Kinetics and Regulation of the Myofibrillar Adenosine Triphosphatase

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1. The steady-state kinetic behaviour of the ATPase (adenosine triphosphatase) of intact myofibrils was studied in the presence of both high and low concentrations of Ca^{2+} (0.25 mM and <10 nM respectively). 2. Kinetic data were collected over the initial linear phase of the assay, which lasts for 20–60 s. To obtain consistent data we found it necessary to use either fresh myofibril preparations or preparations that had been stored in the presence of thiol compounds. 3. When assayed in the presence of 0.25 mM- Ca^{2+} , the myofibrillar ATPase obeyed Michaelis–Menten kinetics over the range 0.03–5.0 mM-MgATP (K_m $16 \pm 6 \mu\text{M}$, V $0.4 \pm 0.1 \mu\text{mol/min per mg}$). 4. At low Ca^{2+} concentrations (<10 nM) the myofibrillar ATPase displayed pronounced substrate inhibition, which was not observed at high Ca^{2+} concentrations. Thus increasing the MgATP concentration had the net effect of decreasing the ATPase activity at low Ca^{2+} relative to that at high Ca^{2+} . This preferential effect of MgATP on the low- Ca^{2+} ATPase may be important in Ca^{2+} control. 5. The substrate inhibition that was observed at low Ca^{2+} was lost on storage or thiol modification of the myofibrils. 6. Under physiological conditions (2 mM-MgATP, I 0.15, pH 7.0), the ATPase of fresh and thiol-protected myofibrils displayed approx. 100-fold activation by Ca^{2+} .

The myofibril-catalysed hydrolysis of MgATP has been the subject of vigorous investigation for about 25 years. Early studies were carried out on intact myofibrils (Weber & Hasselbach, 1954; Perry & Grey, 1956; Bendall, 1961), whereas more recent ones have dealt with the isolated protein components of the myofibrillar ATPase (Eisenberg & Moos, 1967, 1968, 1970; Eisenberg *et al.*, 1968; Lymn & Taylor, 1971; Bremel & Weber, 1972; White & Taylor, 1976). Among the model systems made up from the component proteins are reconstituted actomyosin, acto-heavy meromyosin and acto-subfragment 1. A comparison with intact myofibrils reveals a difference in ATPase behaviour. Although several investigators have reported that progress curves for assays of the myofibrillar ATPase are non-linear (Weber & Hasselbach, 1954; Bendall, 1961; Bowen *et al.*, 1963), this behaviour has not been reported for the model systems. Although substrate-activation of the myofibrillar ATPase has been observed by Bendall (1972), this type of behaviour, too, has not been reported for the model systems. It seems apparent that the model systems

Abbreviation and terms used: ATPase, adenosine triphosphatase. The term 'calcium sensitivity' is defined in the context of this paper as the ratio of the myofibrillar ATPase activity under activating conditions to that under relaxing conditions.

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are not completely equivalent to myofibrils and that further studies on intact myofibrils are required.

We have begun a re-examination of the ATPase of intact myofibrils with the intention of explaining some of these peculiarities in kinetic behaviour. As this work has progressed, it has become apparent that a number of variables exert a significant effect on the kinetics. Some of the important variables are: the method of myofibril preparation and storage, duration of the assay, the free Ca^{2+} concentration, the ionic strength, and the range of MgATP concentrations over which assays are carried out. Control over these variables leads to reproducible kinetic results which explain a number of discrepancies between earlier reports in the literature. These results lead to a better understanding of the Ca^{2+} -sensitivity of the myofibrillar ATPase.

Materials and Methods

Materials

Glutathione, *N*-ethylmaleimide, Na_2ATP (Sigma grade), EGTA and Triton X-100 were supplied by Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Dithiothreitol was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A. All other chemicals were of reagent grade.

