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Chromium induced structural changes in biomolecules

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Abstract. Mechanistic insight has been gained into the diverse roles played by some chromium(III) ions in biosystems. Experimental evidence to show that chromium may serve to assemble insulin rather than insulin-receptor units has been obtained. A proposal that stabilisation of insulin by Cr(III) may be responsible for the beneficial role of Cr(III) in glucose metabolism has also been made. Although one of the well-known industrial applications of chromium(III) salts has been in the stabilisation of the collagen in skin to produce leather, it has remained difficult to provide a molecular basis to tanning. Results of model studies involving the reactions of collagen with a series of complexes of chromium(III) have been presented and an attempt to provide a molecular basis to the stabilisation of collagen by the metal ion have been made. In view of the growing concern about the ecological consequences as well as the occupational hazards of using chromium, abnormalities induced by a series of chromium complexes on the proliferation of human lymphocytes have been investigated. Evidence for apoptosis by Cr(III) has been obtained. Although chromium plays diverse functional roles in biosystems, a perspective discussion of some common features in the nature of interactions of the metal ion with biomolecules has been attempted.

Keywords. Biotoxicity; apoptosis; chromium(III) complexes; chromium–protein complex.

1. Introduction

The biological roles played by chromium have remained active areas of study, though conflicting reports of the biofunction of chromium(III) salts are being made (Mertz 1974; Held *et al* 1984; Dessi *et al* 1989; Langford 1990; Salinikov 1992). Whereas the therapeutic utility of some chromium(III) salts in the control of diabetes is discussed on the one hand, the environmental consequences of the discharge of industrial waste waters into water bodies and land are talked about on the other (Mertz 1979; Bellavere and Gorbi 1981). This in part arises from a somewhat inadequate mechanistic insight into the nature of the interactions of the chromium ion with biomolecules. General discussions on the nature of interactions of metal ions with biomolecules relate to the nature and site of bonding. Coordinate interactions of metal ions with biosystems have been focussed on (Siegel 1976). Such interactions are thought to involve mostly side-chain groups of biomolecules. Some general predictions are possible of the roles of metal ions in biological systems. Short range interactions and crosslinking phenomena in biosystems induced by metal ions may also bring about changes in tertiary and quaternary structures,

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which in turn markedly modulate the functions of biomolecules. In this work, we have made an attempt to highlight some of these structural and morphological changes in biomolecules brought about by chromium(III) salts. Three biomolecules have been included in this work to describe the general phenomena of metal ion-induced quaternary and morphological changes in biosystems. The three systems studied have ranged from a small molecular weight protein like insulin to a whole cell such as a human lymphocyte. The similarities observed in the interactions of chromium with the three seemingly unrelated systems are the types of molecular assemblies promoted by the metal ion.

Numerous experimental and clinical investigations supporting the utility of some Cr(III) salts in the control of diabetes have been reported (Mertz *et al* 1974a; Anderson *et al* 1983; Aguilar *et al* 1993; Morris *et al* 1995). The role of chromium(III) bearing peptides in the glucose tolerance factor (GTF) was reported early (Anderson *et al* 1978). This was, however, questioned in a later study. Arguments were advanced that the free peptides from the baker's yeast rather than the Cr(III)-bearing peptide may be responsible for the GTF function (Held *et al* 1984). Questions were raised as to whether GTF contained the Cr(III) centre or not (Held *et al* 1984). However, an experimental study was reported demonstrating that some Cr(III)-containing insulin adducts were 2·5 times more efficient in controlling diabetes in experimental albino rats (Govindaraju *et al* 1989). In this context, it seems appropriate to discuss the possible roles of Cr(III) in the control of diabetes. A hypothesis was advanced that chromium may serve to assemble insulin and protein receptor units through the formation of a core structure, as in figure 1 (Mertz *et al* 1974b). This hypothesis raises a fundamental question as to how a kinetically inert metal ion like Cr(III) participates in biocatalysis implicating the core structure given in figure 1. The proposed hypothesis could be easily examined by investigating the efficacy of some specially designed macrocyclic complexes of Cr(III) in the control of diabetes. If the proposed hypothesis of Mertz were right (Mertz *et al* 1974b), a stable Cr(III) complex in which a macrocyclic tetradentate ligand is equatorially coordinated may not participate in glucose metabolism effectively. However, our previous studies have shown that the insulin derivatives of the complexes *trans*-diaqua N,N'*bis*(salicylideneamino)ethanechromium(III), *trans*-Cr(salen)$(H_2O)_2^+$ and *trans*-diaquatetramethyltetraazacyclotetradeca[14] tetraenechromium(III), *trans*-Cr(Me_4-[14] tetraene N_4)$(H_2O)_2^{3+}$ participate in the control of diabetes in albino rats (Govindaraju *et al* 1989). Insulin derivatives of Cr(III)–Schiff base complexes have been subjected to chymotrypsin-promoted hydrolysis. Chromium(III)-bearing peptides have been isolated and their amino acid

