Magnesium Ion Dependent Rabbit Skeletal Muscle Myosin Guanosine and Thioguanosine Triphosphatase Mechanism and a Novel Guanosine Diphosphatase Reaction†

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ABSTRACT: The mechanism of the Mg^{2+}-dependent myosin subfragment 1 catalyzed hydrolysis of GTP and 2-amino-6-mercapto-9-β-ribofuranosylpurine 5′-triphosphate (thioGTP) has been investigated by rapid-reaction techniques. The myosin was isolated from rabbit skeletal muscle. The steady-state intermediate of these reactions consists predominantly of a protein–substrate complex unlike the myosin subfragment 1 ATPase reaction which has a protein–products complex as the principal steady-state component. The mechanism of GTP hydrolysis catalyzed by subfragment 1 has other marked differences from the ATPase mechanism. The second-order rate constant of binding of GTP to subfragment 1 is tenfold greater than that for GDP binding. The dissociation rate constant of GDP from subfragment 1 is 0.06 s^{-1} compared with the subfragment 1 catalytic center activity for GTP hydrolysis of 0.5 s^{-1} at pH 8.0 and 20 °C. This shows that GDP bound to subfragment 1 forms a complex which is not kinetically competent to be an intermediate of the GTPase mechanism. GDP is hydrolyzed in the presence of subfragment 1 to GMP and P_i. The subfragment 1 GTPase mechanism has a number of features in common with that of the elongation factor Tu GTPase of the protein biosynthetic system of *Escherichia coli*.

Chromophoric nucleotides derived from 2-amino-6-mercapto-9-β-ribofuranosylpurine (thioguanosine) have been used to measure properties of muscle proteins. For example, Bagshaw et al. (1972) used thioguanosine nucleotides to study the rate constants of certain steps in the ATPase mechanism of myosin subfragment 1 and intermediates of rabbit skeletal subfragment 1 thioGTPase[1] can be characterized by their absorption (Eccleston & Trentham, 1977) and circular dichroic spectra (J. F. Eccleston and P. M. Bayley, unpublished work). It is important, therefore, to see if intermediates of the subfragment 1 thioGTPase can be related to those of the subfragment 1 ATPase.

Bagshaw et al. (1974) proposed the mechanism shown in eq 1 for the Mg^{2+}-dependent ATPase of myosin subfragment 1. k_{+i} and k_{-i} are forward and reverse rate constants, and

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

K_i is the equilibrium constant of the ith step. Values for the rate constants or equilibrium constants at 21 °C in 0.1 M KCl, 5 mM $MgCl_2$, 50 mM Tris adjusted to pH 8.0 with HCl are K_1, 4.5×10^3 M^{-1}; k_{+2}, 400 s^{-1}; k_{-2}, 2×10^{-6} s^{-1}; K_3, 9; $k_{+3} > 160$ s^{-1}; k_{+4}, 0.06 s^{-1}; $K_5 > 1.5$ mM; k_{+6}, 1.4 s^{-1}; k_{-6}, 400 s^{-1}; K_7, 2.7×10^{-4} M (Trentham et al., 1976). Recent work has refined the rate constants of the fast steps 1–3 (Johnson & Taylor, 1978; Geeves et al., 1979; Chock et al., 1979), though eq 1 suffices for the present purposes.

Essential features of this mechanism are (a) two-step binding of ATP and ADP; (b) a rapidly reversible cleavage step; (c) a steady-state intermediate whose principal component is a subfragment 1–products complex; and (d) a subfragment 1–products isomerization which is rate limiting in the overall hydrolysis.

Eccleston & Trentham (1977) have shown that the hydrolysis of thioITP proceeds by a mechanism similar to ATP hydrolysis, although rate constants for particular steps may be different. However, the results presented here show that the subfragment 1 catalyzed hydrolysis of thioGTP differs in certain respects from that of ATP and thioITP. Since thioGTP and thioITP differ structurally due to the 2-amino group of the former and the same group is one of the differences between GTP and ATP, the subfragment 1 GTPase mechanism has also been studied.

Energy transduction involving nucleotide triphosphate hydrolysis is usually linked to ATP hydrolysis. However, this is not always the case and it is interesting to consider how the myosin subfragment 1 catalyzed GTP hydrolysis compares with systems in which GTPase activity occurs such as in protein biosynthesis.

Materials and Methods

Proteins. Subfragment 1 was prepared from myosin extracted from rabbit skeletal muscle and characterized as described by Eccleston & Trentham (1977). For the quenched-flow experiments, the concentration of active sites of subfragment 1 was determined by titrating the protein with increasing known concentrations of ATP in a stopped-flow fluorometer and measuring the amplitude of the rapid increase in fluorescence on binding of ATP to subfragment 1. The amplitude of the observed fluorescence change increases linearly with ATP concentration until it equals that of subfragment 1 at which point the amplitude remains constant (Chock & Eisenberg, 1979). The subfragment 1 protein concentration was measured by using $E_{1cm}^{1\%} = 7.7$ at 280 nm and mol wt 115 000 (Lowey et al., 1969). Comparison of active site to total protein concentration showed the subfragment 1 was typically 80% active. Subfragment 1 A1 and A2 isoenzymes were prepared by the method of Weeds &

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[1] Abbreviations used: thioITP, 6-mercapto-9-β-ribofuranosylpurine 5′-triphosphate; thioGTP, 2-amino-6-mercapto-9-β-ribofuranosylpurine 5′-triphosphate; NTP, nucleoside 5′-triphosphate; ATPase, adenosine triphosphatase; GTPase, guanosine triphosphatase; butyl-PBD, 5-(4-biphenylyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EF-Tu, elongation factor Tu.

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Taylor (1975) following chymotryptic digestion of myosin.

Actin was extracted from acetone dried powder (Barany et al., 1954) with 20 vol of 0.1 mM ATP, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, and 2 mM Tris adjusted to pH 8.0 with HCl for 45 min at 0 °C. The filtrate from this extraction was treated with 50 mM KCl and 5 mM $MgCl_2$ for 1 h at 20 °C and then with 0.6 M KCl for a further 2 h at 20 °C (Spudich & Watt, 1971). F-actin was removed by centrifugation and the pellet depolymerized in 0.2 mM ATP, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, and 2 mM Tris adjusted to pH 8.0 with HCl. The G-actin was polymerized with 50 mM KCl, 2 mM $MgCl_2$ and the resulting F-actin washed by repeated centrifugation and suspension in 50 mM KCl, 2 mM $MgCl_2$, 2 mM Tris adjusted to pH 8.0 with HCl until excess ATP had been removed as determined by absence of 260 nm absorption in the supernatant. The concentration of actin was determined by using $E_{1cm}^{1\%} = 11.08$ (West et al., 1967) at 280 nm and mol wt of 42000 (Elzinga et al., 1973). The absence of excess ATP in the actin was also established in linked assays in the presence of subfragment 1 and an NTP-regenerating system (see below).

