Elementary Processes of the Magnesium Ion-Dependent Adenosine Triphosphatase Activity of Heavy Meromyosin

A TRANSIENT KINETIC APPROACH TO THE STUDY OF KINASES AND ADENOSINE TRIPHOSPHATASES AND A COLORIMETRIC INORGANIC PHOSPHATE ASSAY *IN SITU*

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Transient kinetic studies of Mg^{2+} -dependent heavy-meromyosin ATPase (adenosine triphosphatase) were done by monitoring the release of both ADP and P_i into the reaction medium by using linked assay systems. The release of P_i was monitored by its quantitative transfer to ADP, with concomitant reduction of NAD^+ in the presence of D-glyceraldehyde 3-phosphate, D-glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase. The dissociation rates of the products, ADP and P_i , from heavy meromyosin were shown to be faster than the rate-controlling process, which occurs after the initial bond cleavage of ATP. The chromophoric ATP analogue, 6-mercapto-9- β -D-ribofuranosylpurine 5'-triphosphate (thioATP) was used as a substrate and spectral changes associated with a single turnover of heavy meromyosin could be assigned to elementary processes of the mechanism. It was shown that the dissociation rate of thioADP was not the rate-controlling process of the thioATPase, whose catalytic-centre activity was 7.6 times that of the ATPase at pH8. The dissociation rate of ADP from heavy meromyosin was measured by using thioATP as displacing agent and was found to be $2.3s^{-1}$, which is about 50 times the catalytic-centre activity of the ATPase at pH8. Transient kinetic studies with chromophoric adenosine phosphate analogues have general application for kinases and ATPases both in characterizing the chemical states of the intermediates and in delineating the elementary processes of the enzyme mechanism.

The elucidation of the mechanism of the actin-activated Mg^{2+} -dependent ATPase* of myosin is essential for understanding the dynamics of the contractile system at the molecular level. Both transient and steady-state kinetic methods have been used to analyse the ATPase of myosin and its proteolytic subfragments (Eisenberg & Moos, 1970; Lymn & Taylor, 1970, and references therein). The experiments described below were designed to obtain more direct insight into this mechanism by using two different approaches. First, the rates of release of ADP and P_i produced from ATP during the first turnover of the heavy-meromyosin Mg^{2+} -dependent ATPase were compared with the lower limit of the dissociation rates of ADP and P_i from heavy meromyosin by using linked assay systems. The actin-activated ATPase is too rapid to be studied by this method. As a second approach, single turnovers

*Abbreviations: ATPase, adenosine triphosphatase; thioATP, 6-mercapto-9- β -D-ribofuranosylpurine 5'-triphosphate.

of heavy meromyosin have been examined by using the chromophoric ATP analogue, thioATP, whose potential for analysing many aspects of the actomyosin system has been described (Murphy & Morales, 1970; Tokiwa & Morales, 1971). Because there is a spectral change when thioATP interacts with heavy meromyosin it is possible to investigate a single turnover of the heavy-meromyosin ATPase and so analyse its elementary processes. This method of studying the kinetics of an enzyme in which there is a spectral change on interaction between the enzyme and either substrate or product, but no spectral change on conversion of substrate into product, has great potential for the study of kinases and ATPases.

Experimental

Proteins

Heavy meromyosin. Myosin was extracted from rabbit back and leg muscles and tryptic heavy meromyosin was prepared as described previously (Lowey

et al., 1969). The heavy meromyosin was concentrated by precipitation with ammonium sulphate at 55% saturation, and the precipitate was dissolved in dithiothreitol (0.1 mM) and triethanolamine (200 mM) adjusted to pH 8.0 with HCl. Ammonium sulphate was removed by chromatography on Sephadex G-50 (coarse grade) with the same pH 8.0 buffer. The overall yield of heavy meromyosin from myosin was 20–30%. Protein concentrations were determined by using $E_{280}^{1\%} = 6.47 \text{ cm}^{-1}$ (Young *et al.*, 1965), and small corrections for light-scattering made by measuring the extinction at wavelengths up to 400 nm and extrapolating back to 280 nm. Molar concentrations of subfragment 1 heads are quoted as μM -sites alongside the concentrations (mg/ml) of heavy meromyosin on the basis that each heavy meromyosin molecule (mol.wt. 340000) contains two subfragment 1 heads (Lowey *et al.*, 1969). Glass-distilled water was used throughout, and heavy meromyosin solutions always had dithiothreitol present.

