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OBSERVATIONS ON THE KINETICS, SUBUNIT COMPOSITION, AND SULFHYDRYL REACTIVITY OF MYOSIN FROM *PHYSARUM POLYCEPHALUM*

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Summary

A highly purified preparation of myosin from *Physarum polycephalum* has been shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis to contain heavy chains and only one molecular weight class of light chains, of approx. 15 000 daltons. Kinetic investigations of the Ca^{2+}-ATPase and Mg^{2+}-ATPase (ATP phosphohydrolases, EC 3.6.1.3) at pH 8.0 gave K_m and V values of 17.3 μM and 1.25 μmol P_i/min per mg, and 2.4 μM and 0.12 μmol P_i/min per mg, respectively. Adenylyl imidodiphosphate, a β-γ-imido ATP analog, inhibited the ATPase activity of *Physarum* myosin competitively with K_i values equal to 350 and 12 μM in the presence of Ca^{2+} and Mg^{2+}, respectively. The ATPase activity of *Physarum* myosin was inhibited at a very low rate ($t_{1/2}$ = 24 h) by the ATP analog, 6,6′-dithiobis(inosinyl imidodiphosphate), with concentrations of inhibitor previously shown to inactivate ($t_{1/2} \approx 10$ min) skeletal and cardiac myosins rapidly by reacting with key cysteines.

Recently, myosin has been purified from vertebrate non-muscle tissue, e.g. fibroblasts [1] and glial cells [2], as well as from lower organisms, e.g. *Dictyostelium discoideum* [3] and *Physarum polycephalum* [4—6]. These myosins possess ATPase activity and share many of the properties of myosin purified from muscle tissues. However, they vary in their specific activities, the number and kinds of low-molecular-weight light chains, and their amino acid compositions [7, 8]. In this study, myosin from the acellular slime mold *Phy. polycephalum* was examined for its subunit composition and its reaction with the ATP analogs, 6,6′-dithiobis(inosinyl imidodiphosphate) and adenylyl

Abbreviations used are: $(sIMP\text{-}PNP)_2$, 6,6′-dithiobis(inosinyl imidodiphosphate); TES, *N*-tris-(hydroxylmethylmethyl)2-aminoethanesulfonic acid; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

imidodiphosphate, as a basis for further comparison with myosins from higher organisms.

Physarum myosin was purified from the crude actomyosin preparation of Adelman and Taylor [4]. Freshly collected plasmodia* were washed with deionized water and then homogenized in one volume of 50 mM $Na_4P_2O_7$, 10 mM Tris/maleate buffer (pH 6.8). After 1 h, the homogenate was centrifuged and the supernatant was brought to 45% saturation with saturated $(NH_4)_2SO_4$ (pH 6.5). After centrifugation, the pellet was redissolved in 10 mM Tris/maleate buffer (pH 6.2) and clarified by centrifugation. The supernatant was brought to 35% saturation with saturated $(NH_4)_2SO_4$ (pH 6.0) and the precipitate was collected by centrifugation. The precipitate was redissolved in 0.5 M KCl/0.1 mM dithioerythritol/0.1 mM EDTA/10 mM Tris (pH 8.0) using an homogenizer to give a solution of 15 mg/ml, and clarified by centrifugation. The supernatant was then chromatographed on Sepharose 4 B (Fig. 1) to

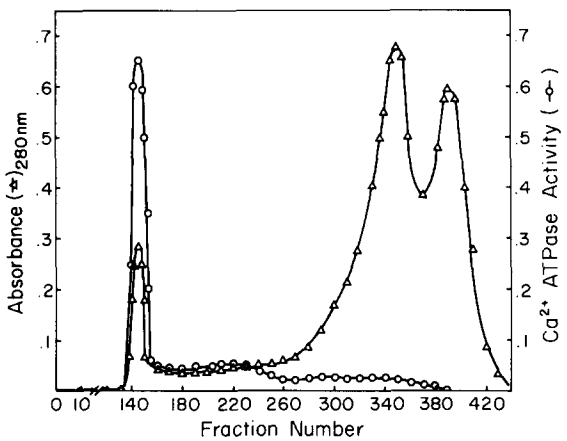

Fig. 1. Chromatography of the 0—35% $(NH_4)_2SO_4$ fraction on Sepharose 4B column. The column (10 x 70 cm) was equilibrated and eluted with 0.5 M KCl, 0.1 mM dithioerythritol, 0.1 mM EDTA, and 10 mM Tris (pH 8.0) at 4°C. The protein (approx. 1.5 g) was applied in 100 ml. Fractions of 15 ml were collected at a flow rate of 90 ml/h and were assayed for protein (△—△) and Ca^{2+}-ATPase activity (○—○).

