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The Magnesium-Ion-Dependent Adenosine Triphosphatase of Bovine Cardiac Myosin and its Subfragment-1

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The kinetics of the Mg^{2+}-dependent ATPase (adenosine triphosphatase) activity of bovine cardiac myosin and its papain subfragment-1 were studied by using steady-state and pre-steady-state techniques, and results were compared with published values for the corresponding processes in the ATPase mechanism of rabbit skeletal-muscle myosin subfragment-1. The catalytic-centre activity for cardiac subfragment-1 is $0.019\,s^{-1}$, which is less than one-third of that determined for the rabbit protein. The ATP-induced isomerization process, measured from enhancement of protein fluorescence on substrate binding, is similarly decreased in rate, as is also the isomerization process associated with ADP release. However, the equilibrium constant for ATP cleavage, measured by quenched-flow by using [γ-^{32}P]ATP, shows little difference in the two species. Other experiments were carried out to investigate the rate of association of actin with subfragment-1 by light-scattering changes and also the rate of dissociation of the complex by ATP. The dissociation rate increases with increasing substrate concentration, to a maximum at high ATP concentrations, with a rate constant of about $2000\,s^{-1}$. It appears that isomerization processes which may involve conformational changes have substantially lower rate constants for the cardiac proteins, whereas equilibrium constants for substrate binding and cleavage are not significantly different. These differences may be related to the functional properties of these myosins in their different muscle types. Kinetic heterogeneity has been detected in both steady-state and transient processes, and this is discussed in relation to the apparent chemical homogeneity of cardiac myosin.

There has been considerable effort in recent years to understand the mechanism of hydrolysis of Mg^{2+}–ATP by myosin and actomyosin to correlate the kinetic cycle with the mechanical cycle of cross-bridge movement in active muscle. Both steady-state and pre-steady-state techniques have been used in a number of laboratories (Eisenberg & Moos, 1970*a*,*b*; Lymn & Taylor, 1970, 1971; Tonomura, 1968, 1972). Our current ideas of the mechanism are based on the studies of Lymn & Taylor (1971, and references therein) and have been extended by Bagshaw *et al.* (1974). This model for the myosin ATPase (adenosine triphosphatase) is a seven-step process in which the kinetic constants for most of the intermediates have been characterized (Bagshaw *et al.*, 1974; Mannherz *et al.*, 1974). Much of the work has been carried out by using proteolytic fragments of rabbit fast-twitch-muscle myosin, chiefly subfragment-1 produced by papain cleavage. This is the smallest fragment of myosin which retains both ATPase activity and actin binding (Lowey *et al.*, 1969), but the preparation is heterogeneous, particularly in its light-chain content (Weeds & Lowey, 1971). A further problem associated with these studies is that the rate constants of the transient reactions measured at room temperature for a number of steps in the hydrolytic process are near the limits of resolution of currently available rapid-reaction equipment. For these reasons we considered that a comparative study of myosin and its proteolytic subfragments from a slower-contracting muscle might be of value. Barany (1967) has studied the relationship between maximal speed of shortening and steady-state ATPase activity of both myosin and actomyosin for a number of different muscle types (fast-twitch, slow-twitch and slow tonic) from different animals and has shown a proportionality between the enzymic activity and the speed of shortening. Thus in a muscle with a slower speed of shortening, the steady-state ATPase activity is lower, and the rates of the transient processes might also be expected to decrease. Muscles with slower contraction speeds are generally those required for maintenance of posture, the slow-twitch muscles, or those required for sustained action, such as cardiac muscle. Heart muscle was chosen for the present study because of its ready availability in large amounts and because its subunit composition has been established (Weeds & Pope, 1971). There has been no detailed investigation of the ATPase activity of cardiac myosin, and in the present experiments we have attempted to show whether the mechanism is similar

to that of fast-twitch myosin and whether differences exist in particular rate constants.

Rabbit fast-twitch-muscle myosin appears to be a mixture of isoenzymes, judging from the presence of non-stoicheiometric amounts of the two related 'alkali' light chains (termed LC1 and LC3) (Weeds *et al.*, 1975). (These 'alkali' light chains cannot be removed without loss of ATPase activity and their presence is essential for enzymic function; Weeds & Lowey, 1971.) Slow-twitch muscle (e.g. soleus muscle) may also contain isoenzymic populations of myosin by the same criteria (Weeds, 1976). However, cardiac myosin is more homogeneous in having only one type of 'alkali' light chain (Weeds, 1975). This light chain, termed LC1, has an apparent mol.wt. on gels in the presence of sodium dodecyl sulphate of 27000 (Sarkar *et al.*, 1971). In addition, cardiac myosin contains another light chain of apparent mol.wt. 19000, termed LC2 (Sarkar *et al.*, 1971). This LC2 light chain is virtually absent from cardiac subfragment-1 (Weeds & Frank, 1972), whereas the corresponding LC2 light chain from fast-twitch-muscle myosin (often called the 'DTNB light chain') is degraded to a variable extent (Weeds & Lowey, 1971). Thus subfragment-1 from cardiac myosin is more homogeneous by this criterion also. Further, the heavy chains of subfragment-1 prepared from fast-twitch myosin are frequently 'nicked' by the proteinase, resulting in additional bands on polyacrylamide gels in the presence of sodium dodecyl sulphate (Stone & Perry, 1973; Margossian & Lowey, 1973). Cardiac myosin is more resistant to proteolytic cleavage (Tada *et al.*, 1969) and the subfragment-1 heavy chain is less degraded (Weeds & Frank, 1972).

The present paper describes the preparation of bovine cardiac subfragment-1 by using soluble papain and an investigation of its kinetic properties by rapid-reaction techniques. The present paper seeks to compare measurements of the activity of this protein with similar measurements made on rabbit subfragment-1 (Bagshaw *et al.*, 1974, and references therein; Koretz & Taylor, 1975). A detailed analysis of the interpretation of the kinetic results is omitted, since this has been reported in detail elsewhere (Bagshaw *et al.*, 1974; Taylor, 1974). Thus the results presented here are compared with those obtained for rabbit fast-twitch myosin and its subfragments, and they are interpreted in terms of the published model. The rate constants are numbered according to the kinetic scheme of Bagshaw & Trentham (1974) set out in eqn. (1) below:

where M denotes subfragment-1 and the asterisks are used to distinguish different intermediates (and also indicate species of enhanced fluorescence relative to M).

We have not attempted to measure the rate constants for all the reaction processes outlined above, but have concentrated particularly on the ATP association and cleavage steps. Some experiments have been carried out on the dissociation of actin–subfragment-1 complex by ATP to demonstrate a rate-limiting first-order process at high ATP concentrations.

Rate constants for cardiac myosin or its subfragment-1 are denoted as k_{+1} etc. and corresponding values for rabbit fast-twitch subfragment-1 are listed as k'_{+1} etc. Equilibrium constants use the same notation, e.g. $K_1 = k_{+1}/k_{-1}$ (cardiac) and $K'_1 = k'_{+1}/k'_{-1}$ (rabbit).

Materials and Methods

Preparation of bovine cardiac myosin

The method is based on that of Tada *et al.* (1969). Ox hearts were cooled in ice within about 15min after the death of the animal. Fat and connective tissue were removed and the muscle was minced through 4mm holes in an electric mincer. The mince (600–1000g) was dispersed in 3vol. of a solution containing 0.3M-KCl, 0.15M-KH_2PO_4 and 2mM-ATP, adjusted to pH6.5 with 2M-NaOH, and stirred continuously for 10min at below 4°C. Extraction was terminated by straining the suspension through two layers of cheesecloth and then filtering through a 2cm bed of cellulose (Whatman 3MM paper blended in the extracting solution). This procedure removed small particles of meat and fat, giving a clear solution, which was diluted by the slow addition of 12vol. of ice-cold water to precipitate the myosin. The precipitate was washed with 40mM-KCl/20mM-potassium phosphate, pH6.5, and collected by centrifugation at 20000*g* for 10min. Myosin was dissolved in 0.6M-KCl/10mM-potassium phosphate, pH6.5, and the volume adjusted to give about 10mg/ml in 0.3M-KCl/10mM-potassium phosphate at the same pH. This solution was centrifuged at 40000*g* for 45min to remove actomyosin and other contaminating material. The myosin was reprecipitated by addition of 6–7vol. of ice-cold water, allowed to settle and collected by centrifugation at 20000*g* for 10min. Once again the precipitate was dissolved in 0.6M-KCl/20mM-potassium phosphate, pH6.5,

$$\mathrm{M+ATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{M\cdot ATP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{M^{*}\cdot ATP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{M^{**}\cdot ADP\cdot P_i} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}}$$
$$\mathrm{M^{*}\cdot ADP\cdot P_i} \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} \mathrm{M^{*}\cdot ADP+P_i} \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} \mathrm{M\cdot ADP} \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} \mathrm{M+ADP} \quad (1)$$

and the myosin centrifuged at 40000*g* to clarify the solution. Actin was present at less than 1% (w/w), as determined by densitometry of sodium dodecyl sulphate-containing gels. The overall yield of myosin was 8–12 g/kg of muscle mince. Myosin was stored at −20°C at a concentration of 8–12 mg/ml in a solution containing 50% (v/v) glycerol, 0.6 M-KCl, 20 mM-potassium phosphate, pH 7.0, 1 mM-$MgCl_2$ and 1 mM-EDTA.

