A Method for Determining the Positional Isotope Exchange in a Nucleoside Triphosphate: Cyclization of Nucleoside Triphosphate by Dicyclohexylcarbodiimide†

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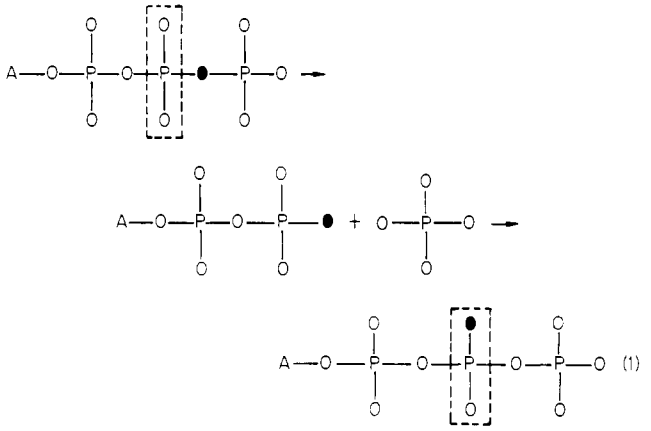
ABSTRACT: The preparations of [$\beta\gamma$-^{18}O,γ-$^{18}O_3$]ATP and [$\beta\gamma$-^{18}O,β-$^{18}O_2$]ATP and a procedure for studying the positional isotopic exchange method of Midelfort and Rose [Midelfort, C. F., & Rose, I. A. (1976) *J. Biol. Chem. 251*, 5881–5887] are described for detection of transient ATP cleavage to ADP in enzyme active sites. ATP is cyclized by reaction with dicyclohexylcarbodiimide in anhydrous pyridine to give adenosine 5′-trimetaphosphate. This rapidly undergoes ring opening on adding water to re-form ATP. This results in the randomization of the β- and γ-phosphates. The new γ-phosphate is hydrolyzed to P_i by using glycerokinase and D-glyceraldehyde. Mass spectral analysis of the ^{18}O distribution in this P_i allows the extent of positional isotopic exchange to be determined. Procedures suitable for use with GTP are also described.

The oxygen-isotope scrambling method of Midelfort & Rose (1976), recently reviewed by Rose (1979), provides an elegant method for studying the cleavage and re-formation of ATP in an enzyme active site. Midelfort & Rose (1976) labeled this bridging oxygen with oxygen-18, while the β-nonbridging oxygens were oxygen-16. If, on incubation with the enzyme, this ATP is cleaved to ADP and either P_i (bound in the active site) or covalently bound phosphate, the free β-oxygens and the $\beta\gamma$-oxygen now may become equivalent, so that re-formation of ATP occurs with the bridging oxygen unlabeled:[1]

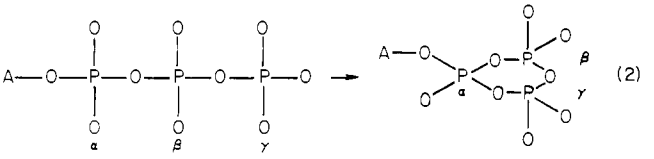


(1)

The fact the $\beta\gamma$-bridging and β-nonbridging oxygens have exchanged positions ("scrambled") gives conclusive evidence that this cleavage and re-formation of ATP can occur. This type of experiment is applicable to many enzymic reactions involving ATP and is one of the best methods for determining if cleavage of a single ATP molecule can occur alone in the active site or with other components of the system.

However, analysis of the two isotopic isomers of ATP in eq 1 is not straightforward: relative amounts of isotope in the $\beta\gamma$-bridging position and in the β-nonbridging positions must be measured. Midelfort & Rose (1976) found the extent of the scrambling by a series of enzymic reactions to isolate the β-phosphate moiety of ATP as inorganic phosphate containing the β-nonbridging oxygens (enclosed by the dashed line in eq 1) but not the $\beta\gamma$-bridging oxygens. Mass spectral analysis of this P_i as trimethyl phosphate showed the extent to which isotopic oxygen had been transferred to the β position. NMR analysis has been used by Lowe & Sproat (1978a) and by J. Bock and M. Cohn (personal communication), using the ^{18}O shift in the ^{31}P resonance (Cohn & Hu, 1978; Lowe & Sproat, 1978a,b) to look directly at the ATP product. The NMR method is limited by the amount of material available, while the enzymic method of analysis is restricted to ATP and so is not applicable to other nucleoside triphosphates.