Sources of enzymes. Pyruvate kinase from rabbit muscle was prepared by the method of Tietz & Ochoa (1962). Phosphoglycerate kinase from yeast (Scopes, 1971) and lactate dehydrogenase from pig muscle (Stinson & Gutfreund, 1971) were given by Dr. R. A. Stinson. Hexokinase and glucose 6-phosphate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). D-Glyceraldehyde 3-phosphate dehydrogenase from pig muscle (given by Miss P. J. Harrigan) was prepared by repeated crystallizations from EDTA (5 mM), thioglycolic acid (5 mM) and ammonium sulphate, and chromatography on Sephadex G-75 to remove traces of haemoglobin and myoglobin. NAD^+ (1 mM) was present for the final crystallization. The enzyme catalysed the reduction of $170 \mu\text{mol}$ of NAD^+ /min per mg measured by the assay system described by Trentham (1971a). For the phosphate assay experiments the crystalline enzyme was dissolved in EDTA (5 mM) at pH 7.0 and chromatographed in this solvent on Sephadex G-25 (fine grade) to remove ammonium sulphate and thioglycolic acid. The pig muscle enzyme was particularly suitable for the P_i assay since the essential thiol group appeared to be less susceptible to oxidation than other sources of the dehydrogenase so that the assays could be performed in the absence of a protecting thiol that might possibly form an adduct with D-glyceraldehyde 3-phosphate, which was also a reagent of the phosphate assay medium.

Reagents

6-Mercapto-9- β -D-ribofuranosylpurine 5'-triphosphate (thioATP). The monophosphate was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). It was also prepared from the parent

nucleoside by the method used for formycin 5'-phosphate synthesis except that the acid treatment was done under reflux at pH 3.4 instead of pH 2 (Ward *et al.*, 1969b), and purified on DEAE-cellulose [conditions described by Murphy *et al.* (1970) for thioATP synthesis; method 2]. The triphosphate was prepared by both methods described by Murphy *et al.* (1970); in each case the DEAE-cellulose column was used for purification. After removal of HCO_3^- by repeated evaporation thioATP was stored frozen at -15°C . ThioATP was assayed from the extinction change at 340 nm when NADP^+ was reduced in the presence of glucose, hexokinase and glucose 6-phosphate dehydrogenase, and from its absorption spectrum. In a high-voltage electrophoresis system at pH 3.5 (pyridine acetate buffer) it appeared as a single spot when the supporting medium (Whatman no. 1 paper) was sprayed for phosphate (Brooks *et al.*, 1959) and *cis*-diol (Baddiley *et al.*, 1956). Electrophoresis at pH 9.2 (borate buffer) showed the absence of any 2'- or 3'-phosphate substitution. The concentration recorded in the experiments was measured from NADP^+ reduction and was 93% of that determined by the absorption measurement. Our control experiments together with Murphy & Morales' (1970) observation that the monophosphate has no measurable interaction with myosin suggests that an impurity, if present, had no effect. ThioATP solutions always had dithiothreitol present.

NAD^+ (grade II; C. F. Boehringer und Soehne, G.m.b.H.) was further purified for the phosphate assay experiments by chromatography on a Dowex 1 (Cl^- form) column. NAD^+ was eluted with 10 mM-HCl. The NAD^+ solution was concentrated by freeze-drying and stored frozen at -15°C . The purification removed an impurity with a relatively high E_{340} . D-Glyceraldehyde 3-phosphate was prepared as its calcium salt by the method of Szewczuk *et al.* (1961). Calcium was exchanged for protons by using Dowex 50 (H^+ form) cation-exchange resin. D-Glyceraldehyde 3-phosphate as prepared by the method of Szewczuk *et al.* (1961) contained about 2% (molar ratio) of P_i , and we were unable to purify it further by fractional precipitation of P_i as magnesium ammonium phosphate. All other reagents were A.R. grade where possible.

