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EFFECTS OF THERMAL DENATURATION ON METAL BINDING AND ULTRASTRUCTURE IN COLLAGEN FIBRILS

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SUMMARY

A study was made of the effect of thermal shrinkage on intact human Achilles tendon with regard to (a) cation binding selectivity and (b) fibril ultrastructure (electron microscopy). The results indicated that the dependence of metal binding on treatment temperature was not a simple one and that at least two types of binding sites are present.

A general increase in binding strength and loss of selectivity by heating to 70 °C in water, but not higher, suggested the existence of a temperature-dependent activated state of the matrix. From a simple model of this activation, an upper limit to the activation energy was estimated to be about 23 cal/g dry tendon.

Electron micrographs showed that at about 60 °C shrinkage begins at localized regions along the fibrils and at their ends. At higher temperatures this "melting" spreads to the entire fibril, leaving it expanded, with only traces of cross-banding.

INTRODUCTION

In a recent study of the binding of many metal ions to tendon collagen[1], we reported apparent correlations between binding strength and both ionic size and ionization potential. In particular, it was noted that tendon collagen preferentially bound ions of approximately 0.6 and 1.2 Å in ionic radius, and that the log of bound ion concentration was proportional to the ionization potential of the metal ion. It remains to be determined how these features relate to the biophysical state of the native collagen matrix. They may be, for example, merely a reflection of the amino acid composition of the tissue.

We report here a combined metal binding and electron microscopic study of tendon collagen which has been hydrothermally denatured at different temperatures from 40 to 90 °C. Using what is known about collagen shrinkage it was hoped that such a study would indicate the way in which the metal binding depends upon the

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"crystalline state" of the matrix. Conversely, both the binding data and the electron micrographs should contain new information about the incompletely understood process of shrinkage in native collagen. This process is characterized by melting of the hydrated crystalline matrix by heat, resulting in a randomly coiled structure, contracted along the fibril direction and expanded perpendicular to it[2]. It is caused most likely by the breaking of intramolecular hydrogen bonds and labile intermolecular covalent bonds[3,4]. Thermodynamically, the shrinkage behavior of matrix collagen can be fairly well accounted for by either the theory of first order reaction rates[5,6] or by crystalline polymer melting theory (*i.e.* as a phase transition)[7]. In any case, theory and experiment seem to agree best only for derived systems involving extensive sample pre-treatments and/or unphysiological solvent environments.

There seems to have been little in the literature on the effect of the shrinkage process on metal binding in collagen. Gustavson[2] noted an increase in chromium binding by hide collagen after heat shrinkage, especially when non-ionic compounds are used. Only one electron microscopic study was located, that of Nutting and Boraski[8], which describes the appearance of heat-denatured cowhide collagen fibrils. The present study shows that the dependence of metal binding on treatment temperature is not a simple one and that at least two types of binding site are present. Electron micrographs of heat-treated tendon collagen show that shrinkage begins at localized areas along the fibril, the ultrastructural manifestations being very similar to that in the gross tissue. Treatment with formaldehyde indicated that this is most likely not an artifact of dehydration in the preparation.

MATERIALS AND METHODS

Denaturation

Samples of normal human Achilles tendon were cut into 5-mm pieces and thoroughly hydrated in distilled deionized water. Aliquots were transferred to constant temperature baths (distilled deionized water) of 40, 50, 60, 70, 80 and 90 °C for 1 h and then removed to room temperature baths for 10 min. The last three aliquots showed marked shrinkage to the eye.

Cation binding

A portion of each temperature aliquot was then stirred in a solution containing the following metal ions as nitrates in the same concentration (0.00423 M): Li^+, Na^+, K^+, Rb^+, Cs^+, Ag^+, Be^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, and Pb^{2+}. The total ionic strength of this solution was 0.16 M and the pH adjusted to 5.4 with NaOH. After 24 h exposure to the ionic solution, the samples were washed in several changes of distilled deionized water for 1 h, air dried and later processed for analysis by arc emission spectroscopy as reported previously[9].