Preparation of myofibrils

Rabbit myofibrils were prepared at 2°C by a method similar to that described by Perry & Grey

(1956). Fresh back muscle (100–150g) was minced and homogenized for 2min in a Waring Blendor with 10vol. (v/w) of 25mM-KCl/20mM-Tris, adjusted to pH7.0 with 1M-HCl at 2°C. The suspension was centrifuged for 15min at 1000g, the supernatant discarded, and the sediment re-homogenized in 200ml of the same buffer. The suspension was centrifuged in transparent 50ml tubes for 15min at 1000g, and the creamy top layer, which consisted of myofibrils free of connective tissue and cell debris, was carefully removed. After dispersion by glass hand-homogenizer in 1 litre of 0.1M-KCl/20mM-Tris/HCl, pH7.0, the suspension was centrifuged for 15min at 1000g. [For purposes of comparison, one preparation included 1% (w/w) Triton X-100 in the buffer at this step.] Redispersion and centrifugation were repeated two or three times; the sediment of washed myofibrils (20–50mg of protein/ml) was suspended in about 200ml of 0.1M-KCl/20mM-Tris/HCl, pH7.0, and the suspension was stored at 4°C in a stoppered bottle under an atmosphere of toluene. The original storage buffer was later modified by the inclusion of 10mM-dithiothreitol. Protein concentration was determined by the biuret method of Kabat & Mayer (1961) by using myofibrils which had been washed free of buffer with 0.1M-KCl. Preparations were occasionally examined by phase-contrast microscopy, and the inclusion of a 1% Triton X-100 wash step was found to have no effect on the appearance of the preparation.

ATPase assays

Assay conditions. Several considerations were applied to the choice of the ionic conditions for the assays; 4mM-Mg²⁺ was used to keep ATP in the form of MgATP and to keep the free Mg²⁺ concentration essentially constant. A moderate KCl concentration was selected to prevent non-specific electrostatic interactions, and a small amount of buffer was included to prevent undesirable pH transients. On the basis of these considerations and the work of Portzehl *et al.* (1969), the standard 'relaxing buffer' (10nM-Ca²⁺) consisted of 0.05M-KCl, 4mM-MgCl₂, 2mM-Tris/HCl and 1mM-EGTA, pH7.0 (adjusted at 25°C with 0.1M-HCl), with various concentrations of MgATP. The standard 'activating buffer' was of the same composition, but with the EGTA replaced by 0.25mM-CaCl₂. In studies on the effect of ionic strength, the standard buffers were modified by varying the KCl concentration.

MgATP substrate. Solutions of Na₂ATP (about 200mM) were adjusted to pH7.0 with 1M-KOH and the concentration was checked spectrophotometrically by using a molar absorption coefficient of 15400M⁻¹·cm⁻¹. Sufficient 0.5M-MgCl₂ was added to make the Mg²⁺ concentration equimolar with the ATP, the pH again adjusted to 7.0 with 1M-KOH, and the volume of the solution adjusted to give a

final concentration of 100mM-MgATP. Nucleotide concentration and pH were then re-checked. Solutions were stored at -20°C for up to 2 weeks without appreciable decomposition.

Apparatus. Assays were carried out with a Radiometer TTT 1c Titrator equipped with an SBR 2c Titrigraph, a syringe drive and a thermostatically controlled titration vessel with magnetic stirrer. An auxiliary 30rev./min motor was used on the Titrigraph chart drive, and titrant was delivered from precision gas-tight syringes equipped with a T-valve system for reloading under air-tight conditions. Assays were carried out at 25.0±0.1°C in a total volume of 10ml, pH7.0, with 0.01M-KOH titrant. A proportional band setting of 0.1 was used.

Calibrations. The feasibility of carrying out assays over a short period of time was evaluated in several ways. First, the mixing time of the vessel was checked by dye injection and found to be about 2s. Second, the assay slope over the 0–20s time interval was checked as a function of enzyme concentration. Calibration curves of slope versus enzyme concentration were linear up to a titrant delivery rate of 1.0μl/s in activating buffer (0.4μl/s in relaxing buffer). In this regard, it was observed that the response time of the apparatus varied with the buffer capacity of the assay solution that was used. For instance, the half-time for titration of a standard portion of acid in the relaxing buffer was roughly twice the half-time for activating buffer, which had only 30% as much buffer capacity. Thus the limiting ATPase velocity that could be followed was dependent on the assay buffer that was used. Experimental conditions were selected accordingly.

