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Preparation and Characterization of Frog Muscle Myosin Subfragment 1 and Actin

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The preparation, structural and steady-state kinetic characteristics of contractile proteins from the leg muscle of frogs *Rana temporaria* and *Rana pipiens* are described. Actin and myosin from the two frog species are indistinguishable. The proteins have structural and steady-state kinetic properties similar to those from rabbit fast-twitch skeletal muscle. Chymotrypsin digestion of frog myosin or myofibrils in the presence of EDTA yields subfragment 1, which is separated by chromatography into two components that are distinguished by their alkali light-chain content.

Rabbit muscle has been the subject of extensive biochemical studies on the composition, structure and kinetic properties of the proteins involved in contraction. However, comparison with data from mechanical and energetic experiments is difficult, because most of these physiological studies have been performed on frog muscle. To make direct comparisons possible, we have examined the biochemical properties of frog skeletal-muscle myosin, under conditions of pH, ionic strength and temperature that approximate as far as is feasible to those in the intact muscle. The first step in this study was to obtain pure active protein from frog muscle in sufficient quantities for the kinetic experiments. A method of isolation was devised similar to that of Weeds (1976). In the present paper we describe the method of isolation and the steady-state ATPase activity of the myosin thus obtained. In the following paper (Ferenczi *et al.*, 1978) the elementary processes of the Mg^{2+}-dependent ATPase of frog myosin and subfragment 1 are investigated by using transient kinetic techniques.

Methods

Preparation of frog myofibrils and myosin

The frog species used was mainly *Rana temporaria*, but some experiments were carried out on *Rana pipiens*. *R. temporaria* were obtained from T. Gerard and Co., East Preston, West Sussex, U.K., and *R. pipiens* from Steinhiber Co., Oshkosh, WI, U.S.A. Animals were kept for up to 5 weeks in ½ in-deep water tanks at 4°C. Between five and 12 frogs were used for each protein preparation. All operations were carried out at 0–4°C in the presence of 100 μM-dithiothreitol. Animals were pithed and the musculature of the thigh and lower legs was quickly removed and kept on ice. Each frog provided between 3 and 5 g wet wt. of muscle. The muscle was blended in 3 vol. of 40 mM-NaCl / 1 mM-$MgCl_2$ / 100 μM-EDTA / 5 mM-sodium phosphate (pH 7.0) / 10 μM-phenylmethanesulphonyl fluoride / 100 μM-sodium azide (solution A) in a Sorvall Omnimixer or MSE Ato-Mix. After centrifugation at 18000*g* for 10 min the supernatant was discarded and the myofibrils were blended in 6 vol. of solution A. This operation was repeated with 10 vol. of solution A, and the myofibrils were finally resuspended in 200 ml (approx. 6 vol.) of 0.6 M-NaCl/10 mM-$MgCl_2$/10 mM-ATP/1.5 mM-sodium pyrophosphate/50 mM-Tris/HCl (pH 7.5). After centrifugation at 190000*g* for 3 h the top two-thirds of the supernatant (containing myosin) was dialysed overnight against 10 mM-NaCl/1 mM-$MgCl_2$/10 mM-sodium phosphate (pH 6.4) to precipitate the myosin. In some cases precipitation occurred only when the pH value was decreased to 6.2. Myosin was centrifuged at 25000*g* for 15 min, and the pellet washed three times to remove nucleotides by resuspending in 10 vol. of the dialysis solution, and repeating the centrifugation. The myosin was then dissolved in 0.6 M-NaCl/10 mM-sodium phosphate buffer (pH 7.0) containing 1 mM-$MgCl_2$. The recovery

Abbreviation used: ATPase, adenosine 5′-triphosphatase.

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of myosin varied between 2% and 5% of the wet weight of muscle.

Myofibrils required for kinetic experiments were collected after the 10min centrifugation step at 18000*g*, washed six times in 6vol. of solution A with repeated centrifugation and filtered through two layers of cheesecloth. Protein concentration was determined by assay of a sample of a myofibril suspension by the biuret method (Gornall *et al.*, 1949), with bovine serum albumin as standard.

Digestion of myosin

Subfragment 1 was obtained by using papain or α-chymotrypsin. Since the chymotryptic digestion gave the least-degraded fraction, this preparation was used for kinetic experiments. Heavy meromyosin was obtained by using trypsin or α-chymotrypsin (Weeds & Taylor, 1975; Bagshaw, 1977); gel electrophoresis of this material gave a band pattern similar to that obtained from heavy meromyosin from rabbit fast-twitch muscle, except for the changed mobility of the alkali-1 light chain (see Fig. 1). All steps of the protein preparations were done at 0–4°C. Digestions were carried out on myofibrils, on synthetic myosin filaments or on monomeric myosin in 0.6M-NaCl. The usual subfragment-1 preparation was obtained by redissolving myosin at about 10mg/ml in 0.6M-NaCl/10mM-sodium phosphate (pH7.0) / 1mM-$MgCl_2$ / 5mM-EDTA/1mM-dithiothreitol. The solution was stirred gently and chymotrypsin was added to give a final concentration of 100μg/ml. Digestion was terminated after 45 min by addition of phenylmethanesulphonyl fluoride to 100μM. After dialysis against 10mM-NaCl/50mM-Tris/HCl (pH7.0)/1mM-$MgCl_2$ the undigested myosin and other insoluble fragments were removed by centrifugation at 38000*g* for 15min.