Myosin concentrations were estimated by absorbance, by using a value for $E^{1\%}_{280} = 5.60\,cm^{-1}$, determined for skeletal myosin (Small *et al.*, 1962). Concentrations of myosin subfragment-1 'heads' are quoted as μM, based on an equivalent weight of 235000 (mol.wt. of the two-headed molecule is 470000; Gershman *et al.*, 1969; Godfrey & Harrington, 1970).

Preparation of subfragment-1

Synthetic myosin filaments were prepared by dialysis of myosin at 12–18 mg/ml into 0.2 M-NaCl/10 mM-sodium phosphate/1 mM-EDTA/1 mM-dithiothreitol, pH 6.5. These synthetic filaments were digested with papain at 0.01 mg/ml for 10 min at 22°C, digestion being terminated by addition of sodium iodoacetate to a final concentration of 1 mM. The digest was cooled in ice and dialysed against 40 mM-NaCl/10 mM-sodium phosphate, pH 6.5, to aggregate further the undigested myosin and rods. After centrifugation at 40000*g* for 45 min, the supernatant was dialysed against 50 mM-Tris/HCl, pH 8.0, before application to a column (40 cm × 2.5 cm) of DEAE-cellulose (Whatman DE-52), equilibrated in the same buffer. The protein was eluted with a linear gradient of NaCl (0–0.5 M) in the same buffer at a flow rate of about 60 ml/h, and fractions (12 ml) were collected (Lowey *et al.*, 1969). The first peak eluted was subfragment-1, which was concentrated to about 2–4 mg/ml by ultrafiltration by using an Amicon UM10 membrane filter, then by $(NH_4)_2SO_4$ precipitation, the 40–55%-saturation fraction being collected. All manipulations were carried out at 4°C.

As an alternative method, subfragment-1 was produced by the direct digestion of myofibrils, by a procedure similar to that of Cooke (1972). After digestion, the myofibrils were washed extensively with dilute salt buffer (40 mM-NaCl/10 mM-sodium phosphate, pH 6.5) and the subfragment-1 was released by suspending the myofibrils in 50 mM-Tris/HCl/1 mM-$MgCl_2$/1 mM-sodium pyrophosphate, pH 8.0. After removal of the myofibrils by centrifugation at 20000*g* for 10 min, the supernatant was applied to the DEAE-cellulose column for fractionation as described above. [The enzyme activity of the papain was determined as described previously (Lowey *et al.*, 1969), the concentration used in the digestions being calculated by assuming a standard specific activity of 15 μmol of benzoylarginine ethyl ester hydrolysed/min per mg.]

The concentration of subfragment-1 was determined by using the Folin reagent (Lowry *et al.*, 1951) or by extinction, by using a value of $6.4\,cm^{-1}$ for $E^{1\%}_{280}$ (Tada *et al.*, 1969). Concentrations of sites are quoted as μM on the basis of a mol.wt. of 115000.

Preparation of actin from rabbit skeletal muscle

Rabbit actin was prepared as described by Straub (1942), and purified by a modification of the method of Mommaerts (1952). The acetone-dried powder was extracted at 0°C with 0.1 mM-ATP/2.5 mM-Tris/HCl, pH 8.0, and the solution obtained after filtration clarified by centrifugation at 80000*g* for 1 h. Actin was polymerized with 0.7 mM-$MgCl_2$/10 mM-NaCl for 1 h at 25°C and pelleted by centrifugation at 160000*g* for 3 h. The pellets were washed to remove excess of nucleotide and resuspended by gentle homogenization in 30 mM-NaCl/1 mM-$MgCl_2$/10 mM-imidazole/HCl, pH 7.0, containing 0.1 mM-NaN_3. The concentration was estimated by using an extinction coefficient $E^{1\%}_{280} = 11.0\,cm^{-1}$ (West *et al.*, 1967).

ATPase assays

(*a*) *Hydrolysis of* [γ-^{32}*P*]*ATP*. Hydrolysis rates were determined by incubating subfragment-1 (5 μM) in 0.1 M-KCl/5 mM-$MgCl_2$/50 mM-Tris/HCl, pH 8.0, with 50 μM-[γ-^{32}P]ATP, removing samples at various times between 0 and 3 min and quenching with an equal volume of 7% (v/v) $HClO_4$. The [^{32}P]P_i liberated was separated from substrate by t.l.c. on polyethyleneimine-cellulose (Polygram CEL 300 PEI; Machery-Nagel and Co., 516 Düren, Germany) by using 0.5 M-K_2HPO_4 adjusted to pH 3.5 with HCl as described by Bagshaw & Trentham (1973), 20 μl samples being applied. After drying, the positions of the markers and the solvent front were located by illumination with u.v. light and the sheets cut into 1 cm strips. ([^{32}P]P_i runs at the salt front, coincident with the AMP marker.) These strips were counted by using a Beckman scintillation counter, in vials containing 4 ml of a mixture of 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(5-phenyloxazol-2-yl)-benzene in toluene/Beckman BiosolvTM (4:1, v/v).

The results obtained give the ratio of P_i to unhydrolysed ATP directly.

(*b*) *Spectrophotometric linked assay*. The reaction mixture contained 0.1 mg of pyruvate kinase/ml, 0.1 mg of lactate dehydrogenase/ml, 0.1 mM-NADH, 0.5 mM-phosphoenolpyruvate and subfragment-1 (about 2 μM) or 2 μM-myosin in a final volume of 2 ml. The buffer contained 0.1 M-KCl (for subfragment-1) or 0.5 M-KCl (for myosin), 50 mM-Tris/HCl, pH 8.0,

and 5mM-$MgCl_2$. Reaction was initiated by addition of 10μl of ATP solution and assayed over a range of ATP concentrations from 2μM to 2mM. The decrease in E_{340} was followed in a Perkin–Elmer 124 double-beam spectrophotometer by using a tungsten light-source, connected to a Kontron pen recorder. Corrections were made for the volume of successive ATP additions, and the reaction temperature was 25°C.

(*c*) *pH-stat assays.* ATPase activities of subfragment-1 in the presence of actin were followed in a pH-stat at pH7.6 as described by Weeds & Taylor (1975). The assay mixture for actin activation contained 30mM-KCl, 2.0mM-$MgCl_2$ and 2.0mM-ATP (the temperature was 25°C).

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate

For this, 5% and 10% polyacrylamide gels were prepared as described previously (Weeds & Taylor, 1975) and run in a buffer containing 0.1M-Tris/Bicine [*NN*-bis-(2-hydroxyethyl)glycine] and 0.1% sodium dodecyl sulphate at pH8.3, at a constant voltage of 70V. Staining and destaining were carried out as described by Weeds *et al.* (1975).

Rapid-reaction equipment

The stopped-flow fluorescence apparatus built by Dr. A. R. Fersht has been described (Fersht *et al.*, 1975). Protein solutions were excited by light at 300nm and light emitted above 320nm was observed at 90° to the incident beam. For light-scattering experiments, light at 350nm was scattered by the protein solutions and observed at 90° to the incident beam. The output was displayed on a Tektronix storage oscilloscope type 564B and recorded on Polaroid film. End points were obtained by triggering the oscilloscope a second time when the transient phase was complete. Measurements were taken directly from the Polaroid print and plotted on a logarithmic scale to obtain the reaction rates.

The stopped-flow apparatus was driven by a pneumatic piston which provided a constant value for the dead-time of the instrument under a given set of conditions. The light-scattering signal changes were very large compared with background noise, and rates up to $800s^{-1}$ proved to be very reproducible. The dead-time of the instrument was calculated from a plot of reaction velocity against the logarithm of the signal amplitude under the conditions used for the actin–subfragment-1 dissociation experiments, and was 0.6ms. By using this value, rates of over $800s^{-1}$ were obtained by extrapolation from the diminution of the signal amplitude. The validity of this approach for measuring very rapid rate constants was demonstrated in control experiments, as described in the Results section.

The quenched-flow apparatus was built by Dr. W. McClure, Max-Planck Institut für Biophysikalische Chemie, Göttingen (W. McClure, unpublished work). The reaction mixture was quenched, after passing through a delay tube, with 15% $HClO_4$. [^{32}P]P_i was separated from [γ-^{32}P]ATP by t.l.c. as described above.

All solutions used for rapid-reaction work were made in degassed water and proteins were clarified by centrifugation or by passage through Millipore filters.

