The Biosynthesis of Plasmodial Myosin during Starvation of *Physarum polycephalum*

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The actomyosin protein complex of *Physarum polycephalum* was prepared from vegetative and starved plasmodia. The yield of actomyosin per unit wet wt. was the same from both types of plasmodia. Myosin was resolved from the complex by gel filtration and purified by ion-exchange chromatography. The Ca^{2+} -stimulated adenosine triphosphatase activities of myosin preparations from vegetative and starved plasmodia were not appreciably different. Synthesis of myosin *de novo* was shown to occur during the starvation phase of the life-cycle by the isolation of labelled myosin preparations from plasmodia starved in the presence of $[2\text{-}^{14}\text{C}]\text{glycine}$. Fractionation of polyacrylamide gels after gel filtration of labelled myosin confirmed the presence of label in the adenosine triphosphatase-active myosin band. It is concluded that during starvation myosin synthesis continues although there is a net loss of approx. 50% of the total protein. Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of *Physarum* myosin showed the presence of low-molecular-weight components of the molecule, similar to those of muscle myosins. The content and composition of the free amino acid pool of *Physarum* was measured at various time-intervals during the vegetative and starvation phases of the life-cycle.

Physarum polycephalum is a plasmodial slime mould that requires haem for growth (Daniel & Baldwin, 1964). During the growth phase of the life-cycle the slime mould consists of a multinucleate mass of protoplasm of indefinite shape; however, under certain environmental conditions the plasmodium can undergo extensive differentiation either by sporulation or sclerotial formation (Rusch, 1970). Like other differentiating micro-organisms a period of starvation is necessary for morphogenesis (Baldwin & Rusch, 1965; Sussman & Sussman, 1969). During this starvation period more than 50% of the plasmodial protein is degraded (Daniel, 1966), and possibly 'expendable' proteins are the source of amino acids used to synthesize new proteins needed later in development. Protein synthesis *de novo* also occurs, since labelled amino acids are incorporated into protein during this phase of the morphogenetic sequence (Sauer *et al.*, 1969). Changes in the activity of certain soluble enzymes have been observed during morphogenesis to the spherule form: some decrease in activity whereas others increase in activity (Hüttermann *et al.*, 1970). The increase in activity of both glutamate dehydrogenase and phosphodiesterase during spherulation has been shown to be due to synthesis *de novo* by density labelling experiments with ^3H -labelled amino acids (Hüttermann *et al.*, 1971; Hüttermann, 1972).

As part of a general study of protein turnover in *P. polycephalum* during starvation, with the aim of determining whether selective degradation occurs, we have investigated the fate of the myosin moiety of

the actomyosin-like protein complex present in *Physarum* plasmodia. Myosin is presumably concerned in the migration movements of the plasmodium during starvation (Rusch, 1970), and in particular we wished to determine (a) whether the protein is formed *de novo* during starvation, and (b), if synthesis *de novo* occurs, whether the new protein formed is the same or a different species to the vegetative protein. The actomyosin complex has been extracted at high ionic strength from *Physarum* by a variety of workers (Hatano & Tazawa, 1968; Nachmias *et al.*, 1970; Nachmias & Ingram, 1970), and further resolved into myosin- and actin-containing fragments (Hatano & Ohnuma, 1970; Nachmias, 1972a). However, in our hands the method of Adelman & Taylor (1969a), employing a relatively low-ionic-strength pyrophosphate-rich extraction buffer, gave the cleanest preparations of the complex, a myosin-containing fraction being resolved by gel chromatography, and further purified by ion-exchange chromatography (Adelman & Taylor, 1969b).

As a preliminary to this work we have also looked at changes in the size and composition of the free amino acid pool of *Physarum* during growth and starvation, since the extensive protein turnover occurring during starvation might be expected to be reflected in the pool contents during this period.

Materials and Methods

Physarum polycephalum strain M3C, originally obtained from Dr. H. P. Rusch, The McArdle

Laboratory for Cancer Research, University of Wisconsin, Madison, Wis., U.S.A., was maintained as a shaken suspension of microplasmodia by serial transfer every 3 days into 40 ml of the semi-defined medium of Chin & Bernstein (1968) in 125 ml Erlenmeyer flasks. Plasmodia for the extraction of myosin were grown in 500 ml volumes of the same medium in 1 litre Erlenmeyer flasks; these were sown with 10 ml of a 3-day stock culture and incubated in a New Brunswick Psychrotherm G-27 incubator-shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 25°C and 200 rev./min for 3 days. The yield of plasmodia/flask was 20–30 g wet wt.

