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Chromium(III)-Induced Structural Changes and Self-Assembly of Collagen

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Rat tail tendon (RTT) collagen has been reacted with a homologous series of chromium(III) complexes viz., $(H_2O)_4Cr(OH)_2Cr(H_2O)_4^{4+}$ 1 (dimer), $Cr_3(OH)_4(H_2O)_9^{5+}$ 2 (trimer), and $Cr_4(OH)_4(O)_2(H_2O)_{12}^{4+}$ 3 (tetramer), and the structural alterations brought about by these complexes have been investigated through atomic force microscopy (AFM) and circular dichroism (CD) studies. Examination of Cr(III)-treated tendons using AFM revealed changes in the D-periodicity of collagen, which may arise due to differences in the topological distribution of various Cr(III) complexes. Evidence for organisation of monomeric collagen into quarter staggered fibrils in the presence of Cr(III) dimer, 1, has been obtained. The quaternary structural changes induced by chromium in the protein have been correlated to the conformational changes of collagen in the absence of denaturation. © 2001 Academic Press

Key Words: **collagen; chromium (III) complexes; atomic force microscope; D-periodicity; conformation; aggregation.**

Collagens are the major structural components of the extracellular matrix, accounting for over 30% of the total protein content of the body (1, 2). Till date nearly 19 different collagen types have been identified (3). Collagen monomer, which is a triple helix, is formed by three individual α-chains made up of repeating Gly-X-Y triplets. Each α-chain is a left-handed poly-Pro-II helix and the three chains intertwine with a one residue shift into a right-handed triple helical coiled coil (4–6). Skin contains mainly type I collagen, which exhibits a fibrillar organisation with a periodicity of a little less than 70 nm. The axial periodicity observed from electron microscopic and AFM investigations is the result of quarter staggered arrangement of collagen monomers (7–11). The crosslinking of collagenous matrix by the different polymeric chromium(III) species present in basic chromium sulphate (BCS) has been shown to impart hydrothermal stability as well as stability against enzymatic degradation to collagen (12). The mechanisms underlying the stabilisation of the collagenous matrix by chromium at the molecular level are yet to be elucidated. Structural alterations to collagenase by the different chromium(III) complexes viz., dimer, 1, trimer, 2, and tetramer, 3, present in BCS leading to inhibition of collagenase activity have been demonstrated by us recently (13).

In order to study the interaction of different chromium(III) complexes with collagen at a molecular level and correlate it to the stabilisation of the matrix, RTT collagen has been treated with different Cr(III) complexes viz. dimer, 1, trimer, 2, and tetramer, 3, employed in the tanning of collagen matrix and structural changes brought about have been addressed using AFM and CD techniques. Through CD studies the potency of different chromium(III) complexes to induce conformational changes have been worked out. While dimer, 1, and trimer, 2, induced aggregation without denaturing collagen, the tetramer, 3, was without any effect. The dimer, 1, was also shown to bring about self-assembly of collagen molecules from acetic acid medium into quarter staggered collagen fibrils. Though trimer, 2, was effective in forming a fibrous network, the quarter staggered arrangement was not favoured. Tetramer, 3, did not induce any effect corroborating with CD spectra. These studies indicate that the extent of stabilisation of collagen matrix by the various chromium(III) complexes are due to their ability to effect structural changes in collagen at the molecular level as well as ability to quarter stagger the collagen molecules from solution.

MATERIALS AND METHODS

Treatment of rat tail tendon with the different Cr(III) complexes. Rat tail tendons teased from six-month-old albino rats were washed extensively in 0.9% saline and stored at less than 4°C. The tendons

