Journal of Supramolecular Structure 3:154–161 (1975)

ISOTOPIC PROBES OF CATALYTIC STEPS OF MYOSIN ADENOSINE TRIPHOSPHATASE

Robert G. Wolcott and Paul D. Boyer

Molecular Biology Institute and Department of Chemistry, University of California, Los Angeles

A new approach to the direct estimation of the value of the off constant for dissociation of ATP from myosin subfragment 1 (S1) has been developed. From measurements of the extremely slow rate of release of [^{32}P]-ATP formed from $^{32}P_i$ by S1 catalysis and the amount of rapidly formed [^{32}P]-ATP tightly bound to S1, the value of the off constant is approximately 2.8×10^{-4} sec^{-1} at pH 7.4.

The concentration dependencies for $P_i \rightleftharpoons H^{18}OH$ exchange and for $^{32}P_i$ incorporation into myosin-bound ATP give direct measurements of the dissociation constant of P_i from S1. Both approaches show that the enzyme has a very low affinity for P_i, with an apparent K_d of > 400 mM.

Measurement of the average number of water oxygens incorporated into P_i released from ATP by S1-catalyzed hydrolysis in the presence of Mg^{2+} suggests that the hydrolytic step reverses an average of at least 5.5 times for each ATP cleaved. With the Ca^{2+}-activated hydrolysis, less than one oxygen from water appears in each P_i released. This finding is indicative of a possible isotope effect in the attack of water on the terminal phosphoryl group of ATP.

INTRODUCTION

The nature of the catalytic process whereby myosin or its active subfragments hydrolyzes ATP has been the subject of intense investigation. Some of the major steps in the process have been elucidated, and the presently generally accepted sequence is shown in Fig. 1.

Values of the various constants, cited in references 1–6, are as follows:

$K_1 = 4.5 \times 10^3$ M^{-1}; $k_2 = 400$ sec^{-1}, $k_{-2} = 10^{-3}$ sec^{-1};

$K_3 = 9$, $k_3 = 160$ sec^{-1}; $k_{-3} = 18$ sec^{-1}; $k_4 = 0.06$ sec^{-1}

(rate limiting in 0.1 M KCl, 50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 21°C);
$K_6K_7 = 10^{-6}$ M.

The standard free energy of hydrolysis of ATP is about −31.6 kJ/mole (7), but may be larger depending on the concentrations of ADP and P_i present. The equilibrium constant for the hydrolytic step (step 3 in Fig. 1) represents a free energy change of only

Dr. Wolcott's present address is the Department of Chemistry, California State University, Northridge, California 91324.

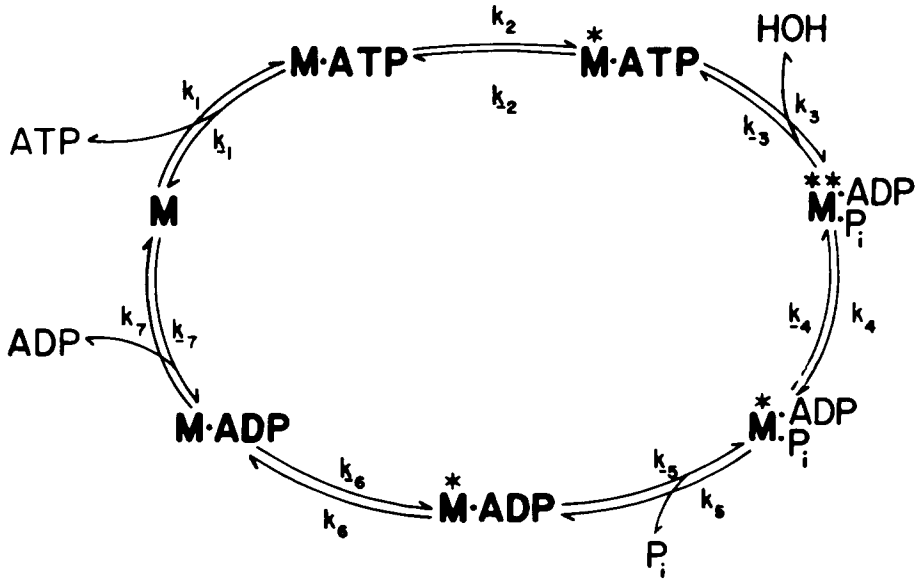

Fig. 1. The myosin hydrolytic sequence. M, myosin or active subfragments.