In this paper, an alternative method of analysis is described and its use is illustrated. Reaction of ATP with dicyclohexylcarbodiimide forms adenosine trimetaphosphate (**1**) (Glonek et al., 1974):



(2)

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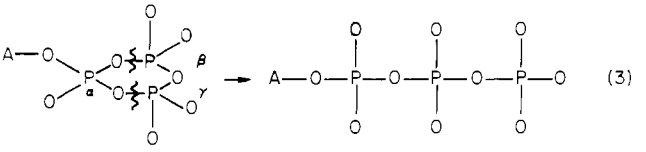
In this species the β- and γ-phosphates are equivalent, so that on hydrolysis ATP is re-formed with 50% of the original β-phosphate now in the γ position:



(3)

Enzymic cleavage of the product ATP by glycerokinase and D-glyceraldehyde gives ADP and P_i, 50% of which is derived from the γ-phosphate of original ATP and 50% of which is derived from the β-phosphate. This P_i derived from the β-phosphate contains the β-nonbridging oxygens but not the $\beta\gamma$-bridging oxygen, so that mass spectral analysis determines the extent to which isotope has exchanged positions as in eq 1.

The method described in this paper enables the applications of the "scrambling" experiments to be extended. Because the basis of this method is chemical, it is suitable for use with other nucleoside triphosphates, apart from ATP, and procedures are described for both GTP and ATP. The method is applicable

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[1] In this paper ● represents an oxygen labeled with ^{18}O. In molecular structures, the protonation state, negative charges, and π bonds are omitted to avoid confusion.

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when only a small amount of labeled material is available. In the following paper (Geeves et al., 1980), the method is used in an investigation of ATP hydrolysis by myosin. In this case not only is the amount of ATP limited by the requirement of having protein in excess of ATP but also the ATP available for analysis is reduced, due to the unfavorable equilibrium constant between protein-bound ATP and protein-bound products.

Experimental Procedures

^{18}O-Enriched water was purchased from Monsanto Research Corp. D-Glyceraldehyde was from Koch-Light Laboratories, England. Diadenosine pentaphosphate, glycerokinase (from *Escherichia coli*), nucleoside diphosphate kinase (from yeast), sodium adenosine 5′-diphosphate, carbonyldiimidazole, and hexamethylphosphoramide were from Sigma Chemical Co. $[^{18}O_4]KH_2PO_4$ was prepared by using the method of Hackney et al. (1980).

$[\beta\gamma\text{-}^{18}O,\gamma\text{-}^{18}O_3]$ATP was prepared by a modified version of the method of Ott et al. (1967), using carbonyldiimidazole to activate ADP, followed by reaction with $[^{18}O_4]P_i$. ADP (250 μmol) was converted to its pyridine salt on a column of Dowex 50 (10 × 1 cm diameter) in the pyridine form. Tributylamine (60 μL, 250 μmol) was added and water was removed by rotary evaporation. The ADP was made anhydrous by repeated removal of anhydrous dimethylformamide and pyridine. The ADP was dissolved finally in anhydrous hexamethylphosphoramide (4 mL). Carbonyldiimidazole (250 mg) was dissolved in this solution and the sealed flask was left for 12 h. Excess carbonyldiimidazole was removed by adding anhydrous methanol (100 μL) and leaving for 3 h. The tributylamine form of $[^{18}O_4]P_i$ (250 μmol) was prepared as described for ADP. The final gum was dissolved in the hexamethylphosphoramide solution of activated ADP and the mixture was left 12 h in a sealed flask. Water (20 mL) and triethylamine (100 μL) were added, and after 30 min the solution was adjusted to pH 8.0. The ATP was purified on a column of DEAE-cellulose (30 × 3 cm diameter), preequilibrated with 10 mM triethylammonium bicarbonate. Elution was by a gradient (1.2 L) of triethylammonium bicarbonate from 10 to 400 mM. Fractions containing ATP were pooled, and salt was removed by rotary evaporation of water and then several batches of methanol. The ATP was stored as an aqueous solution frozen at −20 °C and was repurified by ion-exchange chromatography immediately prior to use, to give a final yield of 50%.