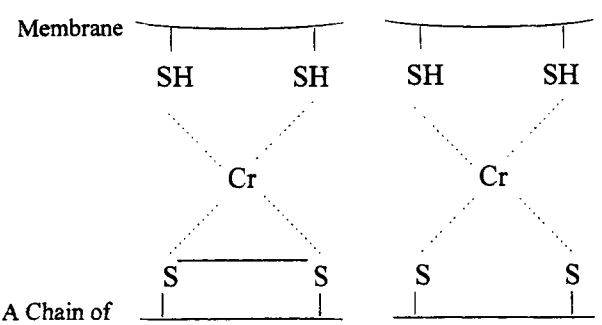

Figure 1. Proposed hypothetical structure of chromium assembly of insulin and receptor units.

composition analysed. Based on such studies it is possible to invoke chromium-induced assembly of insulin in solution. Study of the ternary system Cr(III)–insulin–chymotrypsin has provided convincing experimental evidence for the stabilising influence of Cr(III) on insulin against chymotrypsin-promoted hydrolysis.

Studies on ternary systems involving protein-metal-proteinases are of fundamental relevance not only to biochemical but also to industrial systems. An example of an ill-understood ternary system with industrial relevance has been collagen–chromium(III)– collagenase. An insight into the structural changes imposed by Cr(III) salts on collagen is fundamental to the understanding of tanning on a molecular basis. While tanning is easily described, molecular explanations for the process have not been forthcoming. Questions remain as to whether (a) chromium blocks the functional sites in collagen for collagenase action, or (b) deactivated collagenases or (c) interactions with chromium alter the conformation of collagen such that enzyme-substrate recognitions are not feasible. It is also probable that more than one of the above causes may be responsible for the observed stability of the collagen-chromium complex against collagenase. The need for better understanding of the structural changes involved in chrome tanning has already been identified (Wilkinson1987; Germann 1995). An approach to understanding of tanning on a molecular basis demands the knowledge of the structural changes imposed by specific chromium(III) species involved in the matrix stabilization of collagen against enzymatic hydrolysis and heat.

Since tanning implies widespread use and wasteful discharge of polyhydroxysulphatochromium(III) complexes, the ecological consequences of leather processing have long been receiving much attention (Gauglhofer 1986; Chandrasekaran *et al* 1989; Rao 1991). The occupational hazards associated with constant exposure to chromium(III) salts and the adverse effects of Cr(III) on human self-defence pathways have attained significance (Snow and Xu 1991). We reported recently the effect of some Cr(III) complexes on the proliferation of human lymphocytes (Rajaram *et al* 1995). In this work, we further discuss our results, which demonstrate the apoptosis caused by some selected Cr(III) complexes. The need to take into account the molecular structure of Cr(III) species prior to meaningful discussions on the biotoxic potential of chromium compounds has been discussed.

In all the three functions of chromium discussed in this study, questions regarding transport of chromium into biomolecules as well as the resulting structural changes need to be better resolved.