Subfragment 1 was adsorbed on a DEAE-cellulose (Whatman DE-52) column equilibrated in the same buffer, and separation from other supernatant protein was typically achieved by elution with a linear NaCl gradient also in the same buffer at a flow rate of 30ml/h (see the legend to Fig. 2 for details). The volume of the elution solvent was adjusted according to the degree of fractionation required of subfragment 1 into its isoenzymes; for example, see the legend to Fig. 2.

Preparation of frog actin

Washed frog myofibrils were suspended in 0.6M-NaCl / 10mM-sodium phosphate (pH7.0) / 1mM-$MgCl_2$/0.1mM-dithiothreitol and centrifuged in the presence of 2mM-MgATP for 30min at 38000*g* to clarify the suspension. Pellets were discarded and the concentration of MgATP was increased to 10mM before centrifugation for 3h at 190000*g*. The supernatant was used for myosin preparation, and pellets were rinsed and homogenized in 15ml of 400μM-ATP and 2mM-imidazole/HCl buffer, pH7.0, to depolymerize the actin. Actin was dialysed overnight against the same solution and centrifuged at 70000*g* for 1h to remove F-actin. Repolymerization of the G-actin was achieved by dialysis against 30mM-KCl/5mM-imidazole / HCl (pH7.0) / 1mM-$MgCl_2$ / 100μM-sodium azide. Actin concentrations were determined by using $A^{1\%}_{280} = 11.0 cm^{-1}$ (West *et al.*, 1967). The yield was 0.1–0.2% of the wet weight of muscle. All frog protein preparations were used within 4 days of dissection.

Preparation of rabbit muscle proteins

Rabbit myosin was prepared as described by Weeds & Hartley (1968) and actin as described by Taylor & Weeds (1976) by the method of Straub (1942) and Feuer *et al.* (1948).

Determination of absorption coefficients of frog myosin and subfragment 1

Protein concentrations were determined by synthetic-boundary centrifugation in a Beckman analytical ultracentrifuge by using Rayleigh interference optics to measure the interference fringe shift. A refractive-index increment of 41 fringes for a 1% solution was assumed (Babul & Stellwagen, 1969). Proteins were dissolved in 0.6M-NaCl/50mM-Tris/HCl (pH8.0)/1mM-$MgCl_2$ and centrifuged at 10000 rev./min in the analytical ultracentrifuge. Absorbance measurements were made on the same protein solution. Values for the absorption coefficient obtained were $A^{1\%}_{280}$ $5.6 cm^{-1}$ for myosin and $A^{1\%}_{280}$ $7.1 cm^{-1}$ for subfragment 1.

Protein concentrations were determined from the A_{280} measurements corrected for light-scattering by linear extrapolation of the absorbance measured between 400nm and 320nm to 280nm. Molecular-weight values assumed for the proteins were 470000 for myosin (Gershman *et al.*, 1969), 115000 for subfragment 1 (Lowey *et al.*, 1969) and 42000 for actin (Elzinga *et al.*, 1973).

Sedimentation studies

Sedimentation coefficients of frog myosin and subfragment 1 were compared with those of rabbit myosin and subfragment 1 by using a Beckman model E analytical ultracentrifuge with schlieren optics. Boundaries obtained at 56000rev./min for the frog proteins had the same observed sedimentation coefficients as those of rabbit proteins, but the frog proteins frequently showed additional boundaries characteristic of aggregated material.

Polyacrylamide-gel electrophoresis

Gel electrophoresis was carried out as described previously and the protein components stained with Coomassie Brilliant Blue (Weeds, 1976). Stoicheiometry of proteins in gels was measured by densitometry of the bands in a Joyce–Loebl densitometer. The relative intensities of the bands were divided by the apparent molecular-weight values to obtain molar ratios.

ATPase activities

Although 100 μM-dithiothreitol was generally present in all ATPase assay solutions because it appeared to help maintain protein stability, it was not essential for ATPase activity.

Ca^{2+}-dependent ATPase activity was measured by using a Radiometer pH-stat, in a solution containing 30 mM-KCl/10 mM-$CaCl_2$/1 mM-ATP at 25°C under an N_2 atmosphere; 10 mM-NaOH was used to maintain the pH at 8.0.