−5.4 kJ/mole, while the free energy changes of steps 4–7 have all been found to have either positive or small negative values. A very large negative free energy change thus appears likely in the binding of ATP. Direct measurement of release of ATP from myosin · ATP allows a quantitative estimation of this free energy change.

Myosin or its active subfragments will catalyze an apparent reversal of ATP hydrolysis (6, 8). Quantitation of the rate and extent of the apparent reversal provides a direct means of assessing the value of the rate-limiting step in the reverse reaction process. Since formation of bound [^{32}P]-ATP from ADP and $^{32}P_i$ is relatively rapid with myosin, the rate-limiting step for overall reversal apparently occurs in the ATP release step or steps; i.e. in either step 1 or step 2 of Fig. 1.

We report here preliminary results of isotopic studies of the reversal of the catalytic sequence. It is apparent from Fig. 1 that there is the possibility of exchange of phosphorus between ATP and P_i and of oxygen among ATP, HOH, and P_i. The isotopes ^{32}P and ^{18}O were used as probes of the catalytic interconversion of these molecules.

EXPERIMENTAL

Materials

Subfragment 1 (S1) of myosin was prepared by the method of Cooke (9) and was freed of impurities by KCl gradient elution chromatography on Whatman DE–23. The absence of actin was demonstrated by SDS gel electrophoresis. ATP and ADP were purchased from Boehringer-Mannheim; ^{18}O-phosphate was from Miles Laboratories, and $^{32}P_i$ was from ICN.

Estimation of (^{32}P)-ATP Formation

In order to ensure equilibrium among all components, S1 (2–5 mg) was incubated about 5 min at 23°C in 1.00 ml of solution containing 50 mM KCl, 50 mM Tris-P_i, pH 7.4, 2.5 mM ATP or ADP, and 2.5 mM $MgCl_2$ prior to addition of $^{32}P_i$. When ADP was used, an ATP trap consisting of 25 mM glucose and 5 μg of hexokinase was included to remove any ATP present as a contaminant in the ADP. In those experiments where the phosphate concentration was varied, ionic strength was maintained constant by appropriate additions of Tris-Cl or Tris-SO_4 buffer.

The preliminary incubation was followed by addition of a trace of $^{32}P_i$, and the mixture was further incubated at 23°C for varying lengths of time. Quenching was by rapid addition of cold perchloric acid; if the experiment involved ADP, the quench solution also contained about 2.5 μmoles of cold ATP as carrier. The quenched mixture was centrifuged to remove precipitated protein, then neutralized with KOH, and again centrifuged. An aliquot of the supernatant was removed for determination of specific activity of the medium P_i and the remainder was chromatographed on Dowex–1 (Cl–form). The ATP fraction was nuetralized with concentrated ammonium hydroxide to pH 7–8.

The portion of the radioactivity in the ATP fraction that was present as (^{32}P)-ATP was determined by treatment of part of the ATP pool with hexokinase, glucose, and $McCl_2$. This procedure transfers the terminal phosphoryl group of ATP to glucose to produce heat- and acid-stable glucose-6-phosphate.

After acid hydrolysis, the acid-molybdate-extractable radioactivity was measured by scintillation counting. With suitable controls, the procedure allowed quantitation of the very small amount of (^{32}P)-ATP formed in the reaction mixture.

^{18}O Incorporation and Exchange

Reaction mixtures and conditions were as described above, except that $^{32}P_i$ was omitted and the isotope was present as ^{18}O in the P_i of the medium or in the solvent H_2O. In the latter case, no medium P_i was present initially, and buffering was by Tris-Cl. In either case, the ^{18}O content of the P_i in the medium after reaction was assessed by extraction as the molybdate complex followed by purification, conversion of phosphate oxygens to CO_2, and mass spectrometer analysis (10).

