Thermal stability of human-fibroblast-collagenase-cleavage products of type-I and type-III collagens

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Rat skin type-I and type-III collagens were degraded by human fibroblast collagenase at a temperature below the 'melting' temperature for the two resulting fragments, namely the *N*-terminal three-fourths, TC^A, and the *C*-terminal one-fourth, TC^B. The specific cleavage of the collagen was confirmed by electrophoresis and determination of molecular length by electron microscopy. The two fragments were separated by gel filtration and the thermal stabilities of the isolated fragments were determined. For type-I collagen, the 'melting' temperatures of the two fragments were found to differ by only 0.5 °C and were 4.5–5.0 °C below that of the uncleaved molecule. The 'melting' temperatures of the uncleaved molecule and the *N*-terminal fragment were independent of the extent of *N*-terminal intramolecular cross-linking. For type-III collagen, the 'melting' temperatures of the fragments were found to differ by 1.3 °C. The small fragments of the two types of collagen 'melted' at the same temperature, whereas the large type-III fragment 'melted' at a slightly higher temperature than did the large type-I fragment. Reduction of the disulphide bonds located in the *C*-terminal type-III fragment did not affect the thermal stability of this fragment. The thermal stability of uncleaved type-III collagen was found to be variable, but the reason for this is not known at present.

INTRODUCTION

The unique specificity of mammalian collagenases for the triple helix of collagen gives these enzymes prime interest in relation to extracellular collagen breakdown. Collagenase obtained from various tissues and cells degrades interstitial collagen (types I, II and III) by one specific cleavage that results in two triple-helical fragments, constituting the *N*-terminal three-fourths, TC^A, and the *C*-terminal one-fourth, TC^B, of the molecule (Gross *et al.*, 1980). The thermal stability of the resulting fragments is determining for the conformation of these. When these fragments denature, they become susceptible to other proteinases than collagenase. These other proteinases, which are also able to attack the non-helical telopeptide region of the collagen molecule, may be responsible for the final degradation of the collagen (Sellers & Murphy, 1981). Therefore the thermal stability of the collagenase-degradation fragments is determining for their susceptibility to these other proteinases. In tissues collagen molecules are cross-linked, and in understanding the turnover of tissue collagens it is essential to know how the thermal stability of the fragments is influenced by the collagen cross-linking.

In an earlier study, it was found that the thermal stabilities of the large and the small fragments were quite different (Sakai & Gross, 1967). The 'melting' temperatures of the TC^A and TC^B fragments, resulting from cleavage of calf skin collagen by tadpole collagenase, were 4 °C and 7 °C respectively below that of the uncleaved molecule.

In the present paper, type-I and type-III collagens from rat skin were degraded by human fibroblast collagenase, and the cleavage products were separated by gel filtration. The thermal stabilities of isolated fragments with differing extents of intramolecular cross-linking were determined. The 'melting' temperatures of the fragments were found to be 4–5 °C lower than that of the

uncleaved molecule. But, in contrast with the results reported by Sakai & Gross (1967), the thermal stabilities of the fragments were found to be similar.

EXPERIMENTAL

Reagents

Pepsin (crystallized and freeze-dried) was purchased from Sigma Chemical Co. Sephacryl S-400 and CM-32 CM-cellulose were Pharmacia Fine Chemicals and Whatman products respectively. L-Cystine (Merck) was reagent grade. Other reagents used were analytical grade.

Collagen preparation

Collagen was prepared from the dorsal skin of 60-day-old male Wistar rats. Neutral-salt-soluble collagen was obtained from normal rats, whereas lathyrus neutral-salt-soluble type-I and type-III collagens were obtained from β -aminopropionitrile-treated rats (Danielsen, 1982a). Acetic acid-extracted type-I collagen from normal rats was re-precipitated twice, and each dissolution was followed by centrifugation to precipitate undissolved material (Danielsen, 1981). The freeze-dried acetic acid-extracted collagen that was stored in liquid N₂ was dissolved in 5 mM-acetic acid and filtered through a 0.8 μ m-pore-size membrane filter.

Preparation of collagenase

The collagenase in serum-containing human fibroblast culture medium was concentrated by (NH₄)₂SO₄ precipitation and CM-cellulose chromatography, according to the procedures described by Stricklin *et al.* (1977). The chromatography fractions containing collagenase were concentrated by ultrafiltration (Amicon, PM10 filter) and stored in liquid N₂.

