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ANALYSIS OF THE REDUCIBLE COMPONENTS OF THE MUSCLE PROTEIN, CONNECTIN: ABSENCE OF LYSINE-DERIVED CROSS-LINKS

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Summary

After exhaustive salt extractions of rabbit and human skeletal muscle, the amino acid compositions of the residual proteins were similar to those reported for connectin. Complete removal of collagen contamination was achieved only after treatment of the connectin preparations with bacterial collagenase. On reduction with KB^3H_4, the small amounts of lysine-derived reducible cross-links that were present in the initial connectin preparations were completely absent after treatment with collagenase. In adult human connectin some hexitol-lysine derivatives were present after reduction. These results indicate that, in contrast to previous reports, connectin does not participate in the same lysyl oxidase-mediated cross-linking system that occurs in collagen and elastin.

INTRODUCTION

Connectin is an intractable protein that has been isolated from skeletal and cardiac muscle (1-3). This protein was proposed as a third structural filament in muscle that is responsible for the mechanical continuity of the fibre by forming a fine network of filaments interconnecting the Z lines. These proposals were based on visualization in the electron microscope of filamentous networks in ghost muscle fibres (2,4) that appeared to have similar properties and amino acid composition to those of connectin (2).

Analysis of the products of reduction with NaB^3H_4 of urea-SDS extracted connectin from chicken skeletal muscle led to the conclusion that the protein contained lysine-derived cross-links (5) and, hence, utilized the same lysyl oxidase dependent cross-linking system that occurs in collagen and elastin (6,7). Subsequently, a study of human skeletal muscle connectin prepared without the use of denaturants indicated that the reducible components

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undergo an age-related decrease in amount (8), similar to that previously determined for collagen (9-11).

In view of the potential importance of this type of cross-linking in muscle, particularly in relation to myofibrillar assembly and the effects of ageing and certain nutritional deficiencies, we have investigated the reducible components of connectin from several different species, the results of which are presented in this report.

MATERIALS and METHODS

Connectin preparation. Mixed skeletal muscles from 2-year-old rabbits, breast muscle from 16-week-old chickens and cardiac muscle from a 2-year-old steer were freshly obtained at slaughter and human psoas muscle (from 39-year-old, male) was obtained within 24 h post mortem. The extraction procedures were based on those of Fujii and Kurosu (8) and were all carried out at 4°C. Samples (100 g wet wt) were minced and homogenized in a Waring blender with 5 vols (v/w) of 25mM-KCl-40mM-borate buffer containing 15mM-mercaptoethanol. After centrifuging at 2000 g for 30 min, the residue was resuspended in 10 volumes of 0.1M-KCl-40mM-borate (pH 7.0)-15mM mercaptoethanol and stirred for 16 h. Fibrous material was removed by passing through cheesecloth and the myofibrillar suspension was centrifuged at 5000 g for 20 min. The residue was extracted by stirring with 0.5M-KCl-10mM-$Na_4P_2O_7$-50mM-NaH_2PO_4(pH 6) containing 15mM-mercaptoethanol for 3 h and was centrifuged at 5000 g for 20 min. The residue was subjected to three further extractions with the 0.5M-KCl buffer and any fibrous material that aggregated during this procedure was removed by filtration. Finally, the residue was stirred with 0.6M-KI-60mM-$Na_2S_2O_3$ for 12 h and after centrifugation this extraction was repeated three times. For cardiac muscle a further extraction with 1.1M-KI-60mM-$Na_2S_2O_3$ was carried out. The insoluble material was washed exhaustively with water and freeze-dried. The collagenous material removed at various stages of the extraction was combined and washed successively with 0.5M-KCl buffer, pH 6, water, 0.6M-KI-60mM-$Na_2S_2O_3$ and finally 0.15M-NaCl-10mM-phosphate, pH 7.5.

Treatment of connectin with collagenase (Sigma, Type V) at an enzyme: substrate ratio of 1:20 was carried out in 40mM-Tris/HCl (pH 7.6)-1mM-$CaCl_2$ -10mM-N-ethylmaleimide maintained for 4 h at 37°C in an orbital shaker. The insoluble material was washed successively with incubation buffer and water and was then freeze-dried.

Borohydride reduction. Samples of connectin and intramuscular collagen were equilibrated with 0.15M-NaCl-10mM-phosphate (pH 7.5) and were reduced with KB^3H_4 at a 1:30 borohydride:protein ratio (w/w) as described previously (10). The reduced proteins were hydrolysed in 6M-HCl for 24 h at 108°C.

Analytical procedures. Amino acid compositions were determined with a Locarte analyser after hydrolysis of the samples in 6M-HCl in sealed, evacuated tubes at 108°C for 24 h. For N^{τ}-methyl-histidine analyses, interference by the relatively large amounts of histidine was obviated by preliminary treatment or a portion of the hydrolysate with histidine decarboxylase for 1 h in acetate buffer, pH 4.5.