Nucleotides. ThioGTP was synthesized from thioguanosine as described by Eccleston & Trentham (1977). [γ-^{32}P]-ThioGTP and [γ-^{32}P]GTP were prepared by the method of Glynn & Chappell (1964) by using, in a typical synthesis, 10 μmol of the unlabeled nucleoside 5′-triphosphate and 2 mCi of [^{32}P]P_i. The nucleotides were purified on a Dowex 1 column as described by Glynn & Chappell (1964) except that they were eluted with 0.4 M HCl. TLC of the products on a PEI-cellulose plate eluted with 0.75 M potassium phosphate adjusted to pH 3.4 with HCl showed that the nucleotides [γ-^{32}P]thioGTP and [γ-^{32}P]GTP were at least 97% pure with respect to ^{32}P radioactivity.

Other Reagents. Other enzymes were purchased from Boehringer Corp. Ltd. All other reagents were analytical reagent grade where possible and used without further purification. Water was doubly distilled.

Rapid Reaction Equipment. The quenched-flow apparatus used has been described by Gutfreund (1969) and the stopped-flow apparatus for measuring absorption or fluorescence changes by Gutfreund (1972) and Bagshaw et al. (1972), respectively. Data from stopped-flow experiments were either recorded photographically or collected and processed as described by Hardman et al. (1978) as is illustrated in Figure 4.

The concentrations of proteins and nucleotides given for the stopped-flow experiments are those in the reaction chamber after mixing, so that the actual syringe concentrations are double these values.

Other Methods. Reaction mixtures from quenched-flow experiments were quenched into an equal volume of 10% perchloric acid, rapidly neutralized with 4 M sodium acetate and separated on PEI (poly(ethylenimine))-cellulose plates (Polygram Cel 300 PEI/U_{254}; Macherey, Nagel and Co., Düren, West Germany). NTP and P_i spots (the latter running identically with AMP) were cut out, eluted with 0.5 mL of 0.1 M HCl and ^{32}P counted after the addition of 15 mL of butyl-PBD scintillation fluid (Bagshaw & Trentham, 1973).

Steady-state rates of acto-subfragment 1 catalyzed NTP hydrolysis were measured by following the rate of oxidation of NADH in a system containing 100 μM NTP, 1 mM phosphoenolpyruvate, 0.15 mM NADH, 10 μg/mL rabbit muscle pyruvate kinase, 10 μg/mL lactate dehydrogenase, 2 mM $MgCl_2$, 10 mM KCl, 0.1 mM dithiothreitol, 10 mM Tris adjusted to pH 8.0 with HCl, subfragment 1 (concentrations as appropriate), and F-actin at concentrations indicated in the text. For reactions involving thioGTP, NADH oxidation was followed at 366 nm by using ϵ_{366} 3.3 × 10^3 L mol^{-1} cm^{-1} for NADH in order to avoid excessively high absorbances, since thioGTP absorbs maximally at 342 nm.

Steady-state rates of acto-subfragment 1 catalyzed GTP hydrolysis were also measured under nitrogen atmosphere at pH 8.0 and 25 °C by using a Radiometer pH stat. The solutions contained 4 mM GTP, 5 mM $MgCl_2$, 16 mM KCl, 0.125 mg/mL subfragment 1, and actin in the range 0–1.53 mg/mL.

High voltage electrophoresis in 0.5% pyridine–5% acetic acid at pH 3.5 was carried out as described by Eccleston & Trentham (1977). Nucleotides and P_i were detected as described therein. The time course of [^{14}C]GDP hydrolysis was followed by using autoradiography.

^{31}P nuclear magnetic resonance spectra were recorded at 145.7 MHz on a Bruker WH 360/180 spectrometer, equipped with a deuterium field lock, and operating in the Fourier transform mode. The sample solution (2 mL) was in a 1-cm diameter tube equipped with a Teflon Vortex suppressor and was maintained at 22 °C. For each spectrum, 30 transients were summed by using 10000 Hz spectral width, a pulse width of 23 μs, and an acquisition time of 0.82 s. A sensitivity enhancement exponential function gave a line broadening of 10 Hz.

Theory. For the purpose of the present analysis, the following scheme is sufficient to describe the binding of ATP and GTP to subfragment 1

$$M + ATP \xrightarrow{k_a} MA$$

$$M + GTP \xrightarrow{k_b} MG$$

where MA and MG represent the steady-state complexes of the subfragment 1 ATPase and GTPase, respectively, at 20 °C. Reaction conditions were chosen so that [ATP], [GTP] ≫ [M], and the concentration ranges of ATP and GTP were such that both reactions were first order in ATP and GTP, respectively. So k_a and k_b are second-order rate constants.

It follows that

$$d[M]/dt = -(k_a[ATP] + k_b[GTP])[M]$$

and

$$[M] = [M]_0 e^{-(k_a[ATP]+k_b[GTP])t}$$

where $[M]_0$ is the initial concentration of subfragment 1

$$d[MA]/dt = k_a[ATP][M] = k_a[ATP][M]_0 e^{-(k_a[ATP]+k_b[GTP])t}$$

so that

$$[MA] = \frac{k_a[ATP][M]_0}{k_a[ATP] + k_b[GTP]}(1 - e^{-(k_a[ATP]+k_b[GTP])t})$$

The fluorescence change observed on mixing ATP and GTP with subfragment 1 is due to the formation of MA since MG and M have the same fluorescence (see Results). Werber et al. (1972) reported that the binding of GTP induces a 4% fluorescence change in heavy meromyosin (cf. ATP, 17% change). Such a change, if present, would not affect the value of k_{obsd} (eq 2). However, the amplitude of the fluorescence change is dependent on the fluorescence of MA and MG.

It follows that the rate constant of the exponential increase in fluorescence, k_{obsd}, for simultaneous ATP and GTP binding is given by

$$k_{obsd} = k_a[ATP] + k_b[GTP] \quad (2)$$

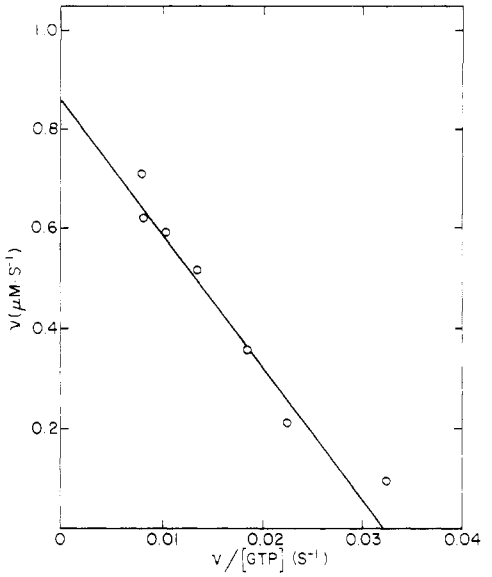

FIGURE 1: Hydrolysis of GTP catalyzed by subfragment 1 at 20 °C and pH 8.0. From a plot of velocity, v, against $v/[GTP]$, the catalytic center activity and K_m for GTP were calculated. The reaction mixture contained 0.1 mM NADH, 1 mM phosphoenolpyruvate, 33 μg/mL pyruvate kinase, 33 μg/mL lactate dehydrogenase, 5 mM $MgCl_2$, 0.1 M KCl, 50 mM Tris adjusted to pH 8.0 with HCl and 2 μM subfragment 1. The decrease in A_{340} was measured and the reaction was initiated by the addition of GTP to a final concentration of 2.8–94 μM.

