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LOW-TEMPERATURE EXTRACTION OF α-ACTININ-LIKE PROTEIN FROM THE I–Z–I BRUSH OF STRIATED MUSCLE*

E. J. BRISKEY AND T. FUKAZAWA**

Muscle Biology Laboratory, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisc. (U.S.A.)

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SUMMARY

1. I–Z–I brushes were subjected to eleven mild, short-term, low-temperature extractions. The ultrastructure of the I–Z–I brushes was observed after three, five and eleven extractions. The protein in each extract was checked for α-actinin-like characteristics.

2. There was little apparent ultrastructural change through Extract III, except for some loosening of the I-filament and Z-band structure. Only a small amount of the intermediate weight component of α-actinin-like protein was found in these extracts. This protein did not affect the superprecipitation of an actomyosin system at low ionic strength. Between Extractions V and XI, the Z-bands disappeared and the I-filaments became individually separated. As the Z-bands disappeared, a heavy component of α-actinin-like protein was solubilized, with a progressive increase in the solubilization of a light weight 6-S component of α-actinin-like protein during the latter extractions. Both the heavy- and light-weight components accelerated the superprecipitation of different kinds of actomyosin, with the heavy component being effective on all preparations, even though at low concentrations, it did not influence the viscosity of F-actin.

3. Reconstituted actomyosin, with its F-actin moiety coming from I-filaments after all of the α-actinin-like protein had been extracted, still showed a marked response to both the heavy and light components of α-actinin-like protein.

INTRODUCTION

α-Actinin[2] is thought to play a role in the structure of the Z-band and its attachment to the I-filaments[2–4]. In a partially purified form, it consists[5] of at least three components with $s^{\circ}_{20,w}$ sedimentation coefficients of 6, 10 and 25. The 6-S component, based on immunochemical techniques[2], and trypsin digestion[4] has been postulated to exist in the Z-band. The original preparatory procedures involved 4–9 h room temperature extractions with considerable tendency to aggregate.

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** While a visiting scholar in the Muscle Biology Laboratory, University of Wisconsin; permanent address: Department of Animal Science, Hokkaido University, Sapporo, Japan.

Our approach, therefore, was to prepare I–Z–I brushes (Z-bands with I-filaments on both sides) which were completely free of myosin A, and to exhaustively extract these I–Z–I brushes[6,7] with mild solvents at 0°. We followed the changes in fine structure of the I–Z–I brush[7], as the Z-lines started to disappear and left only single loose I-filaments. Simultaneously we evaluated the actinin-like portion of the extracted proteins.

METHODS

Rabbits were given sodium pentobarbital (90 mg) and α-tubocurarine chloride (1.5 mg) prior to exsanguination. All skeletal muscles were excised immediately, immersed in ice, and coarsely (2 mm) ground. All subsequent preparation, extraction and handling procedures were carried out at 0–1°.

Preparation of I–Z–I brushes

The separate preparation of I–Z–I brushes in bulk was performed by the procedure of FUKAZAWA *et al.*[6], which essentially consisted of the preparation of myofibrils, exhaustive removal of myosin A, and extensive and special washing and handling procedures.

Extraction of I–Z–I brushes

Each extraction of the I–Z–I brushes consisted of the following: (a) Addition of 0.5 vol. of a solution which was 0.2 mM ATP and 0.2 mM ascorbate (pH 7.5). (b) Stirring slowly for 30 min. (c) Centrifugation at 37000 × *g* for 60 min.

The supernatant after each centrifugation was given its respective extract number and the residue was subjected to a repetition of the extraction procedure.

Electron microscopic observations

I–Z–I brushes were centrifuged at 3000 × *g* for 15 min and washed once with 0.1 M phosphate buffer (pH 7.0). Particles of the I–Z–I brushes were then immersed in cold 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After the glutaraldehyde was drawn off the particles, they were washed twice with 0.1 M phosphate buffer (pH 7.0) and fixed for 2 h with cold 1 % OsO_4, buffered with veronal acetate (pH 7.3). The fixed particles were then dehydrated in graded alcohol and propyleneoxide and embedded in an Epon 812 mixture. Sections were cut with a glass knife in a Porter-Blum MT-2 ultramicrotome and stained with a 1 % solution of uranyl acetate and undiluted lead citrate[8]. All electron micrographs were taken with a Hitachi Hu-11 B instrument.