Spectroscopic measurements

The extinctions for protein concentration and P_i assay (Fig. 1) were measured in a 1 cm light-path cuvette of a Zeiss PMQ II spectrophotometer with a Servoscribe RE 511 recorder (Kelvin Electronics Co.). Measurements of ATPase and thioATPase activity by using the ADP linked assay system were made in the 1 cm light-path thermostatically controlled cuvette in a Uvispek H700 spectrophotometer.

meter (Hilger and Watts Ltd.) with a Gilford recording attachment and a Sunvic recorder (type 10S).

Spectroscopic methods with the stopped-flow apparatus were described by Trentham (1971a). Reactions were studied at room temperature ($21 \pm 2^\circ\text{C}$) with careful control of temperature when relative rates were measured (see, e.g., Figs. 7a and 7b). For the P_i -release experiments (Figs. 2a, 2b and 2c) the syringe containing the P_i assay medium had 5.5 times the cross-sectional area of the other syringe. This was to forestall possible effects caused by large concentration changes in the assay system, but subsequent experiments showed that these precautions were unnecessary and the experiments can be performed equally well with syringes of equal diameter. In the legends to the figures and the text the reagent concentrations recorded are those of the reaction chamber except where otherwise stated. The 100% transmission line does not in general reflect the transmission of the solutions before mixing.

E_{340} of $6.22\text{mm}^{-1}\cdot\text{cm}^{-1}$ was used for NADH and NADPH (Horecker & Kornberg, 1948).

Results and Discussion

Transient kinetics studied by linked assay systems

Production of free ADP was followed spectrophotometrically from the oxidation of NADH in a solution containing NADH, phosphoenolpyruvate, Mg^{2+} , pyruvate kinase and lactate dehydrogenase. Production of free P_i was measured from the reduction of NAD^+ in a solution containing NAD^+ , D-glyceraldehyde 3-phosphate, ADP, D-glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase. The difficulty in operating these linked assay systems for transient kinetic studies is that the rates of disappearance or formation of NADH are slow compared with the maximum turnover rates of the linking enzymes because of the high K_m values of some of the substrates, in particular pyruvate in the first system (Pesce *et al.*, 1964; Stinson & Gutfreund, 1971) and P_i in the second (Furfine & Velick, 1965). The ADP assay system has been described (Imamura *et al.*, 1966). The P_i assay system for transient kinetic studies is novel. The equations describing these linked assays are:

Quantitative conversion (>99%) of NAD^+ into NADH occurred when portions of P_i were added to the linked assay system (Fig. 1). There are two requirements for the P_i linked assay system in studying the first turnover of the ATPase; it must be sensitive to P_i when $[\text{P}_i] \ll [\text{heavy meromyosin sites}]$ and at these low P_i concentrations it must be capable of monitoring P_i production more rapidly than the turnover rate of heavy meromyosin. The two factors that limited the rate of NADH production were the D-glyceraldehyde 3-phosphate dehydrogenase and D-glyceraldehyde 3-phosphate concentrations. High concentrations of D-glyceraldehyde 3-phosphate are needed, first to displace the overall equilibrium favourably and secondly because only 4% of D-glyceraldehyde 3-phosphate is in the catalytically active aldehyde form and the dehydration of the inactive diol to the aldehyde is a relatively slow process (Trentham *et al.*, 1969). Fig. 2(a) shows the rate of NADH production when $10\mu\text{M}\text{-P}_i$ was added to the assay system.

When heavy meromyosin ($10\mu\text{M}\text{-sites}$) was mixed with the phosphate assay medium and about $50\mu\text{M}\text{-ATP}$ (generated as a result of P_i contamination in the reagents), no rapid transient phase of P_i release was observed (Fig. 2b). In a control experiment a pool of $10\mu\text{M}\text{-P}_i$ was generated by incubating heavy meromyosin with $10\mu\text{M}\text{-ATP}$ and this solution was then mixed with the assay medium. As expected, the presence of free P_i led to rapid NADH production, followed by the steady-state rate (Fig. 2c, Table 1). Thus Fig. 2(b) shows that the rate-determining process of the Mg^{2+} -dependent ATPase of heavy meromyosin is either a process before P_i release in the reaction pathway or P_i release itself (experiments described below exclude the latter possibility) and that this rate-determining step cannot be determined solely by ADP dissociation from an ADP-heavy meromyosin complex.