Electron microscopy

The remainder of the aliquot in each case was prepared for electron microscopy by mincing, washing and dispersion in distilled deionized water by 10 kcycles/s sound. Portions of the supernatants were dried on collodion coated grids and stained with sodium phosphotungstate (pH 7.4)[10]. Additional tendon samples were used to test this technique for artifact using formaldehyde (10% buffered formalin) both before

and after 1 h denaturation at 70 °C preceeding examination on the electron microscope (RCA EMU 3-D). The fixation time was 24 h in each case.

RESULTS

Effects of denaturation temperature on cation binding

For the lowest treatment temperatures the relative cation concentrations bound to the matrix were approximately the same as for the room temperature experiments previously reported[1]. For all temperatures both the univalent and divalent cations were divided into two groups, those avidly bound and those relatively weakly bound, the avidly bound ions once again being either in the 0.6 or 1.2 Å ionic size ranges. As can be seen in Fig. 1a, the divalent ion uptake varied more or

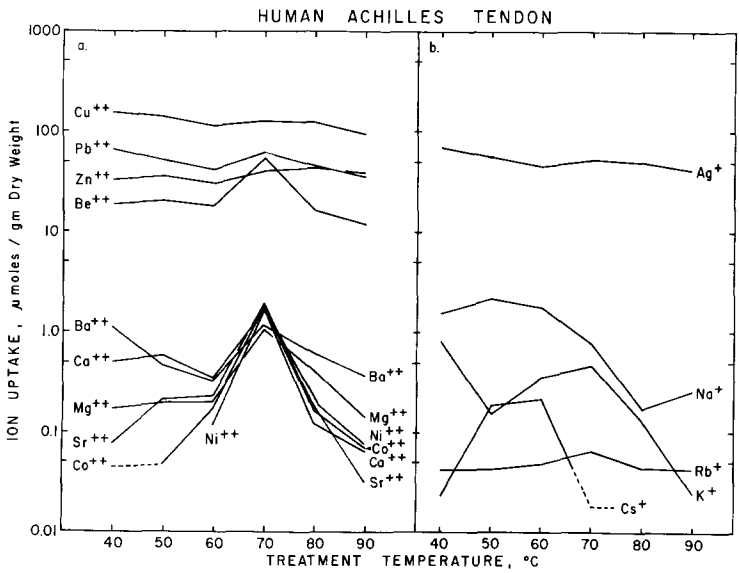
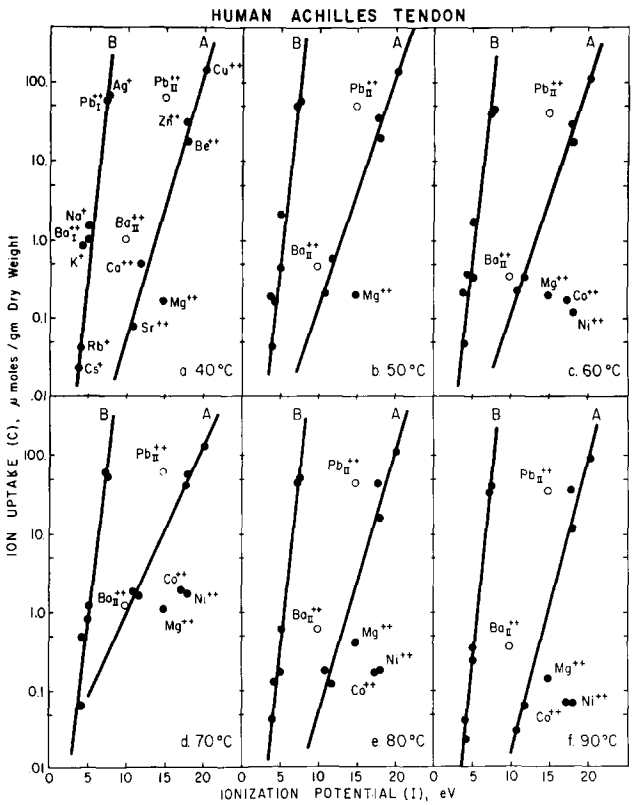