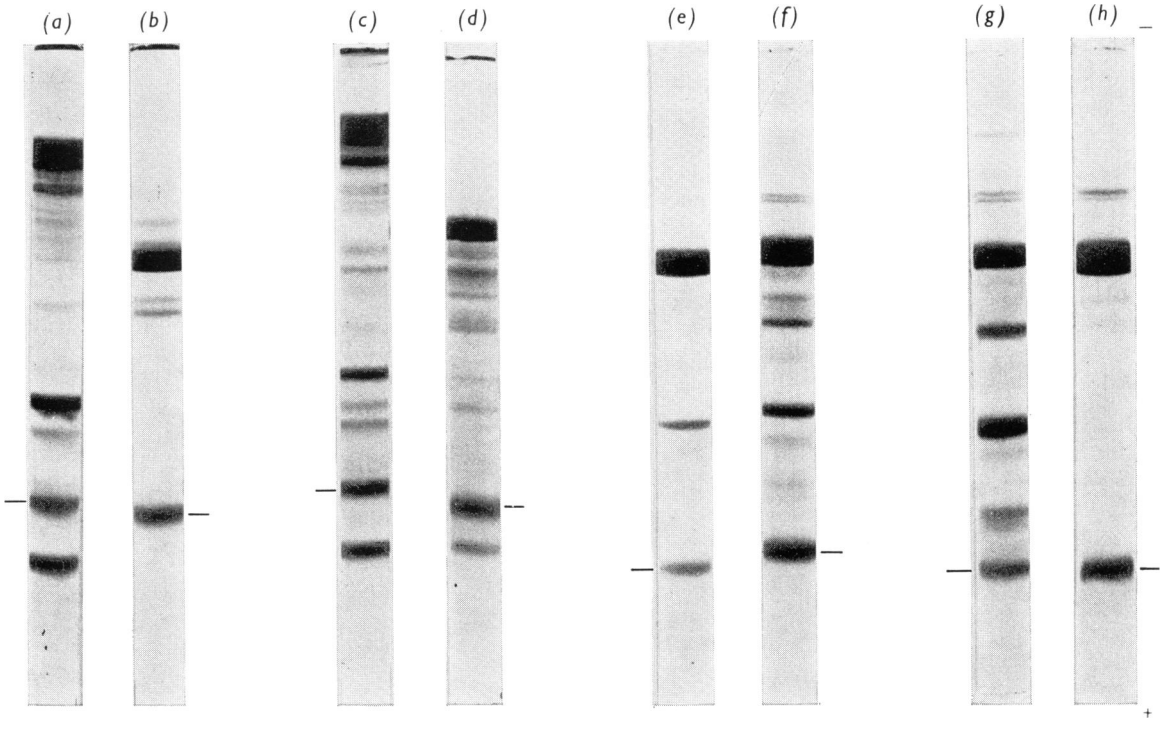
Reagents

ATP, ADP, NADH, phosphoenolpyruvate and *N*-benzoyl-L-arginine ethyl ester were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pyruvate kinase and lactate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; papain was from Worthington Chemical Corp. (Freehold, NJ, U.S.A.). [γ-^{32}P]ATP was from The Radiochemical Centre (Amersham, Bucks., U.K.). Adenine nucleotide concentrations were determined spectrophotometrically from $\varepsilon = 15400 litre \cdot mol^{-1} \cdot cm^{-1}$ at 259nm.

Results

Subfragment-1 preparation

Lowey *et al.* (1969) demonstrated the action of papain on rabbit skeletal-muscle myosin to produce various functional fragments of the molecule. Using gels containing sodium dodecyl sulphate to analyse the constituent polypeptides of subfragment-1, Stone & Perry (1973) showed this fragment to be a complex mixture of polypeptide chains derived by cleavage of the heavy chains, together with three light chains, two of which showed evidence of degradation. By contrast, bovine cardiac subfragment-1 consisted essentially of a single heavy chain of apparent mol.wt. 90000 and a single slightly degraded light chain of apparent mol.wt. 25000 (Weeds & Frank, 1972; Taylor, 1974). From a series of small-scale digestions using different conditions and protein concentrations, the methods outlined above were developed to give a high yield of subfragment-1 with little contaminating heavy meromyosin. As with papain cleavage of rabbit myosin (Lowey *et al.*, 1969), digestion in 0.6M-NaCl yielded both subfragment-1 and heavy meromyosin (Fig. 1), whereas digestion of insoluble myosin produced only subfragment-1 in the supernatant fraction after dialysis to low ionic strength. This subfragment-1 gave a simple band pattern on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Plate 1). Some



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of subfragment-1 preparations in the presence of sodium dodecyl sulphate

Proteins (up to 100μg) were submitted to electrophoresis on 10% (*a–d*) or 7.5% (*e–h*) polyacrylamide gels in 0.1% sodium dodecyl sulphate, 0.1M-Tris/Bicine, pH8.3. (*a*) Bovine cardiac actomyosin used as a marker to indicate the positions of the light chains and any contaminating actin; (*b*) cardiac subfragment-1 prepared from an insoluble myosin digest, run at the same time as marker to demonstrate the slight degradation of the LC1 light chain; (*c*) bovine cardiac actomyosin marker; (*d*) cardiac subfragment-1 prepared from an insoluble myosin digest showing the presence of some 19000 LC2 light chain; (*e*) subfragment-1 prepared from cardiac myofibrils by digestion with papain at 0.015mg/ml for 15min at 23°C; (*f*), subfragment-1 prepared by digestion of cardiac myofibrils at 0.15mg/ml papain for 15min at 2°C; (*g*) $(NH_4)_2SO_4$ (0–40% satn.) precipitate from subfragment-1 preparation shown in (*e*), containing also actin and other contaminants; (*h*) $(NH_4)_2SO_4$ (40–55% satn.) precipitate containing subfragment-1 and a slight contamination by heavy meromyosin. The horizontal lines mark the positions of the LC1 light chain and its papain fragments. Samples applied to the tops of the gels migrate towards the anode.

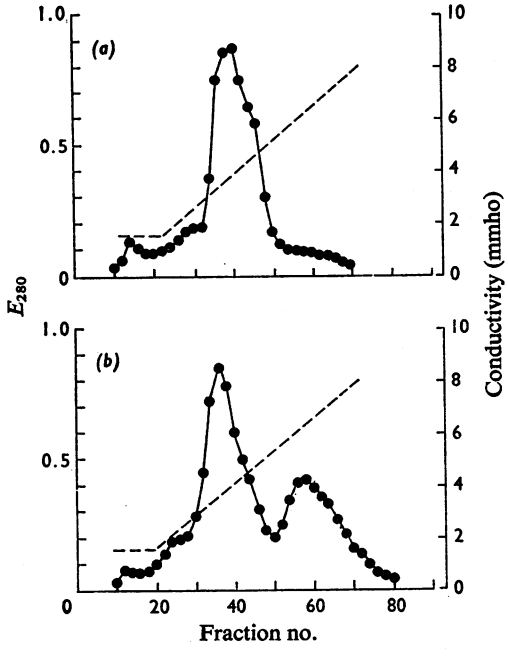


Fig. 1. *Chromatography of myosin fragments on DEAE-cellulose*

Papain digests of cardiac myosin were carried out in either 0.2M-NaCl (insoluble digest, *a*), or 0.6M-NaCl (soluble digest, *b*). After digestion, the proteins were dialysed to $I = 0.05$ mol/litre and insoluble material was removed by centrifugation at 40000*g*. The supernatant fractions were applied to a column (40cm×2.5cm) of Whatman DE-52, equilibrated in 0.05M-Tris/HCl, pH8.0, (4°C) and fragments were eluted with a linear gradient of NaCl (0–0.5M) in the same buffer (fraction size = 12ml). In the insoluble digest (*a*), only subfragment-1 was present, whereas the soluble digest (*b*) gave a second peak containing both heavy meromyosin and subfragment-2. ●, E_{280}; ----, conductivity.

variation between different preparations occurred, notably in the yield of subfragment-1 obtained and in the extent of preservation of the LC2 light chain.

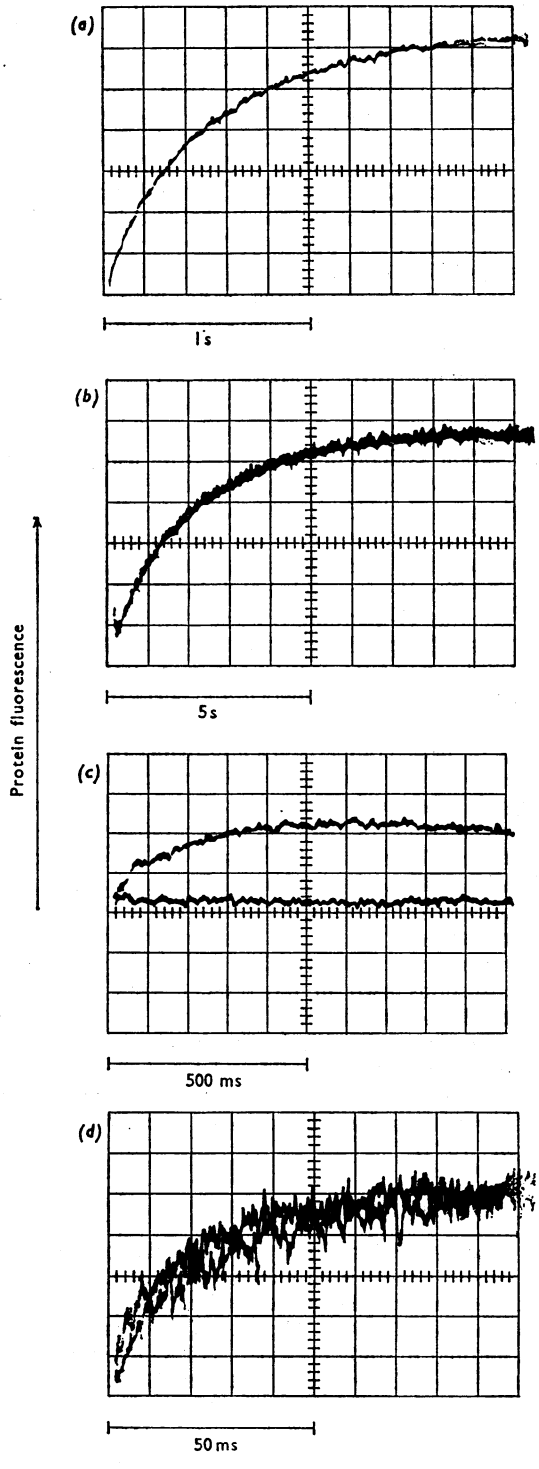
Subfragment-1 produced from myofibrils has two advantages over that prepared from myosin. First, the preparative method isolates only the subfragment-1 that binds to actin after exposure to papain, and that is also released by magnesium pyrophosphate, thus selecting only functionally competent molecules. Second, the procedure is very rapid and obviates the need to prepare myosin. Further, the yield of subfragment-1, calculated on the basis of a given mass of muscle, was greater than that of the conventional preparation. Gel electrophoresis showed that the product was similar to that obtained from myosin, though the LC2 light chain appeared to be less frequently preserved (Plate 1).