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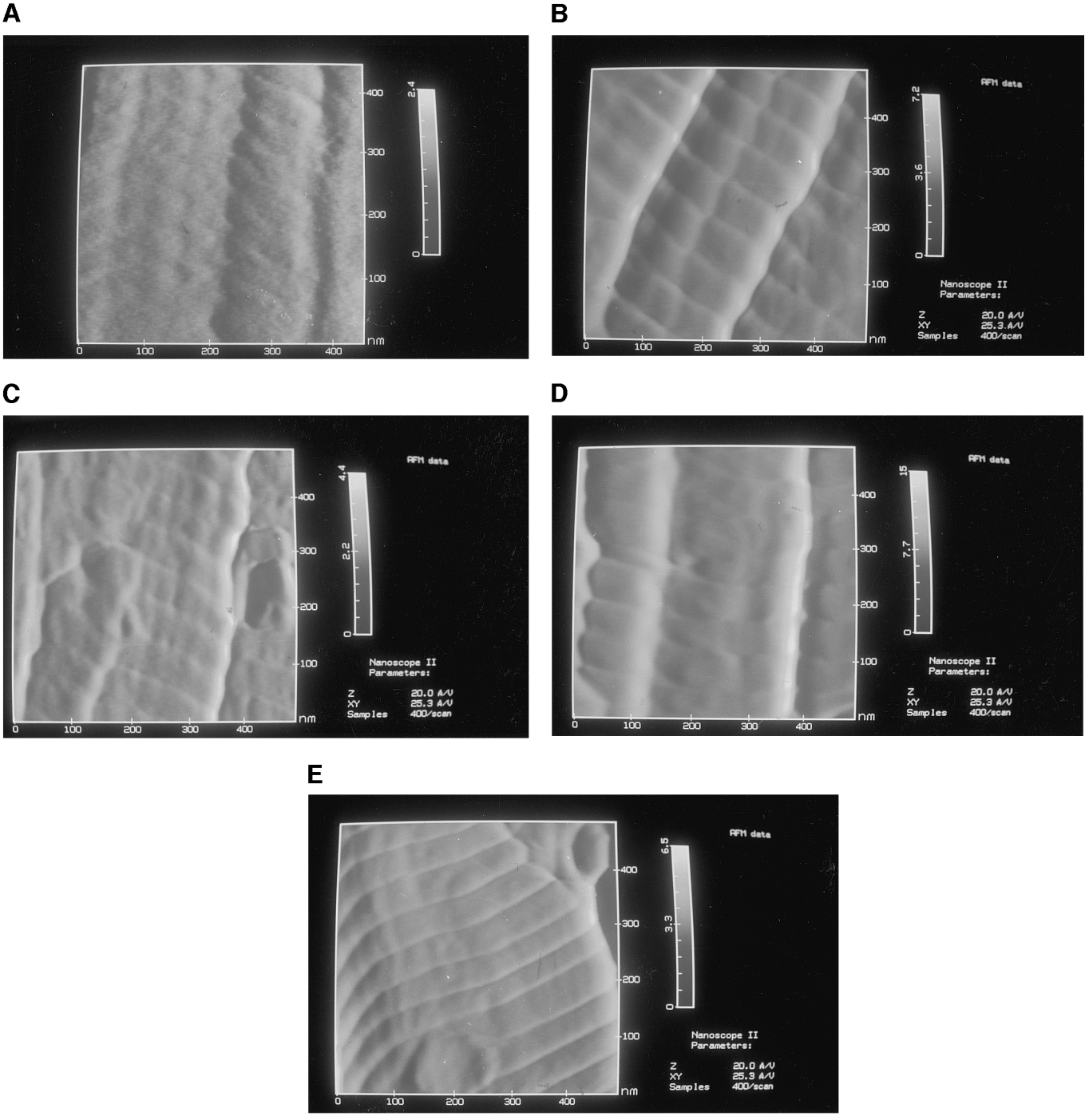

FIG. 1. 0.5 × 0.5 μm AFM images of rat tail tendon treated with different Cr(III) complexes. (A) Control, (B) BCS, (C) dimer, (D) trimer, and (E) tetramer.

were washed in 1:100 Triton X-100 for 10 min to remove extraneous matter followed by washing in running distilled water. The samples were fixed on the stainless steel disk using an adhesive. Native RTT fibres after washing were treated with the different Cr(III) complexes in BCS viz. dimer, 1, trimer, 2, and tetramer, 3, prepared using standard methods (14–16). After incubation for 24 h, the pH was slowly raised to 4.0. The fibres were washed extensively with distilled water and stored wet.

