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The Magnesium Ion-Dependent Adenosine Triphosphatase of Myosin

TWO-STEP PROCESSES OF ADENOSINE TRIPHOSPHATE ASSOCIATION AND ADENOSINE DIPHOSPHATE DISSOCIATION

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The kinetics of protein-fluorescence change when rabbit skeletal myosin subfragment 1 is mixed with ATP or adenosine 5′-(3-thiotriphosphate) in the presence of Mg^{2+} are incompatible with a simple bimolecular association process. A substrate-induced conformation change with $\Delta G^0 < -24 kJ \cdot mol^{-1}$ (i.e. ΔG^0 could be more negative) at pH 8 and 21°C is proposed as the additional step in the binding of ATP. The postulated binding mechanism is $M + ATP \rightleftharpoons M \cdot ATP \rightleftharpoons M^* \cdot ATP$, where the association constant for the first step, K_1, is $4.5 \times 10^3 M^{-1}$ at I 0.14M and the rate of isomerization is $400 s^{-1}$. In the presence of Mg^{2+}, ADP binds in a similar fashion to ATP, the rate of the conformation change also being $400 s^{-1}$, but with ΔG^0 for that process being $-14 kJ \cdot mol^{-1}$. The effect of increasing ionic strength is to decrease K_1, the kinetics of the conformation change being essentially unaltered. Alternative schemes involving a two-step binding process for ATP to subfragment 1 are possible. These are not excluded by the experimental results, although they are perhaps less likely because they imply uncharacteristically slow bimolecular association rate constants.

Recently much attention has been given to characterizing by structural and kinetic means the intermediates associated with the Mg^{2+}-dependent ATPase‡ of myosin and its proteolytic subfragments (Tonomura, 1972; Weber & Murray, 1973). Besides the intrinsic interest in the ATPase mechanism further objectives of these studies are to provide a basis for analysis of the actomyosin ATPase mechanism and to characterize the cross-bridge positions of myosin of relaxed and contracting muscle. By rapid-reaction-kinetics methods four intermediates have been characterized during the catalysis of ATP hydrolysis by heavy meromyosin (Trentham *et al.*, 1972). The work described in the present paper is particularly concerned with the processes involved in the binding of the substrate ATP and with the release of product ADP, although the latter is dealt with more fully by Bagshaw & Trentham (1974).

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‡ Abbreviations: ATPase, adenosine 5′-triphosphatase; ATP(β,γ-NH), 5′-adenylylimidodiphosphate; ATP(γS), adenosine 5′-(3-thiotriphosphate); ATP(α,β-CH_2), α,β-methylene-adenosine 5′-triphosphate.

The protein used for these studies was subfragment 1 prepared from myosin of rabbit skeletal muscle. Subfragment 1 and heavy meromyosin are the products of proteolytic digestion of myosin containing one and two ATPase sites/molecule respectively (Lowey *et al.*, 1969).

An important feature of the ATPase mechanism is that when ATP is mixed with heavy meromyosin, there occurs a transient in the formation of the product–protein complex equal to about 0.8 mol/mol of ATPase site. At ATP concentrations less than 0.1 mM the cleavage of ATP occurs with an apparent second-order rate constant of 1×10^6–$2 \times 10^6 M^{-1} \cdot s^{-1}$, but at high ATP concentrations the observed cleavage rate becomes independent of ATP concentration and approximately equal to $160 s^{-1}$ (Lymn & Taylor, 1971). So under the latter conditions the cleavage rate is probably controlled by the cleavage itself or by some process between cleavage and binary ATP–protein-complex formation. Such a process could be a substrate-induced conformation change, and the aim of the present work is to see whether such a process can be characterized and, if so, whether there is a reversal of the conformation change concomitant with ADP release.

Analogues of ATP that bind to myosin but are cleaved either relatively slowly or not at all provide one possible approach to distinguish conformational

changes from cleavage, because it should then be possible to identify intermediates characteristic of uncleaved ATP bound to the protein. Two such analogues are ATP(β,γ-NH) and ATP(γS) (Bagshaw *et al.*, 1972). In addition the binding characteristics of ADP are likely to give information about the nature of the ATP-binding process. Protein fluorescence provides a spectroscopic signal by which binding of ATP analogues to the protein can be measured (Bagshaw *et. al.*, 1972). In the earlier studies it was reported that the rate of the fluorescence enhancement of subfragment 1 when mixed with ATP became too fast to measure ($>700 s^{-1}$) at high ATP concentrations. This is incorrect; an apparent 100% loss of observable protein-fluorescence change resulted from decreased signal-to-noise ratio of the apparatus in the 1–10ms range. The capacity of the stopped-flow spectrofluorimeter to resolve fast reaction rates is described here in detail, since this is critical for data evaluation.

Materials and Methods

Proteins

Subfragment 1 was prepared from myosin extracted from rabbit skeletal muscle and characterized as described by Bagshaw & Trentham (1973).

Nucleotides

Sodium salts of ATP and ADP were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. ATP(α,β-CH_2) was obtained from Miles Inc., Kankakee, Ill., U.S.A. These nucleotides were used without further purification.

ATP(γS) was prepared essentially as described by Goody & Eckstein (1971) except that the condensation was carried out in dimethylformamide instead of pyridine and the carbamoylethyl-ATP(γS) intermediate was partially purified on DEAE-cellulose before deprotection, which was carried out in 1M-NaOH for 2h at 21°C rather than for 10min at 100°C.

ATP(β,γ-NH) was prepared enzymically by a method based on that of Rodbell *et al.* (1971) scaled up 1000-fold and purified as described by Yount *et al.* (1971).

The concentration and purity of nucleotides were determined as described by Bagshaw & Trentham (1974). All the nucleotides were found to be pure within the limits of the assay sensitivity, except ADP, which contained trace amounts of a material that was probably AMP (Bagshaw & Trentham, 1974).

Other reagents

All other reagents were analytical-reagent grade where possible and used without further purification. Water was double distilled.

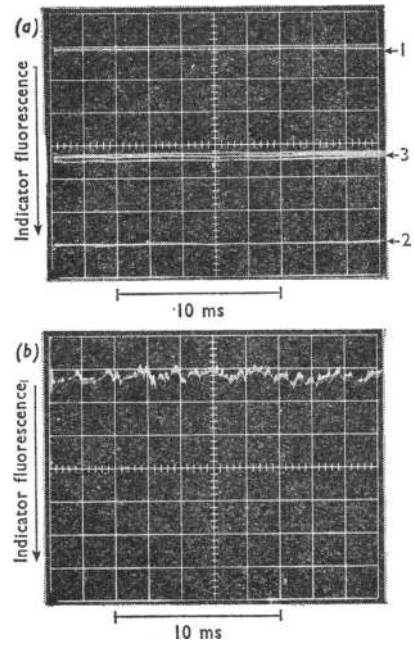

Fig. 1. *Mixing efficiency of the stopped-flow apparatus*

Excitation was at 359nm and the emitted light between 420 and 540nm was analysed. (*a*) Trace 1 corresponds to zero fluorescence and practically equalled the fluorescence of 9μM-4-methylumbelliferone at pH6.2. Trace 2 represents the fluorescence of 9μM-4-methylumbelliferone at pH8.7. Traces 3, comprising three almost superimposed traces, represent the fluorescence of 9μM-4-methylumbelliferone at pH7.7 and were obtained by mixing the umbelliferone in 0.10M-sodium pyrophosphate adjusted to pH8.7 with HCl and 4μM-subfragment 1 (to simulate typical viscosity conditions) with 0.10M-sodium pyrophosphate adjusted to pH6.2 with HCl. (*b*) is an oscilloscope trace of the same reaction as (*a*) trace 3 with the ordinate amplified 20-fold so that 1 major division represents 1.6% of the total fluorescence change in the reaction.

