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Preparation of the Enzymatically Active Subfragment of Myosin by Proteolysis of Myofibrils

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Myofibrils, when submitted to proteolysis, yield several fragments, some of which remain bound to actin. There are separated by pyrophosphate treatment and isolated by preparative centrifugation. When trypsin treatment is performed in the presence of 1 mM $CaCl_2$, two types of products are obtained, which can be separated by chromatography on DEAE-cellulose. However, when proteolysis is performed with 10 mM EDTA in the medium, only one enzymatically active fragment is isolated. It is characterized by its sedimentation coefficient, α-helix content, amino-acid composition, actin-binding properties in the absence of ATP and actin-activation of its enzymatic activity. It is thus shown that the EDTA type fragment is similar to heavy-meromyosin subfragment 1 obtained by direct treatment of myosin or heavy meromyosin with papain or trypsin.

Bàlint *et al.* [1] have shown that traces of alkaline earth ions deeply influence the pattern of trypsin fragmentation of the myosin molecule. If proteolysis is performed in the presence of calcium, or magnesium at concentrations above 0.1 μM and 0.1 mM, respectively, only fragments of light meromyosin are obtained, whereas the helical part of the heavy meromyosin, subfragment 2, is not split from the parent molecule. The elimination of alkaline earth metal ions from the incubation medium during tryptic digestion by addition of EDTA results in the production of subfragment 2 as well as the usual digestion products of light meromyosin. The same results are obtained when myofibrils are submitted to proteolysis instead of isolated myosin. The authors concluded that the heavy meromyosin part of myosin contains sites of high affinity for calcium and magnesium in the region sensitive to trypsin attack. Binding of these ions modifies the structure of the protein so that splitting of heavy meromyosin into subfragments 1 and 2 becomes impossible, at least if low concentrations of trypsin are used.

In the experiments described above, only the helical fragments of myosin were isolated by alcohol fractionation and identified. We found it worthwhile to complete these experiments with the characterization of the fragments which remain specifically bound to actin when myofibrils are taken as starting material.

In the present report we described a procedure to isolate these actin-bound proteins. The fragment resulting from proteolysis in the presence of EDTA is further characterized by its sedimentation coefficient, α-helix content, amino-acid composition, actin-binding properties and enzymatic activity. The conclusion is reached that this fragment is similar to subfragment 1 obtained by direct proteolysis of myosin or heavy meromyosin as described in the literature [2–8].

A similar method for subfragment 1 preparation has been recently published, taking myofibrils as starting material but using papain as proteolytic enzyme [9]. The results obtained by this procedure will be discussed in a later section of this article.

EXPERIMENTAL PROCEDURE

Biochemical Techniques

Myofibrils were prepared according to the procedure of Perry [10], from rabbit skeletal muscles, and actin according to Spudich and Watt [11].

Protein concentrations were determined by the micro-kjeldahl technique or by absorbance measurements at 280 nm standardized by micro-kjeldahl determinations.

Adenosine triphosphatase activities were measured at 25 °C according to the procedure of Ehrlich *et al.* [12].

Isolation Procedure for Actin-Bound Proteins in Myofibrillar Digests

Proteolysis was performed on suspensions of myofibrils stored in 0.1 M KCl, containing between 10 and 15 mg · ml^{-1} protein. Before addition of trypsin, the pH of the suspension was brought to 8.2 by addition of 0.5 M NaOH. Either EDTA or $CaCl_2$ were added to the suspension, the respective final concentrations being 10 and 1 mM. From this step on, 1 mM dithiothreitol (final concentration) was added to all solutions.

Proteolysis was started by addition of trypsin (Worthington or Sigma) in a weight ratio of 1:120. The reaction took place at room temperature for 1 h with continuous stirring, and was stopped by addition of soybean trypsin inhibitor to twice the amount of trypsin.

The digest was then centrifuged for 90 min at $100000 \times g$ and the precipitate, containing the proteolytic fragments of myosin bound to actin, resuspended using a Potter homogenizer. Helicoidal fragments resulting from proteolysis of myofibrils were solubilized in the washing medium containing 0.5 M. KCl, borate buffer pH 8.2 and 1 mM dithiothreitol.

The actin and the fragments bound to it were separated by a 90-min centrifugation at $100000 \times g$. The pellet was resuspended by homogenization in the dissociation medium containing buffered KCl as above, 10 mM sodium pyrophosphate and 5 mM $MgCl_2$.

This suspension was left overnight in the cold with gentle stirrring, then centrifuged for 3 h at $100000 \times g$ to eliminate actin.

