and the subsequent hydrolysis are relatively straightforward and fast. Furthermore, the reactions described in this paper are suitable for studying the positional isotope exchange of other nucleoside triphosphates, in particular GTP. In the following paper (Geeves et al., 1980), use is made of this approach to study the cleavage reaction of ATP by myosin.

Acknowledgments

I thank Vinka Parmakovich, Slavica Sporer, and Vincent Saltmach of the Columbia University Chemistry Department for performing the mass spectral analyses.

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Mechanism of Adenosine 5′-Triphosphate Cleavage by Myosin: Studies with Oxygen-18-Labeled Adenosine 5′-Triphosphate†

Michael A. Geeves,‡ Martin R. Webb,* C. Fred Midelfort,§ and David R. Trentham

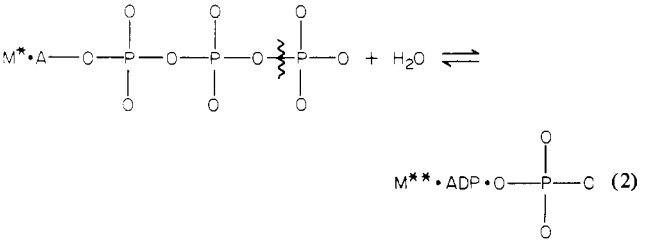
ABSTRACT: During the hydrolysis of MgATP catalyzed by myosin, ATP bound to the protein undergoes a reaction such that the β-nonbridge oxygen atoms exchange position with the βγ-bridge oxygen atom. The extent of this exchange was variable but averaged 45% for ATP that had been bound for 2 s at the myosin subfragment 1 active site at ionic strength 0.08 M, pH 8.0, and 22 °C. This result proves that ATP cleavage in the myosin active site is readily reversible. The result also suggests that the β-phosphate of ADP that must be formed in this cleavage step is highly constrained in the protein.

In recent years considerable progress has been made in the elucidation of the myosin and actomyosin ATPase mechanisms. These have been reviewed by Trentham et al. (1976) and by Taylor (1979). The Mg^{2+}-dependent ATPase of myosin can be described by a seven-step mechanism:

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

where M represents myosin or subfragment 1, the head of the myosin molecule obtained by proteolysis. k_{+i} and k_{-i} are forward and reverse rate constants and K_i is the equilibrium constant for the ith step. The rate-determining step is step 4, while M*·ATP and M**·ADP·P_i are the main steady-state intermediates whose interconversion (step 3) is relatively fast.

Step 3 is generally considered to involve ATP cleavage (i.e., the breaking of the covalent bond between ADP and the γ-phosphate), but the exact nature of this step has not been determined. For example, it is not known which step in eq 1 involves the attack of water on ATP. There are several ways in which the actual hydrolytic cleavage might occur. In the absence of any evidence for a phosphoenzyme (Sartorelli et al., 1966), it is most likely that step 3 of eq 1 represents the straightforward displacement of ADP from the γ-phosphate by a water oxygen:[1]



An alternative possibility is that M**·ADP·P_i represents a

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[1] The protonation state, negative charges, and π bonds are not shown in phosphoric residues to avoid confusion in molecular structures. Oxygen-16 is represented by O; oxygen-18 is represented by ●.

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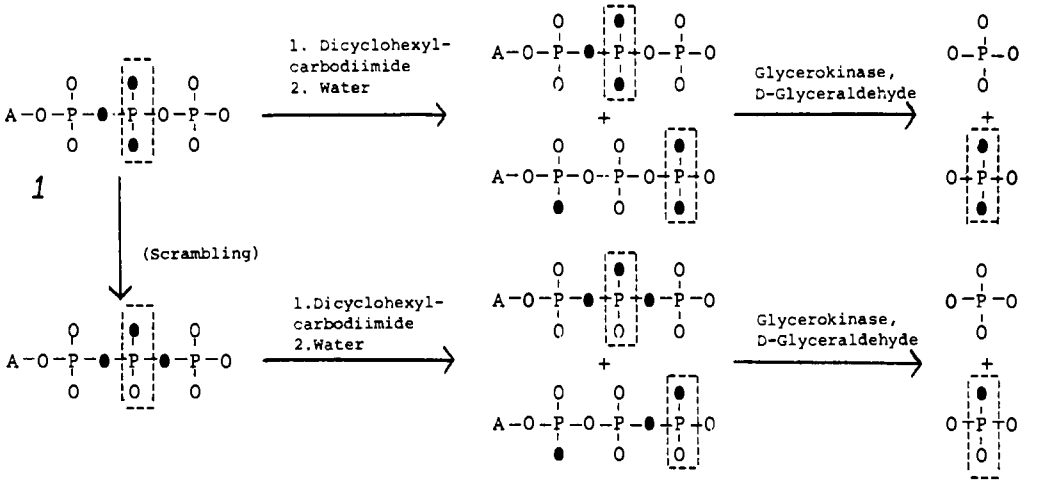
Scheme I

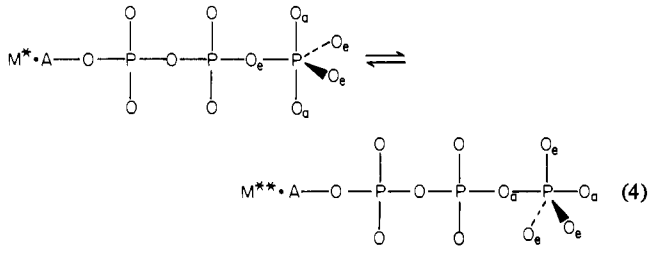


metaphosphate intermediate, as proposed by Lowe & Sproat (1978b) for phosphate transfer by pyruvate kinase. However, the oxygen-18 evidence of Trentham (1977) and the results showing rapid oxygen exchange argue strongly against this possibility.

