Reconstitution of the folding pathway of collagen in a cell-free system: formation of correctly aligned and hydroxylated triple helices

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We describe here a cell-free system which will carry out the initial stages in the synthesis, post-translational modification and assembly of type-X collagen. The mRNA coding for bovine type-X collagen was synthesized *in vitro* and translated in a rabbit reticulocyte lysate to yield a protein that was collagenase sensitive and could be immunoprecipitated with antibodies raised to purified avian type-X collagen. When type-X collagen was synthesized in the absence of added microsomes or in the presence of canine pancreas microsomes, the translation products showed partial resistance to digestion with pepsin but were completely degraded with a mixture of chymotrypsin and trypsin, suggesting that only incorrectly aligned non-native collagen molecules were synthesized under these conditions. When the protein was

synthesized in the presence of microsomes derived from avian fibroblasts or a human fibrosarcoma cell line, the translocated product migrated as a diffuse band characteristic of hydroxylated collagen. The synthesized polypeptides were also resistant to both pepsin and trypsin/chymotrypsin digestion, demonstrating the formation of correctly aligned native collagen. Furthermore, the collagen polypeptides assembled into higher-order structures, possibly trimers, which were stabilized by interchain disulphide bonds. The collagen helix synthesized *in vitro* had a melting temperature of 41 °C which is comparable with the protein synthesized *in vivo*, further demonstrating that the polypeptides were hydroxylated and that the triple helix formed was correctly aligned.

INTRODUCTION

Although the modifications to collagen polypeptides that occur within the endoplasmic reticulum are understood, little is known about the co-ordination of the various stages in the folding and assembly of this protein or of the role of accessory proteins in this process. To address this problem we have studied the synthesis of bovine type-X collagen. Type-X collagen is a small non-fibrillar collagen (molecular mass 59 kDa) consisting of three identical chains, which is synthesized exclusively in the epiphyseal growth plate during chondrocyte hypertrophy (Schmid and Linsenmayer, 1983, 1985; Kielty et al., 1985; Gibson and Flint, 1985; Kwan et al., 1986). A cDNA clone coding for the bovine protein (Thomas et al., 1991a) has been isolated and the deduced amino acid sequence reveals that the protein shares a number of properties with other collagen molecules. Thus the bovine protein consists of three distinct domains: a short N-terminal domain of 38 amino acids, a collagenous triple-helical domain of 463 amino acids and a non-collagenous C-terminal domain of 155 amino acids. Unlike the fibrillar collagens, the N- and C-terminal domains of type X are not cleaved and are present in the mature protein. There are no cysteine residues in the N-terminal domain and only one in the C-terminal domain, which indicates that intramolecular disulphide bonds do not form in these domains, unlike in other collagen polypeptides. However, there are two cysteine residues, separated by only two amino acids, within the triple-helical domain which have been shown biochemically to form interchain disulphide bonds between individual polypeptides within the collagen trimer. The formation of this disulphide bond is a property of bovine type-X collagen and is not found in human (Marriott et al., 1991; Thomas et al., 1991b; Apte et al., 1992) or avian (Gibson et al., 1983; Kielty et al., 1984; LuValle et al., 1988) type-X collagen.

As many of the modifications to collagen occur as the protein

is being synthesized, any system used to study the folding and assembly of this molecule must involve *de novo* protein synthesis. Ideally such a system should be amenable to manipulation, so that the effects of various agents on the folding pathway can be ascertained. We describe such a system here which, for the first time, reconstructs the initial stages in the synthesis, modification, folding and assembly of collagen *in vitro*. As is the case *in vivo*, the protein synthesized was hydroxylated and trimerized to form correctly aligned triple helices linked by interchain disulphide bonds.

MATERIALS AND METHODS

Materials

Restriction enzymes, T7 RNA polymerase, nucleotides and endoglycosidase H were purchased from Boehringer-Mannheim (Lewes, East Sussex, U.K.). Nuclease-treated rabbit reticulocyte lysate, amino acids minus methionine, RNasin ribonuclease inhibitor, and pGEM-3Z were purchased from Promega (Southampton, Hants., U.K.). L-[³⁵S]Methionine and Amplify were purchased from Amersham International (Amersham, Bucks., U.K.). Pepsin, chymotrypsin type I-S, trypsin type III and bacterial collagenase type 1A were from Sigma (Poole, Dorset, U.K.). Collagenase form III was from Advance Biofacture (Lynbrook, NJ, U.S.A.). Proteinase K was from NBL (Cramlington, Northd., U.K.). Cell-culture media were from Gibco/BRL (Paisley, Renfrewshire, Scotland, U.K.). HT-1080 human fibrosarcoma cells (A.T.C.C. CCL121) were from the American Type Culture Collection (Rockville, MD, U.S.A.).