Measurement of protein-fluorescence changes

Measurements were made at room temperature (20–23°C) by using the fluorescence stopped-flow apparatus described previously (Bagshaw *et al.*, 1972). Protein fluorescence was observed by excitation with light at 300nm and detecting the emitted radiation through Schott filters type W.G. 345 and U.G. 11 (not 21611 as earlier reported). This filter system transmits light between 320nm and 390nm with maximum transmission at 350nm. Since the conclusions of the present paper are based in part on the capacity to measure reaction rates of the order of $400 s^{-1}$, values of the dead time and mixing efficiency of the apparatus are required (Gutfreund, 1972, p. 172). The dead-time was found to be 1ms by Whitaker *et al.* (1974). The

mixing efficiency can be investigated by examining any residual signal of a reaction that is too fast to measure. A pH indicator with suitable fluorescence properties provides a convenient system, since second-order association rates of protons and OH^- ions with bases and acids are 10^{10}–10^{11} $M^{-1}\cdot s^{-1}$ (Eigen & Kustin, 1960). The maximum change of fluorescence with change in pH occurs when the pH equals the pK of the indicator. The anion of 4-methylumbelliferone (pK_a 7.6) when excited at 359nm exhibits maximum fluorescence at 457nm (Chen, 1968). Fig. 1 shows the time-course of the fluorescence when 9μM-4-methylumbelliferone in 0.10M-sodium pyrophosphate, pH8.7, was mixed with 0.10M-sodium pyrophosphate, pH6.2, to give a final pH of 7.7. The fluorescence change was over 99% complete at 1ms. Even in the presence of 4μM-subfragment 1, which could possibly retard efficient mixing, the reaction trace was horizontal and corresponded to the end-point of the reaction. The signal-to-noise ratio was such that 1% of the total fluorescence change would have been detected. We conclude that the reactants were efficiently mixed before observation starts in the experiments concerning nucleotide binding to subfragment 1 described below.

Signal-to-noise ratios for nucleotide binding to subfragment 1 were considerably lower in reactions occurring in the 1–10ms range compared with those over 10ms, owing to the necessity of using smaller time-constants in the apparatus. Since the apparatus was being used at the limits of resolution appropriate blank traces are presented to allow critical assessment of the signals.

Theory

This section provides a theoretical basis for interpretation of the magnitude and rate of the protein-fluorescence changes that occur when ATP or the ATP analogues bind to subfragment 1. The main objective is to describe the kinetic models that we consider to be the most reasonable explanation for the experimental observations. For this reason boundary conditions are chosen that relate to the experiments that follow. Alternative kinetic models are discussed briefly, principally from the viewpoint of emphasizing how these models can be distinguished experimentally from the proposed model. Distinction between alternative models is not always clear-cut on the basis of presently available evidence and points of ambiguity are indicated where relevant.

It is simplest to consider first the situation of an ATP analogue which binds to but is not cleaved by subfragment 1 and is a competitive inhibitor of the ATPase with respect to ATP.

The general treatment of one- and two-step binding mechanisms of a competitive inhibitor, X, to an enzyme, E, is presented by Gutfreund (1972, chapter 8) and Viale (1971), and a good example of how alternative mechanisms can be distinguished is afforded by the results obtained from studies of *Escherichia coli* alkaline phosphatase (Halford *et al.*, 1969; Halford, 1971, 1972).

The one-step mechanism (eqn. 1):

$$E+X \underset{k_{-p}}{\overset{k_{+p}}{\rightleftharpoons}} EX \qquad (1)$$

where EX is the binary complex and k_{+p}, k_{-p} are rate constants, is characterized by an exponential decay in [E] when $[X]_0 \gg [E]_0$, where the subscripts refer to time. $k_{obs.}$, the rate of the exponential process of EX formation, is described by eqn. (2):

$$k_{obs.} = k_{+p}[X]_0 + k_{-p} \qquad (2)$$

If eqn. (1) is modified by the fact that EX can break down at a rate k_{+r} as might be appropriate for a substrate analogue with the kinetic properties of ATP(γS), then:

$$k_{obs.} = k_{+p}[X]_0 + k_{-p} + k_{+r} \qquad (3)$$

The important feature of eqn. (1) with or without a step involving EX breakdown is that the one-step binding mechanism is untenable if $k_{obs.}$ does not increase linearly with $[X]_0$. The principal experimental result of the present paper is that $k_{obs.}$ does not increase linearly with ATP analogue concentration, and this provides the main evidence for rejection of the one-step mechanism.

Two-step mechanisms of inhibitor binding generally involve a unimolecular rearrangement of either E, X or EX in addition to the bimolecular step. Isomerization of EX to another conformational state *EX* is considered first (eqn. 4). This is the mechanism that we consider is the predominant kinetic pathway of the binding of ATP or ATP analogues to subfragment 1. Isomerization of E or X is dealt with in the Discussion section.

$$E+X \underset{k_{-p}}{\overset{k_{+p}}{\rightleftharpoons}} EX \underset{k_{-q}}{\overset{k_{+q}}{\rightleftharpoons}} EX \qquad (4)$$

A simplification of the kinetic analysis is to let the bimolecular process occur much more rapidly than the unimolecular isomerization. This means that, when E and X are mixed, EX is rapidly formed and, if $[X]_0$ is much greater than $[E]_0$, *EX* is formed in an exponential process whose rate, k_{EX}, is described by eqn. (5):

$$k_{EX} = \frac{k_{+q}}{1+1/K[X]_0} + k_{-q} \qquad (5)$$

where K, the association constant $= k_{+p}/k_{-p}$.

A useful rearrangement of eqn. (5) for graphical analysis is:

$$1/(k_{EX}-k_{-q}) = (1/k_{+q})+(1/Kk_{+q}[X]_0) \quad (6)$$

Eqn. (5) simplifies to:

$$k_{EX} = Kk_{+q}[X]_0+k_{-q} \quad (7)$$

when $K[X]_0 \ll 1$ and to:

$$k_{EX} = k_{+q}+k_{-q} \quad (8)$$

when $K[X]_0 \gg 1$. So this alternative of eqn. (4) is only distinguishable from eqn. (1) when $K[X]_0 \geqslant 1$. It may not always be possible to satisfy this experimental condition.

Another problem brought out by this treatment of eqn. (4) is that, although an expression has been obtained for k_{EX}, this does not necessarily reflect the rate of the observed protein-fluorescence change when an ATP analogue binds to subfragment 1, since the fluorescence of EX may also differ from that of E. If this is the case then, at high $[X]_0$ when $K[X]_0$ is much greater than 1, the rate of the fluorescence change will be biphasic, the rapid phase representing the rate of EX formation and the slow phase *EX* formation with concomitant change in [EX]. If k_{-q} is much less than k_{+q} so that $[EX]_\infty = [E]_0$, then the amplitude of the total fluorescence change will be constant whether the rate of the change is monophasic or biphasic. However, the amplitude of the slower phase will decrease with increasing $[X_0]$, if the fluorescence of EX is intermediate between that of E and *EX*.

When eqn. (4) is modified by the fact that *EX* can break down at a rate k_{+r} where $k_{+r} \ll k_{+q}$ as might be appropriate for ATP(γS), this leads to eqn. (9):

$$E+X \underset{k_{-p}}{\overset{k_{+p}}{\rightleftharpoons}} EX \underset{k_{-q}}{\overset{k_{+q}}{\rightleftharpoons}} EX \xrightarrow{k_{+r}} \quad (9)$$

Eqns. (5), (6), (7) and (8) are modified respectively to:

$$k_{EX} = \frac{k_{+q}}{1+1/K[X]_0}+k_{-q}+k_{+r} \quad (10)$$

$$1/(k_{EX}-k_{-q}-k_{+r}) = (1/k_{+q})+(1/Kk_{+q}[X]_0) \quad (11)$$

$$k_{EX} = Kk_{+q}[X]_0+k_{-q}+k_{+r} \quad (12)$$

when $K[X]_0 \ll 1$ and to:

$$k_{EX} = k_{+q}+k_{-q}+k_{+r} \quad (13)$$

when $K[X]_0 \gg 1$.

Finally eqn. (14) is considered which relates specifically to ATP binding and cleavage, so that here X is the substrate and *EP* is an enzyme–product complex which breaks down slowly to result in the overall hydrolysis of ATP.

$$E+X \underset{k_{-p}}{\overset{k_{+p}}{\rightleftharpoons}} EX \underset{k_{-q}}{\overset{k_{+q}}{\rightleftharpoons}} EX \underset{k_{-r}}{\overset{k_{+r}}{\rightleftharpoons}} EP \xrightarrow{k_{+s}} \quad (14)$$

A simplification of the kinetic analysis of eqn. (14) is to let both the bimolecular process and the *EX* to *EP* transformation occur much more rapidly than the EX to *EX* isomerization and *EP* decay. When $[X]_0 \gg [E]_0$, first-order rate constants, k_{EX} and k_{EP} (eqn. 15), describe the exponential rates of formation of *EX* and *EP*, which are equal since *EX* and *EP* are always in equilibrium with each other.

$$k_{EX} = k_{EP} = k_{+q}/(1+1/K[X]_0) + (k_{-q}k_{-r}+k_{+r}k_{+s})/(k_{+r}+k_{-r}) \quad (15)$$

Provided that *EX* and *EP* but not EX have protein fluorescence different from that of E, the rate of the observed fluorescence change, $k_{obs.}$, will be monophasic and equal to k_{EX}. Rearranging eqn. (15) gives:

$$1/\{k_{obs.}-(k_{-q}k_{-r}+k_{+r}k_{+s})/(k_{+r}+k_{-r})\} = (1/k_{+q})+(1/Kk_{+q}[X]_0) \quad (16)$$

The fluorescence of *EX* and *EP* need not be equal; indeed only the production of the equilibrium mixture is followed and the individual fluorescence yields of *EX* and *EP* cannot be evaluated directly.