It has been proposed that water enters the reaction scheme prior to step 3 (Young et al., 1974). In terms of eq 1, M*·ATP represents protein-bound ATP with a pentacoordinate terminal phosphate, the extra oxygen being derived from water. In this hypothesis, step 3 becomes a pseudorotation in which the pentacoordinate structure changes, and step 4 involves bond cleavage of protein-bound ATP (eq 3). According to this class

$$M\cdot ATP + H_2O \overset{2}{\rightleftharpoons} \text{“}M^*\cdot ATP\text{”} \overset{3}{\rightleftharpoons} \text{“}M^{**}\cdot ADP\cdot P_i\text{”} \overset{4}{\rightleftharpoons} M^*\cdot ADP\cdot P_i \quad (3)$$

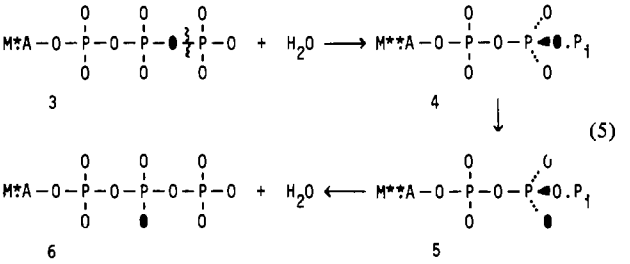
of mechanism “M**·ADP·P_i” is proposed to correspond to a hydrated state of protein-bound ATP and step 3 can be written more explicitly as



(4)

Empirical rules govern which bonds may take up apical (a) or equatorial (e) coordination; the most important of these is that electron-deficient atoms preferentially take up apical positions (Westheimer, 1968) and bonds are formed and broken at an apical position. Isolation of the nucleotide from M*·ATP and M**·ADP·P_i involves quenching the reaction into protein denaturant—generally 1 N acid. When this happens, an apical bond breaks so that according to the Young et al. (1974) mechanism M*·ATP yields ATP while M**·ADP·P_i can yield ADP and P_i to give the observed mixture of ATP, ADP, and P_i.

It is the purpose of this work to investigate ATP cleavage and to distinguish between classes of mechanism of the Mg^{2+}-dependent myosin ATPase. A study of the interconversion of the β-oxygens (nonbridging) and the $\beta\gamma$-oxygen (bridging) of ATP by myosin is described. When ADP is cleaved from the γ-phosphate of ATP, it is possible for these two types of oxygen to become equivalent. If ATP then reforms, these oxygens may exchange positions (“scramble”), a process that can be distinguished by ^{18}O labeling (Midelfort & Rose, 1976). The principle is illustrated in eq 5:



(5)

This process could occur if ADP is cleaved from the terminal phosphate in step 3 of eq 1. The step following this is slow, so that ATP can re-form after undergoing scrambling as in eq 5. However, if ATP cleavage is step 4 (as in the proposed mechanism of eq 3), then there is no chance of the oxygens scrambling since step 4 is rate limiting and essentially irreversible.

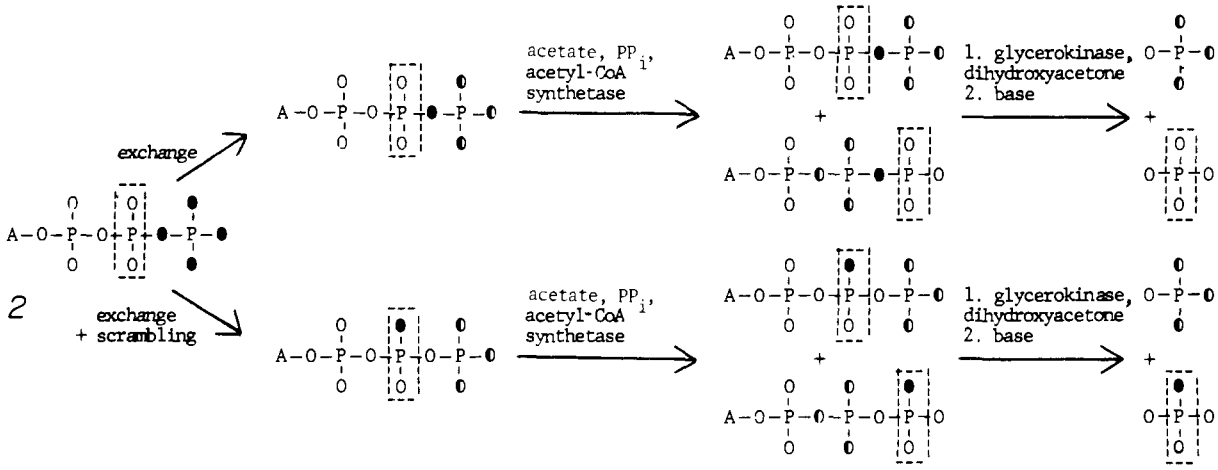
An experimental result which shows evidence of scrambling rules out a pseudorotation mechanism as being a sufficient description of step 3 in eq 1. However, scrambling will not necessarily be complete in the case of the hydrolysis mechanism of eq 2 because the β-phosphate group of ADP in M**·ADP·P_i may be so tightly coordinated to the protein that rotational freedom of the β-phosphate group is restricted.

