Biochem. J. (1984) **222**, 663–668
Printed in Great Britain

Reconstituted collagen fibrils

Fibrillar and molecular stability of the collagen upon maturation *in vitro*

Carl Christian DANIELSEN
Department of Connective Tissue Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C, Denmark

(*Received 19 March 1984/Accepted 29 May 1984*)

During the maturation *in vitro* of reconstituted collagen fibrils prepared from rat skin, the mechanical and thermal stability of collagen increased and the pepsin-solubility decreased. At the same time a larger fraction of the pepsin-soluble collagen attained a lower molecular thermal stability that resulted in a biphasic thermal transition of the soluble collagen. Type-I collagen, with a similar biphasic thermal transition, was isolated from acid-insoluble rat skin collagen.

Reconstituted collagen fibrils attain increasing mechanical and thermal stability during maturation *in vitro* when incubated in air at 37°C (Danielsen, 1981*a*,*b*). These changes in stability are similar to those occurring in collagenous tissues during aging *in vivo*.

Usually the 'helix-to-random coil' transition upon heating of soluble collagen shows a symmetrical transition curve, but some preparations of acid-soluble rat skin collagen have a distinct skewness in the denaturation profile (Danielsen, 1982*a*). The acid-soluble collagen is supposed to represent the more mature and cross-linked part of fibrillar collagen that is extractable by neutral-salt solutions and dilute acids (Robins, 1980). The changed denaturation profile for this collagen fraction may reflect a conformation or structural change of the collagen molecule during age-related stabilization of the fibrils. Therefore an investigation of the relationship between changes in the denaturation profile of the molecular collagen and the extent of stabilization of reconstituted collagen fibrils that were matured *in vitro* was performed.

Materials and methods

Materials

Pepsin (crystallized and freeze-dried) was purchased from Sigma Chemical Co. The DEAE-cellulose ion-exchanger used was Whatman DE-52.

Reconstitution and maturation of collagen fibrils

Collagen fibrils were reconstituted and matured in accordance with a previously described procedure (Danielsen, 1981*a*). Briefly, a stock preparation of purified acid-soluble collagen was obtained from the dorsal skin of 60-day-old male Wistar rats. The collagen was reconstituted into fibrils by gradual heating of neutral solutions of the collagen. The collagen fibrils were dried to membranes within 11 days after aggregation. The membranes were then cut into 4mm-wide strips appropriate for mechanical testing. Eight groups of strips were matured for different time periods (11–150 days after aggregation) by incubation in air at 37°C. The maturation was stopped by transferring the strips to liquid N_2. Immediately after the completion of the aggregation, a portion of collagen fibrils was precipitated by centrifugation and stored in liquid N_2 until the analyses were performed.

Mechanical testing and determination of thermal stability of the fibrils

The mechanical strength of the collagen membranes that were matured for different time periods after aggregation was determined in accordance with the previously described procedures (Danielsen, 1981*a*). The thermal stability of the collagen membranes was determined as the area shrinkage without tension during heating (AS_T) and the shrinkage temperature (T_s) was calculated as the temperature for 50% of this area shrinkage (Danielsen, 1981*b*).

Solubilization and isolation of collagen

Samples of the stock preparation of acid-soluble collagen and of the reconstituted collagen fibrils that were matured for 0–150 days were incubated with stirring in 0.5M-acetic acid at 1:10 pepsin/col-

lagen weight ratio at 4°C for 1 week. After the incubation the suspensions were centrifuged (50000*g* for ½h). The resulting supernatants were dialysed against 0.15M-$CaCl_2$/0.05M-Tris/HCl buffer, pH8, and re-centrifuged (1h), and NaCl was added to give 4M. The precipitated collagen was dissolved with 5mM-acetic acid and centrifuged (50000*g* for 1h). The solubility of a sample was defined by the amount of hydroxyproline in the resulting supernatant divided by the total amount of hydroxyproline in the supernatant and the pooled precipitates.

