Z-Nin, a New High Molecular Weight Protein Required for Reconstitution of the Z-Disk†

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The materials released from myofibrils by Ca²⁺-activated factor (CAF) were fractionated by using a combination of Sepharose 6B and Bio-gel A 50m columns. To investigate the exact constituents of Z-disks on the basis of success or failure of reconstitution of Z-disks, reconstitution of Z-disks by incubating individual fractions with Z-disk-extracted fiber bundles was carried out, and the proteins in each fraction were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

From the electron micrographs of the reconstituted Z-disks, SDS-polyacrylamide gel electrophoretic analysis and amino acid analysis of the isolated proteins from the gels, it was clear that the proteins required for the reconstitution of the Z-disk, in other words, the principal constituents of the Z-disk, were Z-nin, a new high molecular weight protein having a subunit molecular weight of 300,000~400,000 dalton mentioned in this report, α-actinin and tropomyosin.

In our previous reports,¹² to investigate the exact protein composition of Z-disk, were described reconstitutions of Z-disk by incubating proteins released from myofibrils by Ca²⁺-activated factor (CAF) with Z-disk-extracted fiber bundles. From electron micrograph of the Z-disk reconstituted and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the proteins required for reconstitution of Z-disk, in other words, the principal constituents of Z-disk were presumed to be α-actinin, tropomyosin and unidentified protein having large molecular weight.²)

This paper describes further fractionation of the fraction containing three proteins mentioned above, reconstitution of Z-disk by incubating individual fractions with Z-disk-extracted fiber bundles and amino acid composition of the proteins required for reconstitution of Z-disk.

MATERIALS AND METHODS

Rabbit muscle was used for all experiments reported in this paper. Rabbits were anesthetized with sodium pentobarbital (90 mg) and d-tubocurarine chloride (1.5 mg) just before exsanguination.

Preparation of CAF and myofibrils. Crude CAF was prepared from rabbit longissimus dorsi muscle, and myofibrils were made from back and leg muscle immediately after death by the procedures described by Busch et al.³) Crude CAF was partially purified by the procedure described in the previous paper.¹)

Preparation and fractionation of the materials released from myofibrils by CAF. Myofibrils were incubated with partially purified CAF and the materials released from myofibrils were fractionated by using Sepharose 6B column under the conditions described in the previous paper.²) The materials in the Fraction A eluted first from Sepharose 6B column were collected and concentrated to an appropriate volume with Collodion-Bags (Sartorius-Membranfilter GmbH). The concentrated solution was fractionated by gel permeation chromatography on a 1.2×80 cm Bio-gel A 50 m column. The column was eluted with a buffer of 100 mM KCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA and 5 mM β-mercaptoethanol (β-MCE),

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the eluate was collected in 2.0 ml fractions, and the optical density of the fractions was read at 280 nm. Individual fractions were lyophilized with sucrose added. Lyophilized materials were resolved and dialyzed by the procedure described in the previous paper, and subjected to reconstitution of Z-disk.

Preparation of Z-disk-extracted fiber bundles. Rabbit psoas muscle prepared immediately after death was glycерinated in the usual manner for 60 ~ 80 weeks, and the Z-disks were extracted for 12 days with a solution of 2 mM Tris-HCl (pH 7.6) and 1 mM dithiothreitol (DTT) as described by Stromer et al.4)

Reconstitution of Z-disk. The Z-disk-extracted fiber bundles were incubated for 72 hr with the materials in each fraction at 0°C in 100 mM KCl, 2 mM Tris-HCl (pH 7.6), 1 mM DTT, 2.5 mM Ca²⁺ and 1 mM NaN₃. After incubation, the fiber bundles were washed with the same solution without protein and fixed for electron microscopic examination.

Electron microscopy. Electron microscopic observation was carried out by the method described in the previous paper. Specimens were examined with Hitachi H-300 electron microscope operated at 75 kV.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was conducted by the procedures described by Weber and Osborn. The electrophoresis was performed for 3.5 hr at 8 mA per gel on 8 cm gels containing 5% polyacrylamide. An Ozumur densitometer with a 610 nm filter was used to scan the destained gels.