Titration standardization. Calibration of the assay was accomplished by titration of the protons liberated during hydrolysis of known amounts of ATP (0.1–1.0μmol) by amounts of myofibrils (20–50mg of protein) sufficient to give total hydrolysis within 2min.

Assay procedure. Myofibrils (0.8–1.0mg under activating conditions and 8–10mg under relaxing conditions) were pre-equilibrated with buffer in a total volume of 10ml, until the baseline remained steady. Assays were initiated by the addition of portions of concentrated MgATP (3–500μl, 100mM), the pH of which had been carefully adjusted to pH7.0 beforehand. Initial pH imbalances that arose occasionally were rapidly adjusted by manual titration with 0.1M-KOH or 0.1M-HCl from micro-meter syringes. The assay slope was obtained over the first 20–40s, during which period the response was linear. Assays were generally carried out in triplicate. Over the range of ATP concentrations used (0.03–5.0mM), less than 20% of the ATP was hydrolysed during the assay period. ATPase progress curves of extended duration were also obtained, in which up to 90% of the ATP was hydrolysed.

Modification with *N*-ethylmaleimide. Myofibrils (10–20 mg/ml) suspended in 0.1 M-KCl/10 mM-potassium phosphate buffer, pH 7.0, were added to an equal volume of the same buffer containing *N*-ethylmaleimide sufficient to give final concentrations from 0.05 to 1.2 mM. After 30 min the reaction was quenched by addition of 200 mM-dithiothreitol to give a final concentration of 4 mM. Controls were carried out to ensure that the quenching of *N*-ethylmaleimide with dithiothreitol was instantaneous. ATPase assays were carried out within 1 h of modification.

Data analysis. In the kinetic studies, the initial velocity of the ATPase at each substrate concentration was obtained as the mean of a triplicate set of assays. The precision of each set was estimated from the standard deviation. Velocities were plotted in double-reciprocal form, and linear regression (where applicable) was carried out by an unweighted least-squares procedure by the criteria of Cornish-Bowden (1976). K_m and V values were obtained for the fitted line, and the goodness of fit was determined by the correlation coefficient (r). Theoretical ATPase progress curves were obtained by the use of the integrated rate equation:

$$V \cdot t = P + K_m \ln[A/(A - P)]$$

(Walker & Schmidt, 1944), in which A represents the initial substrate concentration, P represents the product concentration at time t after the start of the assay, V represents the maximal velocity, and K_m represents the Michaelis constant. The known values of A and t and the calculated values of K_m and V from initial-velocity kinetic studies were inserted into the equation. Manual iteration was carried out on a programmable calculator until a value of P was found that produced convergence of both sides of the equation to within 0.01 %.

It was frequently observed that the mean values of assays differed more between sets than would be expected on the basis of the precision obtained within a particular set. Care was thus taken to design experiments so that comparative data could be obtained in the same set. The significance of effects caused by various treatments on the myofibrils was estimated by an independent two-tailed t test (Magar, 1972).

Results

ATPase kinetics of fresh myofibrils

It was observed, in agreement with earlier reports (Weber & Hasselbach, 1954; Bendall, 1961; Bowen *et al.*, 1963), that progress curves for the myofibrillar ATPase deviated from linearity over the early stages of the reaction. After an initial period of 20–60 s (dependent on ATP concentration), during which the progress curve was linear, the rate of ATP hydrolysis fell noticeably. Comparison of the

Fig. 1. Progress curve for the myofibrillar ATPase. Fresh myofibrils were assayed under the conditions described in the legend to Table 1. The initial MgATP concentration was 0.049 mM. Error bars indicate the s.d. for triplicate data. The theoretical assay curve was calculated from the K_m and V values determined from initial-rate studies of the same batch of myofibrils (see the Materials and Methods section).

