Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 633-640

EXISTENCE OF LYSINE-DERIVED CROSS-LINKING IN CONNECTIN, AN ELASTIC PROTEIN IN MUSCLE

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Received October 30, 1981

SUMMARY: In the course of isolating and identifying the reducible compounds of connectin fibrils from chicken breast muscle, we found the presence of the lysine-derived cross-link, aldimine form of lysinonorleucine. The failure to detect this compound by Robins and Rucklidge (1980) might be due to treatment of the samples with a crude collagenase preparation, which resulted in complete digestion of connectin. The results from the present study strongly indicate that connectin participates in the lysyl oxidase-mediated cross-linking system which occurs in collagen and elastin.

Connectin is an elastic protein in muscle which is responsible for passive elasticity and mechanical continuity of myofibrils (1,2). This protein has been isolated from both skeletal and cardiac muscles of various species of vertebrates (1-3) and shown to be located between the Z lines of myofibrils, forming a filamentous network (4,5). In a previous study we reported that connectin from chicken breast muscle contains reducible compounds, aldimine forms of lysinonorleucine and histidino-hydroxymerodesmosine, derived from lysine and hydroxylysine aldehydes (6). This finding led to the conclusion that connectin shares the same lysyl oxidase mediated cross-linking system as collagen and elastin. In addition to this report we also showed a significant decrease in the amounts of the reducible cross-links of connectin with

0006-291X/82/020627-08$01.00/0

age, which suggest that the conversion of the reducible cross-links derived from lysine and hydroxylysine aldehydes to non-reducible compounds is an essential step in the maturation of connectin fibrils, similar to collagen fibrils (7).

In contrast to these observations, Robins and Rucklidge have recently reported that no lysine-derived reducible cross-links were detected in connectins obtained from both skeletal and cardiac muscles, although some hexitol-lysine derivatives were present (8). These connectins were shown to be separated from collagenous proteins by treating the initial connectin preparations with collagenase, and they also implied that lysinonorleucine may have been mis-identified due to the presence of hexitol-lysines in the samples. These compounds elute near lysinonorleucine in some chromatographic systems.

The objective of this study was to resolve the discrepancies between the two laboratories, and the results shown below may explain the findings of Robins and Rucklidge.

MATERIALS AND METHODS

Preparation of connectin: High molecular weight connectin was prepared from chicken breast muscle, essentially according to the procedure of Wang et al. (9). Intact chicken breast muscle was directly extracted for 2 min at 100°C with an equal volume of an SDS solution containing 10% SDS, 40mM DTT, 10mM EDTA and 0.1M Tris-HCl buffer, pH 8.0. After being centrifuged for 20 min at 15,000g, the supernatant was subjected to gel filtration on a Bio-gel A-50m column (2.5cm^{2}x 90cm). The eluting solution consisted of 0.1M Tris-glysine (pH 8.8), 5mM EDTA, 0.1% SDS and 0.5mM DTT. The first peaks, containing about a one million dalton protein band on a 3% polyacrylamide gels, were collected and dialyzed against water for several days and then lyophilized. This preparation directly isolated from muscle, Wang's titin, are tentatingly called connectin (T) (cf.ref.10).

Chicken breast myofibrils were throughly extracted with Hasselbach-Schneider solution, water, 0.6M KI, water, 1N acetic acid and water as previously reported (10). The insoluble residue was extracted with an SDS solution, and high molecular weight connectin, connectin (C), was isolated by gel filtration as described for connectin (T).

Reduction with NaB^3H_4 and cross-link analysis: The dry samples of connectin (T,C) and human skeletal muscle collagen were reduced with NaB^3H_4 (11), and lyophilized after dialysis against a large volume of 0.1M acetic acid. The tritiated proteins were hydrolyzed in 3N HCl at 107°C for 48h, and the hydrolysates were evaporated to dryness. Cross-link analysis

was carried out on a 0.9 x 58cm column of Aminex A-4 resin using a 9-chamber gradient with 0.25M sodium citrate, pH 2.9 and 0.4M sodium citrate buffers, pH 8.3 (12, 13, 14). Duplicate samples were also run on a shorter 0.9 x 18cm column of Aminex A-5 resine using 0.35M sodium citrate buffer, pH 5.15 (12, 13). In addition, the major reducible compound of connectin eluting in the basic region of the chromatogram and NaB^3H_4-reduced cross-link, lysinonorleucine, purified from skeletal muscle collagen were run on 0.9 x 58cm column of Aminex A-4 resin using 0.35M sodium citrate buffer, pH 5.28.