Experimental Principle

In order to be able to determine the extent of scrambling, it is necessary to prepare ATP with the oxygen isotope in the $\beta\gamma$-bridging postion different from that in the β-nonbridging position. In this paper two such labeled ATP species are used, [$\alpha\beta$-^{18}O,β-$^{18}O_2$]ATP (**1**, Scheme I) and [$\beta\gamma$-^{18}O,γ-$^{18}O_3$]ATP (**2**, Scheme II). Each of these ATP species was incubated for 2 s with myosin subfragment 1 in molar excess (except for experiment 4). ATP binds rapidly to the protein, producing the steady-state intermediates (described as M*·ATP and M**·ADP·P_i in eq 1). However, the ATPase turnover time is such that almost no product is released within the 2 s. In addition, ATP binds so tightly to myosin that no ATP is released from the protein during the 2-s reaction time (Cardon & Boyer, 1978; Goody et al., 1977). After 2 s, the intermediates will be effectively at equilibrium with only ~10% of the nucleotide isolatable at ATP. It is this small amount of ATP that has to be analyzed to determine whether or not scrambling has occurred.

The reaction mixture was quenched in acid and the ATP was purified. In order to find the amount of scrambling at

Scheme II



2 s, the β-phosphate moiety containing the β-nonbridging oxygens but *not* the βγ-bridging oxygens is isolated for mass spectral analysis of the ^{18}O content. Two distinct methods of performing this analysis are used. One set of experiments is outlined in Scheme I. The oxygen-18 atoms initially are in nonbridging oxygens (enclosed by the dashed line). If the ATP undergoes scrambling, then some of the oxygen-18 will be transferred to the βγ-bridge and one nonbridging oxygen will become oxygen-16. Thus, the extent of scrambling is reflected in the proportion of ATP molecules in which the nonbridging oxygens, enclosed in the dashed line, have gone from two to one ^{18}O per molecule. The moiety enclosed by the dashed line is converted to P_i for mass spectral analysis as trimethyl phosphate in order to determine this extent.

The ATP is treated by the method of Webb (1980): the β- and γ-phosphates are randomly interconverted by treatment with dicyclohexylcarbodiimide to give adenosine trimetaphosphate, which readily is hydrolyzed back to ATP. The labeling pattern in this product ATP is shown in Scheme I, with the original β-oxygens enclosed by the dashed line. Now some of these oxygens are on the γ-phosphate, which can be cleaved from ATP by using D-glyceraldehyde and glycerokinase. This gives P_i containing the γ-oxygens of ATP (Webb, 1980) so that the pattern of labeling in the P_i is as shown in Scheme I. After methylation, chemical ionization–mass spectrometry gives the ratio of doubly to singly labeled species and therefore the extent of scrambling.

The analysis procedure for a second set of experiments is outlined in Scheme II and is as described by Midelfort & Rose (1976). In this case the ATP is labeled with oxygen-18 in the βγ-bridge and in the γ position (**2**, Scheme II). The β-oxygens, enclosed by the dashed line, are unlabeled. After incubation of this ATP with myosin subfragment 1 for 2 s and acid quench, the ATP remaining was purified. This ATP will have undergone extensive intermediate exchange (Bagshaw et al., 1975); the γ-oxygens exchange almost completely with water oxygens, so that the product ATP will have isotopic labeling as shown in Scheme II. The γ-oxygens are represented by ◐ to indicate that they are now only partially labeled. Scrambling results in the transfer of oxygen-18 from the βγ-bridge to the β-nonbridging positions, so that the β-oxygens will contain ^{18}O if scrambling occurs.

Fifty percent of the ATP is analyzed to find out the extent of scrambling. The ATP is treated with acetyl-CoA synthetase, acetyl-CoA, and a small amount of pyrophosphate. This allows the β- and γ-phosphates to interchange. Now half of the original β-oxygens, still enclosed by the dashed line, are on the γ-phosphate. Treatment of this ATP with dihydroxyacetone and glycerokinase, followed by alkaline hydrolysis, yields P_i with the pattern of labeling as shown in Scheme II. This P_i is methylated prior to mass spectrometry (Midelfort & Rose, 1976). The extent of scrambling is determined by the ratio of singly labeled to unlabeled P_i, after correction for the presence of material containing residual ^{18}O from the γ-phosphate (i.e., that left after intermediate exchange: ◐). In order to determine this residual ^{18}O, we treated the remaining 50% of the ATP with dihydroxyacetone and glycerokinase, followed by alkali. The resulting P_i is methylated and analyzed by mass spectrometry.

The experimental protocol requires dilution of the ATP during its isolation to provide sufficient material in the workup procedure. The dilution factor can be determined from the ^{18}O content of the β-phosphate group (of the [β-$^{18}O_1$]ADP formed in the glycerokinase reaction). This β-phosphate group may be isolated as P_i by acid hydrolysis of the ADP (Midelfort & Rose, 1976).

