Developmental changes in the type I procollagen processing pathway in chick-embryo cornea

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Type I procollagen processing in chick-embryo corneas was studied at days 12, 14 and 17 of development. Pulse-chase experiments and electrophoretic analysis of salt-soluble extracts showed developmental changes in the processing pathway. A kinetic model was fitted to the data to determine rate constants for processing of both *N*- and *C*-propeptides. Data for pro α 1(I)-chain processing and pro α 2(I)-chain processing were fitted separately (where pro means procollagen). Between days 12 and 17 the relative flux through the pC-collagen (procollagen chain lacking the *N*-propeptide) and pN-collagen (procollagen chain lacking the *C*-propeptide) pathways increased approx. 4-fold. Pro α 1(I) chains and pro α 2(I) chains were processed by slightly different routes. Variations in the rate constants were compared with electron-microscopic measurements of collagen fibril diameters at each stage of development. Diameters increased by less than 10% over the period from 12 to 17 days. It was concluded that fibril diameters are relatively insensitive to the pathway of procollagen processing in the salt-soluble pool.

INTRODUCTION

The cornea is a tissue that exemplifies the relationship between structure and function in the extracellular matrix. Optical transparency is determined by the regular arrangement of narrow, uniform-diameter, collagen fibrils (Cox *et al.*, 1970; Craig & Parry, 1981). The mechanisms that control the diameters and organization of collagen fibrils in cornea are poorly understood (Birk & Trelstad, 1984).

Several factors may be involved in collagen-fibril-diameter control. These include interactions between different genetic types of collagen (Lapière *et al.*, 1977; Adachi & Hayashi, 1986; Birk *et al.*, 1988, 1990; Wotton *et al.*, 1988; Mendler *et al.*, 1989), interactions with proteoglycans (Vogel & Trotter, 1987; Scott, 1988; Scott & Haigh, 1988) or enzyme processing of procollagen precursors (Hulmes, 1983; Miyahara *et al.*, 1984; Fleischmajer, 1986; Chapman, 1989*a,b*). A number of observations suggest that fibril diameter may be affected by the pathway of procollagen processing, i.e. whether the *N*-terminal or *C*-terminal propeptides are removed first (Miyahara *et al.*, 1984). Immunoelectron microscopy of connective tissues suggests a link between narrow-diameter fibrils and the persistence of the *N*-propeptide (Fleischmajer *et al.*, 1983, 1987*a,b*). However, persistence of the *N*-propeptide is also associated with the formation of pleomorphic fibrils, both *in vivo* (Holbrook & Byers, 1982) and *in vitro* (Hulmes *et al.*, 1989*a,b*).

Here we examine the relation between procollagen processing and collagen fibril diameter in developing chick-embryo cornea. A kinetic model was devised to determine rate constants for removal of *N*- and *C*-propeptides from type I procollagen. We show that, although fibril diameters remain almost constant, there are marked changes in the pathway of procollagen processing in the salt-soluble pool. A preliminary report of this work has appeared elsewhere (Mellor *et al.*, 1990).

EXPERIMENTAL

Materials

L-[5-³H]Proline (1.11 TBq/mmol) was obtained from Amersham International, Aylesbury, Bucks., U.K. L-4-[G-³H]-Hydroxyproline (Hyp; 196 GBq/mmol) was from du Pont, Stevenage, Herts., U.K. Dowex AG50W-X8 (200–400 mesh) resin was from Bio-Rad, Hemel Hempstead, Herts., U.K. Hanks balanced salt solution (HBSS), penicillin/streptomycin (PS; 10000 units/ml and 10000 µg/ml respectively) and minimal essential medium (MEM) were from GIBCO, Paisley, Renfrewshire, Scotland, U.K. Phenylmethanesulphonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), β -aminopropionitrile (BAPN, fumarate salt) and Tris (Trizma base) were from Sigma, Poole, Dorset, U.K. Pepsin (porcine gastric mucosal enzyme) was from Boehringer Mannheim, Lewes, East Sussex, U.K. Cacodylic acid and paraformaldehyde were from Agar Aids, Stansted, Essex, U.K. All other chemicals (analytical grade) were from BDH, Poole, Dorset, U.K.

