BBA 46237

A CONTRACTILE PROTEIN POSSESSING Ca^{2+} SENSITIVITY (NATURAL ACTOMYOSIN) FROM LEUCOCYTES

ITS EXTRACTION AND SOME OF ITS PROPERTIES*

NOBUHIKO SHIBATA, NORIYUKI TATSUMI, KAZUHIKO TANAKA, YOSHIMI OKAMURA AND NOBUYUKI SENDA

Center for Adult Diseases, Osaka (Japan)

(Received June 28th, 1971)

SUMMARY

1. Methods have been developed for the isolation of contractile protein from equine leucocytes resembling natural actomyosin of the smooth muscle.

2. This protein exhibited ATPase (EC 3.6.1.3) activity and underwent superprecipitation at low ionic strength in the presence of ATP, Mg^{2+} and Ca^{2+}, but revealed no superprecipitation in the absence of either Ca^{2+} or Mg^{2+} even in the presence of ATP. Superprecipitation of this protein was enhanced by an increase of Ca^{2+} concentration in a medium containing Mg^{2+} and its intensity was closely associated with enhancement of the Mg^{2+}-dependent ATPase (EC 3.6.1.3) activity of this protein by an increase of Ca^{2+}. The free Ca^{2+} concentration at which the Mg^{2+}-dependent ATPase activity of the protein was half activated was about $1 \cdot 10^{-6}$ M. Superprecipitation of this protein was enhanced by an increase of Mg^{2+} concentration in a medium containing Ca^{2+}. In this case, too, the superprecipitation was closely associated with enhancement of the ATPase activity of this protein by increasing Mg^{2+} with a constant Ca^{2+} concentration. The ATPase activity was half activated in the presence of about 3 mM Mg^{2+}.

3. Electron micrographs of the protein showed thick and thin filaments at low ionic strength and a relatively high ATP level conditions in which muscular actomyosin was dissociated into myosin aggregates and fibrous actin. At a relatively low ATP level, both thin and thick filaments were aggregated with each other, as in actomyosin from the muscle, which seemed to be subject to superprecipitation.

INTRODUCTION

On the basis of the study of the motile form and function of leucocytes, we have suggested that leucocytes are most likely to possess a motile organ, a mechanochemical system coupled with ATP[1], of which the essential substance may be a kind of acto-

Abbreviations: EGTA, 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane; KIU, trypsin Kallikrein inhibitory unit.

* An outline of this paper was reported at the 8th Annual Meeting of the Japan Society of the Reticuloendothelial System Research in Osaka, Japan, 1970.

myosin-like contractile protein similar to that of the muscle[2]. A contractile protein was first isolated by us from equine leucocytes[3] in 1967. The protein had various biophysical and biochemical properties characteristic of actomyosin from the muscle. However, it did not show any Ca^{2+} sensitivity in superprecipitation, unlike natural actomyosin or myosin B from muscle. This finding posed the problem of whether "native tropomyosin", consisting of tropomyosin and troponin[4,5], in the presence of which Ca^{2+} effects the interaction of myosin and actin from the muscle, was substantially absent in leucocytes or inactivated during the extraction process.

Thus, an attempt was made to extract a contractile protein from leucocytes possessing Ca^{2+} sensitivity similar to natural actomyosin.

EXPERIMENTAL PROCEDURE

Isolation of leucocytes from whole blood

Leucocytes were collected from fresh equine arterial blood. The isolation procedure of leucocytes from whole blood was the same as that employed previously except for some points[3]. The modifications were the use of blood suspending solution containing dithiothreitol and Trasylol[6,7] of a strong inhibitor for protease. That is, 10.0 g of EDTA (tetrasodium salt), 8.5 g of NaCl, 3.6 g of cysteine, 2 mg of dithiothreitol and 10^5 KIU (1 KIU corresponds to 0.14 μg of Trasylol) of Trasylol dissolved in 10 ml of 0.15 M saline solution were dissolved in 1 l of distilled water into which 9 l of the blood was mixed.

The volume of leucocyte buffy coat collected from a starting volume of 18 l of blood was 40–50 ml. As in the previous experiment[3], the leucocytes ($1.0 \cdot 10^4$–$1.3 \cdot 10^4$/mm^3 in whole blood) were concentrated to $1.0 \cdot 10^6$–$1.3 \cdot 10^6$/mm^3 in the buffy coat at the final step of isolation. Contamination with red cells in the buffy coat was less than 5 %, and that of platelets was negligible. About 90 % of the collected leucocytes were usually counted to be neutrophiles on the smear preparation.

Extraction of contractile protein possessing Ca^{2+} sensitivity from leucocytes

All procedures were conducted at about 4°. The buffy coat of leucocytes was first mixed with ¼ vol. of Trasylol in 0.15 M NaCl (10^5 KIU in 10 ml), 2 mM dithiothreitol and 2.5 vol. of the extraction solution containing 1 mM ATP and 20 mM histidine buffer (pH 7.0). The final ionic strength of this mixture was about 0.05. The leucocytes were homogenized in a Virtis homogenizer at half the maximum speed and, further, in a Potter–Elvehjem homogenizer for 30 sec each. This homogenization procedure was very important for obtaining contractile protein possessing significant sensitivity for Ca^{2+} in superprecipitation, so it was carried out very carefully. The contractile protein prepared without the above care with regard to the homogenation demonstrated only a partial Ca^{2+} sensitivity, although it was never devoid of Ca^{2+} sensitivity. The resulting homogenate was not as viscous as the leucocyte homogenate prepared by the previous procedure[3]. The homogenate was then centrifuged at 40000 $\times$ *g* for 1 h to remove cell fragments and corpuscular material, leaving a pink supernatant fluid. This supernatant was dialysed for 2 days against about 25 vol. of 5 mM histidine buffer (pH 7.0), containing 0.05 M KCl and 2 mM dithiothreitol in order to remove ATP. The resultant flocculent precipitate was collected by centrifugation at 3000 $\times$ *g* for 20 min. The sediment was dissolved in an