Avian tendon fibroblast cell culture

Avian tendons were dissected from 17-day embryos and digested with bacterial collagenase type 1A (17 units/mg, 9.4 mg/g of tendon) and trypsin (0.25 %, w/v) in serum-free culture medium

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for 60 min at 37 °C. Fibroblasts were washed in Krebs solution containing 10 % fetal calf serum and filtered through Whatman no. 1 filter paper in a 25 mm filter unit using a syringe. The released fibroblasts were plated out at a cell density of 1×10^6 per ml. The culture was maintained with Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, penicillin (200 i.u./ml), streptomycin (200 µg/ml) and glutamine (2 mM).

Human fibrosarcoma cell culture

HT-1080 cells were maintained in minimal essential medium with Earle's salts and glutamine supplemented with 10 % fetal calf serum, sodium pyruvate (1 mM), glutamine (2 mM), minimal essential medium non-essential amino acids (1×), minimal essential medium vitamins (1×) and gentamycin (50 µg/ml).

Preparation of microsomal membranes

Microsomal membranes were prepared from canine pancreas as described previously (Austen et al., 1984). Avian fibroblast and HT-1080 microsomes were prepared as follows. Confluent cells were harvested using 0.25 % (w/v) trypsin and pelleted by centrifugation. The cells were washed twice with ice-cold 50 mM Tris/HCl, pH 7.4, containing 0.25 M sucrose, 25 mM KCl and 0.5 mM MgCl₂. The cells were resuspended in the wash buffer at a concentration of 6×10^7 – 1×10^8 cells/ml. The suspension was homogenized using a tight-fitting Dounce homogenizer (50 strokes). The homogenate was centrifuged to yield the post-nuclear supernatant which was subsequently centrifuged at 150 000 *g* for 10 min at 4 °C. The resulting pellet was resuspended in 25 % (w/v) sucrose containing 0.3 mM phenylmethanesulphonyl fluoride to give a typical *A*₂₈₀ value of 50 units/ml. Canine pancreas and HT-1080 microsomes were treated with staphylococcal nuclease as described (Walter and Blobel, 1983).

Construction of recombinant plasmid

The coding sequence for bovine type-X collagen was assembled from three clones originally isolated in λ bacteriophage (Thomas et al., 1991a). All manipulations of the DNA clones were carried out as described (Sambrook et al., 1989). An *Eco*RI fragment from BX26'2 was first ligated into the *Eco*RI site of pGEM-3Z. This construct was digested with *Xho*I and *Kpn*I and ligated with a 961 bp *Xho*I–*Kpn*I fragment isolated from BX10'10 which had the internal *Eco*RI site removed by site-directed mutagenesis. The resulting construct was digested with *Kpn*I and ligated with a 1488 bp *Kpn*I fragment isolated from BX2.131. The resulting clones were screened for the correct orientation of this fragment which completed the assembly of the entire coding region of type-X collagen in pGEM-3Z. This plasmid was designated pGEM-BX.

Cell-free transcription

Transcription was carried out essentially as described elsewhere (Gurevich et al., 1991). The vector pGEM-BX containing the complete cDNA for bovine type-X collagen (10 µg) was linearized with *Sal*I to give the full-length transcript. Transcription was initiated with T7 RNA polymerase and carried out for 4 h at 37 °C. Subsequently, the mixture was extracted once with phenol/chloroform and once with chloroform. After ethanol

precipitation the RNA was resuspended in 50 µl of RNAase-free water.

Cell-free translation

Transcribed RNA was translated using a nuclease-treated rabbit reticulocyte lysate. The translation mixture (final volume 25 µl) contained 18 µl of reticulocyte lysate, 0.5 µl of 1 mM amino acids (minus methionine), 15 µCi of [³⁵S]methionine, 1 µl of transcribed RNA and, where indicated, supplemented with 1 µl of nuclease-treated dog pancreas, nuclease-treated HT-1080 or avian fibroblast microsomes. Translations were performed in the absence or presence of 3 mM GSSG. Where necessary, translations were carried out in the presence of 2 mM αα'-dipyridyl to inhibit proline and lysine hydroxylation. Unless indicated otherwise, translations without microsomes were carried out at 30 °C for 60 min, those containing canine pancreas microsomes were carried out at 23 °C for 2 h and those containing avian tendon fibroblast or HT-1080 microsomes at 30 °C for 2 h.