Mg^{2+}-dependent ATPase activity was determined at 2°C from the time course of $[^{32}P]P_i$ formation from [γ-^{32}P]ATP, which was prepared by the method of Glynn & Chappell (1964). Samples of protein and ATP were quenched with an equal volume of 6% $HClO_4$ after reaction for different periods of time. The pH of the ice-cold quenched reaction mixture was immediately raised to 4.3 by the addition of 4 M-sodium acetate, pH 8.9, containing 20 mM-ATP marker. The mixture was centrifuged for 1 min in a bench centrifuge to remove precipitated protein, and the relative amounts of $[^{32}P]P_i$ and [γ-^{32}P]ATP were determined after t.l.c. as described by Bagshaw & Trentham (1973).

Mg^{2+}-dependent actomyosin ATPase activity was measured at 0°C at various NaCl or KCl concentrations by using frog myosin and rabbit actin. Actin and myosin were mixed in 0.25 M-NaCl/5 mM-$MgCl_2$/100 μM-dithiothreitol/15 mM-Tris/HCl (pH 7.0) and then diluted to the appropriate ionic strength. Mixing myosin filaments with F-actin led to unreproducible results. [γ-^{32}P]ATP (0.5 mM or 1 mM) was added to initiate the reaction in the appropriate solvent. Samples were taken at various times (generally six in all) and the reaction was stopped by quenching into an equal volume of 1 M-HCl/0.35 M-NaH_2PO_4. A sample of this mixture was dissolved in 40 vol. of Bray's (1960) scintillation fluid and counted for total ^{32}P. A further sample was treated with an equal volume of an activated-charcoal suspension, which adsorbed the nucleotide, and after centrifugation the supernatant was assayed for $[^{32}P]P_i$. From these data the steady-state rates of P_i formation were evaluated. At 0–2°C actin-activated Mg^{2+}-dependent subfragment-1 ATPase activity was measured similarly, except that there was no need to premix the proteins at high ionic strength.

At 25°C, the effect of actin on the Mg^{2+}-dependent ATPase of frog muscle proteins was assayed by the pH-stat method.

Presentation of averaged values

Averaged values are presented as the means ± 1 S.E.M. for the numbers of observations (n) indicated in parentheses.

Reagents

ATP, trypsin and α-chymotrypsin were obtained from Boehringer/Mannheim G.m.b.H., Mannheim. West Germany. Papain was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. $[^{32}P]P_i$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The water used in this work was glass-double-distilled and all other chemicals were analytical grade.

Results

Structural characterization of frog muscle proteins

Myosin isolated from frog muscle was compared with rabbit skeletal-muscle myosin by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate (Fig. 1). Both preparations showed a strong-staining heavy-chain band and three main light chains, though additional components were present in the heavy-chain region of the gel for frog myosin, possibly caused by degradation of heavy chains. Both preparations contained contaminating actin. The light chains have been classified by analogy with rabbit myosin (Weeds & Lowey, 1971). Although no detailed chemical characterization of these light chains has been undertaken, the cysteine chymotryptic peptide characteristic of the alkali light chains (Weeds & Lowey, 1971; Weeds, 1976) was isolated from frog light chains. The apparent molecular weights of the three frog light chains determined from the mobility of the components on gel electrophoresis in the presence of sodium dodecyl sulphate are 21000, 18500 and 16500 respectively. Densitometry of the light-chain bands gives stoicheiometries based on these molecular weights of 0.76:2.00:1.08, assuming that there is 2 mol of the 18500-mol.wt. light chain/mol of myosin (Weeds & Lowey, 1971). That this assumption is justified is indicated by the fact that the densitometry shows 2.2 ± 0.3 18500-mol.wt. light chains per 2 heavy chains of the myosin molecule.

These gels show that frog myosin contains contaminating actin and traces of the regulatory proteins. Densitometry of the actin band indicates that the

