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Difference in thermal stability of type-I and type-III collagen from rat skin

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Type-I and type-III collagens were obtained by differential salt fractionation of neutral-salt-soluble collagen from rat skin. Their thermal stabilities were determined by u.v. difference spectroscopy. The 'melting' temperature (T_m) in 5mM-acetic acid of type-III collagen was almost 2°C above that of type-I collagen. Intramolecular covalent cross-linking had no effect on the thermal stability.

Hydroxyproline is assumed to stabilize the triple-helical structure of collagen (for review, see Ramachandran & Ramakrishnan, 1976). This hypothesis is supported by the correlation found between hydroxyproline content and thermal stability for several different vertebrate collagens (Burjanadze, 1979). However, the hypothesis is challenged by results reported for type-III collagen, which contains approx. 20% more hydroxyproline residues than does type-I collagen (Byers *et al.*, 1974; Fujii & Kühn, 1975). No differences were found in the thermal stabilities of type-I and type-III collagen from rat skin (Byers *et al.*, 1974) and calf skin (Fujii & Kühn, 1975) and of type-I and type-III procollagen from cultured human fibroblasts (Peltonen *et al.*, 1980).

The 'melting' temperature (T_m) of collagen can be determined with high precision (S.D. of differences of duplicate determinations is 0.10°C) by a denaturation procedure monitored by u.v. difference spectroscopy (Danielsen, 1982). This procedure makes it possible to determine minor differences in thermal stability of different collagens. In the present work, in order to elucidate the hypothesis of the stabilizing effect of hydroxyproline on the molecular structure of collagen, the thermal stabilities of type-I and type-III collagens were investigated by this method.

Materials and methods

Preparation of collagen

Collagen was prepared from the dorsal skin of 60-day-old normal and lathyritic male Wistar rats. The lathyritic rats had received 1mg of β-aminoproprionitrile fumarate/g body wt. intraperitoneally each day for 3 weeks before being killed. Neutral-salt-soluble collagen was extracted by homogenization in 1M-NaCl/0.05M-sodium phosphate buffer, pH7.5, containing 20mM-EDTA and 10μM-phenylmethanesulphonyl fluoride, as described previously (Danielsen, 1981). The extracted collagen was precipitated with 4M-NaCl. The pellet was washed three times by resuspension in 4M-NaCl, after which the collagen was redissolved in 0.06M-sodium acetate buffer, pH4.8. The collagen was then reprecipitated by addition of 0.2vol. of 30% (w/v) NaCl, redissolved in 5mM-acetic acid, diluted 1:1 with 2M-NaCl/0.1M-Tris/HCl, pH7.4, and, after adjustment of pH to 7.4 with 1M-NaOH, was fractionated essentially by the method of Chung & Miller (1974) by sequential addition of NaCl to give 1.7M, 1.9M and 2.5M. The 1.7M-NaCl-precipitated fraction was dissolved in 1M-NaCl/0.05M-Tris/HCl, pH7.4, as described above, and precipitated once again with 1.7M-NaCl. Finally, the collagen fractions were dissolved in 5mM-acetic acid. Each redissolution of precipitated collagen was followed by centrifugation (50000*g*, 1h). All extraction and purification procedures were performed below 5°C.

Gel electrophoresis

Collagen was dissolved in a solution of 0.2% (w/v) sodium dodecyl sulphate and 2M-urea in 0.01M-sodium phosphate buffer, pH7.2, to which was added 10% (v/v) glycerol and 0.005% (w/v) Bromophenol Blue. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Furthmayr & Timpl, 1971) was carried out in 5%-acrylamide gels at 6mA/tube for 6h at room temperature with and without prior reduction by 2-mercaptoethanol. Gel staining with Coomassie Blue and destaining were performed by the method of Clark (1976).

Absorbance–temperature transitions

Determination of thermal stability and production of denaturation profiles were performed by

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the procedures previously described in detail (Danielsen, 1982). Briefly, the 'melting' of collagen was measured by recording the absorption difference at 227nm between the sample and the reference during gradual heating of the sample, the temperature gradient (0.24°C/min) being linear. Sample and reference contained the same solution of collagen (0.15–0.3mg/ml in 5mM-acetic acid); 12.5 data points/°C were recorded. The 'melting' temperature (T_m) was calculated as the temperature for 50% of the transition absorption change. Denaturation profiles (the first derivative of the absorbance versus temperature) were smoothed by fitting the first derivative of orthogonal polynomials to a segment of 15 data points around each point.