Starvation was accomplished by using 14-cm-diameter Petri dishes, each containing approx. 50 ml of the CaCO₃-buffered niacin-containing salts solution defined by Daniel & Rusch (1962), modified as described by Sauer *et al.* (1969), and solidified by the addition of 3% (w/v) Bacto-Agar (Difco Laboratories, Detroit, Mich., U.S.A.). Microplasmodia from late-exponential-phase shaken cultures were harvested aseptically by decantation and low-speed centrifugation (40g for 5 min), washed once by suspension in 2–3 vol. of sporulation salts solution followed by sedimentation by low-speed centrifugation, and resuspended in 1 vol. of sporulation salts solution. Between 5 and 10 g wet wt. was pipetted aseptically on to the surface of each sporulation plate, and the plates were incubated in the dark at 22°C for 4–5 days. To obtain the quantities of starved plasmodia necessary for myosin isolation (50–100 g wet wt.), 24–30 plates were inoculated for each experiment and, after the incubation period, the starved plasmodia were scraped from the surface of the agar and pooled. Control plates were induced to sporulate by a 6 h illumination period.

Myosin preparation

Myosin was extracted and purified from both vegetative and starved plasmodia essentially by the method of Adelman & Taylor (1969*a,b*). Freshly harvested plasmodia (vegetative or starved) were washed twice by suspension in ice-cold distilled water, followed by centrifugation at 365g for 5 min. This and all subsequent procedures were carried out at 4°C. The wet weight of plasmodia used for each experiment was determined after washing, and homogenates were prepared by using a ratio of 1 ml of extraction medium (10 mM-Tris-maleate–50 mM-Na₄P₂O₇ buffer, pH 6.8) to 1 g of washed plasmodia. The plasmodial mass was first slurried with the extraction solution by three passes in a Waring blender, and the slurry was then further homogenized by ten passes in a 50 ml-capacity Teflon-glass homogenizer. Homogenates were stored for about 1 h with gentle mixing before centrifugation in the Spinco no. 30 rotor for 45 min at 60 000g ($r_{av.} = 7.8$ cm), and the volume

(Eml) of the supernatant was noted (approx. 1.5 ml/g of washed plasmodia). Extracts were brought to 45% (w/v) saturation with (NH₄)₂SO₄ (special enzyme grade; Mann Research Laboratories, New York, N.Y., U.S.A.), the pH was adjusted to 6.5 and, after 30 min, the precipitate was centrifuged down (no. 30 rotor; 45 min at 60 000g). The pellets were resuspended in 0.20 Eml of 10 mM-Tris-maleate buffer, pH 6.2, by using a Teflon homogenizer, and the solution was clarified (no. 30 rotor; 45 min at 60 000g). The supernatant was then brought to 35% (w/v) saturation with (NH₄)₂SO₄, the pH was adjusted to 6.0 and the precipitate formed after 30 min was spun down [no. 65 rotor; 30 min at 170 000g ($r_{av.} = 5.7$ cm)]. Pellets were resuspended in 10 mM-Tris-HCl buffer (pH 7.8)–2% (w/v) sucrose–5 mM-ATP to a concentration of approx. 10 mg of protein/ml and clarified (no. 30 rotor; 30 min at 60 000g).

Crude actomyosin preparations were desalted by passage down a Sephadex G-25 column (5 cm × approx. 55 cm, giving a sample volume/bed volume ratio of about 1:10), equilibrated and eluted with TAM buffer (1 mM-Tris-HCl–1 mM-ATP–5 mM-2-mercaptoethanol buffer, pH 7.8). The column was eluted under downward flow at approx. 60 ml/h and fractions (13 ml) were assayed for protein content and ATPase* activity. Chromatography on Sephadex G-200 was carried out with two or three columns in series (2.5 cm × 75–95 cm) equilibrated and eluted with TAM buffer, pH 7.8. Fractions (6.5 ml) were collected at an upward flow rate of 10 ml/h and assayed by the same parameters as in the preceding step. ATP was added to the pooled Ca²⁺-stimulated ATPase enzyme to a final concentration of 1 mM and the preparations were applied to a bed (2 cm × 16 cm) of Bio-Rad Cellex-D DEAE-cellulose equilibrated with 10 mM-Tris-HCl buffer, pH 7.8. The column was first eluted with 100 ml of 50 mM-KCl–10 mM-Tris-HCl buffer, pH 7.8, at a flow rate of approx. 60 ml/h. The enzyme was then eluted with 100 ml of 175 mM-KCl–10 mM-Tris-HCl buffer, pH 7.8, at a flow rate of approx. 30 ml/h. Fractions (6.5 ml) collected during the enzyme elution stage were assayed as described above.