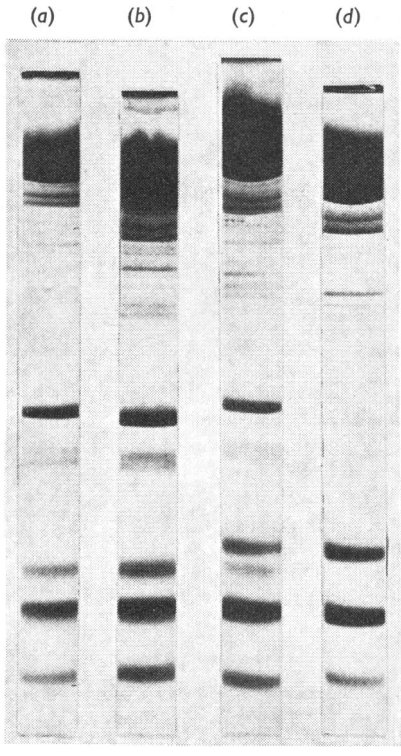

Fig. 1. *Polyacrylamide-gel electrophoresis of frog and rabbit myosins in the presence of sodium dodecyl sulphate*
Proteins were submitted to electrophoresis on 7.5% (w/v) polacrylamide gels in 0.1% sodium dodecyl sulphate, 0.1 M-Tris/*NN*-bis-(2-hydroxyethyl)glycine, pH 8.3; (*a*) 80 μg of frog myosin; (*b*) 160 μg of frog myosin; (*c*) mixture of 80 μg each of frog and rabbit myosin; (*d*) 120 μg of rabbit myosin. The two smaller light chains of frog myosin co-migrate with the equivalent light chains of rabbit myosin, and the largest frog light chain has a mobility greater than the alkali-1 light chain of rabbit myosin.

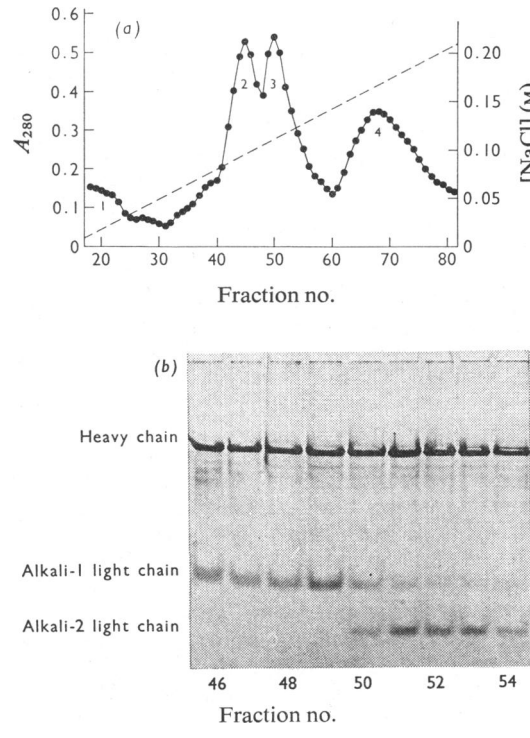

Fig. 2. *Fractionation of frog subfragment* 1
(*a*) Elution profile of subfragment 1 after a chymotryptic digestion of myosin. The supernatant from a digest (see the Methods section) was applied to a column (1.6 cm × 28 cm) of DEAE-cellulose (DE-52) that had been equilibrated with the same solution. Protein (110 mg) was loaded on the column and the bound protein was eluted with a gradient of 10–210 mM-NaCl in a total volume of 400 ml at a flow rate of 30 ml/h. The eluate was monitored at A_{280}. The peaks 2 and 3 contain subfragment 1 (see *b*) and the peak 4 single- and/or double-headed heavy meromyosin. (*b*) Polyacrylamide-gel electrophoresis of protein eluted in (*a*). Polyacrylamide-gel electrophoresis of the fractions eluted was carried out in a slab gel, 7.5% acrylamide, in the presence of 0.1% sodium dodecyl sulphate. Separation of the subfragment-1 peaks is correlated with the sequential appearance of the alkali-1 and alkali-2 light chains. The 18500-mol.wt. light chain cannot be detected.

degree of contamination is about 0.8 mol/mol of myosin, i.e. 0.4 mol of actin/subfragment-1 'head'. This is equivalent to actin contamination of about 7% by weight. Contaminating actin and other proteins could be removed by further purification, either by $(NH_4)_2SO_4$ precipitation and collecting the 40–55%-satn. fraction, or by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) by using a pyrophosphate gradient as described by Richards *et al.* (1967) for rabbit myosin. However, further purification did not increase the Ca^{2+}-dependent ATPase activity of the myosin, and recoveries of protein were poor.

Myofibrils were also prepared from individual muscles, including semitendinosus, gastrocnemius and sartorius. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate showed no significant differences in the light-chain pattern nor was there any appreciable variation in the light-chain ratios determined by densitometry.

Subfragment 1 prepared from frog myosin by chymotryptic digestion gave a single heavy-chain band on gel electrophoresis together with two light-chain bands corresponding to the alkali light chains. Digestion in the absence of free bivalent cations

produced subfragment 1 devoid of 18500-mol.wt. light chains, as has also been observed for chymotryptic subfragment 1 from rabbit myosin (Weeds & Taylor, 1975). In the DEAE-cellulose-chromatography step, subfragment 1 containing the alkali-1 light chain was eluted first, followed by subfragment 1 containing the alkali-2 light chain (Fig. 2) as observed in the preparation of rabbit subfragment 1 by Weeds & Taylor (1975).

Actin prepared from frog muscle gave a single band on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate which co-migrated with rabbit actin (Fig. 3).