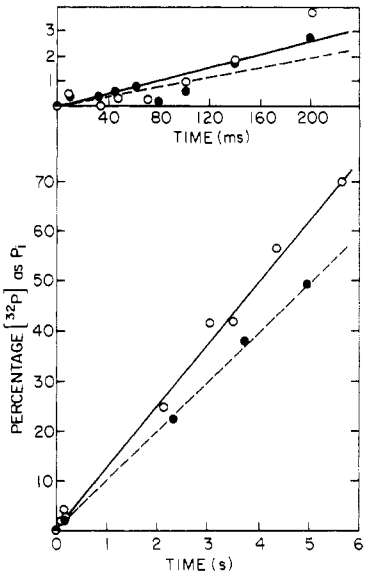

FIGURE 2: Rate of $[^{32}P]P_i$ production in the reaction of subfragment 1 with $[\gamma\text{-}^{32}P]$thioGTP and with $[\gamma\text{-}^{32}P]$GTP in the quenched-flow apparatus at 20 °C. (●---●) Reaction mixture contained 10 μM subfragment 1, 50 μM $[\gamma\text{-}^{32}P]$thioGTP, 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.1 M Mes adjusted to pH 6.5 with KOH. (○—○) Reaction mixture contained 10 μM subfragment 1, 50 μM $[\gamma\text{-}^{32}P]$GTP, 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.1 M Tris adjusted to pH 8.0 with HCl. The points are experimental values. The lines in the top graph are the steady-state rates determined from the lower graph.

Similar analyses are valid when, for example, the kinetics of GDP are measured through a study of the simultaneous binding of thioITP and GDP (Figure 4).

Results

Rate of Cleavage of ThioGTP and GTP by Subfragment 1. Both thioGTP and GTP are substrates for subfragment 1 in the presence of Mg^{2+}. With thioGTP as substrate, the K_m is 22 μM and k_{cat} is 0.35 s^{-1} at 20 °C and pH 8.0 (Eccleston & Trentham, 1977). With GTP as substrate, K_m and k_{cat} are 27 μM and 0.5 s^{-1}, respectively (Figure 1).

The predominant steady-state intermediate of the Mg^{2+}-dependent ATPase and thioITPase of subfragment 1 at pH 8 and 20 °C is a protein–products complex (i.e., M**·ADP·P_i in eq 1) (Lymn & Taylor, 1970; Eccleston & Trentham, 1977). Evidence for this was provided by following the time course of $[^{32}P]P_i$ production on rapidly mixing subfragment 1 with a several-fold molar excess of $[\gamma\text{-}^{32}P]$ATP. Rapid P_i formation was observed prior to steady-state P_i release. The same type of experiment was carried out to determine whether this phenomenon also occurred in the subfragment 1 catalyzed hydrolysis of thioGTP. In a typical experiment, 10 μM subfragment 1 was rapidly mixed with 50 μM $[\gamma\text{-}^{32}P]$thioGTP in a quenched-flow apparatus and the reaction stopped at times between 10 and 200 ms, and at approximately 1-s intervals between 2 and 5 s. Figure 2 shows that, in the reaction of subfragment 1 with thioGTP in 0.1 M KCl at pH 6.5, the rate of production of $[^{32}P]P_i$ during the first 200 ms was identical with the steady-state rate. Extrapolating this rate back to zero time shows there was no rapid $[^{32}P]P_i$ production. This behavior is in marked contrast to the result with ATP. Thus there is no rapid phase of thioGTP cleavage and M*·thioGTP (by analogy with eq 1) rather than M**·thioGDP·P_i is the steady-state intermediate of the thioGTPase.

Since lowering the pH from 8.0 to 6.9 and the temperature to 3 °C decreases the equilibrium constant of the cleavage step, K_3, of the ATPase from 9 to 1 (Taylor, 1977), the hydrolysis of thioGTP was also followed at pH 8 by using the same conditions as at pH 6.5 except that 0.1 M Tris adjusted to pH 8.0 with HCl replaced 0.1 M Mes. Again, no rapid $[^{32}P]P_i$ production was detected.

The time course of GTP hydrolysis was similarly investigated. Figure 2 shows that, in 0.1 M KCl and 0.1 M Tris adjusted to pH 8.0 with HCl, no rapid $[^{32}P]P_i$ production occurred on rapidly mixing 10 μM subfragment 1 with 50 μM $[\gamma\text{-}^{32}P]$GTP. An identical result was obtained at pH 6.5 in 20 mM KCl.

At pH 8.0 and 0.1 M KCl, the reaction of subfragment 1 with excess thioITP exhibited rapid P_i formation (Eccleston & Trentham, 1977). This result was reproduced, but no transient phase was detected when the pH was changed to 6.5 or when the KCl concentration was reduced to 20 mM.

An alternative approach to characterizing the nature of the steady-state intermediate of an enzyme-catalyzed reaction is by means of a single turnover experiment in which substrate is mixed with a molar excess of enzyme and the reaction stopped after substrate binding but before product release has occurred. Bagshaw & Trentham (1973) used this technique in order to measure the equilibrium constant of the cleavage step of subfragment 1 catalyzed ATP hydrolysis (K_3, eq 1). ATP binding to subfragment 1 is fast (1.2×10^6 M^{-1} s^{-1}) and product release slow (0.05 s^{-1}) so that, when $[\gamma\text{-}^{32}P]$ATP is mixed with a molar excess subfragment 1, the relative amount of M*·ATP and M**·ADP·P_i can be measured by stopping the reaction at 2 s with acid. This method of characterizing the nature of protein-bound nucleotide is more difficult to make with the subfragment 1 catalyzed hydrolysis of thioGTP and GTP since both nucleotides bind to subfragment 1 with a slower rate constant than ATP and the turnover rate is an order of magnitude greater than that for ATP hydrolysis. Thus larger corrections are needed to allow for unbound nucleoside triphosphate and for P_i released into the medium as product.

A 20 μM solution of subfragment 1 was mixed with 5 μM $[\gamma\text{-}^{32}P]$thioGTP at pH 6.5 in 0.1 M KCl and the total amount

Table I: Concentration of Free ThioGTP and Subfragment 1 Bound ThioGTP during a Single Turnover of Subfragment 1 ThioGTPase[a]

quenching time (s)	% of ^{32}P present as [γ-^{32}P]thioGTP: total thioGTP	free thioGTP
0	98.0	
2.2	68.5	
60	2.7	
2.3 cold chase		26.8

[a] The experiment was carried out with 20 μM subfragment 1 and 5 μM [γ-^{32}P]thioGTP (concentrations after mixing) at 18 °C in 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.1 M Mes adjusted to pH 6.5 with KOH. The analysis of [γ-^{32}P]thioGTP remaining at various times is described under Materials and Methods. The cold chase experiment gives a measure of free thioGTP present at 2.3 s. In this experiment, the reaction solution was added to 1.3 mM MgATP in the same solvent, and then the mixture was quenched with acid after 60 s. Any M*·thioGTP present at 2.3 s is presumed to hydrolyze, while free thioGTP is prevented from binding to subfragment 1 by the large pool of ATP (Bagshaw & Trentham, 1973).