Collagenase degradation

Collagenase was activated at 37 °C for 10 min in a solution saturated with L-cystine before combination with collagen. The final concentration of 50 mM-CaCl₂ (pH 7.5) in the enzyme/substrate mixture was achieved by mixing collagen solutions with 300 mM-CaCl₂/100 mM-Tris/HCl buffer, pH 7.5. Solid L-cystine was added to saturation and the mixture was incubated at 26 °C for 7 days. Microbial growth was inhibited by addition of a drop of toluene. After incubation, the reaction mixture was dialysed against 5 mM-acetic acid at 4 °C.

A solution of collagenase-treated type-III collagen in 50 mM-CaCl₂/50 mM-Tris/HCl, pH 7.4, was divided into two samples. One sample was dialysed against 5 mM-acetic acid. The other sample was made 5% (v/v) with respect to 2-mercaptoethanol and re-incubated at 26 °C for 18 h, and then dialysed against 5 mM-acetic acid/1 mM-2-mercaptoethanol. The thermal transition of these samples was then determined without separation of the fragments by gel filtration.

Gel filtration

To collagen samples dissolved in 5 mM-acetic acid was added $\frac{1}{10}$ vol. of 1 M-CaCl₂/1 M-Tris/HCl buffer, pH 7.5, and the pH was adjusted to 7.4 with 1 M-NaOH. Before gel filtration, the type-III collagen samples were made 5% (v/v) with respect to 2-mercaptoethanol and incubated for 18 h at room temperature. Portions (6–9 ml) of the collagen samples were applied to a Sephacryl S-400 column (1.6 cm × 95 cm) that was equilibrated and irrigated with 50 mM-CaCl₂/50 mM-Tris/HCl buffer, pH 7.4 at 4 °C. In the case of type-III collagen, the buffer contained 1 mM-2-mercaptoethanol. The flow rate was 12 ml/h, and the eluate was collected in 5 ml fractions. Relevant fractions were pooled, dialysed against 5 mM-acetic acid and freeze-dried.

Denaturation studies

The thermal stability of collagen components was determined in 5 mM-acetic acid solutions containing 0.1–0.2 mg of collagen/ml. In most experiments the type-III collagen solutions also contained 1 mM-2-mercaptoethanol.

Absorbance–temperature transitions. Determination of the thermal stability by u.v. difference spectroscopy and calculation of smoothed denaturation profiles were performed by the procedures previously described (Danielsen, 1982b, 1984).

Ellipticity–temperature transitions. Ellipticity was measured at 227 nm with a Cary 60 spectropolarimeter. A cell with 1 mm light-path was employed, and the temperature was increased at the rate of 0.3 °C/min. In order to minimize the damaging effect of u.v. irradiation of collagen (Hayashi *et al.*, 1979), the u.v. exposure of the sample was reduced by protecting the sample from the beam between the intermittently performed recordings of ellipticity.

SDS/polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out at room temperature according to a previously described procedure (Danielsen, 1982a), based on that of Furthmayr & Timpl (1971), in 5% (w/v) acrylamide at 6 mA/tube until the tracking dye (Bromophenol Blue) was 1 cm from the gel bottom.

Electron microscopy

The collagen molecules were made observable by application of the rotary-shadowing technique (Shotton *et al.*, 1979). The collagen samples, dissolved in 5 mM-acetic acid [the type-III collagen samples also contained 5% (v/v) 2-mercaptoethanol], were mixed with glycerol to give a final concentration of 65% (v/v) glycerol. The final collagen concentration was 1 µg/ml. By using the mica sandwich technique (Mould *et al.*, 1985), each sample solution was spread into a thin layer between two pieces of freshly cleaved mica, and one of the mica pieces was transferred to the rotating stage of Balzers freeze-etch chamber and dried. While rotating, the samples were shadowed with a platinum/carbon mixture (95% w/w) at a 6° shadow angle to a thickness of 0.58 nm. Thereafter, the samples were coated with a supporting film of carbon. Balzers electron-bombardment guns and quartz film-thickness monitor were used. The carbon films were floated on to distilled water and picked up on bare 400-mesh copper grids. The specimens were photographed in a Jeol Jem 100S electron microscope operated at 80 kV at a magnification of 21 000. The magnification was calibrated from photographs of a grid with standard gratings that were taken under the same electron optical conditions. Length calculations of the molecules were performed by projecting the electron-micrographic negatives on to a graphic tablet (Hewlett–Packard 9111A linked to a Hewlett–Packard 9816S computer) and tracing the images of those molecules (70–150 molecules for each sample) that had both ends visible. The final calibrated magnification of the molecules during this tracing was about 250 000.