Fractionation of the extracts

Each extract was fractionated into α-actinin and troponin–tropomyosin containing proteins by the procedure of EBASHI AND EBASHI[9]. The portion which precipitated with 22.5 g $(NH_4)_2SO_4$ per 100 ml of extract was called the crude extract. After the $(NH_4)_2SO_4$ was removed by dialysis against 1 mM $KHCO_3$, the protein solution was adjusted to 3.3 M KCl by the addition of solid KCl. This is the procedure of EBASHI AND EBASHI[1] to precipitate α-actinin from crude extracts and from actin preparations[1,10] and represents the crude α-actinin preparation in our studies. The

α-actinin was separated into light, intermediate and heavy fractions according to NONOMURA[5].

Preparation of well-washed myosin B

Myosin B was prepared essentially according to SZENT-GYORGYI[11] with slight modification[12] and converted to desensitized well-washed actomyosin by the procedures of PERRY *et al.*[13].

Preparation of trypsin-treated myosin B

Myosin B was prepared essentially according to SZENT-GYORGYI[11] and subjected to trypsin treatment, within 3 days of preparation, by the procedure of WATANABE AND STAPRANS[14]. Myosin B suspensions (1 mg protein per ml in 0.1 M KCl and 20 mM Tris–maleate buffer (pH 7.0)) were incubated with trypsin (10 μg/ml) for 10 min at 25°. The reaction was terminated by the addition of trypsin inhibitor (20 μg/ml). Subsequently the suspensions were washed twice with 0.1 M KCl and adjusted to 0.6 M KCl with 3 M KCl solution. All trypsin-treated myosin B samples were used within 2 days of preparation.

Actin

Most of the actin preparations were obtained by the procedure of SERAYDARIAN *et al.*[10]. In some instances the acetone powders were prepared from I-filaments after the eleventh extraction. This procedure provided an actin powder completely free of all Z-band constituents.

Viscosity

The relative viscosity was estimated with an Ostwald viscometer.

Myosin

This protein was prepared according to the procedure of PERRY[15] with minor modifications. The purification steps involved dissolution in 0.2 M KCl, and centrifugation at 20000 × *g* for 1 h.

Reconstituted actomyosin

Actomyosin was prepared by mixing polymerized actin and myosin in the proportion of 1:4 (w/w) at 0.1 M KCl. After centrifugation the sedimented actomyosin was washed once with 0.1 M KCl and subsequently adjusted to 0.4 M KCl by dialysis or by direct addition of a 3 M KCl solution.

Protein concentration

All protein concentrations were determined by the biuret method[16] after calibration against a nitrogen analysis (micro-Kjeldahl). Bovine serum albumin preparations were also used as standards.

Turbidimetric test for superprecipitation

Superprecipitation was assayed by the turbidimetric test of EBASHI[17] and as modified by YASUI AND WATANABE[18] and SERAYDARIAN *et al.*[10]. This was done in 1 cm × 1 cm glass cells of 4 cm height, in the optical path of the Beckman DU spectrophotometer (660-nm wavelength) in the Gilford arrangement for recording,

at 27°, with magnetic stirring during the addition of the components of the reaction mixture. The composition of the reaction mixture will be specified in the description of individual experiments.

Ultracentrifugal analysis

All samples were given ultracentrifugal inspection in a Spinco Model E ultracentrifuge. The conditions of these observations will likewise be described in the appropriate sections.

RESULTS AND DISCUSSION

I–Z–I Brushes

The complete study of ultrastructural changes during extraction has been reported elsewhere[7] in connection with the removal of the calcium-sensitizing proteins. Through Extract III we see little ultrastructural change, except for some loosening of the I-filament and Z-band structure. Fig. 1 shows the ultrastructure of the I–Z–I brush after five extractions. Extensive loosening is apparent in the I- and Z-bands, however, the zigzag configuration and integrity of the Z-band are still distinct. After eleven extractions (Fig. 2) the Z-bands have disappeared and the I-filaments are all individually separated. Thus, we can see that the major change in the Z-band takes place from Extract V to XI, although Extracts III and IV bring about some loosening in the Z-band; Extracts I, II and III have their primary influence on the loosening of the I-bands, with only a slight easing of tension in the Z and I–Z regions.