$[\alpha\beta\text{-}^{18}O,\beta\text{-}^{18}O_2]$ATP was prepared by using this process sequentially. First AMP and $[^{18}O_4]P_i$ were coupled to give $[\alpha\beta\text{-}^{18}O,\beta\text{-}^{18}O_3]$ADP. Reaction of this with unlabeled P_i gave the required product, in a final yield of 20%.

Interconversion of the β- and γ-phosphate moieties of ATP was achieved as follows. $[\beta\gamma\text{-}^{18}O,\gamma\text{-}^{18}O_3]$ATP (12 μmol) was converted to its free acid on a Dowex 50 column (10 × 1 cm diameter) in its H^+ form. Tributylamine (3 μL, 12 μmol) was added and water was removed by rotary evaporation. The ATP was made anhydrous by repeated removal of anhydrous dimethylformamide and pyridine. The ATP was finally dissolved in anhydrous pyridine (2 mL). Dicyclohexylcarbodiimide (100 μmol) was added and the solution was left 15 min at room temperature, unlabeled water (20 mL) was added, and after 15 min the urea precipitate was removed by filtration. The filtrate containing the product ATP in 90% yield was evaporated to dryness and then dissolved in water (2.0 mL) for NMR spectroscopy or treatment with glycerokinase.

$[\alpha\beta\text{-}^{18}O,\beta\text{-}^{18}O_2]$ATP (3 μmol, containing 10 μmol of unlabeled ATP) was treated likewise in order to interconvert its β- and γ-phosphates. Unlabeled ATP (12 μmol) was treated with dicyclohexylcarbodiimide (100 μmol) as above to test for the position of attack by water on the adenosine trimetaphosphate. After 15 min, the solution was treated with ^{18}O-enriched water (0.5 mL) and left for a further 15 min. Unlabeled water (20 mL) was added and the solution was prepared for NMR as above. The conversion of the γ-phosphate of ATP to trimethyl phosphate proceeded as follows. The solution of ATP (2 mL) was made 50 mM in ammonium bicarbonate (pH 8.3), 20 mM in $MgCl_2$, and 10 μM in diadenosine pentaphosphate (to inhibit any adenylate kinase impurity). D-Glyceraldehyde (1 mg) was added, followed by glycerokinase (20 units) (Hayashi & Lin, 1967). The formation of ADP and disappearance of ATP were monitored by coupled enzyme assays. After ~90% reaction, $[^{32}P]P_i$ (0.1 μCi) was added as a marker and the P_i was purified on a column of DEAE-cellulose (15 × 1.5 cm diameter). Elution was by a gradient (200 mL) of triethylammonium bicarbonate (10–400 mM). The P_i had the salt removed by repeated rotary evaporation of methanol from it. It was converted to its free acid as described above (for ATP). Water was removed by rotary evaporation and the P_i was taken up in methanol (100 μL containing 10% water). The γ-phosphate of GTP was converted to P_i as described above, except that the glycerokinase incubation solution also contained nucleoside diphosphate kinase (20 units) and ADP (1 mM).

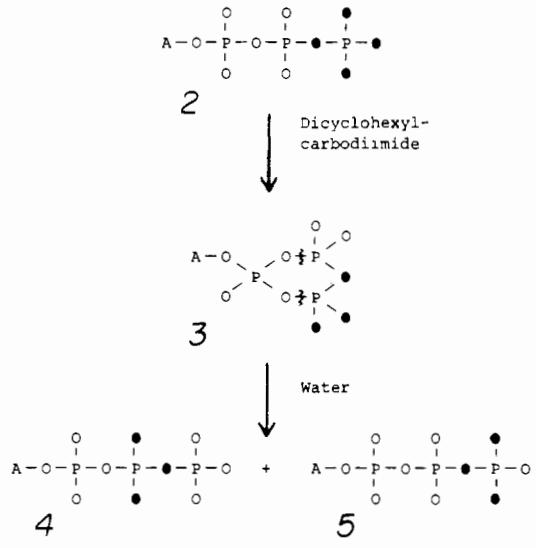
The P_i was methylated by addition of a large excess of diazomethane in ether (2 mL). The ethereal solution of trimethyl phosphate was concentrated prior to analysis on a gas chromatograph–mass spectrometer (Finnigan Model 3300). The column (180 × 0.4 cm) was 0.5% Carbowax 20M and 6% OV-101 (both from BDH Chemicals Ltd., England) supported on Gas-Chrom Q (Applied Science Laboratories, Inc.) treated with Silyl-8 (Pierce Chemical Co.). Elution by a temperature program of 50–150 °C at 4 °C min^{-1} gave trimethyl phosphate free of any significant contamination. Chemical ionization with methane produced the parent ions without fragmentation. The signal intensities at m/e 141, 143, and 145 were continuously monitored and then integrated to obtain the relative amounts of phosphate with zero, one, and two ^{18}O atoms per molecule.