Preparation of reconstituted collagen samples for AFM. The substrate consists of freshly cleaved mica fixed to 15 mm steel disks with an adhesive. For studies involving fibrillar collagen, soluble collagen was prepared from RTT according to the method of Chandrakasan *et al.* (17). Collagen solution in 0.05 M acetic acid was dialysed against 5 mM acetic acid overnight at 4°C. The solution was then centrifuged at 100,000*g* for 1 h in an ultracentrifuge to sediment large aggregates. The supernatant from this centrifugation contains soluble

collagen. Collagen fibrils were prepared in the presence of 0.02 and 0.13 M of NaCl and phosphate buffer (pH 7.2), respectively (18). The substrate was then placed over the sample, removed after 30 min, washed with water, drained and dried. For studies involving self-assembly of collagen in the presence of Cr(III) complexes viz. dimer, 1, trimer, 2, tetramer, 3, and BCS, collagen solution in 5 mM acetic acid (0.5 mg/mL) was mixed gently with different Cr(III) complexes to give a final concentration of 0.001 M as Cr(III). After incubation for 30 min at 27°C, samples were adsorbed to the substrate.

AFM imaging. Images were captured using a Nanoscope II AFM (Digital instruments, Santa Barbara, CA) with a 'J' scale scanner (horizontal ranges of 1 μm). Commercially available 100 μm silicon nitride cantilevers (Digital Instruments) were used with 0.58 N/m nominal spring constant. The tip, a couple of micron long and less than 100 Å in diameter was located on the free end of the cantilever. The discs with the sample were mounted on the sample holder. The instrument was used on contact mode with scan rate varying from 0.1 to 150 Hz. The tunneling current range is +50 nA and the applied force was less than 100 nN. Samples were qualitatively examined from eight independent, randomly selected 500 × 500 nm fields. D-band measurements were made directly from screen displays using the Nanoscope II software.

Circular dichroism studies. The collagen content was estimated by a standard procedure (19). Stock solutions of different Cr(III) complexes viz. dimer, 1, trimer, 2, tetramer, 3, and BCS were prepared. Collagen was titrated with Cr(III) complexes to give final Cr(III) concentrations of 100–500 μM. The ionic strength was maintained at 0.1 M using lithium perchlorate. Collagen was incubated in the presence of Cr(III) complexes at 25°C for 18 h and the spectra were recorded at 25°C using a J-715 Jasco spectropolarimeter. A scan speed of 20 nm/min was used with an average of 5 scans per sample. A slit width of 1 nm and a time constant of 1 s were used. 1 mm cell was used for the experiments. A reference spectrum containing acetic acid and lithium perchlorate was also recorded. The CD spectra of the samples were obtained after subtracting from the reference. Spectra were expressed in terms of mean residue ellipticity [θ_{MRW}] using mean residue weight of 91.2 for collagen (20).

RESULTS

Atomic Force Microscopic Investigations

The sample discs containing the tendons, both control and chromium(III) treated, were scanned over an area of a few square microns. A minimum of 20 scans per sample at eight different locations was carried out. The topographic image in height mode of control RTT fibre is shown in Fig. 1A with distinctive D-periodicity of 64 ± 1 nm. The AFM image of collagen exhibits alternating grooves and ridges. This agrees well with earlier observations on native type I collagen (21). Type I collagen shows a fibrillar organisation in which the fibrils have the characteristic transverse structure with a periodicity of a little less than 70 nm; 67 nm if measured by X-ray diffraction, and 64 nm if measured by electron microscopy. The chromium(III) complexes treated RTT also exhibit ridges and grooves with differences in the periodicity of the ridge and groove as shown in Figs. 1B–1E, for BCS-, dimer-, 1, trimer-, 2, and tetramer-, 3, treated RTT, respectively (Table 1). Marked variation in the D-period with respect to native (nearly 8 nm) has been observed in the case of dimer-, 1, treated RTT fibre. For trimer-, 2, and BCS-treated RTT fibre the values were similar viz. 60 ± 1 and 60 ± 2 nm, respectively, and were only marginally different from that of native RTT fibre. The D-periodicity of tetramer-, 3 treated RTT fibre was also similar to that of native RTT (61 ± 3 nm). The observed trend can be corroborated with results of TEM investigations from previous studies in which the self-staining ability of the heavy metal chromium has been exploited (22).