No differences were observed on gel electrophoresis between proteins prepared from *R. temporaria* and *R. pipiens*.

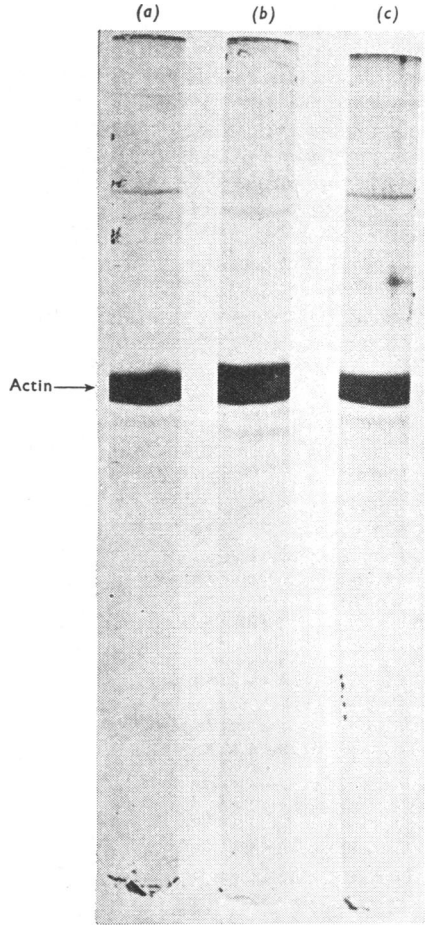

Fig. 3. *Polyacrylamide-gel electrophoresis* (10%, *w/v*) *of frog and rabbit actin in* 0.1% *sodium dodecyl sulphate*
In (*a*) frog and rabbit actin, in (*b*) 24 μg of rabbit actin and in (*c*) 23 μg of frog actin was applied to the gels.

Enzymic characterization of frog myosin and subfragment 1

The ATPase activities of frog myosin and subfragment 1 were determined under a number of different conditions. Subfragment 1 used in these experiments was not fractionated, but isolated from a small DEAE-cellulose column without separating the two isoenzymes.

Ca^{2+}-dependent ATPase activity. The ATPase activity of frog myosin measured at a concentration of 3 μg/ml under the conditions described in the Methods section was 1.37 ± 0.1 μmol of ATP hydrolysed/min per mg of protein ($k_{cat.} = 5.4 s^{-1}$). At higher enzyme concentrations, the enzymic activity was observed to decrease and was less than half this value at concentrations above 100 μg/ml. Further, the enzymic activity decreased by about 25% per day even though the myosin was stored on ice. This loss of activity was due to aggregation and denaturation of the protein.

Subfragment 1 had an ATPase activity, under similar conditions, of 0.67 ± 0.1 μmol of ATP hydrolysed/min per mg of protein ($k_{cat.} = 1.3 s^{-1}$), which is less than 25% of the value found for myosin. This loss of activity reflects inactivation of the protein during preparation and purification of the subfragment 1 and demonstrates the difficulties inherent in these studies on frog myosin and its proteolytic subfragments. Measurements of the Ca^{2+}-dependent ATPase activity of frog subfragment 1 were made between 10°C and 25°C, giving a Q_{10} value of 2.3. Table 1 records these results together with other Ca^{2+}-dependent ATPase activities for comparison.

Table 1. *Ca^{2+}-dependent ATPase activity in* 30 *mM-KCl at* 23 ± 2°*C*
$k_{cat.}$ values were calculated from the observed rates of ATP hydrolysis (μmol of ATP/min per mg of protein) by using the protein molecular weights given in the Methods section and assuming 2 mol of active sites/mol of myosin and 1 mol of active sites/mol of subfragment 1. The myosin content of the myofibrils was assumed to be 50% by weight (Ebashi *et al.*, 1969).

Protein	pH	$k_{cat.}$ (s^{-1})
Frog subfragment 1	8.0	1.3
Frog myosin	8.0	5.4
Frog myosin (sartorius)*	7.4	2.9
Frog myofibrils	8.0	4.8
Rabbit skeletal-muscle subfragment 1†	8.0	8.1
Rabbit skeletal-muscle myosin‡	7.6	2.0

* Barany (1967).
† Weeds & Taylor (1975).
‡ Weeds & Lowey (1971).

To check whether the ATPase activity of frog myosin altered during its purification, for example as a result of protein inactivation, the Ca^{2+}-dependent ATPase was measured for frog myofibrils (Table 1). Activities comparable with those obtained for myosin were observed assuming that the myosin content of the myofibrils was 50% by weight (Ebashi *et al.*, 1969).