A collagen membrane that was prepared and matured for 85 days, as described above, was subjected to more extensive pepsin digestion in 0.5M-acetic acid at 4°C for 4 days. Pepsin was added to give a pepsin/collagen weight ratio of 1:5 at the start of the incubation and added again after 2 days' incubation to give the same weight ratio.

The insoluble residue resulting from the extraction of 40g (wet wt.) of rat skin with 0.5M-acetic acid from the 60-day-old rats (Danielsen, 1981*a*) was re-homogenized in 100ml of 0.5M-acetic acid, combined with 100mg of pepsin and incubated with stirring at 4°C for 1 week. The incubation mixture was then centrifuged (50000*g* for 1h) and the resulting supernatant dialysed against 0.15M-$CaCl_2$/0.05M-Tris/HCl buffer, pH7.5. After re-centrifugation (50000*g* for 1h), the collagen in the supernatant was precipitated by the addition of NaCl to give 4M and dissolved in 5mM-acetic acid. Thereafter the collagen solution was diluted 1:1 with a 0.4M-NaCl/0.1M-Tris/HCl buffer, pH7.4, and chromatographed on a DEAE-cellulose column by the procedure of Miller (1971). The breakthrough fractions were pooled, dialysed against 5mM-acetic acid and diluted 1:1 with 2M-NaCl/0.1M-Tris/HCl buffer, pH7.4, and, after adjustment of pH to 7.4 with 1M-NaOH, the collagen was fractionated by the method based on that of Chung & Miller (1974) by sequential addition of NaCl to give 1.7M, 2.5M and 4M. The 4M-NaCl-precipitated fraction was subjected to DEAE-cellulose chromatography by the procedure of Bentz *et al.* (1978). The collagen was dissolved in 2M-urea/20mM-NaCl/0.05M-Tris/HCl buffer, pH8.6, and applied to a column that was equilibrated with the same buffer at 15°C. The absorbed collagen was eluted with 100mM-NaCl.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out in 5% (w/v) acrylamide gels in the presence of sodium dodecyl sulphate at 6mA/tube for 6h at room temperature according to a previously described procedure (Danielsen, 1982*b*) based on that of Furthmayr & Timpl (1971).

Absorbance temperature transitions

Duplicate determinations of the thermal stability of molecular collagen and production of smoothed denaturation profiles were performed by the procedures described in detail previously (Danielsen, 1982*a*). Briefly, the 'melting' of collagen was measured by recording the absorption difference at 227nm between identical sample and reference collagen solutions (0.10–0.25mg/ml in 5mM-acetic acid) during gradual heating of the sample (0.24°C/min). For comparative purposes, the reproduced denaturation profiles (the first derivative of the absorbance versus temperature) were normalized to an area of one unit by dividing the first derivative by the total transition absorption change. The temperature for each successive 5% absorption change in the total transition absorption change was calculated. The fraction of collagen that 'melted' below a certain temperature was calculated from these data by interpolation. The 'melting' temperature (T_m) was defined as the temperature at which 50% of the transition absorption change had occurred.

Results and discussion

The stability of the reconstituted collagen fibrils increased during the maturation (Fig. 1). The mechanical stiffness (and strength) of the collagen membranes increased 3-fold from the 11th to the 150th day of maturation. The area shrinkage without tension during heating and the fraction of

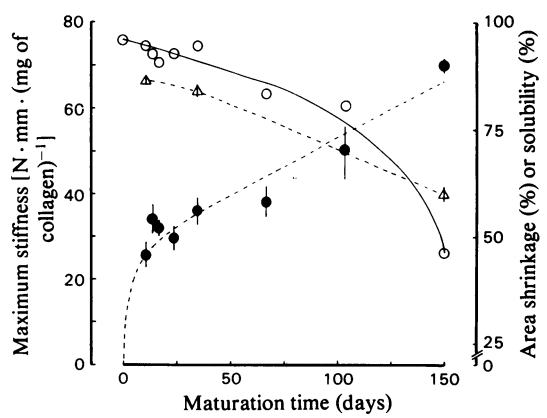

Fig. 1. *Stabilization of reconstituted collagen fibrils upon maturation*

The collagen fibrils were incubated at 37°C in air and after various times removed for determination of mechanical strength (maximum stiffness, ●), percentage area shrinkage during heating (AS_T, △) and solubility by limited peptic digestion (%, ○). (Vertical bars indicate ± S.E.M.)