TABLE 1

D-Period Values Obtained from Analysis of AFM Images of Various Chromium(III) Complexes Treated Rat Tail Tendon Fibres

Nature of treatment	D-period (in nm)
None	64 ± 1
BCS	60 ± 2
Dimer, 1	56 ± 1
Trimer, 2	60 ± 1
Tetramer, 3	61 ± 3

Note. The values are a mean ± SD of eight values.

Formation of aggregates of collagen monomers in acetic acid medium when incubated with Cr(III) complexes was observed through AFM investigations. The images of the different chromium(III) complexes induced aggregates are shown in Figs. 2A–2C. Though aggregation of collagen was found to be induced by dimer, 1, trimer, 2, and BCS, the dimer assembled collagen molecules exhibited characteristic D-period of 64 ± 2 nm (Fig. 2B). On the other hand, BCS- and trimer-, 2, induced aggregation of collagen molecules presents a fibrous network without exhibiting the distinct D-periodicity (Figs. 2A and 2C). The values of D-periodicity are listed in Table 2. The assembly of collagen molecules into quarter staggered fibrils with 64 nm period from acetic acid solution can only be observed when the ionic strength and pH are adjusted to that of physiological values (23).

Circular Dichroism Studies

To study the potential effect of Cr(III) on the structure of collagen during tanning, we studied the interaction of collagen with different Cr(III) complexes in the peptide bond region. The triple helical conformation of collagen presents a circular dichroic spectrum characterised by a positive band at 223 nm and a negative band at 198 nm. The CD spectrum of native soluble collagen is in agreement with that reported earlier (20). Figures 3A–3D show the average spectrum of collagen in the presence of BCS, dimer, 1, trimer, 2, and tetramer, 3, respectively, in the concentration range of 100–500 μM. It is evident that in the presence of increasing concentration of the Cr(III) complexes viz. dimer, 1, trimer, 2, and BCS there is a decrease in the