Experimental Procedures

Materials

Subfragment 1 was prepared by papain digestion from myosin extracted from rabbit skeletal muscle essentially as described by Lowey et al. (1969). [βγ-^{18}O, γ-$^{18}O_3$]ATP was prepared as described by Midelfort & Rose (1976). Its ^{18}O content was determined to be 91.3% enriched in the four labeled positions by mass spectroscopy of the γ-phosphate as trimethyl phosphate (Midelfort & Rose, 1976). [αβ-^{18}O,β-$^{18}O_2$]ATP was prepared as described by Webb (1980), and its ^{18}O content was determined by ^{31}P NMR using the upfield shift in ^{31}P resonance due to substitution of ^{16}O by ^{18}O (Cohn & Hu, 1978; Lowe & Sproat, 1978a). The three labeled positions were 80.0% enriched. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964) using [^{32}P]P_i from the Radiochemical Centre, Amersham, Bucks, U.K. All other chemicals were of reagent grade.

Methods

Experiment 1. A total of 2.9 μmol of [αβ-^{18}O,β-$^{18}O_2$]ATP (in 3 mL) was mixed with 5.0 μmol of subfragment 1 (in 23 mL), both in 50 mM KCl, 2 mM $MgCl_2$, and 50 mM Tris, adjusted to pH 8.0 by HCl. After 2 s the reaction was quenched by addition of 7% $HClO_4$ (10 mL) at 0 °C. (Mixing was done as rapidly as possible by using manual additions of ATP and acid from syringes to the stirred protein solution.) Unlabeled carrier ATP (4.4 μmol) was added with the acid.

The pH was raised to 4 by addition of 0.6 M potassium acetate and 3 M KOH. Protein was removed by centrifugation and the supernatant plus washings were collected. The ATP was purified by ion-exchange chromatography on a column of DEAE-cellulose (30 × 2 cm diameter). Elution was by a gradient of triethylammonium bicarbonate (2 L) from 10 to 400 mM. The product ATP was concentrated by rotary evaporation, and buffer was removed by evaporation of several batches of methanol from it. The ATP was analyzed for oxygen-18 distribution as described by Webb (1980) and outlined in Scheme I. In this and the following experiments, two sets of results were taken for each sample. In all cases the reproducibility was satisfactory. For example, the average variation in the ratio of peak intensities for m/e 145:143 was 2.5% in experiments 1 and 2.

Experiment 2. A similar procedure was used as in experiment 1 except that this reaction was done at low ionic strength. The buffer was 2 mM $MgCl_2$ and 10 mM Tris, adjusted to pH 8.0 with HCl. The same preparations of ^{18}O-labeled ATP and subfragment 1 were used in experiments 1 and 2.

Experiment 3. [$\beta\gamma$-^{18}O,γ-$^{18}O_3$]ATP (12 μM, 1.5 μmol) plus trace [γ-^{32}P]ATP were mixed at 20 °C with 18 μM subfragment 1 in 50 mM KCl, 2 mM $MgCl_2$, and 50 mM Tris, adjusted to pH 8.0 by HCl. The reaction was carried out in several stages by using 4 mL of reactants at a time in a quenched-flow apparatus [a modification of the instrument described by Gutfreund (1969)]. Each sample was quenched at 2 s into an equal volume of 7% $HClO_4$ at 0 °C. The pH was raised to 4 by addition of 0.6 M potassium acetate and 3 M KOH. Protein was removed by centrifugation and the supernatant plus washings were collected. Unlabeled carrier ATP (1.5 μmol) was added to the mixture containing labeled ATP (0.106 μmol, determined from the total ^{32}P and K_3 which was measured as described below). The ATP was purified by ion-exchange chromatography as described above and analyzed as described in Midelfort & Rose (1976) and outlined in Scheme II.

Experiment 4. The same procedure was carried out as in experiment 3, except that 20 μM [$\beta\gamma$-^{18}O,γ-$^{18}O_3$]ATP (4.00 μmol) and trace [γ-^{32}P]ATP were mixed with 19 μM subfragment 1. In this experiment the excess ATP present meant that some ATP was free in solution at the time of quenching; hence K_3 could not be determined directly. As indicated above, the dilution of ATP with unlabeled ATP prior to the DEAE column must be measured for the experimental analysis. Consequently, the ADP formed after the glycerokinase treatment of a sample of this column-purified ATP was hydrolyzed in 1 N HCl. The ^{18}O content of the P_i isolated from the β-phosphate group of the ADP showed that the ATP had been diluted to 10.1-fold.

Determination of K_3. The equilibrium constant for step 3, M*·ATP ⇌ M**·ADP·P_i at 50 mM KCl, 2 mM $MgCl_2$, and 50 mM Tris, pH 8.0, was determined by the method of Bagshaw & Trentham (1973). After [γ-^{32}P]ATP was mixed with a molar excess of subfragment 1 and the solution was acid quenched after 2 s in 7% $HClO_4$, the ratio of [γ-^{32}P]ATP to [^{32}P]P_i was determined by using chromatography on poly(ethylenimine) plates. K_3 was 8.4. In the low salt conditions of experiment 2, K_3 = 3.5 (M. A. Geeves and D. R. Trentham, unpublished experiments).

Results

Chemical ionization–mass spectrometry of trimethyl phosphate, enriched with oxygen-18, gives rise to a series of molecular ion peaks, m/e 141, 143, 145, 147, and 149, corresponding to zero, one, two, three, and four ^{18}O/molecule. The peak intensities give the relative amount of each species.