separate the actomyosin from the remainder of the proteins. The first peak, an enriched myosin-actomyosin preparation, was collected. This solution (approx. 250 ml) was placed in dialysis tubing and concentrated for 12 h at 4°C by burying in sucrose (Schwarz-Man, enzyme grade). This solution was then dialyzed against 100 vols. of 0.5 M KCl, 0.5 mM ATP, 0.5 mM $MgCl_2$, 0.5 mM dithioerythritol, and 10 mM TES (pH 7.0) for 5 h and then made 5 mM in ATP, 5 mM in $MgCl_2$, and 5 mM in dithioerythritol. The solution was spun for 1.5 h at 100 000 x *g* to remove most of the actin and the supernatant concentrated to 3—6 ml using dialysis tubing and sucrose as described above. The solution was then rechromatographed on Sepharose 4-B in the presence of Mg^{2+}-ATP to dissociate the remaining actin from the myosin (Fig. 2). The fractions indi-

*Plasmodia were grown on moistened 9-cm filter paper placed on a layer of rolled oats which were spread over a second piece of filter paper. This was supported on a layer of moistened glass beads within a covered petri dish (9 x 1.5 cm). This technique (details in ref. 21) allowed the harvesting of large amounts of plasmodia (2—3 g/petri dish) directly from the filter paper without contamination from the culture medium.

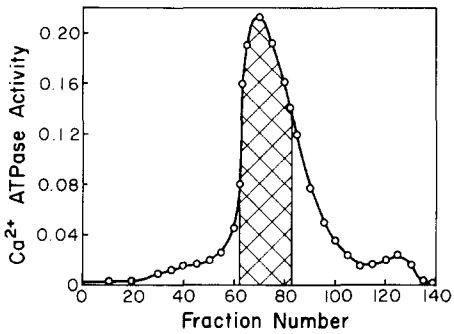

Fig. 2. Chromatography of the ATP-dissociated enriched myosin-actomyosin preparation. The Sepharose 4-B column (2.5 x 90 cm) was equilibrated and eluted with 0.5 M KCl, 0.5 mM ATP, 0.5 mM $MgCl_2$, 0.5 mM dithioerythritol, and 10 mM TES (pH 7.0) at 4° C. The protein (approx. 4 mg/ml) in 5 ml was applied in sucrose to the column. Fractions of 3.5 ml were collected at a flow rate of 14 ml/h. Ca^{2+}-ATPase activity (○——○) was measured at 25° C. The cross-hatched fractions were pooled.

cated by the cross-hatched area (Fig. 2) were pooled and concentrated by use of sucrose and dialysis tubing. Sucrose was removed by further dialysis against the appropriate buffer. Seven preparations of enzyme by this method gave an average yield of 2—3 mg myosin/100 g plasmodia with a specific activity of 1.2—1.3 μmol P_i/min per mg protein (assay conditions: 0.5 M KCl, 2 mM $CaCl_2$, 2 mM ATP, 50 mM Tris, pH 8.0 at 25°C). This activity is comparable to or higher than other reported values [4- 6].

The subunit composition of the purified *Physarum* myosin was examined. The electrophoresis of the myosin in 10% sodium dodecyl sulfate polyacrylamide gels show a high-molecular-weight polypeptide chain which just entered the gel and one major low-molecular-weight fast-moving band (labeled A in Fig. 3) which is presumably a light chain. The latter band has a molecular weight of approximately 15 000, based on comparison of its mobility with that of the light chains of skeletal myosin (data not shown). There is a small shoulder on this fast-moving band which may indicate a trace amount of higher-molecular-weight protein. The small protein band (B) of molecular weight 45 000 is probably actin. There are also small amounts of two higher-molecular-weight bands of unknown origin ahead of the heavy chains. The myosin of *Physarum* is thus composed predominantly of the heavy chains and one class (based on molecular weight) of light chains.