The final step in the purification of slime-mould myosin was a refractionation by salt precipitation. DEAE-enzyme preparations were brought to 45% (w/v) saturation with (NH₄)₂SO₄, the pH was adjusted to 6.5 and the precipitate formed after 30 min was collected by centrifugation [no. 40 rotor; 30 min at 50 000g ($r_{av.} = 5.9$ cm)]. The supernatant was brought to 60% (w/v) saturation with (NH₄)₂SO₄ and after 30 min the precipitate was centrifuged down (no. 40 rotor; 30 min at 50 000g). The pellets were resuspended in 0.5 M-KCl–10 mM-Tris-HCl buffer, pH 7.4, dialysed overnight against the same buffer and finally clarified (no. 65 rotor; 30 min at 170 000g).

* Abbreviation: ATPase, adenosine triphosphatase.

Disc gel electrophoresis

Native acrylamide gels were polymerized in glass tubes 120 mm long $\times$ 6 mm internal diameter. The separating gel contained 7% (w/v) acrylamide and 0.2% (w/v) bismethylenecrylamide, and was buffered with Tris-HCl at pH 8.9. The stacking gel contained 2.5% (w/v) acrylamide and 0.6% (w/v) bismethylenecrylamide, and was buffered with Tris-HCl at pH 6.7. Samples (100–200 μ g) were applied in 20% (w/v) sucrose, and electrophoresis was carried out in a Hoefer apparatus (Hoefer Scientific Instruments, San Francisco, Calif., U.S.A.) at room temperature, with 5 mM-Tris–40 mM-glycine as the running buffer at pH 8.3. The buffer front was indicated with tracking dye (0.1% Bromophenol Blue), a drop of which was added to the cathode buffer chamber. Gels were run at a constant current of 1 mA/tube until the tracking dye had just entered the running gel, and the current was then raised to 2 mA/tube for the duration of the run. Gels were fixed in 20% (w/v) trichloroacetic acid for 30 min, stained for 1 h in a 1:20 dilution in 20% (w/v) trichloroacetic acid of an aq. 1% (w/v) solution of Coomassie Blue and de-stained in 10% (w/v) trichloroacetic acid.

For sodium dodecyl sulphate–polyacrylamide-gel electrophoresis myosin preparations were dialysed against 8 M-urea–0.1% (w/v) sodium dodecyl sulphate–0.1% (v/v) 2-mercaptoethanol–10 mM-Tris-HCl buffer, pH 7.4. For each gel 20–100 μ g of protein was mixed with 100–500 μ l of a sample buffer containing 3% (w/v) sodium dodecyl sulphate, 2% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 20 mM-Tris-HCl buffer, pH 8.0, and heated in a boiling-water bath for 1.5 min. Polyacrylamide gels (10%, w/v), 0.1% (w/v) with respect to sodium dodecyl sulphate, were made as described by Laemmli (1970). Gels were run at 2 mA/tube for approx. 6 h, fixed and stained by the method of Weber & Osborn (1969), and de-stained in a mixture of 10% (v/v) acetic acid and 10% (v/v) methanol.

Molecular weights were determined from a calibration curve constructed by using as protein standards: bovine serum albumin (mol.wt. 67 000), ovalbumin (mol.wt. 45 000), bovine pancreas chymotrypsinogen A (mol.wt. 25 000), sperm-whale myoglobin (mol.wt. 17 800) and horse heart cytochrome *c* (mol.wt. 12 400) (all from Mann Research Laboratories), carboxypeptidase A (mol.wt. 34 000) (from Worthington Biochemical Corp., Freehold, N.J., U.S.A.), bovine pancreas trypsin (mol.wt. 23 000) (from Sigma Chemical Co., St. Louis, Mo., U.S.A.) and lysozyme (mol.wt. 14 400) (from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.).

Stained gels were scanned with a Vicon linear gel scanner (Brinkmann Instruments Inc., Westbury, N.Y., U.S.A.). Gels containing radioactive material were fractionated by passage through a Savant Autogeldivider, and fractions were collected for liquid-

scintillation counting of radioactivity in glass vials; 10 ml of a Triton X-100–toluene (3:1, v/v) scintillation 'cocktail' was added to each and the radioactivities of the samples were counted in a Beckman LS200 liquid-scintillation spectrometer.

Localization of Ca^{2+} -stimulated ATPase activity in native gels was carried out by a modification of the method of Weinbaum & Markman (1966). Gels were incubated for 2–4 h at room temperature in a solution containing 0.8 mM-ATP, 10 mM- CaCl_2 , 0.12% (w/v) $\text{Pb}(\text{NO}_3)_2$ and 40 mM-Tris–maleate buffer, pH 8.0, and, after being washed in distilled water, placed in 0.1 M-Tris–maleate buffer, pH 8.0. When H_2S was bubbled through the suspending buffer, a precipitate of PbS was produced on the gel in the region occupied by the enzyme.