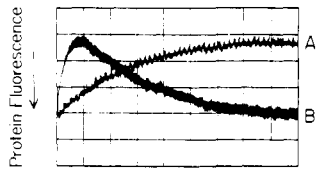

FIGURE 3: Stopped-flow spectrophotometric record of protein fluorescence during a single turnover of the subfragment 1 thioGTPase at 20 °C and pH 6.5. Concentrations of subfragment 1, thioGTP, and solvent conditions are as in Table I. Trace A was recorded at 50 ms/div and trace B at 0.5 s/div.

of [γ-^{32}P]thioGTP and [^{32}P]P_i present at 2.2 s was measured. [^{32}P]P_i present at 2.2 s was 31.5% of the total radioactivity (Table I) and arises from either [^{32}P]M**·thioGDP·P_i or [^{32}P]P_i in the medium. The amount of P_i released into the medium can be calculated from a knowledge of the kinetic parameters. The second-order rate constant for the association of subfragment 1 with thioGTP is 1.6×10^5 M^{-1} s^{-1} (Eccleston & Trentham, 1977) which leads to a pseudo-first-order rate constant of 3.2 s^{-1} in this experiment (eq 3).

$$\mathrm{M} + \mathrm{thioGTP} \xrightarrow{3.2\ s^{-1}} (\mathrm{M^*\cdot thioGTP} \rightleftharpoons \mathrm{M^{**}\cdot thioGDP\cdot P_i}) \xrightarrow{0.20\ s^{-1}} \mathrm{M} + \mathrm{GDP} + \mathrm{P_i} \quad (3)$$

A stopped-flow experiment of the same reaction mixture gave the spectrofluorometric record shown in Figure 3. Numerical analysis of these records showed that, while the association rate constant (the rapid phase) was in good agreement with the value of 3.2 s^{-1}, the rate constant of the slower phase was best fitted by a value of 0.20 s^{-1} (cf. the k_{cat} value of 0.6 s^{-1} recorded by Eccleston & Trentham, 1977). If the rate constants (3.2 and 0.20 s^{-1}) are assigned to steps of the thioGTPase (eq 3), where 0.20 s^{-1} is the overall rate constant of breakdown of the M*·thioGTP–M**·thioGDP·P_i intermediate, then 31% of the total ^{32}P would have been released into the medium as [^{32}P]P_i at 2.2 s. This is good agreement with the 31.5% thioGTP breakdown found (Table I). Thus, M**·thioGDP·P_i was essentially absent (<5% total nucleotide) at 2.2 s, while M*·thioGTP comprised 41.7% of the total nucleotide at that time (Table I), indicating that M*·thioGTP is the predominant intermediate. This supports the transient kinetic result (Figure 2) that the initial rate of thioGTP cleavage equals the steady-state hydrolysis rate.

Rate of Binding of GTP to Subfragment 1 and Acto-Subfragment 1. Measurement of the rate of binding of GTP to subfragment 1 presents certain difficulties. No protein fluorescence change could be detected when 2 μM subfragment 1 was mixed with 80 μM GTP in a stopped-flow fluorometer at pH 8.0 (which contrasts with the fluorescent enhancement when ATP binds). The binding of ATP to subfragment 1 is accompanied by the release of 0.22 mol of protons/mol of subfragment 1 in 0.5 M KCl, 5 mM $MgCl_2$ at pH 8.0 and 21 °C (Koretz & Taylor, 1975) and so the reaction can be followed by the use of a suitable pH indicator. Under these conditions, using phenol red as indicator, we reproduced this result on mixing 20 μM ATP with 10 μM subfragment 1. However, when GTP was substituted for ATP, transient proton release prior to steady-state GTP hydrolysis was barely detectable so that neither the rate nor amplitude of the small transient phase could be measured with sufficient accuracy.

Since no direct signal was available to follow the binding of GTP to subfragment 1, the effect of GTP on the binding of ATP to subfragment 1 was measured. It is shown in the theory section that when two ligands are simultaneously mixed with protein, the observed association rate constant is equal to the sum of the rate constants of the two ligands binding to the protein separately. Hence the difference between the observed rate constants of the fluorescence changes caused by ATP and GTP binding simultaneously and by the same concentration of ATP alone equals the association rate constant of GTP binding to subfragment 1.

The effect of GTP on the binding of 10 μM ATP to 2 μM subfragment 1 was measured in 0.1 M KCl, 5 mM $MgCl_2$, and 50 mM Tris at pH 8.0. For example, the rate of ATP binding to subfragment 1 was increased from 12.4 to 21.6 s^{-1} by the presence of 80 μM GTP at 20 °C. This corresponds to an observed association rate constant of GTP to subfragment 1 of 1.15×10^5 M^{-1} s^{-1}. The mean observed association rate constant of GTP binding to subfragment 1 over the range 40 μM to 160 μM GTP was 1.0 (±0.15 SD) $\times 10^5$ M^{-1} s^{-1}.

The rate of binding of GTP to acto-subfragment 1 was measured at 20 °C in the stopped-flow fluorometer by following the decrease in scattered light at 350 nm on dissociation of acto-subfragment 1 by GTP at 20 °C. Acto-subfragment 1 (5 μM subfragment 1 and 7.5 μM F-actin) was mixed with 25–400 μM GTP in 0.1 M KCl, 5 mM $MgCl_2$, and 50 mM Tris adjusted to pH 8.0 with HCl. Exponential rate processes were observed whose rate constants were directly proportional to GTP concentration. The line drawn through a graph of the observed rate constants vs. GTP concentration passed through the origin. Thus an apparent second-order rate constant for the binding could be calculated and was 1.1×10^5 M^{-1} s^{-1}. Further the apparent rate constant for the reverse process was zero (<1 s^{-1} within the limits of experimental error), since otherwise the line would not have passed through the origin.

Interaction of GDP with Subfragment 1. The rate of binding of GDP to subfragment 1 was also determined in a competition experiment. Figure 4 shows that, when 100 μM GDP and 10 μM thioITP were mixed with subfragment 1, the rate of binding of thioITP was 8.52 s^{-1} compared with 6.91 s^{-1} when 10 μM thioITP alone was mixed with subfragment 1. By using the same treatment of results as described for GTP, the second-order association constant was calculated to be 1.5×10^4 M^{-1} s^{-1}.