appropriate volume of 2.4 M KCl solution containing 2 mM dithiothreitol whose ionic strength was finally adjusted to 0.6 with water. The indissoluble material was removed by centrifugation at 10000 × *g* for 10 min. The supernatant was again dialysed for about 16 h against about 100 vol. of 5 mM histidine buffer (pH 7.0) containing 0.24 M KCl and 2 mM dithiothreitol. The resultant flocculent precipitate was collected by centrifugation at 3000 × *g* for 15 min. The sediment was again dissolved in 0.6 M KCl (pH 7.0) containing 2 mM dithiothreitol. The hardly dissolved material was removed by centrifugation at 33000 × *g* for 30 min. Precipitation and dissolution of the above supernatant fluid was repeated at least twice in KCl solution containing 2 mM dithiothreitol at the final ionic strength of 0.06 and 0.6, respectively. The protein dissolved in 0.6 M KCl was kept at 0° and used for experiments within a day after the extraction. The protein extracted from 40 ml of the buffy coat of leucocytes usually amounted to about 160 mg.

The amount of protein was determined by the method of LOWRY *et al.*[8] using bovine serum albumin as standard.

ATPase activity

The effect of Ca^{2+} on the Mg^{2+}-dependent ATPase (EC 3.6.1.3) activity was determined in the coupled system with pyruvate kinase as an ATP generating system by measuring the time dependence of pyruvate liberation according to the method of REYNARD *et al.*[9]. The reaction medium was composed of 0.4–0.7 mg protein per ml, 6.6 μg pyruvate kinase per ml, 1 mM phosphoenolpyruvate, 10 mM Tris–maleate (pH 7.0), 10 mM $MgCl_2$, different amounts of Ca^{2+} set up by Ca-EGTA buffer (EGTA, 2 mM), 0.06 M KCl and 0.1 mM ATP. The reaction was started by an addition of ATP and was carried out at 37°. At appropriate times, 0.5 ml of the reaction mixture was transferred to a solution containing 0.2 ml of 2.5 mM 2,4-dinitrophenylhydrazin in 3 M HCl to stop the reaction. This coupled system was not influenced by the Mg^{2+} concentration in the medium over the range from 0.5 to 20 mM. The effect of Mg^{2+} on the ATPase (EC 3.6.1.3) activity under a constant Ca^{2+} concentration was likewise determined.

In the determination of the ATPase activity of the reaction medium with 1 mM ATP as substrate, the reaction was initiated by adding ATP and was stopped by adding 5 % trichloroacetic acid at appropriate times. In this case, the coupling system consisting of phosphoenolpyruvate and pyruvate kinase was omitted. Then, the amount of P_i liberated was determined by the method of MARSH[10].

Superprecipitation

The ionic strength of the reaction mixture used for observation of superprecipitation was brought to 0.06 by mixing the protein solution into an appropriate reaction medium indicated in the text. The superprecipitation was followed with a Hitachi UV-VIS spectorophotometer by measuring the change in absorbance at 660 nm in the reaction mixture on addition of ATP at room temperature following the method developed by EBASHI[11]. In some experiments the superprecipitation in the reaction mixture containing the protein was also macroscopically studied.

Viscosimetric determination

This was performed in an Ostwald viscosimeter with 1 ml capacity at 25°. The relative viscosity was measured at pH 7.0 first in 0.6 M KCl (η_{rel}) and then after

addition of 0.01 ml of a solution containing 0.5 M $MgCl_2$ and 0.1 M ATP in 0.6 M KCl (η_{rel} ATP). The sensitivity to ATP was calculated (in %) according to PORTZEHL *et al.*[12] with the formula:

$$\frac{\log_e \eta_{rel} - \log_e \eta_{rel}\text{ATP}}{\log_e \eta_{rel}\text{ATP}} \times 100$$

Ultracentrifuge

A Hitachi Model UCA-I analytical ultracentrifuge was employed for the sedimentation-velocity measurements.

Electronmicrophotographs

Micrographs of the protein were taken by the negative-staining technique of HUXLEY[13]. One drop of an ice-cold preparation of the protein was placed on a microgrid coated with collodion carbon. The preparation was negatively stained with 1 % uranyl acetate, and examined in a Nihon Denshi Model JEM 6C electronmicroscope with an acceleration voltage of 80 kV.

Chemicals

The chemicals used were analytical grade. Trasylol, was obtained from Bayer Leverkusen, Germany and EGTA of a Ca^{2+}-chelating agent from Dojin Igaku Institute, Japan. Various free Ca^{2+} concentrations were set up by means of Ca-EGTA buffer prepared in the manner described by PORTZEHL *et al.*[14]. The water used in preparing the solution was redistilled in a glass vessel.