After thorough dialysis against a 30 mM Tris-HCl buffer pH 7.7, a final purification was performed by centrifugation for 3 h at $100000 \times g$.

Physical Techniques

A Beckman model E analytical ultracentrifuge was used for sedimentation studies, made at 59780 rev./min and 0 °C. Sedimentation coefficients were calculated according to Schachman [13].

Circular dichroism recordings were obtained with a Dichrographe II from Roussel Jouan (Paris) using a scale setting of 1×10^4 absorbance units per 1 cm on the recorder chart. Measurements where made in 0.1-cm cells after dialysis of the protein against a 20 mM phosphate buffer pH 7.5. Protein concentrations ranged between 0.26 and 0.56 mg · ml^{-1}.

Amino-acid analyses were performed with a Beckman Model 121 amino-acid analyzer. 1.5 mg lyophilized protein was hydrolyzed by 6 M HCl for 20 h at 110 °C. After a second lyophilization, the hydrolyzed material was dissolved in 3 ml citrate buffer pH 2.2 and filtered before analysis.

RESULTS

Chromatography of the Products

The two types of preparations were compared by chromatography on DEAE-cellulose (Fig. 1). If proteolysis was performed with EDTA, all of the protein was eluted at a Cl^- concentration of 0.1 M. The absorption coefficient of the peak at 280 nm was equal to 0.77 ml · mg^{-1} · cm^{-1}.

The preparations obtained by proteolysis in the presence of calcium ions gave two well-separated fractions at Cl^- concentrations of 0.1 and 0.16 M. In this case the absorption coefficient of the first peak equalled 0.77 ml · mg^{-1} cm^{-1} and of the second peak, 0.630 ml · mg^{-1} · cm^{-1}.

Ultracentrifugation

On Fig. 2 are compared the sedimentation pattern of the final products of the two kinds of preparations before chromatography. A considerable amount of unsymmetrical components appeared in the preparations obtained by proteolysis in the presence of calcium. If digestion was performed with EDTA, a very homogeneous product was obtained.

The sedimentation constants of eight EDTA-type preparations at different concentrations give an extrapolated $s_{20,w}$ value equal to 5.8 S (Fig. 3).

Circular Dichroism

Circular dichroism spectra of the EDTA preparations exhibit two negative dichroic peaks at 222 and 209 nm. The data obtained at these two wavelengths were recalculated as mean residual molar ellipticities using the expression:

$$\Theta = D^{1\%}_{1\,\mathrm{cm}} \times \overline{w}/10 \times 3300 \text{ (deg. cm}^2\text{ dmol}^{-1}\text{)}$$

[14], where 3300 is the proportionality constant relating the molar circular dichroism to the molar ellipticity, and $\overline{w}_r$ the mean residual weight of the amino acids in the protein, here taken as 115 [15]. Measurements were done at several protein concentrations for each preparation.

The absolute values for the ellipticity at 222 and 209 nm amounted to -13350 ± 550 and -12450 ± 550 deg. cm^2 dmol^{-1}, which represent average values for four preparations. From these values, the percentage of α-helix was calculated by reference to the values obtained for poly(L-glutamic acid) [16]. This amounted to $34 \pm 1\%$ and to $32 \pm 1\%$ for the measurements performed at 222 and 209 nm, respectively.

Amino-Acid Analyses

The amino-acid analyses are summarized Table 1, where comparison is made with data obtained by other authors [3, 7, 17] for subfragment 1.