Incorporation of isotopic label and determination of radioactivity

Labelling of plasmodia during starvation was carried out in 14 cm-diameter Petri dishes each containing 50 ml of sporulation salts–agar supplemented with $[2\text{-}^{14}\text{C}]\text{glycine}$ (Schwarz/Mann Laboratories) (0.1 $\mu\text{Ci/ml}$; 0.5 mM). Radioactivity was measured in trichloroacetic acid-precipitated material filtered on to prewashed glass-fibre papers (Whatman GF/C) and washed with 10% (w/v) trichloroacetic acid at room temperature followed by 70% (v/v) ethanol at 70°C. The filters were dried at 100°C in scintillation vials, mixed with 10 ml of toluene–Spectrafluor (Amersham/Searle Corp., Chicago, Ill., U.S.A.) and their radioactivities counted in a Beckman LS200 liquid-scintillation spectrometer.

Amino acid analyses

The free amino acid contents of vegetative and starving plasmodia were determined at various intervals during the *Physarum* life-cycle. Preliminary experiments showed that centrifugation of microplasmodia did not result in a loss from the amino acid pool, when compared with material harvested by decantation; therefore sedimentation at 365 g for 5 min was adopted as routine for the collection and washing of microplasmodia. Duplicate samples were harvested from 500 ml submerged cultures growing with aeration at 25°C, and assayed for either amino acids or protein. The pellets were washed twice by centrifugation in basal salts (a solution of the inorganic constituents of the growth medium), frozen (–20°C for about 12 h) and, after being thawed, extracted with 90% (v/v) ethanol overnight at 4°C. After a further washing with 1 vol. of 90% (v/v) ethanol, the combined ethanolic extracts were concentrated by evaporation *in vacuo* at 40°C and either they were assayed for total amino acid content by the ninhydrin method (Moore & Stein, 1948) or duplicate

samples were analysed on a Beckman model 116 amino acid analyser. The amount of each amino acid in the extracts was determined by measurement of the peak areas on the traces from the analyser and subsequent reference to standard curves. Starving plasmodia were harvested daily and resuspended in sporulation salts solution, and duplicate samples were extracted and analysed by the same procedure outlined for vegetative plasmodia.

Determination of protein

Protein concentrations in samples of vegetative and starving plasmodia were measured after overnight extraction with equal volumes of 10% (w/v) trichloroacetic acid and acetone. Insoluble material was sedimented by centrifugation, then dissolved in a known total volume of 1M-NaOH and water, and the protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as a standard.

Assay of ATPase activity

Assays of ATPase activity were carried out at room temperature (22°C) in medium containing

Fig. 1. Changes in free amino acid amounts during growth of *P. polycephalum*

Submerged 500ml cultures were grown as described in the Materials and Methods section. Duplicate samples were removed at intervals, and the microplasmodia were sedimented by centrifugation and assayed for total protein and free amino acids: ○, mg of protein/10ml of sample; ●, μg of leucine equivalents/10ml of sample; □, μg of leucine equivalents/mg of protein.

0.6M-KCl, 10mM-Tris-maleate buffer, pH 8.0, 10mM-CaCl₂ and 2mM-ATP, unless otherwise specified. The reaction was initiated by the addition of protein (0.5–1.0 mg) and terminated by adding 0.1ml of 50% (w/v) trichloroacetic acid. P_i was measured by the method of Sumner (1944).

Results

Changes in amino acid pool size and composition during growth and starvation of *P. polycephalum*

The concentration of free amino acids was analysed in growing and starving plasmodia to determine (i) whether loss in protein during starvation was accompanied by an increase in the amino acid pool, and (ii) whether the composition of the pool varied during starvation, possibly as a result of selective utilization of individual amino acids as an energy source.

The total intracellular free amino acid pool, expressed as μg of leucine equivalents/mg of protein, remained essentially constant throughout the 3-day growth period (Fig. 1). During a 4-day starvation period total plasmodial protein declined by approx. 50%, as reported by Daniel (1966); however, the concentration of free amino acids rose during the first 48 h and then declined to the starting value.

Table 1. Composition of the free amino acid pools in growing and starved plasmodia of *P. polycephalum*

Plasmodia were grown and induced to starve as described in the Materials and Methods section. At the times indicated duplicate samples were extracted and assayed for both protein and free amino acids. The concentration of each amino acid in the extracts was assessed by measurement of peak areas on the traces from the amino acid analyser. N.D., Not detectable.