RESULTS AND DISCUSSION

Formation of S1-Bound (^{32}P)-ATP from $^{32}P_i$

Data indicating the formation of (^{32}P)-ATP from $^{32}P_i$ catalyzed by S1 are presented in Table I. When ADP and an ATP trap are present, (^{32}P)-ATP is formed in a quantity sufficient to account for about 1.0–1.3% of the potentially available S1 active sites. The reaction appears to be complete in less than 1 min; other data from our laboratory suggest that the reaction may be half complete in 2–5 sec. If ATP is present, less (^{32}P)-ATP is formed during the initial rapid phase. Only about 0.5–0.7% of the available S1 sites appear to contain (^{32}P)-ATP during the first few seconds of incubation with $^{32}P_i$. Of considerable interest is a continued, very slow increase in the total level of (^{32}P)-ATP present. This suggests that some of the S1-bound (^{32}P)-ATP is slowly released to the medium;

TABLE I. Formation of Bound (^{32}P)-ATP from $^{32}P_i$ by Myosin Subfragment 1[a]

Time incubated after $^{32}P_i$ addition (min)	pmoles (^{32}P)-ATP found with ATP trap[b]	pmoles (^{32}P)-ATP found in presence of free ATP[c]
0.2	120 ± 24	70 ± 2
1.0	– –	95 ± 8
15.0	138 ± 5	121 ± 12

[a]Experiment performed as described in text, using 1.6 mg of S1.
[b]Reaction mix contained 25 mM glucose, 5 μg hexokinase, 2.5 mM $MgCl_2$, and 2.5 mM ADP.
[c]Reaction mix contained 2.5 mM $MgCl_2$ and 2.5 mM ATP.

rebinding of any such (^{32}P)-ATP would be prevented by competition of nonlabeled ATP present for the S1 active site.

Release of (^{32}P)-ATP from S1

The apparent slow release of a fraction of the bound (^{32}P)-ATP to the medium provides a direct method for assessing the value of the rate constant for the slowest step in the reverse direction. The burst size may be taken as a measurement of the concentration of enzyme sites containing bound (^{32}P)-ATP, while the rate of increase of (^{32}P)-ATP concentration in the medium is a measure of the rate at which these S1 molecules lose their bound (^{32}P)-ATP to the medium. A preliminary value of the rate constant computed using these data is 2.8×10^{-4} sec^{-1}, which is about one-fifth as large as the value of 14×10^{-4} sec^{-1} we obtained with myosin (6). This difference might reflect the presence of traces of actin in the myosin preparation; alternatively, because S1 is a proteolytic fragment of myosin, it may not have the same kinetic characteristics as myosin.

The Apparent K_d for P_i

Both the ^{32}P and ^{18}O techniques have been used to estimate the value of the dissociation constant of P_i from $\overset{*}{M}:^{ADP}_{P_i}$ complex. Measurements of the amount of bound (^{32}P)-ATP found after 1 min of incubation with $^{32}P_i$ were made at varying concentrations of P_i, with careful maintenance of ionic strength. The data, presented in Fig. 2, show that there is no saturation of the S1 sites, even up to 400 mM P_i. This indicates that the dissociation constant of P_i from the $\overset{*}{M}:^{ADP}_{P_i}$ complex is very large. Parenthetically, the data also reveal the need for maintaining constant ionic strength: the amount of (^{32}P)-ATP formed at high ionic strength (right plot) are significantly lower than at low ionic strength (left plot).

The same type of result is obtained by the entirely independent method of measuring the extent of medium oxygen exchange (Fig. 2, left plot). In this experiment, the extent of loss of ^{18}O from labeled medium P_i during a fixed time of incubation is determined. Loss of ^{18}O from medium P_i would be predicted from Fig. 1 if the catalytic sequence reverses at least through step 3 (that is, as far as the formation of $\overset{*}{M} \cdot$ ATP from $\overset{*}{M} \cdot$ADP and P_i). The data gain show no leveling off of the observed exchange rate up to 80 mM P_i. This is additional evidence in favor of a very low affinity of S1 for P_i.

Other workers have confirmed the formation of tightly bound (^{32}P)-ATP on myosin and S1, and have presented calculations leading to a value of about 2×10^{-7} sec^{-1} for