Fig. 1. Cation binding concentrations for human Achilles tendon first heated for 1 h in distilled deionized water at different temperatures and then exposed to a mixed solution of 17 ions in equimolar concentrations. (a) Divalent ion results. (b) Univalent ion results. Li^+ and Cd^{2+} were included but not measurable by the spectrographic technique. Note the loss of specificity in the 60 °C and 70 °C samples.

less coherently with treatment temperature, with a sharp increase in binding and loss of specificity in the 70 °C sample. This increase was not sustained with higher treatment temperatures. The only consistent behavior of the univalent ion uptake (Fig. 1b) was perhaps a general decrease with increasing treatment temperature.

Uptake versus ionization potential

In Fig. 2 the log of the uptake is plotted as a function of the ionization potential of the ion. In all cases the data fell quite naturally on two straight lines of differing slope. Pb^{2+} and Ba^{2+} concentrations fit best on Line B when their first ionization potentials were used. Line B had not been distinguished in earlier experiments[1]



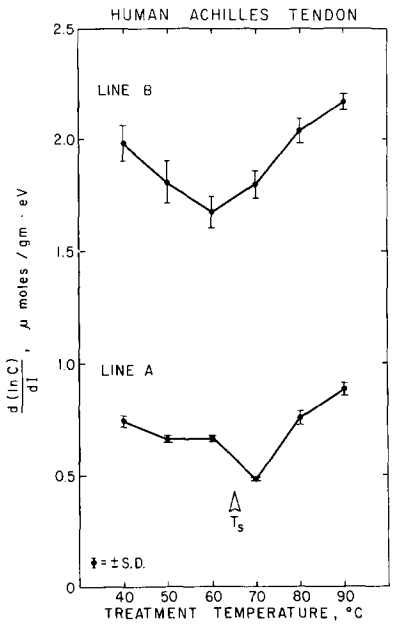


Fig. 2. Data in Fig. 1 plotted as a function of ionization potential of the ion for each temperature of treatment. Ba^{2+} and Pb^{2+} fell on Line B when their first ionization potentials were used. It is likely that Lines A and B correspond to different types of cation-binding site in the intact matrix. These sites remain essentially intact despite thermal denaturation.

Fig. 3. The slopes of Lines A and B from Fig. 2 as a function of treatment temperature. Linear least-squares slopes and standard deviations are used, excluding the data for Ni^{2+}, Co^{2+} and Mg^{2+}. Differences in temperature dependence suggest the distinction of the binding sites for A and B. Note that percentage changes in slope are far more pronounced for Line A.

either because of insufficient data or differing substrates. Mg^{2+}, Ni^{2+} and Co^{2+} seemed to show anomalous behavior (relative to the rest) no matter which ionization potential was used in the plot. Linear least-square fits were made to the data and the slopes plotted in Fig. 3. While the slopes of both lines decreased below and increased above the shrinkage temperature (approx. 65 °C), they differed in their response to shrinkage itself. The slope of Line A decreased further while the Line B slope increased over the shrinkage range. It should be noted that the percentage changes in slopes are much larger for Line A than Line B.