RESULTS

The TC^A and TC^B fragments were separated from each other and from the protein contaminants in the collagenase preparation by the Sephacryl S-400 gel filtration. The electrophoretic migration patterns for the chain components of the isolated fragments were consistent with those resulting from collagenase cleavage of collagen. The yield of TC^A and TC^B fragments was approximately 3:1 when the collagen content was estimated by absorption at 227 nm. The total recovery of collagen after the gel filtration was 80–90%.

The 'melting' temperatures (T_m) of the TC^A and TC^B fragments of type-I collagen were found to be 34.7 °C and 34.2 °C respectively, which are 4.5–5 °C below that of the undegraded tropocollagen molecule (T_m 39.2 °C) (Fig. 1a). These results were confirmed by the c.d. measurements. The thermal stabilities of the collagen components were identical for lathyritic neutral-salt-soluble and acid-soluble type-I collagens.

The thermal transition of a sample containing a mixture of undegraded tropocollagen and TC^A and TC^B fragments from neutral-salt-soluble type-I collagen that had not been subjected to gel filtration was determined. The transition of the undegraded tropocollagen could be clearly discerned from those of the fragments, for which the transitions emerged (Fig. 2). It is known that pH influences the thermal stability of collagen (Dick & Nordwig, 1966). Consistently the pH was found to influence the thermal transition of the undegraded tropocollagen molecules as well as of the fragments. However, the 'melting' profiles for the single fragments

Fig. 1. Denaturation profiles of tropocollagen and the collagen fragments obtained after gel filtration of collagenase-degraded tropocollagen

The denaturation of lathyritic neutral-salt-soluble (a) type-I collagen and (b) type-III collagen was monitored by u.v. difference spectroscopy. —, Undegraded tropocollagen; -----, TC^A fragment; ·····, TC^B fragment.

did not become distinguishable, irrespective of the pH value. Thus variations in the pH did not reveal different 'melting' temperatures of the two fragments.

The 'melting' temperatures of the TC^A and TC^B fragments of type-III collagen that had been reduced before the gel filtration were 35.4 °C and 34.1 °C respectively (Fig. 1b). The 'melting' temperature of the undegraded type-III collagen, which, except for collagenase addition, had been processed in parallel to the enzyme-treated collagen, was 39.3 °C. This 'melting' temperature is considerably lower than that of the stock preparation of type-III collagen. The 'melting' temperature of this stock preparation was 40.9 °C, in agreement with a previously published result (Danielsen, 1982a).

The 'melting' temperature of the samples containing unseparated type-III collagen degradation fragments was 35.1 °C for both the reduced sample and the non-reduced one. This 'melting' temperature corresponds to the calculated 'melting' temperature of a mixture of 75% TC^A and 25% TC^B by using the 'melting' temperatures found for the isolated fragments. In contrast with the reduced sample, the non-reduced one did not give any band with mobility faster than that of the TC^A fragment upon electrophoresis. Therefore the disulphide bonds linking the TC^B fragments together were neither cleaved off during the collagenase treatment nor spontaneously reduced during the subsequent processing.

Electron micrographs of rotary-shadowed type-I and type-III collagen components are shown in Fig. 3. For

Fig. 2. Denaturation profiles of type-I collagen and collagenase-degradation fragments thereof at different pH values

(a) Neutral-salt-soluble collagen in 5 mM-acetic acid, pH 3.5. (b)–(d) Denaturation profiles of partially collagenase-degraded neutral-salt-soluble collagen determined at pH 2.25 (b), pH 3.5 (c) and pH 7.4 (d), which were achieved in solutions of 5 mM-acetic acid/5 mM-HCl, 5 mM-acetic acid and 5 mM-acetic acid diluted 1:1 with 0.3 M-sodium phosphate buffer, pH 7.4, respectively.

both types of collagen, the lengths of the undegraded tropocollagen and TC^A and TC^B fragment molecules were distributed around peaks with mean values ($\pm$ S.D.) of 295($\pm$ 11) nm, 222($\pm$ 11) nm and 63.5($\pm$ 8) nm respectively.

DISCUSSION

Owing to the similarity in 'melting' temperature of the TC^A and TC^B fragments that was found in the present

Fig. 3. Electron micrographs of rotary-shadowed type-I and type-III collagen components

Lathyrctic neutral-salt-soluble [(a)–(c)] type-I and [(d)–(f)] type-III collagen components. (a) and (d) Undegraded tropocollagen; (b) and (e) TC^A fragment; (c) and (f) TC^B fragment. Magnification $\times 60\,000$.

work, it is necessary to separate the two fragments in order to determine their thermal stabilities. The Sephacryl S-400 gel-filtration procedure described in the present work is suitable for this purpose.