RESULTS

Superprecipitation

It is a well-known characteristic of natural actomyosin, the contractile protein of the muscle, to undergo superprecipitation at low ionic strength in the presence of not only Mg^{2+} and ATP but also Ca^{2+} (ref. 11).

As shown in Fig. 1, the extracted protein from leucocytes showed a marked superprecipitation on addition of 0.1 mM ATP in a reaction mixture with low ionic strength (0.06) of 10 mM Tris–maleate buffer (pH 7.0) containing 10 mM $MgCl_2$ and $1 \cdot 10^{-4}$ M Ca^{2+} (Fig. 1, right), but failed to demonstrate superprecipitation in the medium without free Ca^{2+} (addition of 1 mM EGTA alone instead of 1 mM Ca-EGTA buffer) (Fig. 1, middle). That is, immediately after the addition of ATP, turbidity of the reaction mixture rapidly increased in the vessel containing $1 \cdot 10^{-4}$ M Ca^{2+} (Fig. 1, upper right), whereas it decreased to exhibit clearing when Ca^{2+} was eliminated by the addition of only EGTA instead of Ca-EGTA buffer (Fig. 1., upper middle). 20 min after the addition of ATP, the protein showed a marked superprecipitation (Fig. 1, lower right), but it kept clearing in the medium not containing free Ca^{2+} (Fig. 1, lower middle) and no superprecipitation was observed in the control (left) containing no added ATP.

It was evident that the degree of superprecipitation which was measured by an increase in absorbance at 660 nm (Fig. 2) was associated with the concentration of free Ca^{2+} in the medium. That is, a superprecipitation occurred significantly at concentrations above $1 \cdot 10^{-6}$ M Ca^{2+} and increased up to $1 \cdot 10^{-4}$ M Ca^{2+}, whereas the

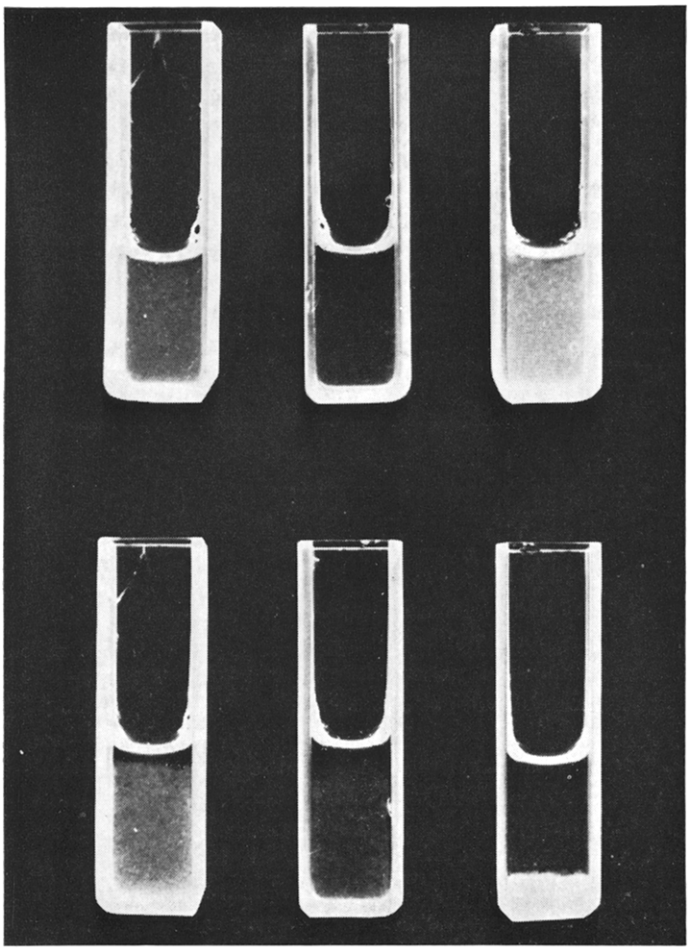


Fig. 1. Superprecipitation of the natural actomyosin-like contractile protein extracted from leucocytes. The upper series shows changes in turbidity just after an addition of ATP. The lower shows sedimentation of the protein 20 min after an addition of ATP. The left vessels in each series are controls for each containing no added ATP. The middle vessels in each series contain 1 mM EGTA instead of Ca-EGTA buffer. The controls contain $1 \cdot 10^{-4}$ M Ca^{2+}. Protein concentration, 1.75 mg/ml; ionic strength, 0.06; $MgCl_2$, 10 mM; Ca^{2+} concentration, $1 \cdot 10^{-4}$ M by 1 mM Ca-EGTA buffer (pH 7.0); Tris–maleate buffer, 10 mM (pH 7.0). ATP concentration used is 0.1 mM. Total vol., 1.5 ml; temp., 20°.

elimination of Ca^{2+} in a medium containing 1.0 mM EGTA resulted in a complete inhibition of superprecipitation.

Fig. 3 shows the effect on superprecipitation of the extracted protein following the addition of $MgCl_2$ to a reaction mixture with low ionic strength (0.06) containing 10 mM Tris–maleate buffer (pH 7.0), 0.1 mM ATP and $1 \cdot 10^{-4}$ M Ca^{2+}. Up to 20 mM $MgCl_2$ added, the superprecipitation was enhanced. In a medium containing 5 mM EDTA, 0.1 mM ATP and $1 \cdot 10^{-4}$ M Ca^{2+}, but no Mg^{2+}, the protein showed no superprecipitation.