Collagenase treatment: Throughly extracted chicken muscle residues were treated with collagenase at an enzyme-substrate ratio of 1:20 according to Robins and Rucklidge (8). The incubation was carried out at 37°C in 40mM Tris-HCl (pH 7.6), 1mM $CaCl_2$ and 10mM N-ethylmaleimide. The collagenases used were TypeV, Sigma and CLSPA, Millipore Corporation.

SDS gel electrophoresis: SDS gel electrophoreses of connectin and collagenase treated connectin were performed according to Werer and Osborn (15), with 3% polyacrylamide gels containing 6M urea.

RESULTS AND DISCUSSION

Connectin is a highly insoluble protein isolated from thoroughly extracted muscle residues (1-3) and largely remains at the top of 5-10% polyacrylamide gels in SDS gel electrophoresis (2). When such connectin aggregates are dispersed in 10% SDS solution containing 0.1% 2-mercaptoethanol, it is observed that slowly migrating doublet or triplet bands appear on SDS gel electrophoresis (10). Recently a similar high molecular weight protein has been isolated from an SDS extract of whole muscle fibers or myofibrils (9), and this doublet band protein designated as titin was shown to exist in muscle residues freed of myosin and actin (16). Subsequently, our comparative studies on amino acid composition, electrophoretic mobilities in polyacrylamide gel electrophoresis and location in myofibrils of titin and connectin clearly demonstrated that both proteins are identical (10). Thus, the present cross-link analysis of connectin has been performed on both preparations showing a doublet, connectin T (titin), or triplet, connectin C, on 3% polyacrylamide gel electrophoresis (10); these connectin preparations had very similar amino acid compositions devoid of any hydroxyproline,