Ultracentrifugal inspection of crude extracts (precipitated by 22.5 g $(NH_4)_2SO_4$ per 100 ml)

Fig. 3 shows the ultracentrifugal sedimentation of the crude Extracts I–XI. The typical α-actinin peak[1,4,10] is not evident until Extract IV. It is probable, as

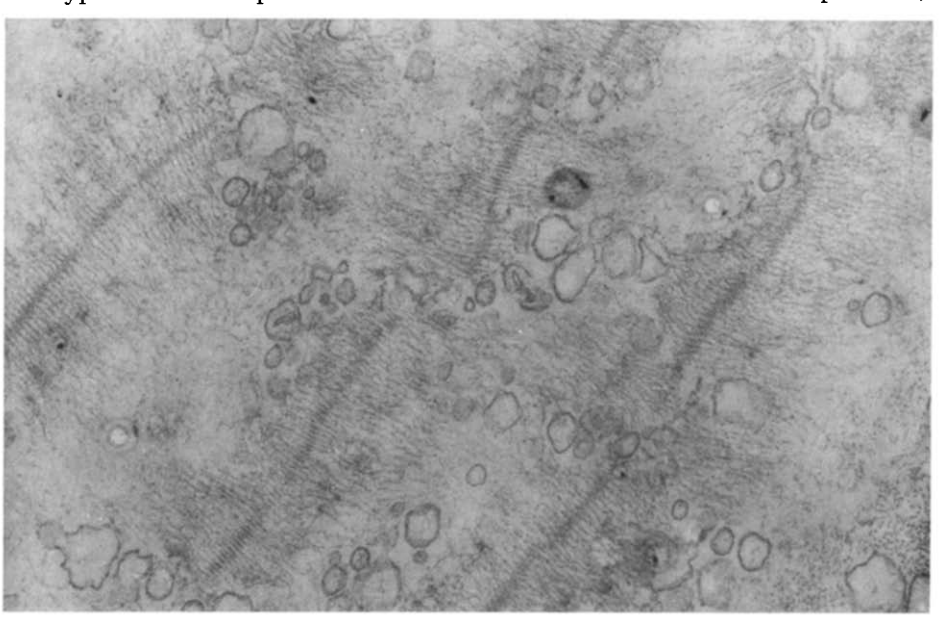


Fig. 1. Ultrastructure of I–Z–I brush after five extractions (× 37800).

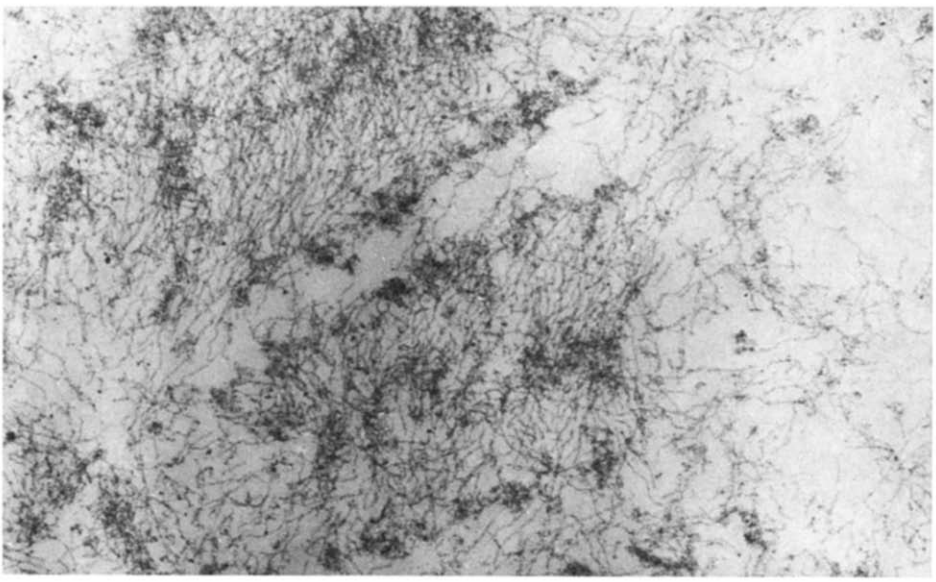


Fig. 2. Ultrastructure of I–Z–I brush after eleven extractions (only I-filaments remaining) (× 90 000).