From the above results, it was evident that the presently extracted protein required not only Mg^{2+} and ATP but also Ca^{2+} for superprecipitation.

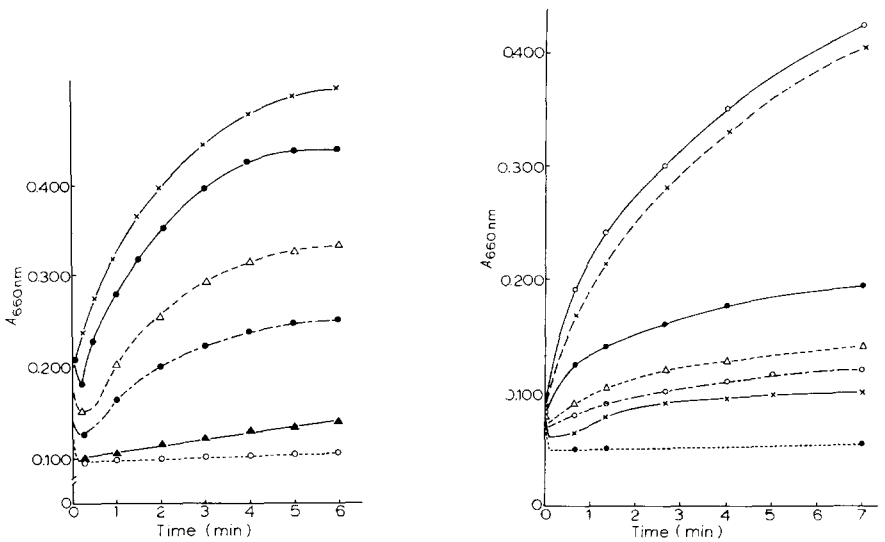


Fig. 2. Effects of Ca^{2+} on superprecipitation at 20° of the extracted protein. Protein concentration, 0.65 mg/ml; ionic strength, 0.06; Tris–maleate buffer, 10 mM (pH 7.0); ATP, 0.1 mM; phosphoenolpyruvate, 1 mM; pyruvate kinase, 10 μg; $MgCl_2$, 10 mM; total vol., 1.5 ml. The reaction was started by the addition of ATP. Free Ca^{2+} concentration was set up by Ca-EGTA buffer, EGTA concentration being 2 mM. Concentrations of Ca^{2+} in the reaction medium were $1 \cdot 10^{-4}$ M (×—×), $1 \cdot 10^{-5}$ M (●—●), $5 \cdot 10^{-6}$ M (△---△), $1 \cdot 10^{-6}$ M (●---●), $1 \cdot 10^{-7}$ M (▲—▲) and nearly zero obtained with only EGTA without added $CaCl_2$ (○---○).

Fig. 3. Effects of added Mg^{2+} on the superprecipitation at 20° of the extracted protein. Protein concentration, 0.45 mg/ml; ionic strength, 0.06; Tris–maleate buffer, 10 mM (pH 7.0); ATP, 0.1 mM; phosphoenolpyruvate, 1 mM; pyruvate kinase, 10 μg; Ca^{2+} concentration, $1 \cdot 10^{-4}$ M (Ca-EGTA buffer); total vol., 1.5 ml. The reaction was started by the addition of ATP. Final concentration of added $MgCl_2$ were 20 mM (○—○), 10 mM (×—×), 5 mM (●—●), 2.5 mM (△---△), 1 mM (○---○), 0.5 mM (×—×). ●---●, with 1 mM EDTA.

ATPase activity

From the above results, it is reasonable to expect that there may be some correlations between the activation of the Mg^{2+}-dependent ATPase activity or the ATPase activity under a constant Ca^{2+} concentration of the protein and the enhancement of superprecipitation due to an elevated concentration of Ca^{2+} or Mg^{2+} in the reaction medium.

The Mg^{2+}-dependent ATPase activity was activated by an elevation of Ca^{2+} concentration in a medium containing the ATP-generating system consisting of phosphoenolpyruvate, pyruvate kinase and Mg^{2+}. It increased rapidly at $1 \cdot 10^{-6}$ M reaching a maximum at $1 \cdot 10^{-4}$ M (Fig. 4), showing correlation with the enhancement of the superprecipitation. That is, the ATPase activity was 0.0207 and 0.0283 μmole pyruvate per mg protein per min without added Ca^{2+} and at $1 \cdot 10^{-4}$ M Ca^{2+}, respectively. The free Ca^{2+} concentration at which the Mg^{2+}-dependent ATPase activity of the protein was half activated was about $1 \cdot 10^{-6}$ M. This value was almost the same as that of muscular actomyosin with Ca^{2+} sensitivity[15] and actomyosin reconstituted

(ref. 16) from myosin and the actin/tropomyosin–troponin complex of the heart or skeletal muscle with regard to the above organs.