If the fluorescence of EX is intermediate between that of E and the equilibrium mixture of *EX* and *EP*, when $K[X]_0 \geqslant 1$, a biphasic process will occur on mixing E and X; the rapid phase representing the rate of EX formation and the slower phase *EX*+*EP* formation with concomitant change in [EX]. The amplitude of the total protein-fluorescence change will be constant if in the steady state $[EX]+[EP] = [E]_0$. This condition holds when:

$$(k_{-q}k_{-r}+k_{+r}k_{+s})/(k_{+r}+k_{-r}) \ll k_{+q}/(1+1/K[X]_0)$$

However, the amplitude of the slower phase will decrease with increasing $[X]_0$, reaching a plateau which will reflect the fluorescence difference of EX and the equilibrium mixture of *EX* and *EP*.

Kinetic analysis of eqn. (14) is more complex if the rate constants of the transformations EX to *EX* and *EX* to *EP* are comparable. The production of *EP* would then lag behind that of *EX*. Resolution of the two processes depends on either the difference in fluorescence of *EX* and *EP* or an independent method such as chemical analysis of the product in *EP*.

The kinetic analyses of eqns. (4), (9) and (14) are more complex if the initial binding equilibrium is not rapidly established relative to the isomerization. For a model in which the value of k_{-p} is comparable to k_{+q} a plot of k_{EX} against $[X]_0$ would not be hyperbolic and the corresponding reciprocal plot would not be linear. In such a situation the values of k_{+p}, k_{-q} and k_{+q} could be extracted from the data by computer curve-fitting techniques.

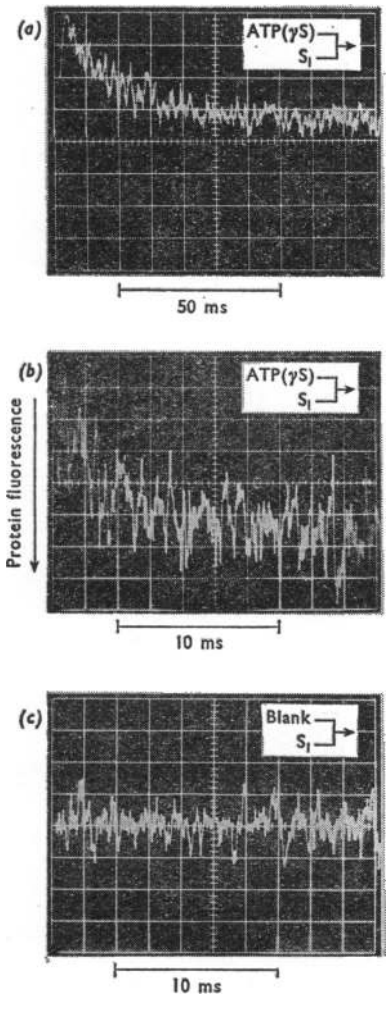

Fig. 2. *Stopped-flow records of the fluorescence of subfragment* 1 *when mixed with ATP*(γS)

One syringe contained 5.0 μM-subfragment 1 (S_1) and the other ATP(γS) at (*a*) 60 μM, (*b*) 600 μM and (*c*) zero concentrations (reaction-chamber concentrations). Both syringes contained 0.10M-KCl–5mM-$MgCl_2$–50mM-Tris adjusted to pH8.0 with HCl.

Results

ATP(γS) binding

Bagshaw *et al.* (1972) carried out kinetic studies of the reaction between ATP(γS) and subfragment 1 and obtained the following results. The apparent second-order rate constants associated with fluorescence change when either ATP(γS) or ATP bind to subfragment 1 in 0.10M-KCl at pH 8.0 are both in the range 1×10^6–$2\times10^6\,M^{-1}\cdot s^{-1}$. The observed cleavage rate of ATP(γS) ($0.25\,s^{-1}$) is, however, about a 1000-fold less than that of ATP. The observed dissociation rate of ATP(γS) from subfragment 1 is less than $0.2\,s^{-1}$. For the purpose of the analysis which follows, the observed dissociation rate was taken as zero (the limit of $0.2\,s^{-1}$ had been set by the experimental sensitivity). Because of the similar second-order rate constants for ATP(γS) and ATP on the one hand, and the large difference in the cleavage rates for the two compounds on the other, ATP(γS) is well suited to differentiate between fluorescent changes owing to binding or cleavage of the substrate.

The rates of the fluorescence change were measured when various concentrations of ATP(γS) were mixed with subfragment 1. Three experimental records are shown, including a reaction trace with no ATP(γS) present (Fig. 2). The traces had a low signal-to-noise ratio when a small time-constant had to be used. Fig. 3(*a*) shows that the observed rate did not increase linearly with ATP(γS) concentration but reached a plateau which was 1000-fold greater than

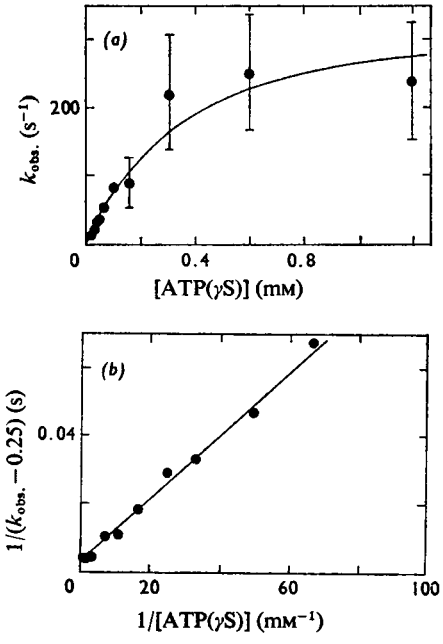

Fig. 3. *Kinetic analysis of the fluoresence enhancement of subfragment* 1 *on interaction with ATP*(γS)

(*a*) Data from traces such as those in Fig. 2 in which $k_{obs.}$ equals the rate of the fluorescence increase analysed as an exponential process. The basis on which the error bars are drawn is described in the text. (*b*) A secondary plot of the data in (*a*) analysed according to eqn. (11) where $k_{obs.} = k_{EX}$. The straight line gives $K = 3.9\times10^3\,M^{-1}$ and $k_{+q} = 300\,s^{-1}$. From these values of K and k_{+q} the solid line in (*a*) was drawn according to eqn. (10).

the cleavage rate of ATP(γS) ($0.25\,s^{-1}$). The value of $k_{obs.}$ at each point in Fig. 3(*a*) is the average of three stopped-flow traces. Above rates of $200\,s^{-1}$ the error in each value of $k_{obs.}$ was $\pm 80\,s^{-1}$, as indicated by the error bars in Fig. 3(*a*). Below values of $k_{obs.} = 200\,s^{-1}$ the error bars were less. The apparent second-order association rate constant obtained from the data at low concentrations of ATP(γS) was $1.2\times10^{6}\,M^{-1}\cdot s^{-1}$. If a one-step mechanism was applicable to the binding of ATP(γS) then the rate of the fluorescence change when [ATP(γS)] = 1.2mM would be $1440\,s^{-1}$. However, the observed rate was $240\,s^{-1}$. Further, if $k_{obs.}$ had been $1440\,s^{-1}$ only 24% of the fluorescence change would have been observed, since the dead-time of the instrument was 1ms. The observed amplitude of the protein fluorescence change at 1.2mM-ATP(γS) was 75% of the maximum and constant amplitude which was observed from 15 to 100μM-ATP(γS). Possible reasons for the 25% decrease in amplitude are discussed below. The experiment described in Fig. 3 was repeated with a different protein preparation and gave the same result. For these reasons a one-step mechanism (eqn. 1) for the binding of ATP(γS) to subfragment 1 is excluded.

However, a graph of $1/(k_{obs.}-0.25)$ against $[ATP(\gamma S)]^{-1}$ is linear within experimental accuracy (Fig. 3*b*). This is compatible with a two-step binding mechanism (eqn. 9) in which results have been plotted according to eqn. (11), where k_{EX}, k_{-q}, k_{+r} and $[X]_0$ correspond to $k_{obs.}$, 0, $0.25\,s^{-1}$ and [ATP(γS)] respectively. Analysis of Fig. 3(*b*) gives $K = 3.9\times10^{3}\,M^{-1}$ and $k_{+q} = 300\,s^{-1}$.