Microsomal vesicles were isolated post-translationally by layering the translation mixture over 50 mM Hepes, pH 7.5, containing 100 mM KCl and 0.5 M sucrose and sedimenting the microsomes by centrifugation at 150 000 *g* for 10 min at 4 °C. The pellet was resuspended in an appropriate buffer for subsequent enzymic digestion.

Immunoprecipitation

Isolated microsomes or translation mixtures were immunoprecipitated using a monoclonal anti-(avian type-X collagen) antibody (kindly donated by Dr. A. P. L. Kwan). Samples were diluted in 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 5 mM EDTA, 0.25 % gelatin, 0.05 % Nonidet P40 and 0.02 % sodium azide. The samples were immunoprecipitated using 30 µl of the monoclonal anti-(avian type-X collagen) antibody attached to activated Sepharose CL-4B (50 %, v/v, suspension) for 16 h at 4 °C. The pelleted Sepharose beads were washed three times in the dilution buffer and resuspended in 40 µl of SDS/PAGE sample buffer.

Proteinase K treatment

Translation mixtures were assayed for translocation by treatment post-translationally with proteinase K as described previously (Bulleid and Freedman, 1988).

Bacterial collagenase digestion

Isolated microsomal vesicles were resuspended in 50 mM Tris/HCl, pH 7.4, containing 5 mM CaCl₂, 1 mM phenylmethanesulphonyl fluoride, 5 mM *N*-ethylmaleimide and 1 % Triton X-100. Portions (5 µl) of resuspended microsomal vesicles or translation mixture were made up to a final volume of 10 or 20 µl respectively with the resuspension buffer without Triton X-100 and incubated with 1 unit of collagenase form III for 4 h at 37 °C. The digestion was terminated by the addition of SDS/PAGE sample buffer.

Pepsin digestion

Isolated microsomal vesicles resuspended in 20 µl of 0.5 M acetic acid in 1 % (v/v) Triton X-100 or translation mixtures adjusted to 0.5 M acetic acid were incubated with pepsin (100 µg/ml) for 16 h at 4 °C. Digestions were stopped by neutralization and addition of 5 vol. of SDS/PAGE sample buffer.

Chymotrypsin/trypsin digestion

Isolated microsomal vesicles were resuspended in 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 10 mM EDTA and 1% Triton X-100. Portions (5 μ l or 10 μ l) of resuspended microsomal vesicles or translation mixture were made up to a final volume of either 10 μ l or 20 μ l respectively with the resuspension buffer without Triton X-100 containing chymotrypsin (250 μ g/ml) and trypsin (100 μ g/ml) and incubated at room temperature for 2 min. The digestion was stopped by the addition of 5 vol. of boiling SDS/PAGE sample buffer and boiling the samples for 3 min.

Heat denaturation

Isolated microsomal vesicles were resuspended in 50 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 10 mM EDTA and 1% Triton X-100, and 10 μ l samples were placed in a DNA thermal cycler. A stepwise temperature gradient was set up from 36 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.

SDS/PAGE

Samples were prepared for electrophoresis by mixing with 5 vol. of SDS/PAGE sample buffer [0.0625 M Tris/HCl, pH 6.8, SDS (2%, w/v), glycerol (10%, w/v) and Bromophenol Blue] with or without 50 mM dithiothreitol and boiled for 5 min. SDS/PAGE was performed by the method of Laemmli (1970). After electrophoresis, gels were processed for autoradiography or fluorography with Amplify and exposed to Kodak X-Omat AR film.

RESULTS

Cell-free synthesis and translocation of type-X collagen

To establish a system capable of synthesizing type-X collagen, we first constructed in the transcription vector pGEM-3Z a cDNA clone which contained the complete coding region. The resulting plasmid, pGEM-BX, was transcribed *in vitro* yielding an RNA transcript which, when translated in a rabbit reticulocyte lysate, gave rise to a single major polypeptide with a relative molecular mass of approximately 70 kDa (Figure 1, lane 1) relative to globular molecular-mass markers. This is an overestimate of the molecular mass of type-X collagen (65.5 kDa from deduced amino acid sequence) because of the anomalous mobility of collagenous proteins relative to globular proteins. The major translation product synthesized in the presence of microsomal membranes was immunoprecipitated with an antibody raised against purified avian type-X collagen, verifying the authenticity of the product (Figure 1, lanes 2–5). To determine whether the polypeptide synthesized was collagenous, the products of translation were treated with bacterial collagenase, an enzyme that specifically digests the triple-helical domain of collagens. The translation products synthesized in both the presence and absence of microsomes were sensitive to digestion with this enzyme, with the C-terminal domain being left undegraded (Figure 2). The N-terminal domain does not contain any methionine residues and therefore was not detected after collagenase treatment.

