THE REVERSAL OF THE MYOSIN AND ACTOMYOSIN ATPase REACTIONS AND THE FREE ENERGY OF ATP BINDING TO MYOSIN

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SUMMARY: Evidence is presented that both myosin and actomyosin in presence of Mg^{2+} and KCl catalyze an incorporation of $^{32}P_i$ into ATP. The rate with actomyosin is about 1/500 the rate of ATP hydrolysis; the rate with myosin is less than 1/100 of that with actomyosin. With myosin, but not with actomyosin, an apparent initial "burst" of $^{32}P_i$ incorporation into ATP is observed. Actin binding thus promotes ATP dissociation. The data with myosin allow estimation of both the amount of enzyme-bound [^{32}P]-ATP present and the rate constant, k_{-1}, for dissociation of the myosin·ATP. From these results and other data a $-\Delta G^\circ$ for ATP binding to myosin of 12-13 kcal/mole may be estimated, with a much lower $-\Delta G^\circ$ for hydrolysis of enzyme-bound ATP. Protein conformational change accompanying ATP binding appears to be the principal means of capture of energy from the overall reaction of ATP cleavage.

A central question about the mechanism of energy-transducing processes associated with ATP is how covalent bond formation or cleavage is coupled to the energy transduction observed. Recent observations with mitochondrial oxidative phosphorylation, together with those reported here on myosin-actomyosin ATPase, favor a conformational coupling hypothesis for oxidative phosphorylation and muscle contraction (1,2). According to this hypothesis, major free energy changes in the overall processes are coupled to protein conformational changes associated with binding or release of ATP. Smaller energy changes occur in the steps where the enzyme-bound ATP and ADP + P_i are interconverted. Results with myosin ATPase offer evidence that protein conformational changes accompany binding of ATP (3-9), but the magnitude of the associated energy changes is not known.

Recently, Hotta and Fujita (10) presented evidence for incorporation of $^{32}P_i$ into ATP by actomyosin, but this observation has been questioned (11). One purpose of this communication is to present data that support an ability

of myosin and actomyosin to catalyze a slow $^{32}P_i \rightleftharpoons$ ATP exchange. Other independent evidence for such a capacity is indicated by brief abstracts of papers presented at meetings (12,13). Our stimulus to check this possibility came from the hypothesis that reversible cleavage of bound ATP to bound ADP and P_i was the source of the ^{18}O exchanges associated with ATP hydrolysis by myosin and actomyosin (1,2,9). If this were the case, some labeling of the bound ATP by $^{32}P_i$ seemed likely, and even quite limited dissociation of any labeled ATP would give rise to a $^{32}P_i \rightleftharpoons$ ATP exchange. Indeed, it seemed possible that by extending the sensitivity of ^{32}P measurement and separation techniques one might observe the rate and characteristics of the reversal of ATP hydrolysis by myosin as well as actomyosin, detect bound ATP species, and derive estimates for the free energy changes of important steps. Such results are reported herein. The results are in harmony with the recent findings of Bagshaw and Trentham who, using quite different approaches, obtained data that give kinetic evidence for the reversible formation of bound ATP from bound ADP and P_i during ATP hydrolysis by myosin subfragments (9).

EXPERIMENTAL: Methods and materials are described in Table and Figure legends.

RESULTS AND DISCUSSION: In preliminary experiments with both myosin and actomyosin, radioactivity added as $^{32}P_i$ appeared in the ATP fraction of column eluates, but amounted to only 10^{-7} to 2×10^{-5} of the total $^{32}P_i$ added. Although the hydrolysis rate in acid was consistent with presence of [^{32}P]-ATP, this test is not definitive. A conversion of the ^{32}P to an acid stable form by hexokinase-glucose gave assurance that the ^{32}P-labeled substance was indeed ATP.* It also added to the assay sensitivity because residual $^{32}P_i$ and acid-hydrolyzable ^{32}P impurities present were removed in the isobutanol-benzene extract after acid molybdate addition. This lowered the background counts in zero time controls to about twice the instrument background.

*The $t_{\frac{1}{2}}$ for hydrolysis of ATP in 0.1 N HCl at 100° is about 8 min and for glucose 6-phosphate is about 2300 min (14,15).