There is a great deal of uncertainty concerning the light-chain subunit

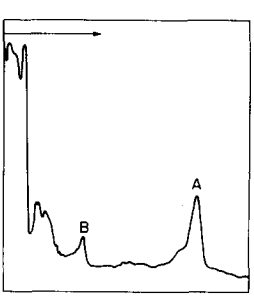

Fig. 3. A gel scan measured at 566 nm of a 10% sodium dodecyl sulfate polyacrylamide gel of purified *Physarum* myosin. The arrow indicates the direction of electrophoresis. Band A is the light chain and band B is actin.

composition of myosin from *Physarum polycephalum*. Nachmias [9] originally reported only one fast-moving band of molecular weight between 13 000 and 15 000 present in *Physarum* myosin. Using a different purification procedure, Nachmias [6] has recently reported two types of light chain, of molecular weights 21 000 and 17 000, with 1 : 2 : 1 stoichiometry of heavy chains to the 21 000- and 17 000-dalton light chains, respectively. The *Physarum* myosin prepared by White and Lascelles [10] gave low-molecular-weight proteins of 24 000, 18 000, 15 000, and 12 000 daltons, with the 15 000-dalton polypeptide in the largest quantity. Adelman [11] reported that *Physarum* myosin is composed primarily of the heavy chain(s) and a 15 000-dalton polypeptide. Also present were several minor components of lower molecular weight, including a doublet of approximately 12 000 daltons. In general, the results of this study agree with these other studies, which also determined subunit structure by sodium dodecyl sulfate polyacrylamide gel electrophoresis, but demonstrated that only one major class of light chains of 15 000 daltons must be present to give maximal ATPase activity.

There could be several explanations for the discrepancy between the multiple-type light chains observed by others and the single-type light chain observed in this study. *Physarum* myosin may only have one class of light chains, and the second class of light chains or other proteins observed may be proteolytic fragments. A single class of light chains based on band pattern on sodium dodecyl sulfate polyacrylamide gels has been found in ovomyosin from sea urchin eggs [12], and myosin from the abalone, *Haliotis discus* [13]. In general, myosins usually have two or three classes of different molecular weight light chains; for example, myosin isolated from the cellular slime mold *Dictyostelium discoideum* has two classes of light chains of 18 000 and 16 000 daltons [3]. It is also possible that in *Physarum* myosin there may be two types of light chains of similar molecular weights which could be resolved on urea polyacrylamide gels as in scallop myosin [14]. It is also possible that there are two classes of light chains in *Physarum* myosin but that one class of light chains which is not essential for ATPase activity was destroyed by proteolysis or else was lost by dissociation during the purification procedure. Other workers have shown that one class of light chains can be removed from rabbit skeletal myosin by Nbs_2 [15] or from scallop myosin by EDTA [14] with no loss of ATPase activity.

The light chains of *Physarum* myosin were further characterized by examining their potential reaction with the purine disulfide analog of ATP, 6,6′-dithiobis(inosinyl imidodiphosphate). This analog, $(sIMP\text{-}PNP)_2$, inactivates skeletal and cardiac myosin ATPase by reacting with key cysteines located on the skeletal alkali light chains and cardiac 21 000-dalton* light chain [16, 17]. In these light chains, the modified cysteines are surrounded by an identical amino acid sequence. Therefore, this analog was used as a probe for homology between light chains as well as between myosins of different cellular types and species.