Microsomal membranes were added to the translation

reactions to reconstitute the initial stages in the translocation, post-translational modification and assembly of collagen. When canine pancreatic microsomes were included in the translation reaction, a polypeptide with a faster mobility was synthesized, demonstrating removal of the signal peptide (Figure 1, lane 2). That the protein was translocated into the lumen of the microsomes was demonstrated by resistance to proteolysis with proteinase K (Figure 3b, lane 3), the protein being digested if the microsomes were solubilized with detergent before treatment with protease (lane 4). Canine pancreas microsomes are unlikely to contain significant levels of the enzymes required to carry out modifications to the collagen polypeptide such as hydroxylation of proline residues by prolyl 4-hydroxylase as they are derived from cells that are not actively secreting collagens. Therefore, to reconstruct these events we isolated microsomes from two cell lines, avian tendon fibroblasts and human fibrosarcoma or HT-1080, cells which are active in the synthesis of certain types of collagen but not type-X collagen and contain all the enzymes necessary for the initial modification of the polypeptide chains (Pihlajaniemi et al., 1981; Alitalo et al., 1980).

When avian tendon fibroblast microsomes were included in the translation reaction, the major polypeptide synthesized migrated as a diffuse band during SDS/PAGE (Figure 1, lanes 4 and 5; Figure 3a, lanes 2 and 3). This polypeptide was shown to be translocated into the lumen of these microsomes by protease resistance (Figure 3a, lane 3). Similar results were obtained when HT-1080 microsomes were included during translation (results not shown). The diffuse pattern of the translocated polypeptide observed in the presence of these microsomes is characteristic of

Figure 1 Cell-free translation of type-X collagen

Collagen type-X RNA was synthesized by *in vitro* transcription of pGEM-BX linearized with *Sa*I using T7 RNA polymerase. Translation of type-X RNA was carried out using a rabbit reticulocyte lysate system supplemented with amino acids (minus methionine), [³⁵S]methionine and canine pancreas microsomes (lanes 2 and 3) or avian fibroblast microsomes (lanes 4 and 5) in either the absence or presence (lanes 3 and 5) of 3 mM GSSG. Translations were carried out at 30 °C for 60 min and terminated by incubation on ice. Lane 1, products of translation in the absence of added microsomes. Type-X collagen synthesized in the presence of microsomes was immunoprecipitated with a monoclonal antibody raised against avian type-X collagen. Proteins were separated by SDS/PAGE through a 10% polyacrylamide gel. The gel was fixed, soaked in Amplify, dried and developed by fluorography. Abbreviations: pX, pre-type-X collagen; X, signal-peptide-cleaved type-X collagen; M, molecular mass.

Figure 2 Collagenase treatment of translation products

Translation of type-X RNA was carried out in either the absence (lanes 1 and 3) or presence (lanes 2 and 4) of avian fibroblast microsomes. Either neat translation mixtures or isolated microsomal vesicles were treated with bacterial collagenase which digests the triple-helical domain (lanes 3 and 4). Proteins were separated by SDS/PAGE through a 15% polyacrylamide gel and the gel was fixed, dried and developed by autoradiography. The position of the C-terminal domain is as indicated. Abbreviation: *M*, molecular mass.

Figure 3 Translocation and protease protection of translation products

Translations were carried out in the absence (lane 1) or presence (lanes 2–4) of either avian fibroblast (a) or canine pancreas (b) microsomes. Proteinase K treatment was carried out at 4 °C in either the absence (lane 3) or presence (lane 4) of Triton X-100. Proteins were separated by SDS/PAGE through either a 10% (a) or 12.5% (b) polyacrylamide gel. The gels were developed by autoradiography. Abbreviation: *M*, molecular mass.

hydroxylated collagen and is thought to be due to differential binding of SDS to the hydroxylated molecule (Cheah et al., 1979a,b). To confirm further that the translation product was

Figure 4 Inhibition of hydroxylation with $\alpha\alpha'$ -dipyridyl

Translation of type-X RNA was carried out in the presence of HT-1080 microsomes in either the absence (lane 1) or presence (lane 2) of 2 mM $\alpha\alpha'$ -dipyridyl. Proteins were separated by SDS/PAGE through a 10% polyacrylamide gel. The gel was fixed and dried, and radiolabelled proteins were revealed by autoradiography. pX, pre-type-X collagen; X, signal-peptide-cleaved type-X collagen; hyd. X, diffuse band containing hydroxylated type-X collagen.

hydroxylated, translations were carried out in the presence of a known inhibitor of prolyl 4-hydroxylase, $\alpha\alpha'$ -dipyridyl (Juva et al., 1966; Margolis and Lukens, 1971). The inclusion of the inhibitor abolished the formation of the diffuse band, the translation products migrating as two sharp bands corresponding to the signal-peptide-cleaved and uncleaved polypeptides (Figure 4, lane 2).