Fertile hen eggs (J strain) were obtained from the AFRC Institute of Animal Physiology and Genetics (Edinburgh), Roslin, Midlothian, Scotland, U.K., and placed in a Western incubator at 37 °C, with a humid atmosphere, until required. On days 12, 14 and 17 of embryonic development, corneas were removed using forceps, washed in HBSS containing 1% (v/v) PS, and either processed for electron microscopy or used for the pulse-chase experiments (see below). Embryonic development was assessed by measuring the length of the third toe from the tip to the middle of the metatarsal joint (Hamburger & Hamilton, 1951). The observed variation in this parameter was small, and values were distinct at each stage of development studied.

Electron microscopy

Corneas were fixed for at least 1 h at room temperature in 4%

Abbreviations used: BAPN, β -aminopropionitrile; col, collagen chain; HBSS, Hanks balanced salt solution; NEM, *N*-ethylmaleimide; MEM, minimal essential medium; pro, procollagen chain; pC, pC-collagen chain, i.e. procollagen chain lacking the *N*-propeptide; pN, pN-collagen chain, i.e. procollagen chain lacking the *C*-propeptide; PMSF, phenylmethanesulphonyl fluoride; PS, penicillin/streptomycin.

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(w/v) formaldehyde/0.1 M-sodium cacodylate, pH 7.4. After sequential staining with 1% (w/v) phosphotungstic acid and 1% (w/v) uranyl acetate, specimens were dehydrated in increasing concentrations of ethanol and embedded in Araldite according to standard procedures. Ultrathin sections were cut using a diamond knife and examined in a JEOL 1200EX transmission electron microscope.

Fibril diameters were measured from electron micrographs with a Joyce-Loebl Magiscan 2. Diameters were measured as the minimum width of fibrils in approximately transverse section, using a program devised by Dr. N. Ward (Department of Medical Biophysics, University of Manchester, Manchester, U.K.). At least 450 diameters were recorded for each stage of development studied. Micrographs were calibrated using a replica line grating (2160 lines/mm).

Pulse-chase experiments

Corneas were pulsed with [^3H]proline and chased for various times in the presence of unlabelled proline by using the following procedure. All incubations were at 37 °C in medium A [MEM containing 1% PS (v/v), ascorbic acid (50 $\mu\text{g}/\text{ml}$) and BAPN (64 $\mu\text{g}/\text{ml}$)] supplemented with either radioactive or unlabelled L-proline. [Ascorbic acid and BAPN were added from freshly prepared 20 $\times$ stock solutions in MEM/1% PS (v/v).] Before the radioactive pulse, corneas were placed in microcentrifuge tubes (three corneas/tube) containing 0.5 ml of medium A. The tubes were gassed with CO_2/air (1:19), sealed and preincubated for 15 min in a 37 °C water bath. After preincubation, a further 0.5 ml of medium A was added containing 100 μCi (3.7 MBq/ml) of [^3H]proline/ml. The tubes were gassed as described above and incubated for a pulse time of 20 min. At the end of the pulse, the corneas were pelleted briefly and the supernatant replaced with 1 ml of medium A containing 1 mg of unlabelled proline/ml. After rapid resuspension the corneas were again pelleted and the medium replaced with medium A containing 0.1 mg of proline/ml. Corneas were then re-suspended and incubated for chase times up to 180 min.

At the end of the chase, 0.1 ml of 250 mM-EDTA/25 mM-NEM/10 mM-PMSF/1 M-Tris/HCl, pH 7.4, was added to each tube and the tubes were vortex-mixed. (NEM and PMSF were freshly added to the Tris/EDTA solution, the latter from a 50 mM-stock solution in propan-2-ol.) The corneas were then pelleted briefly and the supernatant was replaced with 250 μl of 1 M-NaCl/25 mM-EDTA/10 mM-NEM/1 mM-PMSF/50 mM-Tris/HCl, pH 7.4 (measured at room temperature). The corneas were extracted in this buffer by placing the tubes horizontally on a reciprocal shaker at 4 °C for 40 h. Tubes were then centrifuged at 12000 g for 5 min at 4 °C and the extracts collected in the supernatants.

