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Comparative Analysis of the Structure and Thermal Stability of Sea Urchin Peristome and Rat Tail Tendon Collagen

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Abstract We have purified collagen from two distinct sources; the vertebrate, rat tail tendon and an invertebrate, sea urchin adult tissue, the peristome. The collagenous nature of the purification products was confirmed by amino acid compositional analysis. Both preparations had high contents of glycine and proline residues and hydroxyproline was also present. The total pyrrolidine (proline + hydroxyproline) content decreased from 17.9 mole % in rat tail collagen to 12.9 mole % in peristome collagen. Distinctly different circular dichroic spectra were measured for these collagens. Analyses of spectra, measured as a function of temperature, revealed distinct thermal denaturation profiles. The melting temperature for rat tail collagen was 38.5°C, while the corresponding value for peristome collagen was significantly lower at 27°C. A similar thermal denaturation profile was obtained for rat tail collagen in digestion experiments using a 41-kDa gelatinase activity, isolated from sea urchin eggs. These results identify structural differences between a typical, vertebrate type I fibrillar collagen and an echinoderm collagen which serves as a constituent of a mutable connective tissue. These differences may relate to the functional roles played by collagen in these distinctly different tissues. J. Cell. Biochem. 84: 567–574, 2002.

Key words: collagen; thermal stability; mutable; comparative

The importance of the extracellular matrix (ECM) is now widely recognized. While the classical view of the ECM is that of a "cement" responsible for the structural integrity of cells, tissues, and organs, we now recognize that many important cellular properties are dependent upon the ECM. Cell proliferation, migration, and differentiation are all cellular properties influenced by the ECM [Adams and Watt, 1993]. The functional role played by the ECM is dependent upon composition, and collagen has long been recognized as a major constituent protein of the ECM. Indeed, collagen is now known to constitute as much as 25% of the total protein in multicellular animals [Hay, 1991]. Extensive research on vertebrate collagen molecules has led to the identification of fibrillar, non fibrillar, and fibrillar-associated forms. The fibrillar and nonfibrillar forms can be distinguished by the presence of continuous or discontinuous gly–Xaa–Yaa repeats in the triple helical region and the presence of class-specific, conserved flanking regions. These distinct structural features are conserved in nature and this has facilitated the identification of collagen molecules in invertebrates.

The sea urchin embryo has been used both as a paradigm for embryonic development and to investigate the evolution of metazoans. This embryo is characterized by the presence of two extracellular matrices: the hyaline layer on the apical surface and the basal lamina on the basal surface of ectoderm cells. Antisera, prepared against vertebrate ECM components, cross-react with species in both the hyaline layer and basal lamina, demonstrating a commonality in composition between the echinoderm and vertebrate extracellular matrices [Wessel et al., 1984]. In addition, collagen deposition has been

Abbreviations used: PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium docecyl sulfate–polyarylamide gel electrophoresis.

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shown to be essential for gastrulation [Wessel and McClay, 1987; Wessel et al., 1989]. Adult sea urchin tissues also contain collagen and the mutable connective tissues are of particular interest. These tissues, the spine ligament, peristome, and tube foot are mutable connective tissues, which can rapidly change their tensile properties [Motokawa, 1984]. In contrast, vertebrate connective tissues are more static and change occurs over the course of months or years. The molecular basis for mutability is presently unknown and may be related to structural features of individual constituent molecules and/or the organization of these molecules within the mutable tissues [Del Costillo and Smith, 1996; Wilkie, 1996]. Understanding the mechanism, which facilitates rapid changes in tensile properties, will require a detailed biochemical characterization of individual constituent molecules.

In the study reported here, we have used circular dichroism to identify structural differences between a type I vertebrate collagen and the collagen isolated from a mutable connective tissue, the peristome. We chose these molecules because they are separated by a considerable phylogenetic distance, function in organisms that live at quite different temperatures and are derived from static (rat tendon) and mutable (sea urchin peristome) tissues. Characterizing collagen molecules from divergent sources is likely to provide insights into molecular characteristics, which must be preserved in order to ensure functional capabilities. In addition, such comparative studies may enable us to probe the mechanistic differences between collagen function in mutable and non-mutable tissues.