Formation of correctly aligned triple helices

To demonstrate the formation of correctly aligned chains, we took advantage of the fact that triple helices are resistant to degradation by proteolytic enzymes such as pepsin, trypsin and chymotrypsin. As the polypeptides synthesized in the presence of canine pancreas microsomes are unhydroxylated, they are unlikely to form stable triple-helical structures at temperatures higher than the melting temperature of unhydroxylated collagen. Consequently we carried out the translation of type-X collagen in the presence of these microsomes at 23 °C before treatment with proteases. We observed the presence of protease-resistant peptides when the type-X collagen polypeptides synthesized were treated with pepsin (Figure 5, lane 2). However, the polypeptide was completely digested by treatment with a combination of trypsin and chymotrypsin (lane 3). The presence of several resistant peptides after pepsin treatment indicated that the chains were forming incorrectly aligned triple helices. Such structures have previously been shown to be resistant to degradation with pepsin, but are degraded by treatment with trypsin/chymotrypsin (Bruckner and Prockop, 1981). The only molecules that are resistant to degradation with these enzymes are correctly aligned 'native' collagen triple helices. Similar results were obtained when translation was carried out in the absence of added microsomes or in the presence of canine pancreas microsomes at 30 °C (results not shown). However, when the translation was

Figure 5 Post-translational proteolysis of products of translation in the presence of canine pancreas microsomes

Translations were carried out in the presence of canine pancreas microsomes. Products of translation were treated post-translationally with either pepsin (PEP; 100 $\mu\text{g}/\text{ml}$) (lane 2) or a combination of chymotrypsin (250 $\mu\text{g}/\text{ml}$) and trypsin (100 $\mu\text{g}/\text{ml}$) (CT/T) (lane 3). Proteins were separated by SDS/PAGE through a 12.5% polyacrylamide gel. The gel was fixed and dried, and radiolabelled proteins were revealed by autoradiography.

carried out in the presence of avian fibroblast or HT-1080 microsomes at 30 °C and the translation products were treated with trypsin/chymotrypsin, a resistant fragment was formed with a relative molecular mass, compared with globular standards of approximately 47 kDa (Figure 6a, lanes 4 and 7; Figure 6b, lane 3). This corresponds to the molecular mass of the pepsin-resistant fragment produced from digestion of *in vivo* synthesized bovine type-X collagen (Marriott et al., 1991) and demonstrates that correctly aligned triple helices are formed in the presence of these microsomal membranes.

Formation of interchain disulphide bonds

As mentioned previously, bovine type-X collagen forms interchain disulphide bonds within the triple-helical domain (Marriott et al., 1991). To investigate whether we could form such a bond in our cell-free system supplemented with avian fibroblast microsomes, we optimized the redox conditions by the addition of GSSG to allow disulphide bonds to form (Scheele and Jacoby, 1982). We analysed the products of translation by first isolating the microsomes by ultracentrifugation and separating the translocated polypeptides by SDS/PAGE carried out under either reducing conditions (Figure 6a, lanes 2–7) or non-reducing conditions (lanes 8–13). As mentioned previously, in the absence of GSSG a polypeptide was synthesized that was resistant to digestion with either pepsin or trypsin/chymotrypsin (lanes 2–4). Similar results were obtained when the translation was carried out in the presence of GSSG (lanes 5–7). When the translation products were separated under non-reducing conditions, higher-molecular-mass structures were observed particularly for translations carried out in the presence of GSSG. After treatment with chymotrypsin/trypsin, protease-resistant products were observed when samples were separated under non-reducing conditions (lane 13). This indicates that the higher-molecular-mass struc-