Amino acid	Composition (μmol/mg of protein)	
	3-day vegetative plasmodia	3-day starved plasmodia
Pro	3.27	0.54
Ser+Gln+Asn	2.05	1.04
Ala	1.59	2.27
Val	1.19	0.89
Leu	1.03	0.91
Ile	0.49	0.45
Gly	0.30	0.59
Lys	0.27	0.03
Arg	0.20	N.D.
Met	0.18	0.08
His	0.12	0.02
Glu	0.10	0.50
Asp	0.07	0.22

Amino acid analyses of *P. polycephalum* extracts showed that, with the exception of proline, the predominant pool amino acids during growth (serine, alanine, valine and leucine) were also predominant during starvation, and amino acids undetectable during growth (e.g. all the aromatic amino acids) did not appear during starvation (Table 1).

Purification of slime-mould myosin from vegetative and starved plasmodia

Actomyosin was extracted from vegetative and starved plasmodia by the method of Adelman &

Taylor (1969a). Since both slime-mould actomyosin and myosin were soluble at ionic strengths low enough to 'depolymerize' presumptive actin, the separation of the actomyosin into fractions containing the actin and myosin proteins was based on the resolution of the high-molecular-weight myosin from the low-molecular-weight actin subunit by gel chromatography on Sephadex G-200 at low ionic strength. To assure full depolymerization of the actin component, crude slime-mould actomyosin preparations were first desalted by passage over Sephadex G-25.

The yield of actomyosin obtained from either vegetative or starved plasmodia was the same; per g wet

Fig. 2. Elution of labelled *P. polycephalum* myosin from DEAE-cellulose

The column (2cm×16cm) contained DEAE-cellulose (Bio-Rad Cellex-D) equilibrated with 10mM-Tris-HCl buffer, pH7.8. The applied sample consisted of 90ml of ATPase enzyme voided by Sephadex G-200 (0.5mg of protein/ml, with a Ca²⁺-stimulated ATPase specific activity of 1.18μmol of P_i/10min per mg, prepared from plasmodia of specific radioactivity 40612c.p.m./mg of protein) plus 0.9ml of 0.1M-ATP. After elution with 100ml of 50mM-KCl-10mM-Tris-HCl buffer, pH7.8, the enzyme was eluted with 100ml of 175mM-KCl-10mM-Tris-HCl buffer, pH7.8. Fractions (6.5ml) were assayed for protein content (●), Ca²⁺-stimulated ATPase activity (□) and radioactivity (○). The pooled enzyme from the DEAE-cellulose step constituted a total volume of 13ml, containing 0.8mg of protein/ml, with a Ca²⁺-stimulated ATPase specific activity of 2.18μmol of P_i/10min per mg and a specific radioactivity of 27443c.p.m./mg of protein.

wt. of either 3-day vegetative or 4-day starved plasmodia the amount varied in the range 2–5 mg of protein. Protein and Ca^{2+} -stimulated ATPase activity were quantitatively recovered after Sephadex G25-chromatography, and peak fractions, accounting for 50–75% of the input material, were pooled and applied directly to a Sephadex G-200 column. The elution profile after Sephadex G-200 chromatography typically consisted of a protein peak coincident with the column void volume and a shoulder of retarded protein, with the Ca^{2+} -stimulated ATPase activity localized in the voided protein. When pooled enzyme from the Sephadex G-200 step, made 1 mM with respect to ATP, was applied to a DEAE-cellulose column and eluted stepwise, a discrete peak of Ca^{2+} -stimulated ATPase activity with approximately twice the input specific activity was eluted (Fig. 2). Actomyosin and myosin prepared from either vegetative or starved plasmodia had approximately the same Ca^{2+} -stimulated ATPase activity at all stages of the purification procedure (Table 2).

Native 7% (w/v) polyacrylamide gels of myosin preparations from both vegetative and starved plasmodia were characterized by a major protein band, which migrated approximately one-third the length of the gel, together with one or more minor adjacent bands, and another major protein band, which barely entered 7% polyacrylamide gels. This protein gave a strong positive reaction for Ca^{2+} -stimulated ATPase activity, which, together with its inability to migrate far in 7% gels, was indicative of its myosin-like nature. Electrophoresis in 5% (w/v) polyacrylamide gels caused a greater degree of migration of this protein but with concomitant loss of resolution. Increased migration without loss of resolution was obtained by continued electrophoresis of 7% polyacrylamide gels

until the marker dye had left the gel. Under these conditions the ATPase enzyme was detected as a discrete band. The patterns of protein and enzyme activity in myosin preparations from starved plasmodia were similar to those from vegetative plasmodia. They showed no evidence of different species of myosin or of new additional proteins.

The final step in the purification of slime-mould myosin, a $(\text{NH}_4)_2\text{SO}_4$ fractionation of the enzyme from the DEAE-cellulose step, gave a protein preparation that consisted predominantly of the Ca^{2+} -stimulated ATPase-active protein and two other bands. Such preparations had a Ca^{2+} -stimulated ATPase specific activity of 3.5–4.0 μmol of P_i /10 min per mg of protein, which compares favourably with a Ca^{2+} -stimulated ATPase specific activity of 4.16 μmol of P_i /10 min per mg of protein reported for rabbit myosin assayed under the same conditions (Adelman & Taylor, 1969b).