Studies were undertaken to explore the variation in yield of subfragment-1 obtained in these experiments. The results showed that the yield was critically dependent on the presence of bivalent cations (Taylor, 1974). To activate the papain, both dithiothreitol and EDTA were added, and if there was insufficient EDTA present in the added papain to chelate the bivalent cations in the myosin, the yield of subfragment-1 was low. Thus the proteolytic susceptibility of the myosin was altered in the presence of bivalent cations, as has been noted elsewhere (Biro *et al.*, 1972; Yamashita & Mimura, 1974). In the presence of bivalent cations the proportion of heavy meromyosin produced is increased and the LC2 light chain is more extensively preserved (Taylor, 1974). Similar results have been reported for rabbit skeletal-muscle myosin by using chymotrypsin (Weeds & Taylor, 1975) and papain (Margossian *et al.*, 1975). The activity of papain towards its synthetic substrate, benzoylarginine ethyl ester, is not affected by bivalent ions showing that it is the availability of susceptible bonds in the myosin that is affected by bivalent metal ions.

Most of the kinetic experiments described in the present paper were carried out with subfragment-1 prepared from cardiac myosin and purified by DEAE-cellulose chromatography and $(NH_4)_2SO_4$ fractionation. Where comparisons are made between the rates of ATP binding to both subfragment-1 and myosin, the subfragment-1 was prepared from the same myosin preparation. Preparations of subfragment-1 from myofibrils showed no significant kinetic differences when compared with those obtained from myosin, but both types of preparation were analysed in an attempt to understand the reasons for the observed kinetic heterogeneity. The preparations used in the present work contained virtually no intact LC2 light chain as evidenced by gel electrophoresis in the presence of sodium dodecyl sulphate (Plates 1*b* and 1*h*). Any effects that this light chain may have on the ATPase activity will not therefore be detected (see Margossian *et al.*, 1975).

ATP binding

When ATP binds to rabbit skeletal-muscle subfragment-1 there is an enhancement of the intrinsic fluorescence of the protein (Werber *et al.*, 1972; Bagshaw *et al.*, 1972). The rate of this change can be monitored to study the kinetics of ATP binding, and Bagshaw *et al.* (1974) demonstrated that the observed rate tended to a limit of about $400\,s^{-1}$. This is consistent with a two-step binding process, the first part of which is a second-order association (K'_1 =



$4.5 \times 10^3 M^{-1}$), followed by a first-order isomerization step, $k'_{+2} = 400 s^{-1}$ (see eqn. 1).

Addition of ATP to cardiac subfragment-1 produced a rapid increase in protein fluorescence, whose rate could be described by a single exponential (Fig. 2 shows experimental traces). Observed rates calculated from these traces were plotted against ATP concentration (Fig. 3), showing that the fluorescence change tended to a limit at about $100 s^{-1}$. This limiting value may be estimated more accurately from the double-reciprocal plot (Fig. 3*b*). Analysing these results according to the scheme outlined in eqn. (1) gives a value for $k_{+2} = 100 s^{-1}$ from the intercept on the ordinate of Fig. 3(*b*), and the gradient of the line is equal to $1/K_1 k_{+2}$, from which a value of $K_1 = 5 \times 10^3 M^{-1}$ may be calculated. Comparing these results with those of Bagshaw *et al.* (1974) shows little difference in the value of K_1, but the rate of the isomerization process is one-quarter of that obtained for rabbit skeletal-muscle subfragment-1 (see Table 1).

The fluorescence traces could not be fitted by a single exponential for a number of preparations of cardiac subfragment-1, and these preparations appeared to be kinetically heterogeneous in that some of the protein was characterized by a slower rate of ATP association. Certain of these abnormal preparations were almost exclusively of the slower type (Fig. 2*b*), giving values of $K_1 = 7 \times 10^3 M^{-1}$ and $k_{+2} = 5 s^{-1}$. Experiments were therefore carried out to determine the kinetics of ATP association with bovine cardiac myosin, and the records (Fig. 2*c*) corresponded to the faster binding rates, showing that the slower process in these preparations is abnormal and might perhaps be due to damage of the subfragment-1 during the preparation. Although experiments were undertaken to explore the relationship between digestion conditions and the kinetics of the subfragment-1 species produced, no consistent pattern emerged for the production of this abnormal species. However, this kinetic heterogeneity was observed only in the transient phase of ATP binding, the steady-state rates of ATP hydrolysis for the two types of subfragment-1 being similar, as were also the ATPase activities in the presence of actin.

Fig. 2. *Stopped-flow records of the fluorescence enhancement of cardiac myosin or subfragment-1 when mixed with ATP*

One syringe contained 5.0 μM-subfragment-1 (*a*, *b*, *d*) or myosin (*c*) and the other ATP at 20 μM (*a*, *b*, *c*) or 1 mM (*d*) (concentrations after mixing). Both syringes contained 5 mM-$MgCl_2$ and 50 mM-Tris/HCl, pH 8.0, with KCl at 0.1 M (*a*, *b*, *d*) or 0.5 M (*c*). (*a*) and (*d*) are normal subfragment-1 preparations, and (*b*) is the abnormal subfragment-1 species showing slower binding of ATP. The lower trace in (*c*) shows the base fluorescence value. Two traces are shown in (*d*).

The amplitude of the fluorescence change induced by ATP binding to cardiac subfragment-1 was 8–10%, which is half the value observed for rabbit subfragment-1 (Bagshaw *et al.*, 1974). Although the fluorescence change was smaller, the signals were of sufficient amplitude to measure these rates accurately because the maximum rates were only $100 s^{-1}$. Experiments were also carried out to measure the binding of ADP to cardiac subfragment-1 (corresponding to k_{-7} and k_{-6} in eqn. 1). However, the amplitude of the fluorescence change in this case was less than 3%, so that the low signal-to-noise ratio made measurement of association rates both difficult and unreliable, particularly for the normal subfragment-1 species. Nevertheless, experiments carried out with the abnormal slow-binding species gave a second-order rate constant of $2\times 10^4 M^{-1}\cdot s^{-1}$ and a limiting value of about $3 s^{-1}$ at high ADP concentrations when interpreted in the manner described above. These experiments showed that ADP binding also follows a two-step process, as has been described for rabbit skeletal-muscle subfragment-1 by Bagshaw *et al.* (1974). The importance of these particular observations is not in the absolute values obtained, but the fact that the rate of the fluorescence change tends to a plateau for ADP binding indicates that this process is related to nucleotide binding alone and not, as in the case of ATP, to its subsequent chemical cleavage. The existence of a plateau is evidence for an isomerization process after the second-order binding step. Thus these experiments are consistent with those of Bagshaw *et al.* (1974), though the rate of the substrate-induced isomerization process is only one-quarter that observed for rabbit subfragment-1.

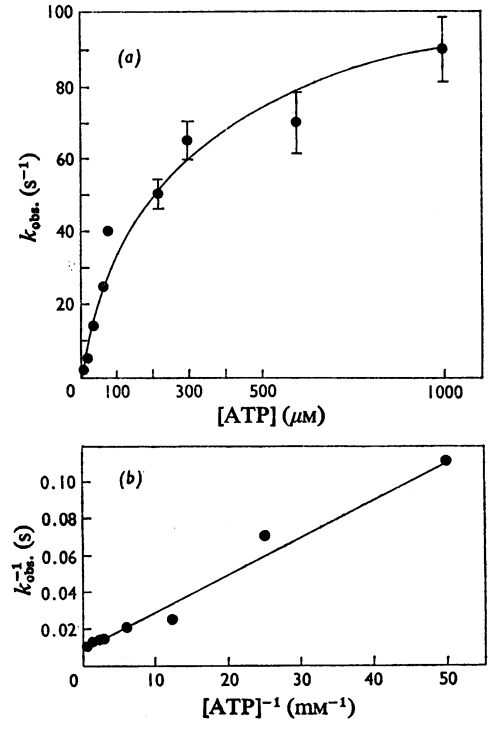


Fig. 3. *Kinetic analysis of the fluorescence enhancement of cardiac subfragment-1 on interaction with ATP*

Data from stopped-flow records for the normal subfragment-1 species (Fig. 2) were analysed as exponential processes to derive $k_{obs.}$, the rate of fluorescence enhancement. (*a*) Plot of $k_{obs.}$ against ATP concentration. (*b*) Double-reciprocal plot used to derive k_{+2}. From this plot, $k_{+2} = 100 s^{-1}$ and $K_1 = 5\times 10^3 M^{-1}$.