Table I: Mass Spectral Data from Scrambling Experiments with [$\alpha\beta$-^{18}O,β-$^{18}O_2$]ATP

expt	origin of trimethyl phosphate	rel peak intensities[a] at 141	143	145
1[b]	from ATP[c]	92.34	4.71	2.95
	predicted for no scrambling[d]	92.18	2.65	5.27
	predicted for complete scrambling[d]	92.97	5.27	1.76
2[b]	from ATP[c]	93.37	2.61	4.01
	predicted for no scrambling[d]	93.25	2.24	4.50
	predicted for complete scrambling[d]	94.00	4.50	1.50

[a] Normalized. Peak m/e 143 is corrected for natural abundance of oxygen-18. [b] See the text for details of myosin subfragment 1 incubation conditions. [c] ATP, after incubation with myosin subfragment 1, was chemically manipulated as in Scheme I before the new γ-phosphate was cleaved to P_i and methylated. [d] Calculated from NMR data which show that the oxygen-18 is 80% enriched, before dilution with unlabeled, carrier ATP.

Table I shows the mass spectral results for trimethyl phosphate from scrambling experiments 1 and 2 with [$\alpha\beta$-^{18}O,β-$^{18}O_2$]ATP. The first line for each incubation shows the experimental data for the phosphate moiety that has been taken through the procedure in Scheme I. The presence of unlabeled ATP as carrier causes the peak at m/e 141 to be high, but as described above, this peak is not used directly in the analysis. Considering first the results for experiment 1, the intensity of m/e 145 arises solely from [$^{18}O_2$]P_i, which is the product from the β-phosphate of unscrambled ATP. However, because the isotopic enrichment was only 80% for each labeled oxygen, [$^{18}O_2$]P_i gives rise to a family of peaks with the ratio m/e 141:143:145 = 4:32:64. This ratio is 0.18:1.48:2.95, when adjusted to the actual m/e 145 peak size. The total intensity of this family of peaks gives the amount of [$^{18}O_1$]P_i from unscrambled ATP. This species gives some intensity at m/e 143; the remainder must be due to the [$^{18}O_1$]P_i produced from the β-phosphate of ATP that has undergone scrambling as in Scheme I. The intensity at m/e 143 due to this species is 4.71 − 1.48 = 3.23. Again, because the ^{18}O enrichment is only 80%, the [$^{18}O_1$]P_i gives rise to a family of peaks with the ratio m/e 141:143 of 0.80:3.23 when adjusted to the actual m/e 143 peak intensity. The total intensity of this family of peaks gives the amount of [$^{18}O_1$]P_i due to scrambled ATP. The ratio of scrambled to unscrambled ATP is given by the ratio of these two families of peaks from [$^{18}O_1$]P_i and [$^{18}O_2$]P_i (0.80 + 3.23):(0.18 + 1.48 + 2.95) = 0.87. Thus, the fraction of ATP molecules that have undergone isotopic transfer is 0.87/(1 + 0.87) = 0.46. Even with complete scrambling, transfer of an oxygen atom from a β-nonbridge to $\beta\gamma$-bridging position will only occur in two-thirds of the ATP molecules, so that the extent of scrambling in experiment 1 is 70%. For comparison, the ^{18}O distributions expected for complete and no scrambling are given in Table I.

Table I also gives the results for experiment 2 which was done at low ionic strength. In this case the extent of scrambling was calculated to be 16%.

When [$\beta\gamma$-^{18}O,γ-$^{18}O_3$]ATP (**2**) is used for the scrambling experiment, an increase in singly labeled P_i gives evidence for the scrambling process, as shown in Scheme II. Table II gives the results of two such experiments. In experiment 3, subfragment 1 was in excess over ATP for the incubation. The first line of data shows the distribution of ^{18}O in the γ-phosphate of the ATP after this incubation. The distribution shows that there was extensive intermediate exchange. With

Table II: Mass Spectral Data for Scrambling Experiments Using [$\beta\gamma$-^{18}O,γ-^{18}O$_3$]ATP

expt	origin of trimethyl phosphate	rel peak intensities[a] at 141	143	145	147
3[b]	γ-phosphate of ATP with no β- to γ-phosphate interchange[c]	99.41	0.27	0.15	0.17
	γ-phosphate of ATP after β- to γ-phosphate interchange[d]	99.29	0.63	0.03	0.05
	corrected for unlabeled pyrophosphate[e]	99.22	0.69	0.03	0.06
	predicted for no scrambling[f]	99.72	0.13	0.07	0.08
	predicted for complete scrambling[g]	97.70	2.15	0.07	0.08
4[b]	γ-phosphate of ATP with no β- to γ-phosphate interchange[h]	96.05	0.29	0.73	2.93
	γ-phosphate of ATP after β- to γ-phosphate interchange[d]	97.78	0.67	0.32	1.23
	corrected for unlabeled pyrophosphate[i]	97.35	0.80	0.38	1.47
	predicted for no scrambling[f]	98.03	0.14	0.36	1.47
	predicted for complete scrambling[j]	96.19	1.98	0.36	1.47