Quantitative conversion of NADH into NAD^+ is well established for the ADP assay system. Fig. 3(a) shows that the rate of NADH disappearance is sufficiently rapid to fulfil the requirements outlined for the linked assay system. When heavy meromyosin was mixed with the assay medium and ATP no rapid transient phase was observed (Fig. 3b) which shows that the rate-determining step precedes ADP release. ADP release itself is excluded, as shown

Fig. 1. Dependence of NADH produced on the concentration of P_i

P_i was added in portions to a solution of D-glyceraldehyde 3-phosphate (0.80 mM), NAD^+ (2.0 mM), $MgCl_2$ (3.3 mM), ADP (0.40 mM), D-glyceraldehyde 3-phosphate dehydrogenase (0.15 mg/ml), phosphoglycerate kinase (2.8 μg/ml) and triethanolamine (80 mM) adjusted with HCl to give a final pH of 8.1 at 25°C.

above (Fig. 2b). In control experiments ADP (either added as free ADP or generated *in situ* from ATP) and an excess of heavy meromyosin were mixed with the assay system. In both cases the presence of ADP led to rapid NADH disappearance followed by the steady-state rate (Fig. 3c, Table 1). Repeating these experiments in the presence of 1 mM- P_i gave identical reaction profiles for NADH disappearance which, together with the lack of P_i inhibition of the ATPase activity, shows that P_i dissociation is not the rate-limiting process.

Comparison of Figs. 3(b) and 3(c) shows that the amplitude of the transient phase in the presence of heavy meromyosin is 80% of the amplitude in its absence. (The amplitude of the transient phase is the extinction difference between the observed trace and the steady-state part of the trace, both extrapolated back to zero time.) Thus at least 80% of the ADP is available for rapid utilization by the assay system. In fact, since the initial rate is only 10 times the steady-state rate, more than 80% of the ADP is available, although the exact amount cannot be calculated because it depends upon a precise description of the kinetic model (Gutfreund, 1965; Trentham, 1971b). Equilibrium binding studies suggest that less than 30% of the ADP is free in solution under these conditions (Morita, 1967; Lowey & Luck, 1969). There-

Fig. 2. Assay of P_i measured by NADH production in a linked assay system recorded at 340 nm at pH 8.0 in a stopped-flow apparatus

One syringe contained in (a) P_i (10 μM: reaction chamber concentrations), (b) heavy meromyosin (1.58 mg/ml, 9.3 μM-sites) [HMM-ATPase], and (c) heavy meromyosin (2.37 mg/ml, 14 μM-sites) and ADP and P_i (both 10.2 μM, generated from ATP) [ADP, P_i , HMM-ATPase] and in all the traces the other syringe contained D-glyceraldehyde 3-phosphate (0.68 mM), NAD^+ (1.7 mM), $MgCl_2$ (2.8 mM), ADP (0.34 mM), D-glyceraldehyde 3-phosphate dehydrogenase (0.38 mg/ml) and phosphoglycerate kinase (2.4 μg/ml) [ATP, P_i assay]. Both syringes contained triethanolamine (80 mM) adjusted to pH 8.0 with HCl.

Fig. 3. Assay of ADP measured by NADH disappearance in a linked assay system recorded at 340 nm and at pH 8.0 in a stopped-flow apparatus

(a) Reaction of ADP with the linked assay system. One syringe contained ADP ($5 \mu\text{M}$; reaction-chamber concentrations) and the other syringe the ADP assay medium, NADH (0.14 mM), phosphoenolpyruvate (2.8 mM), tris (37 mM , pH 8.0), pyruvate kinase (1 mg/ml) and lactate dehydrogenase (0.5 mg/ml) [ADP assay]. Both syringes contained MgCl_2 (3.7 mM). KCl (37 mM) and triethanolamine (37 mM) adjusted to pH 8.0 with HCl. The horizontal lower trace was recorded a few seconds after the end of the reaction. (b) Reaction of heavy meromyosin with ATP in the

presence of the linked assay system. One syringe contained heavy meromyosin (2.5 mg/ml , $15 \mu\text{M}$ -sites) and KCl (25 mM) [HMM-ATPase], and the other ATP ($50 \mu\text{M}$), NADH ($50 \mu\text{M}$), phosphoenolpyruvate (2.8 mM), MgCl_2 (10 mM), pyruvate kinase (1 mg/ml) and lactate dehydrogenase (0.4 mg/ml) [ATP, ADP assay]. Both syringes contained tris (50 mM) adjusted to pH 8.0 with HCl. The record of (c) was obtained as for (a) except that heavy meromyosin (5.3 mg/ml , $31 \mu\text{M}$ -sites) was present in the ADP syringe [ADP, HMM-ATPase]. The upper trace represents the initial rapid conversion of ADP into ATP with concomitant NADH disappearance. ATP is then continuously cycled at the steady-state rate (as shown in the lower traces) until all the NADH is oxidized.