Table 1 Amino acid compositions[a] of connectin preparations

	Rabbit		Human	
	Untreated	Collagenase-treated	Untreated	Collagenase-treated
4-Hyp[b]	16	0	11	0
Asp	96	96	94	97
Thr	56	62	50	54
Ser	54	59	50	59
Glu	116	124	118	126
Pro	69	59	56	48
Gly	137	75	96	71
Ala	82	70	77	82
Cys/2	3	5	4	5
Val	53	59	55	58
Met	19	25	22	25
Ile	48	60	53	61
Leu	69	84	76	78
Tyr	25	36	39	38
Phe	28	37	37	40
Hyl[c]	2.2	0.7	2.8	0.0
Lys	65	77	77	79
His	17	21	24	22
Arg	43	51	58	57

[a] Expressed as residues per 1000 residues without correction for hydrolytic recoveries: tryptophan was not determined.
[b] Hydroxyproline was determined separately by a sensitive colorimetric procedure (12).
[c] Analyses of hydroxylysine were carried out on an extended basic column of the analyser using high loadings corresponding to about 2 mg of protein.

Analyses of the labelled compounds in the $KB^{3}H_{4}$-reduced proteins were performed by fractionation of the hydrolysates on an extended basic column of a Locarte analyser, previously calibrated with authentic collagen cross-linking amino acids (13). The identities of the isolated components were confirmed by molecular weight estimation on a 2 x 140 cm column of Biogel-P2 and by analysis of the products of degradation by periodate (14).

RESULTS

The amino acid compositions of the material remaining after exhaustive extractions of myofibrillar proteins from rabbit skeletal muscle and human psoas muscle are shown in Table 1. Attempts to completely remove the collagen contamination by purely physical means (8) were unsuccessful but treatment of the extracted protein with bacterial collagenase effected total removal of the hydroxyproline (Table 1). Consistent with the removal of collagenous material, the relative proportions of glycine and proline were lower after treatment with collagenase. For human psoas muscle,