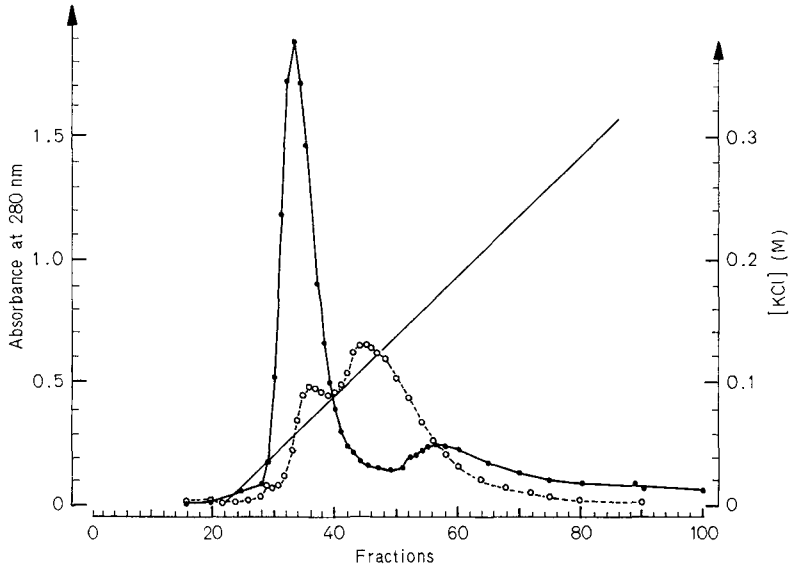

Fig. 1. *DEAE-cellulose chromatography of the enzymatically active fragments obtained by tryptic digestion of myofibrils.* (●——●) Proteolysis performed in the presence of 10 mM EDTA; (○----○) proteolysis performed in the presence of 1 mM $CaCl_2$. Chromatography was carried out at 4 °C on DEAE-cellulose (Whatman DE-52) using a column (30 × 2 cm) equilibrated with 30 mM Tris-HCl pH 7.6. Elution was done with a linear KCl gradient from 0 to 0.4 M at a flow rate of 40 ml/h, collecting 6-ml fractions

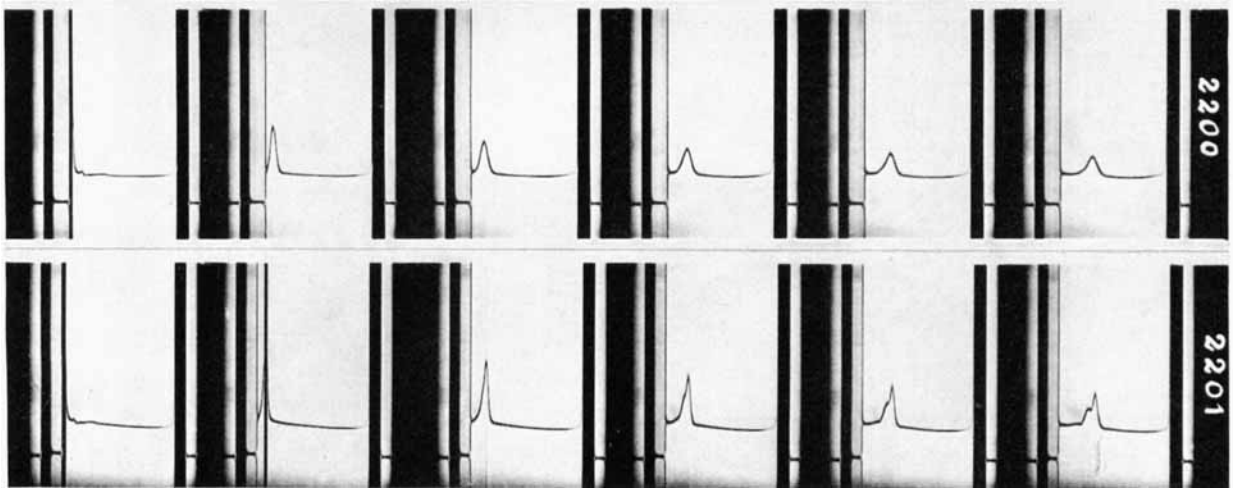

Fig. 2. *Ultracentrifugation diagrams of actin-free residues after dissociation by pyrophosphate.* Upper: fragments obtained after proteolysis in the presence of 10 mM EDTA: Protein concentration: 4.1 mg · ml^{-1}; lower: fragments obtained after proteolysis in the presence of 1 mM $CaCl_2$. Protein concentration: 4.8 mg · ml^{-1}. Both specimens were centrifuged at 59780 rev./min, the pictures being taken every 8 min

All results were re-calculated as a percentage of total amino-acid content, correction being made for tryptophan, proline and cysteine. Our values are the average of four analyses.

Interaction with Actin in the Absence of ATP

Interaction in the absence of ATP was studied by the centrifugation procedure of Young [5]. Increasing amounts of EDTA-type protein were added to a fixed amount of F-actin. The mixtures were then centrifuged for 2 h at 100000 × *g* to sediment the actin · protein complex. The absorbance at 280 nm of the supernatant allows one to calculate the concentration of unbound protein. Fig. 4 shows that all the protein is bound to actin when the weight ratio F actin/protein is equal to 0.38.