Fig. 4. Hydrolysis of thioATP catalysed by heavy meromyosin at 25°C in triethanolamine (100 mM) adjusted to pH 7.9 with HCl

From a plot of velocity (v) against $v/[\text{thioATP}]$, the steady-state kinetic parameters V_{max} (from intercept on the ordinate axis) and K_m (negative gradient) are $0.16 \mu\text{mol}$ of thioATP hydrolysed/min per mg of protein and $5.7 \mu\text{M}$ respectively. The reaction mixture contained NADH ($74 \mu\text{M}$), phosphoenolpyruvate (0.37 mM), pyruvate kinase (0.1 mg/ml), lactate dehydrogenase (0.1 mg/ml), MgCl_2 (9 mM), KCl (70 mM) and heavy meromyosin (0.35 mg/ml , $2.1 \mu\text{M}$ -sites). The decrease in E_{340} was measured and the reaction was initiated by addition of thioATP to a final concentration of $2.4\text{--}106 \mu\text{M}$.

Table 1. Comparison of Mg^{2+} -dependent ATPase and thioATPase activities of heavy meromyosin studied in the present work and by others

The values in rows 4 and 5 are those of Lowey *et al.* (1969) and Eisenberg & Moos (1970) respectively. Catalytic-centre activities in rows 1, 3, 4, 5 and 6 are calculated on the basis that both subfragment 1 heads per heavy meromyosin molecule (mol.wt. 340000) (Lowey *et al.*, 1969) are simultaneously active (Tokiwa & Morales, 1971; Spudich & Watt, 1971). These values should be doubled if only one site per molecule of heavy meromyosin is active at any instant. In row 2 the catalytic-centre activity is calculated from the initial ADP concentration, since it is less than the heavy meromyosin concentration. Since not all the nucleotide will exist as a complex with heavy meromyosin in the steady state (a small fraction will be free in solution or in a complex with pyruvate kinase), this value will tend to be a lower limit. In rows 7 and 8 the catalytic-centre activities are calculated from the exponential decay rates. The catalytic-centre activity of heavy-meromyosin Mg^{2+} -dependent ATPase varies with reaction medium and temperature (Taylor *et al.*, 1970) so that some variation in the activities tabulated is to be expected.

Source of values	Substrate	Catalytic-centre activity (s^{-1})	Temperature ($^{\circ}C$)	Substrate hydrolysed ($\mu\text{mol/min per mg of protein}$)
1 Figs. 2(b) and 2(c)	ATP	0.041	21	0.0145
2 Fig. 3(c)	ATP	0.041	21	
3 Conditions of Fig. 4	ATP	0.058	25	0.0205
4 Actin-activated, pH 7.9	ATP	7	25	2.4
5 Actin-activated, pH 7	ATP	11		4
6 Fig. 4	ThioATP	0.44	25	0.16
7 Fig. 5	ThioATP	0.44	21	
8 Fig. 6(b)	ThioATP	0.55	21	

fore ADP appears to dissociate from its complex with heavy meromyosin more rapidly than the turnover rate of the Mg^{2+} -dependent ATPase, which supports the conclusions above.

Transient kinetics studied by using thioATP

Analysis of the mechanism of an enzyme-catalysed reaction by steady-state kinetic methods when there is no spectral difference between the substrate and product is difficult except by recourse to linked assay or sampling techniques. This is typically the situation with kinases and ATPases. However, provided that the enzyme-substrate or enzyme-product complexes have spectra different from the sum of the spectra of enzyme and either substrate or product, then the transients of the enzyme-catalysed reaction can be observed, and in favourable situations the elementary processes can be resolved along the time axis. This has been discussed in a review on transient kinetic methods by Gutfreund (1971) and demonstrated by using proteolytic enzymes (Barman & Gutfreund, 1966).