MATERIALS AND METHODS

Preparation of Collagen

Sea urchins (*Strongylocentrotus purpuratus*) were purchased from Seacology, Vancouver, Canada. Peristomes were dissected from animals, the epidermis removed by scraping and the tissue cut into small pieces. Rat tails were a gift from Dr. Sean Brosnan (Department of Biochemistry, Memorial University). The tail tendons were removed by dissection and cut into small pieces. Collagen was extracted as described by Burke et al. [1989]. Tissue pieces were homogenized and the homogenate extracted with distilled water for 16 h at 4°C. The pellet was harvested by centrifugation at 10,000*g* for 15 min at 4°C and extracted twice more with distilled water. The final pellet was suspended in 20 ml of 0.5 M acetic acid containing 0.5 mg pepsin (Worthington Biochemical Co.) and digestion allowed to proceed for 6.5 h at 4°C. The pH of the digestion mixture was adjusted to 7.6 with NaOH and insoluble material removed by centrifugation at 15,000*g* for 1 h at 4°C. The supernatant was made 5 M in NaCl and rotated overnight at 4°C. Protease inhibitors, benzamidine hydrochloride, PMSF, and EDTA were present throughout the procedure at concentrations of 20 mM, 50 μg/ml, and 5 mM, respectively. After salting-out with 5M NaCl, collagen was stored in distilled water containing protease inhibitors at the final concentrations indicated above. Protein concentrations were determined using bovine serum albumin as a standard [Lowry et al., 1951].

Amino Acid Analysis

Amino acid compositions were determined following hydrolysis of the collagen preparations for 72 h in 6N HCl at 110°C. Liberated amino acids were fractionated in a Beckman 121 amino acid analyser. Tryptophan content was not determined.

Circular Dichroism Analysis

Aliquots of purified collagen were incubated for 30 min at the indicated temperatures. Incubations were performed in a 0.1 cm pathlength cell and were followed by the collection of 32 spectra. Circular dichroic spectra were collected in the far-ultraviolet region between 210 and 260 nm at the incubation temperature. Spectra were recorded in a model J500-A JASCO spectropolarimeter and processed with a DP-500N data processor.

Digestion of Rat Tail Collagen

Aliquots of rat tail collagen were pre-incubated at the indicated temperatures for 30 min followed by incubation for various times at 15°C in the presence of the 41-kDa collagenase/gelatinase. The digestion reactions were terminated by the addition of an equal volume of ice-cold 20% (w/v) trichloroacetic acid. The digestion products were fractionated in a 8% (w/v) polyacrylamide gel in the presence of SDS [Laemmli, 1970], stained with Coomassie Brilliant Blue R250 and destained. The stained 126-kDa bands were excised and the dye eluted overnight at room temperature in 25% (v/v)

pyridine and the eluted dye quantitated by optical density measurements at 605 nm. Background binding of the stain was determined using gel slices, which did not contain protein, and the appropriate corrections were made.

RESULTS

Using a standard peptic digestion protocol, we have prepared collagen from the adult sea urchin peristome and the rat tail tendon. The purification products were analysed by SDS–PAGE (Fig. 1). Peristome collagen consisted of two species of apparent mol masses 140- and 116-kDa (lane 1), while the rat tail collagen consisted of polypeptide species of apparent mol masses 126- and 123-kDa (lane 2). The high mol mass spices seen in lane 2 are most likely covalently cross-linked forms of the α chains (β forms). When rat tail collagen is heat denatured at 60°C and incubated with a gelatinase, these higher mol mass species disappear as do the α chains at 126- and 123-kDa. Using both densitometric gel analyses and the elution of Coomassie Blue followed by optical density measurements at 605 nm [Fenner et al., 1975], we have determined the relative staining intensities of the peristome collagen polypeptides. The results suggest that the 116- and 140-kDa species are present in 2:1 molar ratio similar to the α_1 and α_2 chains of type I, fibrillar rat tail collagen. The collagenous nature of these preparations was confirmed by amino acid compositional analysis. Both preparations contained amino acid residues diagnostic of collagen. In both preparations, approximately one-third of the residues were glycine and the proline content was high at 11.1 mole % for the rat tail preparation and 8.1 mole % for the peristome preparation (Table I). As expected