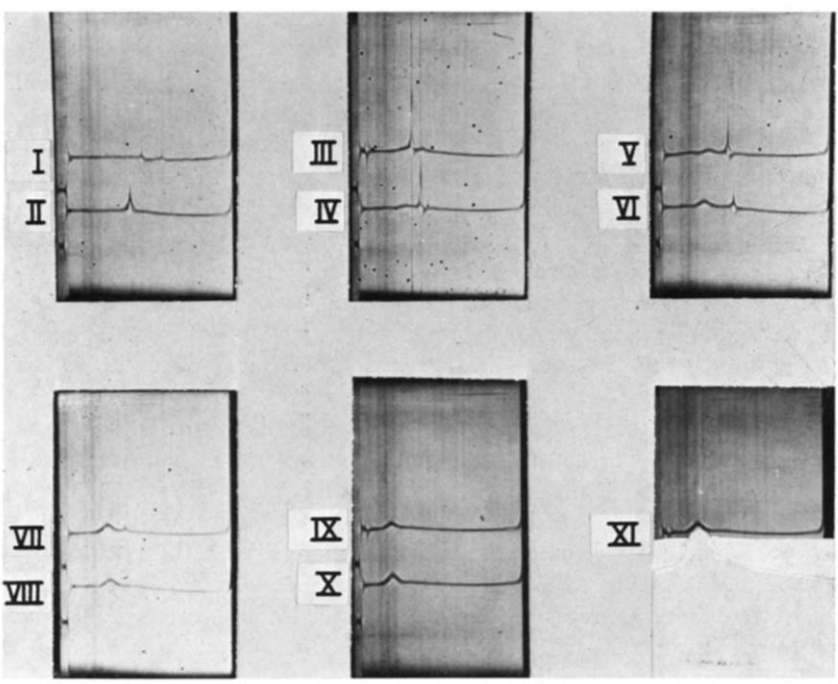


Fig. 3. Ultracentrifugal sedimentation of crude extracts precipitated with 22.5 g $(NH_4)_2SO_4$ per 100 ml. Conditions: 60 000 rev./min; 20°; protein concentration: I, 1.92 mg/ml, II, 3.40 mg/ml, all the rest 6.0 mg/ml; ionic environment, 1 mM $KHCO_3$; pictures each taken 33 min after reaching full speed.

will be pointed out in the next figure, that these first three extracts do contain a small amount of the intermediate weight component of α-actinin-like protein. Another protein or complex with a rather distinct boundary appears in these early extracts. Further studies are being conducted on the nature of this protein, however, at this time we can say that it does not have any calcium-sensitizing activity, it does not precipitate with the addition of 3.3 M KCl nor does it accelerate the onset or extent of the superprecipitation of an actomyosin system. From Extract IV through XI there is an apparent increase in the amount of α-actinin-like protein in the crude extracts. Further, it appears that after Extract VII, there is an increase in the amount of the light-weight component of α-actinin.

Several authors[14,19,20] have used the absorbances at 278, 260 nm and the ratio 278/260 nm to give an indication of the identification of the protein or its composition. An increase in absorbance at 278 nm to approximately 10 is indicative of α-actinin-like protein. Our extracts ranged from 2.58 for Extract II, 2.71 for Extract III, 7.3 for Extract V, and 10.15 for Extracts X and XI.

Separation of α-actinin by 3.3 M KCl treatment of protein precipitated by 22.5 g $(NH_4)_2SO_4$/100 ml

Fig. 4 shows the percentage of α-actinin (3.3 M KCl precipitated) in the $(NH_4)_2SO_4$ (22.5 g/100 ml) precipitated protein from Extracts IV through XI. The amount of the precipitated protein in Extracts I–III was so low that the quantitative separation of α-actinin (3.3 M KCl precipitated) could not be realized. The α-actinin protein ranged from 45 to 95 % of that precipitated by $(NH_4)_2SO_4$; initially, therefore, $(NH_4)_2SO_4$ was precipitating considerable protein other than α-actinin, with several possibilities coming to mind. First of all, some of this fraction could represent protein still undescribed to date; secondly, some could be actin; and thirdly, some could be actin in complex with α-actinin or troponin[21]. Further work on this point is underway, however, we can say that all of the protein remaining in solution after 3.3 M KCl precipitation was totally unreactive in terms of the accepted effect of α-actinin in accelerating superprecipitation or increasing the viscosity of actin. Toward the last extraction the yields of α-actinin have increased and hardly any protein other than α-actinin is being precipitated by $(NH_4)_2SO_4$. α-Actinin-like protein from Extract IV through XI all had absorbance values at 278 nm of 10–11.