High-resolution ^{31}P NMR spectra to determine ^{18}O distribution 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 (2.0 mL) containing 20% D_2O and 10 mM EDTA was made pH 8.8 by addition of Tris. The sample was in a 1 cm diameter sample tube with a Teflon vortex suppressor and was maintained at 22 °C. A 1000-Hz spectral width was used, with a pulse width of 15 μs and an aquisition time of 8.2 s. A sensitivity enhancement exponential function gave a line broadening of 0.1 Hz.

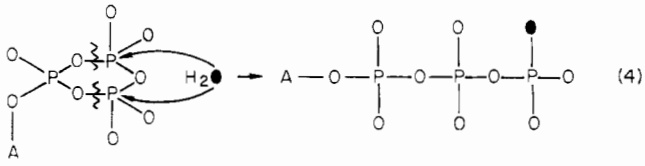
Results and Discussion

The reaction of ATP with dicyclohexylcarbodiimide in anhydrous pyridine was described by Glonek et al. (1974), who proposed that the product was adenosine 5′-trimetaphosphate (**1**). The existence of this species was first suggested by Smith & Khorana (1958) to explain the product distribution of the condensation of nucleoside monophosphates with inorganic phosphate in the presence of dicyclohexylcarbodiimide. However, apart from NMR data of Glonek et al. (1974), there is no direct evidence for the trimetaphosphate species. The assumed trimetaphosphate is very susceptible to nucleophilic attack (Knorre et al., 1976) and, in particular, the ring is rapidly opened by water to re-form ATP. The following experiment was done in order to determine the points of attack of water on the trimetaphosphate. Reaction of unlabeled ATP

Scheme I



with dicyclohexylcarbodiimide in anhydrous pyridine, followed by ring hydrolysis by ^{18}O-labeled water, gave ATP, almost quantitatively, with all isotope in the γ position. The hydrolysis



must proceed solely by attack of water on the phosphorus atoms *not* bound to the adenosine:

In the cyclic species the β- and γ-phosphate species should be equivalent (apart from the asymmetry of the adenosine). Therefore, it seems reasonable to expect the reaction to be able to interchange the β- and γ-phosphate moieties. The exact fates of the P and O atoms were followed by using ^{18}O labeling in order to test this possibility. [$\beta\gamma$-^{18}O,γ-$^{18}O_3$]ATP (**2**) was prepared and treated with dicyclohexylcarbodiimide, followed by water (Scheme I). The product ATP was analyzed by ^{31}P NMR. The distribution of ^{18}O around the β- and γ-phosphorus atoms was consistent with the following distribution of ATP species: 20% unreacted ATP (**2**), 40% **4**, and 40% **5**. The cyclic compound is being hydrolyzed without discrimination between the two positions of cleavage, as shown in Scheme I. A danger of the procedure described above is that incubating nucleoside triphosphates in pyridine can lead to disproportionation (Verheyden et al., 1965). This result indicates that this is not occurring in the short time required for the cyclization, since there is essentially no loss of ATP during the reaction nor any rearrangement beyond that expected from Scheme I.