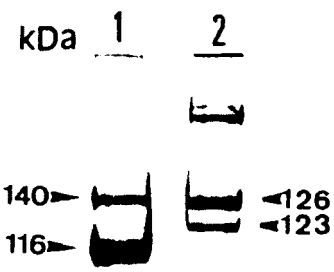

Fig. 1. Electrophoretic analysis of peristome and rat tail tendon collagens. Aliquots (5 μg) of peristome (**lane 1**) or rat tail (**lane 2**) collagen were fractionated in an 8% (w/v) polyarylamide gel in the presence of sodium docecyl sulfate (Laemmli, 1970). The gel was stained with Coomassie Brilliant Blue R250 and destained.

TABLE I. Comparison Between the Amino Acid Compositions of Rat Tail Tendon and Sea Urchin Peristome Collagens

Amino Acid	Rat tail	Peristome
Asx	57.5	81.0
Thr	26.7	47.0
Ser	46.8	76.6
Glx	75.5	96.3
Pro	111.0	81.3
Hy-Pro	68.5	48.5
Gly	309.0	280.6
Ala	104.2	68.3
Val	32.7	36.2
Cys	1.1	1.6
Met	5.8	8.7
Ile	21.7	29.5
Leu	36.1	45.1
Tyr	9.4	16.6
Phen	17.9	15.6
Lys	25.5	9.3
Hy-Lys	6.2	3.8
His	5.3	5.9
Arg	46.9	47.0

Values are presented as amino acid residues/1,000 residues.

both preparations contained hydroxyproline and hydroxylysine. The hydroxyproline content of the peristome preparation was lower than that of the rat tail preparation and this difference was also reflected in the total pyrrolidine contents of these preparations. This result is particularly interesting, since pyrrolidine residues have been implicated as key structural elements in determining the stability of triple helical collagen.

To further explore structural differences between the two collagens, we measured the far ultra-violet circular dichroism spectra of these species. The spectra were strikingly different (Fig. 2). The spectrum for rat tail collagen had a peak of positive ellipticity, maximum at 232 nm, reflective of a collagen-type structure. In contrast, the spectrum for peristome collagen was characterized by a broad peak of negative ellipticity with a maximum at 235 nm. In addition, two small positive peaks of ellipticity were recorded at 227 and 223 nm. This latter feature correlates with a collagen-type structure. We have expanded on our analysis of the structures of rat tail and peristome collagens by measuring circular dichroism spectra as a function of temperature. *Strongylocentrotus purpuratus* grows at a temperature of 12–15°C, while a corresponding physiological temperature for the rat tail would be close to 37°C. Increasing the temperature from 22°C to 37°C, resulted in both an increase in the magnitude of the molar ellipticity and a shift in the