2. Experimental

2.1 *Chromium(III)-insulin interaction*

The preparation and characterisation of insulin derivatives of five chromium complexes viz. aqua-nitrilotriacetatomonooxalatochromiate, $Cr(nta)(ox)(H_2O)^{2-}$, 1, aquaethylenediaminetetra-acetatochromiate, $Cr(edta)(H_2O)_2^-$, 2, *trans*-diaquabis (salicylidineamino) ethanechromium(III), *trans*-$Cr(salen)(H_2O)_2^+$, 3, ethylenediaminetriacetatomonoaquochromium(III), $Cr(edta)(H_2O)$, 4, *trans*-diaquatetramethyltetraazacyclo-tetradeca[14] tetraenechromium(III), *trans*-$Cr(Me_4[14]\text{ tetraene})(H_2O)_2^{3+}$, 5, has been reported earlier (Govindaraju *et al* 1989). The ratio of insulin to chromium has been estimated using standard methods for chromium and protein analyses (Lowry *et al* 1951; Thompson and Gordon 1966; Bradford 1976). The values

of protein:chromium(III) analysed have been generally in the range of 1: 0·30 ± 0·03 within experimental error.

Since the chromium(III) compounds chosen for investigation contain replaceable water ligands and are soluble at body *p*H values, it is possible to evaluate the biological consequences of the Cr(III) compounds without ambiguities resulting from precipitation. Among the various complexes investigated, *trans*-Cr(salen)$(H_2O)_2^+$ and *trans*-Cr(Me$_4$-[14] tetraeneN$_4$)$(H_2O)_2^{3+}$, led to higher improvements in the efficiencies of insulin in the control of blood sugar levels in diabetic albino rats. Whereas 5 units of free insulin/kg of body weight were necessary to effect control on glucose levels, a similar effect could be observed with 2 units equivalent of insulin adducts of 3 and 5. Both these complexes are cationic and yet do not seem to have problems in transport into biosystems as evidenced from the beneficial role of the Cr(III)-insulin adducts in the control of diabetes. It is pointed out that in the case of both 3 and 5, the central metal ion is coordinated to a tetradentate ligand equatorially and in the resulting insulin derivative, Cr(III) is unlikely to participate in the assembly of insulin and the receptor units in the framework of the core structure proposed earlier (Mertz *et al* 1974b). The observed beneficial effects of the Cr(III) insulin derivatives investigated in our studies may well provide arguments against the earlier hypothesis (Mertz *et al* 1974b). Based on our results, further mechanistic insight has been possible.

The Cr(III) derivatives of insulin prepared in this study have been subjected to chymotrypsin-promoted hydrolysis under standard conditions. The resulting protein hydrolysates have been subjected to a chromatographic separation of the various fractions using a gel permeation chromatographic column. Eight fractions of varying molecular weight ranges could be isolated from the chymotrypsin hydrolysates of free insulin. The number of fractions obtained from Cr(III) derivatives of insulin is only three or four. The amino acid compositions of the chromium-containing fractions have been analysed using standard methods.

It is known that insulin contains two histidine residues, both in the B-chain of the molecule. The ratios of any one particular amino acid to histidine content in Cr(III)-bearing peptide fractions offer important information on the role of chromium. The molecular weights of the chromium-containing fractions from the insulin derivatives of 3 and 5 are in the range of 2900–3100 daltons. The various amino acid ratios observed are listed in table 1. A best-fit model for the observed amino acid composition ratios in

Table 1. Composition of various amino acids in Cr(III)-peptides with respect to histidine.

		Insulin derivative of				
Amino acid	Free insulin[a]	1	2	3	4	5
Aspartic acid	1·5[b]	3·3	1·5	6·0	6·0	8·0
Glutamic acid	3·5[b]	8·0	12·5	12·2	12·0	19·5
Serine	2·0	3·0	3·4	4·0	2·5	2·7
Tyrosine	2·0	2·0	1·3	1·6	6·0	4·3
Leucine	3·0	8·0	9·5	2·4	11·0	17·0
Valine	2·5	5·0	2·9	—	6·0	8·9

[a]Calculated on the basis of amino acid composition of insulin

[b]Glutamine and aspargine have been treated as aspartic and glutamic acids for purposes of calculation.

the chromium-bearing peptides from insulin derivatives of complexes 3 and 5 shows that three molecules of insulin may be associated with each chromium atom in the adduct.