Fibril appearance

Fig. 4 is a composite of electron micrographs of sodium phosphotungstate stained fibrils representative of each treatment temperature. The 40 °C and 50 °C samples are seen to be quite normal in appearance. Among the 60 °C fibrils (Fig. 4c)

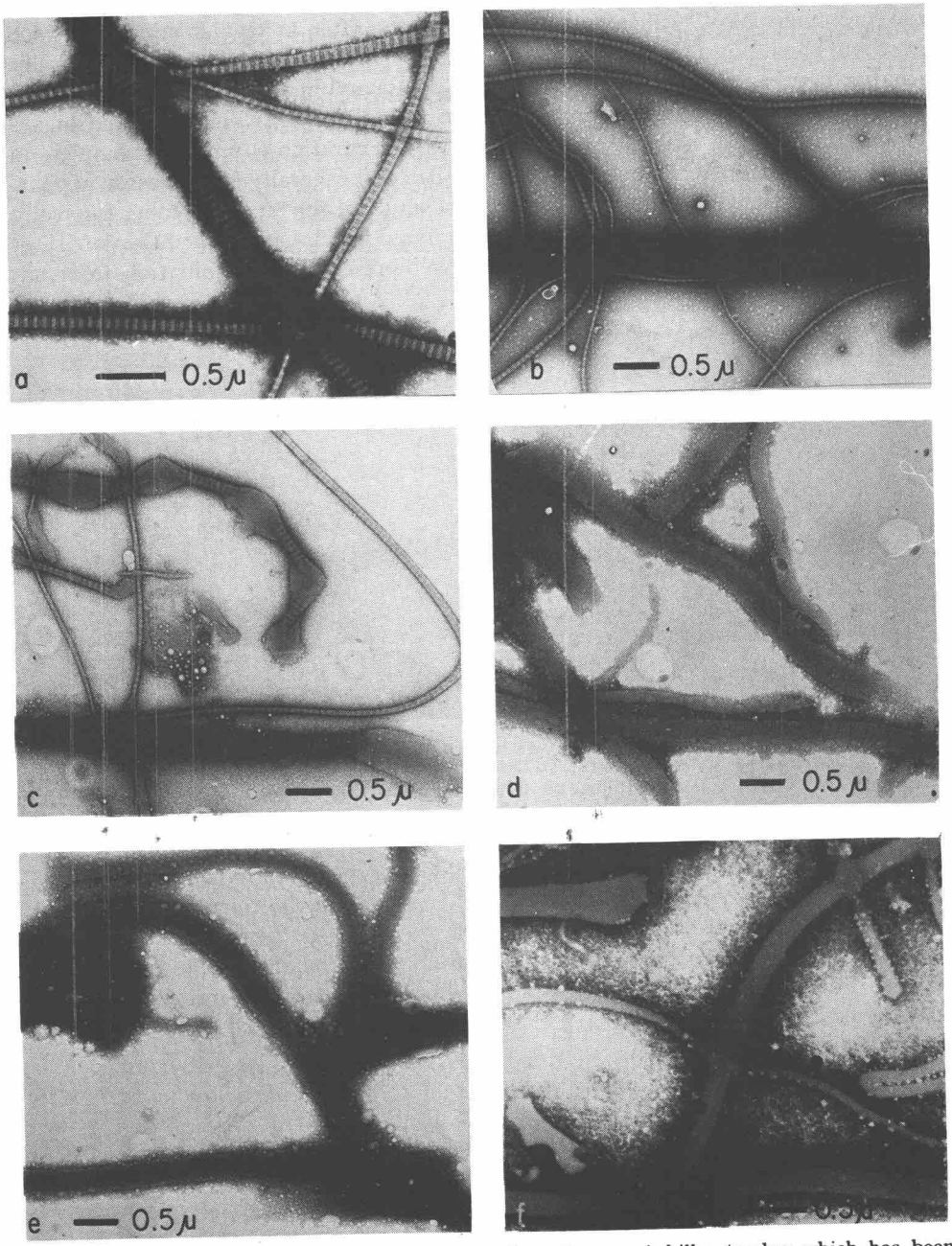


Fig. 4. Electron micrographs of collagen fibrils from human Achilles tendon which has been heated in water for 1 h at: (a) 40 °C, (b) 50 °C, (c) 60 °C, (d) 70 °C, (e) 80 °C and (f) 90 °C. Regional "melting", common in 60 °C samples, gives way to the relatively amorphous structures seen in 70 °C (and higher) treated samples. Samples stained with sodium phosphotungstate (pH 7.4) before drying on the grid.