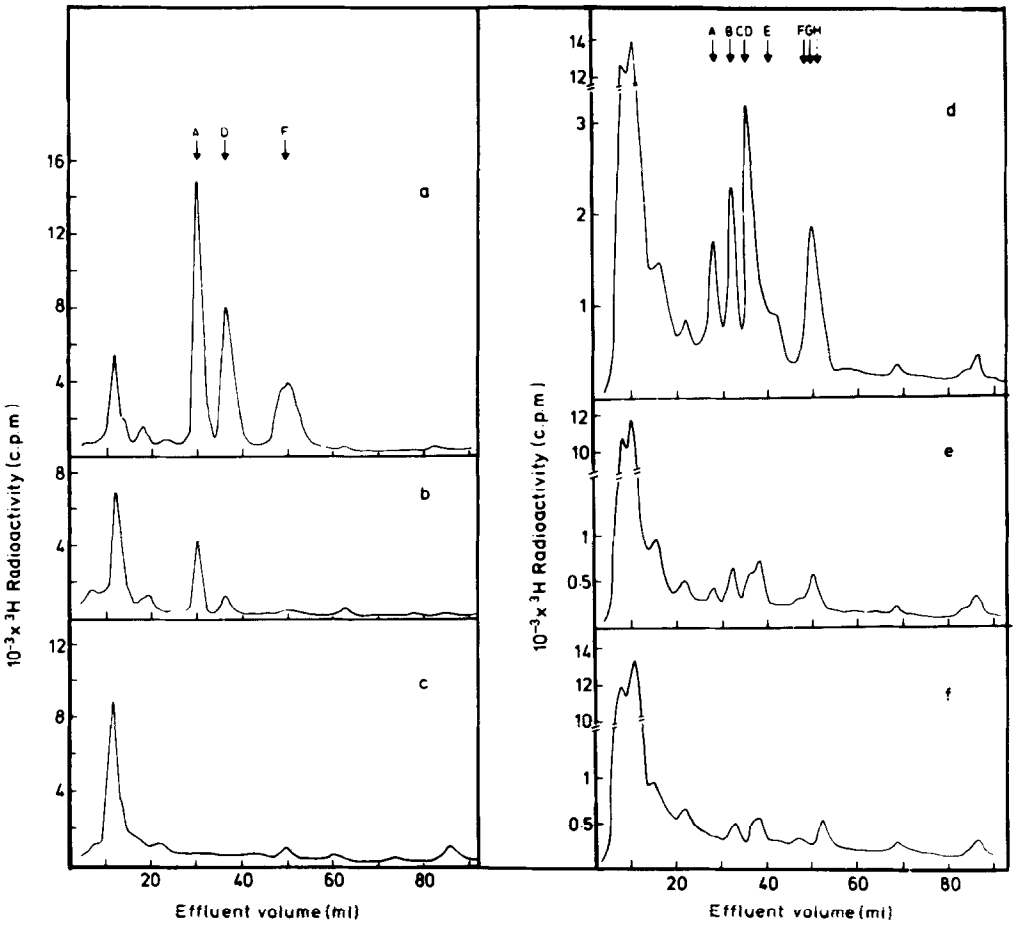

Fig. 1 Chromatography of acid hydrolysates of the KB^3H_4-reduced components of collagen and connectin on a 0.9 x 30 cm column of a Locarte analyser using 0.35M-sodium citrate buffer, pH 5.25 (column temp. 56°C). The radioactive profiles are of (a) intramuscular collagen, (b) connectin prepared by salt extractions and (c) collagenase-treated connectin from rabbit skeletal muscle, and (d) intramuscular collagen, (e) connectin prepared by salt extractions and (f) collagenase-treated connectin from human psoas muscle. Each set of samples, a-c and d-f, were reduced concurrently with the same KB^3H_4 solution for direct comparison. The chromatographic positions indicated are A, dihydroxylysinonorleucine; B, mannitol-lysine; C, glucitol-lysine which co-chromatographs with D, hydroxylysinonorleucine; E and G, anhydro derivatives of hexitol-lysine; F, histidinohydroxymerodesmosine and H, lysinonorleucine.

collagenase treatment resulted in the total removal of hydroxylysine but for rabbit muscle connectin, a small amount of hydroxylysine remained despite the complete absence of hydroxyproline (Table 1). Analyses of

N^{τ}-methyl-histidine revealed the presence of about 0.05 residues per 1000 residues in hydrolysates of collagenase-treated connectin. When collagenase-treated connectin was subjected to SDS-gel electrophoresis (5% acrylamide), the small amount of material that dissolved in the 2% SDS sample buffer exhibited a very high molecular weight.

Analyses of the components labelled by reduction with KB^3H_4 of the connectin preparations and the intramuscular collagens isolated from the same muscles are illustrated in Figure 1. In the young growing rabbit, the three major labelled compounds in the intramuscular collagen were identified as dihydroxylysinonorleucine, hydroxylysinonorleucine and histidino-hydroxymerodesmosine, the latter being an artefact of the reduction procedure (6). In rabbit connectin, the only reducible components in the basic region of the chromatogram were shown to be derived from collagen contamination, as the collagenase-treated sample was completely devoid of labelled components in this region (Fig. 1). Further analysis of the labelled material that eluted with the acid and neutral amino acids (5 - 15 ml) revealed several unidentified components but the reduced cross-link precursors, dihydroxynorleucine and hydroxynorleucine, were entirely absent. In adult human intramuscular collagen, the cross-links were partially masked by hexitol-lysines and their anhydro derivatives that are known to increase with age (9, 15). These components were also detected in the connectin preparation and very small amounts appeared to remain after collagenase treatment. In the latter sample, however, none of the lysine-derived cross-links were present.

Analyses (not shown) of connectin isolated from chicken breast muscle and bovine cardiac muscle gave similar results to those obtained for the rabbit in that none of the known lysine-derived reducible cross-links were detected.

DISCUSSION

The complete absence of lysine-derived, reducible cross-links in connectin from the four species analysed in this study refutes the suggestion

(5,8) that this muscle protein shares the same lysyl oxidase mediated cross-linking system as collagen and elastin. In connectin extracted by urea-SDS treatment lysinonorleucine was proposed as the major reduced cross-link (5). As shown in Fig. 1, lysinonorleucine chromatographs close to one of the anhydro forms of the hexitol-lysines: these anhydro derivatives are produced during acid hydrolysis (15). The hexitol-lysine derivatives were shown to be degraded by periodate whereas lysinonorleucine is stable under these conditions. Reducible lysine-hexose adducts have been shown to be present in erythrocyte membranes and a number of other proteins (16) but these compounds do not constitute intermolecular cross-links. In a study of human connectin extracted without denaturing conditions to preserve labile aldimines, three additional reducible compounds were detected (8), but their involvement in cross-linking was not established. Using similar extraction procedures for human connectin, our analyses showed that only small amounts of hexitol-lysine derivatives were present in the basic region of the chromatogram. In these experiments it was found essential to remove collagen contamination by treatment with collagenase: immunological studies showed that the untreated connectin preparations contained types I, III and V (AB_2) collagens (G.J.R., unpublished results).

The amino acid analyses (Table 1) are similar to the values reported for connectin (1,5). These compositions are not, however, distinctive or characteristic and the question remains as to whether the isolated material represents a discrete molecular species or is a cross-linked aggregate of a number of proteins. If myosin and actin are the only methylated proteins in muscle, then the amounts of N^{τ}-methyl-histidine detected corresponds to about a 10% contamination by myosin or less than 5% contamination by actin. The virtual absence of these proteins in the gel electrophoretograms is indicative of some form of covalent bonding in the protein aggregate. More recently, a number of other muscle proteins that are resistant to high salt extraction have been described. These include the intermediate

filament proteins, desmin and vimentin, that appear to provide lateral interconnection between Z discs (17), and titin, a high molecular weight protein distributed mainly within the Z lines (18). Connectin isolated by the procedures described here may be identical to or be contaminated by a number of these other proteins.

Thus, although further characterization of the isolated protein is necessary, the present results show that cross-linking of these structural components does not involve the copper-dependent enzyme, lysyl oxidase. This enzyme is undoubtedly present in muscle initiating cross-linking of extracellular collagen but its activity within an intracellular compartment containing high concentrations of carnosine and anserine is unlikely, as these peptides are known to chelate copper (19).

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