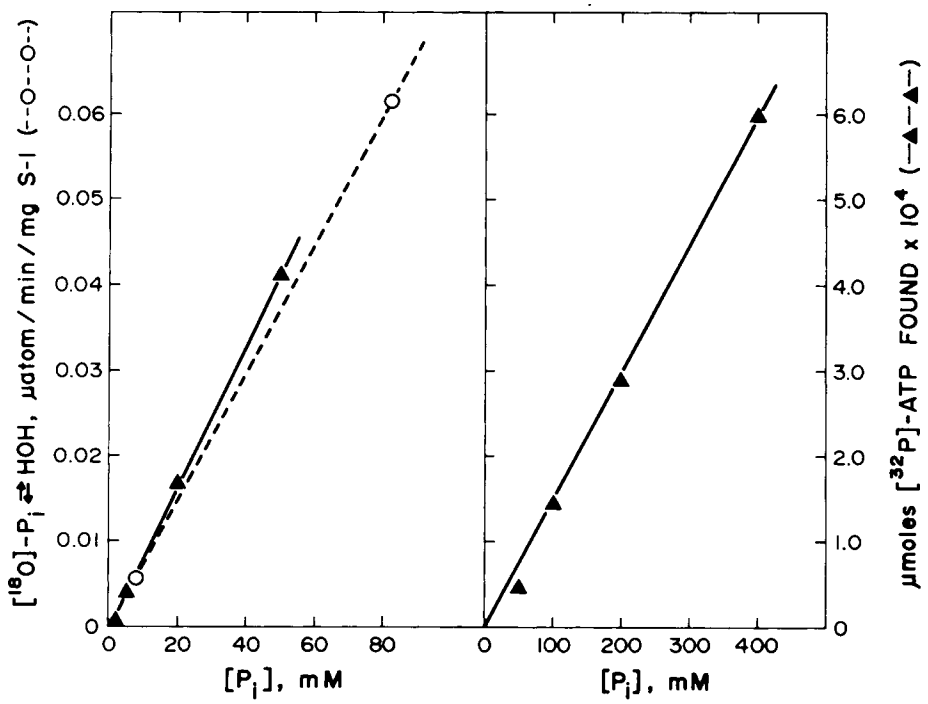

Fig. 2. Effect of P_i concentration on the extent of formation of bound (^{32}P)-ATP and (^{18}O)-P_i ⇌ HOH exchange. Left: 1.91 mg S1, 2.5 mM MgADP, 25 mM glucose, 5 μg hexokinase, pH 7.4, 23°C; ionic strength maintained at 0.21 by appropriate addition of 1 M Tris-Cl, pH 7.4; total volume was 1.00 ml. Right: 1.94 mg S1, otherwise as above, except ionic strength maintained at 1.2 by appropriate addition of 2 M Tris-SO_4, pH 7.4. Radioactivity was introduced as a trace of $^{32}P_i$ and reactions were quenched after 1 min. ^{18}O, when present, was in the P_i of the medium; reactions were quenched after 24 hr. Such samples had 0.02% NaN_3 included to suppress microbial growth.

k_{-2} (8). This value is three orders of magnitude smaller than that given here. They have also reported a value of 7.3 mM (pH 8) or 55 mM (pH 6) for the dissociation constant for P_i. These calculations are based on the equilibrium measurements of the extent of (^{32}P)-ATP formation as a function of $^{32}P_i$ concentration, together with previously reported constants. A difficulty with their approach is the apparent lack of control of ionic strength during variation of the $^{32}P_i$ concentration, which we have shown has a profound effect on the extent of formation of bound (^{32}P)-ATP. We thus feel that the indirect route to the value of k_{-2} used by Mannherz et al. (8) may be subject to error, including errors in previously evaluated constants used in the calculation, as well as in the cited figure for the free energy of ATP hydrolysis (11), which we feel is too large (see reference 7). In addition, their estimates of K_4 and K_5 may need further evaluation. The estimates rely on values of intercepts of double reciprocal plots of

$1/[(^{32}P)\text{-ATP}]$ vs. $1/[^{32}P_i]$. Very small errors in either of the intercepts would result in large errors in values for K_4 and K_5.

The direct measurement of the off constant and the dissociation constant by the isotopic methods described here appear capable of giving satisfactory values. Also, we feel that our direct measurement of the dissociation constant of P_i by two independent methods with control of ionic strength gives a better evaluation than that obtained by Mannherz et al. (8).

Water-Oxygen Incorporation during ATP Cleavage

Careful analysis of the extent of oxygen incorporated from water into the P_i released from ATP by myosin (the "intermediate" exchange) affords an additional probe of the catalytic process. If the steps following cleavage (step 3 in Fig. 1) are rapid compared to the cleavage step, then the model would predict incorporation of only one oxygen from water into the released P_i. However, if one or more of the subsequent steps are slow, then the process occurring at step 3 will have an opportunity to reverse one or more times. This provides a satisfying molecular explanation for the ^{18}O exchanges (4, 6). The dynamic reversal of step 3 would result in more than one oxygen incorporated from water into P_i. The extent of any extra incorporation can be used to compute the apparent average number of times the reaction undergoes reversal. The calculation involves two assumptions; namely, that water has unrestricted access to the catalytic site and that all four oxygens on the bound P_i are chemically equivalent.