[a] Normalized. Peak *m/e* 143 is corrected for natural abundance of oxygen-18. [b] See the text for details of myosin subfragment 1 incubations. The [$\beta\gamma$-^{18}O,γ-^{18}O$_3$]ATP, labeled to an extent of 91.3%, was diluted 15.1-fold in experiment 3 and 10.1-fold in experiment 4 prior to isolation on a DEAE column (see Experimental Procedures). [c] P_i was obtained from the γ-phosphate of ATP, after the incubation with myosin subfragment 1 but prior to β- and γ-phosphate interchange. The mass spectral peaks show the oxygen-18 remaining in the γ-phosphate after intermediate exchange. [d] ATP, after incubation with myosin subfragment 1, was enzymically manipulated as in Scheme II, before the new γ-phosphate was cleaved and methylated. [e] Calculated, as unlabeled pyrophosphate (10% of the molarity of ATP) was added to promote pyrophosphate exchange. [f] Calculated by assuming that the only oxygen-18 observed is that remaining after intermediate exchange. The acetyl-CoA synthetase exchange reaction will remove half of this ^{18}O from the γ-phosphate group. [g] Calculated by assuming half the P_i derived from labeled ATP is from the β-phosphate. Two-thirds of this P_i contains one oxygen-18 (derived from the $\beta\gamma$-bridge oxygen at 91.3% ^{18}O enriched in the initial ATP); the other half contains ^{18}O with the distribution obtained for intermediate exchange. [h] The γ-phosphate of ATP was obtained after incubation with myosin subfragment 1 but prior to β- and γ-phosphate interchange. The mass spectral peaks show significant oxygen-18 remaining. From these data 61% of the isolated ATP was protein bound at the time of quenching and 39% was free in solution. The 61:39 ratio was calculated on the basis that M*·ATP would have zero *m/e* 147 peak due to total intermediate change (cf. sample 1, experiment 3) and hence any *m/e* 147 peak was due to unbound ATP. In this experiment P_i was isolated from M**·ATP·P_i and showed almost total exchange (98.9%). [i] Calculated by adjusting the *m/e* 147 peak intensity (=1.23) to the predicted intensity (=1.47) which is independent of the extent of scrambling. (This infers the pyrophosphate dilution was 16%; cf. footnote *e*.) [j] The predicted intensity of the *m/e* 143 peak takes into account the fact that only 61% of the isolated ATP was M*·ATP; see footnote *h*. Otherwise, the calculation is as in footnote *g*.

no exchange, the *m/e* 147 peak would have been 5.23% from the known dilution of the [^{18}O]ATP with unlabeled material. From these data, the relative peak intensities for no scrambling and complete scrambling can be calculated and are shown in Table II. These are compared with the result obtained for ATP after the PP_i exchange process. Correction is made to this experimental result, to take into account dilution with unlabeled PP_i. The extent of scrambling depends on the intensity of the *m/e* 143 peaks and can be calculated from a comparison of the peak intensity expected for complete and zero scrambling. Thus, the extent of scrambling in experiment 3 is 100(0.69 − 0.13)/(2.15 − 0.13) = 28%.

In experiment 4, the ATP was in excess over subfragment 1, so that during the incubation time some ATP remained unbound and will not therefore undergo either scrambling or oxygen exchange with solvent. This is reflected in the amount of ^{18}O in the γ-phosphate in the first line of data for experiment 4.

The *m/e* 145 and 147 peak ratio should not be effected by scrambling. Thus, this ratio provides an excellent internal check on the analytical procedure. As can be seen, the experimentally determined *m/e* 145 to 147 peak ratio of ATP that has been analyzed for scrambling (0.32:1.32) matches very closely that in which the γ-phosphate group has been removed without PP_i exchange (0.73:2.93). After correction for unlabeled PP_i, the extent of scrambling in experiment 4 can be calculated from the intensity of the *m/e* 143 peak as for experiment 3 and is 36%.

Discussion

The experiments described in this paper show that the β-nonbridge and $\beta\gamma$-bridge oxygens of ATP have partially exchanged positions. This result requires that when bound to myosin, ATP undergoes reversible cleavage to ADP. Before discussing the overall conclusions, it is necessary to discuss the differences between the experiments and between the actual values obtained for the extents of scrambling. Three of the incubations (experiments 1, 3, and 4) of ATP and subfragment 1 had similar buffer conditions at high ionic strength. However, there is variation in the extents of scrambling obtained for these conditions: 28, 36, and 70%, giving an average of 45%.

A number of factors can contribute to the variability in extent of scrambling. Myosin subfragment 1 itself is variable to some degree, both in the kinetics of individual steps and in the percent of the material that is active (Tonomura & Inoue, 1974; Trentham et al., 1976). ^{18}O exchange experiments indicate that there may be two modes of hydrolysis of MgATP by some preparations of myosin (M. R. Webb, D. R. Trentham, K. K. Shukla, and H. M. Levy, unpublished results). Most ATP undergoes hydrolysis with almost complete exchange, although a small but variable percent undergoes hydrolysis with almost no exchange. This may well reflect partially damaged ATPase sites, which would cause differences in the extent of scrambling. The incubation time with subfragment 1 (2 s) is difficult to measure accurately and so may provide some variation between experiments. If some ATP remained unbound during the incubation, the observed extent of scrambling would be decreased. Indeed, this is a potential source of great variation. For example, if 10% of the ATP remained unbound, this would give rise to half the recovered ATP (since ~90% of the bound ATP gives ADP + P_i). Thus, the observed extent of scrambling would be only half the real extent. In experiment 4, this problem could be compensated for, as ATP was sufficiently in excess so that the amount of bound ATP could be calculated. In all other experiments, the protein was present in considerable excess over ATP to minimize the possibility of unbound ATP. In experiment 3, the almost complete intermediate exchange of the γ-oxygens of ATP shows that little or no ATP remained unbound. In all the experiments, the amount of ATP available for analysis is severely limited. The analytical procedure involves considerable manipulation of the small amount of product ATP in order to obtain the mass spectral data.[2] The extent of

scrambling very likely depends on the lifetime of ADP bound to protein in a steady-state complex and on the angular velocity of rotation of the β-oxygens of ADP. Factors such as slight variation in temperature or ionic strength may well affect these two processes differently. One experiment was done at low ionic strength and indeed did show a much lower extent of scrambling. It should be noted that the calculation of the extent of scrambling in experiments 3 and 4 depends on an accurate determination of the isotopic dilution during the workup procedure and particularly on the absence of contamination by P_i in the final steps. The calculation is essentially independent of the dilution in experiments 1 and 2.