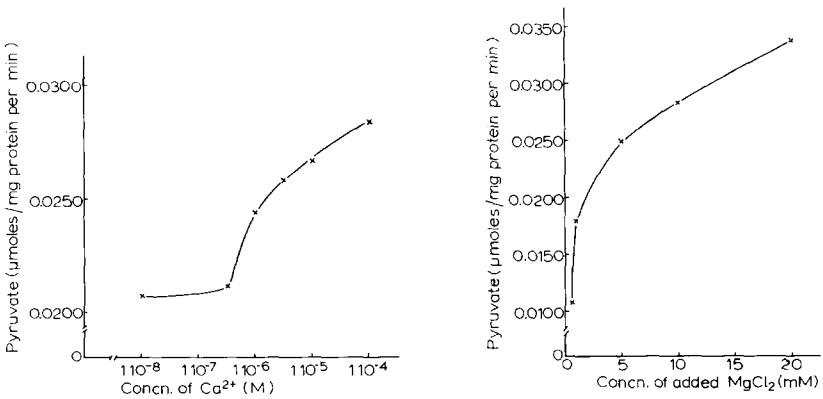


Fig. 4. Effects of Ca^{2+} concentration on the Mg^{2+}-dependent ATPase activity of the extracted protein. Experimental conditions are the same as those in Fig. 2, except that the temperature is 37° and the reaction time is 5 min.

Fig. 5. Effects of added Mg^{2+} on the ATPase activity under a constant Ca^{2+} concentration of the extracted protein. Experimental conditions are the same as those in Fig. 3, except that the temperature is 37° and the reaction time is 5 min.

Up to 20 mM added $MgCl_2$, the ATPase activity of the protein was also activated by an increase of Mg^{2+} concentration in a medium containing the ATP generating system and a constant amount of Ca^{2+} (Fig. 5). The ATPase activity was 0.0107 and 0.0338 μmole pyruvate per mg protein per min at medium concentrations of 0.5 mM and 20 mM $MgCl_2$, respectively. This effect of Mg^{2+} on the ATPase activity was similar to that seen with an arterial contractile protein of the smooth muscle[17].

The extracted protein showed ATPase activity activated by Ca^{2+} and inhibited by Mg^{2+} at high concentration of KCl. At low KCl concentration, the ATPase activity of the protein was slightly activated by Ca^{2+} and Mg^{2+} (Table I).

The Ca^{2+}-activated ATPase activity of actomyosin from striated muscle was to be greater at low ionic strength than at high ionic strength[18], but in the extracted

TABLE I

ATPase ACTIVITY OF NATURAL ACTOMYOSIN LIKE CONTRACTILE PROTEIN FROM LEUCOCYTES AT DIFFERENT KCl CONCENTRATIONS

ATPase activity was measured under the following conditions: 10 mM Tris–maleate buffer (pH 7.0) with or without 1 mM $MgCl_2$ or $CaCl_2$, 1 mM ATP at 37°; protein concn., 1.10 mg/ml; reaction time, 5 min.

KCl concn. (M)	*Split P (μmoles/mg protein per min)*		
	No metal ions added	*$MgCl_2$ added*	*$CaCl_2$ added*
0.06	0.0201	0.0205	0.0268
0.60	0.0293	0.0268	0.0647

protein from leucocytes, the ATPase activity was higher at high ionic strength than at low. The same thing was true for actomyosin from the smooth muscle[19,20].

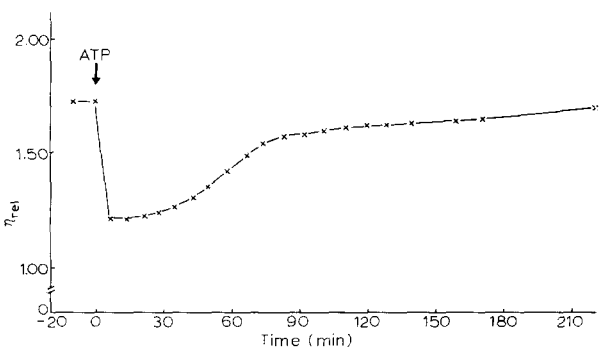
It seems reasonable to postulate that the contractile protein extracted from leucocytes exhibited ATPase activity of the actomyosin type which was activated by Mg^{2+} and Ca^{2+} at low ionic strength, and also ATPase activity of the myosin type which was inhibited by Mg^{2+} and activated by Ca^{2+} at high ionic strength, under conditions in which actomyosin is dissociated into myosin and actin.

Change in viscosity by addition of ATP

The relative viscosity of the protein was decreased by the addition of ATP and Mg^{2+} at high ionic strength. This was followed by a gradual increase in viscosity towards the initial level, probably being brought about by the hydrolysis of ATP (Fig. 6). The sensitivity towards ATP computed according to the formula of PORTZEHL *et al.*[12] was 190 %. This value was almost the same as that obtained for actomyosin from striated muscle[12] or smooth muscle[17].

Analytical ultracentrifugal pattern

The ultracentrifugal pattern of the protein in 10 mM Tris–maleate buffer (pH 7.0) with ionic strength of 0.6 at 20° shows two peaks (Fig. 7) corresponding to 5.8 S and 37 S in the presence of ATP and Mg^{2+}. The former peak corresponded fairly well to that of myosin from striated[21] and smooth muscle[17,20] and the latter nearly to that of F-actin from the smooth muscle[22] or from plasmodium[23].



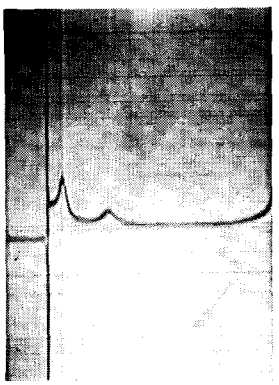


Fig. 6. Effect of ATP on the viscosity of the protein from leucocytes at 25°. Protein concentration, 4.0 mg/ml; ionic strength, 0.6; Tris–maleate buffer, 10 mM (pH 7.0). For details see text.