The method of preparation of [^{18}O]ATP and the cyclization procedure are applicable to GTP also. The preparation of [$\beta\gamma$-^{18}O,γ-$^{18}O_3$]GTP using carbonyldiimidazole-activated GDP and [$^{18}O_4$]P_i gave yields similar to ATP. This GTP reacted with dicyclohexylcarbodiimide, followed by water, to give GTP in an equivalent manner to that in Scheme I. The ^{31}P NMR of the γ-phosphorus of the initial and product GTP are shown in Figure 1 as doublets due to splitting by the β-phosphorus. The initial GTP was labeled to an extent of 99%. The product GTP has almost no material containing four ^{18}O per γ-P, showing that almost all the GTP reacted. The two major peaks are singly and triply labeled material, consistent with the guanine analogues of structures **4** and **5**. The ratio is 43:57, indicating that there is slight discrimination between the cleavage positions shown in structure **3**. The size of the minor peaks (unlabeled GTP and doubly labeled material) means

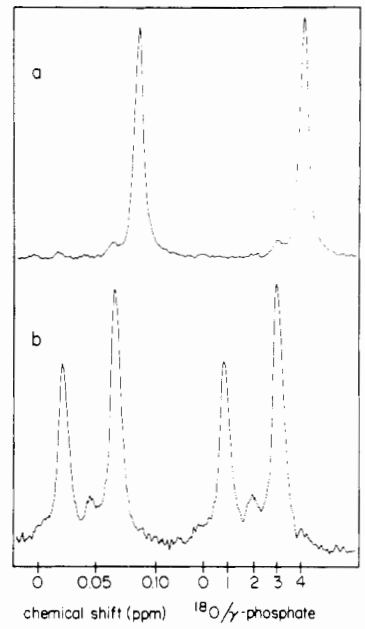
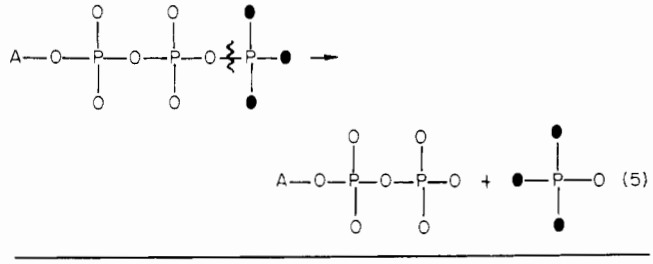


FIGURE 1: ^{31}P NMR spectrum of the γ-phosphorus of [$\beta\gamma$-^{18}O,γ-$^{18}O_3$]GTP 99% enriched with oxygen-18 before (a) and after (b) the cyclization procedure with dicyclohexylcarbodiimide and hydrolysis.

there has been a small loss (4%) of label during the reactions and workup.

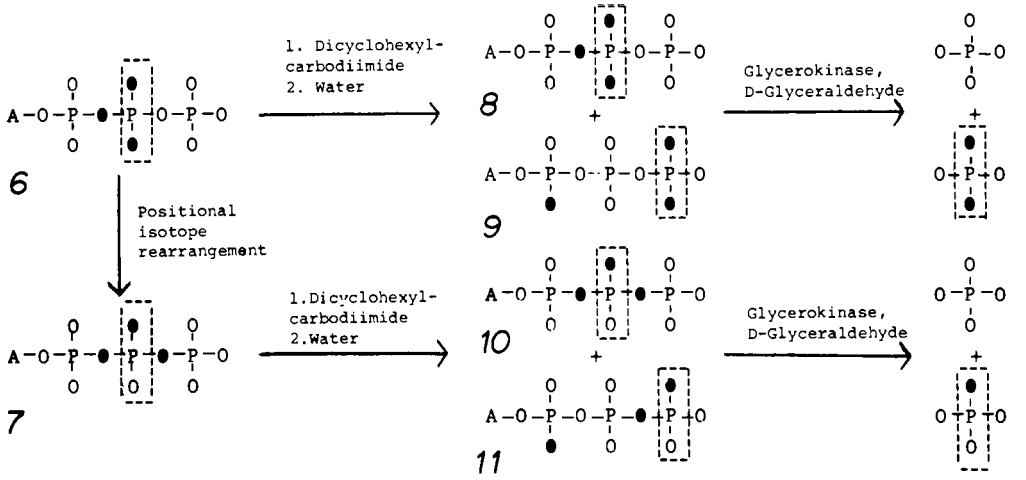
These results show that there is extensive interconversion of the β- and γ-phosphates of both ATP and GTP by using the cyclization procedure. The problems of incomplete interconversion can be circumvented by using a different pattern of labeling in the initial nucleoside triphosphate: [$\alpha\beta$-^{18}O,β-$^{18}O_2$]ATP (**6**).[2] If this undergoes enzyme-catalyzed positional isotope exchange, some of the isotope will be transferred to the $\beta\gamma$ bridge in exchange for an ^{16}O atom, as shown in Scheme II, to give **7**. Positional isotope exchange results in some the β-PO_2 moiety (enclosed by the dashed line) going from doubly to singly labeled. This ATP (now a mixture of **6** and **7**) when taken through the cyclization/hydrolysis procedure should form to equal extent ATP species **9** and **11**, together with some **8** and **10**. The ratio of **11** to **9** reflects the extent of positional isotope exchange in the original ATP.