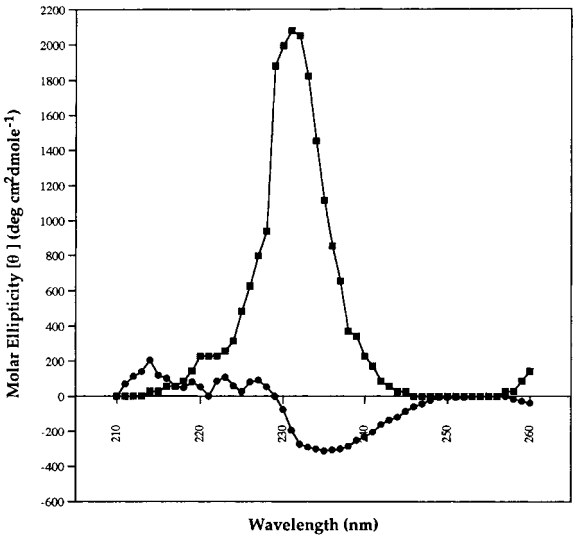

Fig. 2. Circular dichroism analysis of peristome and rat tail tendon collagens. Aliquots of peristome (-●-; 0.43 mg/ml) or rat tail (-■-; 0.4 mg/ml) collagen were incubated at 15°C and 22°C, respectively for 30 min. Following incubation, 32 spectra were collected at the incubation temperature using a J500-A JASCO spectropalorimeter with an attached DP-500N data processor.

wavelength of the maximum value for rat tail collagen (Fig. 3A.) The wavelength of the maximum value shifted from 232 nm at 22°C to 227 nm at 37°C. Increasing the temperature from 37°C to 40°C resulted in a dramatic change in the recorded spectrum. At 40°C, the positive ellipticity was replaced by negative values between 245 and 222 nm without any change in the wavelength at maximum value. This dramatic change in spectrum reflects a shift in conformation from collagen to gelatin. A further increase in negative ellipticity occurred as the temperature increased from 40°C to 60°C. When the circular dichroism data was analysed by plotting ellipticity values at 232 nm vs. temperature, a sharp transition occurred between 37°C and 40°C (Fig. 3B). The melting temperature for rat tail collagen was 38.5°C. When the peristome collagen data were analysed, a similar change in circular dichroism spectra was also seen, but the change occurred at a lower temperature than that seen with rat tail collagen (Fig. 4A). The negative ellipticity increased substantially as the temperature was raised from 25°C to 30°C. The wavelength of the maximum value shifted from 236 nm at 15°C to 230 nm at 60°C. When the molar ellipticity values at 232 nm vs. temperature were plotted, a dramatic decrease was seen between 25 and 30°C reflecting the transition from a collagen-