The dissociation rate of GDP from subfragment 1 could be determined by displacing GDP with either ATP or thioITP as described by Bagshaw et al. (1972). If 100 μM GTP was added to 2 μM subfragment 1 and then mixed with 100 μM thioITP within 1 min, the displacement of the nucleotide from the steady-state intermediate of the subfragment 1 GTPase

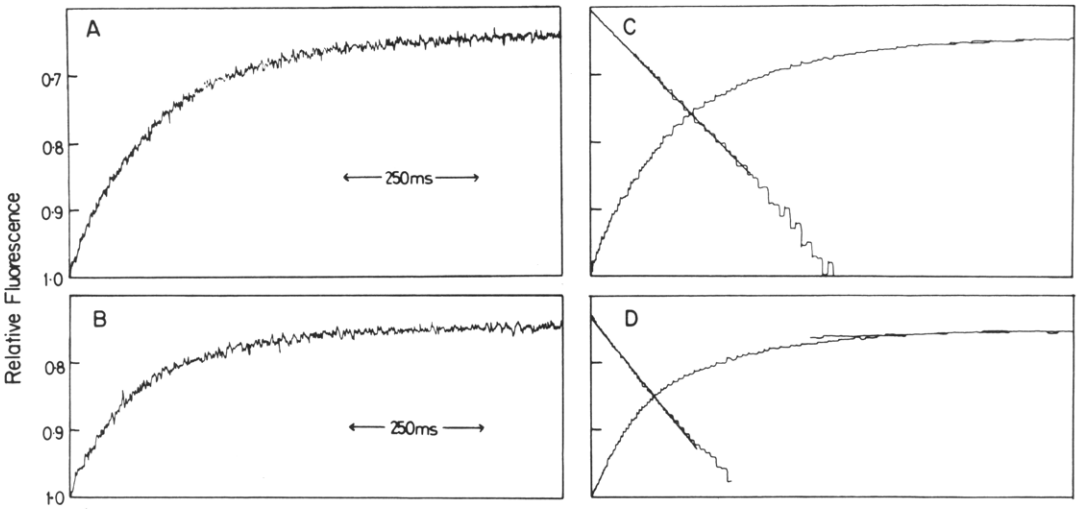

FIGURE 4: Stopped-flow spectrophotometric record of the effect of GDP on the binding of thioITP to subfragment 1 at 20 °C. In A, one syringe contained 2 μM subfragment 1 and the other 10 μM thioITP. In B, one syringe contained 2 μM subfragment 1 and the 10 μM thioITP and 100 μM GDP. All syringes also contained 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 50 mM Tris adjusted to pH 8.0 with HCl. C and D are computed averages of four traces of A and B, respectively. Also shown are log plots of the data and a linear least-squares regression line which gives a value of 6.91 s^{-1} (SD = 0.23 s^{-1}) for C and 8.52 s^{-1} (SD = 0.44 s^{-1}) for D.

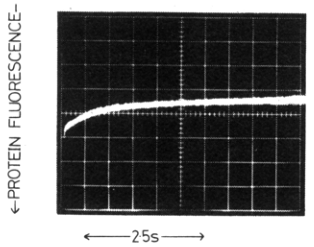

FIGURE 5: Stopped-flow spectrophotometric record of protein fluorescence during the displacement of GTP from its steady-state intermediate with subfragment 1 by thioITP at 20 °C. One syringe contained 2 μM subfragment 1 and 100 μM GTP. The other contained 100 μM thioITP. The reaction was started within 1 min of adding GTP to subfragment 1. Both syringes also contained 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 50 mM Tris adjusted to pH 8.0 with HCl.

could be measured and was 1.04 s^{-1} (Figure 5). If the displacement reaction is performed after all of the GTP has been hydrolyzed to GDP, the rate of displacement of GDP from subfragment 1 is observed. This was 0.06 s^{-1} by using either ATP or thioITP as displacing reagent (Figure 6). An identical process was observed if the subfragment 1–GDP complex was generated by the addition of GDP to subfragment 1 instead of allowing GTP to be hydrolyzed. Kinetic constants associated with the GTP and GDP interactions are summarized in Table II.

Figure 7 shows the binding of thioITP to subfragment 1 which was only partially saturated with GDP. The reaction is biphasic—a fast phase corresponding to the expected rate of binding of thioITP to free subfragment 1 followed by a slow phase which is interpreted as the displacement of GDP from subfragment 1. The amplitude of the total signal was equal to that when thioITP alone binds to subfragment 1. Calculation of the relative amounts of free subfragment 1 and GDP-bound subfragment 1 gives a dissociation constant of GDP from subfragment 1 of 60 μM from Figure 7A and 82 μM from Figure 7B.

This value for the dissociation constant of GDP from subfragment 1 (60–82 μM) differs from the value obtained from the ratio of the dissociation to association rate constants (4 μM). An attempt was made to resolve this discrepancy by measuring the dissociation constant by equilibrium dialysis methods. However, it was found that significant hydrolysis

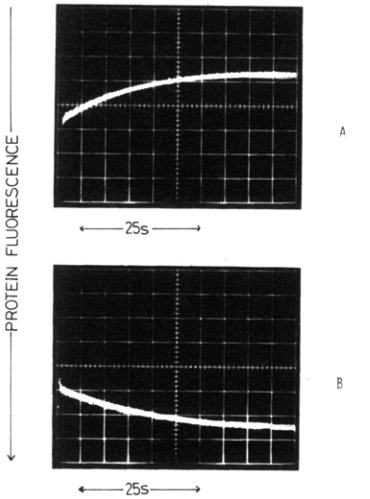

FIGURE 6: The displacement of GDP from subfragment 1 by thioITP and ATP in a stopped-flow spectrophotometer following protein fluorescence at 20 °C, pH 8.0. In A, one syringe contained 2 μM subfragment 1 and 50 μM GTP and the other 50 μM thioITP. In B, one syringe contained 2 μM subfragment 1 and 100 μM GTP and the other 200 μM ATP. The reactions were started 5 min after mixing GTP with subfragment 1 when all GTP had been hydrolyzed to GDP. All syringes also contained 0.1 M KCl, 5 mM $MgCl_2$, and 50 mM Tris adjusted to pH 8.0 with HCl.

Table II: Kinetic Constants for Mg^{2+}-Dependent Rabbit Skeletal Muscle Subfragment 1 GTPase[a]

	k_{ass}[a] (M^{-1} s^{-1})	k_{cat}[b] (s^{-1})	k_{diss}[c] (s^{-1})	K_m[b] (μM)
GTP	1.0×10^5	0.5		27
GDP	1.5×10^4		0.06	

[a] Measured from the effect of GTP and GDP on the rate of binding of ATP or thioITP to subfragment 1 (Figure 4). k_{ass} is the association rate constant. [b] Measured by using a linked assay system containing lactate dehydrogenase and pyruvate kinase (Figure 1). [c] Measured by displacement of GDP from subfragment 1 by ATP or thioITP (Figure 6). k_{diss} is the dissociation rate constant.

of GDP occurred in the presence of subfragment 1 at 20 °C. In a solution containing 94 μM subfragment 1, 48 μM [^{14}C]GDP, 5 mM $MgCl_2$, 0.1 M KCl, and 50 mM Tris adjusted to pH 8.0 with HCl, more than 95% of the GDP was