Amino acid analysis. Amino acid composition of the stained proteins separated by SDS–polyacrylamide gel electrophoresis was determined in accordance with the procedures described by Sreekrishna et al. Each stained band was excised with a razor blade from the SDS-polyacrylamide gel. The coomassie-protein–SDS complex was extracted, dialyzed and lyophilized. The dry sample was hydrolyzed in a sealed evacuated tube for 20 hr at 110°C with 5.7 n HCl. Amino acid analysis was carried out on a Hitachi Model 835 amino acid analyzer.

Protein concentrations. Protein concentrations were measured by the biuret method that was standardized against crystalline bovine serum albumin.

RESULTS

Gel permeation chromatography of the materials eluted first from 6% agarose column

When the materials in the Fraction A eluted first from Sepharose 6B (6% agarose) column were concentrated and applied to Bio-gel A 50 m column, the elution profile shown in Fig. 1 resulted. The elution profile was arbitrarily divided into three fractions, A-A, A-B and A-C as shown Fig. 1. Each fraction was lyophilized with sucrose added and subjected to reconstitution of Z-disk or SDS-polyacrylamide gel electrophoresis.

Reconstitution of Z-disk by incubating the materials in fractions A-A ~ A-C with Z-disk-extracted fiber bundles

Electron micrographs of Z-disk-extracted fiber bundles and Z-disk-reconstituted fiber bundles obtained by incubation with individual fractions A-A, A-B and A-C are shown in Figs. 2 ~ 5. Only the materials in Fraction A-C have been bound to Z-disk region, and the Z-disk extracted from myofibrils with a low ionic strength solution has been almost completely reconstituted. The zig-zag configuration as observed in the intact Z-disk can be seen in the reconstituted Z-disk. When the Z-disk-extracted fiber bundles were incubated with the materials in Fraction A-A or A-B, the increase of the materials accumulated to the Z-disk region was observed, but the reconstitution of Z-disk was not successful.
Protein Composition of Z-Disk

Glycerinated rabbit *psoas* muscle was teased into thin bundles and extracted for 12 days with 2 mM Tris-HCl, pH 7.6, 1 mM DTT at 0°C. This and all other samples for electron microscopy were fixed with glutaraldehyde-OsO₄, embedded in Epon, and stained with uranyl acetate-lead citrate. Each scale mark on electron micrographs indicates 1.0 μ. (× 15,000)

Reconstitution was carried out by incubating the materials in Fraction A-A (0.487 mg/ml) with 3 ~ 4 pieces of Z-disk-extracted fiber bundles (0.1 × 10 mm) for 72 hr in the medium adjusted to 100 mM KCl, 2 mM Tris-HCl, pH 7.6, 1 mM DTT, 2.5 mM Ca²⁺ and 1 mM NaN₃ at 0°C. (× 15,000)

**SDS-polyacrylamide gel electrophoresis of fractions A-A ~ A-C**

The lyophilized materials in Fractions A-A, A-B and A-C were dissolved in 1.0% SDS, 1% β-MCE and 0.01 M sodium phosphate, pH 7.0 by warming at 37°C for 2 hr, and then electrophoresed in 5% polyacrylamide gels in the presence of 0.1% SDS and 0.1 M sodium phosphate, pH 7.0. The electrophoretic patterns of Fractions A-A ~ A-C, and densitometric scan of Fraction A-C are shown in Figs. 6 and 7, respectively. Molecular weights of the protein bands were estimated by using known proteins as standards. From electron microscopic observation and electrophoretic pattern, it is clear that proteins required for the recon-
Reconstitution was carried out by incubating the materials in Fraction A-B (0.487 mg/ml) with 3~4 pieces of Z-disk-extracted fiber bundles (0.1 x 10 mm) under the same conditions as specified for Fig. 3. (x 15,000)

Reconstitution was carried out by incubating the materials in Fraction A-C (0.531 mg/ml) with 3~4 pieces of Z-disk-extracted fiber bundles (0.1 x 10 mm) under the same conditions as specified for Fig. 3. (x 15,000)

Reconstitution of Z-disk were distributed to Fraction A-C. Fraction A-C consisted principally of proteins having subunit molecular weights near 300,000~400,000 (S-1), 100,000 (S-2) and 34,000 (S-3) daltons.