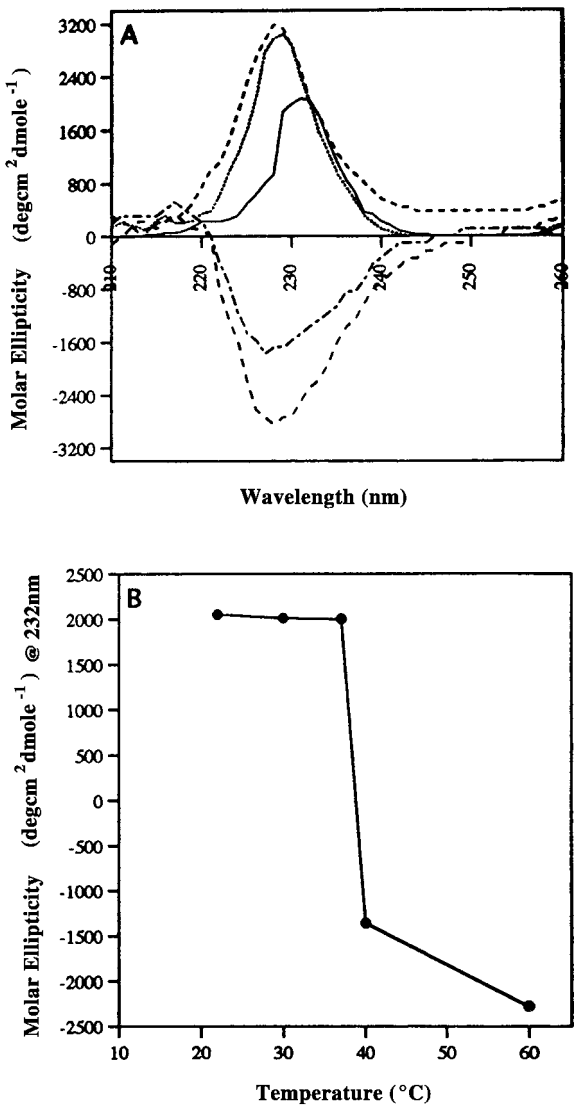

Fig. 3. Circular dichroism spectra of rat tail tendon collagen measured as a function of temperature (**A**) and analysis of the change in molar ellipticity at 232 nm with temperature (**B**). A: Aliquots of rat tail collagen were incubated at 22°C [-; 0.4 mg/ml], 30°C [●●●; 0.33 mg/ml], 37°C [----; 0.18 mg/ml], 40°C [-●-●; 0.11 mg/ml], or 60°C [----; 0.10 mg/ml] for 30 min followed by the collection of 32 spectra between 210 and 260 nm at the incubation temperature. B: Molar ellipticity at 232 nm were plotted as a function of temperature.

type structure to gelatin (Fig. 4B). The melting temperature was determined to be 27°C, which was 11.5°C lower than that recorded for rat tail collagen. Collectively, these results point to structural differences between vertebrate, rat tail tendon collagen, and invertebrate peristome collagen.

We have attempted to confirm the thermal denaturation profile for rat tail collagen in a

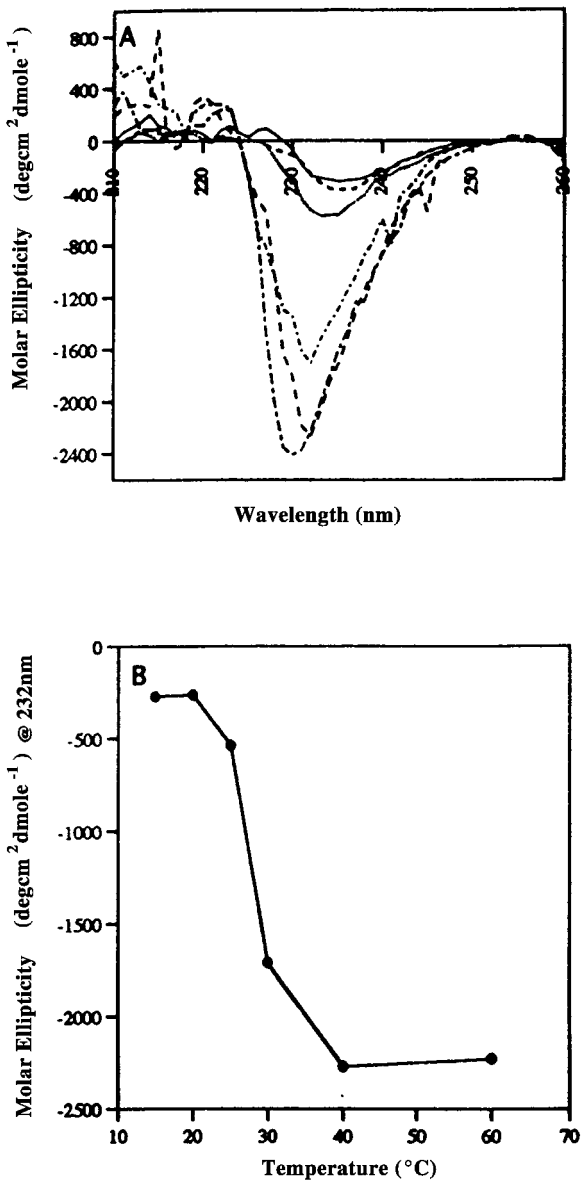

Fig. 4. Circular dichroism spectra of sea urchin, peristome collagen measured as a function of temperature (**A**) and analysis of the change in molar ellipticity at 232 nm with temperature (**B**). A: Aliquots of peristome collagen were incubated at 15°C [-; 0.43 mg/ml], 20°C [– – –; 0.40 mg/ml], 25°C [....; 0.40 mg/ml], 30°C [- · · · -; 0.21 mg/ml], 40°C [- - -; 0.20 mg/ml], or 60°C [-.-.-. 0.20 mg/ml] for 30 min followed by the collection of 32 spectra, between 210 and 260 nm, at the incubation temperature. B: Molar ellipticities at 232 nm were plotted as a function of temperature.