Results and discussion

After reduction with 2-mercaptoethanol, the higher-molecular-weight components of the collagen fraction precipitated by 1.7M-NaCl were mainly converted into a component with mobility similar to that of α_1(I) chains (Figs. 1*a* and 1*b*), indicating the presence of type-III collagen in this fraction (Byers *et al.*, 1974; Fujii & Kühn, 1975). The colour scan of the type-I collagen (2.5M-NaCl-precipitated fraction) is shown in Fig. 1(*c*), and was unchanged upon reduction (results not shown).

Though positioned at a higher temperature, the denaturation profile of the 1.7M-NaCl-precipitated type-III fraction has the same symmetrical appearance as that of the 2.5M-NaCl-precipitated type-I fraction (Fig. 2). This indicates a fairly homogeneous type-III preparation, as also indicated by the electrophoresis.

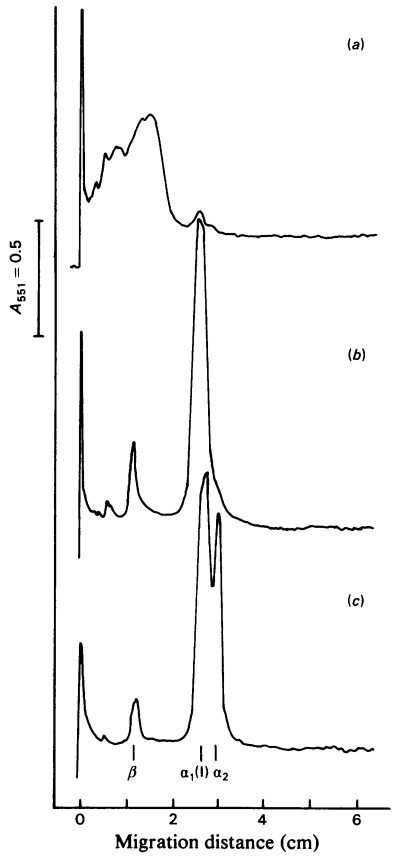

Fig. 1. *Gel electrophoresis of fractionated collagens from lathyritic rat skin*
Scans of Coomassie-Blue-stained gels for the 1.7M-NaCl-precipitated type-III fraction (*a*) before and (*b*) after reduction by 5% (w/v) mercaptoethanol and (*c*) for the 2.5M-NaCl-precipitated type-I fraction.

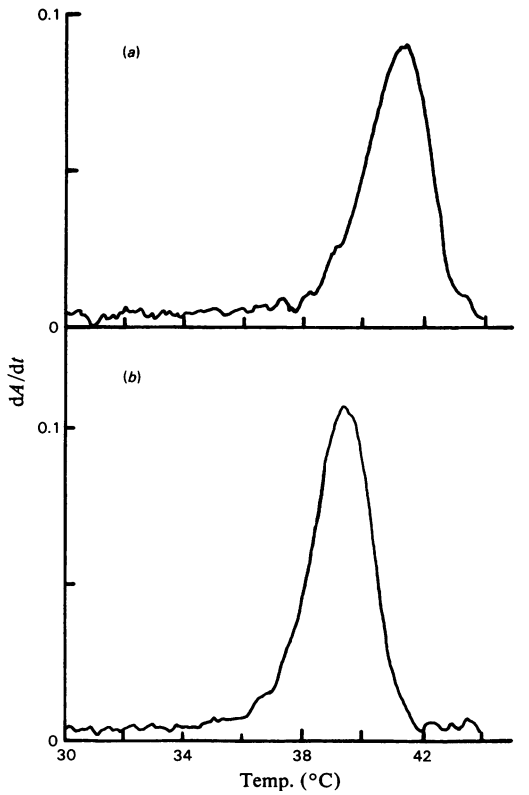

Fig. 2. *Denaturation profiles of fractionated collagens*
The smoothed values of the first derivative of absorbance (dA/dt) are plotted versus temperature (*a*) for the 1.7M-NaCl-precipitated fraction (type-III collagen) and (*b*) for the 2.5M-NaCl-precipitated fraction (type-I collagen) that were obtained from lathyritic rat skin.