ATP binding and cleavage

The above result with ATP(γS) is strong evidence for the binding of ATP to subfragment 1 being a two-step process. If this conclusion is valid then it is necessary that the rate of the fluorescence change when ATP binds to subfragment 1 also reaches a plateau at high ATP concentrations. The rates of the fluorescence change were measured when various concentrations of ATP were mixed with subfragment 1, and three experimental records are shown (Fig. 4). Fig. 5(*a*) shows that the observed rate did not increase linearly with ATP concentration. A graph of $1/k_{obs.}$ against $[ATP]^{-1}$ is linear within experimental accuracy (Fig. 5*b*). This is compatible with a two-step binding mechanism and ATP cleavage (eqn. 14) in which results have been plotted according to eqn. (16) where $[X]_0 = [ATP]$ and $k_{obs.} \geqslant (k_{-q}k_{-r}+k_{+r}k_{+s})/(k_{+r}+k_{-r})$. As discussed below, according to our mechanism for the Mg^{2+}-dependent ATPase, $(k_{-q}k_{-r}+k_{+r}k_{+s})/(k_{+r}+k_{-r}) = 0.055\,s^{-1}$ and is 60-fold less than the smallest value of $k_{obs.}$ recorded in Fig. 5(*a*). Analysis of Fig. 5 gives $K = 4.5\times10^{3}\,M^{-1}$ and $k_{+q} = 400\,s^{-1}$.

The experiment was repeated with 12 separate subfragment-1 preparations at I 0.14M. Values of k_{+q} were generally $400\,s^{-1}$ but occasionally subfragment-1 preparations yielded lower values, the smallest being $125\,s^{-1}$. The value of K was less variable. The plateau in the rate of the protein-fluorescence change was not caused by insufficient Mg^{2+} at high ATP concentrations since $[Mg^{2+}]$ was always in excess of [ATP] and the observed rate was constant at high ATP concentrations when $[Mg^{2+}]$ was increased to 50mM.

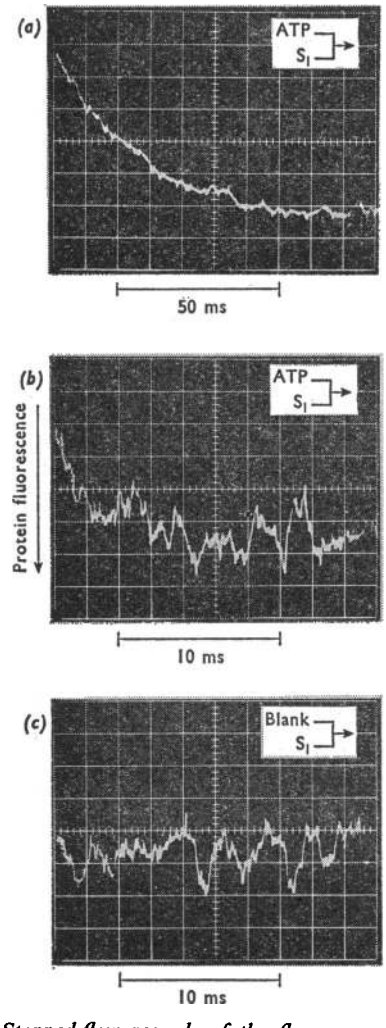

Fig. 4. *Stopped-flow records of the fluorescence of subfragment* 1 *when mixed with ATP*

One syringe contained 3.7μM-subfragment 1 (S_1) and the other ATP at (*a*) 30μM, (*b*) 1mM and (*c*) zero concentration (reaction-chamber concentrations). Both syringes contained 0.10M-KCl–5mM-$MgCl_2$–50mM-Tris adjusted to pH8.0 with HCl.

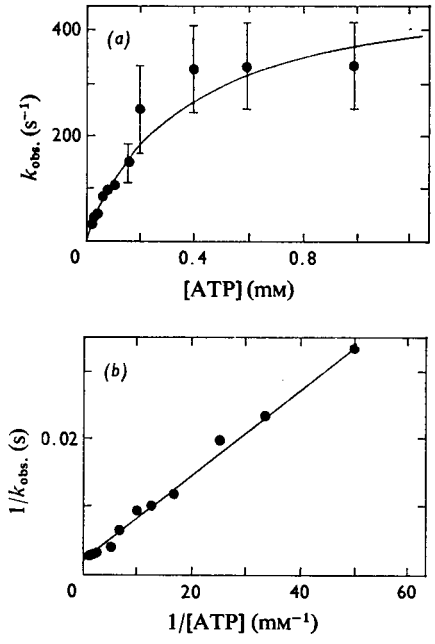

Fig. 5. *Kinetic analysis of the fluorescence enhancement of subfragment* 1 *on interaction with ATP*

(*a*) Data from traces such as those in Fig. 4. The error bars are drawn on the same basis as for ATP(γS). (*b*) A secondary plot of the data in (*a*) analysed according to eqn. (16). The straight line gives $K = 4.5 \times 10^3 M^{-1}$ and $k_{+q} = 400 s^{-1}$. From these values of K and k_{+q} the solid line in (*a*) was drawn according to eqn. (15), where $k_{obs.} = k_{EX}$.

ADP binding

Malik & Martonosi (1971) recorded protein-absorption changes in a stopped-flow apparatus when 1mM-ADP was mixed with heavy meromyosin to measure the association rate of ADP to the protein. For characterization of the mechanism of ADP binding it was preferable to use the protein fluorescence to monitor the binding because of the greater signal-to-noise ratio, and to measure the dependence of the amplitude and rate of the protein-fluorescence change on ADP concentration. Signal-to-noise ratios were similar to those found with ATP(γS) and typical records of oscillograph traces are shown (Fig. 6). Fig. 7(*a*) shows that the observed rate did not increase linearly with ADP concentration, excluding a one-step mechanism for the binding of ADP. However, a graph of $1/(k_{obs.} - 1.4)$ against $[ADP]^{-1}$ is linear within experimental accuracy (Fig. 7*b*). This is compatible with a two-step binding mechanism (eqn. 4) in which the results have been plotted according to eqn. (6) where k_{EX}, k_{-q} and $[X]_0$ correspond to $k_{obs.}$, $1.4 s^{-1}$ and [ADP] respectively. The value $1.4 s^{-1}$ is used because it was the observed rate at which ADP was displaced from subfragment 1 by an excess of ATP (Bagshaw *et al.*, 1972). Analysis of Fig. 7(*b*) gives $K = 3.7 \times 10^3 M^{-1}$ and $k_{+q} = 400 s^{-1}$.

Bagshaw & Trentham (1974) have shown that the binary complex formed by mixing ADP with subfragment 1 is the same as that generated during hydrolysis of ATP catalysed by subfragment 1 and is

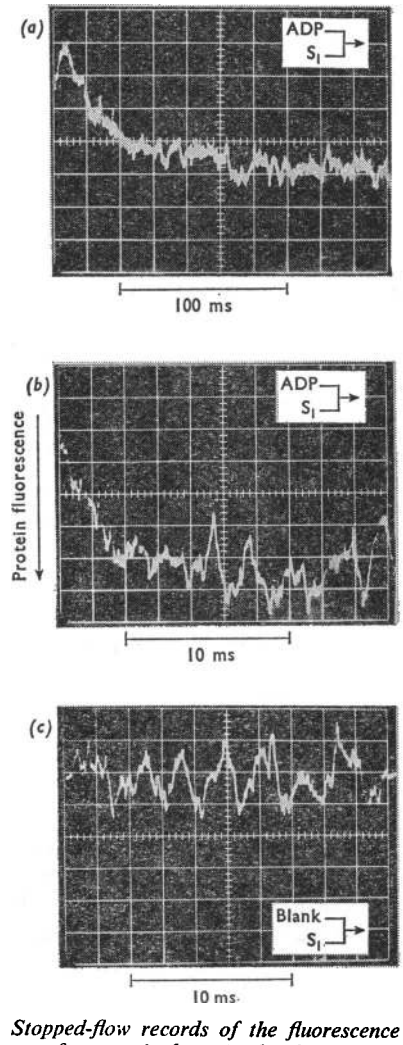

Fig. 6. *Stopped-flow records of the fluorescence of subfragment* 1 *when mixed with ADP*

One syringe contained 4.0μM-subfragment 1 (S_1) and the other ADP at (*a*) 20μM, (*b*) 1.0mM, (*c*) zero concentration (reaction-chamber concentrations). Both syringes contained 0.10M-KCl–5mM-$MgCl_2$–50mM-Tris adjusted to pH8.0 with HCl.

