

## ISOLATION AND CHARACTERIZATION OF A MYOSIN-LIKE PROTEIN FROM BOVINE ADRENAL MEDULLA

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Received 7 February 1977

### 1. Introduction

In recent years it has become apparent that proteins similar to those found in the contractile apparatus of muscle-tissue also occur in a variety of secretory cells [1]. These findings have led to a comparison between the stimulus-secretion coupling of secretory tissues and the excitation-contraction coupling in muscle [2]. Specifically, these similarities include a requirement for  $\text{Ca}^{2+}$  and metabolic energy, perhaps in the form of ATP. Furthermore, both processes can be inhibited in the presence of actin-filament disrupting agents such as cytochalasin B. As these same contractile proteins are involved in the chemical to mechanical energy transformation of skeletal-muscle movement, the idea has developed that an analogous muscle contractile apparatus might be implicated in the process of secretion [2-4]. The suggestion has been made that the release of neurotransmitters by the adrenal medulla is mediated by such a contractile event [5]. Recently an actomyosin-like protein has been described in the adrenal medulla which has  $\text{Mg}^{2+}$ - and  $\text{Ca}^{2+}$ -activated ATPase activity and which displays both the ATP-sensitive super-precipitation and viscometric properties which characterize muscle actomyosin [6,7]. The proposal has also been made that the regulatory role of  $\text{Ca}^{2+}$  in the two processes may be mediated by similar mechanisms [8]. Indeed a calcium-binding protein has recently been isolated and purified from adrenal medulla which shows a striking resem-

blance in its chemical and physical properties to troponin-C from muscle tissue, and may play a role in initiating secretion which is similar to the role of troponin-C in the initiation of muscle contraction [9].

This paper describes a rapid purification procedure for myosin from bovine adrenal medulla. Some physical, chemical and biological properties of the isolated protein are documented. A comparison of this material with myosin from other non-muscle and muscle sources is made.

### 2. Materials and methods

Bovine adrenal glands obtained from a local meat packing plant (Swifts Canadian Co. Ltd) were transported stored on ice and the medullae were separated from the cortices by hand dissection.

#### 2.1. Extraction of myosin-like protein

All procedures were carried out at  $4^{\circ}\text{C}$ . The medullae were weighed and then ground in a standard meat grinder (large sieve) twice. The ground tissue was mixed with 4 vols (v/w) of 35% sucrose, 10 mM imidazole pH 7.0, 1 mM DTT, 0.1 mM  $\text{MgCl}_2$ , 10 mM sodium pyrophosphate and 0.1 M KCl. The suspension was immediately centrifuged at  $25\,000 \times g$  in a preparative Sorval centrifuge for 60 min. The pink, turbid supernatant was then dialysed against 16 volumes of a solution containing 0.05 M KCl, 0.1 mM DTT, 10 mM imidazole-chloride, pH 7.0, 10 mM sodium pyrophosphate, 0.1 mM  $\text{MgCl}_2$  for 10-15 h. The pink pellet was resuspended in 5-10 ml solution B, which consisted of: 0.6 M KCl, 5 mM ATP, 5 mM DTT, 1 mM  $\text{MgCl}_2$ ,

*Abbreviations:* ATPase, adenosine triphosphatase; CD, circular dichroism; DTT, dithiothreitol; SDS, sodium dodecyl sulfate;  $R_F$ , relative mobility

20 mM imidazole-chloride, pH 7.0, and gently homogenized in a Potter homogenizer. The suspension was centrifuged for one hour at  $100\,000 \times g$  and the clear brownish tinted supernatant was immediately placed on a 6% agarose column (Bio-gel A 5 m, 100–200 wet mesh) of dimensions  $2.5 \times 72$  cm that had been pre-washed with 20–30 ml solution B. The column was then eluted with 0.6 M KCl, 1 mM DTT, 0.5 mM ATP, 0.1 mM  $MgCl_2$ , 10 mM imidazole-chloride pH 7.0 at a flow-rate of 12 ml/h. Each fraction contained 4.4 ml. The entire procedure was completed within 48 h from the time of excision of the glands.