The metal centre in Cr(III)-insulin adducts seems to promote self assembly of insulin. Chromium-bearing peptides are rich in aspartic and glutamic acids. Whereas in the free insulin the ratio of asp/his and glu/his are only 1·5 and 3·5, in chromium-bearing fractions from insulin derivatives of 3, the ratios are 6·0 and 12·2 respectively. Analogous values for insulin derivatives of 5 are 8·0 and 19·5 respectively. In other words, the metal centre is situated in a charged patch of carboxylic acid sites. The observed amino acid ratios are best reconciled by invoking the assembly of the multiples of three insulin molecules for each chromium atom.

The published crystal structure of insulin demonstrates the tendency of insulin to self assemble in the presence of zinc(II) salts to afford hexameric structures in a crystalline lattice (Blundell *et al* 1972). There have been reports that zinc promotes the formation of hexameric arrangement of insulin. There are also suggestions that such assemblies of insulin may provide polar channels of charged side-chain amino-acid residues (Blundell *et al* 1972). On the basis of the available data on the amino acid composition of chromium-bearing peptides, it seems appropriate to consider the interactions of such polar channels in insulin with chromium(III) ions. In the light of the known hexameric structures of insulin, it seems attractive to consider the possibility that Cr(III) behaves like Zn(II) in promoting the formation of hexameric structures. In effect, the nature of chromium interactions with insulin seems to have led to intermolecular crosslinks. The increased stability of insulin against chymotrypsin-promoted hydrolysis of the protein may well arise from such cross-linking. It has been established earlier that chymotrypsin-promoted hydrolysis of free insulin occurs at a site near tyrosine. The reduced number of peptide fractions isolated after chymotrypsin-promoted hydrolysis indicates that the hydrolysis pattern of chromium-containing derivatives of insulin may differ significantly from that of free-insulin. It is probable that in chromium-containing insulin derivatives, chymotrypsin-promoted hydrolysis involves sites after the tyrosine or that cleavage does not necessarily occur at sites near all tyrosine residues. A case now exists based on our results to propose an increased long-range order in chromium insulin derivatives.

3. Chromium–collagen interactions

The main emphasis of the established industrial methods of stabilization of a dead connective tissue, viz. skin, is to complex the protein collagen with a mixture of chromium(III) salts contained in an industrial preparation called basic chromium sulphate (BCS). The composition and constituents of BCS have been extensively analysed (Slabbert 1976; Balasubramanian *et al* 1977; Rao 1991). There is now sufficient experimental evidence to show that three important species contained in BCS are of the molecular structures of 6, 7 and 8 given in figure 2 (Rao 1991; Gotsir *et al* 1992). A molecular basis to tanning needs to take into account the possible range of interactions and assign causes for the observed stability of the collagenous matrix against enzymatic hydrolysis and heat-induced dimensional changes. Currently used models for the mechanism of collagen stabilization through interactions with chromium do not adequately resolve these issues.