second assay. We have previously purified a 41 kDa collagen and gelatin cleavage activity in the sea urchin egg [Mayne and Robinson, 1996]. This species was shown to cleave both collagen and gelatin from the sea urchin, while rat tail collagen was largely refractory to cleavage. However, rat tail gelatin was cleaved by this species [Robinson, 1997]. We have, therefore, pre-incubated rat tail collagen at different temperatures followed by incubation at 15°C in the presence of the 41 kDa species for various times. To monitor the collagen to gelatin transition in rat tail collagen, we quantitated cleavage of the 126 kDa polypeptide as a function of preincubation temperature. As the pre-incubation temperature was increased from 15°C to 60°C, there was a dramatic increase in both the rate of cleavage and the percentage of the 126 kDa polypeptide cleaved (Fig. 5). A substantial increase in both the rate of cleavage and the percentage of the 126 kDa polypeptide cleaved, occurred between 30°C and 35°C. These increases reflect the shift from a native collagen to a more gelatin-type structure, which would be susceptible to digestion. At a preincubation temperature of 60°C, almost 90% of the 126 kDa polypeptide was cleaved. These results parallel those obtained by circular dichroism analysis of rat tail collagen.

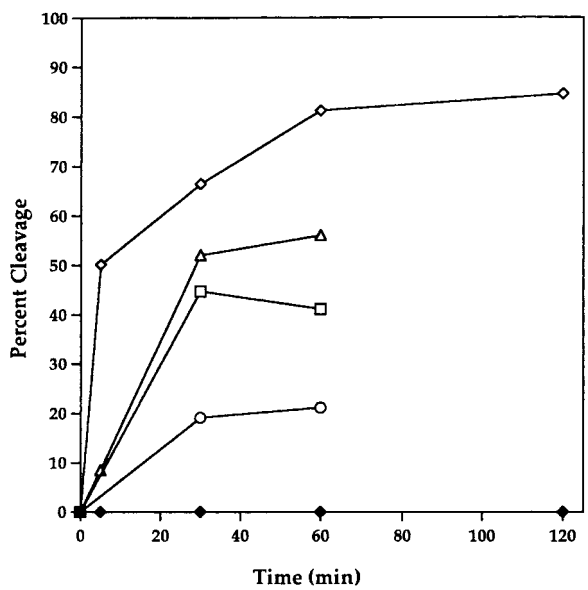

Fig. 5. Quantification of the cleavage of rat tail collagen as a function of temperature. Aliquots (5 μg) of rat tail collagen in 50 mM Tris, pH 8.0, were preincubated at 15°C [-◆-], 30°C [-○-], 35°C [-□-], 44°C [-△-], or 60°C [-◇-] for 30 min. Following pre-incubation, $CaCl_2$ was added to 10 mM, and 1 unit of the 41-kDa collagenase/gelatinase was added followed by incubation at 15°C for the indicated time periods. Digestion of the 126 kDa polypeptide was quantified as indicated in Materials and Methods. Zero percent cleavage was determined at each pre-incubation temperature by processing an aliquot of collagen in the absence of added 41-kDa collagenase/gelatinase.