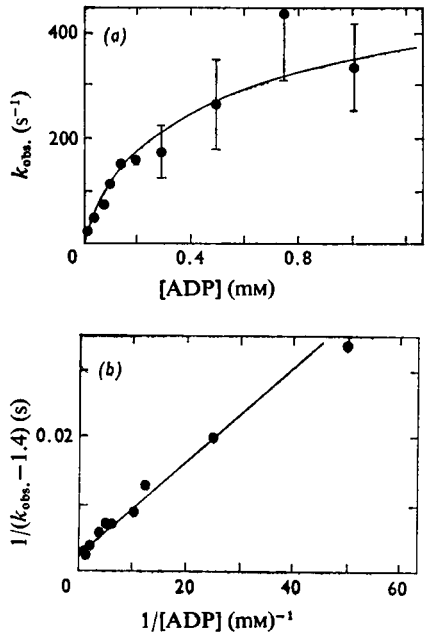

Fig. 7. *Kinetic analysis of the fluorescence enhancement of subfragment* 1 *on interaction with ADP*

(*a*) Data from traces such as those in Fig. 6. The error bars are drawn on the same basis as for ATP(γS). (*b*) A secondary plot of the data in (*a*) analysed according to eqn. (6), where $k_{obs.} = k_{EX}$. The straight line gives $K = 3.7\times10^{3}\,M^{-1}$ and $k_{+q} = 400\,s^{-1}$. From these values of K and k_{+q} the solid line in (*a*) was drawn according to eqn. (5).

an intermediate in the reaction pathway. It is therefore of great interest that ADP binds with a two-step mechanism, since this suggests that the conformation change, which the evidence suggests is associated with ATP binding, is reversed when ADP is released during the ATPase activity.

ATP(β,γ-NH) and ATP(α,β-CH_2)

ATP(β,γ-NH) is an analogue of ATP which is not cleaved, but enhances protein fluorescence when it binds to subfragment 1 (Fig. 8). It can be displaced from subfragment 1 at a rate of $0.02\,s^{-1}$ (Bagshaw *et al.*, 1972). The observed rate of binding increased linearly with ATP(β,γ-NH) concentration (Fig. 9), but a feature of ATP(β,γ-NH) binding is that the apparent second-order rate constant ($6\times10^{4}\,M^{-1}\cdot s^{-1}$) was 30-fold slower than that of ATP. This means that although a one-step binding mechanism (eqn. 1) is theoretically possible, the association rate constant (k_{+p} of eqn. 1) would be abnormally low, as protein–ligand association rates are typically 10^{7}–$10^{8}\,M^{-1}\cdot s^{-1}$ (Gutfreund, 1971). The result (Fig. 9) is also consistent with a two-step binding mechanism (eqn. 4) in which $k_{obs.} = k_{EX}$ and concentrations of ATP-(β,γ-NH) used were such that $K[ATP(\beta,\gamma\text{-NH})]_0 = K[X]_0 \ll 1$, giving rise to eqn. (7). Analysis of Fig. 9 according to eqn. (7) gives $Kk_{+q} = 6\times10^{4}\,M^{-1}\cdot s^{-1}$.

It is of interest to know whether k_{+q} or K has decreased relative to the values determined for ATP, ATP(γS) and ADP. This could best be found out by using higher concentrations of ATP(β,γ-NH) but these were not available. However, another ATP analogue, ATP(α,β-CH_2), was available in sufficient quantity and it does have similar kinetic properties to ATP(β,γ-NH) except that like ATP(γS) it has a slow observed cleavage rate of $0.1\,s^{-1}$ (Mannherz

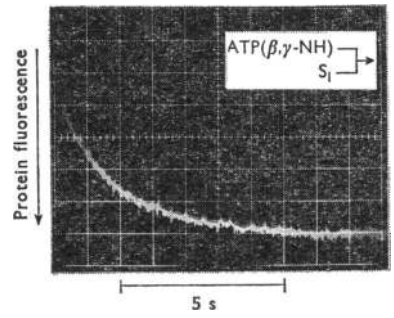

Fig. 8. *Stopped flow record of the fluorescence of subfragment* 1 *when mixed with ATP(β,γ-NH)*

One syringe contained 2.4 μM-subfragment 1 (S_1) and the other 7 μM-ATP(β,γ-NH) (reaction-chamber concentrations). Both syringes contained 0.10 M-KCl–10 mM-$MgCl_2$–0.10 M_2-triethanolamine adjusted to pH 8.0 with HCl.

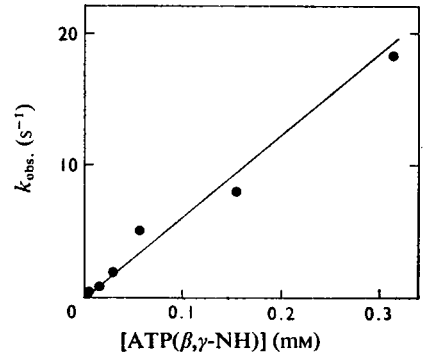

Fig. 9. *Kinetic analysis of the fluorescence enhancement of subfragment* 1 *on interaction with ATP(β,γ-NH)*

Data from traces such as that in Fig. 8 [for ATP(β,γ-NH) concentrations ≥20 μM, 7 μM subfragment 1 was used]. The data were analysed according to eqn. (7), where $k_{obs.} = k_{EX}$. The straight line gives $Kk_{+q} = 6\times10^{4}\,M^{-1}\cdot s^{-1}$.

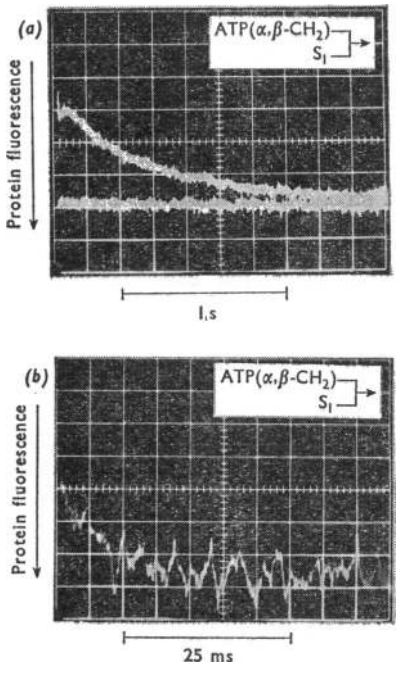

Fig. 10. *Stopped-flow record of the fluorescence of subfragment* 1 *when mixed with ATP(α,β-CH₂)*

One syringe contained 9.6 μM-subfragment 1 (S_1) and the other in (*a*) 100 μM- and in (*b*) 12mM-ATP(α,β-CH_2) (reaction-chamber concentrations). The solvent was 27mM-$MgCl_2$–75mM-KCl–50mM-Tris adjusted to pH7.2 with HCl. The horizontal trace in (*a*) was obtained by triggering the oscilloscope a few seconds after mixing and indicates stability of the protein fluorescence during steady-state hydrolysis of ATP(α,β-CH_2).

et al., 1973). The experiments with ATP(α,β-CH_2) were carried out at pH7.2 because they were part of a complementary research programme relating the kinetics to structural studies (Mannherz *et al.*, 1973). The plateau in the rate of change of the protein fluorescence when high concentrations of ATP were mixed with subfragment 1 was similar at pH7.0 and pH8.0. ATP(α,β-CH_2) enhanced protein fluorescence when mixed with subfragment 1 (Fig. 10). The observed rates, $k_{obs.}$, of the protein-fluorescence change were proportional to [ATP(α,β-CH_2)] in the range 50–200 μM-ATP(α,β-CH_2), giving an apparent second-order rate constant of $1.85\times10^4\,M^{-1}\cdot s^{-1}$. The ordinate intercept of a plot of $k_{obs.}$ versus [ATP(α,β-CH_2)] was $0.2\,s^{-1}$. According to the two-step binding mechanism with slow cleavage of the ATP analogue (eqn. 9), and equating $k_{obs.}$ with k_{EX} and [ATP(α,β-CH_2)] with $[X]_0$, this intercept ($0.2\,s^{-1}$) $= k_{-q}+k_{+r}$, when $K[X]_0 \ll 1$ (eqn. 12). At the highest ATP(α,β-CH_2) concentration used (12mM), $k_{obs.}$ was $145\,s^{-1}$. From eqn. (10) therefore:

$$\frac{k_{+q}}{1+1/K[X]_0}+k_{-q}+k_{+r}=145\,s^{-1}$$

when $[X]_0$ was 12mM. It follows that k_{+q} is at least $145\,s^{-1}$ and so is likely to be comparable with k_{+q} determined for ATP, ATP(γS) and ADP. ATP(α,β-CH_2) and probably therefore ATP(β,γ-NH) have a low value of Kk_{+q} because of the abnormally small value of K. Data on the rates of the observed fluorescence change were very sparse at sufficiently high [ATP(α,β-CH_2)] for evaluation of K and k_{+q}, but the values obtained were $K=46\,M^{-1}$ and $k_{+q}=400\,s^{-1}$.