### 2.2. Other techniques

In order to stabilize the ATPase activity and also to have sufficiently high concentration to conduct physical measurements it was necessary to concentrate the dilute protein solutions resulting from the gel-filtration chromatography. This was performed by dialysis against solid sucrose (Mann Ultra Pure) which was subsequently removed by dialysis against a high ionic strength solvent consisting of 0.6 M KCl, 10 mM imidazole-chloride, pH 7.0, 1 mM DTT.

Protein concentrations were routinely measured by the method of Lowry [10] using crystallized bovine serum albumin (Sigma) as a standard.

Ultraviolet circular dichroism (CD) measurements were made with a Cary model 60 recording spectropolarimeter equipped with a Cary model 6001 CD accessory according to methodology described by Oikawa et al. [11].

SDS–Polyacrylamide gel electrophoresis on 5%, 7.5% and 10% gels was performed maintaining a constant ratio of bis-acrylamide to acrylamide as described by Shapiro [12].

Ultracentrifuge measurements were performed in a Beckman Spinco model E analytical ultracentrifuge equipped with a photoelectric scanner, multiplex accessory and high intensity light source. Low-speed sedimentation equilibrium runs were carried out as outlined in the procedural manual of Chervenka [13].

Amino acid analyses were made on a Beckman 121 amino acid analyser. Duplicate samples were hydrolyzed in constant boiling 6 N HCl for 24 h, 48 h and 72 h. The values reported are averaged ones for the three hydrolysis times. Threonine and serine values were obtained by extrapolation to zero-time.

Myosin ATPase activity was measured colorimetri-

cally following the liberation of inorganic phosphate ions on hydrolysis of ATP, by the method of Fiske and Subbarow [14]. The hydrolysis was allowed to proceed for the times of between 5–20 min at either 23°C or 37°C. Assay conditions were 2.5 mM ATP, 0.6 M KCl, 10 mM imidazole-chloride, pH 7.0, and either 10 mM  $MgCl_2$  or 10 mM  $CaCl_2$ .

### 3. Results

A typical elution profile from the Bio-gel A 5 m column recorded from the optical density at 280 nm is shown in fig.1. Two peaks were observed routinely. The first peak was small and sharp and eluted just after the void volume. The second peak was large and broad with a distinct shoulder which was constant in all experiments or volumes loaded onto the column. No overlap between peaks occurred.

Due to the presence of nucleotides in the buffer, as well as the known property of skeletal myosin to bind nucleotides in preparative isolation, the elution profile was also measured at 260 nm. Material in peak I possessed an  $A_{260\text{ nm}}:A_{280\text{ nm}}$  ratio of 1.0:1.1, indicating the presence of both protein and nucleotides. The  $A_{260\text{ nm}}:A_{280\text{ nm}}$  ratio did not change after dialysis against three changes of 100 vols of high ionic strength solvent, over a time period of 24 h. Precipitation of

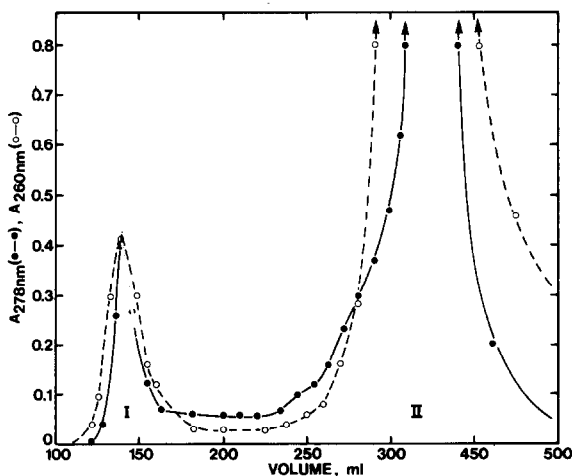


Fig.1. Final purification of adrenal medulla myosin on Bio-gel A 5 m. (●—●) Absorbance at 280 nm. (○- - ○) Absorbance at 260 nm. Conditions are as described in the text.