Table 1. *Comparison of the rate constants and equilibrium constants for cardiac subfragment-1 with those for rabbit skeletal-muscle subfragment-1*

Rate constants and equilibrium constants for cardiac subfragment-1 are taken from this work. Comparable results for rabbit subfragment-1 were taken as follows: (*a*) Bagshaw *et al.* (1974); Bagshaw & Trentham (1973, 1974); (*b*) Weeds & Taylor (1975); (*c*) H. White (personal communication). The ratio is obtained from k (rabbit)/k (cardiac).

Kinetic process	Cardiac subfragment-1	Rabbit subfragment-1		Ratio
MgATP catalytic-centre activity	$k_{+4} = 0.019 s^{-1}$	$k'_{+4} = 0.06 s^{-1}$	(*a*)	3.2
ATP-induced isomerization	$k_{+2} = 100 s^{-1}$	$k'_{+2} = 400 s^{-1}$	(*a*)	4.0
ADP release	$k_{+6} = 0.3 s^{-1}$	$k'_{+6} = 1.4 s^{-1}$	(*a*)	4.7
Equilibrium constant for ATP binding	$K_1 = 5\times 10^3 M^{-1}$	$K'_1 = 4.5\times 10^3 M^{-1}$	(*a*)	0.9
Equilibrium constant for ATP cleavage	$K_3 = 11$	$K'_3 = 9$	(*a*)	0.8
Catalytic-centre activity for actin-activated ATPase activity	$k_{cat.} = 3.0 s^{-1}$	$k_{cat.} = 40 s^{-1}$	(*b*)	13.0
Actin-subfragment-1 dissociation second-order rate constant	$K''_1\cdot k''_{+2} = 2\times 10^6$–$3.5\times 10^6 M^{-1}\cdot s^{-1}$	$7\times 10^6 M^{-1}\cdot s^{-1}$		2.6
Maximum dissociation rate	$k''_{+2} = 1500$–$2000 s^{-1}$	—		
Equilibrium constant	$K''_1 = 1\times 10^3$–$2\times 10^3 M^{-1}$	—		
Actin-subfragment-1 association second-order rate constant	5×10^5–$10\times 10^5 M^{-1}\cdot s^{-1}$	$1.4\times 10^6 M^{-1}\cdot s^{-1}$	(*c*)	

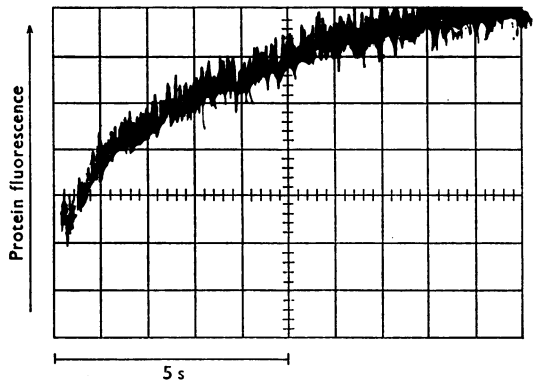


Fig. 4. *Stopped-flow record of fluorescence enhancement of cardiac subfragment-1 when ATP displaces ADP*

One syringe contained 5μM-subfragment-1 and 20μM-ADP, and the other 500μM-ATP (concentrations after mixing). Both syringes contained 0.1M-KCl, 5mM-$MgCl_2$ and 50mM-Tris/HCl, pH8.0. The rate of ADP release was 0.3s^{-1}.

Although it was not possible to measure the rate of ADP binding to cardiac subfragment-1 by using the normal fast-binding species, the rate of ADP release from subfragment-1 could be determined by displacement with ATP. The rate observed for the transition to the higher-fluorescence intermediate was 0.3s^{-1}, and this value was independent of ATP concentration (Fig. 4). Bagshaw *et al.* (1972) showed that the rate of ADP displacement by ATP for rabbit subfragment-1 was also independent of ATP concentration, giving a value of 1.4s^{-1} for k'_{+6} (eqn. 1). Thus the rate k_{+6} for cardiac subfragment-1 is 0.2 times that for rabbit skeletal-muscle subfragment-1, showing that both isomerization processes are decreased in rate to a similar extent.

Transient phosphate production

Lymn & Taylor (1970) observed transient phosphate formation with heavy meromyosin, and the rate of this process increased with ATP concentration to over 160s^{-1} (Lymn & Taylor, 1971). These experiments demonstrated that ATP cleavage was not the rate-limiting step in the enzymic mechanism, but the steady-state rate was controlled by a process related to product release. The limiting rate of the cleavage process has not been determined because of the difficulty in making these measurements by using quench-flow techniques. According to the kinetic model in eqn. (1), cleavage follows the rapid isomerization process (k_{+2}). Since this process in cardiac subfragment-1 is much slower than the

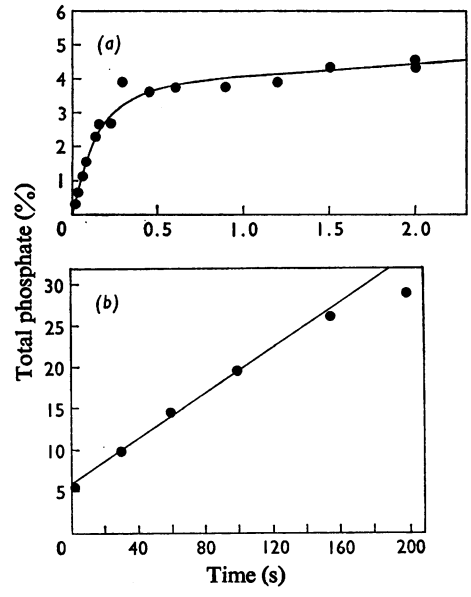


Fig. 5. *Production of* [^{32}P]P_i *from* [γ-^{32}P]*ATP by cardiac myosin*

One syringe contained myosin (5μM heads) and the other 20μM-ATP (concentrations after mixing). Both syringes contained 0.5M-KCl, 5mM-$MgCl_2$ and 50mM-Tris/HCl, pH8.0. (*a*) Initial production of phosphate. (*b*), Steady-state phosphate production.

corresponding step for skeletal subfragment-1, it was important to explore the rate of the cleavage step for the slower species.

Experiments were carried out with both myosin and subfragment-1 from cardiac muscle to compare the rates of the fluorescence change on ATP binding with the transient production of P_i by using [γ-^{32}P]ATP. The observed rates for these two processes were similar under identical conditions, indicating that the cleavage process is not resolved from the isomerization step. Fig. 5 shows an experiment with cardiac myosin. Similar values were obtained for subfragment-1, e.g. with 20μM-ATP and subfragment-1 (5μM), the observed rate for the fluorescence change was 7s^{-1}, and that for the production of P_i was 6s^{-1}. Thus under these conditions, the cleavage process occurs at a rate which is at least as fast as the isomerization process.

A second value of measurements of the transient phase of phosphate production is that the amplitude of this phase provides information about the active-site concentration of the enzyme. The stoicheiometry determined from our standard preparations of cardiac myosin and subfragment-1 was between 0.3 and 0.5mol of P_i/subfragment-1 site, which

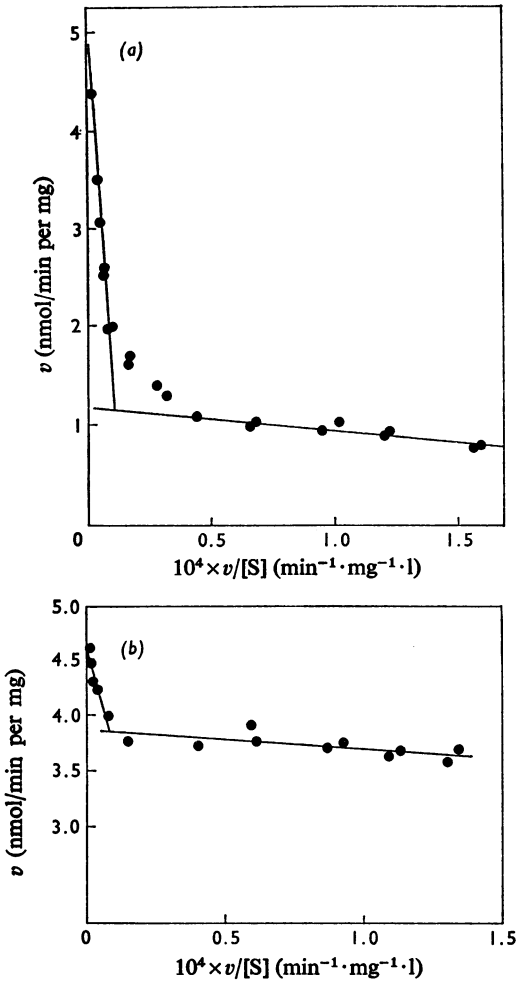


Fig. 6. *Plot of v against v/s for steady-state myosin Mg^{2+}-dependent ATPase activity*

The steady-state ATPase activity was measured by the linked assay system (see the Materials and Methods section) in 0.5M-KCl/5mM-$MgCl_2$/50mM-Tris/HCl, pH 8.0 at 25°C, by using a concentration range of ATP of 5μM–2.5mM. (*a*) Cardiac myosin after centrifugation at 200000*g* for 3h; (*b*) myosin after purification on DEAE-cellulose.

was much lower than the value of 0.75mol of P_i/subfragment-1 head obtained by Lymn & Taylor (1971), who used rabbit heavy meromyosin. However, this low stoicheiometry seemed to be related to the formation of inactive aggregates as described below. After further purification of cardiac myosin, transient amplitudes of 0.8mol of P_i/mol of subfragment-1 sites were observed, comparable with those found for skeletal-muscle myosin.