collagen that was solubilized by limited peptic digestion decreased for the collagen fibrils during the 5 months maturation period. These changes in stability are similar to those reported elsewhere for fibrils matured *in vitro* (Robins & Bailey, 1977; Danielsen, 1981*a*,*b*) and for collagenous tissues aged *in vivo* (Viidik & Busted, 1977; Vogel, 1978). The shrinkage temperature, T_s ($\pm$S.E.M.), was 50.9 ($\pm$0.3), 50.5 ($\pm$0.5) and 48.9 ($\pm$0.5)°C for the collagen membranes matured for 11, 35 and 150 days respectively.

The 'melting' temperature, T_m, of the stock preparation of acid-soluble collagen and of the pepsin-solubilized collagen that was matured in fibrillar form for 0–150 days was between 39.4 and 39.8°C. The height of the denaturation profiles for the matured and solubilized collagen fibrils changed inversely below and above 37°C respectively (Fig. 2), so that the fraction of collagen that 'melted' below 37°C increased with maturation time (Fig. 3). The denaturation profile of the stock preparation of acid-soluble collagen was unaffected by the peptic digestion that was performed.

Owing to the decreasing solubility of the collagen fibrils during maturation, the pepsin-soluble fraction of collagen may not be representative of the total collagen in the collagen fibrils. However, increasing the soluble fraction (to 89%) by a more extensive peptic digestion of the collagen fibrils that were matured for 85 days