In view of the known strong tendency of contractile proteins to interact with each other and the consequent difficulties in obtaining pure preparations, it seemed possible that the contaminating protein bands present in native gels of myosin preparations might be other muscle-type proteins present as impurities in the myosin. In an attempt to characterize these proteins and gain some information on the subunit composition of the plasmodial myosin molecule, salt-fractionated myosin from the DEAE-cellulose step was analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. A number of discrete protein species were revealed by electrophoresis of such preparations on 10% sodium dodecyl sulphate–polyacrylamide gels, ranging in molecular weight from a band of very high but undetermined molecular weight at the top of the gel (band A), through bands of

Table 2. Ca^{2+} -stimulated ATPase activity of myosin preparations from vegetative and starved plasmodia of *P. polycephalum*

Activities are for preparations from 3-day vegetative plasmodia or from plasmodia starved for 4 days on sporulation salts–agar. Values are the means of three separate experiments, corrected for zero-time activity. ATPase assays were carried out at room temperature in KCl concentrations of either 0.60 M or 60 mM, with 10 mM-Tris–maleate buffer, pH 8.0, and 2 mM-ATP. Bivalent cations, as indicated, were present at final concentrations of 10 mM each. Reactions were terminated by the addition of 0.1 ml of 50% (w/v) trichloroacetic acid. N.T. = Not tested.

Enzyme preparation	Concn. of K^+ (M)	Sp. activity (μmol of P_i /10 min per mg of protein)					
		3-day vegetative plasmodia			4-day starved plasmodia		
		No M^{2+}	Mg^{2+}	Ca^{2+}	No M^{2+}	Mg^{2+}	Ca^{2+}
Actomyosin voided from	0.60	0.448	0.475	1.305	0.152	0.000	1.595
Sephadex G-25	0.06	0.232	0.281	0.990	0.000	N.T.	0.980
Enzyme voided from	0.60	0.431	0.443	1.495	0.226	0.336	1.970
Sephadex G-200	0.06	0.301	0.448	1.239	0.000	N.T.	1.300
Myosin from DEAE-cellulose step	0.60	N.T.	N.T.	2.773	N.T.	N.T.	2.851

approximate molecular weights 130000 (band *B*), 44000 (band *C*), 39000 (band *D*) and 24000 (band *E*), to a large band of molecular weight 15000 (band *G*), with a shoulder of approximate molecular weight 18000 (band *F*) and another shoulder of molecular weight about 12000 (band *H*) (Fig. 3). To determine which of these bands constituted the myosin subunits, the ATPase-active protein band was excised from 7% native gels, homogenized with sodium dodecyl sulphate and electrophoresed on 10% sodium dodecyl sulphate–polyacrylamide gels. Such gels were characterized by an identical band pattern in the 12000–18000 and 24000-molecular-weight region to that of sodium dodecyl sulphate–polyacrylamide gels of salt-fractionated myosin from the DEAE-cellulose step, together with a variable amount of the high-molecular-weight protein (band *A*), scarcely capable of entering 10% sodium dodecyl sulphate–polyacrylamide gels. The bands absent from such gels but present in sodium dodecyl sulphate–polyacrylamide gels of salt-fractionated myosin from the DEAE-cellulose step (bands *B*, *C* and *D* in Fig. 3) were assumed to be impurities in the myosin preparations, but were not further characterized.

Fig. 3. Densitometer scan of sodium dodecyl sulphate–polyacrylamide gel after gel electrophoresis of purified *P. polycephalum* myosin preparation

The 45–60%–saturation-(NH_4)₂SO₄ fraction of myosin from the DEAE-cellulose step was dialysed overnight against 8M-urea–1% (w/v) sodium dodecyl sulphate–1% (v/v) 2-mercaptoethanol–10mM-Tris–HCl buffer, pH 7.4. The applied sample consisted of 90 μg of dialysed protein mixed with 100 μl of sample buffer and heated at 100°C for 1.5 min. The 10% (w/v) sodium dodecyl sulphate–polyacrylamide gels were made as described in the Materials and Methods section, and electrophoresis was carried out at 2mA/tube. Gels were fixed and stained by the method of Weber & Osborn (1969). For details of bands (A)–(H) see the text.

Incorporation of labelled amino acid into slime-mould myosin during starvation

The conditions for following [2-¹⁴C]glycine incorporation into myosin during starvation were established by preliminary small-scale experiments. Incorporation of labelled glycine was linear for at least 5 days of starvation on the surface of salts–agar containing [2-¹⁴C]glycine (0.5mM; 0.1 $\mu\text{Ci/ml}$), and these conditions were therefore used for large-scale starvation experiments.