Mg^{2+}-dependent ATPase activity. To compare the Mg^{2+}-dependent ATPase with the turnover of ATP found in living resting muscle, conditions of temperature, pH and ionic strength were chosen to be as close as possible to those used in physiological experiments. Measurements for subfragment 1 were made in 0.1 M-KCl/50 mM-Tris/HCl (pH 7.0 at 0–2°C). Myosin was assayed in 0.5 M-KCl to maintain the monomeric state. Assays were performed with ATP concentrations between 0.1 μM and 10 μM. Fig. 4 shows a Lineweaver–Burk plot of this ATPase activity, and Table 2 summarizes the extrapolated values for $k_{cat.}$ and K_m. Since loss of enzymic activity is observed with time, it was important to assay the active-site concentration at the time of each experiment to determine the specific activity of the protein. As shown by the study of transient P_i liberation described in the following paper (Ferenczi *et al.*, 1978), 33±3% ($n = 12$) of myosin subfragment-1 heads and 32±5% ($n = 9$) of subfragment-1 were active in these experiments. Table 2 shows turnover numbers corrected on this basis for the Mg^{2+}-dependent ATPase of myosin and subfragment-1.

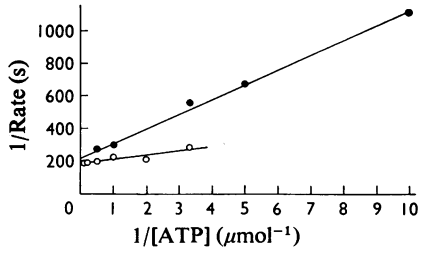

Fig. 4. *Lineweaver–Burk plot of the Mg^{2+}-dependent ATPase of frog myosin and subfragment 1*
The solvent contained 5 mM-$MgCl_2$/50 mM-Tris/HCl, pH 7.0 at 0–2°C, and either (●) myosin in 0.5 M-KCl or (○) subfragment 1 in 0.1 M-KCl. The initial ATP concentration was at least at a 10-fold molar excess over the active subfragment-1 heads.

Actin-activated frog subfragment-1 ATPase activity. This was measured in a solvent of 10 mM-KCl/1 mM-$MgCl_2$ / 0.1 mM-dithiothreitol / 5 mM-morpholinopropanesulphonic acid at pH 7.0 and 0–2°C. The reaction was initiated by the addition of 0.5 mM-[γ-^{32}P]ATP. Activity was measured at several actin concentrations from 0 to 340 μM. Up to 170 μM the ATPase activity increased approximately linearly with actin concentration, but by only a further 10% in the range 170–340 μM-actin. The data could not be meaningfully interpreted in terms of Michaelis–Menten saturation kinetics with respect to actin concentration, although they were insufficiently accurate to exclude the possibility of Michaelis–Menten behaviour. Accordingly the observed $k_{cat.}$ of 1.45 s^{-1} was calculated from the rate of ATP hydrolysis measured at 340 μM-actin. Actin activation was 440-fold. Corrected $k_{cat.}$ was calculated to be 4.5 s^{-1} based on the active-site concentration of subfragment 1 determined by transient P_i formation (see previous section and Ferenczi *et al.*, 1978).

The Mg^{2+}-dependent frog subfragment-1 ATPase was activated identically by rabbit and frog actin. The activity measured with a pH-stat increased 8-fold over an actin concentration range of 0.1–1 mg/ml at pH 8.0 and 25°C in a solvent of 30 mM-KCl/1 mM-ATP/1.5 mM-$MgCl_2$ containing 62 μg of subfragment 1/ml. For both rabbit and frog actin at 1 mg/ml, 3.0 μmol of ATP was hydrolysed/min per mg of subfragment 1, which corresponds to an observed $k_{cat.}$ under these conditions of 6.0 s^{-1}. As at 0°C, saturation kinetics were observed as the actin con-

Table 2. *Steady-state kinetic constants of the Mg^{2+}-dependent ATPase*
K_m values for rabbit skeletal-muscle myosin subfragment 1 are calculated from $K_m = k_{cat.}/k_{ass.}$ where $k_{ass.}$ is the apparent second-order association rate constant of ATP to subfragment 1. The validity of this has been discussed by Taylor (1977). $k_{cat.}$ values for myosin and subfragment 1 have been corrected to allow for inactive protein.

Protein	[KCl] (mM)	pH	T (°C)	$k_{cat.}$ (s^{-1})	$10^7 \times K_m$ (M)
Frog subfragment 1	100	7.0	0–2	0.011	1.6
Frog myosin	500	7.0	0–2	0.004	5.7
Rabbit skeletal-muscle subfragment 1*	50	6.9	3	0.015	0.71
Rabbit skeletal-muscle subfragment 1†	100	8.0	20	0.06	0.33
Rabbit skeletal-muscle myosin‡	35	7.0–8.0	25	0.06	4.1§

* Sleep & Taylor (1976).
† Bagshaw & Trentham (1974).
‡ Schliselfeld (1976).
§ In 25 mM-NaCl; Schliselfeld & Barany (1968).