Inactivation studies using different concentrations of $(sIMP\text{-}PNP)_2$ with *Physarum* myosin turned out to be markedly different from skeletal and

*This light chain has a molecular weight of 21 000 based on its amino acid composition [22] and 27 000 by sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis [23].

cardiac myosin. Whereas both skeletal and cardiac myosin [17, 18] were inactivated in minutes by 10 : 1 ratios of $(sIMP\text{-}PNP)_2$ to enzyme, *Physarum* myosin lost its ATPase activity slowly under such conditions (Fig. 4). Even after 3 days the enzyme still retained 25% of its original activity. This activity was increased approximately 3-fold upon incubation with 5 mM dithioerythritol for 30 min at 0°C, indicating the analog was inactivating by forming mixed disulfides with the enzyme's cysteines. Conditions were altered to see if a greater rate of inactivation with $(sIMP\text{-}PNP)_2$ could be obtained. The substitution of 1.0 mM Ca^{2+} for Mg^{2+} during the inactivation or inactivating at 23°C instead of 0°C did not increase the rate of inactivation. Furthermore, adding $(sIMP\text{-}PNP)_2$ in several aliquots over a 2-h period did not markedly increase the rate of inactivation.

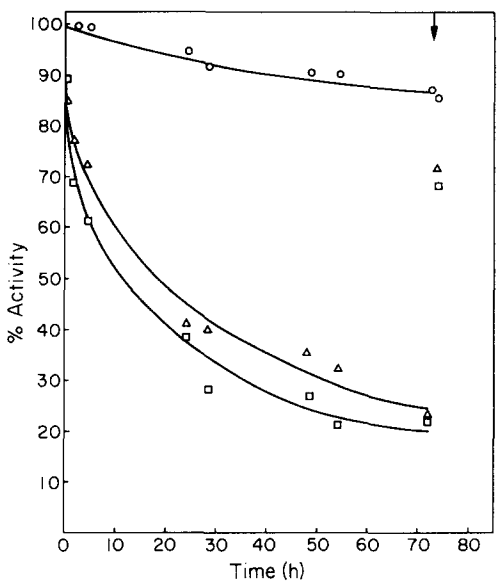

Fig. 4. Inactivation of *Physarum* myosin with $(sIMP\text{-}PNP)_2$. The enzyme (0.16 mg/ml) was incubated in 0.4 M KCl, 1.0 mM $MgCl_2$, 8 mM bicine (pH 8.0) with (△) 110 or (□) 220 μM $(sIMP\text{-}PNP)_2$ at 0° C. At varying times, aliquots were assayed for Ca^{2+}-ATPase activity. The arrow indicates those samples assayed 30 min after the addition of dithioerythritol to give a final concentration of 5 mM. (○), control.

It is possible that $(sIMP\text{-}PNP)_2$ did not react because the PNP linkage was affecting its binding to the enzyme. Accordingly, the kinetics of hydrolysis of ATP in the presence of the β-γ imido analog of ATP, adenylylimidodiphosphate, was examined. Adenylylimidodiphosphate was found to be a competitive inhibitor of both the Ca^{2+}- and Mg^{2+}-ATPase activities. The K_i values for Ca^{2+}-adenylylimidodiphosphate and Mg^{2+}-adenylylimidodiphosphate were 350 and 12 μM, respectively (Table I). As a corollary, the K_m values for the Ca^{2+}- and Mg^{2+}-ATPases were determined to be 17.3 and 2.4 μM, respectively. Therefore, the PNP linkage, at least at the active site, allowed tight binding so that the imidodiphosphate group does not appear responsible for the lack of inactivation by $(sIMP\text{-}PNP)_2$.

The reaction of $(sIMP\text{-}PNP)_2$ at a key cysteine appears to be absent in *Physarum* myosin, although we have confirmed the fact that *p*-chloromercuribenzoate rapidly blocks ATP hydrolysis [19]. Thus, treatment of *Physarum*

TABLE I

KINETIC CONSTANTS OF *PHYSARUM* MYOSIN ATPase INHIBITION BY ADENYLYLIMIDODIPHOSPHATE

Conditions: 0.5 M KCl, 2.0 mM Ca^{2+}, 25 mM Tris (pH 8.0) at 25°C using [γ-^{32}P]ATP. [γ-^{32}P]ATPase assays were performed as described by Yount et al. [26]. Data were analyzed by Michaelis-Menten program on a Wang 700 C programmable calculator.