observed and theoretical time courses (Fig. 1) revealed that the falling phase of the curve was accompanied by deviation from Michaelis–Menten behaviour. Thus the decline in rate could not be explained simply by the fall in substrate concentration that accompanied the assay. Similar decreases in rate were observed in the presence of both Ca^{2+} and EGTA, although the decline was not as abrupt with EGTA. By using the rates obtained over the initial linear period of the reaction in activating buffer (0.25 mM- CaCl_2), a linear double-reciprocal plot was obtained (Fig. 2a). The Michaelis–Menten kinetic parameters from several preparations of myofibrils studied (within 2 days of preparation) in the presence of activating buffer (see the Materials and Methods section) are presented in Table 1. Superprecipitation, i.e. a visible increase in the turbidity of the assay mixture, occurred at all ATP concentrations in this buffer, usually within 5–10 s of the start of the assay.

When myofibrils were assayed in relaxing buffer (i.e. with 1 mM-EGTA, see the Materials and Methods section), the double-reciprocal plot obtained from the initial-rate portion of the assay showed an upward curvature at higher substrate concentrations (Fig. 2b), indicating apparent substrate inhibition (Cleland, 1971). The precise form of this inhibition was somewhat variable. Some preparations ap-

proached a plateau of inhibition at high substrate concentration (Fig. 2*b*), whereas others were inhibited in asymptotic fashion (see Fig. 7, control). Treatment of the myofibrils with Triton X-100 left the inhibition pattern essentially unchanged, suggesting that residual fragments of sarcoplasmic reticulum were not responsible for this kinetic behavior (Orentlicher

et al., 1974). It may be that the exact form of inhibition is sensitive to subtle variations in preparative or assay techniques. Nevertheless, all the preparations fresh or thiol-protected myofibrils that we have examined (five) have exhibited the characteristic upward curvature of the double-reciprocal plot. Thus substrate inhibition appears to be an intrinsic feature of the myofibrillar ATPase under relaxing conditions.

ATPase kinetics of aged myofibrils

When myofibrils were assayed in relaxing buffer, the ATPase activity increased during the first 4–5 days of storage (Fig. 3). As the ATPase activity in activating buffer changed very little over the same period, this increase caused a decrease in calcium-sensitivity. Possible explanations for the modification of the calcium-sensitivity were partial proteolysis of troponin or tropomyosin (Ebashi & Ebashi, 1963) or thiol modification (Mueller, 1966; Parker & Kilbert, 1970; Hartshorne & Daniel, 1970; Daniel & Hartshorne, 1972, 1973). The former explanation was considered to be unlikely, since polyacrylamide-gel electrophoresis showed that the subunits of troponin and tropomyosin remained intact during storage.

The involvement of thiol groups with changes in the ATPase activity was investigated by dividing a fresh myofibril preparation into two portions, one of which was stored at 4°C in 0.1M-KCl/20mM-Tris/HCl, pH 7.0, and the other was stored in the same buffer to which 10mM-dithiothreitol had been added. Samples were withdrawn at intervals, and assays were carried out with 2mM-MgATP under the standard conditions. During storage in both the presence and the absence of dithiothreitol, the ATPase activity in activating buffer remained virtually constant (Fig. 3*a*). During storage in the presence of dithiothreitol, the ATPase activity in relaxing buffer also remained constant, but it irreversibly doubled within 3 days when dithiothreitol was omitted (Fig. 3*b*), suggesting that labile thiol groups were involved. This conclusion is consistent with the results of Daniel & Hartshorne (1972), whose studies on native actomyosin indicate that certain thiol groups of the myosin molecule are essential for Ca²⁺-sensitivity.

Fig. 2. Myofibrillar ATPase velocity of fresh myofibrils (a) Activating buffer. Triplicate assays of myofibrils were carried out as described in the legend to Table 1 (with 0.25mM-CaCl₂). Initial rates were obtained and plotted in double-reciprocal form. (b) Relaxing buffer. Myofibrils were assayed as described in (a) except that 1mM-EGTA was substituted for 0.25mM-CaCl₂ in the assay buffer. The distinction between ordinate values in (a) and (b) should be noted.