Table 3. *Steady-state kinetic constants of the actin-activated Mg^{2+}-dependent ATPases*

$k_{cat.}$ values are obtained assuming that both myosin heads were simultaneously active, which has not been established to be necessarily the case for the actomyosin ATPase. Corrected $k_{cat.}$ values are calculated on the same basis as in Table 2 (i.e. that only 33% of the myosin subfragment-1 heads are active).

Temperature (°C)	Protein	Salt	pH	Observed $k_{cat.}$ (s^{-1})	Corrected $k_{cat.}$ (s^{-1})	K_m for actin (μM)
0–2	Frog subfragment 1	10mM-KCl	7.0	1.45	4.4	
	Frog myosin	100mM-NaCl*	7.0	0.035	0.11	12
	Frog myosin	30mM-NaCl*	7.0	0.03	0.10	<5
	Frog myosin†	30mM-KCl	7.4	0.5	—	—
	Rabbit subfragment 1‡	0	7.0	0.3	—	17
22–25	Frog subfragment 1	30mM-KCl	8.0	17	53	—
	Frog myosin	100mM-KCl	8.0	5.5	17	1.7
	Rabbit subfragment 1§	6mM-KCl	8.0	40	—	22
	Rabbit myosin‖	35mM-KCl	7.4	9.3	—	0.11

* No K^+ other than that present in the actin stock solution was present in the actomyosin ATPase assays. Control experiments with rabbit actomyosin showed that if Na^+ replaced K^+ in the assay medium the activities differed by less than 25%. Further, Eisenberg & Moos (1970) and Schliselfeld (1976) have shown that K^+ is not essential for actomyosin ATPase activity. Myosin was prepared in NaCl rather than KCl as this facilitates analysis of the protein on sodium dodecyl sulphate/polyacrylamide gels.

† Native actomyosin (Barany, 1967).

‡ Eisenberg & Kielley (1972).

§ The K_m for actin was computed from the K_m values of actin for the two subfragment-1 isoenzymes and the ratio of the isoenzymes (Weeds & Taylor, 1975).

‖ Schliselfeld (1976).

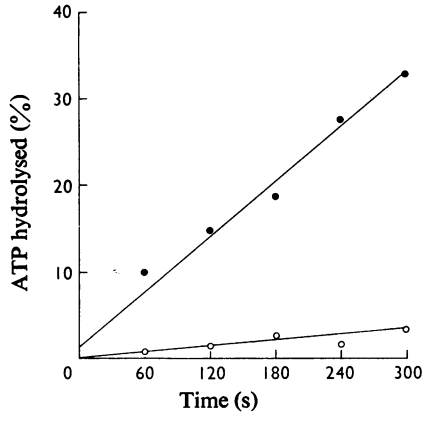

Fig. 5. *Myofibrillar ATPase activity*

The solution at 0–2°C contained a total protein concentration of 1.0mg/ml in 0.1M-KCl/5mM-$MgCl_2$/1mM-[γ^{32}P]ATP/50mM-Tris/HCl at pH 7.0 and either (○) 100μM-EGTA or (●) 500μM-$CaCl_2$.

centration was increased, but the data were insufficiently accurate to be certain that Michaelis–Menten behaviour occurred. At 8mg of rabbit actin/ml the observed $k_{cat.}$ was $17 \pm 4 s^{-1}$ ($n = 4$) so that the corrected $k_{cat.}$ is $53 s^{-1}$. This may be an underestimate in view of the lability of subfragment 1 at room temperature.

Table 4. $k_{cat.}$ *for the Mg^{2+}-dependent frog myofibrillar ATPase*

$k_{cat.}$ for the myofibrillar ATPase has been evaluated on the basis that 50% of the myofibrillar protein is myosin (Ebashi *et al.*, 1969) and that both heads are simultaneously active (see Table 3).

	[KCl] (mM)	pH	T (°C)	$k_{cat.}$ (s^{-1})
+100μM-EGTA	100	7.0	0–2	0.05
+500μM-Ca^{2+}	100	7.0	0–2	0.44
(+Ca^{2+})*	50	7.4	0	0.50

* Barany (1967); Ca^{2+} though not specifically added was clearly present in the solution.

The actin-activated frog myosin ATPase was measured at 0°C and pH 7 (Table 3). A 35-fold actin activation was recorded in 100mM-NaCl. Surprisingly in 30mM-NaCl only 3.3-fold actin activation was observed. However, actin contamination of myosin coupled with the low K_m of actin meant that even with no added actin significant activation probably occurred.