When myosin was purified from plasmodia starved in the presence of [2-¹⁴C]glycine, stepwise elution from DEAE-cellulose gave coincident peaks of protein, ATPase activity and radioactivity (Fig. 2). The specific radioactivity of such preparations (approx. 30000 c.p.m./mg of protein) was comparable with that of the total protein in starved plasmodia (approx. 40000 c.p.m./mg of protein). Gel electrophoresis of the labelled enzyme from the DEAE-cellulose step and subsequent counting of the radioactivities of the gel fractions showed radioactivity in three proteins, one of which corresponded to the Ca²⁺-stimulated ATPase-active band (Fig. 4).

Fig. 4. Distribution of radioactivity in polyacrylamide gels of labelled *P. polycephalum* myosin preparations

The sample consisted of 240 μg of myosin from the DEAE-cellulose step in 175mM-KCl–10mM-Tris–HCl buffer, pH 7.8, plus 10% (w/v) sucrose. Native polyacrylamide gels (7%, w/v) were made as described in the Materials and Methods section, and electrophoresis was carried out at 2mA/tube. Stained gels were scanned and then fractionated by passage through an Autogeldiver. Fractions were collected in scintillation vials, mixed with 10ml of scintillation 'cocktail' and their radioactivities were counted. Upper line, densitometer tracing; lower line, radioactivity. The arrow indicates the location of the ATPase activity.

Discussion

Morphogenesis in slime-moulds is initiated by nutritional deficiency, and metabolism of pre-existing cell constituents results in the loss of a considerable fraction of the original plasmodial dry weight. The high rate of protein turnover allows for extensive protein utilization for maintaining the composition of critical proteins, and for synthesizing new proteins (Smith & Galbraith, 1971). The purpose of the present investigation was to determine whether *P. polycephalum* myosin was 'spared' during the starvation phase of the life-cycle, and whether in fact synthesis of myosin occurred during this period.

No significant differences were found in either the yield or the Ca^{2+} -stimulated ATPase specific activity of myosin preparations from vegetative and starved plasmodia (Table 2). However, since starvation is accompanied by a loss in dry weight, a comparison of the amount of myosin/g dry wt. of vegetative and starved plasmodia is not indicative of either net synthesis or net degradation. A tentative conclusion that can be drawn is that myosin is maintained at a constant concentration/unit of plasmodia during starvation.

The true synthesis *de novo* of certain soluble enzymes during differentiation of *P. polycephalum* has been shown by using equilibrium sedimentation in CsCl density gradients to compare the buoyant densities of enzymes extracted from plasmodia spherulating in the presence of deuterated amino acids with the densities of the same enzymes in extracts of vegetative plasmodia (Hüttermann *et al.*, 1971; Hüttermann, 1972). This method was not applicable to myosin, since CsCl, at the high concentration needed to band the enzyme, interfered with the assay of enzyme activity.

Incorporation of a labelled amino acid into *Physarum* plasmodia during starvation was first reported by Sauer *et al.* (1969). Hatano & Ohnuma (1970) found glycine present in *Physarum* myosin to the extent of $43 \text{ mol}/10^5 \text{ g}$ of protein, and preliminary work showed glycine to have a relatively low pool content during starvation (Table 1). With appropriate concentrations of $[2\text{-}^{14}\text{C}]$ glycine, label incorporation into plasmodia was linear over a 5-day starvation period with a final uptake of approx. 12% of the total label, and the specific radioactivity of myosin preparations from labelled plasmodia approximated to the specific radioactivity of total plasmodial protein (Fig. 2), indicating a true synthesis *de novo*. In addition, gel electrophoresis of labelled myosin preparations showed the same band pattern as vegetative preparations, with label localized in three proteins, one of which corresponded to the ATPase-active band at the top of the gel (Fig. 4).

Our results indicate that synthesis of myosin *de novo* occurs at a time of intense protein degradation by the organism. This is in keeping with the proposed role for

myosin as a causative agent of the vigorous cytoplasmic streaming associated with starving plasmodia of *P. polycephalum* (Rusch, 1970). The turnover of myosin was not measured in the present work; however, the fact that the specific activity of the enzyme remains the same during starvation as that in vegetative plasmodia suggests that the rate of synthesis is sufficient to balance the rate of degradation.