Actin Activation of the Enzymatic Activity of the EDTA-Type Preparation

Enzymatic activities were measured at low ionic strength in the presence of 0.1 mM $MgCl_2$ and 1 mM

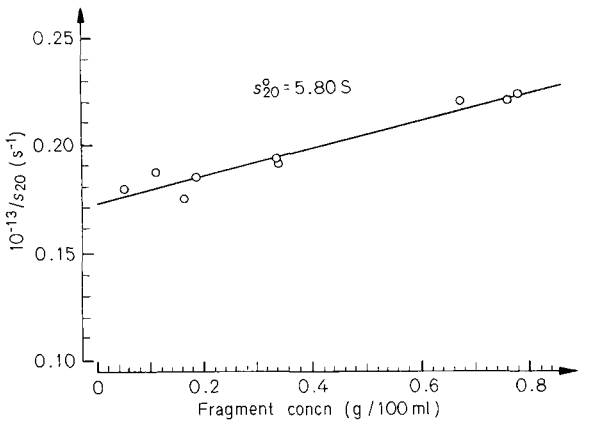

Fig. 3. *Concentration dependence of the sedimentation coefficients of the fragments obtained by proteolysis of myofibrils in the presence of 10 mM EDTA*

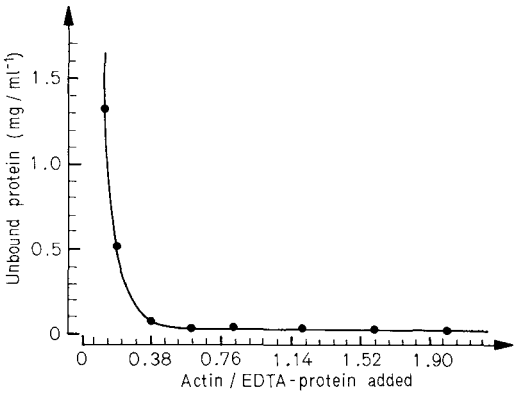

Fig. 4. *Interaction of the EDTA-type protein with actin in the absence of ATP.* Experimental conditions: F-actin: 0.25 mg · ml^{-1} in all experiments, EDTA-type protein: varies from 0.12 to 2.10 mg · ml^{-1}, in the following medium: 50 mM KCl, 5 mM Tris-HCl pH 7.9, 0.5 mM $MgCl_2$ at 4 °C

Table 1. *Amino-acid composition of EDTA-preparation as compared to values obtained elsewhere for subfragment 1*

Amino acid	Amount in EDTA prepn	Values obtained by		
		Lowey [7]	Young [3]	Mueller [17]
	% total	%		
Lysine	8.8	9.4	11.3	8.3
Histidine	2.2	2.1	1.9	1.7
Arginine	4.25	4.0	3.5	3.2
Aspartic acid	10.3	9.9	10.7	11.3
Threonine	6.1	5.7	5.5	6.1
Serine	5.6	4.8	4.4	5.3
Glutamic acid	14.1	13.7	15.7	16.8
Glycine	6.9	7.1	6.1	6.8
Alanine	9.2	8.2	8.4	9.3
Valine	7.05	6.4	5.3	4.9
Methionine	3.15	3.3	3.4	3.5
Isoleucine	5.15	6.2	5.8	4.8
Leucine	8.5	8.8	10.7	9.2
Tyrosine	3.4	4.0	3.0	3.3
Phenylalanine	5.1	6.1	4.9	5.5

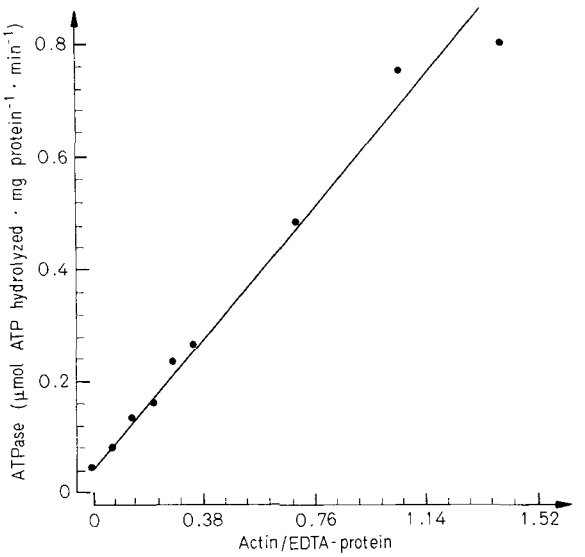

Fig. 5. *Enzymatic activity of EDTA-type protein in the presence of increasing actin concentrations.* The solvent is the same as in Fig. 4, with 1 mM ATP and at 25 °C. F-actin concentrations: from 0.02 to 0.4 mg · ml^{-1}. EDTA protein: 0.25 mg · ml^{-1}

ATP. Addition of actin increased considerably the ATPase activity of the EDTA preparation (Fig. 5).