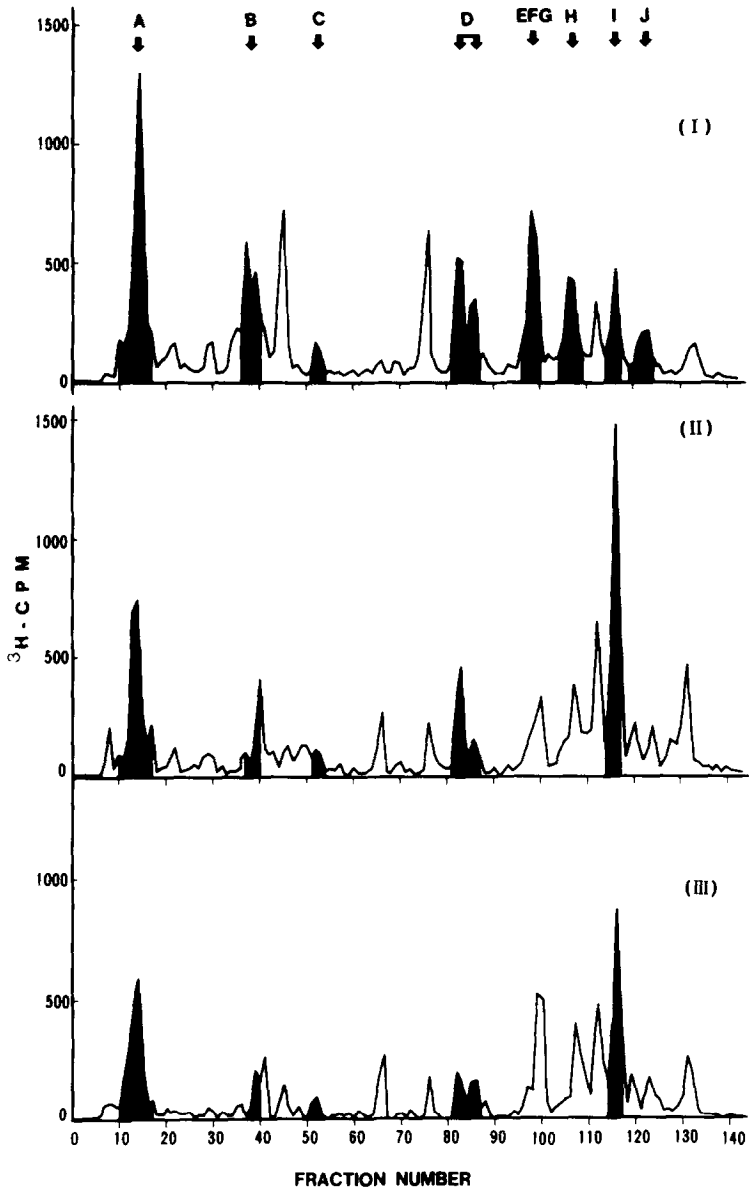


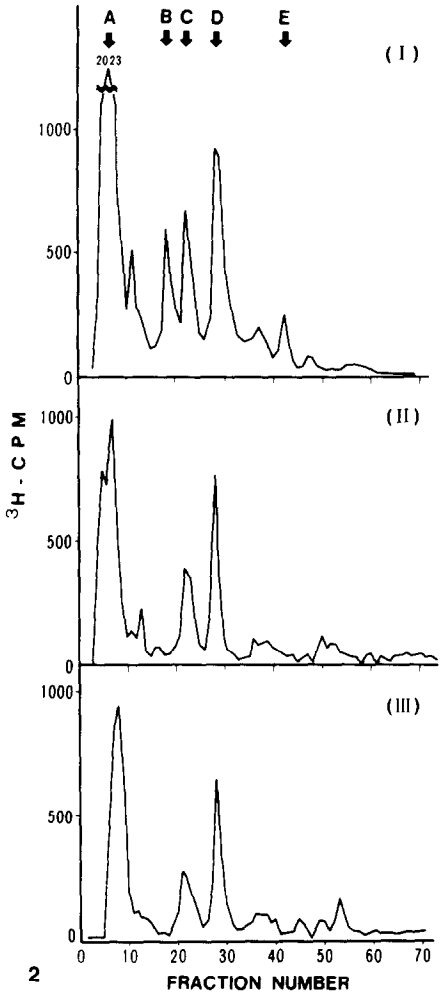
Fig. 1. Chromatographic patterns of the radioactive components in acid hydrolysates of NaB^{3}H$_4$-reduced human skeletal muscle collagen (I), connectin C (II) and connectin T (III) fractionated on a 0.9 x 58 cm column of Aminex A-4 resin using a 9-chamber gradient with sodium citrate buffer. The peaks are: A, unknown (fall through); B, dihydroxynorleucine; C, hydroxynorleucine; D, N$^{\varepsilon}$-hexosylhydroxylysine; E, N$^{\varepsilon}$-hexosyllysine; F, aldol histidine; G, dihydroxylysinonorleucine; H, hydroxylysinonorleucine; I, lysinonorleucine; J, histidino-hydroxymerodesmosine.

indicating that there is no collagen contamination. Fig.1 shows the chromatographic patterns of the radioactive components in

acid hydrolysates of NaB^3H_4-reduced connectins (C,T) and intramuscular collagen. Quite a number of radioactive components were present in both connectin preparations, C and T, and those radioactive profiles were found to be almost identical. Also, the radioactive profile of reduced connectin resembled that of reduced collagen as previously demonstrated (6,7). In connectin, radioactive peaks were observed in the elution positions of the reduced cross-link precursors, dihydroxylysinonorleucine and hydroxylysinonorleucine, N^{ε}-hexosylhydroxylysine and a reduced cross-link, lysinonorleucine. In addition, connectin was found to contain some unidentified radioactive peaks appearing just prior to the elution of lysinonorleucine (6,7).

Although all amino acid and cross-link compounds are eluted from the above column, duplicate samples were run on a second chromatographic system in order to obtain better separation and identification of the basic compounds [Fig.2]. In the chromatogram of connectin, the most prominent peak in the basic region was shown to appear in the elution position of the reduced cross-link, lysinonorleucine.

In order to further establish that the major radioactive compound eluting in the basic region of the chromatogram of connectin is lysinonorleucine, the radioactive compound from connectin and the NaB^3H_4-reduced cross-link, lysinonorleucine purified from skeletal muscle collagen, were separately run on a third chromatographic system (Fig.3.). The elution positions of both compounds were found to be exactly the same. Thus, the present systematic analysis of the cross-links of connectin using three different chromatographic systems strongly support our previous report that connectin contains lysine-derived cross-links (6,7) and excludes the possibility that the major reducible cross-link of connectin identified as aldimine form of lysino-