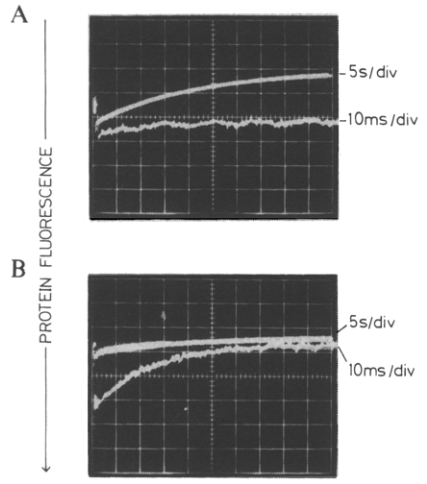

FIGURE 7: Stopped-flow spectrophotometric record of protein fluorescence during the binding of thioITP to subfragment 1 partially saturated with GDP at 20 °C. In A, one syringe contained 2 μM subfragment 1 and 100 μM GDP and the other 100 μM thioITP. In B, one syringe contained 2 μM subfragment 1 and 10 μM GDP and the other 100 μM thioITP. All syringes also contained 0.1 M KCl, 5 mM $MgCl_2$, and 50 mM Tris adjusted to pH 8.0 with HCl. In both experiments, the reactions were carried out within 10 min of adding GDP to subfragment 1. Note that the concentrations given are those in the reaction chamber (see Material and Methods). Calculations of GDP dissociation constants are based on syringe concentrations, i.e., 200 μM GDP (A), 20 μM GDP (B), and 4 μM subfragment 1.

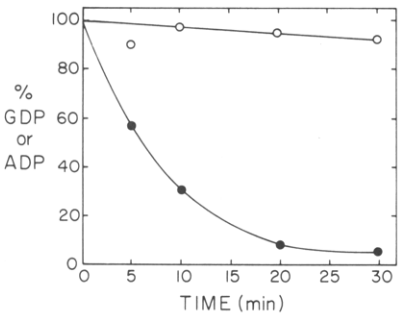

FIGURE 8: The hydrolysis of GDP and ADP in the presence of subfragment 1 at pH 8.0 and 20 °C. The reaction mixture contained 94 μM subfragment 1, 48 μM [^{14}C]GDP or [^{14}C]ADP, 5 mM $MgCl_2$, 0.1 M KCl, 50 mM Tris adjusted to pH 8.0 with HCl. Samples (0.1 mL) were removed at intervals and added to 0.1 mL of cold 7% perchloric acid and neutralized by the additon of 0.05 mL of 4 M sodium acetate. The precipitated protein was removed by centrifugation and thin-layer chromatography performed on the supernatant on PEI-cellulose eluting with 0.75 M KH_2PO_4 adjusted to pH 3.4 with HCl. The radioactive material was detected by autoradiography using Kodirex film and radioactive bands were eluted with 0.5 mL of 0.1 M HCl and counted after the addition of 15 mL of butyl-PBD scintillation fluid. The remaining [^{14}C]GDP and [^{14}C]ADP were calculated as a percentage of the total radioactivity and corrected for the presence of a small quantity of impurity in the GDP and ADP.

hydrolyzed at 30 min and 20 °C (Figure 8).

The effect of Ca^{2+} and of EDTA on the hydrolysis of GDP in the presence of subfragment 1 was measured by incubating 10 μM subfragment 1 with 50 μM [^{14}C]GDP at pH 8.0 and 20 °C in the presence of 5 mM $MgCl_2$, or 5 mM $CaCl_2$, or 1 mM EDTA. Samples were removed for analysis as described in Figure 8 at 3-min intervals. The rate of hydrolysis was linear for 15 min. In the presence of 5 mM $MgCl_2$, this rate was 0.19 μmol of GDP hydrolyzed (μmol of subfragment 1)$^{-1}$ min^{-1} compared to a rate of 0.04 min^{-1} in 5 mM $CaCl_2$. No hydrolysis could be detected if 1 mM EDTA replaced $MgCl_2$ or $CaCl_2$.

When [^{14}C]GDP was replaced by an identical concentration of [^{14}C]ADP, less than 10% of ADP was hydrolyzed at 30 min

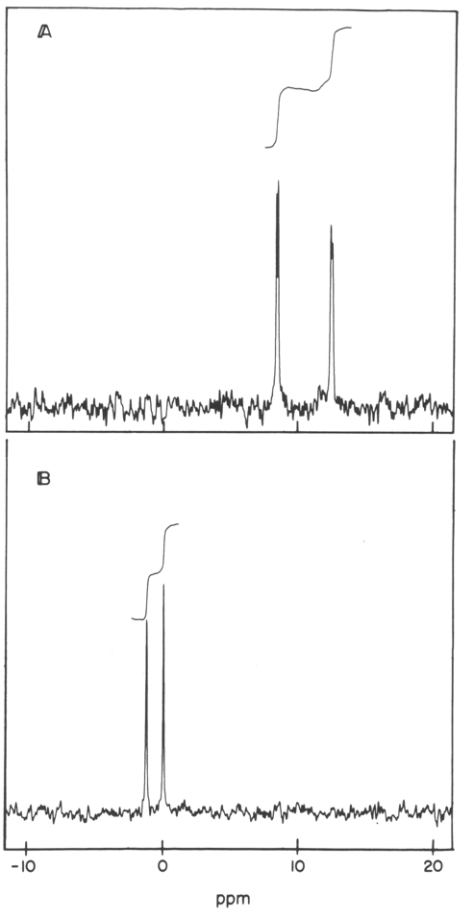

FIGURE 9: ^{31}P nuclear magnetic spectrum of GDP following incubation with subfragment 1 for 8 h at pH 8 and 23 °C. Initially the solutions for both spectra contained 5.3 mM GDP. In B, the solution contained 6.5 mg/mL subfragment 1, while A was a control without subfragment 1. The solvents for both spectra were 10 mM KCl, 7.5 mM $MgCl_2$, 1.0 mM EDTA, 20% deuterium oxide, and 100 mM Tris adjusted to pH 8.0 with HCl. Chemical shifts are referred to P_i in the above solvent. The upper traces in A and B are integrals of the spectra.

(Figure 8). At 0 °C, after 30 min, 7% GDP had been hydrolyzed and no hydrolysis of ADP could be detected. The presence of 0.2 mM ATP in the reaction mixtures inhibited GDP hydrolysis completely. In the absence of subfragment 1, no hydrolysis of GDP could be detected. The solvent for all these experiments was 0.1 M KCl and 50 mM Tris at pH 8.0.

The possibility exists that an extraneous phosphatase was present. It is unlikely that such a phosphatase would co-chromatograph identically with each subfragment 1 isoenzyme. The time course of [^{14}C]GDP hydrolysis catalyzed by the A1 and A2 isoenzymes was followed, as described in the legend to Figure 8. The isoenzymes catalyzed GDP hydrolysis at approximately the same rate (i.e., within a factor of 2).