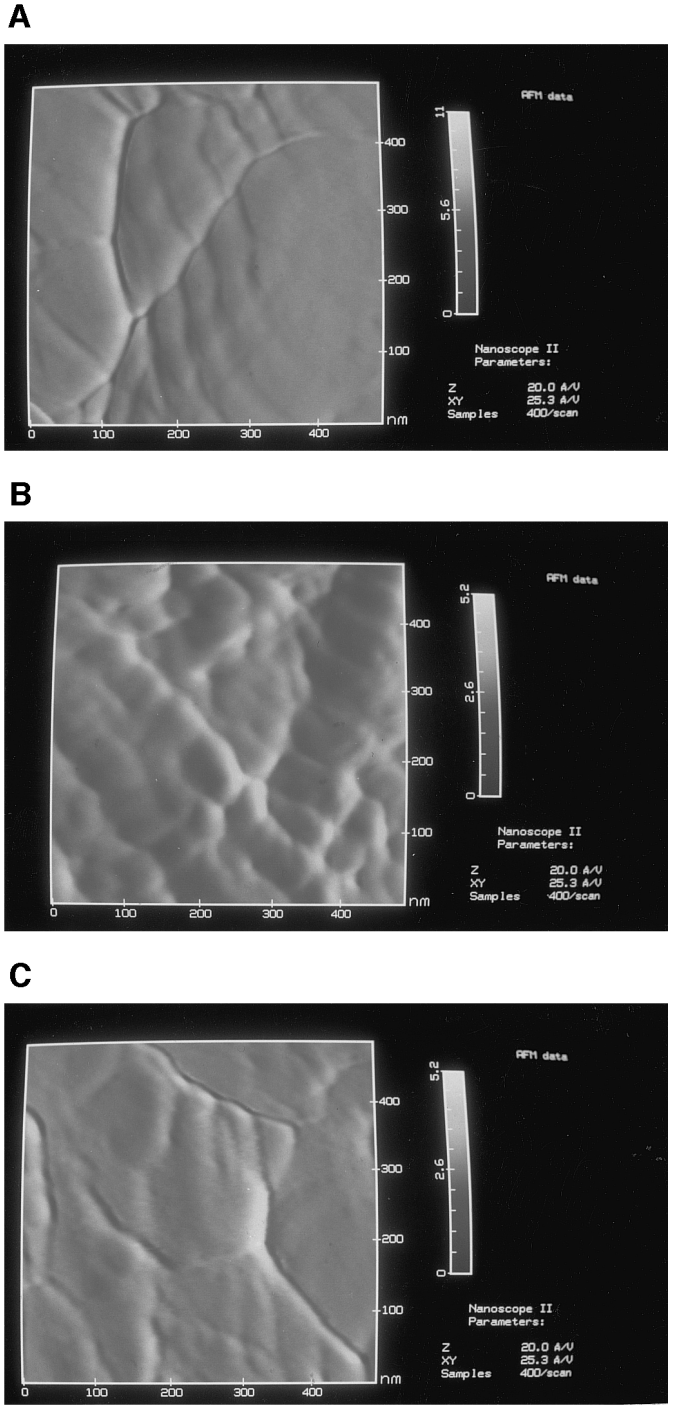

FIG. 2. 0.5 × 0.5 μm AFM images of chromium(III) induced aggregation of collagen monomers. (A) BCS, (B) dimer, and (C) trimer.

dichroic intensities at 223 and 198 nm. The ratio of positive peak intensity over negative peak intensity (Rpn) is used in establishing triple helical conformation in solution. The Rpn values of collagen in the presence of different concentrations of Cr(III) complexes is listed in Table 3. From the table it is observed that the values of Rpn of the treated samples do not change significantly from that for the native triple helical conformation of collagen (0.15 ± 0.02).

DISCUSSION

In the present study, for the first time we have provided evidence for self-assembly of collagen induced by chromium. This study is a step toward understanding the topological distribution and interaction of chromium(III) with the collagenous matrix, which is required to interpret the contributions of metal in stabilising collagen from degradation. The chromium(III) dimer-, 1, induced aggregation of collagen solution (5 mM acetic acid) with characteristic D-periodicity of 64 nm reflective of the quarter staggered arrangement. Thus, Cr(III) dimer, 1, is able to maximize the interaction between the collagen monomers and assemble them into a quarter staggered fibril. In the quarter staggered arrangement the electrostatic interactions are reported to be maximal (24). The dimer, 1, exhibits unique propensity to align the fibrils into quarter stagger, while trimer, 2, and BCS only formed fibrous network of collagen molecules without any characteristic banding pattern. The aggregation whether quarter staggered or fibrous may be due to the interaction of these Cr(III) complexes with that of the ionised carboxyl groups of aspartic and glutamic acids of collagen in 5 mM acetic acid at a pH of 3.8 (25, 26). That the dimer, 1, aligns the molecules in this form is possible only after interaction with the aspartic and glutamic acid residues of the collagen monomers and in a sequential process, aligns these monomers into pentafibril assembly, and further assembles the pentafibril units into a quarter staggered form. The size of the dimer, 1, molecule also seems to be an essentiality for the formation of quarter staggered form since trimer, 2, with dimensions higher than that of dimer is not able to pack the collagen monomers into the quarter staggered form.

From the CD spectral changes of collagen brought about by the various Cr(III) complexes it is seen that the dichroic intensity at 223 and 198 nm decrease in a dose dependent manner. However the Rpn ratio did not deviate much from that obtained for native collagen.

TABLE 2

D-Periodicity of Assembled Collagen Molecules Mediated by Chromium

Cr(III) complex added	D-periodicity (in nm)
BCS	77 ± 1
Dimer, 1	64 ± 2
Trimer, 2	72 ± 1
Tetramer, 3	—