Extraction efficiency

To determine the proportion of newly synthesized collagenous material extracted by the 1 M-NaCl buffer, extracts and pellets were analysed for [^3H]hydroxyproline content, after hydrolysis for 16 h at 116 °C, by chromatography on Dowex AG50W-X8 columns eluted with 1.5 M-HCl (McAnulty & Laurent, 1987). Extraction efficiency was calculated from the [^3H]hydroxyproline present in the extracts as a proportion of total [^3H]hydroxyproline recovered in both extracts and pellets.

SDS/PAGE

Procollagen processing was observed by SDS/PAGE and fluorography. After extraction in 1 M-NaCl with proteinase inhibitors, 20% (w/v) SDS was added to approx. 100 μl of the supernatant to give a final SDS concentration of 2%. Samples were then heated for 3 min at 100 °C and either stored at -20 °C or processed immediately for electrophoresis. Before electro-

phoresis, one-quarter the sample volume of 5 $\times$ reducing sample buffer [15% (v/v) β -mercaptoethanol/50% (v/v) glycerol/2% (w/v) SDS/0.05% (w/v) Bromophenol Blue/0.125 M-Tris/HCl, pH 6.8] was added and the samples were further heated for 3 min at 100 °C. Samples were then immediately applied to discontinuous gels (Laemmli, 1970) containing 4.5% acrylamide in the stacking gel and either 5 or 6% acrylamide in the separating gel. Electrophoresis was carried out for approx. 4 h at 40 mA constant current using a water-cooled LKB vertical electrophoresis unit. In order to improve resolution in the region of the collagen bands, electrophoresis was continued occasionally for 1 h after the tracking dye had reached the bottom of the separating gel.

Pepsin digestion

To investigate the possible contribution from type V collagen, some extracts were digested with pepsin as follows. After buffer exchange for 0.5 M-acetic acid using Amicon C-10 micro-concentrators, pepsin was added to a final concentration of 100 $\mu\text{g}/\text{ml}$ and the samples were incubated at 4 °C for 18 h (Kumamoto & Fessler, 1981). The reaction was stopped by buffer exchange for 1 M-NaCl/25 mM-EDTA/50 mM-Tris/HCl, pH 7.4, and the pepsin-digested samples analysed by electrophoresis as described above.

Fluorography and densitometry

After electrophoresis, gels were processed for fluorography (Skinner & Griswold, 1983) and exposed to pre-flashed (Laskey & Mills, 1975) Kodak XAR-5 film at -70 °C for up to 8 weeks. Fluorograms were analysed with a Joyce-Loebl Chromoscan 3 scanning densitometer. The positions of the pro α 1(I) and pro α 2(I) bands were determined with purified type I procollagen standards from chick-embryo corneal fibroblasts prepared using a procedure for chick-embryo tendon type I procollagen (Mould *et al.*, 1990). The positions of the pC α 1(I), pN α 1(I), α 1(I), pC α 2(I), pN α 2(I) and α 2(I) bands were determined after incubation of corneal type I procollagen with purified procollagen N-proteinase and procollagen C-proteinase, prepared from chick-embryo tendons (Hojima *et al.*, 1989) and the medium of cultured chick-embryo tendons (Hojima *et al.*, 1985) respectively.

The amounts of radioactivity in each band were determined from the integrated peak areas in the densitometric scans. Peak area was found to be proportional to the amount of radioactivity loaded, provided that the maximum peak height did not exceed an absorbance of 1.0. Thus care was taken when exposing the fluorograms to remain within the linear response of the film. In order to convert total ^3H cpm in each band to relative molar quantities, peak areas were normalized by dividing by the corresponding number of proline and hydroxyproline residues per chain. The numbers of residues were as follows (Galloway, 1982): pro α 1(I), 270; pC α 1(I), 245; pN α 1(I), 260; α 1(I), 235; pro α 2(I), 256; pC α 2(I), 237; pN α 2(I), 244; α 2(I), 225. As the pro α 2(I) band migrates only slightly more slowly than the pN α 1(I) band, it was sometimes difficult to resolve these bands for quantification. When this occurred, the combined peak area was divided by an average correction factor of 258. Normalized peak areas were then summed and expressed as a molar proportion for each band. When the pro α 2(I) and pN α 1(I) bands were not resolved, their respective contributions were calculated by using the observed total molar proportions:

$$\text{pro}\alpha 1(\text{I}) + \text{pC}\alpha 1(\text{I}) + \text{pN}\alpha 1(\text{I}) + \alpha 1(\text{I}) = 66.7\%$$

$$\text{pro}\alpha 2(\text{I}) + \text{pC}\alpha 2(\text{I}) + \text{pN}\alpha 2(\text{I}) + \alpha 2(\text{I}) = 33.3\%$$

These total molar proportions were determined from gel tracks where the pro α 2(I) and pN α 1(I) were clearly resolved, when the totals were highly reproducible, i.e. $66.3 \pm 2.8\%$ (s.d., $n = 15$).