Table 1. Kinetic parameters for the myofibrillar ATPase in high-Ca²⁺ buffer

Assays were carried out at 25°C in 0.05M-KCl/4mM-MgCl₂/0.25mM-CaCl₂/2mM-Tris/HCl, pH 7.0, with MgATP concentrations ranging from 0.03 to 5.0mM.

	Myofibril batch no.	1	2	3	Average ± s.d.
K_m (μM)		22	15	11	16 ± 6
V (μmol/min per mg)		0.56	0.37	0.31	0.4 ± 0.1
r (correlation coefficient for linear regression on double-reciprocal plot)		0.98	0.97	0.99	—

Fig. 3. *Effect of aging on the ATPase of myofibrils* ATPase assays were carried out at the intervals indicated on myofibrils which had been stored at 4°C in 0.1 M-KCl/20 mM-Tris/HCl, pH 7.0. Single assays were performed under the standard conditions described in the Materials and Methods section. (a) Activating buffer (0.25 mM- CaCl_2); (b) relaxing buffer (1 mM-EGTA). ●, Myofibrils stored with 10 mM-dithiothreitol; ■, myofibrils from the same batch, stored without dithiothreitol. The distinction between ordinate values in (a) and (b) should be noted.

Fig. 4. *ATPase velocity of aged myofibrils assayed in activating buffer*

Myofibrils were stored in 0.1 M-KCl/20 mM-Tris/HCl, pH 7.0, at 4°C for 5 days before assay. Single assays were carried out as described in the legend to Table 1. (a) Myofibrils stored with 10 mM-dithiothreitol; (b) myofibrils stored without dithiothreitol.

The origins of this aging effect were explored by carrying out kinetic studies on myofibrils that had been stored at 4°C in 0.1 M-KCl/20 mM-Tris/HCl, pH 7.0, for 5 days after preparation. Fig. 4(a) shows that in activating buffer, the double-reciprocal plot for the ATPase of myofibrils stored with 10 mM-dithiothreitol was similar to that obtained with fresh myofibrils. For myofibrils stored in the absence of dithiothreitol, the double-reciprocal plot was apparently non-linear (Fig. 4b), although the large errors at low ATP concentrations tend to obscure this feature. This storage-induced curvature does resemble the apparent substrate activation observed by Bendall (1972), however. For the ATPase measured in relaxing buffer, storage of myofibrils with dithiothreitol preserved the upward curvature of the double-reciprocal plot (Fig. 5a), which was consistently observed for fresh preparations. Omission of dithiothreitol led to a linear double-reciprocal plot (Fig. 5b) and loss of substrate inhibition. Comparison of Figs. 5(a) and 5(b) reveals that the loss of upward curvature in the absence of dithiothreitol resulted in

slightly more than 100% activation at high ATP concentrations. This activation quantitatively explains the increase in ATPase that is observed on storage in the absence of reducing agent (Fig. 3). Since the ATPase in activating buffer is unaffected by aging, the activation (more strictly, removal of substrate inhibition of the relaxed ATPase) causes a decrease in the calcium sensitivity of aged myofibrils.

Results similar to those illustrated in Fig. 3 were obtained by using 5 mM-reduced glutathione instead of dithiothreitol, indicating that glutathione is equally useful for thiol protection. It was also found that myofibrils prepared with reduced glutathione tended to retain substrate inhibition in relaxing buffer, even after being washed free of glutathione with 0.1 M-KCl/20 mM-Tris/HCl, pH 7.0. By contrast, when 5 mM-ascorbic acid was used as the reducing agent, no protective effect was obtained.