GDP hydrolysis catalyzed by subfragment 1 was followed directly in a ^{31}P NMR spectrometer at 23 °C and pH 8. Figure 9 illustrates that, during an 8-h incubation, GDP was stable, while in the presence of subfragment 1 there was complete hydrolysis to GMP and P_i. A standard spectrum of GMP, P_i, and PP_i (absorption at +7.76 ppm) identified the hydrolysis products as GMP and P_i. The standard spectrum showed that equimolar GMP and P_i had relative peak heights of 0.79 to 1, a similar ratio to that shown in Figure 9B. Integration of the peaks in Figure 9B also indicates that the stoichiometry of GMP and P_i is 1 to 1, showing that no breakdown of GMP occurred.

Table III: Actin Activation of Mg^{2+}-Dependent Hydrolysis of ATP, ThioITP, GTP, and ThioGTP Catalyzed by Subfragment 1[a]

analogue	actin K_m (μM)	triphosphatase catalytic center act. (s^{-1}) −actin	+actin	actin activation	$\frac{k_{+3}+k_{-3}}{k_{+4}}$
ATP	nonsaturating (at 20 μM)	0.10	5.20	>52-fold	>51
thioITP	5	0.30	2.12	7.1	6.1
GTP	1.5	0.60	2.45	4.1	3.1
thioGTP	<1.0	0.37	0.75	2.0	1.0

[a] Conditions were as described under Materials and Methods.

In several of the above experiments, GMP was present in the solution due to GDP hydrolysis. It was of interest to find out whether or not GMP was tightly bound to the protein. The experiment described in Figure 7A was repeated, except that 100 μM GMP replaced GDP. In contrast to the result with GDP, the kinetics of thioITP binding to subfragment 1 preincubated with GMP were identical with those in which no GMP was present. This shows that GMP does not bind significantly at the subfragment 1 triphosphatase active site.

ThioGDP and thioIDP bound to subfragment 1 in the presence of $MgCl_2$ exhibit difference spectra relative to the free nucleotides (Eccleston & Trentham, 1977). These spectra decrease with time and eventually disappear. The kinetics of those processes closely matched those of the subfragment 1 catalyzed GDP hydrolysis. The spectral changes can be ascribed to slow subfragment 1 catalyzed hydrolysis of the diphosphates.

Actin Activation of Subfragment 1 GTPase, ThioGTPase, and ThioITPase. The results of the quenched-flow experiments show that an analogue of the M*ATP intermediate is the predominant component in the steady-state of the Mg^{2+}-dependent subfragment 1 thioGTPase and GTPase, and also of the thioITPase in low salt conditions or at pH 6.5. This suggests that either the forward rate constant of the cleavage step, k_{+3}, is small and is rate limiting in the reaction or that k_{+3} is large but the equilibrium constant of the cleavage step, K_3, is much less than 1. Measurement of the maximum rate of activation by actin and of the K_m for actin should resolve this ambiguity as is discussed below.

Actin activation of subfragment 1 NTPases was measured by using the linked-assay system described in the Materials and Methods section and varying the actin concentration between 0 and 20 μM. Values for the effective K_m for actin (defined as the actin concentration required for half-maximal activation) and V_{max} were calculated from double-reciprocal plots of rates of hydrolysis against actin concentration. The results are summarized in Table III. Similar results were obtained for GTP hydrolysis when the reaction was followed in a pH stat.

Discussion

A problem of general interest in muscle contraction is to understand what are the special structural properties of ATP compared with other NTPs which enable it to interact with actomyosin so that muscle contraction occurs. A key facet in this is that the rate constants of the elementary steps of the myosin and actomyosin NTPases vary. Ferenczi et al. (1978b) pointed out that a unique property of ATP as a substrate is that the ratio of the NTPase activities of actomyosin and myosin is greatest for ATP, a property which is borne out by the results of this work.

The hydrolysis step of the myosin ATPase is readily reversible. Furthermore, Taylor (1977) has shown that altering the pH from 8 to 6.9 and lowering the temperature from 21 to 3 °C changes the value of K_3 from 9 to 1 in the case of rabbit muscle subfragment 1. It is not therefore surprising that other NTPs have K_3 values such that the predominant steady-state intermediate of their NTPases is M*·NTP rather than M**·NDP·P_i. So with GTP and thioGTP as substrates of subfragment 1, the steady-state intermediates are predominantly M*·GTP, and M*·thioGTP. As with ATP, K_3 for the thioITPase is sensitive to solvent, so that conditions are readily found for which M*·thioITP is the predominant steady-state intermediate of the thioITPase.

On the other hand, the kinetics of the interaction of GDP with subfragment 1 and the susceptibility of GDP to hydrolysis are surprising. Both give rise to concern in the use of ATP analogues for investigation of the myosin ATPase, although the GDP results show the active site has novel and unexpected properties. It is not clear why, during GTPase activity, the steady-state intermediate is not a GDP–subfragment 1 complex with the catalytic center activity being 0.06 s^{-1} at pH 8. In contrast at low temperature, when ADP dissociation becomes the rate-limiting process of the subfragment 1 ATPase (Bagshaw & Trentham, 1974), M*·ADP is the predominant steady-state intermediate.

A further unusual property of GDP binding to subfragment 1 is that the association rate constant is much less than that of GTP. In contrast the apparent association rate constants (k_{-6}/K_7) of ADP and thioGDP are similar to those of ATP and thioGTP, respectively, though those of thioIDP and thioITP do differ (Bagshaw et al., 1972; Bagshaw & Trentham, 1974; Eccleston & Trentham, 1977). GDP binding probably involves more than one process since the rate constant of 1.5×10^4 M^{-1} s^{-1} is three orders of magnitude less than is typical for diffusion-controlled ligand–protein association (Gutfreund, 1975). It is likely that at least part of the binding involves an isomerism in which the binary complex takes up a conformation appropriate for GDP-catalyzed hydrolysis. This conformation is of interest in view of unexpected hydrolysis, and it might lead to important structural cross-bridge studies, as has for example work with the 5′-adenylyl imidodiphosphate–myosin complex (Goody et al., 1975).

The K_m of ATP is less than that of other nucleotides and the muscle cell contains several millimolar ATP (Dawson et al., 1977) so that it is unlikely that subfragment 1 catalyzed GDP hydrolysis has any physiological significance. However, the unexpected nucleoside diphosphatase activity is consistent with the idea that myosin has evolved from a phosphatase of relatively broad specificity and that selective pressures have eliminated what would be undesirable adenosine diphosphatase activity.

The internal inconsistency between the dissociation constant of GDP determined kinetically and from equilibrium measurements (Figure 7) has already been pointed out. Even when the hydrolysis of GDP is taken into account, the discrepancy is difficult to understand. A second inconsistency arises in the K_m values of GTP. The K_m of GTP is expected to be k_{cat}/K_1k_{+2}. In practice K_m is measured to be 27 μM while k_{cat}/K_1k_{+2} = 5 μM (Table II). This may indicate the mechanism is more complex than the current scheme suggests. However, linked-assays seem inherently to lead to high K_m values for ATP in studies of the myosin ATPase. For example, in studies of cardiac myosin the measured K_m of ATP is 2 μM compared with the value calculated from k_{cat}/K_1k_{+2} of 0.04 μM (Taylor & Weeds, 1976). In contrast, when the K_m of ATP was

determined by direct measurements of [γ-^{32}P]ATP hydrolysis for frog muscle subfragment 1, measured and calculated values of K_m were approximately equal (although for myosin the measured K_m was fourfold greater; Ferenczi et al., 1978a,b).