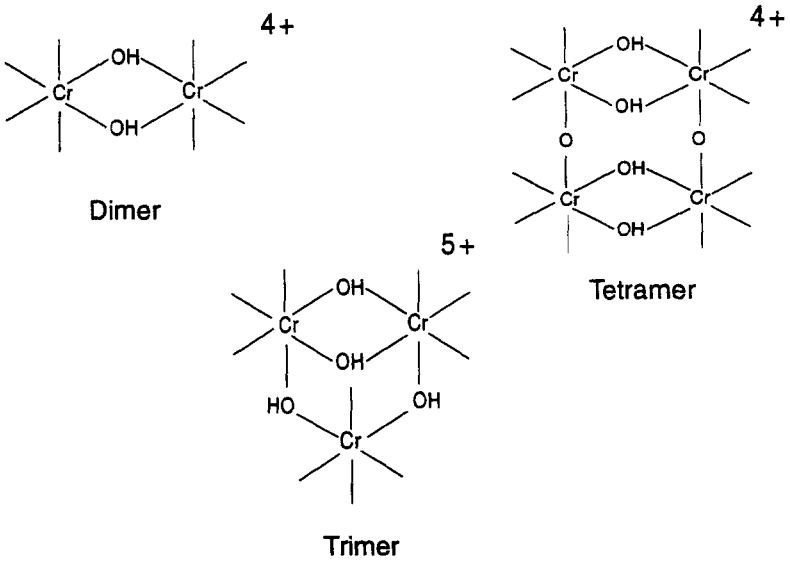

Figure 2. Core structure of polymeric complexes of chromium(III) with relevance to tanning dimer 6, trimer 7 and tetramer 8 with water ligand omitted for convenience.

In this work, an approach has been made to react isolated and well-characterized chromium(III) species with purified rat tail tendon collagen and to examine under transmission electron microscopy any morphological differences in collagen assembly due to interaction with the metal ion. Collagen samples treated with commercial samples of basic chromium sulphate (BCS), dimer, 6, trimer, 7, or tetramer, 8, have been examined under a JEOL-1200 E transmission electron microscope with a magnification of about 35,000. In this approach, self staining by chromium has been used. Generally, a staining technique using heteropolyacids is employed to assess the quaternary structure of collagen (Koller *et al* 1973). Intermolecular and self-assembly of collagen leads to a characteristic quaternary structure which can be easily viewed under a transmission electron microscope. Various levels of organization of collagen in connective tissues are well known (Fleischmajer *et al* 1990) (Figure 3).

The current approach is to investigate the changes brought about by chromium in secondary, tertiary and quaternary structures and discuss their possible consequences on protein stability against heat and enzymatic attack. For assessing the relative stability added by different chromium(III) species, an attempt has also been made to hydrolyse the chromium–collagen adduct using selective cleavage methods using cyanogen bromide. The ease of hydrolysis of Cr(III)–collagen adducts has been assessed as a function of the degree of polymerisation of the chromium species used for stabilization. It is known that CNBr treatment leads to breakdown of proteins at each methionine residue (Bornstein and Piez 1966). Since there are as many as seven residues of methionine in collagen, eight peptides are expected in case of native collagen. Previous studies have shown that native collagen is fragmented into eight residues after CNBr cleavage of collagen (Bornstein and Piez 1966). In this investigation, collagen samples treated previously with dimer, 6, trimer, 7, or tetramer, 8, have been subjected to CNBr cleavage using standard methods (Bronstein and Piez 1966). Polyacrylamide