For heavy meromyosin the purine moiety of the substrate provides scope for introducing the chromophore. With absorption optics the largest and most readily monitored signals are likely to be introduced when a purine moiety contains an ionizing group with pK near neutrality in which the absorption band of either the acid or its conjugate base has its maximum

clear of the protein absorption band at greater than 300nm. One such analogue is 6-mercapto-9- β -ribofuranosylpurine and transient kinetic studies of the heavy-meromyosin Mg^{2+} -dependent ATPase were done by using 6-mercapto-9- β -ribofuranosylpurine 5'-triphosphate (thioATP) and the 5'-diphosphate (thioADP). ThioATP causes contraction of both myofibrils and glycerinated muscle fibres; it interacts with myosin under mild oxidizing conditions and is postulated to form a unique disulphide bridge between each myosin subfragment 1 head and the adenosine analogue (Murphy & Morales, 1970).

Since thioADP is a substrate for pyruvate kinase the K_m for thioATP at pH 8 to heavy meromyosin could be measured and was $5.7\mu\text{M}$ (Fig. 4). The catalytic-centre activity of the Mg^{2+} -dependent heavy-meromyosin thioATPase was $0.44s^{-1}$ and of the ATPase was $0.058s^{-1}$ in identical assay conditions, assuming that all the myosin subfragment 1 heads had simultaneously active sites.

Extinction coefficient changes of the order of $10^4\text{M}^{-1}\cdot\text{cm}^{-1}$ at 330nm are associated with the ionization at $pK 8.1$ on the purine moiety of thioATP (eqn. 1). The half-time of this extinction change at pH 8 was shown to be less than the time-resolution of the stopped-flow machine (dead time 3ms).

When thioATP was mixed with an excess of heavy-meromyosin active sites, the reaction profile displayed many notable features (Fig. 5). The profile was in two distinct exponential phases, which were well resolved. The observed rate constants for the two phases were

Fig. 6. Displacement of thioADP and 'thioATP' from heavy meromyosin by ATP

(a) Stopped-flow spectrophotometric record at 330 nm of the reaction of an equilibrium mixture of heavy meromyosin and thioADP with a large excess of ATP. One syringe contained heavy meromyosin (2.6 mg/ml, 15 μ M-sites; reaction-chamber concentrations) and thioADP (15 μ M, generated *in situ* from thioATP [thioADP, HMM-ATPase,] and the other ATP (1 mM). Both syringes contained KCl (80 mM), MgCl₂ (10 mM) and triethanolamine (100 mM) adjusted to pH 8.0 with HCl. (b), as (a) except that the heavy-meromyosin syringe contained thioATP (5 μ M) ['thioATP', HMM-ATPase] that was continuously regenerated by phosphoenolpyruvate (0.5 mM) and pyruvate kinase (0.5 mg/ml).

and 50 μ M-ADP were mixed with heavy meromyosin (28 μ M-sites), the rate of the spectral change (24 s⁻¹) was similar to that observed in the absence of ADP. Thus the dissociation rate of ADP is approx. 2.3 s⁻¹, which is 50 times the turnover rate of the Mg²⁺-dependent ATPase.

The conclusion that the rate constant of ADP dissociation from heavy meromyosin is faster than the Mg²⁺-dependent ATPase turnover rate differs from that of Taylor *et al.* (1970), which was deduced from their rapid gel-filtration experiments. The transport of interacting systems by using zonal

Fig. 7. Displacement of ADP from heavy meromyosin by thioATP

(a). Stopped-flow spectrophotometric record at 340 nm (the E_{330} was large) of the reaction of an equilibrium mixture of heavy meromyosin and ADP with thioATP. One syringe contained heavy meromyosin (4.8 mg/ml, 28 μ M-sites; reaction-chamber concentrations) and ADP (50 μ M, generated *in situ* from ATP) [ADP, P_i, HMM-ATPase,] and the other thioATP (75 μ M). Both syringes contained KCl (80 mM), MgCl₂ (10 mM) and triethanolamine (200 mM) adjusted to pH 8.0 with HCl. (b), as (a) except that ADP was omitted from the heavy-meromyosin syringe [HMM-ATPase].

methods is complex and this makes analysis of their results very difficult (Nichol *et al.*, 1964). Moreover, other of their experiments suggested that ADP produced by catalytic hydrolysis of ATP was apparently being released more slowly than the turnover rate.