A study of actin activation of an NTPase has relevance both in relation to the ability of the NTP to support contraction and because it helps distinguish alternate kinetic mechanisms of the NTPase. Equation 4 contains steps which are likely to be important in controlling the steady-state Mg·NTPase activity of myosin subfragment 1.

$$\text{M*·NTP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \text{M**·NDP·P}_i \xrightarrow{k_{+4}} \text{subfragment 1} + \text{products} \quad (4)$$

The catalytic center activity of the NTPase is then given by eq 5

$$k_{cat} = \frac{k_{+3}k_{+4}}{k_{+3} + k_{-3} + k_{+4}} \quad (5)$$

and the steady-state concentration ratio of the intermediates is given by

$$\frac{[\text{M*·NTP}]}{[\text{M**·NDP·P}_i]} = \frac{k_{+4} + k_{-3}}{k_{+3}}$$

so that [M*·NTP] will predominate over [M**·NDP·P$_i$] if $k_{+4} > k_{+3}$ or if $k_{-3} > k_{+3}$. It is of interest to know which of these two inequalities is responsible for M*·NTP being the steady-state intermediate of the NTPase. Studies with myosin ATPase have shown that actin enhances ATPase activity by combining with M**·ADP·P$_i$, causing rapid product dissociation and by-passing the step controlled by k_{+4} (Lymn & Taylor, 1971). Thus, a simplistic view is that, if actin activates an NTPase several-fold, it is because k_{+4} is rate limiting, while no activation indicates k_{+3} is rate limiting. Therefore if actin activates the NTPase α-fold, this is because the catalytic center activity becomes k_{+3} in the presence of actin and so from eq 5

$$k_{+3} = \frac{\alpha k_{+3}k_{+4}}{k_{+3} + k_{-3} + k_{+4}}$$

and thus

$$\frac{k_{+3} + k_{-3}}{k_{+4}} = (\alpha - 1)$$

Following this treatment, it is seen that, with the exception of ATP, $k_{+3} + k_{-3}$ is comparable to k_{+4} for each of the triphosphates examined (Table III).

The small K_m values of actin in the actomyosin NTPases relative to that in the ATPase can be understood as follows. The factor by which the rate constant for the breakdown of the myosin–products complex has to be raised for this breakdown to be no longer rate limiting during NTPase activity is much less for ATP analogues than for ATP itself. The observed rate constant for the breakdown is actin concentration dependent. Consequently maximal actomyosin NTPase activity is observed at lower actin concentrations for ATP analogues than for ATP.

At saturating analogue concentration, the amplitude of transient P$_i$ formation in the experiments described in Figure 2 is given by $k_{+3}(k_{+3} + k_{-3})[M_0]/(k_{+3} + k_{-3} + k_{+4})^2$, where $[M_0]$ is the protein active site concentration. This expression reduces to $0.76k_{+3}[M_0]/(k_{+3} + k_{-3})$ in the case of GTP if $(k_{+3} + k_{-3})/k_{+4} = 3.1$ (Table III). Since a transient of $0.3[M_0]$ would have been detected, it follows that $k_{-3} > 1.5k_{+3}$.

Thus, estimates of k_{+3}, k_{-3}, and k_{+4} can be made based on this analysis. For GTP $k_{+3} = 2.45$ s^{-1} (from the actin activated k_{cat}), $k_{-3} > 3.67$ s^{-1} and $k_{+4} > 2.0$ s^{-1} (Table III). It follows that both inequalities $k_{+4} > k_{+3}$ and $k_{-3} > k_{+3}$ probably contribute to M*·GTP rather than M**·GDP·P$_i$ being the steady-state intermediate of the subfragment 1 GTPase.

In principle the analysis of the rate constants k_{+3}, k_{-3}, and k_{+4} could be taken further, for example, if oxygen exchange experiments were carried out (for review, see Trentham et al., 1976; Sleep et al., 1978). The limited data available show that intermediate oxygen exchange occurs with GTP as substrate but to much less extent than with ATP as substrate (Young & Koshland, 1963). This reflects that kinetic partitioning between M**·GDP·P$_i$ to M*·GTP or subfragment 1 plus products favors the latter to a much greater extent than when ATP is substrate.

Qualitatively, and so far as comparisons are meaningful quantitatively, the results obtained here are compatible with the studies of Hasselbach (1957) and Weber (1969). They investigated the steady-state kinetic properties of the myosin, actomyosin, and myofibrillar GTPases and examined myofibrillar contraction and relaxation in the presence of GTP. Seidel (1975) reported transient P$_i$ formation on mixing GTP with rabbit muscle myosin at pH 7.5 and 20 °C. The absence of experiments in the millisecond time range means that his interpretation is open to question. The data reported here do not rule out the absence of transient P$_i$ formation, though it is unlikely to be in excess of 0.3 mol/mol of subfragment 1 active site in the pH range 6.5–8.

Although biological energy transduction is usually linked to ATP hydrolysis, the hydrolysis of GTP often plays an important role in such systems. The binding of aminoacyl-tRNA to the triplet code of messenger RNA and to the ribosome is accompanied by GTPase activity of the elongation factor, EF-Tu (Thompson & Stone, 1977). It has been proposed that energy input is required in this process to ensure high accuracy of messenger RNA translation (Hopfield, 1974).

The GTPase site of EF-Tu has similarities to that of myosin. Using spectroscopic techniques and substituting Mn^{2+}, a paramagnetic probe, for Mg^{2+}, Bagshaw & Reed (1976) and Wilson & Cohn (1977) have shown that the metal ions in the MnADP–subfragment 1 and MnGDP–EF-Tu complexes exist in similar environments, in that, for example, at most only one water molecule in the inner metal coordination sphere is freely exchangeable with the solvent. The relatively tight binding of GDP to myosin subfragment 1 reported here can be compared with the dissociation constant of GDP from EF-Tu of 3×10^{-9} M (Miller & Weissbach, 1977). ThioGDP binds to EF-Tu with a similar perturbation of its spectrum to that occurring when it binds to subfragment 1 (J. F. Eccleston and D. A. Miller, unpublished work).

Studies of the amino acid sequence (Laursen et al., 1977) and tertiary structure of EF-Tu are well advanced and the nucleotide binding site has been described (Morikawa et al., 1978). The similarities between myosin and EF-Tu outlined above may provide a basis for using the structure of the nucleotide binding site of EF-Tu as a model for the myosin site. Changes in the absorption, circular dichroism, and fluorescent spectra and the pK of the 6-mercapto group in different conformational states of EF-Tu–nucleotide complexes will be able to be interpreted with a definitive structure as a reference point.

Acknowledgments

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