Figure 6 Triple helix and interchain disulphide bond formation

Translations were carried out in the presence of avian fibroblast (a) or HT-1080 (b) microsomes. For some of the translations carried out with avian fibroblast microsomes, GSSG (3 mM) was also included to allow the formation of disulphide bonds (a, lanes 6, 7, 12 and 13). For translations carried out in the presence of HT-1080 microsomes, a reticulocyte lysate preparation was used which did not contain added dithiothreitol and therefore did not require the addition of GSSG to allow disulphide bond formation to occur. Microsomes were isolated by centrifugation and the products of translation were either untreated (a, lanes 2, 5, 8 and 11; b, lanes 2 and 4) or treated post-translationally with pepsin (P; 100 $\mu\text{g}/\text{ml}$) (a, lanes 3, 6, 9 and 12) or a combination of chymotrypsin (250 $\mu\text{g}/\text{ml}$) and trypsin (100 $\mu\text{g}/\text{ml}$) (C) (a, lanes 4, 7, 10 and 13; b, lanes 3 and 5). Samples were separated by SDS/PAGE through a 10% polyacrylamide gel under either reducing (a, lanes 1–7; b, lanes 1–3) or non-reducing (a, lanes 8–13; b, lanes 4 and 5) conditions. Gels were fixed, soaked in Amplify, dried, and radiolabelled proteins were revealed by fluorography. Abbreviation: *M*, molecular mass.

tures represented correctly aligned helices, which are probably dimers and trimers of type-X collagen. The presence of these structures when the translation products were separated under non-reducing conditions and their absence when they were

Figure 7 Heat denaturation of collagen triple helix

Translations were carried out in the presence of HT-1080 microsomes. Microsomes were isolated by centrifugation through a sucrose cushion, solubilized and either analysed directly by SDS/PAGE (lane 2) or heated to a set temperature as indicated before being digested with a combination of chymotrypsin (250 $\mu\text{g}/\text{ml}$) and trypsin (100 $\mu\text{g}/\text{ml}$) (lanes 3–10). The samples were analysed by SDS/PAGE through a 10% polyacrylamide gel. Gels were fixed, soaked in Amplify, dried, and radiolabelled proteins were revealed by fluorography. Abbreviation used: *M*, molecular mass.

separated under reducing conditions indicates that interchain disulphide bonds had formed. Similar results were obtained with translation products synthesized in the presence of HT-1080 microsomes (Figure 6b). Here translation was carried out in a rabbit reticulocyte lysate that had been prepared in the absence of added dithiothreitol (Bulleid and Freedman, 1988). Products of translation were separated under either reducing (lanes 2 and 3) or non-reducing conditions (lanes 4 and 5). The formation of higher-molecular-mass structures which were partially resistant to digestion with chymotrypsin/trypsin again indicated the formation of correctly aligned helices which were stabilized by interchain disulphide bonds within the triple-helical domain.

Thermal stability of the *in vitro* synthesized collagen

A further indication of whether the collagen polypeptides synthesized *in vitro* were hydroxylated and folded correctly was the stability of the triple helix to temperature. Unhydroxylated polypeptides form triple helices which are unstable at physiological temperatures and in the case of type-I collagen have a melting temperature of approx. 27 °C. There is evidence that suggests that smaller collagens such as type-X cannot form helices if the chains are unhydroxylated (Clark and Richards, 1988). In contrast, hydroxylated avian type-X collagen synthesized *in vivo* has a melting temperature of 47 °C (Schmid and Linsenmayer, 1984). To characterize further the collagen synthesized *in vitro*, we determined the temperature at which the helix dissociates by incubating the translation products at a variety of temperatures and then treating them with trypsin/chymotrypsin. The product formed in the presence of HT-1080 microsomes was sensitive to digestion at temperatures above 41 °C (Figure 7). Similar results were obtained with the product synthesized in the presence of avian fibroblast microsomes (results not shown).

This clearly demonstrates the formation of triple-helical molecules stabilized by the hydroxylation of proline and lysine residues. The slightly lower melting temperature of the helix formed *in vitro* most likely reflects incomplete hydroxylation before helix formation.

DISCUSSION

The main aim of this study was to develop a cell-free system that would carry out the initial stages in the synthesis, modification, folding and assembly of collagen. Cell-free systems capable of the synthesis of collagen have been reported previously (Cheah et al., 1979a,b; Ouellette et al., 1981; Monson, 1983; Castagnola et al., 1986; Adams, 1987). These studies demonstrated that procollagen polypeptides synthesized *in vitro* were unhydroxylated but could be hydroxylated provided that purified prolyl 4-hydroxylase was added post-translationally (Cheah et al., 1979a,b). The inclusion of canine pancreas microsomes during translation resulted in translocation of the synthesized collagen polypeptide with subsequent removal of the signal peptide (Castagnola et al., 1986). Two reports (Ouellette et al., 1981; Monson, 1983) demonstrated that the collagen chains synthesized *in vitro*, even in the absence of microsomes, could form pepsin-resistant helices. We have confirmed the observation that pepsin-resistant molecules can be formed in the absence of added microsomes but suggest that these structures are likely to arise from non-specific association of the triple-helical domains as they are not resistant to digestion with a combination of trypsin and chymotrypsin.