DISCUSSION

Collagens are now recognized as ubiquitous structural components of the ECM. These proteins can engage in self-association reactions, which generate linear fibrillar or more dispersed mesh-like structures. In addition, collagen molecules possess domains that facilitate interactions with glycoprotein and proteoglycan components of the ECM. Collagen molecules are characterized by the presence of Gly–Xaa–Yaa repeats, which may be continuous (fibrillar collagen) or discontinuous (non-fibrillar collagen). The high contents of both glycine and proline facilitates the formation of a unique and triple helical structure, which is stabilized by interstrand hydrogen bonding mediated by hydroxyproline residues. The unique amino acid composition and structural features of vertebrate collagen have facilitated the identification and isolation of collagen from invertebrate sources [Adams, 1978]. In the sea urchin, both embryonic and adult tissues have been shown to contain collagen. A cDNA clone, isolated from a gastrula-stage sea urchin library, encodes a polypeptide homologous to the $\alpha 2$ chain of vertebrate type I collagen [Exposito et al., 1992], while a type IV gene is expressed in sea urchin primary mesenchyme cells [Venkatesan et al., 1986; Exposito et al., 1993, 1994]. In addition, fibrillar-type collagen has been identified in adult, sea urchin tissues [Burke et al., 1989; Tomita et al., 1994].

Echinoderms appear to be unique in possessing rapidly mutable connective tissues [Motokawa, 1984]. Tube foot, spine ligament, and peristome are all adult tissues, which can rapidly adjust their mechanical properties. While these tissues appear to be neurally regulated, there is no clear understanding of the mechanism responsible for their mutability, although changes in the mechanical properties of collagen fibers are likely involved [Del Costillo and Smith, 1996; Wilkie, 1996]. We and others have been engaged in a characterization of collagen molecules isolated from mutable sea urchin tissues [Pucci-Minafra et al., 1978; Burke et al., 1989; Trotter and Koob, 1994; Robinson, 1997]. Measurements of the mean periodicity in native fibers of tube foot and peristome revealed values of 55.1 and 44.4 nm, respectively [Burke et al., 1989]. These values differ from the 67 nm periodicity detected in vertebrate, type I fibrillar collagen. In contrast, measurements of the axial periodicity of acid soluble, reconstituted collagen fibers from Aristotle's Lanternae of the sea urchin, *Paracentrotus lividus*, revealed a value of 64 nm [Pucci-Minafra et al., 1978]. These results suggest that the packaging of collagen into fibers in mutable tissues may differ from one tissue to another. In addition, differences in amino acid composition between sea urchin spine ligament and vertebrate type I collagens were also evident, when the former collagen was compared with collagens from mutable tissues in the star fish and sea cucumber [Trotter and Koob, 1994]. Furthermore, limited biochemical analysis of collagens, isolated from mutable sea urchin tissues, have revealed both similarities and differences between these collagens and vertebrate, type I collagen [Trotter and Koob, 1994; Robinson, 1997]. Collectively, these results indicate that both biochemical and structural differences exist between collagens from different mutable connective tissues as well as between these collagens and vertebrate type I collagen.

In the study reported here, we have extended previous work and analyzed the structure and thermal stability of peristome collagen. The collagenous nature of the isolated protein was confirmed by both amino acid compositional analysis (Table I), and its cleavage by a 41-kDa sea urchin collagenase [Robinson, 1997]. The circular dichroism analyses, reported here, were designed to compare the secondary structural features of peristome collagen to those of type I rat tail tendon collagen and determine their melting temperatures. The collagens used in this study were released from their native fibers by pepsin digestion, a treatment expected to maintain the triple helical structure. The spectrum of rat tail tendon collagen, measured at 22°C, was positive with a peak at 232 nm, while the peristome collagen spectrum measured at 15°C, was largely negative with a peak at 235 nm (Fig. 2). As the temperature was raised from 22°C to 37°C, the peak of maximum elliplicity for rat tail collagen shifted from 232 to 228 nm (Fig. 3A) close to the value of 221 nm reported for vertebrate, triple helical type I collagen [Hayashi et al., 1979]. The distinctly different spectra recorded for rat tail and peristome collagens suggest differing secondary structural features. These spectra were reproducibility recorded at 15°C and 22°C suggesting that they reflect the correct native state of these

collagens. Furthermore, the significant changes to these spectra induced by increasing temperatures also support the conclusion that they reflect the native conformations of these proteins. In both cases, the shift towards large negative elliplicity values with increasing temperature suggests denaturation of the proteins.