To determine whether the mode of preparation had an initial effect on the ATPase activity measured under relaxing conditions, myofibrils were prepared in several ways and assayed within 1 day of preparation. Table 2 shows the results of these experiments. Analysis of the results by *t* test showed that ATPase activity under neither relaxing conditions

Fig. 5. ATPase velocity of aged myofibrils assayed in relaxing buffer

Myofibrils were stored in 0.1 M-KCl/20 mM-Tris/HCl, pH 7.0, at 4°C for 5 days before assay. Triplicate assays were carried out as described in the legend to Fig. 2(b). (a) Myofibrils stored with 10 mM-dithiothreitol. The dashed line indicates the extrapolation of the plot from low MgATP concentrations. (b) Myofibrils stored without dithiothreitol.

nor activating conditions was significantly affected by the inclusion of reducing agents during the preparative steps.

ATPase of *N*-ethylmaleimide-modified myofibrils

Chemical modification of myofibrillar thiol groups was carried out as a direct test for the relationship between thiol groups and ATPase behaviour. Fig. 6 shows that addition of an increasing concentration

Fig. 6. Effect of *N*-ethylmaleimide on the ATPase of myofibrils

Modification with *N*-ethylmaleimide was carried out as described in the Materials and Methods section. Single ATPase assays were carried out as described in the legend to Table 2. ■, ATPase in activating buffer (100% = 0.27 μmol of ATP cleaved/min per mg). ●, ATPase in relaxing buffer (100% = 0.043 μmol of ATP cleaved/min per mg).

Table 2. ATPase activity of myofibrils freshly prepared in the presence and absence of reducing agents

Assays were carried out at 25°C in 0.05 M-KCl/4 mM-MgCl₂/2 mM-Tris/HCl/2 mM-MgATP, pH 7.0. In addition, low-Ca²⁺ assays contained 1 mM-EGTA and high-Ca²⁺ assays contained 0.25 mM-CaCl₂. The difference between groups (A) and (B) was found to be insignificant at the 90% confidence level.

Type of ATPase activity	Myofibrils prepared without reducing agent (A)		Myofibrils prepared with reducing agent (B)			Overall average of (A) and (B) $\pm$ s.d.
			Batch 3	Batch 4	Batch 5	
	Batch 1	Batch 2	Dithiothreitol (0.5mm)	Glutathione (5mm)	Ascorbic acid (5mm)	
ATPase in high Ca ²⁺ (μ mol/min per mg)	0.63	0.586	0.40	0.475	0.54	0.53 $\pm$ 0.09
ATPase in low Ca ²⁺ (μ mol/min per mg)	0.03	0.04	0.023	0.038	0.035	0.033 $\pm$ 0.007
ATPase ratio (high Ca ²⁺ /low Ca ²⁺)	20.9	14.5	17.3	12.5	15.4	16 $\pm$ 3

Fig. 7. Effect of *N*-ethylmaleimide on the ATPase velocity of myofibrils in relaxing buffer

Myofibrils were modified with 0.5 mM *N*-ethylmaleimide as described in the Materials and Methods section. Triplicate assays were carried out in the relaxing buffer (1 mM-EGTA) described in the legend for Fig. 2(b). ○, *N*-Ethylmaleimide-treated myofibrils; □, control.

of *N*-ethylmaleimide produced activation of the ATPase in relaxing buffer without affecting the ATPase in activating buffer, an effect resembling that of aging. Sufficiently high concentrations of *N*-ethylmaleimide would presumably activate the ATPase in relaxing buffer by as much as 630%, corresponding to a total loss of Ca^{2+} -sensitivity. The kinetic data illustrated in Fig. 7 show that treatment with 0.5 mM *N*-ethylmaleimide produced qualitatively the same effect on the myofibrillar ATPase under relaxing conditions as did aging, namely the elimination of substrate inhibition at high MgATP concentration. *N*-Ethylmaleimide treatment also activated the ATPase at low MgATP concentration, indicating a possible modification of thiol groups in addition to those affected by aging.

ATPase activity and ionic strength

ATPase activities under relaxing and activating conditions were studied as a function of KCl concentration under the standard assay conditions ([MgATP] = 2 mM). Fig. 8 shows that the ATPase in activating buffer was little affected by ionic strength up to the physiological value (I in enzymic assays = [KCl] + 0.02), whereas the ATPase in relaxing buffer was markedly inhibited with increasing ionic strength.