There are a number of reasons why values of less than unity can be obtained for the stoicheiometry, and these have been discussed by Trentham *et al.* (1976), but a principal cause is the presence of inactive enzyme. Attempts were made to explore this possibility by using the steady-state ATPase assay, since results of experiments carried out by the radiochemical assay procedure have given values lower than those obtained by the linked assay system. One difference in the two techniques was the range of substrate concentrations used; ATP was used at micromolar concentrations in the radiochemical assays, whereas the linked assay used up to millimolar concentrations. When steady-state ATPase assays were carried out by the linked assay system over a concentration range of ATP from 20μM to 2.5mM, the activity plots were biphasic (Fig. 6). Similar observations have been made for heavy meromyosin from rabbit muscle by Lymn & Taylor (1970). In the experiment on stored cardiac myosin shown in Fig. 6, values for $V_{max.}$ of 1.18 and 4.9nmol/min per mg (0.005s^{-1} and 0.019s^{-1}) were obtained, with corresponding values for K_m of 2 and 360μM respectively. After re-precipitation, this cardiac myosin was redissolved and clarified by centrifugation at 200000*g* for 3h and the assay then repeated. This time the $V_{max.}$ values of the two phases were 0.009 and 0.019s^{-1}, with corresponding K_m values of 4 and 200μM. For subfragment-1 the turnover rate at high ATP concentrations was 0.017s^{-1}, whereas at low concentrations the rate was less than half this value as measured by either the linked assay or [^{32}P]P_i release. Thus the low stoicheiometry appeared to be due in part to the presence of molecules whose activity was impaired at low substrate concentrations.

Attempts were made to purify cardiac myosin on DEAE-cellulose as described by Richards *et al.* (1967). Much of the protein did not bind, but the myosin that was eluted in the salt gradient gave a more-linear Hofstee plot with $V_{max.} = 0.018 s^{-1}$ (Fig. 6*b*), and this preparation gave the transient amplitude of 0.8mol of P_i/mol of myosin sites reported above.

The heterogeneity observed in the steady-state assays was also reflected in the results of analytical ultracentrifugation, which showed an additional boundary in front of the main myosin peak indicative of aggregated protein, and the bulk of this aggregated protein was not retarded on the DEAE-cellulose column. However, examination of this myosin fraction in the ultracentrifuge in the presence of ATP did not show any reversal of the aggregation as might have been expected from the effect of ATP concentration on the ATPase activity.

Examination of cardiac subfragment-1 in the analytical ultracentrifuge also showed evidence of aggregates, which appeared to form more rapidly when the protein was stored at low ionic strength.

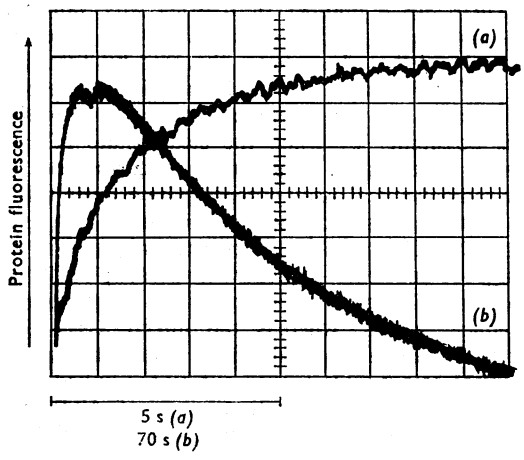


Fig. 7. *Stopped-flow records of fluorescence enhancement of cardiac subfragment-1 during a single turnover of the ATPase*

One syringe contained 18μM subfragment-1 and the other 5μM-ATP (concentrations after mixing). Both syringes contained 0.1M-KCl, 5mM-$MgCl_2$ and 50mM-Tris/HCl, pH8.0. The time-scale in trace (*a*) is 1s per division, and that in trace (*b*) is 14s per division.

Although this aggregation occurred less estensively on storage in 0.1M-NaCl/50mM-Tris/HCl (pH8.0)/5mM-$MgCl_2$, it could not be eliminated altogether.

With freshly purified proteins the maximum amplitude of the transient phosphate production was 0.8mol of P_i/mol of myosin sites. Assuming that all the myosin present was fully active, values of less than unity can be obtained if the ATP is hydrolysed by two different pathways as proposed by Tonomura (1972), or if the cleavage process is readily reversible so that an equilibrium mixture of M*·ATP and M**·ADP·P_i is generated as the steady-state complex (Bagshaw & Trentham, 1973; Bagshaw *et al.*, 1975). From measurements of the amount of ATP unhydrolysed at a time when the binding reaction was complete, but before product release had occurred, Bagshaw & Trentham (1973) obtained a value for K'_3 of 9. Similar experiments have been carried out with cardiac subfragment-1, by following the time-course of ATP cleavage in a single turnover when subfragment-1 (18μM) was mixed with 5μM-ATP. After 20s, when all the ATP was bound to the subfragment-1 as monitored from the fluorescence enhancement (Fig. 7), there was still 10% of the ATP unhydrolysed, and this substrate decayed subsequently at a rate corresponding to the steady-state rate (Fig. 8). The equilibrium concentration of ATP present in this experiment permits a value for K_3 to be calculated.

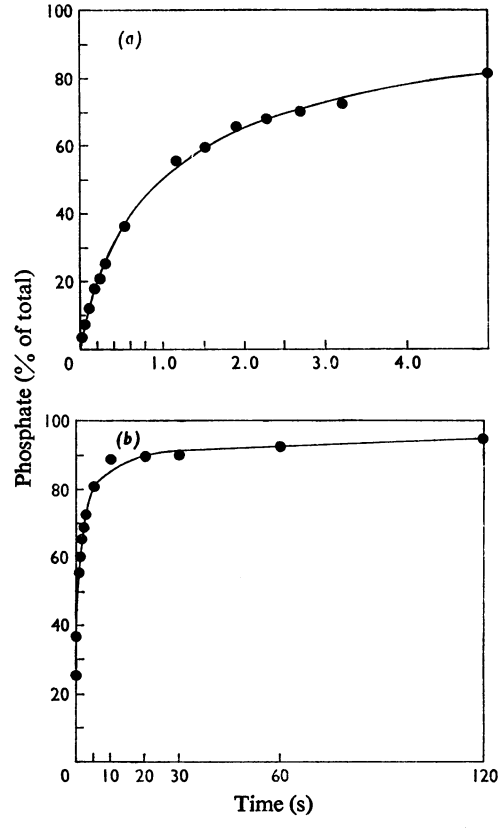


Fig. 8. *Production of* [^{32}P]P_i *from* [γ-^{32}P]*ATP during a single turnover by subfragment-1*

One syringe contained 50μM-subfragment-1 and the other 5μM-ATP (concentrations after mixing). Both syringes contained 0.1M-KCl, 5mM-$MgCl_2$ and 50mM-Tris/HCl, pH8.0. (*a*) Initial phases of P_i production; (*b*) as above, plus steady-state rate. Some of the early time-points in (*b*) are omitted for clarity.

Fig. 8 shows that equilibrium is attained when 92% of the ATP has been hydrolysed, giving a value for K_3 of 11. This value is similar to that obtained for skeletal-muscle subfragment-1 (Bagshaw & Trentham, 1973). When this reversibility is taken into account, the amplitude of the transient phosphate production indicates that the protein is 90% active. Thus it seems probable that in these preparations all the active subfragment-1 heads are able to hydrolyse ATP by a mechanism involving transient phosphate production, and there is no evidence to suggest that a large proportion of these will hydrolyse the substrate by a different route not involving transient phosphate release, as has been suggested by Tonomura & Inoue (1974). However, we would not claim that these experiments rule out possible

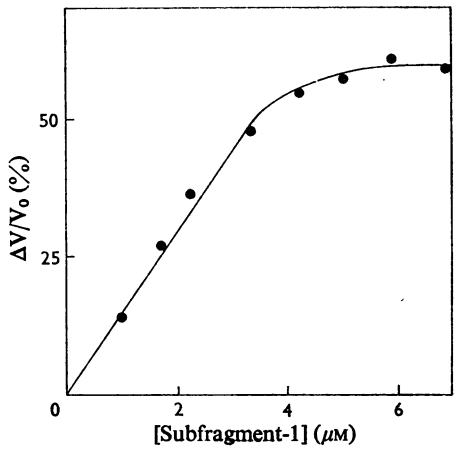


Fig. 9. *Actin–subfragment-1 association determined by increase in light-scattering*

The amplitude of the change in light-scattering (ΔV) obtained with increasing subfragment-1 concentrations for a constant concentration of actin (5 μM). One syringe contained actin and the other subfragment-1, and both syringes contained 0.1 M-KCl, 5 mM-$MgCl_2$ and 50 mM-Tris/HCl, pH 8.0.

co-operative interactions between the two subfragment-1 heads.