The electron microscopy confirms that the collagen was cleaved into a one-fourth and a three-fourths fragment by the collagenase cleavage performed. The added lengths of the two fragments correspond, within experimental error, to the total length of the collagen molecule. This indicates that the triple-helical structure of the collagen molecule is retained throughout the entire length of the fragments during the collagenase cleavage and the subsequent gel filtration performed in the present work.

Sakai & Gross (1967) found that the 'melting' temperature of the TC^B fragment from tadpole-collagenase-digested calf skin collagen was 3 °C below that of the TC^A fragment, whereas the present results show that the 'melting' temperatures of the two fragments differ by only 0.5 °C. This discrepancy of the results was not expected, since tadpole collagenase and human fibroblast collagenase cleave collagen at the same locus (Gross *et al.*, 1980), and the amino acid compositions of calf skin and rat skin collagen and collagenase-degradation fragments thereof are very similar (Kang *et al.*, 1966; Sakai & Gross, 1967).

No dissimilarity in the thermal stability of the TC^A and TC^B fragments was revealed at the different pH values tested in the present work. The discrepancy of the results is therefore not obvious from the different buffers (pH

values) or the different techniques that were used for the determination of thermal stability in the present work and in that of Sakai & Gross (1967). Sakai & Gross (1967) observed a double-banded pattern of α_1^B - and α_2^B -chains upon electrophoresis. This pattern could be caused by heterogeneity or a broader specificity of the applied tadpole collagenase preparation and might cause the relatively lower thermal stability that they found for the TC^B fragment. Compared with human fibroblast collagenase, tadpole collagenase has less specificity with regard to hydrolysis of peptide bonds in oligopeptides (Weingarten & Feder, 1986). However, it still remains to be established whether the broader catalytic activity of tadpole collagenase might result in additional cleavage sites in the native collagen molecule that could have consequences for the thermal stability of the fragments.

In previous work, the 'melting' temperature of collagen was found to be independent of the extent of intramolecular cross-linking when the extent of this cross-linking was estimated by the ratio of monomer (α -chains) to monomer plus polymer (β - and γ -chains) collagen components by electrophoresis of denatured collagen (Danielsen, 1982a). In agreement with this finding, the thermal stabilities of lathyrctic neutral-salt-soluble (93 % monomer) and acid-soluble collagen (35 % monomer) were, in the present work, found to be identical. The intramolecular cross-links are located in the N-terminal telopeptides of the collagen molecule (Bailey & Robins, 1976). After the collagenase treatment, the ratios of monomer to monomer plus dimer N-terminal chain fragments [$\alpha^A/(\alpha^A + \beta^A)$] were 97 % and 52 % for the lathyrctic neutral-salt-soluble and acid-soluble collagen respectively. However, also, the thermal stability of the TC^A fragment was independent of the different proportions of the monomer and polymer components that were obtained by isolating the TC^A fragments from the lathyrctic neutral-salt-soluble and acid-soluble collagens.

The thermal stability of type-III collagen is not influenced by reduction of the disulphide bonds when this reduction is performed in 5 mM-acetic acid (Danielsen, 1982a, and unpublished work). However, the thermal stability of the collagen is diminished when the disulphide bonds are reduced during incubation in a neutral CaCl₂ buffer (C. C. Danielsen, unpublished work). This finding may explain the diminished thermal stability that was observed for the type-III collagen processed in parallel with the collagenase-treated sample. At present it is not known whether the change of thermal stability is due to incubation in a neutral buffer or to the extent of the completion of disulphide-bond cleavage. However, the variable thermal stability of type-III collagen may explain why the thermal stability of this collagen type was found to be higher (Danielsen, 1982a) than that reported by other groups (Byers *et al.*, 1974; Fujii & Kühn, 1975; Peltonen *et al.*, 1980). The present results do not indicate that the thermal stability of the TC^B fragment containing the disulphide bonds is influenced by reduction.

Many genetic defects of interstitial collagens, some of which cause severe clinical disorders, are known (Prockop & Kivirikko, 1984; Uitto *et al.*, 1986). The techniques described in the present work are applicable to estimating changes in the triple-helical stability induced by mutations of collagen in pathological specimens. By these techniques it is possible to determine in which part of the

collagen molecule, corresponding to either of the two collagenase-cleavage fragments, the changing stability takes place and is affected by mutation.

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