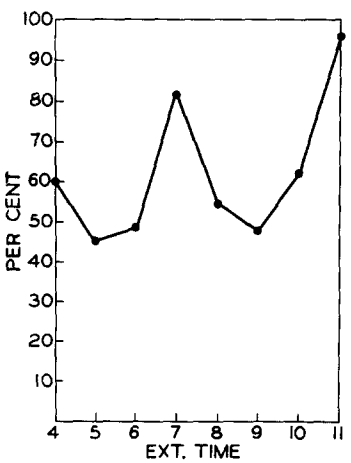
Fig. 5 shows the ultracentrifugal analysis of α-actinin-like protein precipitated by 3.3 M KCl treatment of protein from the various extracts that had precipitated by 22.5 g $(NH_4)_2SO_4$ per 100 ml. These crude α-actinin preparations, which were precipitated by 3.3 M KCl, show little light-weight component (6 S) until after the V–Vl extracts; the light-weight component becomes more distinct in extracts near and including X and XI. When the α-actinin preparations were pooled into three groups: (A) α-actinin from Extracts I–III, (B) α-actinin from Extracts IV–IX and (C) α-actinin from Extracts X–XI, it can be noted (Fig. 6) that pooled α-actinin A has a predominance of intermediate component[5], while pooled α-actinin B has intermediate, a predominance of heavy- and some light-weight component. The last two extracts, or pooled α-actinin C has the appearance of being richer in the light-weight component (6 S) than does either A or B.

Effect of pooled α-actinin preparations on superprecipitation of trypsin-treated myosin B

When tested with trypsin-treated myosin B, the first three α-actinin preparations, pooled together as α-actinin A, are not effective in accelerating the onset or extent of superprecipitation (Fig. 7). Pooled α-actinin Preparations IV–IX, as α-actinin B, are the most effective, with still a substantial effect remaining with α-actinin C (pooled preparations X–XI).

Separation of pooled α-actinin into light, intermediate and heavy components

Crude α-actinin preparations from Extracts IV–IX were substantial enough so that when they were combined we had an adequate quantity to separate into



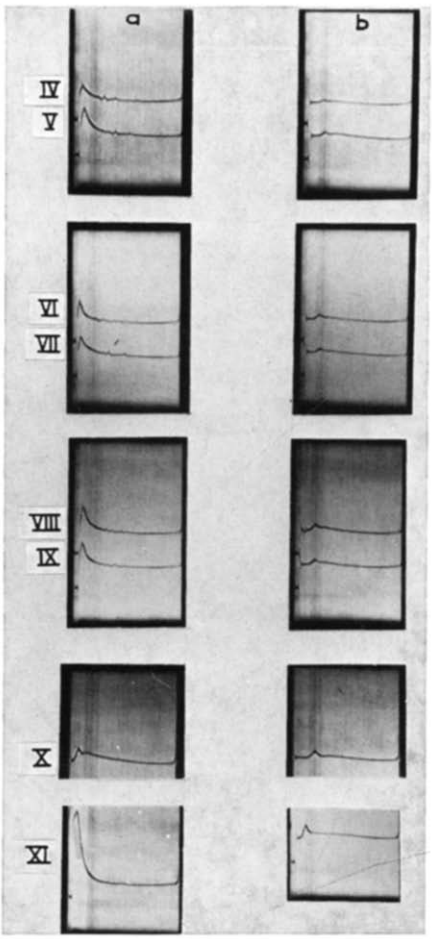


Fig. 4. Separation of α-actinin by 3.3 M KCl treatment of protein precipitated by 22.5 g $(NH_4)_2SO_4$ per 100 ml. Per cent indicates that percentage of the $(NH_4)_2SO_4$ precipitated protein that now precipitates under 3.3 M KCl conditions. Ext. time indicates extraction number as described in METHODS.