For myosin at pH 8 and 25°C rabbit actin at concentrations up to 8mg/ml was added to a stirred solution containing myosin in 100mM-KCl and the reaction was initiated by the addition of ATP to give a final concentration of 1mM-ATP and 1.5mM-$MgCl_2$. There was variability in the results, presum-

ably due to the fact that filamentous proteins were being mixed. The observed $k_{cat.}$ was $5.5 \pm 0.8 s^{-1}$ ($n = 4$). Assuming Michaelis–Menten behaviour the K_m for actin was $1.7 \pm 0.4 \mu M$ ($n = 3$).

Mg^{2+}-dependent myofibrillar ATPase activity. This was measured and showed sensitivity to Ca^{2+} (Fig. 5), and the results are recorded in Table 4. $k_{cat.}$ for the myofibrillar ATPase in the absence of Ca^{2+} (i.e. +EGTA) is considerably higher than that of the subfragment-1 ATPase. In part, this can be accounted for if subfragment 1 is only partially active. However, it seems likely that the major part of the difference in activities arises because the myofibrillar system is not completely Ca^{2+}-sensitive and some actin-activated ATPase activity is present even in the presence of EGTA.

Discussion

By many criteria frog myosin and its subfragment 1 are very similar to those proteins obtained from rabbit fast-twitch muscles. On gel electrophoresis the band pattern is very similar, showing the presence of three light chains. The molar ratio of the 18500-mol.wt. light chain to the sum of the alkali light chains 1 and 2 is 1:1, as in rabbit skeletal-muscle myosin. The ratio of alkali light chain 1/light chain 2 differs from that in rabbit, as has been found with other species (Lowey & Risby, 1971; Sarkar, 1972). Slight differences in the mobilities of the light chains suggest possible differences in molecular weight, though it must be remembered that the alkali-1 light chain gives an anomalously high molecular weight by this method (Frank & Weeds, 1974). The extent of actin contamination found in frog myosin was variable, but densitometry of gels showed that the contamination was generally 3–4% by weight (a molar ratio of about 0.35 mol of actin/mol of myosin), but occasionally, as in Fig. 1, as high as 7% by weight. At this value, the effect on the steady-state Mg^{2+}-dependent ATPase activity of myosin in 0.5M-KCl is expected to be small. Further, subfragment 1 was free from actin contamination as judged by gel electrophoresis, and the differences observed in enzymic activities can be accounted for by ionic-strength effects and variable inactivation of the proteins during storage.

The main problem found in working with frog myosin and its proteolytic subfragments was the marked lability of the proteins as compared with rabbit myosin. This lability could be minimized, but not eliminated, by using the proteins within 3 days of dissection and by carrying out all manipulations at 0–4°C. As a further precaution, dithiothreitol was present at all stages of the preparation and storage to prevent disulphide formation and consequent aggregation. Further purification of the proteins could be achieved by ion-exchange chromatography or $(NH_4)_2SO_4$ precipitation, but these procedures had to be weighed against the concomitant loss of activity during the time required. Myosin obtained within 24h of frog dissection gave consistently higher Ca^{2+}-dependent and actin-activated ATPase activities than myosin, that had undergone further purification. Sedimentation-velocity studies showed that storage of the myosin resulted in considerable aggregation, suggesting that inactivation was due to this. Comparable effects have been observed with rabbit cardiac-muscle myosin subfragment 1 (Taylor & Weeds, 1976). Lability of myosin from other poikilotherms has been reported by Johnston & Goldspink (1975), which contrasts with the relative stability of myosins from homoeotherms.

A feature of the actin-activated frog ATPases is that the activity of subfragment 1 is higher than that of myosin, particularly at 0–2°C. As discussed in the following paper, the values of the ATPase activity of working muscle at 0°C correspond to those of the actin–subfragment-1 ATPase. It is likely that the abnormally low actomyosin ATPase arises because of the failure of the disorganized actin and myosin filaments to interact effectively. Although this has been recognized to be a problem when working with rabbit skeletal-muscle proteins, it can be overcome, as has been demonstrated by Schliselfeld (1976). It may be that the greater stability of rabbit myosin and the higher temperature used by Schliselfeld (1976) enabled him to achieve a favourably organized actomyosin lattice. Native actomyosin (Barany, 1967) and myofibrils have a similar ATPase activity that is intermediate between those of reconstituted actomyosin and actin–subfragment 1 (Tables 3 and 4).

Our data are in general agreement with those of Barany (1967), particularly when the problem of protein lability is taken into account. The kinetic constants of the rabbit proteins have been included in the Tables for comparison with those of the frog. There is general similarity between the two sets of constants, though the higher $k_{cat.}$ at 0–2°C of the actin-activated frog subfragment-1 ATPase is noteworthy. It is probable that the activation energy of this ATPase is significantly less than its counterpart in the rabbit (150kJ/mol; White & Taylor, 1976).

The physiological implications of the steady-state kinetic results are discussed in the following paper (Ferenczi *et al.*, 1978) along with the transient kinetic results.

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