That plasmodial myosin can be localized on 7% polyacrylamide native gels suggests differences, either in shape and size or charge, between the plasmodial molecule and striated-muscle myosin. However, there are no obvious marked differences in shape factors between *Physarum* myosin and vertebrate muscle myosins. Both proteins behave similarly on an Agarose 4B column (Adelman & Taylor, 1969b) and in the analytical ultracentrifuge (Adelman & Taylor, 1969b; Hatano & Ohnuma, 1970), and both negatively stained *Physarum* myosin-enriched actomyosin (Nachmias & Ingram, 1970) and metal-shadowed *Physarum* myosin (Hatano & Takahashi, 1971) show an extended 'tail' portion of the molecule at least 120 nm long. On the other hand, *Physarum* myosin is strikingly different in its solubility properties from muscle myosins (Adelman & Taylor, 1969b), and evidence suggests that this may be due to a charge distribution along the extended part of the molecule different from that characteristic of muscle myosins (Nachmias, 1972b). Whether this is sufficient to account for differences in electrophoretic mobilities between slime-mould and muscle myosins remains to be seen.

Salt-fractionated myosin preparations from the DEAE-cellulose step had a Ca^{2+} -stimulated ATPase specific activity comparable with that of rabbit muscle myosin. Sodium dodecyl sulphate-polyacrylamide gels of such preparations were characterized by a very distinctive band pattern in the 12000–18000-molecular-weight region, together with a protein peak of molecular weight 24000 and a limited number of high-molecular-weight peaks (Fig. 3). The same pattern of bands of molecular weight 12000–18000 and 24000, together with a high-molecular-weight protein barely able to enter 10% sodium dodecyl sulphate-polyacrylamide gels, was obtained on sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of the excised ATPase-active band from native polyacrylamide gels, suggesting that these bands constitute the subunits of the plasmodial myosin molecule; the high-molecular-weight protein banding at the top of the gel presumably corresponding to the heavy chains. The impurities present in salt-fractionated myosin preparations from the DEAE-cellulose step and detected on sodium dodecyl sulphate-polyacrylamide gels were not further characterized, and whether or not they represent contamination of the myosin with slime-mould actin [calculated by Adelman & Taylor (1969b)]

to have a molecular weight of between 37000 and 44000] or the slime-mould equivalents of troponin or tropomyosin, if they exist, is for the moment open to speculation.

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References

- Adelman, M. R. & Taylor, E. W. (1969a) *Biochemistry* **8**, 4964-4975
- Adelman, M. R. & Taylor, E. W. (1969b) *Biochemistry* **8**, 4976-4988
- Baldwin, H. H. & Rusch, H. P. (1965) *Annu. Rev. Biochem.* **34**, 565-594
- Chin, B. & Bernstein, I. A. (1968) *J. Bacteriol.* **96**, 330-337
- Daniel, J. W. (1966) in *Cell Synchrony* (Cameron, I. L. & Padilla, G. M., eds.), pp. 117-152, Academic Press, London and New York
- Daniel, J. W. & Baldwin, H. H. (1964) *Methods Cell Physiol.* **1**, 9-41
- Daniel, J. W. & Rusch, H. P. (1962) *J. Bacteriol.* **83**, 234-240
- Hatano, S. & Ohnuma, J. (1970) *Biochim. Biophys. Acta* **205**, 110-120
- Hatano, S. & Takahashi, K. (1971) *J. Mechanochem. Cell Motility* **1**, 7-14
- Hatano, S. & Tazawa, M. (1968) *Biochim. Biophys. Acta* **154**, 507-519
- Hüttermann, A. (1972) *Arch. Mikrobiol.* **83**, 155-164
- Hüttermann, A., Porter, M. T. & Rusch, H. P. (1970) *Arch. Mikrobiol.* **74**, 283-291
- Hüttermann, A., Elsevier, S. M. & Eschrich, W. (1971) *Arch. Mikrobiol.* **77**, 74-85
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Moore, S. & Stein, W. H. (1948) *J. Biol. Chem.* **176**, 367-388
- Nachmias, V. T. (1972a) *J. Cell Biol.* **52**, 648-663
- Nachmias, V. T. (1972b) *Proc. Nat. Acad. Sci. U.S.* **69**, 2011-2014
- Nachmias, V. T. & Ingram, W. C. (1970) *Science* **170**, 743-745
- Nachmias, V. T., Huxley, H. E. & Kessler, D. (1970) *J. Mol. Biol.* **50**, 83-90
- Rusch, H. P. (1970) *Advan. Cell Biol.* **1**, 297-327
- Sauer, H. W., Babcock, K. L. & Rusch, H. P. (1969) *Exp. Cell Res.* **57**, 319-327
- Smith, J. E. & Galbraith, J. C. (1971) *Advan. Microbial Physiol.* **5**, 45-134
- Sumner, J. B. (1944) *Science* **100**, 413-414
- Sussman, M. & Sussman, R. R. (1969) *Symp. Soc. Gen. Microbiol.* **10**, 403-435
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412
- Weinbaum, G. & Markman, R. (1966) *Biochim. Biophys. Acta* **124**, 207-209