Fig. 5. Ultracentrifugal sedimentation of α-actinin (3.3 M KCl precipitated) in the $(NH_4)_2SO_4$ (22.5 g/100 ml) precipitated protein from Extract IV through XI. Conditions: 60000 rev./min; 20°; 1 mM $KHCO_3$; picture, time after reaching full speed, 9 (a) and 25 min (b). Protein concentrations: IV, 3.32 mg/ml; V, 5.28 mg/ml; VI, 4.4 mg/ml; VII, 5.5 mg/ml; VIII, 2.32 mg/ml; IX, 6.0 mg/ml; X, 10.82 mg/ml; XI, 15.52 mg/ml.

light, intermediate and heavy components according to the procedure of NONOMURA[5]. Fig. 6 shows the sedimentation of these separated fractions or components. As can be seen, there appears to be little over-lap between the light and intermediate α-actinins, however, the contamination in each would appear to be minor—unless there is an interaction between them which would be still evident in our low-temperature preparation—but more easily resolved in the warm-temperature-extract of NONOMURA[5]. For the moment we wish to consider the possibility that these preparations are a step closer to their native state, than are room-temperature-extracted α-actinin preparations[1], because of the mild conditions to which our proteins have been subjected. We do not imply with this statement that each of the light, intermediate and heavy components from our preparations are necessarily more homogeneous—but instead feel that they may be held together more tenaciously or be combined more rigorously with other proteins, than in the case of their respective counterparts from room-temperature-extracted α-actinin. In preliminary studies the light-weight component has a sedimentation coefficient of approx. 6 S, as in the case of room-temperature-extracted α-actinin[5]. However, with room-temperature-extracted α-actinin, the inter-

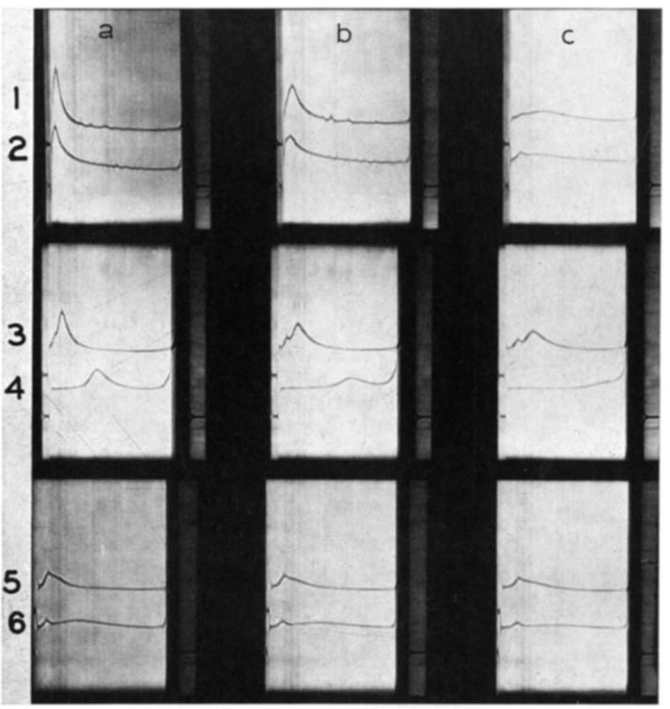


Fig. 6. Ultracentrifugal sedimentation of α-actinin from three pooled groups of extracts and light, intermediate and heavy components in the second pooled group of extracts. 1. α-Actinin from Extracts I–III. 2. α-Actinin from Extracts IV–IX. 3. Intermediate component (NONOMURA[5] and METHODS). 4. Heavy component (NONOMURA[5] and METHODS). 5. Light component (6 S) (NONOMURA[5] and METHODS). 6. α-Actinin from Extracts X and XI. Conditions: 60000 rev./min; 20°; 1 mM $KHCO_3$. Protein concentration, 1 and 2 = 8 mg/ml; 3 = 5.5 mg/ml; 4 = 6.95 mg/ml; 5 = 2.8 mg/ml; 6 = 8.65 mg/ml. Pictures 1 and 2: (a), 50000 rev./min; (b), 60000 rev./min; (c), 8 min after full speed. Pictures 3 and 4: (a), 4; (b), 8; (c), 12 min after full speed. Pictures 5 and 6: (a), 8; (b), 12; (c), 16 min after full speed.

mediate and heavy components have sedimentation coefficients of 10 and 25 S, respectively, whereas ours appear considerably heavier than 10 and 25 S.