The present experiments suggest that the cleavage of ATP by cardiac myosin and its subfragment-1 occurs by a mechanism similar to that described by Bagshaw & Trentham (1973) for skeletal-muscle subfragment-1, and we have interpreted our results according to their model, namely that the cleavage process appears to be in rapid reversible equilibrium. Further, the isomerization step associated with ATP binding is relatively irreversible, resulting in a large negative standard free-energy change on binding ATP. The value of k_{-2} cannot be determined by these techniques, but for rabbit subfragment-1 it has been estimated by other methods (Mannherz *et al.*, 1974; Walcott & Boyer, 1975).

Interaction with actin

Addition of ATP to actomyosin causes a rapid dissociation of the complex, and this process has been studied for actin–heavy meromyosin by Lymn & Taylor (1971) using turbidity change as a monitor. Their results showed a linear increase in rate until it became too fast to measure. Since the rate constants for ATP association with cardiac subfragment-1 were lower than the corresponding values for rabbit subfragment-1, the kinetics of actin-subfragment-1 dissociation were investigated to explore the limiting value. To maximize the signal change, light-scattering was measured instead of turbidity (see the Materials and Methods section). Fig. 9 shows that the change in light-scattering was a linear function of the occupancy of the actin sites.

The conditions used for these experiments were similar to those used for the fluorescence measurements, and subfragment-1 was added in slight excess over the actin sites to ensure saturation. Fig. 10 shows a number of traces obtained with different concentrations of ATP. In most cases these traces were fitted by single exponentials, though as with the fluorescence traces some preparations gave biphasic responses. The observed rates of dissociation increased linearly with ATP concentration up to 200 μM-ATP, with a second-order rate constant of $2\times10^6\,M^{-1}\cdot s^{-1}$. However, in contrast with the results with rabbit skeletal-muscle heavy meromyosin, a plateau in the observed rate constant was observed at high ATP concentrations. Fig. 11 shows a Hofstee plot of a set of experiments with cardiac subfragment-1, where rates in excess of $800\,s^{-1}$ have been determined by extrapolation from the diminution of the signal amplitude. The extrapolated value for the maximum rate of dissociation is about $2200\,s^{-1}$, and the slope gives a binding constant of $1.8\times10^3\,M^{-1}$. Measurements were made on six preparations of cardiac subfragment-1 and the results gave values for the apparent second-order dissociation rate constant in the range 2×10^6–$3.5\times10^6\,M^{-1}\cdot s^{-1}$, with a maximum rate in the range 1500–$2000\,s^{-1}$. If a two-step binding process is assumed by analogy with ATP binding to myosin alone, then $k''_{+2} = 1500$–$2000\,s^{-1}$, and $K''_1 = 1\times10^3$–$2\times10^3\,M^{-1}$ (where K'' and k'' apply to actin subfragment-1).

As a control that the observed plateau was not an instrument artifact, a single preparation of rabbit skeletal-muscle actin subfragment-1 was also analysed for the rate of dissociation by ATP. The second-order rate constant obtained in this experiment was $7\times10^6\,M^{-1}\cdot s^{-1}$; the rate increased linearly with ATP concentration to $2000\,s^{-1}$ without any limiting value being observed and the signal eventually disappeared. Thus the magnitude of the limiting value for rabbit actin–subfragment-1 dissociation is beyond resolution by this procedure.

Eccleston *et al.* (1975) have examined the binding of ATP to arterial actomyosin, which has a very low specific activity for its ATPase. By measuring the rate of the turbidity change with increasing ATP concentrations, they also showed that the observed rate reached a plateau, but with a value of $120\,s^{-1}$. Eccleston *et al.* (1975) discuss two kinetic schemes to account for their observation, and conclude a preference for a mechanism of the form:

$$AM + ATP \underset{}{\overset{k''_{+1}}{\rightleftharpoons}} AM\cdot ATP \overset{k''_{+2}}{\rightleftharpoons}$$

$$AM^*\cdot ATP \overset{k''_{+3}}{\rightleftharpoons} M^*\cdot ATP + A$$

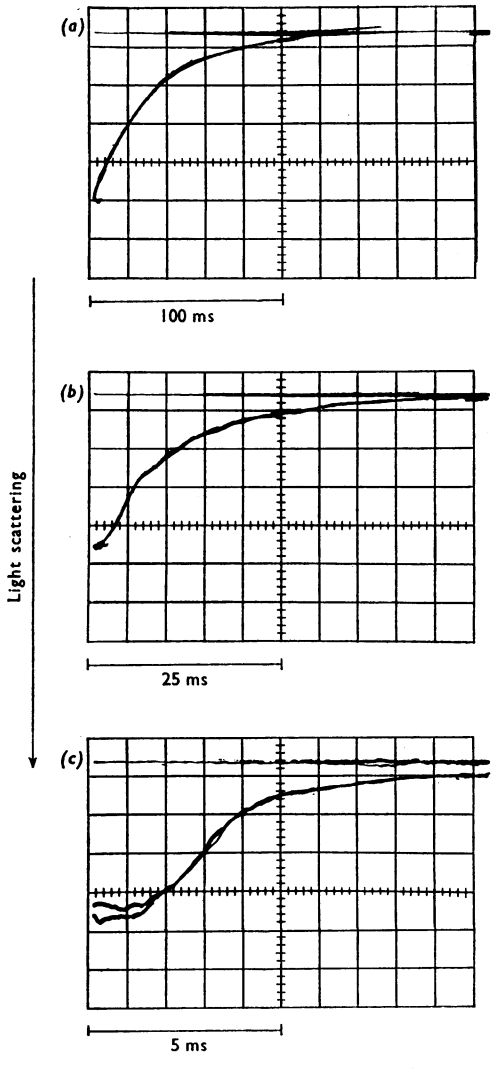


Fig. 10. *Stopped-flow records of the ATP-induced dissociation of actin–subfragment*-1

One syringe contained 5μM-actin and 5.5μM-subfragment-1, and the other contained ATP at 20μM (*a*), 60μM (*b*) or 300μM (*c*) (concentrations after mixing). Both syringes contained 0.1M-KCl, 5mM-$MgCl_2$ and 50mM-Tris/HCl, pH8.0. Each record shows two traces which virtually superimpose, and the upper horizontal line shows the light-scattering signal at the end of the transient phase. The apparent lag in (*c*) is due to 1.5ms of continuous flow after the oscilloscope has been triggered.

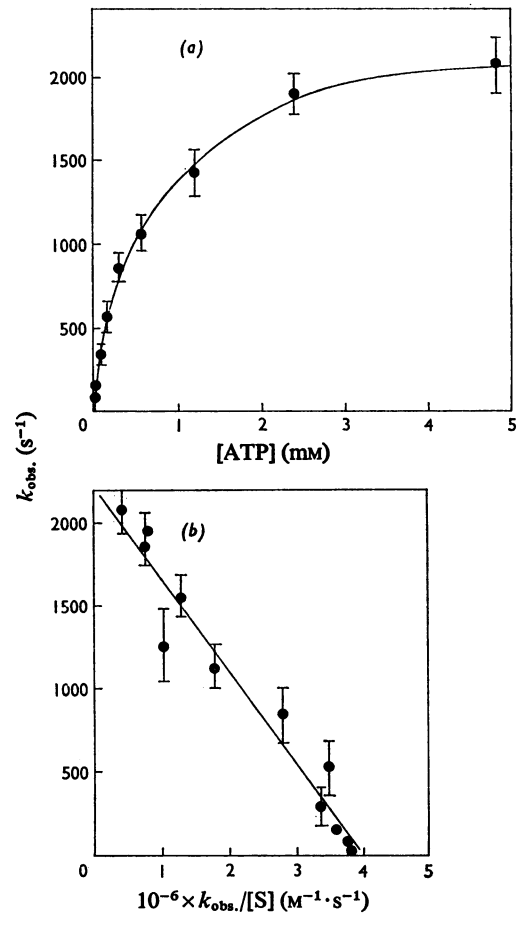


Fig. 11. *Kinetic analysis of actin-subfragment*-1 *dissociation by ATP*

(*a*) Data from traces such as those in Fig. 10 analysed as exponential processes to derive $k_{obs.}$, the rate of light-scattering decrease. (*b*) Secondary plot of $k_{obs.}$ against $k_{obs.}/[ATP]$ to obtain the maximum dissociation rate constant. Rates in excess of 800s^{-1} were obtained from the diminution in amplitude of the scattering signal (see the Materials and Methods section).

where 'A' denotes actin, and the rate of 120s^{-1} corresponds to k''_{+2}. If this model is accepted for our experiments with cardiac subfragment-1, then the value of $k''_{+2} = 1500$–$2000 s^{-1}$ would apply to the isomerization step indicated in the equation above and not to the dissociation process itself. This is another example of a process being controlled in rate by the preceding isomerization step, just as cleavage in the myosin ATPase is controlled by the substrate-induced isomerization.