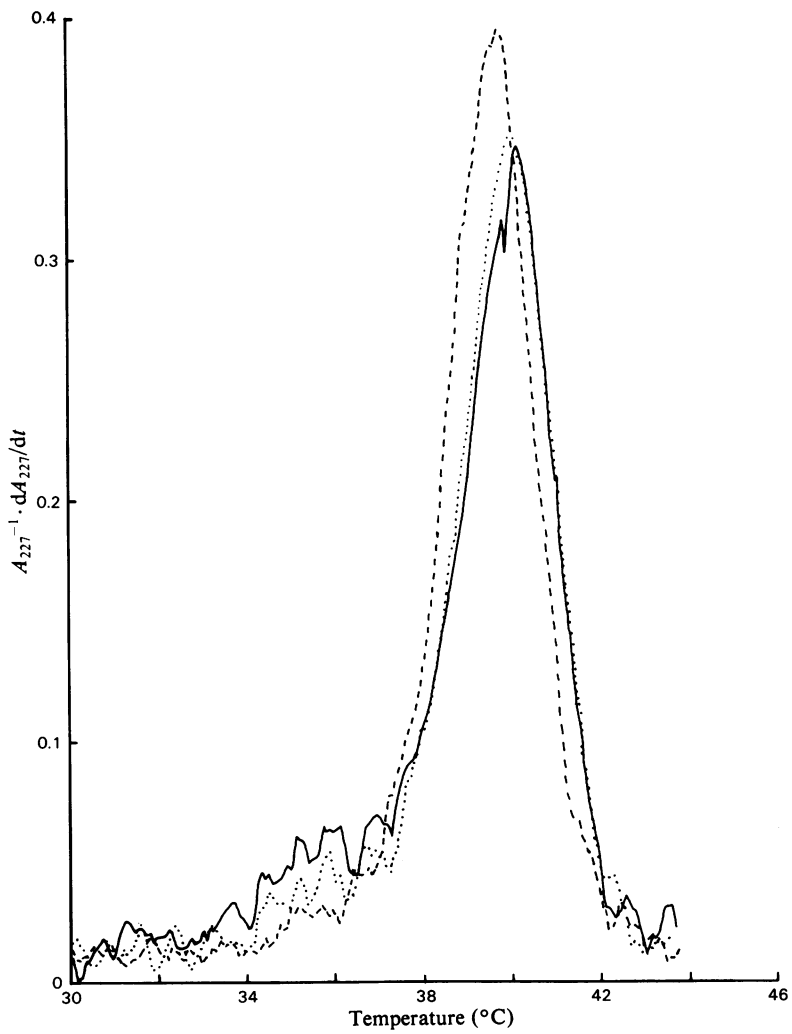

Fig. 2. *Denaturation profiles of pepsin-solubilized collagen*
The collagen is the pepsin-soluble part of the reconstituted collagen fibrils that were matured at 37°C in atmospheric air for 0 (------), 67 (·····) and 150 days (——).

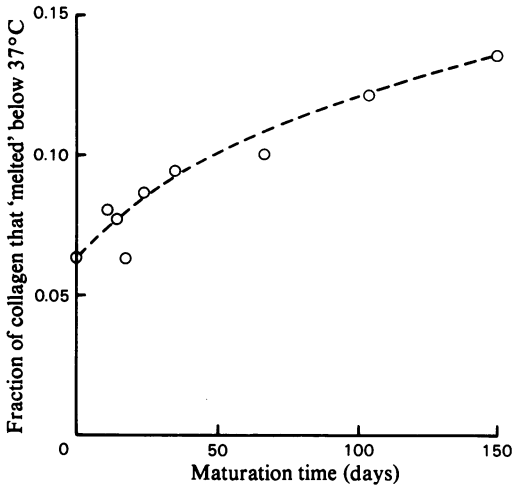

Fig. 3. *Fraction of pepsin-solubilized collagen that 'melted' below 37°C*

The fractions represent the transition absorption change at temperatures below 37°C relative to the total transition absorption change during thermal denaturation for the pepsin-soluble part of the reconstituted collagen fibrils that were incubated at 37°C in atmospheric air for 0–150 days. (The standard deviation of the difference of duplicate determinations is 0.0063.)

resulted in a pronounced transition between 34 and 36°C (Fig. 4). The fraction of soluble collagen from this experiment that 'melted' below 37°C was approximately 3-fold higher than the corresponding fraction of the collagen from which the fibrils were prepared. Therefore the change in the denaturation profiles that was observed after maturation of the collagen in fibrillar form represents a molecular destabilization of a fraction of the collagen during the maturation. The destabilization revealed as diminished molecular thermal stability of the matured collagen may either be a molecular change occurring during the maturation or reflect an alteration of the collagen that was induced by the subsequently performed peptic digestion. If this possible alteration is induced by the peptic digestion, then the collagen matured *in vitro* must be more prone for such an alteration, since the thermal stability of acid-soluble collagen was unaffected by the digestion procedures that were applied.

The electrophoresis of the pepsin-solubilized collagen fraction of the fibrils indicated that degradation of the collagen chains could not account for the changed denaturation pattern (Fig. 5ii).