Metal present	ATP		Adenylylimidodiphosphate	
	K_m (μM)	V (μmol P_i/min per mg)	Concn. range (μM)	K_i* (μM)
Ca^{2+}	17.3 ± 1	1.25 ± .03	56 —100	350 ± 40
Mg^{2+}	2.4 ± 0.2	0.12 ± .005	3.5— 14.6	12 ± 1

*The adenylylimidodiphosphate inhibition was competitive in the presence of both Ca^{2+} and Mg^{2+} although the Ca^{2+} data results are subject to a relatively large error because of the narrow range of adenylylimidodiphosphate concentrations used to determine the K_i.

myosin with only a 2-fold molar excess of *p*-chloromercuribenzoate at 0°C for 2 h gives 89% loss of the Ca^{2+}-ATPase activity [21]. *Physarum* myosin has very few cysteines [19] (less than 4.6 mol/mol of myosin**, compared to either skeletal myosin [20] (41.1 mol of cysteine/mol of myosin) or cardiac myosin [20] (36.2 mol of cysteine/mol of myosin). Therefore, the apparent lack of reactivity of $(sIMP\text{-}PNP)_2$ with *Physarum* myosin may reflect the absence of key cysteines rather than differences in the function of its light chains. It is also possible that the light chains of *Physarum* myosin were modified by $(sIMP\text{-}PNP)_2$ without having an effect on ATP cleavage. Additional work is needed on the number, amino acid composition, and function of the *Physarum* myosin light chains to answer these questions.

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References

1 Adelstein, R.S., Conti, M., Johnson, G., Paten, I. and Pollard, T.D. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3693—3697
2 Ash, J. (1975) J. Biol. Chem. 250, 3560—3566
3 Clarke, M. and Spudich, J. (1974) J. Mol. Biol. 86, 209—222
4 Adelman, M.R. and Taylor, E.W. (1969) Biochemistry 8, 4976—4988
5 Hatano, S. and Tazawa, M. (1968) Biochim. Biophys. Acta 154, 507—519
6 Nachmias, V.T. (1974) J. Cell Biol. 62, 54—65
7 Weltman, J. and Dowben, R. (1973) Proc. Natl. Acad. Sci. U.S. 70, 3230—3234
8 Kessler, D., Nachmias, V. and Loewy, A. (1976) J. Cell Biol. 69, 393—406
9 Nachmias, V.T. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 607—612
10 White, H. and Lascelles, J. (1973) Biochem. J. 135, 639—647
11 Adelman, M.R. (1975) Biophys. Soc. Abstr. 15, 161a
12 Mabuchi, I. (1973) J. Cell Biol. 59, 542—547
13 Azuma, N., Asakura, A. and Yagi, K. (1975) J. Biochem. 77, 973—981
14 Szent-Gyorgyi, A.G., Szent-Kiralyi, E.M. and Kendrick-Jones, J. (1973) J. Mol. Biol. 74, 179—203
15 Weeds, A.G. and Lowey, S. (1971) J. Mol. Biol. 61, 701—725
16 Wagner, P.W. and Yount, R.G. (1975) Biochemistry 14, 1908—1914
17 Greene, L.E. and Yount, R.G. submitted for publication
18 Yount, R.G., Frye, J.S. and O'Keefe, K.R. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 113—118

**These values were calculated using a molecular weight of 460 000 for *Physarum* myosin [4] and 470 000 for skeletal [24] and cardiac myosins [25].

19 Hatano, S. and Ohnuma, J. (1970) Biochim. Biophys. Acta 205, 110—120
20 Barany, M., Gaetjens, E., Barany, K. and Karp, E. (1964) Arch. Biochem. Biophys. 106, 280—293
21 Greene, L.E. (1975) Ph.D. Dissertation
22 Weeds, A.G. and Frank, G. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 9—14
23 Lowey, S. and Risby, D. (1971) Nature 234, 81—84
24 Gersham, L.C., Stracher, A. and Driezen, P. (1969) J. Biol. Chem. 244, 2726—2736
25 Wolodko, W. and Kay, C. (1975) Can. J. Biochem. 53, 164—174
26 Yount, R.G., Ojala, D. and Babcock, D. (1971) Biochemistry 10, 2490—2496