (N. J. Bulleid and R. Wilson, unpublished work). However, this does not explain why inter-chain disulphide bond formation within the C-telopeptide is prevented when translation is carried out in the presence of dog pancreas microsomes which do not contain any prolyl 4-hydroxylase. An alternative explanation is that hydroxylation of proline residues is necessary to stabilize the association of the C-telopeptides. The self-association of these peptides may be crucially dependent upon the presence of hydroxyproline rather than proline within the sequence.

These results are in contrast to those obtained for type-I procollagen where the telopeptide was shown to be important for trimer assembly [8]. Here purified C-propeptides were denatured and refolded in an *in vitro* assay. Only C-propeptides containing the C-telopeptides were capable of refolding and forming disulphide-linked trimers, indicating that these sequences are required for the initial association of the procollagen chains. We cannot rule out that the C-telopeptides may be having an indirect role in the initial association, for example by ensuring the correct folding of the C-propeptide domain. However, we can rule out a direct role in the initial association as this would result in the cysteine residues being brought together in close proximity, which would presumably lead to the formation of inter-chain disulphide bonds.

Using this system we were also able to ask the question, is the formation of inter-chain disulphide bonds necessary for correct folding and assembly of procollagen? From our results it is clear that the procollagen chains are capable of associating, folding and forming correctly aligned triple helices without inter-chain disulphides forming within either the C-propeptide or C-telopeptide. This is best illustrated by the *cys2teloCS* mutant which cannot form inter-chain disulphide bonds within the C-propeptide or C-telopeptide. This mutant was still able to form disulphide-linked trimers and a triple helix, but only when α,α' -dipyridyl was not included during translation.

If inter-chain disulphide bonds are not required for folding of procollagen then what is their role? One possible explanation is

that they are required to maintain the stability of procollagen once this protein has been secreted from the cell. Alternatively they may be required specifically in type-III procollagen to prevent the C-telopeptide from unfolding once the C-propeptide has been cleaved. Our results indicate that the C-telopeptide does not associate unless part of the triple-helical domain is hydroxylated, therefore, there may be a requirement for a covalent linkage to prevent this peptide from dissociating. From studies of type-I collagen [26], the C-telopeptide of type-III collagen presumably plays a crucial role during fibril formation, therefore, any unfolding of this peptide would have drastic effects on the ability of the molecule to form fibrils and fulfil its biological function.

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