The differing secondary structural features of peristome and rat tail collagen was also reflected in their thermal denaturation profiles. Collagen to gelatin transition temperatures have been reported for chick embryo tendon collagen (Brown et al., 1972), bovine type I collagen [Shimizu et al., 1990], lathyritic rat skin collagen [Brodsky-Doyle et al., 1976], rat tail tendon collagen [Gross, 1964], and type I collagen from chick embryo [Hayashi et al., 1979]. In all these studies, melting temperatures between 37°C and 42°C were reported. In addition, a considerably lower melting temperature of 23.1°C has been reported for collagen isolated from a non-mutable sea urchin tissue, the test [Shimizu et al., 1990]. Our measurement of the melting temperature for rat tail collagen gave a value of 38.5°C (Fig. 3B), which is in good agreement with values published for collagens from similar sources. The mutable peristome tissue of the sea urchin, *Strongylocentrotus purpuratus*, is exposed to sea water at temperatures of 12–15°C and peristome collagen had a melting temperature of 27.0°C. In addition to secondary structural differences, rat tail and peristome collagens differ in their contents of pyrrolidine residues. Rat tail collagen contains a total of 17.9 mole % proline plus hydroxyproline, while peristome collagen contains 12.9 mole % pyrrolidine residues (Table I). Total pyrrolidine residue content may play a role in determining the thermal stability of collagen [Josse and Harrington, 1964]. However, no quantitative relationship has been defined, and collagens with small differences in pyrrolidine residue content may show large differences in thermal stability. For example, sea urchin test collagen has a total pyrrolidine residue content of 20.4 mole %, while bovine type I collagen has a total content of 21.9 mole %. Despite this small difference, the melting temperatures for these collagens differ by 16.7°C (23.1°C and 39.8°C for sea urchin test and bovine collagens, respectively; Shimizu et al., 1990). In addition, the sea urchin peristome and test collagens differ significantly in total pyrrolidine content (20.4 and 12.9 mole %, respectively), while their melting temperatures differ only by 3.9° C. It seems likely that total pyrrolidine content alone cannot account for the observed differences in the thermal stabilities of collagens. The distinctly different melting temperatures measured for rat tail and peristome collagens are complex properties of these molecules, which most likely depend upon a number of factors including primary and secondary structure. The differences in thermal stability are reflective of the ambient temperatures at which the sea urchin and the rat live. In addition, differences in thermal stability may also be related to phylogenetic distances between vertebrates and invertebrates as well as the mutability of some sea urchin tissues. In terms of mutability, it is worth noting that Shimizu et al. [1990] have reported a melting temperature of 23.1°C for collagen isolated from the non-mutable test of the sea urchin *Asthenosoma ijimai*. Furthermore, we have determined the melting temperature for collagen isolated from the test of the sea urchin *Strongylocentrotus purpuratus*. A value of 24.0° C was obtained [Eckenweber and Robinson, unpublished data]. These results demonstrate that collagen molecules, possessing a low melting temperature, are not a unique feature of mutable tissues.

Rapid changes in the mechanical properties of a connective tissue could be facilitated by changes in interactions between constituent molecules occurring on a short time scale. Intuitively, the relatively low temperatures required to disrupt interchain interactions in collagen molecules from mutable tissues could help to facilitate the rapid changes in intermolecular interactions required for mutability. However, it is clear that low melting temperature alone is not sufficient to explain the mutable nature of some echinoderm tissues. Mutability may also depend upon rapid changes in interactions between collagen and other components of the tissue. A detailed definition of both the composition of mutable tissues and intermolecular interactions, occurring within these tissues, will be required before a detailed understanding of the mechanism of mutability emerges.

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