Conclusions

The transient kinetic experiments by Taylor and his co-workers (Lymn & Taylor, 1970, and references therein) provide an excellent framework for deducing the mechanism of heavy-meromyosin ATPase action and ultimately relating this enzyme mechanism to the molecular events in muscle contraction. As they have been careful to point out, the mechanisms

that they proposed are the simplest consistent with available experimental evidence. Their mechanism for heavy-meromyosin Mg^{2+} -dependent ATPase has been expanded to include the results of these experiments:

where E represents heavy meromyosin, $E \cdot ATP$, $E^* \cdot ADP \cdot P_i$, $E \cdot ADP \cdot P_i$ and $E \cdot ADP$ are enzyme-substrate and enzyme-product complexes, and k_{+1} etc. are rate constants. Estimates of the rate constants at pH 8 and room temperature are k_{+1} (or $k_{+1}k_{+2}/k_{-1}$), $2.4 \times 10^6 M^{-1} \cdot s^{-1}$; $k_{+2} > 125 s^{-1}$ (Lynn & Taylor, 1970); k_{+3} , $0.04 s^{-1}$; k_{+5} , $2.3 s^{-1}$.

It is stressed that the estimate of k_{+5} is based on a single type of experiment, and analysis by other methods (e.g. relaxation techniques, use of other chromophoric reagents) is required before this rate constant can be confidently accepted. The transient experiments described here and the lack of recorded inhibition by P_i of ATPase action suggest that k_{+4} is rapid. The reverse rate constants should be susceptible to analysis by rapid-reaction and isotope-exchange techniques but in the chemical bond-breaking/conformation-change steps they are likely to be much less than the forward rate constants. Whether the process involved in the $E^* \cdot ADP \cdot P_i$ -to- $E \cdot ADP \cdot P_i$ transformation is covalent-bond breaking and/or conformation change is not clear. If it is the latter, one process of the mechanism must generate the E^* conformation. Morita's (1967) spectral data suggest that this could be identified with the $E \cdot ATP$ -to- $E^* \cdot ADP \cdot P_i$ transformation.

If this mechanism for the Mg^{2+} -dependent heavy-meromyosin ATPase is correct, then, in the relaxed state of muscle, where the ATP concentration is 3 orders of magnitude greater than the K_m of ATP, the predominant form of myosin must be $E^* \cdot ADP \cdot P_i$ and the turnover rate of the ATPase $\approx k_{+3}$. The question therefore arises whether bond cleavage is required to produce the relaxed state as characterized by the X-ray-diffraction patterns (Huxley, 1969), since the binding of ATP alone is sufficient to dissociate actomyosin (Lynn & Taylor, 1971; Yount *et al.*, 1971). Further kinetic and X-ray studies with for example 5'-adenylyl imidodiphosphate (Yount *et al.*, 1971) are required to answer this question. It is noteworthy that actin activation appears to accelerate at least two processes of the myosin ATPase, since the rate of the actin-activated Mg^{2+} -dependent ATPase (Table 1) is greater than both k_{+3} and k_{+5} .

The fact that a spectral change is induced by the

thioADP-heavy meromyosin interaction, which perhaps parallels that observed when ADP and heavy meromyosin are mixed (Morita, 1967), suggests that this interaction is important, as is shown by the capacity of ADP to promote oxygen exchange between P_i and water in the presence of heavy meromyosin (Swanson & Yount, 1965).

The transient kinetic approach provides a method for analysing the mechanism of kinases and ATPases, in particular the actin-activated ATPase of myosin. In addition to elucidation of the elementary processes, characterization of the chemical states of the intermediates is possible, which will reflect the protein structure. It is likely that 2-amino-6-mercapto-9- β -ribofuranosylpurine analogues will be particularly useful in this respect, since the absorption band is well removed from that of protein (Fox *et al.*, 1958) and they should quench protein fluorescence. The fluorescent properties of various adenosine analogues that are suitable for such studies have been described (Ward *et al.*, 1969a).

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