Scheme 1. Kinetic model for procollagen processing via pC-collagen and pN-collagen pathways

Rate constants (k_1 , k_2 , k_3 and k_4) are indicated.

Fig. 1. Histograms of collagen fibril diameters in developing chick-embryo cornea

Mean diameters ($\pm$ S.D.) are: (a) 12 days, 19.8 ± 2.8 nm; (b) 14 days, 20.2 ± 3.0 nm; (c) 17 days, 21.7 ± 3.0 nm.

Kinetic model

To determine rate constants for *N*- and *C*-terminal procollagen processing, the following kinetic model (Atkins, 1969) was devised. Processing of both $\text{pro}\alpha 1(\text{I})$ and $\text{pro}\alpha 2(\text{I})$ chains was characterized by four rate constants (Scheme 1). At steady state, the amounts of each ^3H -labelled procollagen chain and its processing products were determined by the following linear differential equations, where **bold type** indicates the radioactively labelled species:

$$d[\text{pro}]/dt = -(k_1 + k_2)[\text{pro}] \quad (1)$$

$$d[\text{pC}]/dt = k_1[\text{pro}] - k_3[\text{pC}] \quad (2)$$

$$d[\text{pN}]/dt = k_2[\text{pro}] - k_4[\text{pN}] \quad (3)$$

$$d[\text{col}]/dt = k_3[\text{pC}] + k_4[\text{pN}] \quad (4)$$

The solutions to these equations are as follows:

$$[\text{pro}] = A_0 \exp(-(k_1 + k_2)t) \quad (5)$$

$$[\text{pC}] = A_0 [k_1 / (k_3 - k_1 - k_2)] \{ \exp[-(k_1 + k_2)t] - \exp(-k_3 t) \} \quad (6)$$

$$[\text{pN}] = A_0 [k_2 / (k_4 - k_1 - k_2)] \{ \exp[-(k_1 + k_2)t] - \exp(-k_4 t) \} \quad (7)$$

$$[\text{col}] = A_0 - [\text{pro}] - [\text{pC}] - [\text{pN}] \quad (8)$$

where A_0 is the initial molar proportion for $\text{pro}\alpha 1(\text{I})$ or $\text{pro}\alpha 2(\text{I})$.

As the data for $\text{pro}\alpha 1(\text{I})$ - and $\text{pro}\alpha 2(\text{I})$ -chain processing were analysed separately, two sets of four rate constants were required to describe processing of the procollagen molecule. For each chain, the kinetic model was fitted to all the experimental data (i.e. procollagen, pC-collagen, pN-collagen and collagen) simultaneously, using a gradient method (Marquadt, 1963), with data points weighted by the reciprocal of the variance. Generally, the data for each chase time represented the average for three to eight experimental observations. Occasionally, when only one observation was available, a minimum weighting was used for the curve-fitting. When fitting the model, initial values for $\text{pro}\alpha 1(\text{I})$ and $\text{pro}\alpha 2(\text{I})$ molar proportions were fixed at 66.7 and 33.3%, respectively.

RESULTS

Fibril diameters

Fig. 1 shows the observed diameters of corneal collagen fibrils at different stages of embryonic development. The distribution of diameters is sharp, and the average diameter (approx. 20 nm) increases by less than 10% from day 12 to day 17.