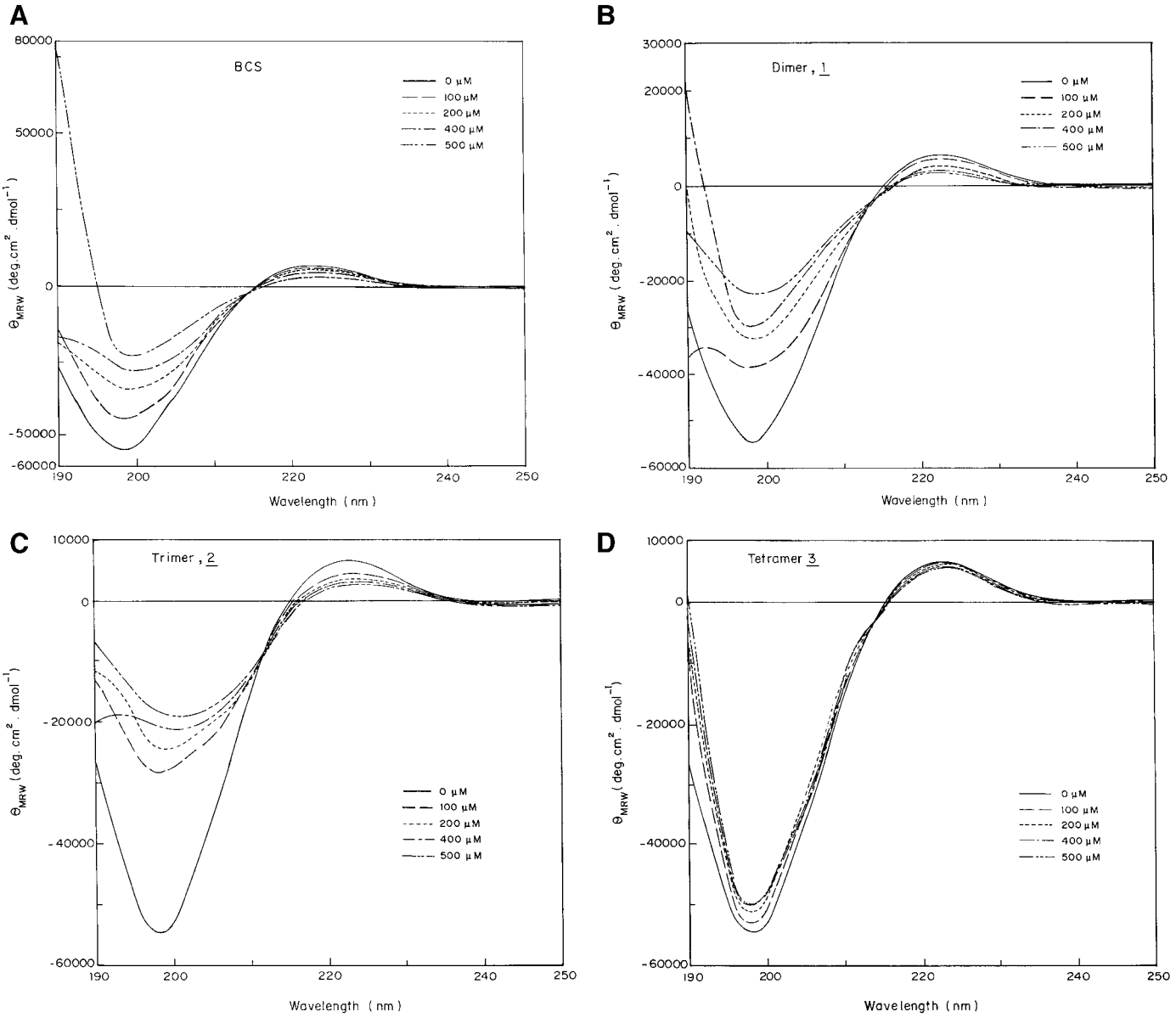

FIG. 3. CD spectra corresponding to Cr(III) titration of collagen in 5 mM acetic acid with a protein concentration of 0.5 mg/mL and pathlength 1 mm in the presence of different Cr(III) complexes. (A) BCS, (B) dimer, (C) trimer, and (D) tetramer at concentrations of 0 μM (—), 100 μM (- - -), 200 μM (· · ·), 400 μM (– · – ·), and 500 μM (– · · –) as Cr(III).