Table 1. *Kinetic and protein fluorescence characteristics of the two-step binding mechanism*

Comparison of K, k_{+q} and Kk_{+q} obtained from the rates of protein fluorescence changes when ATP or ATP analogues were mixed with subfragment 1 based on two-step binding mechanisms and analysis of the data of Figs. 3, 5, 7 and 9. Equilibrium constants K_1, K_7 and rate constants k_{+2} and k_{-6} are defined in the proposed subfragment-1 Mg^{2+}-dependent ATPase mechanism (eqn. 17). The relative fluorescence represents the fluorescence of the proteins with the nucleotide bound relative to that of unliganded protein. For ATP(γS) and ATP(β,γ-NH) the relative fluorescence values, which are accurate to about 2% [e.g. $= 1.10\pm0.02$ for ATP(γS)], were measured from stopped-flow traces at nucleotide concentrations less than $1/K$, such as Figs. 2(*a*) and 8. For ATP and ADP the values are taken from Bagshaw & Trentham (1974). In the stopped-flow apparatus the values for ATP and ADP were 1.16 and 1.06 respectively. The lower values probably reflect the different optical properties of the two spectrofluorimeters.

Nucleotide	Kk_{+q} ($M^{-1}\cdot s^{-1}$)	K (M^{-1})	k_{+q} (s^{-1})	Relative fluorescence
ATP(γS)	1.2×10^6	3.9×10^3	300	1.10
ATP	1.8×10^6	4.5×10^3 ($=K_1$)	400 ($=k_{+2}$)	1.18
ADP	1.5×10^6	3.7×10^3 ($=K_7^{-1}$)	400 ($=k_{-6}$)	1.067
ATP(β,γ-NH)	6×10^4		>20	1.10

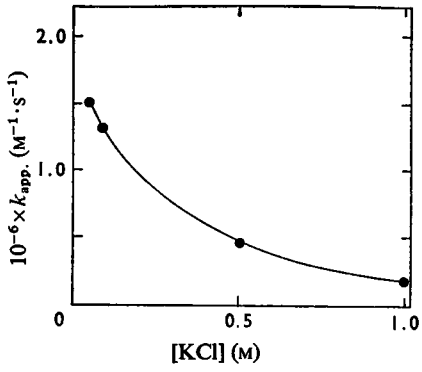

Fig. 11. *Dependence of the apparent second-order association rate constant of ATP and subfragment* 1 *on KCl concentration*

Variation of the apparent second-order association rate constant, $k_{app.}$, on KCl concentration when 9.8μM-subfragment 1 was mixed with 20μM-ATP in the presence of 5mM-$MgCl_2$–20mM-Tris adjusted to pH8.0 with HCl.

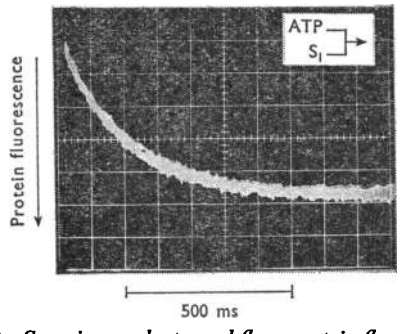

Fig. 12. *Superimposed stopped-flow protein-fluorescence traces demonstrating the lack of salt specificity when increasing ionic strength retards the rate of the interaction of ATP and subfragment* 1

One syringe contained 5μM-subfragment 1 (S_1) and the other 20μM-ATP (reaction-chamber concentrations). Both syringes contained 5mM-$MgCl_2$–50mM-Tris adjusted to pH8.0 with HCl. First a trace was obtained in the presence of 0.50M-$(CH_3)_4N^+Br^-$–50mM-KCl, and then a second trace, which superimposed the first, was obtained when this salt was replaced by 0.55M-KCl.

Table 1 summarizes the kinetic results when ATP or ATP analogues were mixed with subfragment 1.

Specificity and effect of KCl concentration on K and k_{+q}

High KCl concentrations have been shown to decrease the apparent second-order rate constant obtained from transient P_i-release studies when ATP is mixed with heavy meromyosin (Lymn & Taylor, 1970). If the two-step binding mechanism for ATP is correct (eqn. 14), the apparent second-order rate constant equals Kk_{+q}, so that increasing the KCl concentration may be decreasing K or k_{+q}. When 20μM-ATP was mixed with subfragment 1, the apparent second-order rate constant of the protein fluorescence change decreased tenfold over a 20-fold increase in KCl concentration (Fig. 11). On the other hand, when the experiment described in Fig. 11 was repeated with 5mM-ATP so that the plateau of $k_{obs.}$, corresponding to k_{+q}, had been reached, $k_{obs.}$ (= 210s^{-1}) was the same in both 0.1M- and 1.0M-KCl. This shows that increasing [KCl] decreases K. The effect is not specific for KCl, since the rate of the protein fluorescence change was unaltered when 0.5M-$(CH_3)_4N^+Br^-$ plus 0.05M-KCl replaced 0.55M-KCl in the reaction medium (Fig. 12).

Amplitudes of protein-fluorescence changes

The observed protein-fluorescence enhancements induced by ATP and ATP analogues are listed in Table 1.

Since ATP induces an 18% increase in subfragment-1 fluorescence on binding and cleavage, whereas analogues such as ATP(α,β-CH_2) and ATP(γS) result in a 10% enhancement on binding, it is probable that there is a significant fluorescence change associated with the cleavage step. On a one-step mechanism for the binding of ATP to subfragment 1 the fluorescence change due to cleavage would occur at a plateau rate at high ATP concentrations. The remainder of the fluorescence change which is associated with binding would become too fast to measure. This mechanism can be distinguished from eqn. (14), in which the fluorescence of EX equals that of E since, according to the latter scheme, the whole of the fluorescence change would be a monophasic exponential whose observed rate constant would reach a plateau at high ATP concentrations.

In the interpretation of the data of Figs. 4 and 5 it is necessary therefore to measure the amplitude of the signal observed in the stopped-flow apparatus as a function of [ATP]. If it remains constant throughout the range $K[ATP]_0 < 1$ to $K[ATP]_0 > 1$, it provides additional evidence that the binding process is contributing to the observed fluorescence change whose rate reaches a plateau at high ATP concentrations.

Numerous experiments were performed to investigate this point, particularly in view of our initial incorrect conclusion that the whole of the fluorescence change became too fast to measure (Bagshaw *et al.*, 1972). Variation was obtained with different

protein preparations and in about one-third of the experiments the amplitude fell by about 30% as the ATP concentration was increased over a range typified by Fig. 5 (i.e. 20μM to 1mM). However, in a majority of the experiments the amplitude was invariant within experimental sensitivity. Fig. 13 illustrates this in an experiment in which the rate of the fluorescent change increased 20-fold for a 100-fold increase in ATP concentration and covered a concentration range $K[ATP]_0 < 1$ to $K[ATP]_0 > 1$. On balance these studies of fluorescence amplitudes provide additional support for the two-step mechanism for ATP binding, since the observed fluorescence signal at high ATP concentrations was greater than the 8% change expected on cleavage alone.

A decrease in the fluorescence amplitude at high ATP concentration does not rule out a mechanism of the type given in eqn. (14) because the fluorescence of the initially formed binary complex (EX of eqns. 4, 9 and 14) may be slightly enhanced. However, there are other possible causes of decreased amplitude. First, the rate of the observed process when [nucleotide] $> 1/K$ approaches the resolution limit of the stopped-flow apparatus and, although corrections were made for loss of signal within the instrument dead-time, uncertainty in the value of the dead-time introduces errors. For example, 67% or 55% of a $400s^{-1}$ process would be observed depending on whether the dead-time was 1 or 1.5ms. Secondly, high concentrations of nucleotide may absorb some of the exciting light; this was particularly true of nucleotides synthesized by methods involving evaporation of triethylamine–bicarbonate buffers in the final step. Thirdly, the low signal-to-noise ratios of traces such as Figs. 2(*b*), 4(*b*) and 6(*b*) introduces error. For these reasons the fluorescence of EX (or M·ATP and M·ADP of eqn. 17) cannot be evaluated precisely, but it is in the range 1.00–1.03 relative to free E (or M of eqn. 17).