Fig. 8. Influence of ionic strength on the ATPase of myofibrils

Duplicate assays were carried out according to the legend to Table 2, and average values of the ATPase activity were plotted. ■, ATPase in activating buffer (100% = 0.56 μmol of ATP cleaved/min per mg); ●, ATPase in relaxing buffer (100% = 0.048 μmol of ATP cleaved/min per mg); ----, ATPase ratio (relaxed/activated × 100%).

The minimum activity of the ATPase in relaxing buffer was approached at about 0.15 M-KCl, which corresponds to a total ionic strength of about 0.17. At this ionic strength the ATPase activity in relaxing buffer was decreased to 1% of that obtained in activating buffer (Fig. 8); thus a calcium sensitivity of 100 was obtained. The ATPase activities of myofibrils that had been stored with glutathione for up to 20 days exhibited similar calcium sensitivity and ionic-strength dependence.

Discussion

In this study of the myofibrillar ATPase, we have restricted our analysis to the steady-state conditions associated with the early linear phase of the assay. This phase, which lasts 20 s or longer, is distinct from the transient 'burst' of the actomyosin ATPase, which is complete within 50 ms and involves a single turnover per myosin molecule (Lymn & Taylor, 1971). Assuming that myofibrillar protein is about 50% myosin by weight (Potter, 1974), it is estimated that the early phase of the myofibrillar ATPase involves at least 25 turnovers of each myosin molecule (at low concentrations of Ca^{2+} and MgATP), and usually hundreds of turnovers (at high concentrations of Ca^{2+} and MgATP). This linear phase probably corresponds to the rapid initial rate of ATP hydro-

lysis which was originally observed by Weber & Hasselbach (1954) and carefully documented by Bendall (1961).

The linearity of the ATPase progress curve over this number of turnovers suggests that a steady state is attained within the first 2 s of the reaction and that the Michaelis–Menten equation is suitable for fitting the data. Examination of the ATPase activity of fresh myofibrils under activating conditions, i.e. in the presence of 0.25 mM-Ca^{2+} , provides further justification for this approach. The observation that the double-reciprocal plot is linear over a wide range of MgATP concentrations suggests that the factors that eventually lead to the curvature in the assays do not influence the kinetic behaviour in the initial-rate region. Preliminary inhibition studies show that insufficient product inhibition occurs to explain this curvature. Bendall (1961) has suggested that contraction of the myofibrils may contribute to this effect, and we have observed that contraction indeed occurs during the course of the assay. Native actomyosin, which undergoes superprecipitation under comparable conditions, displays the same sort of deviation from a linear assay curve (C. C. Goodno, unpublished work). By contrast, we are unaware of any reports of this type of behaviour for systems such as acto-heavy meromyosin and acto-subfragment 1, which fail to undergo superprecipitation or contraction.

The linear double-reciprocal plots for myofibrils under activating conditions yield values of K_m and V (Table 1) in the same range as those reported under similar conditions for myofibrils (Bendall, 1972), for native actomyosin (Levy & Ryan, 1966) and for acto-heavy meromyosin (Eisenberg & Moos, 1967). Although Bendall (1972) has obtained curved double-reciprocal plots, which seem to indicate substrate activation in myofibrils, our results suggest that this curvature may have arisen from a process related to the aging of the preparation. Hence the apparent substrate activation observed by Bendall (1972) may not be an intrinsic feature of myofibrils.

Under relaxing conditions ($[\text{Ca}^{2+}] < 10\text{ nM}$) the kinetic pattern is indicative of substrate inhibition in the region above $50\text{ }\mu\text{M-MgATP}$. Similar behaviour is shown by myofibrils (Weber, 1969), native actomyosin (Levy & Ryan, 1965; Chaplain, 1966) and calcium-regulated acto-subfragment 1 (Bremel *et al.*, 1973). This type of behaviour has not been reported for unregulated acto-subfragment 1 or acto-heavy meromyosin (Eisenberg & Moos, 1967, 1968), except at high ratios of subfragment 1 to actin monomers (Bremel *et al.*, 1973). Several possible explanations may be advanced for the substrate inhibition in myofibrils. The simplest explanation is that formation of dead-end complexes of the type enzyme $\cdot$ (MgATP) $_2$ may occur (Cornish-Bowden, 1976). This mechanism predicts that the double-reciprocal plot will make an