The CD spectral changes in collagen with only small deviations in Rpn ratio in the presence of Cr(III) complexes is indicative of conformational changes in the collagen molecule and without denaturation. Such a conclusion can be drawn from early studies (27–29). Partially denatured collagen, was found to give CD spectra with lower intensity, red shifted crossover points, and a higher ratio of the intensity of short wavelength band to the intensity of the long wavelength band. On complete denaturation the positive peak at 223 disappears completely and the negative band has been found to be red shifted. In the Cr(III) treated collagen there is no significant red shift of the negative band nor is there any disappearance of the positive band at 223 nm. Thus the decrease in dichroic intensity or in other words, the change in the CD of collagen in the presence of Cr(III) is not due to denaturation or loss of triple helicity.

Another explanation for the changes in the CD spectra in the presence of BCS, dimer, 1, and trimer, 2, may be based on aggregation of collagen brought about by the Cr(III) complexes. Very early studies on the interaction of collagen with glycoproteins using CD have been attempted by Franzblau *et al.* (30). Decrease in the mean residue ellipticity of collagen with increase in the concentration of glycoprotein has been observed

TABLE 3

Circular Dichroism Data of Soluble Collagen in 5 mM Acetic Acid in the Presence of Increasing Concentrations of BCS, Dimer, Trimer, and Tetramer

Cr(III) μM	Max (θ_{MRW}), nm $\times$ 10^3 (deg cm^2 dmol^{-1})	Min ($-\theta_{MRW}$), nm $\times$ 10^3 (deg cm^2 dmol^{-1})	Rpn
Native collagen	223 (6.59)	198.2 (54.57)	0.121
BCS			
100	223 (5.85)	198 (46.12)	0.127
200	223.2 (5.13)	198.2 (32.84)	0.156
300	223 (4.63)	199.6 (32.68)	0.141
400	223.4 (3.86)	199.8 (27.51)	0.140
500	223.6 (3.29)	198.6 (20.7)	0.158
Dimer, 1			
100	223.2 (6.02)	198.8 (36.80)	0.163
200	223.2 (4.44)	198.4 (32.84)	0.135
300	223 (4.22)	198 (30.56)	0.138
400	223.2 (4.06)	198.2 (29.46)	0.137
500	223.6 (3.39)	198.6 (22.21)	0.152
Trimer, 2			
100	223 (4.68)	198.4 (28.49)	0.164
200	223.2 (3.33)	198.4 (24.78)	0.134
300	223.8 (3.02)	198.6 (22.88)	0.132
400	223 (2.64)	198.8 (21.79)	0.121
500	223.6 (2.53)	198.2 (18.05)	0.135
Tetramer, 3			
100	222.8 (6.81)	198 (54.71)	0.124
200	222.6 (6.05)	198.4 (53.45)	0.113
300	222.8 (6.59)	198.6 (52.11)	0.126
400	223 (6.98)	198.2 (51.07)	0.136
500	223 (6.07)	197.4 (50.13)	0.121

taking into consideration the ellipticity at 198 nm and this decrease has been explained in terms of aggregation of collagen brought about by the presence of glycoprotein. The results obtained on the one hand, indicates that the collagen is not denatured and on the other hand indicates aggregation of the collagen molecules in the presence of Cr(III) complexes due to coordination of chromium with the aspartic and glutamic acids in collagen.

The collagenous matrix of tendon/skin presents a wide range of pore size dimensions. While molecular dimensions of the complexes limit access to the reaction sites on the protein, varying reactivities of the Cr(III) complexes may play a role in the extent of crosslinking collagen. The observed differences in D-periodicity may also be explained in terms of displacement of water involved in hydration network of collagen by chromium. Recent crystal structure studies have revealed water bridges as critical elements in connecting adjacent triple helices (31, 32). Decreases in D-periodicity have also been observed during different stages of removal of structural water on heating (33). Smaller size of the dimer, 1, compared to trimer, 2, (34) which permits greater accessibility into the collagenous matrix may also be a factor in the extent of displacement of water. The tetramer, 3, on the other hand, because of its poor thermodynamic affinity and greater kinetic lability does not bring about changes in D-periodicity. The poor reactivity of the tetramer has been reported earlier (16, 34, 35).

Thus the results of AFM and CD studies indicate that the collagen monomers can be aggregated and stabilised into quarter stagger aggregates in acidic medium in the presence of dimer, 1, suggesting a role for dimer in the long range ordering of collagen fibrils without denaturating of the protein.

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