Table I

Incorporation of [^{32}P]-P_i into ATP in Presence of Actomyosin[a]

Experiment No.	Time after $^{32}P_i$ addition	nmoles [^{32}P]-P_i incorproated into ATP
1	1-2 sec	0.006
2	1 min	0.36
3	5 min	1.91

[a]A 1 ml reaction volume at 24° contained 1.6 mg actomyosin (20), 50 mM Tris-P_i, pH 7.4, 60 mM KCl, 10 mM Mg acetate and 5 mM ATP. After 5 min, 1-2 mCi of $^{32}P_i$ (0.03 µmole) were added and the reaction was allowed to proceed for varying lengths of time. After quenching with $HClO_4$, removing protein, neutralizing with KOH and removing $KClO_4$, nucleotides were adsorbed on a 1.5 ml Dowex-1-Cl^- column. The column was eluted sequentially with 3 ml of 0.2 M Tris·Cl, pH 7.5, 10 ml of 60 mM HCl, and 3.5-4 ml of 1 M HCl. The last eluate, containing about 90% of the ATP, was neutralized with conc. NH_4OH to pH 8. One-half was incubated with 10 mM $MgCl_2$, 25 mM glucose and 50 µg of hexokinase for 1 hour at 24°. Both halves were made 1 M in HCl and heated 15 min at 100°. The $^{32}P_i$ was removed by successive extractions as the acid molybdate complex (21), with a chase of cold P_i. [^{32}P]-ATP originally present was estimated as the counts converted to acid-stable form by hexokinase.

Table I shows the apparent reversal of ATP cleavage catalyzed by actomyosin actively hydrolyzing ATP in presence of Mg^{2+}. Comparison of the 1 and 5 minute incubation values indicates a reverse reaction rate of about 240 pmoles of [^{32}P]-ATP synthesized/min/mg actomyosin. The net rate of ATP hydrolysis under our experimental conditions was about 120 nmoles/min/mg, or about 500 times greater than the reverse reaction. In these and other similar experiments, no initial "burst" of ATP synthesis was observed indicating that less than 1/1000 of the active sites contained bound ATP.

The continued appearance of [^{32}P]-ATP with time suggested that the ^{32}P-labeling of ATP represented ATP released to the medium. However, further assessment appeared desirable because the total amount of [^{32}P]-ATP formed was

less than the amount of actomyosin on a molar basis. The results of an ultrafiltration experiment are presented in Table II. They show that all or nearly all of the [^{32}P]-ATP synthesized was free in the medium.

Data from similar experiments on the reversal of ATP hydrolysis by myosin are shown in Fig. 1. In contrast to the actomyosin behavior, with myosin a

Table II

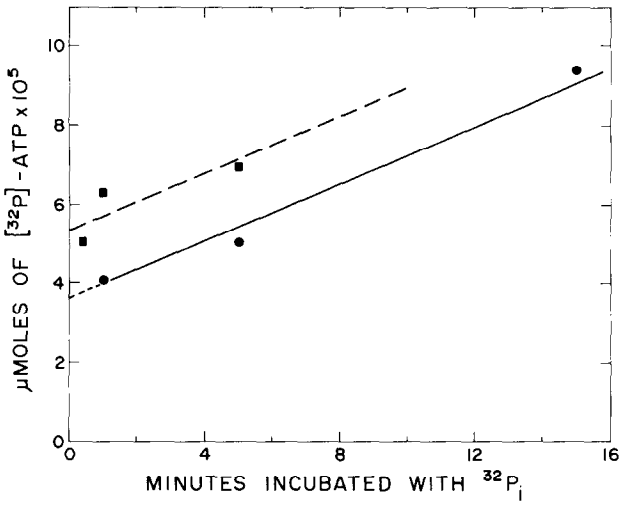
Ultrafiltration of [^{32}P]-ATP Formed by Actomyosin[a]

Solution	cpm/ml
Ultrafiltrate	21,300
Remaining actomyosin solution	20,600

[a]A 1 ml reaction volume like that reported with Table I, was placed in an ultrafiltration apparatus (Schleicher and Schuell). Ultrafiltration was carried out for 45 min after $^{32}P_i$ addition, at which time about 0.75 ml of ultrafiltrate had been collected, and [^{32}P]-ATP in the ultrafiltrate and remaining actomyosin solution were estimated as described with Table I.

measurable initial burst of [^{32}P]-ATP formation is observed. Although there is some scatter in the data, a reasonable estimate is that this burst amounts to about 0.005 ATP/ATPase site. The burst appears to be complete in less than 18 sec, which is less than the turnover time for myosin under these conditions. It is followed by a steady synthesis of [^{32}P]-ATP whose rate is about 2 pmole/min/mg myosin, or about 1/120 of the rate observed with actomyosin.

A pertinent question is whether contaminating proteins in the myosin preparation could be causing the observed burst and reversal. A thermal inactivation experiment, which took advantage of the unusually high heat lability of myosin (16), was performed to help evaluate this possibility. As noted in



Legend for Fig. 1.

Time Course of $[^{32}P]$-ATP Formation by Myosin. Measurements were made as described with Table I, but with two different myosin preparations (22) (indicated by squares and circles) using 2.1 mg of protein.

Table III

Comparison of Heat Inactivation of Myosin ATPase and $^{32}P_i \rightarrow$ ATP Activities[a]

Sample	Ca^{2+}-ATPase μmoles/min/mg	pmoles of $[^{32}P]$-ATP/15 min
Stock myosin	0.408	129
Myosin heated 10 min @ 40°	0.137	27
Percent activity loss	67	78

[a]Myosin (22) was heated under N_2 (16); then aliquots were assayed by standard methods (Ca^{2+}-ATPase) and according to the procedure given with Table I ($[^{32}P]$-ATP).