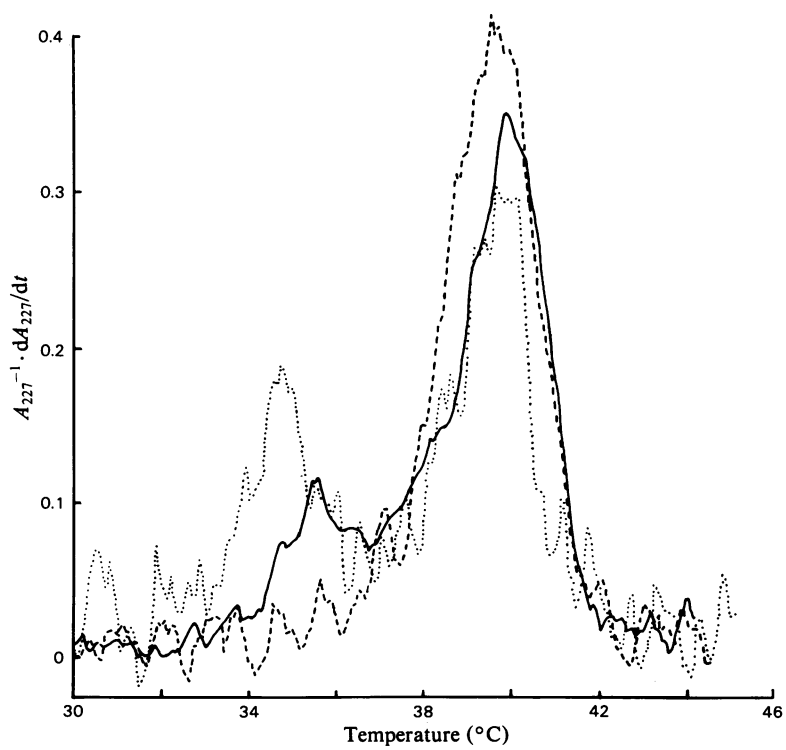

Fig. 4. *Denaturation profiles of soluble collagens isolated from reconstituted and native fibrils*

Pepsin-solubilized collagen from reconstituted collagen fibrils that were incubated at 37°C in atmospheric air for 85 days (——), pepsin-solubilized acetic acid-insoluble collagen from skin that was precipitated by 4M-NaCl and unadsorbed on the ion-exchanger during the DEAE-cellulose chromatography by the procedure of Bentz *et al.* (1978) (·····), and the stock preparation of acid-soluble collagen not subjected to heat aggregation (------).