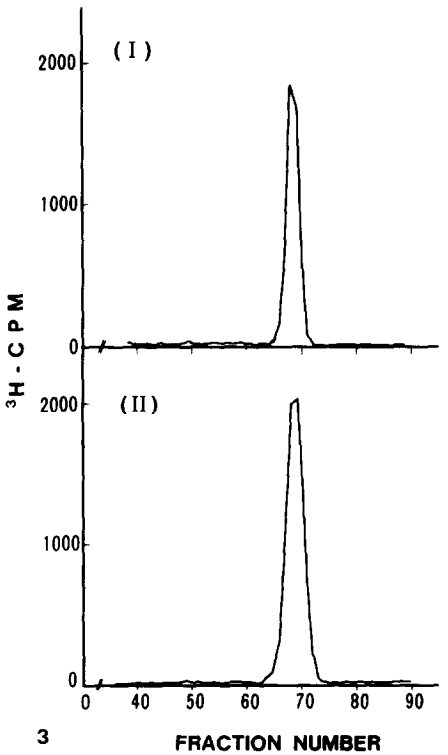


Fig. 2. Chromatographic patterns of the radioactive components in acid hydrolysates of NaB^{3}H$_4$-reduced human skeletal muscle collagen (I), connectin C (II) and connectin T (III) fractionated on a 0.9 x 18 cm column of Aminex A-5 resin using 0.35M sodium citrate buffer, pH 5.15. The peaks are: A, unknown (fall through); B, dihydroxylysinonorleucine; C, hydroxylysinonorleucine; D, lysinonorleucine; E, histidino-hydroxymerodesmosine.

Fig. 3. Chromatographic patterns of lysinonorleucine purified from skeletal muscle collagen (I) and the major radioactive compound of connectin C (II) eluting in the basic region of the chromatogram (Fig. 2) fractionated on a 0.9 x 58 cm column with 0.35M sodium citrate, pH 5.28.

norleucine may be one of the anhydro derivatives produced from the hexitol-lysines during acid hydrolysis (8).

In order to determine why Robins and Rucklidge failed to detect the lysine-derived cross-links of connectin, we have examined

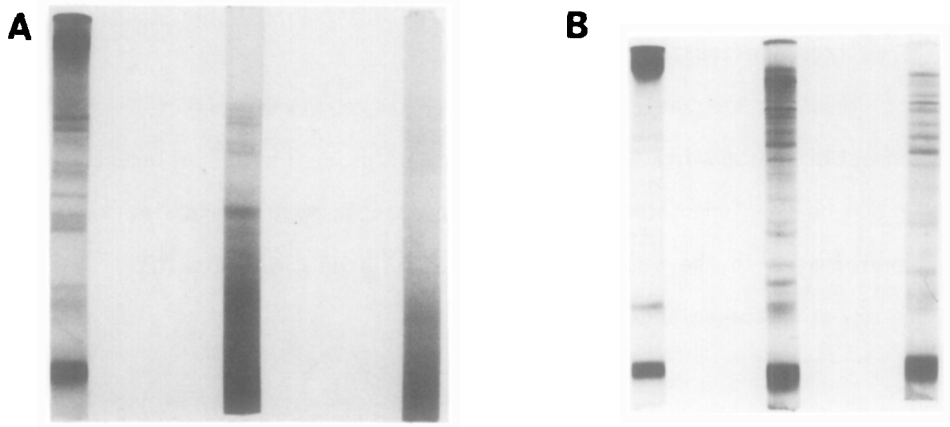


Fig. 4. Effect of collagenase treatment on the throughly extracted residues of chicken skeletal muscle. A, collagenase Type V (Sigma); B, collagenase CLSPA (Millipore). Incubated for 0 (left), 20 min (middle) and 60 min (right) with collagenase, 1/20 protein weight ratio in 1mM $CaCl_2$, 10mM N-ethylmaleimide and 40mM Tris-HCl, pH 7.6 at 30°C. SDS gel electrophoresis was done in 3% polyacrylamide gels.

the effects of collagenase treatment on connectin. One of their major points was that this enzyme removed contaminating collagen along with the cross-links. We used the same collagenase preparation, Sigma Type V. It is apparent from Fig. 4A that treatment of muscle residues with collagenase, Sigma Type V, results in a complete digestion of the high molecular weight connectin. It is generally recognized that a crude collagenase preparation is contaminated with other proteases, and we recently reported that high molecular weight connectin is quite sensitive to proteolytic enzymes (17). Furthermore, it should be noted that even a highly purified collagenase preparation, CLSPA Millipore Corp., was found to split connectin into several peptides of high molecular weight (Fig. 4B). Whether this splitting of connectin was due to contaminating proteases or not remains uncertain. Therefore, it is unlikely that the collagenase-digested preparations obtained by Robins and Rucklidge were intact connectin fibrils containing lysine-derived cross-links and their precursors. In fact, lysine-derived reducible

cross-links were shown in their initial connectin preparations prior to collagenase treatment (8).

Thus, on the basis of a detailed cross-link analysis of connectin and showing the unreliability of using impure collagenase, it can be concluded that connectin, an elastic muscle protein, participates in the same lysyl oxidase-mediated cross-linking system as collagen and elastin.

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