All the results agree with the conclusion that scrambling occurs, but at a slow rate since scrambling is incomplete during the 2-s incubation time. The fact that scrambling does occur to a significant extent makes the pseudorotation mechanism as formulated in eq 3 untenable.[3] The cleavage of ATP to ADP must be reversible. The experiments in this paper give the first direct evidence for this reversible cleavage. Previous evidence is based on the acid quench of protein-bound nucleotide. In such experiments ADP is released but may have been bound to the γ-phosphate in a labile species. Oxygen-18 exchange experiments give direct information about the involvement of water in the ATPase, not on the cleavage of ADP from ATP. While not consistent with "M**·ADP·P_i" being a pentacovalent phosphorus species, the results in this paper do not rule out M**·ADP·P_i being protein-bound ADP and metaphosphate, although the evidence referred to in the introduction makes this mechanism unlikely. The most likely explanation is that step 3 of eq 1 is a simple hydrolysis. The γ-phosphate of ATP is reversibly transferred to water to give M**·ADP·P_i as written in eq 2.

The rate of scrambling may be quantified by using a rate constant k_r for the randomization of orientation of the β-oxygens of ADP in M**·ADP·P_i. Substrate ATP binds rapidly to myosin, and because k_{+3} and k_{-3} are large, it rapidly forms the equilibrium with M**·ADP·P_i. In this first-formed products complex, the β-oxygens are held in an orientation (as in structure **4**, eq 5), such that the same oxygen will form the $\beta\gamma$-bridge when ATP re-forms. k_r represents the process by which the β-oxygens are released from this orientation and rotate freely to give a random distribution of the oxygen label (eq 6). This new products complex, M**·ADP′·P_i (differing

$$\begin{array}{ccc} M^{*}\cdot ATP & \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} & M^{**}\cdot ADP\cdot P_i \\ & & \downarrow k_r \\ M^{*}\cdot ATP' & \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} & M^{**}\cdot ADP'\cdot P_i \end{array} \qquad (6)$$

from M**·ADP·P_i only in the position of oxygen-18 atoms) is an equilibrium mixture of **4** and **5** (eq 5) in a ratio of 1:2. (Step 4 is not shown as we are only concerned with material that remains in the steady-state complexes.) As soon as M**·ADP′·P_i forms, it gives an equilibrium proportion of M*·ATP′, which represents ATP with fully scrambled label (**3** and **6** in a ratio of 1:2). After 2 s, the reaction is quenched and the extent of scrambling (E) is given by M*·ATP′/(M*·ATP′ + M*·ATP). Because the equilibrium of step 3 is rapid, E = M**·ADP′·P_i/(M**·ADP′·P_i + M**·ADP·P_i). The rate of randomization is

$$-\frac{d[M^{**}\cdot ADP\cdot P_i + M^{*}\cdot ATP]}{dt} = k_r[M^{**}\cdot ADP\cdot P_i]$$

Thus

$$-\frac{1 + K_3}{K_3}\frac{d[M^{**}\cdot ADP\cdot P_i]}{dt} = k_r[M^{**}\cdot ADP\cdot P_i]$$

and so

$$-\frac{1 + K_3}{K_3}\ln\frac{M^{**}\cdot ADP\cdot P_i}{M^{**}\cdot ADP'\cdot P_i + M^{**}\cdot ADP\cdot P_i} = k_r t$$

and at t = 2 s

$$k_r = -\frac{1 + K_3}{2K_3}\ln(1 - E)$$

At the higher ionic strength conditions, K_3 = 8.4 and E = 0.45, so that k_r = 0.33 s^{-1}. At low ionic strength, K_3 = 3.5 and E = 0.16, giving k_r = 0.11 s^{-1}.

In contrast to this slow positional isotope exchange, the results in Table II show that water oxygen exchange with the γ-phosphate of ATP occurs much more rapidly. The difference in rates for the two exchange processes was also observed by Wimmer & Rose (1977) analyzing the interaction of ATP with ATP synthase of chloroplast lamellae. They suggest that the ADP is restricted from free rotation by binding of the β-phosphate.