Fig. 7. Ultracentrifugal pattern of the extracted protein. The centrifugation was carried out at 54 200 rev./min in 10 mM Tris–maleate buffer (pH 7.0) containing 6.5 mg protein per ml, 0.6 M KCl, 10 mM $MgCl_2$, and 5 mM ATP. Sedimentation proceeds from left to right. The photograph was taken at 25 min after top speed was reached.

Electronmicroscopic findings

The protein at low ionic strength was studied by electronmicroscopy 1 min after an addition of the protein solution.

The protein dissolved in 0.6 M KCl was added to 10 mM Tris–maleate buffer (pH 7.0) containing 10 mM $MgCl_2$ and either 5 mM ATP and 1 mM EGTA or 0.1 mM ATP and 0.1 mM $CaCl_2$. The final KCl concentration in these preparations was 0.06 M.

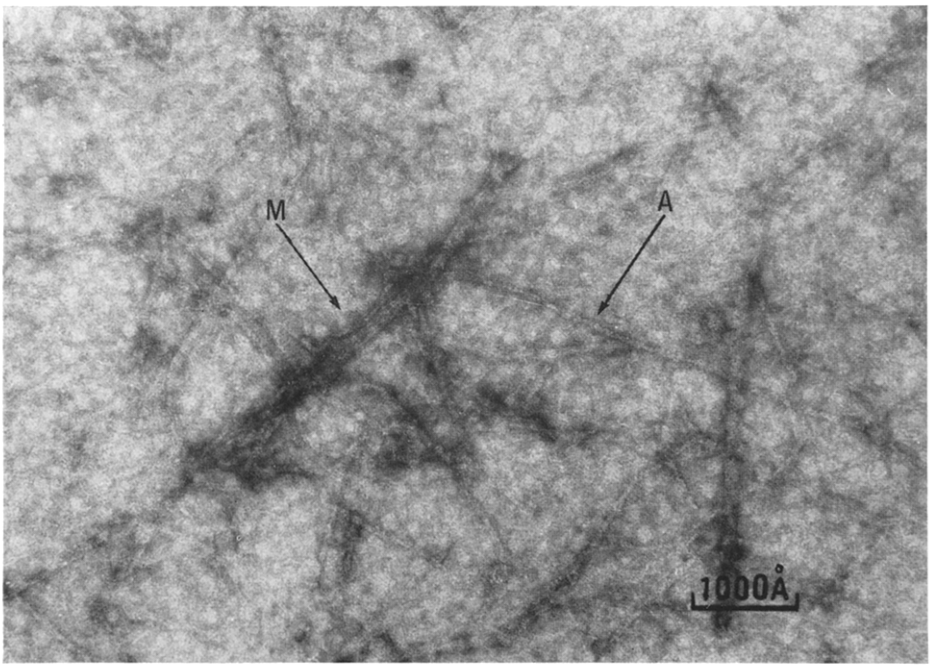


Fig. 8. Electron micrograph of the extracted protein in clearing phase at low ionic strength. The preparation contained 0.15 mg of protein per ml, 0.06 M KCl, 10 mM $MgCl_2$, 1 mM EGTA, 5 mM ATP and 10 mM Tris–maleate buffer, pH 7.0.

In the preparation containing 5 mM ATP and 1 mM EGTA, the protein showed a clearing response. In the electron micrograph of this preparation, thick (M) and thin (A) filaments were seen about 150 Å and 80 Å in diameter, respectively (Fig. 8). It is suggested that the former corresponds to myosin aggregates and the latter to F-actin filaments.

In the preparation containing 0.1 mM ATP and 0.1 mM $CaCl_2$, electron micrographs of negatively stained material showed the formation of many large aggregates, apparently due to joining together both myosin aggregates and F-actin filaments, as observed in the superprecipitation of skeletal actomyosin[24,25] (Fig. 9).

DISCUSSION

From the above experimental findings, it may be concluded that the protein under investigation corresponds to a so-called natural actomyosin-like contractile protein of leucocytes.

The viscosity of the extracted protein was somewhat lower than that of actomyosin from striated muscle reported by MOMMAERTS[26]. In this point, there arises an argument that the extracted protein should not be called natural actomyosin. Reconstitution of myosin- and actin-like protein obtained separately from the extracted protein of leucocytes demonstrated the superprecipitation characteristic of synthetic actomyosin[27]. This finding, together with biophysical and biochemical features