Amino acid composition of proteins S-1 ~ S-3

Star diagrams of the amino acid composition of the isolated proteins S-1 ~ S-3 and total number of amino acids of the protein S-1 that was standardized at 1000 residues are shown in Figs. 8~10 and Table I. In each figure, star diagrams of the amino acid composition of the proteins indispensable for identifying the isolated proteins with known myofibrillar proteins, i.e. titin and connectin (Fig. 8), z-actinin (Fig. 9) and tropomyosin (Fig. 10), are also shown. As shown in Fig. 8,
Protein Composition of Z-Disk

Fig. 6. SDS-Polyacrylamide Gel Electrophoresis of Fractions A-A, A-B and A-C. Amount of protein loaded onto each gel was about 30 μg. See Fig. 1 for definition of Fractions A-A, A-B and A-C. (a) Fraction A-A; (b) Fraction A-B; (c) Fraction A-C.

Fig. 7. Densitometric Scan of SDS-Polyacrylamide Gel of Fraction A-C. This is scan of gel (c) referred to in Fig. 6. Arrow indicates origin.

Fig. 8. Star Diagrams of Amino Acid Compositions of Protein S-1, Titin and Connectin. Each diagram is formed by plotting the number of residues of each amino acid standardized at 100 residues of leucine and joining up the points obtained. (a) protein S-1; (b) titin, prepared by the procedure described by Wang et al.; (c) titin, taken from the paper by Maruyama; (d) connectin, taken from the paper by Maruyama.

Fig. 9. Star Diagrams of Amino Acid Compositions of Protein S-2 and α-Actinin. (a) protein S-2; (b) α-actinin, purified from rabbit, electrophoresed, extracted, hydrolyzed and analyzed by the procedure described in the text; (c) α-actinin, taken from the paper by Robson et al.
protein S-1 is clearly different from titin or connectin. The most remarkable differences were that protein S-1 had lower amount of proline content than that of titin or connectin, as well as lower contents of lysine and arginine. As shown in Figs. 9 and 10, amino acid compositions of proteins S-2 and S-3 seem to be close to the compositions of α-actinin and tropomyosin, respectively. Higher contents of serine and glycine of proteins S-2 and S-3 may be derived from polyacrylamide gels. A slight difference of the star diagram of the tropomyosin isolated from the gels (Fig. 10-b) from other two diagrams seems to be due to some contaminants of troponin T.

**Mobility of protein S-1 and titin on SDS-polyacrylamide gel**

SDS–polyacrylamide gel electrophoretic patterns of protein S-1 and titin are shown in Fig. 11. Higher mobility of protein S-1 on SDS-polyacrylamide gel as compared with titin is another line of evidence that protein S-1 is different from titin.

From the results obtained in this report, protein S-1 having subunit molecular weight 300,000 ~ 400,000 dalton is a new high molecular weight protein different from myofibrillar proteins reported up to now and is indispensable for the reconstitution of Z-disk. To indicate these, we have named the protein Z-nin.

**DISCUSSION**

Our previous papers \(^{1,2}\) described the reconstitution of Z-disk by incubating some pro-
Protein Composition of Z-Disk

Proteins released from myofibrils by CAF with Z-disk-extracted fiber bundles. From electron micrograph of the Z-disk reconstituted and SDS-polyacrylamide gel electrophoresis, it was indicated that the proteins in Fraction A eluted first from 6% agarose column were essential for the reconstitution of Z-disk. In this paper, the proteins in Fraction A were further fractionated by using Bio-gel A 50m column and reconstitution of Z-disk by incubating individual fractions with Z-disk-extracted fiber bundles were carried. The results obtained indicated that the proteins in Fraction A-C (Fig. 1) have bound to Z-disk region in the Z-disk-extracted fiber bundles and Z-disk extracted from myofibrils with a low ionic strength solution has been almost completely reconstituted.