The values for the rate constants for rotation of the β-phosphate can be compared with the rate constant for a similar randomization of the P_i oxygens obtained from intermediate exchange results. All oxygens of P_i are equally likely to undergo exchange with those of water (Sleep et al., 1978).[4] ATP labeled with ^{18}O in the γ position binds to myosin to give M*·ATP, which is rapidly cleaved to M**·ADP·P_i. This first-formed products complex may be considered as having the P_i oxygens in an orientation so that, on re-forming ATP, the same oxygen that entered the P_i leaves it:

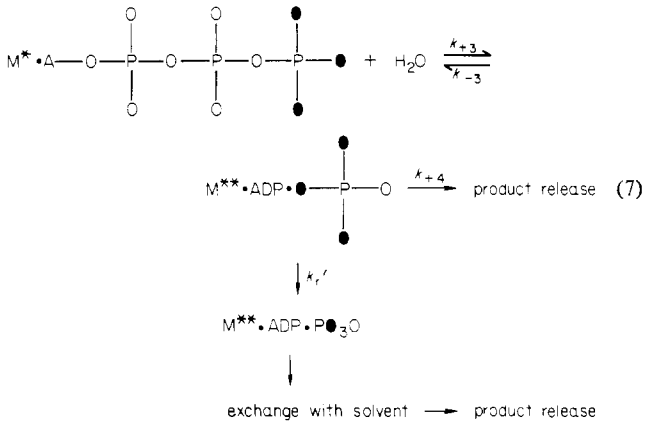
[2] An attempt to analyze the ATP product directly by nuclear magnetic resonance, using the ^{18}O shift in ^{31}P resonance (Cohn & Hu, 1978; Lowe & Sproat, 1978a), failed due to the insufficient material available to obtain the high-resolution spectrum required.

[3] An alternative explanation of the observed slow rotation at the β-phosphate allows the pseudorotation mechanism to remain tenable. This requires an extra, reversible step in the kinetic mechanism of eq 1 following the steady-state intermediate "M**·ADP·P_i", which can be the pentacovalent intermediate of eq 4:

$$M^{**}\cdot ADP\cdot P_i \underset{k_{-a}}{\overset{k_{+a}}{\rightleftharpoons}} M^{+}\cdot ADP\cdot P_i \rightarrow M^{*}\cdot ADP\cdot P_i$$

In this scheme it is assumed that the randomization of the β-phosphate occurs rapidly at M+·ADP·P_i, which represents the cleaved products. We know from transient kinetic experiments (Taylor, 1979) that P_i formation is monophasic and relatively rapid and that the interconversion of the steady-state intermediates is readily reversible, so that if M+·ADP·P_i was a significant proportion of the steady-state intermediates, both k_{+a} and k_{-a} must be fast. However, the slow scrambling of protein-bound ATP is incompatible with this rapid interconversion of M**·ADP·P_i and M+·ADP·P_i so that it must be that [M**·ADP·P_i] ≫ [M+·ADP·P_i]. From this it follows that $k_{+a} \ll k_{-a}$ and k_{+a} approximately equals k_r, the rate constant for randomization of β-oxygens of M**·ADP·P_i, since this k_r (defined more exactly in the text) is controlled by the flux of M**·ADP·P_i to M+·ADP·P_i. It should be noted that the model suggested above is not compatible with the "refractory state" model of Eisenberg et al. (1972) in which M**·ADP·P_i is the refractory state. This incompatibility is because in the refractory state model, k_{+a} must equal the catalytic center activity of the actomyosin ATPase (20 s^{-1} at pH 8.0) which is 2 orders of magnitude greater than the value predicted in this model.

[4] This evidence of oxygen equivalent based on medium exchange is in constrast to the conclusions of Shukla & Levy (1978) based on intermediate exchange. However, more recent work has shown that the intermediate exchange by actomyosin is indeed random (Sleep et al., 1980; M. R. Webb, D. R. Trentham, K. K. Shukla, and H. M. Levy, unpublished result). Actomyosin gives only partial exchange unlike the almost complete exchange in the absence of actin. It is therefore a more sensitive test of oxygen equivalence.



This first-formed M**·ADP·P_i has two possible fates apart from re-forming unchanged M*·ATP. It can release products (governed by k_{+4}) or the P_i can rotate in the active site, so that the random exchange can occur. This irreversible process of randomization of the oxygen orientation (so that now any oxygen will leave on re-forming ATP) is governed by the rate constant k_r'. Because intermediate exchange is random, almost all M**·ADP·P_i (>98%) must take this randomization pathway. In practice this means that $k_r' > 50k_{+4}$. In the case of myosin, k_{+4} is 0.06 s^{-1}, so that $k_r' > 3$ s^{-1}. It is likely that the actomyosin ATPase mechanism also has M**·ADP·P_i as an intermediate on its reaction pathway (Taylor, 1979). Under conditions in which the breakdown of M**·ADP·P_i to products is at 2 s^{-1} (i.e., apparent k_{+4} = 2 s^{-1}), random exchange is observed[4] so that $k_r' > 100$ s^{-1}.

The rate constants k_r and k_r' represent the rates at which the oxygens of ADP and P_i become randomized in orientation. Presumably this process is a release of coordination of the oxygens, followed by rotation. In the case of ADP, all three β-oxygens must be free from coordination for rotation to occur. With P_i, complete freedom from coordination would allow rotation. However, rotation of three oxygens can occur if one remains coordinated. This one oxygen must then be released as one of the others becomes coordinated. Rotation now achieves complete randomization.

The difference between k_r and k_r' shows that there is a constraint on ADP very different from that of P_i. This may be due to the tighter coordination of ADP in the active site. Indeed, coordination of the β-oxygens of ATP to electrophilic sites (one of which may well be Mg^{2+}) would increase the lability of the ($\beta\gamma$-O)–(γ-P) bond, thus helping the rapid cleavage of ATP.

Acknowledgments

We thank Vinka Parmakovich, Slavica Sporer, and Vincent Saltmach of the Columbia University Chemistry Department for performing the mass spectral analyses.

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