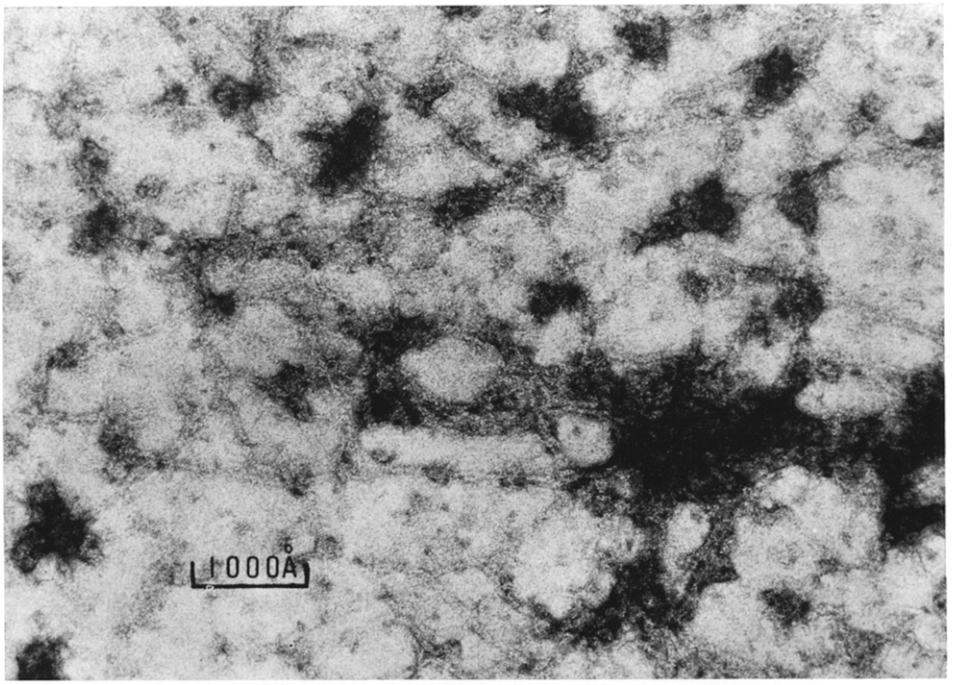


Fig. 9. Electron micrograph of the extracted protein superprecipitated at low ionic strength. The preparation contained 0.15 mg of protein per ml, 0.06 M KCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP and 10 mM Tris–maleate buffer (pH 7.0).

of the protein described already in results, may indicate that the extracted protein actually corresponds to natural actomyosin from muscle.

In order to extract the natural actomyosin-like protein from leucocytes, leucocytes were homogenized in a solution with low ionic strength containing ATP and histidine buffer (pH 7.0) instead of Weber–Edsall's solution[40] with high ionic strength used for the extraction of skeletal myosin B. This procedure was based on the finding that the actomyosin-like contractile protein extracted previously by us from leucocytes exhibited biophysical and biochemical features similar to those of carotid myosin B, except for the absence of Ca^{2+} sensitivity[3]. Superprecipitation of the carotid myosin B was accelerated by increasing the concentration of added Mg^{2+} (ref. 17). The contractile protein with Ca^{2+} sensitivity from carotids was capable of being extracted with a low ionic strength solution containing ATP and histidine buffer (pH 7.0)[17,28]. Furthermore, the Ca^{2+}-sensitizing protein contained in natural actomyosin, a so-called troponin, is reported to be very labile to proteinase digestion[29] and, since the ATPase of myosin is an SH enzyme[30], Trasylol, a natural proteinase inhibitor, and dithiothreitol, an SH protector, were added to the above extracting solution.

The protein thus prepared exhibited the Ca^{2+} sensitivity in superprecipitation (Figs. 1 and 2) and the Mg^{2+}-dependent ATPase activity (Fig. 4), as well as biophysical and biochemical properties characteristic of myosin B or natural actomyosin from muscle.

In natural actomyosin or myosin B from striated muscle the optimal Mg^{2+}

concentration necessary for superprecipitation is considerably low[31], whereas it was very high in the extracted protein from leucocytes (Fig. 3), just as observed in carotid smooth muscle[17]. Furthermore, as described in RESULTS, the extracted protein demonstrated features resembling actomyosin from smooth muscle in the behaviour of the ATPase and analytical ultracentrifugal pattern. Thus, the presently extracted contractile protein from leucocytes seems to be similar to natural actomyosin from smooth muscle rather than that from striated muscle.

In the analytical ultracentrifugal pattern (Fig. 7), the $s^{\circ}_{20,w}$ value of the retarding peak of the extracted protein was almost the same as that of the retarding peak of an actomyosin-like protein extracted previously from leucocytes[3], but the value of the foregoing peak in Fig. 7 was somewhat greater than that of the foregoing peak of the protein prepared by the previous method[3]. This increase in s value of the foregoing component observed in the extracted protein having Ca^{2+} sensitivity, may be explained by the assumption that the F-actin of the protein may be endowed with a native tropomyosin-like substance.

The contractile protein has been extracted, so far, not only from muscle but also from free cells, for example from platelets[32], plasmodium[33], fibroblasts[34], sarcoma cells[35], and leucocytes[3], *etc.*. These contractile proteins, however, showed requirement for Mg^{2+}, ATP and no Ca^{2+} unlike natural actomyosin from muscle, which has a Ca^{2+} requirement for superprecipitation. Therefore, it has been posed that the question of whether or not the contractile protein of free cells other than muscle is different from that of muscle is decided on the basis of a need for a Ca^{2+} concentration in the superprecipitation. From the nature of the contractile protein presently extracted from leucocytes, it is suggested that even the contractile protein of free cells may be susceptible to Ca^{2+} as observed in the contractile protein from the muscle. HATANO[36] demonstrated that the movement of plasmodial fragments was subject to the specific effect of Ca^{2+} following caffeine treatment, although the contractile protein possessing Ca^{2+} sensitivity for the superprecipitation had not been isolated from plasmodium.

From findings obtained in morphological, physiological and phylogenetic studies concerning the motility of leucocytes, we have reported that the motile form and function of leucocytes are coordinately controlled by ATP and that leucocytes possess a motor organ consisting of an actomyosin-like contractile protein acting as a mechanochemical system coupled with ATP[2,37–39]. In this paper, it has become evident that the contractile protein of leucocytes, as well as of the natural actomyosin of smooth muscle, exhibits the contraction and relaxation regulated by concentrations of free Ca^{2+} in the presence of ATP and Mg^{2+}. It is conceivable that the motor organ of leucocytes consists of natural actomyosin-like contractile protein similar to that of muscle and that the movement of leucocytes is achieved by contraction and relaxation of the natural actomyosin-like contractile protein.