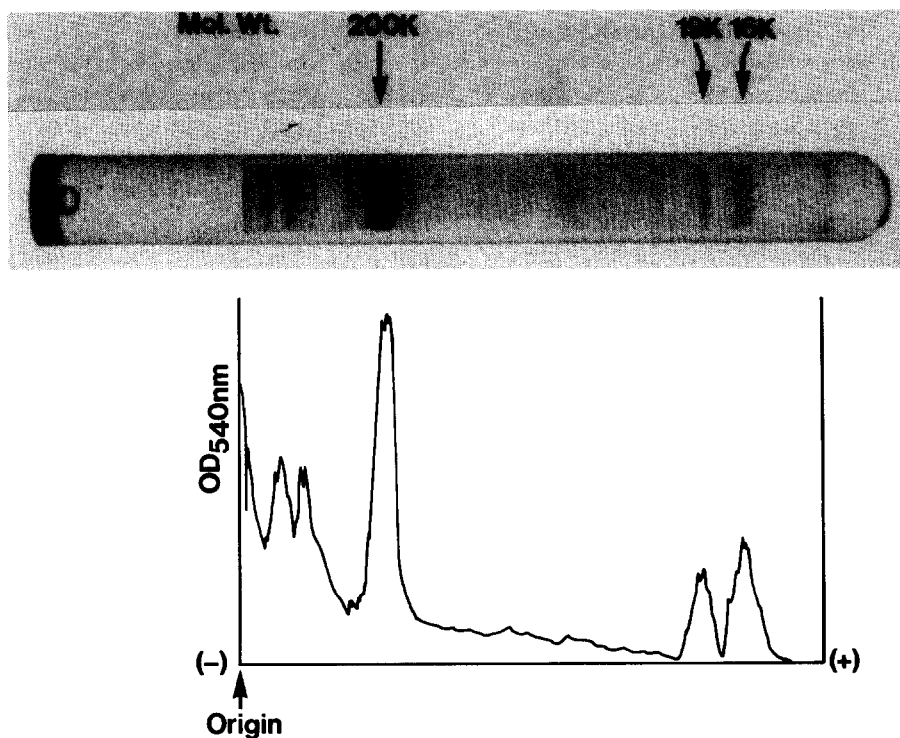


Fig.2. 5% Polyacrylamide-SDS gel electrophoresis of 50  $\mu$ g of adrenal-medulla myosin, also a densitometric scan of the Coomassie Blue stained gel. Run conditions are as quoted in the text.

the protein by dialysis against low ionic strength solvent, and redissolution did not change the  $A_{260\text{ nm}}:A_{280\text{ nm}}$  ratio either. These results would suggest that much of the nucleotide is intrinsically bound to the protein. The  $A_{260\text{ nm}}:A_{280\text{ nm}}$  ratio of peak II material was 1.5:1.7 indicating that nucleotides were predominantly present. This result is in agreement with the suggestion of Pollard [15] who showed that peak II was mainly composed of KI-ATP.

Since nucleotides were present it was decided that the protein concentration of peak I and the shoulder of peak II should be determined by the procedure of Lowry [10] employing column buffer as the solvent blank, to correct for the anomalous colour reaction of nucleotides.

Peak I fractions were dialyzed for 12 h against  $2 \times 100$  vol. high ionic strength myosin solvent and concentrated to approximately 1.5 mg/ml. Five percent SDS-polyacrylamide gels (fig.2) showed the presence of two low mol. wt bands of approximately 19 000 and 16 000 as determined by comparative  $R_f$  mea-

surements of skeletal muscle myosin light chains of 25 000, 18 000 and 16 000 [16]. The major band on the gel was that of approximately 200 000 mol. wt which co-migrated with the high mol. wt band of muscle. There were also some higher molecular weight components present, which were probably aggregates of the 200 000 dalton entity. By these criteria, material in peak I was tentatively identified as a myosin-like protein.