Each time the cleavage occurs, one oxygen from water is incorporated into the P_i bound in the enzyme complex. If the catalytic sequence reverses and if all four P_i oxygens are equivalent, then the probability of retention of the oxygen atom just gained is 3/4. The subsequent cleavage introduces another water oxygen, and again if reversal occurs, the probability of retention of the total water oxygen content is 3/4. This leads to an expression giving the expected number of water oxygens as a function of the number of times the cleavage step has operated:

$$\frac{\text{water oxygens}}{P_i \text{ molecule}} = 1 + \sum_{n} \left(\frac{3}{4}\right)^{n-1},$$

where n = the number of times the cleavage step has operated. Table II lists the values obtained from this formula.

Measurements of the number of water oxygens incorporated from $H^{18}OH$ into the P_i cleaved from ATP under conditions as used for the $^{32}P_i$ incorporation experiments are shown in Table III. We find incorporation of 0.92 ± 0.028 oxygen/P_i using Ca^{2+}, while 3.16 ± 0.063 oxygen/P_i are found using Mg^{2+}. Where Ca^{2+} replaces Mg^{2+}, the steady-state rate of ATP cleavage is increased due to a marked increase in the value of k_4. Thus, with Ca^{2+} as the metal ion, little or no equilibration is possible at the cleavage step, in contrast to the situation prevailing with Mg^{2+} as the metal ion.

If no equilibration occurs at step 3, one would expect to find 1.00 water oxygen in each P_i released. We find a slightly lower amount. Since adequate internal standards

TABLE II. Effect of the Number of Cleavages on the Water-Oxygen Content of Released P_i

Number of cleavages (n)	Water-Oxygens/P_i released
1	1.00
2	1.75
3	2.31
4	2.73
5	3.05
6	3.29
7	3.47
8	3.60

TABLE III. Incorporation of Water-Oxygen Into P_i Cleaved from ATP by S1[a]

Metal ion (2.5 mM)	Water-oxygens/P_i
Ca^{2+}	0.92 ± 0.028
Mg^{2+}	3.16 ± 0.063

[a]Triplicate determinations were performed as described in text.

were used and since the precision of the data was good, we feel that this may reflect an isotope effect during the attack by water on the bound ATP. If this is the case, then the data would suggest that P–O bond formation contributes to rate limitation in the cleavage step.

The data with Mg^{2+} as the metal ion suggest that the cleavage step reverses several times before the P_i is released from the enzyme. Correction of the observed number of water oxygens in each P_i released for the apparent isotope effect and reference to Table III show that the cleavage has occurred an average of at least 6.5 times, and hence that each P_i shuttles back and forth in step 3 an average of at least 5.5 times before release. The data of Bagshaw and Trentham (4) suggest an even larger number of reversals. Our approach would give a minimum value, since restricted access of water to the site or nonequivalence of the four P_i oxygens could limit oxygen incorporation.

ACKNOWLEDGMENTS

This work was supported by grant no. GB-363-44X from the National Science Foundation.

REFERENCES

1. Lymn, R. W., and Taylor, E. W. Biochemistry 9:2975 (1970).
2. Lymn, R. W., and Taylor, E. W. Biochemistry 10:4617 (1971).
3. Bagshaw, C. R., Eccleston, J. F., Trentham, D. R., Yates, D. W., and Goody, R. S. Cold Spring Harbor Symp. Quant. Biol. 37:127 (1972).
4. Bagshaw, C. R., and Trentham, D. R. Biochem. J. 133:323 (1973).
5. Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., and Trentham, D. R. Biochem. J. 141:351 (1974).
6. Wolcott, R. G., and Boyer, P. D. Biochem. Biophys. Res. Commun. 57:709 (1974).
7. Rosing, J., and Slater, E. C. Biochim. Biophys. Acta 267:275 (1972).
8. Mannherz, H. G., Schenck, H., and Goody, R. S. Eur. J. Biochem. 48:287 (1974).
9. Cooke, R. Biophys. Res. Commun. 49:1021 (1972).
10. Boyer, P. D., and Bryan, D. M. Methods Enzymol. 10:60 (1967).
11. Alberty, R. A. J. Biol. Chem. 244:3290 (1969).