These data were replotted according to the Lineweaver and Burk procedure (Fig. 6), in order to calculate the maximum activation by extrapolation at infinite actin concentration. This corresponds to an enzymatic activity of 5 μM ATP hydrolyzed · mg^{-1} · min^{-1}, a rate 100-times higher than measured in the absence of actin under the same experimental conditions.

DISCUSSION

The experiments reported here corroborate the conclusions of Bàlint *et al.* [1], namely that the presence of alkaline earth ions profoundly modify the pattern of trypsin fragmentation of the myosin molecule. When proteolysis of myofibrils is performed in the presence of calcium, two kinds of proteins remain bound to actin at the end of the reaction. Although no systematic characterization of these proteins was made here, it seems quite probable that a mixture of heavy meromyosin and its subfragment 1 is obtained. This is suggested by the elution pattern on DEAE-cellulose. Furthermore, the absorption coefficients at 280 nm for these peaks correspond to the published values for these proteins [3, 18, 19]. However, it seemed to us more interesting to characterize the single actin-bound fragment obtained by proteolysis of myofibrils in the presence of EDTA.

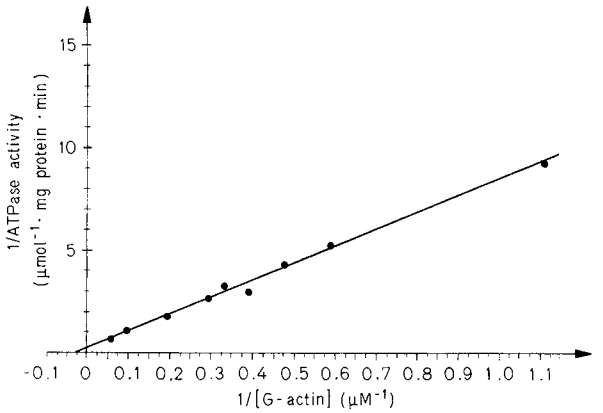

Fig. 6. *Lineweaver and Burk representation of actin activation of the enzymatic activity of the EDTA-type protein*

After dissociation from actin by pyrophosphate treatment in the presence of $MgCl_2$ and separation by preparative ultracentrifugation, the protein thus obtained appears as a single peak by elution on DEAE-cellulose. Ultracentrifugation data also confirm the existence of an homogeneous product, with an extrapolated sedimentation coefficient of 5.80 S. This value corresponds to that of subfragment 1 given in the literature [2,3,6—8,20,21].

The same conclusion must be reached from our data about α-helix content, which averages 33% [7,8] and the amino acid composition very close to the published values for subfragment 1 [3,7,17].

Interaction of the EDTA-type protein with actin further confirms the hypothesis of its identity with subfragment 1. In the experiment described above, all of the protein is bound to actin in the absence of ATP at a weight ratio equal to 0.38. This corresponds to the value published elsewhere [5] for the binding of subfragment 1 to actin where saturation is attained for a mole to mole ratio.

The enhancement of the enzymatic activity by increasing concentrations of actin also corresponds to published data for actin activation of subfragment 1 [20]. When extrapolated to infinite actin concentration, we obtained a 100-fold activation of the ATPase as measured at low ionic strength in the presence of $MgCl_2$.

The preparation procedure of subfragment 1 described here is simple and reliable. The protein we obtained is similar to subfragment 1 resulting from proteolysis of myosin or heavy meromyosin, according to the criteria chosen here for the characterisation of the product.

It is, however, impossible to assume a perfect homogeneity of our preparations since the physical methods used in this work only describe global properties of the molecule.

Papain digestion of myofibrils [9] gives relatively pure subfragment 1 preparations, as characterized by chromatography of the product on Sephadex G-200 and by measurement of its sedimentation and diffusion coefficients. EDTA is not required when digestion is performed by short-time papain treatment, since in this case subfragment 1 is specifically split from the helical part of the myosin molecule [7]:

We are thankful to Dr R. Cardinaud in whose laboratory the amino-acid analyses were performed. The procedure described here was successfully used to prepare subfragment 1 from swine cardiac actomyosin by Gy. Fekete, at the Biochemistry Group, Eövös Loránd, University, Budapest. This work was partly supported by a grant from the Muscular Dystrophy Association of America.

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