To characterize this protein in greater detail low-speed sedimentation equilibrium experiments were performed. The sample was dialyzed against 0.6 M KCl, 10 mM Tris-HCl pH 7.0, 1 mM DTT, 10 mM sodium pyrophosphate, 10 mM EDTA. Runs employed the photoelectric scanner and were carried out at 4°C with slight overspeeding (10%) then reduction to 3200 rev/min. Plots of the natural logarithm of optical density versus the square of the distance from the centre of rotation showed very drastic upward curvature indicative of a readily aggregating system. From the limiting slopes of several of these plots the

molecular weight of the lowest species present was judged to lie in the range 450 000–500 000. Molecular weights were also measured in a 5 M guanidine-HCl solvent. Again aggregation still was apparent but the limiting slope approach yielded a subunit mol. wt close to 200 000. The tendency of these chains to aggregate, even in the presence of 5 M guanidine hydrochloride, is no doubt accounting for the higher aggregates observed in the SDS gels.

Amino acid analyses were performed on three separate preparations of adrenal medulla myosin with times of acid hydrolysis of 24 h, 48 h and 72 h. Values for the labile residues were obtained by extrapolation to zero-time. The results are displayed in table 1. It is apparent that 11 residues fall within the known range of muscle myosin while another 4 residues (Lys, Ser, Glu, Pro) fall within the expanded limits of blood platelet and muscle myosin, while only 1 residue, viz. Gly, is removed from these limits.

ATPase activity of peak I material was determined

under several different assay conditions (table 2). It is clear from these results that medulla myosin ATPase is similar to most other non-muscle and muscle myosins in having a  $\text{Ca}^{2+}$ -activated and  $\text{Mg}^{2+}$ -inhibited ATPase in a high ionic strength solvent. In agreement with similar data obtained with non-muscle myosins, the activity was relatively low as compared to muscle myosins. A preliminary report [26] on bovine adrenal medulla myosin prepared by a different preparative procedure including an ammonium sulfate fractionation step, indicated on ATPase activity comparable to these figures.

The far ultraviolet CD-spectrum of adrenal medulla myosin is typical of a protein containing  $\alpha$ -helical regions, with two negative dichroic peaks located at 221 nm and 207 nm. The ellipticity values at these wavelengths were  $-23\ 400$  and  $-22\ 600$  deg.  $\text{cm}^2 \cdot \text{dmol}^{-1}$  respectively, which compare favourably with figures obtained for muscle myosin in this laboratory [11].

Table 1  
Comparison of amino acid composition of myosin from bovine adrenal medulla, blood platelets and rabbit muscle

Amino acid	Residues/ $10^5$ g protein				
	Adrenal medulla <sup>a</sup>	Equine platelet [17]	Human platelet [18]	Rabbit skeletal [19]	Rabbit cardiac [19]
Lys	59.5	52	79.7	85–92	84–91
His	16.0	16	14.2	15–16	14–16
Arg	49.9	43	44.3	41–43	47
Asp	81.0	96	84.4	85	81–92
Thr	43.4	49	38.6	41–44	38–44
Ser	59.6	140	23.3	36–41	36–41
Glu	111.3	97	180.2	155–157	147–169
Pro	38.5	51	22.5	22	22–26
Gly	86.1	71	52.8	39–43	37–43
Ala	73.7	41	76.6	75–78	71–76
Cys	—	24	7.7	9	7–8
Val	48.8	46	37.0	42–47	33–42
Met	20.9	16	21.5	22–23	20–23
Ile	36.2	35	33.7	42–43	28–40
Leu	80.9	58	96.0	78–81	80–93
Tyr	23.7	24	17.7	18–20	15–18
Phe	34.3	24	31.1	27–29	26–31
Try	—	17	12.3	4–7	3–6
Ammonia	—	15	112.3	78	—

<sup>a</sup>This study, hydrolysis conditions are as described in the text. Indicated values represent the mean of six determinations.