Effect of components of α-actinin-like protein on relative viscosity of F-actin

The light-weight component markedly increases the relative viscosity of F-actin (Fig. 8). Unlike the experiments of GOLL *et al.*[4], we did not have to add the

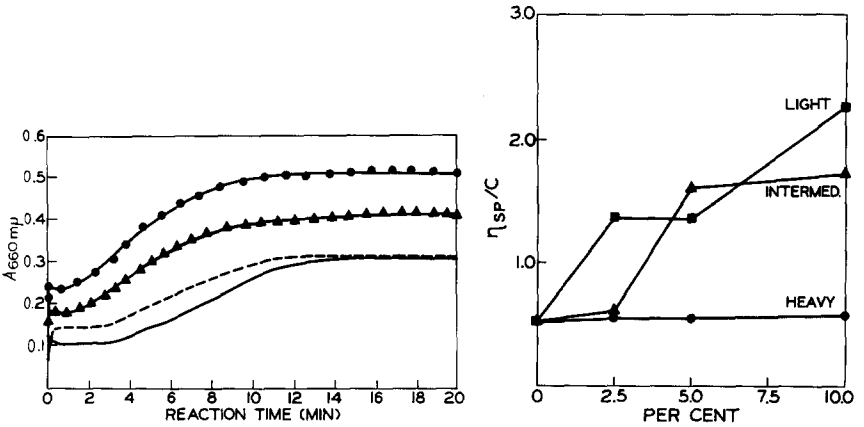


Fig. 7. Effect of pooled α-actinin preparations on superprecipitation of trypsin-treated myosin B. ——, control; - - - -, α-actinin from Extracts I–III; ●—●, α-actinin from Extracts IV–IX; ▲—▲, α-actinin from Extracts X and XI. Protein concentration, 0.30 mg/ml; KCl, 0.03 M; Tris–maleate buffer (pH 7.0), 20 mM; $MgCl_2$, 1 mM; ATP, 1 mM; temp. 25°.

Fig. 8. Effect of components of α-actinin-like protein on relative viscosity. Flow time of water, 66.9 sec (20°). Actin 1.63 mg/ml in 0.1 M KCl and 10 mM Tris–maleate buffer (pH 7.0); per cent indicates α-actinin component as a percentage of actin (w/w). Light, intermediate and heavy components separated according to NONOMURA[5].

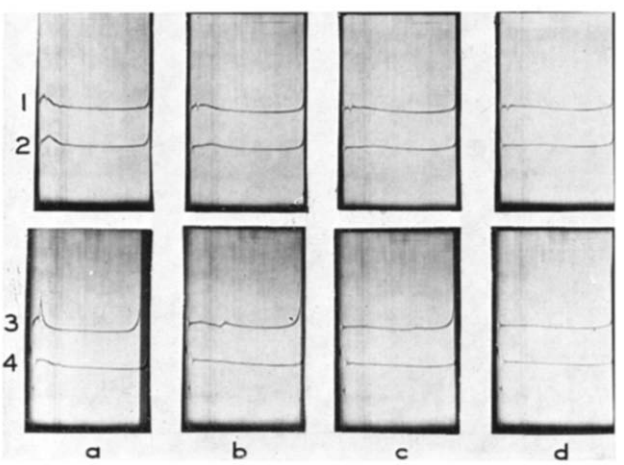


Fig. 9. Ultracentrifugal sedimentation of F-actin in the presence of light, intermediate, and heavy components of α-actinin. Conditions: 60000 rev./min; 20°; 0.1 M KCl and 10 mM Tris–maleate buffer (pH 7.0); actin, 4 mg/ml; α-actinin or component, 1.2 mg/ml. Time after reaching full speed: (a), 0; (b), 4; (c), 8; (d), 12 min. 1. Light component. 2. Intermediate component. 3. Heavy component. 4. Crude α-actinin.

α-actinin to the actin before polymerization. In fact, the effect on viscosity was so pronounced that we were forced to limit our study to a maximum of 10 % on weight/weight basis. The intermediate component, when added to F-actin at the 5 and 10 % level had significant effects on the relative viscosity of the actin. Conversely the heavy components did not alter the relative viscosity of F-actin at these concentrations and conditions. It is conceivable that at higher protein concentrations or lower ionic strengths, the heavy component may be effective. Nevertheless,