To enable the collagen chains to fold correctly in our system, we included microsomal membranes derived from cell lines active in the synthesis of collagen. Both avian fibroblast and HT-1080 microsomes were capable of hydroxylating type-X collagen and formed triple helices that were resistant to digestion with trypsin/chymotrypsin. The HT-1080 cell line (Rasheed et al., 1973) is derived from a human fibrosarcoma and has the advantage that it is an immortalized line and microsomes isolated from this line reproducibly carried out the hydroxylation and folding of collagen. In contrast, avian tendon fibroblasts are a primary cell line, and, although they may be passaged several times, the microsomes isolated from different passages varied in their ability to hydroxylate the nascent collagen chains (R. B. Middleton and N. J. Bulleid, unpublished work). Both these microsomal preparations were capable of carrying out the initial translocation, signal peptide cleavage, hydroxylation, folding, trimerization and triple-helix formation of type-X collagen, yielding a collagen molecule that shared the properties of the *in vivo* synthesized protein, such as protease resistance and temperature of denaturation of the triple-helical domain. The low yield of product obtained precludes the use of this system to produce sufficient quantities of protein for physical characterization.

The ability of these microsomal preparations to carry out the modification, folding and assembly of collagen demonstrates that they contain the enzymes and any accessory proteins or chaperones necessary to carry out these processes. There have been reports that proteins resident within the endoplasmic reticulum are required to facilitate the folding of collagen and may play a role in preventing individual chains from associating before the formation of the correctly aligned triple helix (Chessler and Byers, 1992; Nakai et al., 1992). One likely candidate for such a role is colligin or hsp47, a collagen-binding protein located in the endoplasmic reticulum which can be co-immunoprecipitated with nascent collagen chains (Nakai et al., 1990). This protein has been shown to bind to both denatured

collagen (Nagata et al., 1986; Nandan et al., 1990) and nascent procollagen chains, binding being disrupted at low pH (Saga et al., 1987; Nakai et al., 1992). Recently it has been shown that immunoglobulin heavy-chain binding protein is associated with malfolded collagen chains synthesized in cell lines expressing forms of the protein that carry mutations within the C-terminal domain (Chessler and Byers, 1993). In contrast, procollagen chains carrying mutations within other parts of the molecule expressed in different cell lines are not associated with this binding protein but are associated with the enzyme prolyl 4-hydroxylase (Chessler and Byers, 1992). Other proteins that have been suggested to have a role in collagen folding within the endoplasmic reticulum include protein disulphide-isomerase in disulphide-bond formation (Koivu and Myllyla, 1987) and peptidyl-prolyl *cis-trans*-isomerase (Steinmann et al., 1991). Whether all these proteins are involved in collagen biosynthesis remains to be clearly established. However, the development of this cell-free system, which can carry out the initial stages in the modification and folding of collagen, will enable us to determine the time course of events during collagen folding and to determine which accessory proteins are associated with the nascent chains before their assembly into triple helices.

We thank Alvin Kwan for the type-X antibody and Terrig Thomas for help in constructing the full-length cDNA clone. We also thank Karl Kadler for critical reading of the manuscript. This work was supported by The Royal Society and a grant from The Wellcome Trust (ref. 32706).