Pulse-chase observations

Processing of type I procollagen at different stages of development is indicated in Fig. 2, which shows representative densitometric scans of fluorograms at chase times of 0, 10 and 180 min. At the start of the chase, data from all stages of development are similar. The data are also similar after 180 min, when procollagen chains have been almost fully processed to collagen chains, though small amounts of pN-collagen chains persist. At intermediate chase times, differences in the pathway of procollagen processing are apparent. For example, Fig. 2 shows similar amounts of pC-collagen and pN-collagen chains in day 12 corneas after a 10 min chase. After the same chase time, similar data were obtained in day 14 corneas. In day 17 corneas, however, pC-collagen is the predominant processing intermediate at 10 min chase. These observations suggest that the pathway of procollagen processing varies with development.

Fig. 2. Densitometric scans of fluorograms obtained after 0, 10 and 180 min of chase

(a) 12 Days; (b) 17 days.

Fig. 3. Pulse-chase data for procollagen processing in 12-day-chick-embryo corneas

The molar proportion of each component is shown as a function of chase time. Error bars are $\pm$ S.D. Continuous lines are fitted curves based on the kinetic model, with rate constants from Table 1. (a) Pro α 1(I)-chain processing; (b) pro α 2(I)-chain processing.

To examine these observations further, data were compared over a range of chase times, as shown in Figs. 3–5. Whereas pC-collagen was observed at intermediate chase times at all stages of development, pN-collagen was much more significant at days 12 and 14. Hence developmental changes in relative C- and N-terminal processing were apparent.

Processing rate constants

In order to quantify developmental changes in processing rates, the kinetic model was fitted to the data in Figs. 3–5, where the continuous lines show the best fit obtained at each stage of development. Rate constants determined from the kinetic model are listed in Table 1. On days 12 and 14 of embryonic development, the corresponding rate constants are similar. On day 17, however, there are marked decreases in both k_2 and k_4 , with a small reduction in k_3 . The reduction in k_2 corresponds to increasing processing via the pC-collagen pathway (see the Discussion).

Extraction efficiency

To determine whether the pulse-chase observations were representative of all the procollagen processed in the corneas, extracts and pellets were analysed for [3 H]hydroxyproline content, using [3 H]proline and [3 H]hydroxyproline standards (results not shown). The average percentage of total [3 H]hydroxyproline radioactivity in the extracts for various chase times and all stages of development was $62 \pm 14\%$ (S.D., $n = 23$).

Pepsin digestion

To determine the possible contribution to the fluorograms from type V collagen, extracts were treated with pepsin to remove non-triple helical regions. At each stage of development, almost all the radioactivity in the pepsinized extracts migrated in the positions of the α 1(I) and α 2(I) chains, with less than 2% in the regions expected for pepsin-treated α 1(V) and α 2(V) chains (McLaughlin *et al.*, 1989; results not shown).

Fig. 4. Pulse-chase data for procollagen processing in 14-day-chick-embryo corneas

The molar proportion of each component is shown as a function of chase time. Error bars are $\pm$ s.d. Continuous lines are fitted curves based on the kinetic model, with rate constants from Table 1. (a) Pro α 1(I)-chain processing; (b) pro α 2(I)-chain processing.

DISCUSSION

Collagen fibril diameter and procollagen processing pathway

The small and uniform diameter of collagen fibrils in cornea has been observed in several species (Hay *et al.*, 1979; Craig & Parry, 1981). Unlike the situation in other connective tissues, fibril diameters in cornea remain essentially constant throughout embryonic and adult life (Parry & Craig, 1984). The maintenance of small diameters is necessary for corneal transparency (Cox *et al.*, 1970). The diameters of fibrils observed here in developing chick-embryo cornea are consistent with these general observations.

Whereas fibril diameters remain essentially constant, there are large changes in the pathway of type I procollagen processing. The flux through the pC-collagen or pN-collagen pathway (Scheme 1) is determined by the product of the procollagen precursor concentration and the corresponding rate constant (k_1 or k_2). As both pathways begin with the same precursor, the relative flux through the pC-collagen and pN-collagen pathways

is determined by the ratio of rate constants, k_1/k_2 . Between days 12 and 17 of chick-embryo corneal development, this ratio increases approx. 4-fold (Table 2). If fibril diameters were controlled by the relative rates of C- and N-terminal processing, then developing cornea is a tissue where the ratio k_1/k_2 should remain unchanged. The large changes observed in k_1/k_2 suggest that fibril diameters are relatively insensitive to the pathway of procollagen processing, at least in the salt-soluble pool.