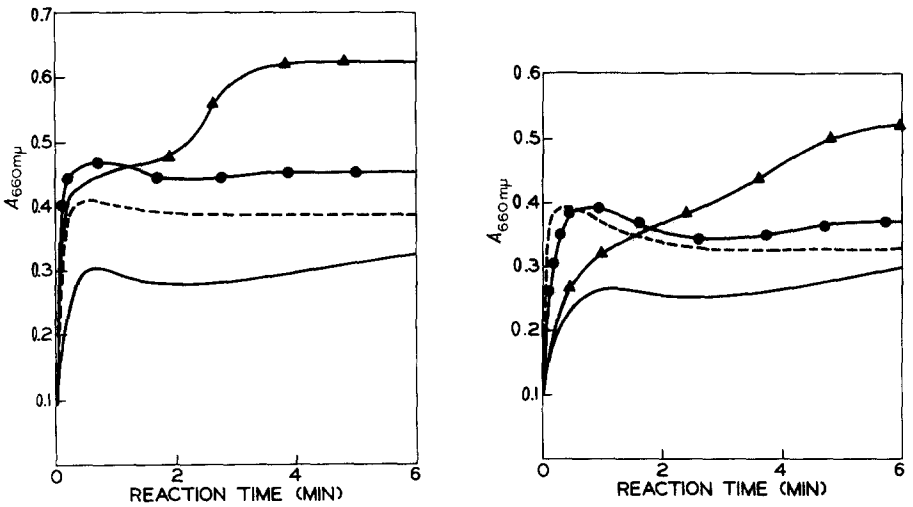


Fig. 10. Effect of components of α-actinin-like protein upon superprecipitation of reconstituted actomyosin. ——, control; ●—●, light; - - - - -, intermediate; ▲—▲, heavy components. Actomyosin, 0.288 mg/ml; actinin component, 96 μg/ml; KCl, 0.04 M; Tris–maleate buffer (pH 7.0), 20 mM; $MgCl_2$, 1 mM; ATP, 1 mM; temp. 25°.

Fig. 11. Effect of components of α-actinin-like protein upon superprecipitation of desensitized actomyosin. ——, control; - - - - -, light; ●—●, intermediate; ▲—▲, heavy components of α-actinin. Actomyosin, 0.288 mg/ml; actinin component, 96 μg/ml; KCl, 0.04 M; Tris–maleate buffer (pH 7.0), 20 mM; $MgCl_2$, 1 mM; ATP, 1 mM; temp. 25°.

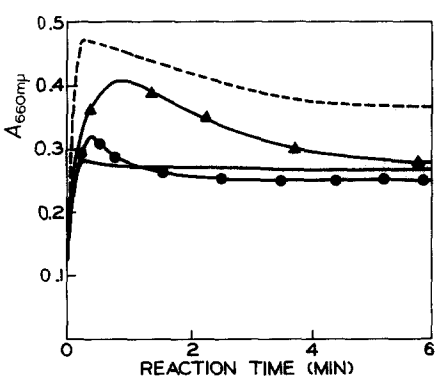


Fig. 12. Effect of components of α-actinin-like protein upon superprecipitation of actomyosin reconstituted with actin from I-filaments after eleven extractions of the I–Z–I brush. ——, control; - - - - -, light; ●—●, intermediate; ▲—▲, heavy component of α-actinin. Actomyosin, 0.63 mg/ml; actinin component, 0.2 mg/ml; KCl, 0.03 M; Tris–maleate buffer (pH 7.0), 20 mM; $MgCl_2$, 1 mM; ATP, 1 mM.

to support the lack of effect of the heavy component on relative viscosity, we can note in Fig. 9, that upon analytical centrifugation, in the presence of F-actin, it does not seem to combine with or make the F-actin crumble in the cell as in the case of light, intermediate or crude α-actinin.

Effect of components of α-actinin-like protein upon superprecipitation of different kinds of actomyosin

As can be seen in Fig. 10, when the components were tested on reconstituted actomyosin, the most pronounced effect was noted with the heavy component. When, however, these components were added to desensitized actomyosin (Fig.11) although the light component was most immediately effective, the heavy component greatly influenced the extent of superprecipitation.

There is no question of the importance of the kind of actin that is employed in the superprecipitation test. We are in the process of studying the actin from the single I-filaments, left after removing eleven extracts. This actin is of particular interest because it would appear to be free of all Z-band constituents. Actomyosin was, therefore, reconstituted with actin from these single filaments. Fig. 12 shows the effect of light, intermediate and heavy components upon the superprecipitation of this actomyosin. While the light component was most effective, it is particularly interesting that the heavy component, again has a rather marked accelerating effect immediately after the ATP has been added.

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