As shown in the SDS–polyacrylamide gel electrophoretic patterns, Fraction A-C consisted principally of proteins having subunit molecular weights near 300,000–400,000, 100,000 and 34,000 daltons. We tentatively named these proteins S-1, S-2 and S-3, respectively. To identify these proteins, stained proteins were extracted from gels, hydrolyzed and subjected to amino acid analysis. The presence of high molecular weight proteins including protein S-1 in the Z-disk region has been suggested by many workers.2,8–11 Especially, titin10 and connectin11 are representative because of their higher contents in myofibrils, and the possibility that titin was identical with connectin was suggested by Maruyama11 and Maruyama et al.12 So it is very important to study whether protein S-1 is identical with titin or connectin, or not. Amino acid analysis and SDS–polyacrylamide gel electrophoretic analysis showed clearly that protein S-1 was different from titin or connectin. And protein S-1 was easily solubilized from myofibrils by CAF, whereas connectin has not yet been obtained in non-denaturing reagent. From these results, protein S-1 seems to be a new high molecular weight protein different from myofibrillar proteins reported up to now. Judging from the yield of the proteins in Fraction A-C and densitometric scan of the gel A-C, yield of the protein S-1 is estimated to be about 2–4 mg from 1000 mg of myofibrils. Consequently, allowing for preparative losses of 50%, rabbit skeletal muscle seems to contain 0.4–0.8% of its myofibrillar protein as protein S-1. This is slightly lower than the amount of α-actinin contained in the muscle.13,14 The success or failure of the reconstitution of Z-disk depends on the presence or absence of this new protein S-1. To indicate these, we have named the protein Z-nin. On the basis of subunit molecular weights and amino acid analysis, the proteins S-2 and S-3 are identical with α-actinin and tropomyosin, respectively. Higher contents of serine and glycine of the proteins S-2 and S-3 as compared with the values of α-actinin and tropomyosin appeared on the references13,15 may be derived from acrylamide gels as indicated by Kyte.16 α-Actinin has been established as a Z-disk protein by antibody labeling experiments by Masaki et al.17 and Schollmeyer et al.18 There is still some controversy as to whether or not tropomyosin is a constituent of Z-disk. The presence of tropomyosin in Z-disk was reported by Huxley,19 Corsi and Perry20 and Suzuki et al.,2 and on the other hand, was denied in the papers written by Masaki et al.,17 Schollmeyer et al.18 and others.4,21 Recently, Sainsbury and Bullard22 reported the presence of a new proline-rich protein having same subunit molecular weight with tropomyosin in insect Z-disk and indicated that most of the preceding evidence for tropomyosin as a Z-disk constituent was based on the identification of tropomyosin by its subunit weight obtained from SDS–polyacrylamide gel electrophoresis. However, protein S-3 in this report is identical with tropomyosin on the basis of amino acid analysis in addition to the subunit molecular weight, and proline content of the protein S-3 is quite lower as compared with the new protein reported by Sainsbury and Bullard.22 The difference in the amino acid composition of the protein having same subunit molecular weight in Z-disk protein may be due to the difference
between insect and vertebrate muscles. Our result presents a new evidence that tropomyosin is a constituent of Z-disk. The reconstituted Z-disk, SDS-polyacrylamide gel electrophoresis and amino acid analysis of the isolated proteins, the proteins required for the reconstitution of Z-disk, in other words, the principal constituents of Z-disk are Z-nin, a new high molecular weight protein named in this report, α-actinin and tropomyosin. In addition to the principal proteins proposed in this report, proteins easily digested by CAF such as desmin and other proteins such as synemin, eu-actinin and 220,000 dalton protein can be counted for the constituents of Z-disk. Further study of the Z-disk constituents including Z-nin is now under way in our laboratory.

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