Fig. 9. Influence of MgATP concentration on Ca^{2+} -sensitivity

The ATPase ratios (activated/relaxed) were calculated from the data in Figs. 4(a) and 5(a).

asymptotic approach to the ordinate, but such a form of inhibition is not consistently observed for myofibrils (cf. Fig. 2b and Fig. 7, control). The second possibility is that co-operativity occurs in the regulated actin filament, as suggested by the 'rigor hypothesis' of Bremel & Weber (1972) and Bremel *et al.* (1973). Substrate inhibition of myofibrils, indeed, occurs over the same range of MgATP concentration over which dissociation of rigor linkages occurs in regulated acto-subfragment 1 (Bremel *et al.*, 1973). However, the formation of these linkages is not blocked by 0.1 mM-Ca^{2+} , which we find sufficient to block substrate inhibition in myofibrils. Thus it appears that the rigor hypothesis requires a certain amount of extrapolation to fit our kinetic data.

Yet another possible explanation is found in the co-operative behaviour of the myosin molecule. Nihei & Filipenko (1975) have observed biphasic kinetic behaviour of myosin and heavy meromyosin over the same range of MgATP concentration where we have observed substrate inhibition of myofibrils. Moreover, Highsmith (1978) has observed variations in the binding of heavy meromyosin to actin which are possibly indicative of negative co-operativity between myosin heads. Thus co-operative behaviour on the part of myosin may be a contributing factor in substrate inhibition.

The foregoing observations might be applied to the physiological condition as follows. The ATP concentration inside the muscle cell is in the range $2\text{--}5\text{ mM}$ (Long, 1961; Kraeling & Rampacek, 1977), and this ATP is maintained in the form of MgATP by an excess of Mg^{2+} (Long, 1961). Under relaxing conditions, where the Ca^{2+} concentration is low ($\leq 0.1\text{ }\mu\text{M}$), the MgATP concentration is more than adequate to decrease the myofibrillar ATPase

activity by substrate inhibition. On activation, the intracellular Ca^{2+} concentration rises to about $10\mu\text{M}$, and the substrate inhibition is relieved, since inhibition does not occur in the presence of an increased concentration of Ca^{2+} . The switching off of substrate inhibition results in almost a doubling of the the calcium activation of the ATPase (Fig. 9). Thus it would appear that substrate inhibition is integral to the overall mechanism of Ca^{2+} control.

When the effect of ionic strength is superimposed on substrate inhibition (at 2mM-MgATP), one observes a further enhancement of Ca^{2+} -sensitivity. Within the physiological range of ionic strength (comparable with about 0.15M-KCl), the activated ATPase (high Ca^{2+}) activity remains relatively constant, but the relaxed ATPase (low Ca^{2+}) falls to about 10% of its reference value at low ionic strength (Fig. 8). Thus the relaxed ATPase is suppressed to about 1% of the value of the activated ATPase (Ca^{2+} -sensitivity = 100), and the catalytic rate per myosin molecule turns out to be about 0.05s^{-1} . This value compares with reported values ranging from 0.08 to 0.11s^{-1} for isolated myosin (Lymn & Taylor, 1971; Bagshaw & Trentham, 1974; Schlüsselfeld, 1976). Thus, when Ca^{2+} concentration is low (i.e. under relaxing conditions), the physiological ionic strength and concentration of MgATP completely suppress actin activation of the myofibrillar myosin ATPase. The 100-fold activation that is observed under the same conditions may approach the physiological extent of activation in mammalian muscle. It is well known that the rate of oxygen consumption provides an estimate of the degree of activation *in vivo*, and Asmussen (1965) has observed that the oxygen consumption of athletes increases approx. 100-fold during maximal muscular exertion.

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