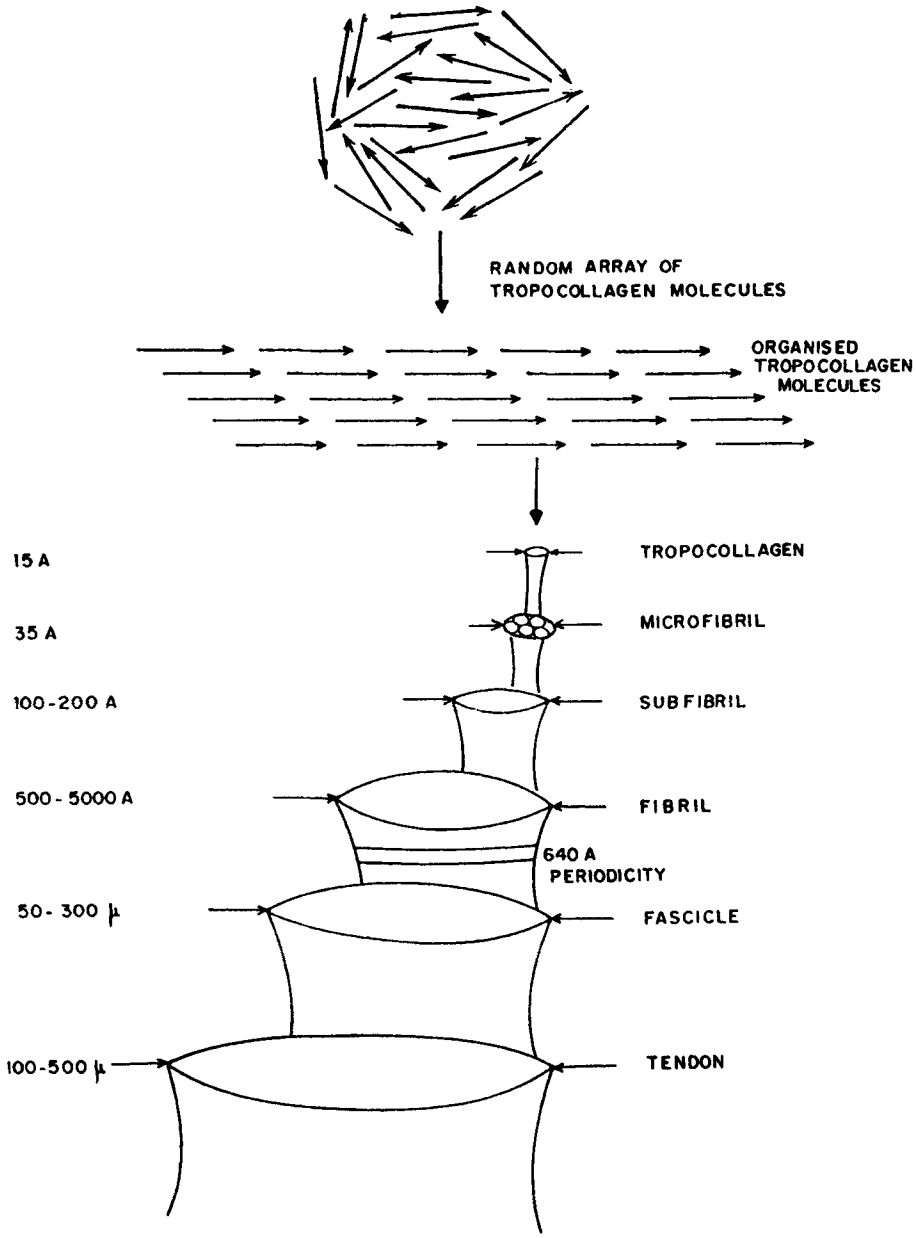

Figure 3. Hierarchial ordering of collagen in connective tissue.

gel electrophoresis (PAGE) has been performed on CNBr hydrolysates employing standard procedures (Laemmli 1970).

Transmission electron microscopic investigations made in this study show that it may be possible to probe the morphological structures of collagen-chromium compound without recourse to staining with heteropolyacids. Significant morphological changes are observed in collagen samples depending on the degree of polymerisation of the chromium species used for stabilisation (figure 4). It has been shown previously that

the tetramer 8, exhibits poor affinity to collagen and is an inefficient tanning species. The transmission electron micrograph of rat tail tendon treated with 8, does not seem to exhibit evidence of staining by chromium. This in part may be due to the poor uptake of the tetramer by collagen as reported earlier (Chandrasekaran *et al* 1989; Rao 1991). The banding pattern of collagen is a characteristic feature of the quaternary structure of the protein. A repeat sequence of 640 Å units has been observed in native collagen and this has been attributed to the intermolecular organization of the 3000 Å long protein (Hodge and Schmitt 1960). Preliminary results of this study seem to favour the hypothesis that Cr(III) ions may modulate the quaternary structure of the protein differently depending on the degree of polymerisation and the molecular structure of the Cr(III) species. At this stage it may be appropriate to review briefly the status of knowledge on structural organization of collagen. The protein exhibits unique primary, secondary, tertiary and quaternary structures. The repeat sequence of the triplet Gly-Pro/Hypro-X pattern has been fundamental to the stability of the three-dimensional architecture of the molecule. Interchain and intrachain hydrogen bonds involving peptide residues are numerous and the triple helical structure of the protein has been well resolved (Ramachandran 1967).