however, many have swollen amorphous segments, often at regular intervals, which are about three times the normal fibril width and have only vestiges of banding. The denaturation has apparently begun at these points and in the cut ends although the gross specimen did not exhibit obvious signs of shrinkage. In the 70 °C and higher temperature samples (Figs 4d, 4e and 4f), the denaturation is virtually complete in 1 h, the vast majority of the fibrils being swollen and generally featureless in appearance. In fibrils which maintain traces of cross banding, the repeat distance is usually reduced. On each grid a very few fibrils were found which had escaped the denaturation for reasons not understood, unless they represented contamination from untreated tissue.

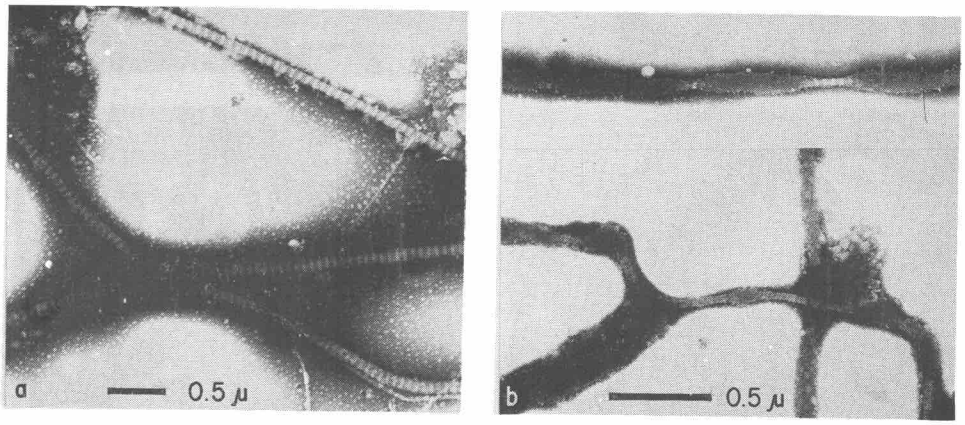


Fig. 5. Collagen fibrils from human Achilles tendon (a) fixed in formaldehyde and then heated for 1 h in water at 70 °C, (b) heated in water for 1 h at 70 °C and then fixed in formaldehyde. Since formaldehyde is able to stabilize the fibrils, it is likely that the forms in (b) and in Fig. 4 closely resemble those in the hydrated state.

Tendon which had been fixed in formaldehyde and then exposed to the 70 °C bath for 1 h (Fig. 5a) shows little if any fibril denaturation. On the other hand, fibrils fixed in formaldehyde after exposure to the 70 °C bath (Fig. 5b) have the same denatured forms as in the unfixed, heat-shrunk material. Since the cross-linking induced by formaldehyde is able to stabilize fibrils against denaturation, it is likely that the heat-shrunk forms in Fig. 4 closely resemble the hydrated fibrils and are not an artifact of preparation.

DISCUSSION

Although it appears that the total ion binding capacity of the tendon collagen matrix is not drastically changed by hydrothermal shrinkage, relative selectivities are shifted. The binding of the less strongly binding divalent cations (also Be^{2+}, and possible K^+ and Rb^+) shows a marked peak after exposure to 70 °C denaturation. This behavior is not to be expected from the simple disordering of a crystalline matrix, but is suggestive of a more complex process, perhaps involving a temperature dependent activated state of the matrix.

If the linear relationship between ion uptake and ionization potential is a result of coordination complexing to a particular type of site, as we have suggested

before[1], then in these experiments two binding sites are distinguished. The integrity of Lines A and B despite denaturation shows that the corresponding sites remain intact although hydrogen bonds and covalent intermolecular bonds are disrupted. Thus they are probably associated with the main amino acid chains in collagen and are not destroyed by random coiling of the protein. The distinctiveness of the sites is emphasized by the behavior of their slopes as a function of temperature (Fig. 3). The anomalous behavior of Co^{2+} and Ni^{2+} is most likely due to their preference for charged carboxyl side groups rather than other N and O coordinating sites[11,12].