The 'melting' temperature (T_m) of type-III collagen was almost 2°C above that of type-I collagen (Table 1), in contrast with the results reported by others for type-I and type-III collagen (Byers *et al.*, 1974; Fujii & Kühn, 1975) and procollagen (Peltonen *et al.*, 1980). It is possible that the different purification and isolation procedures used in the present study and in the previous reports can explain the conflicting results. The collagens used in the present denaturation experiments were prepared and isolated under mild conditions in the cold. Compared with type-I collagen, type-III collagen is reported to be more susceptible to the purification and isolation procedures applied (Burke *et al.*, 1977; Herrmann *et al.*, 1980). It remains, however, to be established that this would influence thermal stability.

Intramolecular covalent cross-linking in collagen is provided by the aldol condensation product (Bailey & Robins, 1976) and by disulphide bonds (type-III collagen) (Chung & Miller, 1974). Variations in covalent cross-linking were found not to affect thermal stability of collagens in the present study. Firstly, the thermal stability of collagen from lathyritic rats was not different from the stability of collagen from normal rats (Table 1). As far as is known, the only significant effect of β-aminopropionitrile is an inhibition of lysyl oxidase (Bornstein & Traub, 1979), resulting in a decrease in the polymeric collagen components (i.e. in the interchain linkages), as shown in Table 1. Secondly, the T_m of this type-III collagen from lathyritic rats was unchanged after 70h incubation at room temperature in the presence of 1mM-mercaptoethanol. The mercaptoethanol was retained during the subsequent denaturation experiment. Electrophoresis showed that mercaptoethanol (1mM) reduced the disulphide bonds of type-III collagen significantly. Thus, neither the aldol condensation product nor the disulphide intramolecular bonds affect the thermal stability of the type-I and type-III collagens.

Table 1. *'Melting' temperature (T_m) and proportions of monomer (α-chains) and polymer (β- and γ-chains) components of normal and lathyritic collagens*

The colour-scan areas for the different peaks of the 1.7M-NaCl-precipitated type-III fractions were evaluated after reduction by 5% mercaptoethanol. The T_m and area values are means of duplicate determinations.

Collagen	T_m (°C)	Colour-scan area (%) α	Colour-scan area (%) β+γ
Type I, normal	39.1	68	32
Type III, normal	41.0	69	31
Type I, lathyritic	39.2	93	7
Type III, lathyritic	41.0	84	16

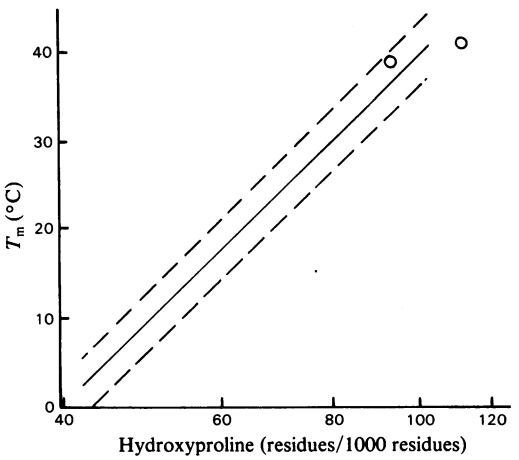

Fig. 3. *'Melting' temperature (T_m) versus hydroxyproline content for interstitial collagens of vertebrates*

The solid line represents the best fit of T_m on log(hydroxyproline content) and the dashed lines indicate the range of the experimental points (based on data compiled by Burjanadze, 1979). The T_m values for type-I and type-III collagen found in the present study are plotted against the hydroxyproline contents [from Burjanadze (1979) and Byers *et al.* (1974) respectively] (○).

As indicated in Fig. 3, the present results are consistent with the hypothesis that the thermal stability of collagens is related to the hydroxyproline content.

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