REFERENCES

1 K. FUKUSHIMA, N. SENDA, S. ISHIGAMI, J. ENDO, M. ISHII, Y. MURAKAMI, K. NISHIAN AND Y. UEDA, *Med. J. Osaka Univ.*, 5 (1954) 231.
2 N. SENDA AND H. TAMURA, *Annu. Rep. Center Adult Dis., Osaka*, I (1961) 1.
3 N. SENDA, N. SHIBATA, N. TATSUMI, K. KONDO AND K. HAMADA, *Biochim. Biophys. Acta*, 181 (1969) 191.
4 S. EBASHI AND F. EBASHI, *J. Biochem. Tokyo*, 55 (1964) 604.
5 S. EBASHI AND A. KODAMA, *J. Biochem. Tokyo*, 60 (1966) 733.

6 E. WERLE, Report, *Int. Symp. on New Aspects of Trasylol Therapy, Munich, 1968*, Vol. 3 F. K. Schattauer Verlag, Stuttgart–New York, 1970, p. 51.
7 N. BACK AND H. WILKENS, Report, *Int. Symp. on New Aspects of Trasylol Therapy, Munich 1968*, Vol. 3, F. K. Schattauer Verlag, Stuttgart–New York, 1970, p. 63.
8 O. H. LOWRY, N. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265
9 A. M. REYNARD, L. F. HASS, D. D. JACOBSEN AND P. D. BOYER, *J. Biol. Chem.*, 236 (1961) 2277
10 B. B. MARSH, *Biochim. Biophys. Acta*, 32 (1959) 357.
11 S. EBASHI, *J. Biochem. Tokyo*, 50 (1961) 236.
12 H. PORTZEHL, G. SHRAMM AND H. H. WEBER, *Z. Naturforsch.*, 5b (1950) 61.
13 H. E. HUXLEY, *J. Mol. Biol.*, 7 (1963) 281.
14 H. PORTZEHL, P. C. CALDWELL AND J. C. RÜEGG, *Biochim. Biophys. Acta*, 79 (1964) 581.
15 A. WEBER AND S. WINICUR, *J. Biol. Chem.*, 236 (1961) 3198.
16 A. M. KATZ, D. I. REPKE AND B. R. COHEN, *Circulation Res.*, 19 (1966) 1062.
17 N. SHIBATA AND T. YAMAGAMI, *J. Jap. Coll. Angiol.*, 9 (1969) 176.
18 W. HASSELBACH, *Z. Naturforsch.*, 7b (1952) 163.
19 D. M. NEEDHAM AND J. M. CAWKWELL, *Biochem. J.*, 63 (1956) 337.
20 R. H. SCHIRMER, *Biochem. Z.*, 343 (1965) 269.
21 P. JOHNSON AND A. J. ROWE, *Biochem. J.*, 74 (1960) 432.
22 L. LASZT AND G. HAMOIR, *Biochim. Biophys. Acta*, 50 (1960) 430.
23 S. HATANO, T. TOTSUKA AND F. OOSAWA, *Biochim. Biophys. Acta*, 140 (1967) 109.
24 N. IKEMOTO, S. KITAGAWA AND J. GERGELY, *Biochem. Z.*, 345 (1966) 410.
25 K. TAKAHASHI AND T. YASUI, *J. Biochem. Tokyo*, 62 (1967) 131.
26 W. H. F. M. MOMMAERTS, *J. Gen. Physiol.*, 36 (1948) 361.
27 N. SHIBATA, N. TATSUMI, Y. MORI, K. TANAKA AND N. SENDA, in preparation.
28 J. RÜEGG, E. STRASSNER AND R. H. SHIRMER, *Biochem. Z.*, 343 (1965) 70.
29 S. EBASHI, H. IWAKURA, H. NAKAJIMA, R. NAKAMURA AND Y. OOI, *Biochem. Z.*, 345 (1966) 201
30 F. DICKENS AND G. E. GLOCK, *Biochim. Biophys. Acta*, 7 (1951) 578.
31 K. MARUYAMA AND S. WATANABE, *J. Biol. Chem.*, 237 (1962) 3437.
32 M. BETTEX-GALLAND AND E. F. LUSCHER, *Biochim. Biophys. Acta*, 49 (1961) 530.
33 S. HATANO AND M. TAZAWA, *Biochim. Biophys. Acta*, 154 (1968) 507.
34 H. HOFFMANN-BERLING, *Biochim. Biophys. Acta*, 14 (1954) 172.
35 H. HOFFMANN-BERLING, *Biochim. Biophys. Acta*, 19 (1956) 453.
36 S. HATANO, *Exp. Cell Res.*, 61 (1970) 199.
37 N. SENDA, *Sang*, 25 (1954) 707.
38 N. SENDA, *Rev. Hematol.*, 9 (1954) 418.
39 N. SENDA AND H. TAMURA, *Proc. 8th Int. Congr. Haematol., Tokyo, 1960*, Vol. 2, Pan Pacific Press, Tokyo, 1962, p. 826.
40 K. MEYER AND H. H. WEBER, *Biochem. Z.*, 266 (1933) 137.