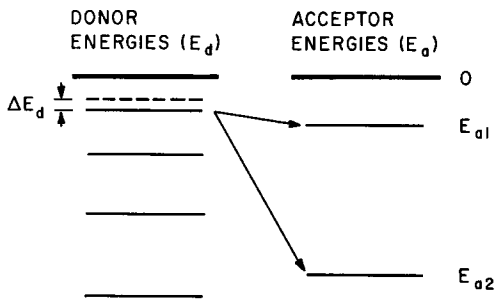


Fig. 6. Simplified representation of unidentate complexation of a metal ion (acceptor) and protein active site (donor) by electron transfer. Note that a small shift in donor energy, E_d (such as might occur in thermal denaturation) would result in a large percent change in binding energy ($E_d - E_a$) for acceptors with shallow unoccupied subshells.

The sensitivity of the weakly bound divalent cations to the thermal exposure at intermediate temperatures can be explained on the basis of a conformational rearrangement of the protein in the neighborhood of the binding sites. In a simplified scheme of unidentate coordination complexation a free electron pair is contributed by the donor (protein active site) and shared by the acceptor (metal ion) in an unfilled sub-shell. In Fig. 6 the binding energy *(E)* of the complex is represented as the difference between donor and acceptor electron energy levels. A small shift (ΔE_d) in donor energy, such as might be encountered in the conformational changes due to the breaking of hydrogen bonds, would result in a far greater percentage change in bond energy for weakly binding ions (shallow acceptors, E_{a_1}) than for strongly binding ions (deep acceptors, E_{a_2}). Thus, tendon denatured in water at 70 °C for 1 h can be characterized as being in an activated state for metal complexing to Site A. This activation would be relaxed at higher treatment temperatures (or longer exposure times) because of the likelihood of reorganization of the denatured chains into a more stable configuration and the remaking of hydrogen bonds. An approximate upper limit for the change in donor energy (ΔE_d) due to the 70 °C per 1 h denaturation can be obtained by assuming

$$\Delta E_d \simeq E \equiv E_d - E_{a_1} \qquad (1)$$

Where E is the binding energy of the severely affected Site A ions. From Fig. 6 and Eqn 1 it can be seen that

$$\Delta E_d < \tfrac{1}{2} E_{a_1} \simeq 5 \text{ eV}$$

Assuming a total binding capacity in these experiments of about 0.2 mmoles/g

(Fig. 1), the change in donor energy, as reflected in the changes in ionic binding, is less than about 23 cal per g dry tendon. This value is close to the heat of fusion measured by Flory and Garrett[7] suggesting that the conformational changes affecting metal binding in matrix collagen are in fact strongly coupled to the melting of the entire crystalline matrix itself.

The changes at the fibrillar level of organization as noted in the electron micrographs are: (1) spread out over 10–20 °C in thermal treatment temperature, (2) spatially localized at first in certain zones along the fibril and at its ends, and (3) characterized finally by a 3-fold expansion in width and loss of all but traces of banding. These changes almost exactly imitate the changes in gross specimens of tendon during shrinkage[2]. Similar observations were reported by Nutting and Boraski[8] for dilute dispersions of cowhide corium collagen heated for 5 min at 63 °C and by cowhide collagen merely swelled in $BaCl_2$. The factors which predispose certain regions of the fibril to be more sensitive to denaturation have yet to be determined. It is worthy of note that the fibrils treated at 70 °C for 1 h, while appearing completely denatured on the electron microscope, had metal binding behavior quite different from undenatured, incompletely denatured, and other denatured samples.

ACKNOWLEDGEMENTS

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