The tertiary structure of the protein influenced by covalent and other crosslinks has been vital to the stability of the connective tissue protein. Lateral and longitudinal aggregation of collagen leads to its quaternary structure. Long-range ordering of the protein promotes a hierarchial ordering leading to the formation of pentafibrils, fibres and fibre bundles (figure 3). It is relevant to discuss the topological distribution of

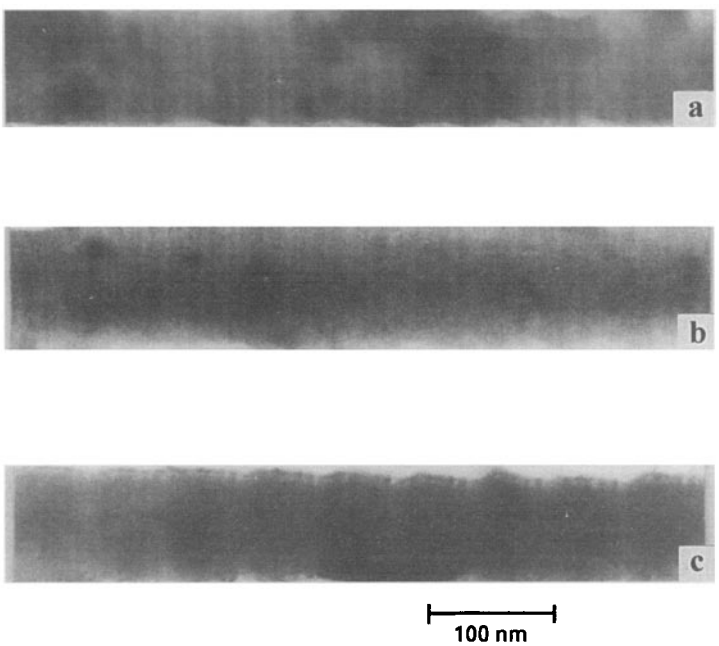

Figure 4. Transmission electron micrographs of rat tail tendon fibre treated with basic chromium sulphate (**a**), dimer (**b**) and trimer (**c**). The micrographs of RTT treated with tetramer, 8, do not show evidence for any bonding pattern, possibly due to poor uptake of Cr(III) resulting in absence of staining.

chromium in the collagen lattice. The microfibril of collagen is believed to consist of a pentagonal array of tropocollagen molecules with typical diagonal distances of 35 Å. The intermolecular distance of tropocollagen in such arrays being small (< 10 Å), it is appropriate to assess whether the molecular dimensions of the chromium species could limit access of the metal ion to all possible functional sites in collagen for coordination to Cr(III). The differences observed in the morphological structure of chromium-collagen adducts with variation in the degree of polymerisation of chromium, viz. dimer, trimer and tetramer, can partially be explained on the basis of such considerations as topological distribution of Cr, kinetic lability and charge.

The hydrothermal stability of rat tail tendon collagen treated with isolated species of dimer, 6, trimer, 7 and tetramer, 8, has been examined. The thermal shrinkage of rat tail tendon collagen treated with BCS, 6, 7, and 8 occurred at 104, 102, 87 and 68°C, respectively. The untreated rat tail collagen underwent shrinkage at 60°C. In other words, the chromium–collagen adduct of the tetramer, 8, does not exhibit any increased hydrothermal stability in relation to rat tail tendon collagen. This corroborates our earlier finding that tetramer, 8, contributes to more than 60% of chromium in the spent chrome tanning solution (Chandrasekaran *et al* 1989; Rao 1991). Since the results presented in this study are more revelatory in nature, than rigorous, discussions need to be limited at this stage to the morphological structures and stabilization of the protein against CNBr cleavages.