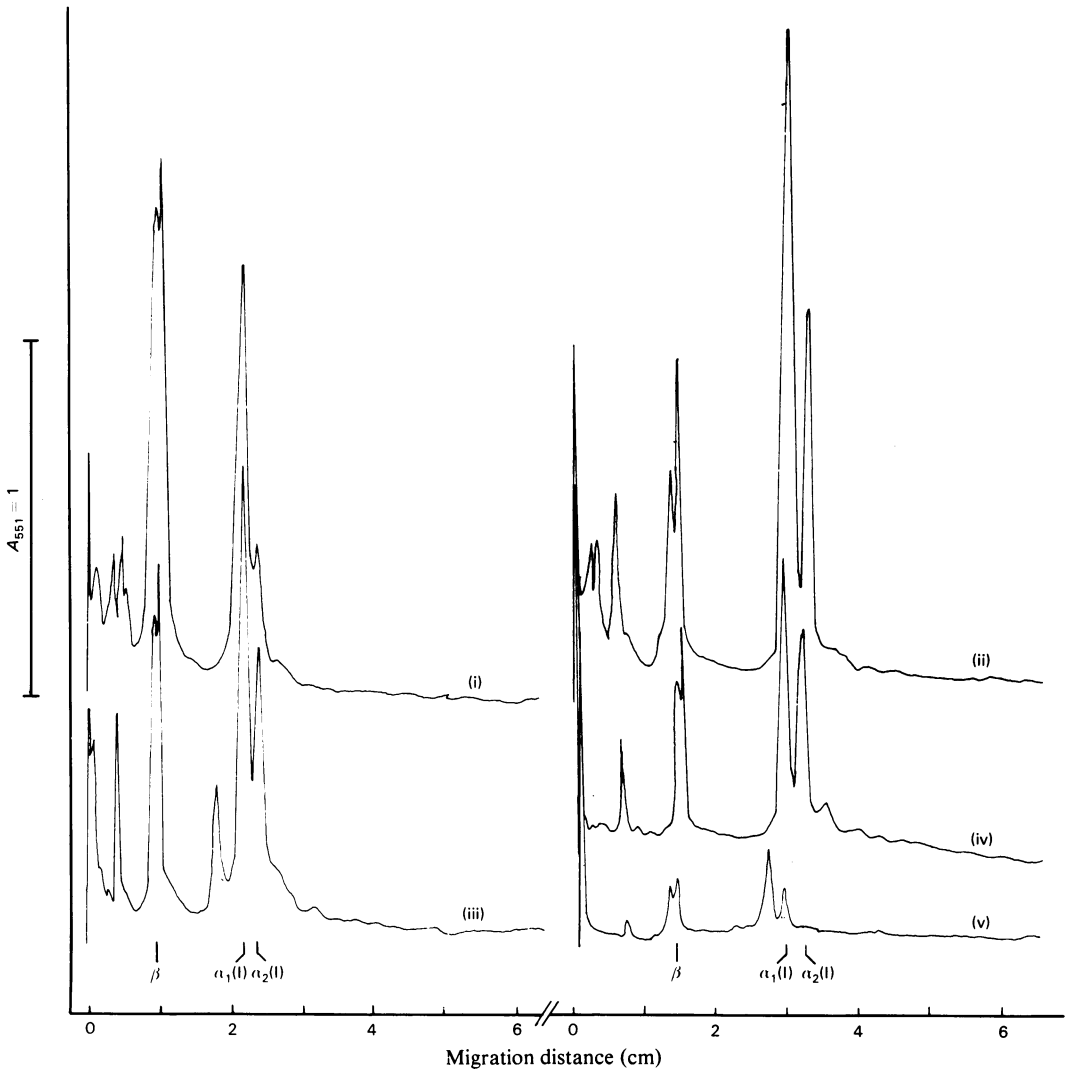

Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of soluble collagens isolated from reconstituted and native fibrils*

(i) Acetic acid-soluble collagen. (ii) Pepsin-solubilized collagen from reconstituted collagen fibrils incubated at 37°C in atmospheric air for 85 days. (iii)–(v) Pepsin-solubilized acetic acid-insoluble collagen from skin that was precipitated by 4M-NaCl (iii) and that on subjection to DEAE-cellulose chromatography by the procedure of Bentz *et al.* (1978) was separated in collagen unadsorbed (iv) and adsorbed (v) on the ion-exchanger.

The pepsin-solubilized acetic acid-insoluble skin collagen that was precipitated by 4M-NaCl during the sequential precipitation by 1.7M-, 2.5M- and 4M-NaCl constituted 7% of the solubilized collagen and contained in addition to type-I collagen an electrophoretic band with mobility similar to that of a type-V collagen chain (Brown & Weiss, 1979) (Fig. 5iii). The latter collagen type was adsorbed on the ion-exchanger during the DEAE-cellulose chromatography by the procedure of Bentz *et al.* (1978) (Fig. 5v). The denaturation profile of the resulting type-I collagen fraction (Fig. 5iv) was similar to that of the collagen isolated from the collagen fibrils matured *in vitro* (Fig. 4). This similarity suggests that a modification of collagen is occurring both *in vitro* and *in vivo*.

Attempts to isolate collagen with a biphasic thermal transition from skin gave the highest yield for peptic digests of the acetic acid-insoluble fraction. They have been unsuccessful for neutral-

salt extracts. This indicates that the changed denaturation pattern is associated with the least-soluble fractions of skin collagen. A diminished molecular stability of the acid-insoluble collagen constituting the oldest fraction of collagen in skin may have implications for the enzymic degradation of the collagen during the collagen turnover *in vivo*. However, the relationship between the changed denaturation pattern of the molecular collagen and the changing mechanical and thermal stability of collagenous tissues during aging remains to be elucidated.

The conformational or structural changes underlying the decreased thermal stability of a fraction of the collagen are unknown. As charged amino acid residues contribute to the molecular stability (Dick & Nordwig, 1966; Rauterberg & Kühn, 1968), a blockade or exposure of charged residues could affect the thermal stability of the collagen. The local relaxations of the triple-helical structure, as revealed by the proteolytic-probe technique (Ryhänen *et al.*, 1983) may also result in collagen molecules with locally confined defects in the triple-helical structure if the relaxations of the helix are not fully reversible.

This work was supported by grants from the Danish Medical Research Council (J. nos. 12-2227 and 12-3932).

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