Table 2  
Enzymic activity of bovine adrenal medulla myosin, and comparison with myosins from other sources

Type of myosin	ATPase activity ( $\mu\text{mol Pi/min/mg protein}$ )			Temperature ( $^{\circ}\text{C}$ ) of assay	Reference
	K <sup>+</sup> EDTA	Ca <sup>2+</sup>	Mg <sup>2+</sup>		
Striated-muscle:					
Rabbit muscle	5.5	1.0	0.01	37	[20]
Smooth-muscle:					
Horse esophagus	1.37	0.8	0.03	37	[21]
Vertebrate cytoplasmic:					
Rat brain		0.27	0.03	37	[22]
Human platelets	0.55	0.44	0.02	37	[23]
Physarum	0.03	0.87	0.03	20	[24]
Acanthamoeba	3.5	0.4	0.05	29	[25]
Adrenal medulla	0.16	0.25	0.04	37	<sup>a</sup>

<sup>a</sup>This study, ATPase measurements were made as described in the text. Indicated values are averaged ones.

#### 4. Discussion

The isolation procedure adopted for adrenal medulla myosin was a synthesis of two previously established techniques for the extraction of non-muscle myosin like proteins. In preliminary experiments the procedure of Clarke and Spudich [27] without modification yielded turbid peak I material which possessed a low enzymic activity. Due to the excessive turbidity, SDS—polyacrylamide gels were of poor quality; however, no major contaminants seemed to be present and the turbidity was assumed to be caused by protein denaturation.

This prompted the introduction of a number of modifications as outlined by Pollard [15] with the object of decreasing this denaturation. Basically three modification steps were employed. Firstly, Tris—HCl, which is known to decrease the enzymic activity of muscle myosin was replaced by an imidazole buffer. Sulfhydryl groups were protected by introduction of a high concentration of DTT. Lastly, sodium pyrophosphate, which is known to stabilize muscle myosin, was incorporated in the first homogenization; this was subsequently removed in later steps to prevent ATPase inhibition. Employing these procedures a viable

preparation of adrenal medulla myosin could be obtained within 48 h from the time of excision of the glands. This preparative procedure yielded 5–6 mg purified protein/200 g wet medulla.

The isolated protein has been implicated to be a myosin on the basis of the following observations. From both SDS—polyacrylamide gels and sedimentation equilibrium studies, the native molecule has a molecular weight, and a substructure closely paralleling that of skeletal myosin. Its circular dichroism spectrum is similar in shape and amplitude to that of skeletal myosin as is its responsiveness to ATP and Ca<sup>2+</sup>. The ATPase activity of the protein, like its skeletal counterpart, is activated by Ca<sup>2+</sup> and inhibited by Mg<sup>2+</sup>. However, in this regard, its efficiency is greatly reduced from that of the muscle enzyme, which may well reflect its known less efficient structural organization in the non-muscle cell. The amino acid composition also falls within the range of classification of a myosin, although in this connection it is to be noted that it more closely resembles the blood-platelet myosin or non-muscle type. The precise physiological role of this myosin-like protein in exocytosis must await further studies of its interaction with other contractile-type proteins isolated from this tissue and with cofactors such as calcium and ATP.

## Acknowledgements

The authors wish to thank Mr A. Keri, Mr V. Ledsham, Mr K. Oikawa and Mr M. Natriss for their excellent technical assistance. Special thanks are due to Mr V. Jones of Swift Canadian Co. Ltd, for the generous supply of fresh bovine-adrenal glands. This investigation was supported by the Medical Research Council of Canada.

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