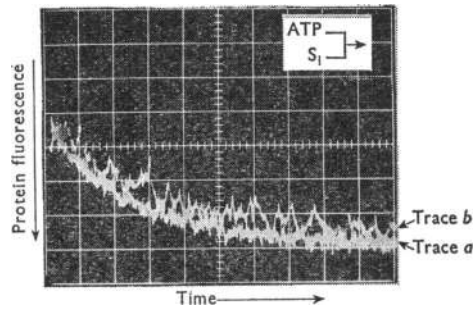

Fig. 13. *Comparison of protein-fluorescence enhancement when different concentrations of ATP interact with subfragment* 1

One syringe contained 4.35μM-subfragment 1 (S_1) and the other 5μM-ATP (trace *a*) and 500μM-ATP (trace *b*) (reaction-chamber concentrations). Both syringes contained 0.10M-KCl–5mM-$MgCl_2$–50mM-Tris adjusted to pH8.0 with HCl. Scan speeds for trace (*a*) = 100ms/division and for trace (*b*) = 5ms/division.

Discussion

Eqn. (17) (eqn. 7 of Bagshaw & Trentham, 1974) describes a mechanism of the Mg^{2+}-dependent ATPase of myosin subfragment 1. It includes two-step processes for ATP binding and ADP release. Although this mechanism is consistent with presently available kinetic evidence it is not unique, and possible alternatives are discussed below. An additional isomerization of $M^{**}\cdot ADP\cdot P_i$ is required to accommodate the results of $H_2{}^{18}O$ incorporation into P_i through the exchange reaction (Bagshaw & Trentham, 1973). The nomenclature for eqn. (17) and its relation to the nomenclature of earlier mechanisms has been described (Table 1 of Bagshaw & Trentham, 1974) and is summarized here: M represents subfragment 1 and M·ATP etc. intermediates of the ATPase mechanism; stars help to distinguish intermediates and also denote intermediates with greater protein fluorescence than M; k_{+i}, k_{-i} and K_i ($= k_{+i}/k_{-i}$) are the forward and reverse rate constants and equilibrium constant respectively of the ith step in the mechanism.

Values for the rate constants or equilibrium constants at 21°C in a reaction medium of 0.1M-KCl–5mM-$MgCl_2$–50mM-Tris adjusted to pH8.0 with HCl are, according to this mechanism (eqn. 17): K_1, $4.5\times10^3M^{-1}$; k_{+2}, $400s^{-1}$; $k_{-2} < 0.02s^{-1}$; K_3, 9; $k_{+3} \geq 160s^{-1}$; k_{+4}, $0.06s^{-1}$; $K_5 > 1.5$mM; k_{+6}, $1.4s^{-1}$; k_{-6}, $400s^{-1}$; K_7, 2.7×10^{-4}M; $k_{-4} > 3\times10^{-9}s^{-1}$, since the overall equilibrium constant [$=(k_{+1}k_{+2}\cdots k_{+7})/(k_{-1}k_{-2}\cdots k_{-7})$] is 1.7×10^7M under these conditions (Alberty, 1969). Sources for these constants

$$M + ATP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} M\cdot ATP \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} M^{*}\cdot ATP \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} M^{**}\cdot ADP\cdot P_i \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}}$$
$$M^{*}\cdot ADP\cdot P_i \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} M^{*}\cdot ADP + P_i \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} M\cdot ADP \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} M + ADP \qquad (17)$$

are: K_1, k_{+2}, k_{-6}, K_7 (Table 1); k_{-2}, K_3 (Bagshaw & Trentham, 1973); k_{+3} (Lymn & Taylor, 1971, and discussion below); k_{+4}, K_5 (Bagshaw & Trentham, 1974); k_{+6} (Bagshaw *et al.*, 1972). In assessing the value of these constants, the experimental error and variability between different protein preparations should be borne in mind and discrepancies within a factor of 2 may well be insignificant. $(k_{-q}k_{-r}+k_{+r}k_{+s})/(k_{+r}+k_{-r})$, which was required to evaluate the data of Fig. 5(*a*) according to eqn. (16), corresponds to:

$$\frac{k_{-2}k_{-3}+k_{+3}k_{+4}}{k_{+3}+k_{-3}} = \frac{k_{-2}}{1+K_3}+\frac{k_{+4}}{1+1/K_3}$$

and was $0.055\,s^{-1}$. ΔG^0 for the isomerization of M·ATP to M*·ATP $(=-RT\ln k_{+2}/k_{-2})$ is $<-24\,kJ\cdot mol^{-1}$ (i.e. ΔG^0 could be more negative), and ΔG^0 for the isomerization of M·ADP to M*·ADP $=-14\,kJ\cdot mol^{-1}$. This compares with $\Delta G^0=-4.5\,kJ\cdot mol^{-1}$ for the cleavage step.

Protein fluorescence relative to that of subfragment 1 of the intermediates of eqn. (17) is estimated to be: M·ATP and M·ADP, 1.00 to 1.03; M*·ATP, 1.10; M**·ADP·P_i, 1.19; M*·ADP, 1.067. These values are taken from the above results and those of Bagshaw & Trentham (1974). The assignment of 1.10 to M*·ATP is tentative and is based on the protein fluorescence of the complexes formed from the interaction of ATP(γS) and ATP(β,γ-NH) with subfragment 1 (Table 1). The relative fluorescence of the steady-state intermediate is 1.18 (Table 1). The assignment of 1.19 to M**·ADP·P_i is based on the the fact that the steady-state intermediate at 21°C and pH8 comprises 87% M**·ADP·P_i, 9% M*·ATP and 4% M*·ADP (Bagshaw & Trentham, 1974). The fluorescence of M*·ATP and M*·ADP has been assigned, so the relative fluorescence of M**·ADP·P_i can be calculated. The fluorescence of M*·ADP·P_i is unknown, since it cannot, as yet, be generated in sufficient concentration for analysis either during ATPase activity or by mixing ADP and P_i with subfragment 1 (Bagshaw & Trentham, 1974). Insofar as direct comparisons can be made, the relative fluorescence of intermediates agrees with the values of Werber *et al.* (1972) and Mandelkow & Mandelkow (1973).

In addition to the experiments reported here evidence in favour of a two-step binding mechanism is afforded by the H^+-release experiments of Bagshaw & Trentham (1974). E. W. Taylor & J. F. Koretz (personal communication) also favour a two-step binding mechanism based on their H^+ release experiments. Further evidence comes from studies with bovine cardiac myosin subfragment 1 which showed that observed rates of protein fluorescence enhancement reached a plateau when ATP or ATP analogues were mixed with the protein (R. S. Taylor, A. G. Weeds, C. R. Bagshaw & D. R. Trentham, unpublished work). The saturation rates with the cardiac protein were lower and a more favourable signal-to-noise ratio was obtained in experiments analogous to those described in Fig. 2(*b*), 4(*b*) and 6(*b*).

Two-step binding mechanisms could involve substrate or protein isomerization in addition to or instead of isomerization of the binary complex. The kinetic results are incompatible with substrate isomerization because, as noted above in relation to bovine cardiac subfragment 1, the observed saturation rates which depend on the isomerization rate constant are specific to the source of the myosin. Further, isomerization of reactive and inactive forms of substrate reveals itself as a rate-limiting phenomenon in substrate binding at low rather than high substrate concentrations, when the reactive form becomes deficient, in contrast with what is observed here (Gutfreund, 1972, p. 63).