REFERENCES

- Adams, S. L. (1987) *Methods Enzymol.* **144**, 84–95
- Alitalo, K., Vaheri, A., Krieg, T. and Timpl, R. (1980) *Eur. J. Biochem.* **109**, 247–255
- Apte, S. S., Seldin, M. F., Hayashi, M. and Olsen, B. R. (1992) *Eur. J. Biochem.* **206**, 217–224
- Austen, B. M., Kadhobhai, M. A., Herman-Taylor, J. and Ridd, D. H. (1984) *Biochem. J.* **224**, 317–325
- Bruckner, P. and Prockop, D. J. (1981) *Anal. Biochem.* **110**, 360–368
- Bulleid, N. J. and Freedman, R. B. (1988) *Biochem. J.* **254**, 805–810
- Castagnola, P., Cancedda, F. D. and Cancedda, R. (1986) *Biochem. Biophys. Res. Commun.* **136**, 109–115
- Cheah, K. S. E., Grant, M. E. and Jackson, D. S. (1979a) *Biochem. Biophys. Res. Commun.* **91**, 1025–1031
- Cheah, K. S. E., Grant, M. E. and Jackson, D. S. (1979b) *Biochem. J.* **182**, 81–93
- Chessler, S. D. and Byers, P. H. (1992) *J. Biol. Chem.* **267**, 7751–7757
- Chessler, S. D. and Byers, P. H. (1993) *J. Biol. Chem.* **268**, 18226–18233
- Clark, C. C. and Richards, C. F. (1988) *Biochem. J.* **250**, 65–70
- Gibson, G. J. and Flint, M. H. (1985) *J. Cell Biol.* **101**, 277–284
- Gibson, G. J., Keilty, C. M., Garner, C., Schor, S. L. and Grant, M. E. (1983) *Biochem. J.* **211**, 417–426
- Gurevich, V. V., Pokrovskaya, I. D., Obukhova, T. A. and Zozulya, S. A. (1991) *Anal. Biochem.* **195**, 207–213
- Juva, K., Prockop, D. J., Cooper, G. W. and Lash, J. W. (1966) *Science* **152**, 92–94
- Keilty, C. M., Hulmes, D. J. S., Schor, S. L. and Grant, M. E. (1984) *FEBS Lett.* **169**, 179–184
- Keilty, C. M., Kwan, A. P. L., Holmes, D. F., Schor, S. L. and Grant, M. E. (1985) *Biochem. J.* **27**, 545–554
- Koivu, J. and Myllyla, R. (1987) *J. Biol. Chem.* **262**, 6159–6164
- Kwan, A. P. L., Freemont, A. J. and Grant, M. E. (1986) *Biosci. Rep.* **6**, 155–162
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- LuValle, P., Ninomiya, Y., Rosenblum, N. D. and Olsen, B. R. (1988) *J. Biol. Chem.* **263**, 18378–18385
- Margolis, R. L. and Lukens, L. N. (1971) *Arch. Biochem. Biophys.* **147**, 612–618
- Marriott, A., Ayad, S. and Grant, M. E. (1991) *J. Cell Sci.* **99**, 641–649
- Monson, J. M. (1983) *Collagen Relat. Res.* **3**, 1–12
- Nagata, K., Saga, S. and Yamada, K. M. (1986) *J. Cell Biol.* **103**, 223–229
- Nakai, A., Hirayoshi, K. and Nagata, K. (1990) *J. Biol. Chem.* **265**, 992–999
- Nakai, A., Satoh, M., Hirayoshi, K. and Nagata, K. (1992) *J. Cell Biol.* **117**, 903–914
- Nandan, D., Ball, E. H. and Sanwal, B. D. (1990) *Biochem. Cell Biol.* **68**, 1057–1061
- Ouellette, L. A., Paglia, L. M. and Martin, G. R. (1981) *Collagen Relat. Res.* **1**, 327–335
- Pihlajaniemi, T., Myllyla, R., Alitalo, K., Vaheri, A. and Kivirikko, K. I. (1981) *Biochemistry* **20**, 7409–7415
- Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P. and Gardner, M. B. (1973) *Cancer* **33**, 1027–1033
- Saga, S., Nagata, K., Chen, W.-T. and Yamada, K. M. (1987) *J. Cell Biol.* **105**, 517–527
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Schmid, T. M. and Linsenmayer, T. F. (1983) *J. Biol. Chem.* **258**, 9504–9509
- Schmid, T. M. and Linsenmayer, T. F. (1984) *Biochemistry* **23**, 553–558
- Schmid, T. M. and Linsenmayer, T. F. (1985) *J. Cell Biol.* **100**, 598–605
- Scheele, G. and Jacoby, R. (1982) *J. Biol. Chem.* **257**, 12277–12282
- Steinmann, B., Bruckner, P. and Superti-Furga, A. (1991) *J. Biol. Chem.* **266**, 1299–1303
- Thomas, J. T., Kwan, A. P. L., Grant, M. E. and Boot-Handford, R. P. (1991a) *Biochem. J.* **273**, 141–148
- Thomas, J. T., Cresswell, C. J., Rash, B., Nicolai, H., Jones, T., Solomon, E., Grant, M. E. and Boot-Handford, R. P. (1991b) *Biochem. J.* **280**, 617–623
- Walter, P. and Blobel, G. (1983) *Methods Enzymol.* **96**, 84–93