The number of bands in the CNBr cleavage product on PAGE varies significantly depending on the nature of the chromium(III) species used for stabilization. Whereas reaction with dimer 6, led to a relatively more stable collagen sample which resisted CNBr cleavage, complexation to the tetramer 8 does not seem to afford significant stability. It is of interest that independent studies have identified the tetramer to be a poor tanning type species (Rao 1991). The poor tanning ability has been traced to its anomalous kinetic lability for aqua ligand substitution (Rao 1991). On the other hand, the trimer 7 afforded substantial stability of collagen. This in part may be attributed to changes in the tertiary and quaternary structure of collagen imposed by interactions of chromium(III) ions with the protein. Although alterations in secondary structures of the protein are feasible due to the influence of chromium on solute–solvent (collagen–water) as against solute–solute (collagen–collagen) interactions, discussions on these need to await further data. This study has, however, shown that the molecular structures of chromium species are of significance. The conventional treatment of chrome-tanning mechanisms based on broad analysis of chromium(III) binding to the side chain aspartic and glutamic acids of collagen molecule overlooks many important details. Investigations on the molecular basis for tanning are ongoing. These aim at directed synthesis of chromium compounds with near total utilization of the metal ion during tanning based on species selection. The environmental consequences of chromium-based tanning methods have also prompted our studies on chromium-induced abnormalities in the phytoagglutinin-induced proliferation of human lymphocytes in laboratory cultures.

4. Chromium interactions in lymphocyte proliferation

The experimental details of the studies on chromium-induced abnormalities in cell proliferation have already been presented elsewhere (Rajaram *et al* 1995). The effect of

six chromium compounds on cell proliferation of lymphocytes has been investigated. The six compounds have included Cr(edta)(H_2O), 2, *trans*-[Cr(salen)(H_2O)$_2$]ClO_4, 3, hexaaquachromium(III) chloride, 9, trisoxalatochromiate, 10, *trans*-diaquabis (salicylideneamino)propanechromium(III) perchlorate, *trans*-[Cr(salprn)(H_2O)$_2$]ClO_4 11, and sodium chromate, 12. Apoptosis has been reported when the compounds 3, 11 and 12 are incorporated into a lymphocyte cell culture in our laboratory (Rajaram *et al* 1995). Significant morphological changes are observed when the three chromium species are incorporated into the cell lines in micro quantities. When the Cr(III) species exhibits solubility at body *p*H values, evidence for the transport of the metal ion into the cell has been obtained regardless of the charge on the molecule. On the other hand, many of the soluble chromium compounds including 2 and 10 do not seem to cause any adverse effects in lymphocyte proliferation. This observation has called for a need to take into account the molecular structure of chromium species in any discussion on metal ion toxicity rather than to generalize the biological consequences of exposure to chromium(III) salts without references to the ligand environment around the metal ion.

5. General treatment of chromium interactions with biosystems

The observed Cr(III)-induced apoptosis has raised many fundamental issues. Since apoptosis is programmed death of cells, the possible effect of chromium on signalling pathways in cell growth is of importance. It is necessary to investigate the structural changes imposed by chromium on biomarker proteins. Possibilities such as the formation of protein–DNA–chromium adducts have been considered as probable causes for the adverse effects of chromium (Wedrychonski *et al* 1985; Costa 1991). Discussions on chromium-induced changes in biosystems need to take into account probable stabilization of unusual oxidation states like Cr(V) and their structural influence on biomolecules and generation of radical sources (Aiyar *et al* 1990). New experimental and compelling ESR evidence has been obtained for the formation of relatively stable Cr(V) species (Kumari *et al* 1995) on the reaction of Cr(VI) with some proteins. Such unusual species may well catalyse large structural changes in biomolecules. A concerted treatment of structural changes imposed by chromium on biomolecules needs to await further data; nevertheless the results presented in this study demonstrate that the roles of chromium in biosystems could involve the promotion of the assembly of biomolecules and formation of intermolecular crosslinks. The implication of the metal ion in the formation of intermolecular crosslinks and induction of long range order in biomolecules seems to merit further study and analysis.

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