Protein isomerization is not excluded as an alternative mechanism on the available evidence. For example, eqn. (18) is a plausible mechanism for the first two steps of eqn. (17).

$$M \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftharpoons}} M^*+ATP \underset{k'_{-2}}{\overset{k'_{+2}}{\rightleftharpoons}} M^*\cdot ATP \qquad (18)$$

Eqn. (18) leads formally to different kinetics from eqn. (17) (Halford, 1971), but the experiments that we have been able to do are insufficient to make the distinction. The values of the rate constants in eqn. (18) correspond to their counterparts in eqn. (17) as follows: k'_{+1} with k_{+2}, k'_{-2} with k_{-2} and $k'_{+1}k'_{+2}/k'_{-1}$ with K_1k_{+2}.

$$\begin{array}{ccc} M+ATP & \underset{k''_{-2}}{\overset{k''_{+2}}{\rightleftharpoons}} & M^*\cdot ATP \longrightarrow \\ k''_{+1}\Big\uparrow\Big\downarrow k''_{-1} & & \\ M\cdot ATP & & \end{array} \qquad (19)$$

Eqn. (19) illustrates a second alternative, which involves two distinct binding modes of ATP. A plateau in the rate of formation of M*·ATP at high [ATP] will be observed when $k''_{+2}/k''_{-2} > k''_{+1}/k''_{-1}$ but $k''_{+2} < k''_{+1}$. Under these conditions, on mixing M and ATP, the initial phase leads to predominantly M·ATP formation, and the observed saturation rate represents the rate of the transformation of M·ATP to M*·ATP in a flux through M. The values of the rate constants in eqn. (19) correspond to their counterparts in eqn. (17) as follows: $k''_{+2}k''_{-1}/k''_{+1}$ with k_{+2}; k''_{-2} with k_{-2}; and k''_{+1}/k''_{-1} with K_1. Theoretically this substrate-binding scheme is distinguishable from that in eqn. (17) because the initial rate of M*·ATP formation will depend on [ATP] in eqn. (19), but not in eqn. (17) (Viale, 1971).

However, in practice the amplitude of the initial phase of M*·ATP formation is small relative to total M*·ATP formation when $k''_{+2} < k''_{+1}$ and, in any event, the signal of this phase, even if it were observed, could not be assigned specifically to M·*ATP (as opposed to M·ATP) formation by our fluorescence technique.

It should be noted that in the model of ADP binding equivalent to eqn. (19), the complex generated by mixing ADP with subfragment 1 gives M·*ADP. This is the same intermediate as is generated during the steady-state hydrolysis of ATP, which has been shown by Bagshaw & Trentham (1974) to be a necessary feature of any ATPase mechanism.

In a third alternative it is possible to postulate the same two-step binding mechanism for ATP as in eqn. (17), but with the observed second-order rate constant ($1.8\times10^6\,M^{-1}\cdot s^{-1}$) (Table 1) being equal to k_{+1} instead of K_1k_{+2} as would arise if k_{-1} was less than k_{+2}. A feature of this model is that a graph of $k_{obs.}$ against [ATP] deviates from a hyperbola. Intermediate situations are also plausible when k_{-1} is comparable with k_{+2}. A hyperbola is only obtained when $k_{-1} \gg k_{+2}$, and the deviation from the hyperbola becomes more apparent as k_{-1} is decreased. The observed deviation of the points from the solid line of Fig. 5(*a*) may arise because of incomplete equilibration of M and M·ATP before isomerization to M*·ATP, as would arise if k_{+1} was about $10^7\,M^{-1}\cdot s^{-1}$, but the range of experimental error in Fig. 5(*a*) does not allow further analysis of the data. If $k_{-1} < k_{+2}$ or the intermediate situation is correct, there is insufficient information to evaluate K_1. k_{+2} would lie between $330\,s^{-1}$, the maximum observed rate in Fig. 5(*a*), and $400\,s^{-1}$, the value obtained from the graphical analysis which assumes $k_{-1} \gg k_{+2}$. In principal, if the equilibrium of M and M·ATP is incomplete before the isomerization, there would be a lag phase in the protein-fluorescence change. However, the rather low signal-to-noise ratio at high [ATP] coupled with the lack of precise knowledge of the magnitude of M·ATP fluorescence does not mean that the failure to observe a lag phase excludes a model with incomplete equilibration.

A feature that eqn. (19) and eqn. (17) (with $k_{+1} = 1.8\times10^6$ M) have in common but in contrast with eqn. (17) (with $K_1k_{+2} = 1.8\times10^6\,M^{-1}\cdot s^{-1}$) is that the second-order rate constants for ATP binding to subfragment 1 (k''_{+2} in eqn. 19) are one to two orders of magnitude less than typical protein–ligand association rate constants (Gutfreund, 1971). The factor is even greater with the ATP analogues, ATP(β,γ-NH) and ATP(α,β-CH_2), or when myosin from slow muscle (cat soleus) is used (Weeds *et al.*, 1974). For eqn. (18) the second-order rate constant, k''_{+2}, could be $10^8\,M^{-1}\cdot s^{-1}$ for ATP as substrate, but would have to be one to two orders of magnitude less than this for ATP(β,γ-NH) and ATP(α,β-CH_2) as k'_{+1} and k'_{-1} are independent of the substrate. The kinetic studies of P_i binding to subfragment 1 are not compatible with eqn. (18), although the range of experimental error in these studies precludes a firm conclusion (Bagshaw & Trentham, 1974). It is for these reasons that on balance we prefer eqn. (17) to the alternative schemes. As a working hypothesis nucleotide recognition by the protein influences K_1, probably the major effect being on k_{-1}, whereas the kinetics of the isomerization process are specific to the type of muscle and influence k_{+2}. However, low apparent second-order rate constants can arise because rapid equilibria, either of conformation or protonation, partition the protein or the substrate into the reactive and inactive forms. Unequivocal assignment of a precise two-step mechanism will be difficult, although improvements in instrumentation and kinetic analysis of ATP or ATP-analogue binding to subfragment 1 in the presence of actin should remove some of the ambiguity.

The observed rates at which ATP and ATP(β,γ-NH) dissociate from subfragment 1 are $<0.02\,s^{-1}$ and $0.02\,s^{-1}$ respectively (Bagshaw *et al.*, 1972; Bagshaw & Trentham, 1973). This means that in a one-step binding mechanism there is an *a priori* requirement for a lag phase in the formation of M**·ADP·P_i when certain ATP concentrations are mixed with the protein (Bagshaw *et al.*, 1972). For a two-step binding mechanism (eqn. 17) this requirement is not necessary. The rate of formation of M**·ADP·P_i has been measured under conditions in which a lag phase was expected according to a one-step binding mechanism. The rate profile did show some lag-phase character, though there was a systematic deviation from the theoretical lag-phase curve required of the one-step binding mechanism (Bagshaw *et al.*, 1972). If k_{+3} is comparable with k_{+2} then a lag phase in M**·ADP·P_i formation will be evident at high ATP concentrations even in the two-step binding mechanism (eqn. 17). Under these conditions transient H^+ release (controlled by k_{+2} alone; Bagshaw & Trentham, 1974) would not occur coincidently with M**·ADP·P_i formation, and there is some evidence for this (Koretz *et al.*, 1972). Resolving the kinetics of the transformation of M·ATP to M*·ATP and the cleavage step in order to evaluate the separate rate constants, k_{+3} and k_{-3}, remains an important problem of the ATPase mechanism. The approach for measuring the equilibrium constant of the cleavage step, K_3 (=9), is valid on either a one- or a two-step binding mechanism. However, the values of rate constants for the cleavage step described by Bagshaw & Trentham (1973) were applicable only to the one-step binding mechanism, since evaluation of k_{+3} and k_{-3} requires knowledge of the sum, $k_{+3}+k_{-3}$.

If eqn. (17) is valid for subfragment 1, it is important to question whether it is also valid for myosin

and heavy meromyosin. On all points where direct comparisons have been made, the mechanisms for the three proteins appear to be identical. Rates of H^+ release and rapid cleavage of ATP in single turnover experiments or in the transient phase, rates of protein fluorescence change when ATP and protein are mixed, and steady-state rates of ATPase activity are all similar in comparable reaction media (Lymn & Taylor, 1971; Koretz *et al.*, 1972; Bagshaw & Trentham, 1973; Weeds *et al.*, 1974).

A number of workers using a range of techniques have produced evidence for protein conformation changes when ATP, ADP or ATP analogues interact with myosin or its proteolytic subfragments. In most cases major changes are induced by ATP and lead to a conformation of the predominant steady-state intermediate which is $M^{**} \cdot ADP \cdot P_i$ at room temperature. Changes induced by ADP are indicative of the conformation $M^* \cdot ADP$, whereas ATP(γS) and ATP(β,γ-NH) probably induce a conformation equivalent to $M^* \cdot ATP$. Representative examples are described by Cheung & Morales (1969), dos Remedios *et al.* (1972), Morita (1967), Seidel & Gergely (1972), Watterson & Schaub (1973) and Werber *et al.* (1972). The relevance of the work described here is that nucleotide binding to subfragment 1 has been characterized as a two-step process in which the unimolecular step for ATP binding may well involve a major conformation change in view of the large negative free-energy change. A wide range of experiments by Boyer *et al.* (1973, 1974) indicate a striking parallel between the mechanisms of mitochondrial ATP synthesis and the myosin ATPase in that both appear to involve two states of ATP-bound protein with widely differing ATP affinities. This suggests a prominent role of nucleotide binding in energy transduction during muscle contraction and other processes in biology.

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