One disadvantage of using myosin in turbidimetric assays, as described in the experiments of Eccleston *et al.* (1975), is that this protein can undergo self-association. Thus experiments with cardiac

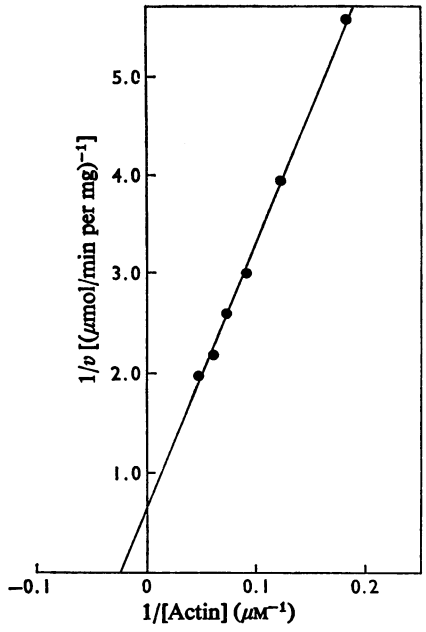


Fig. 12. *Actin-activated ATPase activity of cardiac subfragment-1*

The actin-activated ATPase activity was measured in a pH-stat in 30mM-KCl, 2.0mM-$MgCl_2$ and 2.0mM-ATP at pH7.6 and 25°C, with increasing concentrations of actin. The straight line gives $V_{max.} = 3.0 s^{-1}$, and $K_{app.} = 4.1 \mu$M-actin.

subfragment-1 have the advantage that self-association is unlikely to interfere.

Experiments were also carried out to measure the rate of association between actin and cardiac subfragment-1. By using increasing concentrations of subfragment-1 in excess of actin, the rate of change of light-scattering was followed on mixing. The association reaction should follow pseudo-first-order kinetics under these conditions, giving a single exponential process for the light-scattering change. Measurements of the observed rates by using different subfragment-1 concentrations yielded a second-order rate constant of $10^6 M^{-1} \cdot s^{-1}$. As an alternative approach the method described by Finlayson *et al.* (1969) was followed. In this experiment equal molar concentrations of actin and subfragment-1 were mixed and the change in light-scattering was followed. Since the equilibrium constant for the association process is large, the reverse reaction can be ignored during the early part of the association. For the special case where $[A_0] = [M_0]$, the reaction $A + M \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AM$ gives $k_a t = 1/[M] - 1/[M_0]$, where $[A_0]$ and $[M_0]$ are the concentrations at zero time. Thus a plot of 1/[M] or 1/[AM] against time should be linear. Since the reaction goes nearly to completion, the final voltage change $\Delta V_{max.}$ is proportional to $[M_0]$ and $[M] = [M_0] - [AM] = \Delta V_{max.} - \Delta V$. Thus a plot of $(\Delta V_{max.} - \Delta V)^{-1}$ against t should be linear. Linear plots were obtained within the concentration range used for the proteins (4–10 μM), and the slopes obtained gave a second-order rate constant of $5 \times 10^5 M^{-1} \cdot s^{-1}$. Thus the association rate constant for cardiac subfragment-1 with actin is in the range $5 \times 10^5 - 10 \times 10^5 M^{-1} \cdot s^{-1}$. These values, measured in 0.1M-KCl, compared with published ones for rabbit actomyosin measured in 0.5M-KCl of $1.4 \pm 0.5 \times 10^5 M^{-1} \cdot s^{-1}$ (Finlayson *et al.*, 1969). Comparable values for rabbit actin-sub-fragment-1 association are $1.4 \times 10^6 M^{-1} \cdot s^{-1}$, measured in 100mM-KCl (H. White, personal communication).

Table 1 summarizes all these results and also tabulates the $V_{max.}$ for the steady-state actin-activated ATPase activity (see Fig. 12).

Discussion and Conclusions

In the present paper we have determined the rate constants for a number of the elementary processes in the hydrolysis of ATP by cardiac myosin and its subfragment-1. The results show that not only is the steady-state Mg^{2+}-dependent ATPase activity substantially decreased as compared with subfragment-1 from fast-twitch-muscle myosin, but certain of the transient processes are also much slower. Although in these experiments we have not attempted to compare the rate constants for all the processes outlined in eqn. (1), it appears that those rate constants for cardiac subfragment-1 involving isomerization processes are decreased to approximately one-quarter of the comparable values for skeletal subfragment-1 (Table 1). Thus k_{+2} and k_{+6} are related to isomerization processes in ATP binding and ADP release (Bagshaw *et al.*, 1974), and k_{+4}, the catalytic-centre activity, has also been ascribed to an isomerization (Trentham *et al.*, 1972). By contrast, the equilibrium constants established for ATP association (K_1) and cleavage (K_3) show little difference between the two species. If these isomerization processes can be ascribed to conformational changes in the enzyme–ligand complexes, then it appears that the adaptation of the particular myosin to its physiological role involves regulation not only at the rate-limiting process in the mechanism of ATP hydrolysis, but also at a number of other steps where the enzyme is required to alter its conformation.

A number of other processes in the ATPase cycle involving actin have also been shown to differ between cardiac and skeletal-muscle subfragment-1 species in addition to the catalytic-centre activity ($k_{cat.}$) (Table 1). These include the actin–subfragment-1 associa-

tion and the maximum rate observed for dissociation of actin–subfragment-1 by ATP, which may also be an isomerization process (Eccleston *et al.*, 1975). The maximum rate of dissociation of actin–subfragment-1 from rabbit skeletal muscle has not been determined, since no limiting value was observed in our experiments. If it is assumed that the values for the equilibrium constants are the same for the two species, then the maximum rate for the dissociation of skeletal-muscle actin–subfragment-1 can be estimated from the second-order rate constant ($K''_1 \cdot k''_{+2}$) as between 3500 and 7000 s^{-1}.

It is not clear why these transient processes should all be decreased by a similar extent, but a possible outcome is that the relative concentrations of the different intermediates in the hydrolytic cycle will be maintained at a constant value for myosins from different origins. Hence the free-energy changes, which depend on the relative concentrations of these intermediates, will be comparable for equivalent steps in the kinetic cycle. Since the intermediate states of the cross-bridges in the contractile cycle must be related to chemical intermediates in the kinetic cycle for actomyosin ATPase, the relative balance of the cross-bridge states may thus be preserved in different muscle types. Although these conclusions are based on an incomplete analysis of the cardiac subfragment-1 ATPase, further experiments with this protein and other myosins are needed to test their validity.

One of the reasons for undertaking this kinetic analysis of cardiac subfragment-1 was based on the chemical homogeneity of this protein as compared with subfragment-1 from fast-twitch muscle (see the introduction). The results have revealed a number of problems related to heterogeneity of a different kind. Cardiac myosin and its subfragment-1 readily undergo aggregation as has been shown by sedimentation-velocity centrifugation in the analytical ultracentrifuge, and also by the biphasic Eadie–Hofstee plots (Fig. 6) for the steady-state ATPase activity. Although at high ATP concentrations the catalytic-centre activity is the same as for column-purified myosin, the aggregates are not dispersed, as shown by ultracentrifugation. The presence of this inactive protein at low ATP concentrations gave low values for the active-site concentration as determined from the amplitude of the transient phase of phosphate liberation. However, after further purification of the myosin, transient amplitudes of 0.8 mol of P_i/mol of subfragment-1 sites were observed. These results suggest that both subfragment-1 'heads' are equally active, and proposals of an ATPase mechanism involving two different pathways (Tonomura, 1972) are not required to explain the kinetic data.

Heterogeneity based on protein aggregation and inactivation is easy to detect, but a further type of heterogeneity was encountered with cardiac subfragment-1 related to the kinetics of ATP binding. ATP binding is monitored by enhancement of the protein fluorescence, and although in most cases the kinetics of the process were the same for cardiac subfragment-1 and its parent myosin, there were a number of preparations giving abnormal binding characteristics (compare Figs. 2*a* and 2*b*). In these abnormal species, the rate constant for the isomerization process (k_{+2}) was decreased to about 5% of the expected value, and k_{-6} was similarly decreased, but the steady-state ATPase activity showed no similar impairment. Unfortunately we have been unable to demonstrate differences between the normal and abnormal subfragment-1 preparations based on structural analysis (e.g. by gel electrophoresis), nor was it possible to correlate the occurrence of the abnormal species with particular preparative procedures. Thus the cause of its production remains an enigma, though its existence serves as a warning that heterogeneity of subfragment-1 can occur which is undetectable by conventional steady-state analysis, by gel electrophoresis or other routine procedures.

Although cardiac subfragment-1 showed kinetic heterogeneity by these criteria in spite of its suggested chemical homogeneity (Weeds, 1975), the kinetic analyses described in the present paper suggest that the mechanism of ATP hydrolysis is similar to that proposed by Lymn & Taylor (1970, 1971) and by Bagshaw & Trentham (1974). Attempts to obtain subfragment-1 preparations that are homogeneous by both structural and kinetic criteria have been partially fulfilled by the isolation of two subfragment-1 isoenzymes from rabbit fast-twitch myosin by using chymotryptic cleavage (Weeds & Taylor, 1975). Preliminary results confirm that both isoenzymes have identical rates for the ATP binding (A. G. Weeds & R. S. Taylor, unpublished work) in addition to the steady-state data already published (Weeds & Taylor, 1975).

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