Table III, heating decreased the Ca^{2+}-activated ATPase and $^{32}P_i \rightleftharpoons$ ATP exchange activities in an essentially parallel manner.

These results suggest two facets of the myosin ATPase mechanism: (a) the overall reaction is reversible to a small but still measurable extent, and (b) in the absence of actin, a small but significant apparent burst of [^{32}P]-ATP formation is observed. The magnitude of this burst indicates that there is a relatively low energy barrier to the formation of the bound ATP from $^{32}P_i$ and ADP. This appears to reflect an ability of the protein to favor ATP formation at the catalytic site without energy input from external sources.

The hydrolytic sequence with myosin must include a minimum of three steps: ATP binding, hydrolysis of bound ATP, and release of the ADP and P_i formed. These steps, each of which may be separated into additional experimentally discernible components, may be depicted as in equation (1):

$$\underset{\text{Step 1}}{\overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}}} \qquad \underset{\text{Step 2}}{\overset{\text{HOH } k_2}{\underset{\text{HOH } k_{-2}}{\rightleftharpoons}}} \qquad \underset{\text{Step 3}}{\overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}}}$$

$$\text{E + ATP} \quad \text{E·ATP} \quad \text{E·ADP·P}_i \quad \text{E + ADP + P}_i \qquad (1)$$

$$\Delta G^\circ \qquad -12.7 \frac{\text{kcal}}{\text{mole}} \qquad -1.3 \frac{\text{kcal}}{\text{mole}} \qquad +5 \text{ to } +6 \frac{\text{kcal}}{\text{mole}} \qquad \text{(Estimated } \Delta G^\circ \text{ values with myosin)}$$

A calculation of the reverse rate constant k_{-1} for dissociation of myosin·ATP can be made from the data presented here, using the apparent burst size as a measure of the concentration of bound [^{32}P]-ATP at steady state. This yields a value of 1.0-1.6 x 10^{-3} sec^{-1}. From measurements using myosin subfragments, Bagshaw and Trentham (9) deduced that the reverse rate constant, k_{-1}, was less than 0.02 sec^{-1}, a result obviously in agreement with our determination of a smaller value.

With actomyosin, only a lower limit can be calculated for k_{-1}. Using the lowest initial burst size we could have detected in our experiments, this value is 0.5 sec^{-1}. The reverse rate with myosin is thus very roughly 1/2000 or less

of that with actomyosin. Actin appears to have a powerful stimulating effect on the rate of ATP dissociation, apparently producing a protein conformational change favoring ATP release.

The above data allow estimation of the apparent ΔG° for the reaction myosin + ATP $\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$ myosin·ATP. Using the value of 2.4×10^6 M^{-1} sec^{-1} for k_1 reported for heavy meromyosin in 0.05 M KCl by Lymn and Taylor (17) and our value of 1.3×10^{-3} sec^{-1} for k_{-1}, the apparent free energy change of ATP binding is about -12.7 kcal/mole. The data of Bagshaw and Trentham (9) indicate that the apparent ΔG° for bound ATP hydrolysis (step 2 of equation 1) is only about -1.3 kcal/mole. Other findings also favor a relatively small $-\Delta G^\circ$ for bound ATP cleavage. From our limited data with ADP and $^{32}P_i$ addition, about 0.01 of the catalytic sites are combined with [^{32}P]-ATP, indicating a ΔG° for the cleavage of bound ATP of no more than -2.8 kcal/mole. Also, the large $-\Delta G^\circ$ for step 1 together with a positive ΔG° for step 3 (required by reasonable affinities of myosin for ADP and P_i) point to a small $-\Delta G^\circ$ for step 2. Thus we conclude that the largest $-\Delta G^\circ$ of the overall hydrolytic sequence occurs in the ATP binding step (step 1).

The apparent ΔG° for ATP hydrolysis under conditions like those used in these experiments is about -7.5 to -8 kcal/mole (18,19). The large $-\Delta G^\circ$ associated with the binding reaction and the additional $-\Delta G^\circ$ for the hydrolysis step means that a ΔG° of +5 to +6 kcal/mole accompanies the release of ADP and P_i in step 3 as written; obviously a similar $-\Delta G^\circ$ accompanies ADP and P_i binding.

Free energy changes estimated from our data are apparent ΔG° values for the experimental conditions used. Concentrations of various reactants that prevail in intact muscle would govern actual free energy changes in the contraction and cleavage cycle. Nevertheless, it is apparent that ATP binding must be regarded as a key reaction in energy transduction in muscle.

The coupling of large free energy changes in binding and release steps to a protein conformational change, supported by data reported here, appears to offer a logical and simple manner by which to achieve energy transduction assoc-

iated with cleavage of the "high-energy" phosphate bond of ATP.

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