Secretion and processing of procollagens

Extraction efficiency, as measured by the proportion of total [3 H]hydroxyproline present in the 1 M-NaCl extracts, was limited to approx. 60%. Are these results fully representative of type I procollagen processing in cornea? For isolated chick-embryo corneal cells in culture (Kao *et al.*, 1983), approx. 55% of pulse-labelled type I procollagen is secreted with a first-order rate constant of 0.071 min^{-1} (half-life 10 min), whereas the rate constant for secretion of the remaining 45% is 0.0053 min^{-1} (half-life 130 min). If procollagen processing is entirely extra-

Fig. 5. Pulse-chase data for procollagen processing in 17-day-chick-embryo corneas

The molar proportion of each component is shown as a function of chase time. Error bars are $\pm$ S.D. Continuous lines are fitted curves based on the kinetic model, with rate constants from Table 1. (a) Pro α 1(I)-chain processing; (b) pro α 2(I)-chain processing.

cellular, almost half of the slowly secreted pool should remain intracellular, and hence unprocessed, by the end of the chase (180 min). As there was no evidence here of a slowly processed pool of type I procollagen in whole corneas, either this pool is processed partly intracellularly, or the 1 M-NaCl buffer preferentially extracted the extracellular material. The latter interpretation is consistent with the limited extraction efficiency, which therefore probably relates to the larger pool of more-rapidly-secreted type I procollagen. More experiments are required to study the fate of the slowly secreted pool. It is noteworthy that the combined rate constant ($k_1 + k_2$) for processing of type I procollagen is comparable with the rate constant for secretion of the rapidly secreted pool (Kao *et al.*, 1983).

Secretion of type V procollagen from isolated chick-embryo corneal cells is characterized by a single first-order rate constant of 0.0057 min^{-1} (half-life 120 min; Kao *et al.*, 1983). This

relatively slow secretion of type V procollagen is consistent with the small amounts of type V collagen observed in the experiments described here, in contrast with the relatively high amounts of type V collagen detected in 12 h cultures (McLaughlin *et al.*, 1989).

Significance of the rate constants

Table 2 also shows that the ratio k_1/k_2 is consistently greater for pro α 2(I) chains than for pro α 1(I) chains. Therefore, in chick-embryo cornea, a greater proportion of pro α 2(I) chains are processed via pC-collagen than are pro α 1(I) chains. This difference in processing pathway is consistent with some properties of the purified procollagen N- and C-proteinases. When type I procollagen is processed *in vitro* by procollagen N-proteinase (Berger *et al.*, 1985), one of the two pro α 1(I) chains per molecule is the last chain to be cleaved. In contrast, when procollagen is

Table 1. Type I procollagen processing rate constants in developing chick-embryo cornea

S.D. values are shown in parentheses.

Age (days) ...	Value (min ⁻¹)		
	12	14	17
Proα1(I) processing			
k_1	0.02829 (0.00224)	0.01716 (0.00261)	0.03834 (0.00258)
k_2	0.02070 (0.00219)	0.02325 (0.00249)	0.007242 (0.001625)
k_3	0.1039 (0.0128)	0.1173 (0.0286)	0.03953 (0.00426)
k_4	0.01686 (0.00198)	0.01951 (0.00390)	0.001692 (0.002365)
Proα2(I) processing			
k_1	0.02950 (0.00206)	0.03023 (0.00309)	0.03547 (0.00209)
k_2	0.01662 (0.00137)	0.01212 (0.00230)	0.003792 (0.000811)
k_3	0.08913 (0.00858)	0.09039 (0.01520)	0.03926 (0.00307)
k_4	0.01962 (0.00266)	0.01384 (0.00460)	0.00005 (0.00206)

Table 2. Relative flux through the pC-collagen and pN-collagen pathways (k_1/k_2) and steady-state ratios of pC-collagen to pN-collagen (k_4/k_3) in salt-soluble extracts of developing chick-embryo cornea

Data for pro α 1(I) and pro α 2(I) chain processing are indicated separately. Lower and upper limits were calculated from the rate constants and S.D. values shown in Table 1.

Parameter	Age (days) ...	12	14	17
pC/pN relative flux				
Pro α 1(I) processing		1.14–1.65	0.57–0.95	4.04–7.28
Pro α 2(I) processing		1.53–2.07	1.88–3.39	7.25–12.6
pC/pN steady-state ratio				
Pro α 1(I) processing		0.15–0.34	0.06–0.25	0.0–0.84
Pro α 2(I) processing		0.26–0.57	0.16–0.83	0.0–0.73

processed *in vitro* by procollagen C-proteinase (Njieha *et al.*, 1982), the last chain to be cleaved is the pro α 2(I) chain. Hence, in the presence of both enzymes, more pro α 2(I) chains should follow the pC-collagen pathway than pro α 1(I) chains, as observed in chick-embryo cornea. No attempt was made here to measure differences in processing rates for the two pro α (I) chains in each procollagen molecule; hence the observed rates must be regarded as mean values.

No allowance was made for the effects of the finite pulse time on the kinetic model. Convolution with an appropriate 'top hat' function would broaden the calculated curves, and rate constants determined without such a correction may be somewhat underestimated. It was felt that the accuracy of the experimental data did not warrant such a refinement, which would not affect the observed relative changes in rate constants during embryonic development.

Processing rates are the products of rate constants and the corresponding substrate concentrations. Processing rates may also be determined from Michaelis–Menten kinetics, where

different substrates (e.g. procollagen and pN-collagen) can compete for the same enzymic reaction (e.g. removal of *N*-propeptides). The relation between the two expressions is complex, and there is no requirement for rate constants referring to the same enzymic reaction (e.g. k_1 and k_4) to be equal. Similar reasoning applies for the rate constants k_2 and k_3 .

Steady-state levels and kinetic intermediates

These results have important implications for the steady-state levels of procollagen and its processing intermediates. From eqns. (2) and (3), at steady state for the bulk population:

$$k_1[\text{pro}] = k_3[\text{pC}] \quad (9)$$

$$k_2[\text{pro}] = k_4[\text{pN}] \quad (10)$$

Hence, from eqns. (9) and (10), the steady-state ratio of pC-collagen to pN-collagen is:

$$[\text{pC}]/[\text{pN}] = k_1k_4/k_2k_3 \quad (11)$$

Table 2 shows the steady-state ratios calculated from eqn. (11). At all stages of embryonic development studied, the steady-state levels of pN-collagen exceed those of pC-collagen, in both pro α 1(I) and pro α 2(I) chain processing. Yet pC-collagen is sometimes the major kinetic intermediate in the pulse-chase experiments. There is no conflict between pC-collagen as the major kinetic intermediate and pN-collagen as the major steady-state intermediate. There is evidence that such a situation exists in chick-embryo tendon, where immunoblotting (Fleischmajer *et al.*, 1988) has indicated a pN-collagen/pC-collagen ratio greater than 1, whereas pulse-chase experiments (Leung *et al.*, 1979) show pC-collagen as the major kinetic intermediate.

Implications for corneal morphogenesis

Changes in the values of the rate constants for procollagen processing may reflect corresponding changes in the embryonic development of the chick cornea. Between days 12 and 19, the cornea decreases in thickness, owing to transport of water out of the stroma, resulting in closer packing of collagen fibrils and attainment of adult levels of transparency (Coulombre & Coulombre, 1958; Hay *et al.*, 1979). This stromal condensation may interfere with the synthesis, transport or activity of the procollagen proteinases and other extracellular-matrix components (Kessler & Adar, 1990) involved in processing. Changes in the amounts of matrix macromolecules during chick corneal development have been documented (Hay *et al.*, 1979; Linsenmayer *et al.*, 1984; Funderburgh *et al.*, 1986; Fitch *et al.*, 1988; Bard, 1990).

Developmental changes in procollagen processing pathway may have subtle effects on extracellular-matrix organization. For example, an increased flux through the pC-collagen pathway may allow processing intermediates to diffuse more freely from the cell before completion of processing and deposition of collagen fibrils. The pC-collagen intermediate is known to be more soluble than pN-collagen (Hulmes *et al.*, 1989b). Increased diffusion properties may be important, as the composition and amount of extracellular matrix changes during corneal development. This is a possible mechanism whereby cells could control the site of deposition of collagen fibrils after secretion of procollagen and its processing enzymes into the extracellular matrix.

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