In order to study the ^{18}O distribution in ATP species **9** and **11** by mass spectrometry, it is necessary to cleave the γ-phosphate to form P_i, without loss of isotope. Glycerokinase, in the presence of D-glyceraldehyde, is in effect an ATPase. A likely mechanism is that an oxygen of the *gem*-diol of the hydrate of D-glyceraldehyde is phosphorylated and then P_i is eliminated. Unlabeled ATP was hydrolyzed by this system in ^{18}O-labeled water to show that one oxygen of the resulting P_i is derived from water. P_i formed with only one oxygen labeled. γ-Labeled ATP was hydrolyzed in unlabeled water to show that the other three oxygens of the P_i were derived from the γ-phosphate of ATP without exchange. The P_i contained three ^{18}O with same enrichment as the ATP:



[2] This pattern of labeling also circumvents any problems arising in the analysis due to discrimination in cleavage of the cyclic trimetaphosphate, as occurred with GTP.

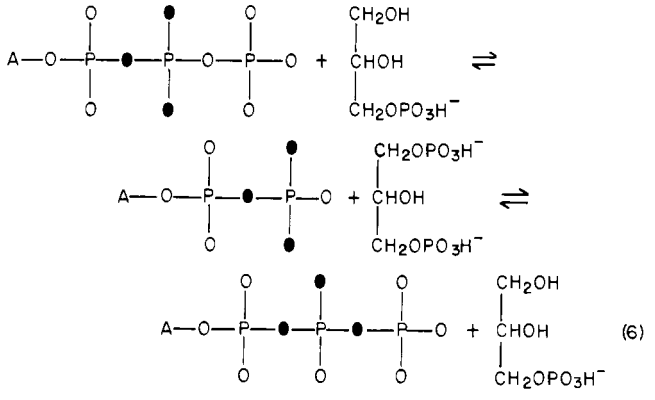
Scheme II



The γ-phosphate of GTP may be cleaved to P_i by this procedure by including nucleoside diphosphate kinase and ADP in the reaction solution, so that the γ-phosphate is transferred to form ATP, which is then a substrate for the glycerokinase. This coupled enzyme system gave P_i with the same isotopic enrichment as the γ-labeled GTP.

The ability of the procedure described above and in Scheme II to detect scrambling (the conversion of **6** to **7**) was tested by using [$\alpha\beta$-^{18}O,β-$^{18}O_2$]ATP, with each labeled position enriched to 75% with ^{18}O as shown by ^{31}P NMR. As a control a portion of this ATP was diluted with unlabeled material and, after cyclization and hydrolysis, it was treated with glycerokinase and D-glyceraldehyde to give P_i. This P_i was methylated for analysis by mass spectrometry. The isotopic distribution is shown in Table I. Because the isotopic enrichment is less than 100%, doubly labeled P_i gives rise to a family of peaks, due to two, one, and zero ^{18}O per molecule. The ratio of *m/e* 145:143 peaks obtained is that expected for 75.5% enrichment, in good agreement with the value for the initial ATP.

Another portion of [$\alpha\beta$-^{18}O,β-$^{18}O_2$]ATP was incubated with 3-phosphoglycerate kinase, phosphoglycerate, and Mg^{2+} so that it must undergo scrambling:



(6)

This ATP, after isolation, was diluted with unlabeled ATP and subjected to the cyclization and hydrolysis procedure. The P_i obtained by the glycerokinase treatment was methylated, and its mass spectral data are shown in Table I. The trimethyl phosphate from this ATP after 3-phosphoglycerate kinase treatment gives an increased intensity at *m/e* 143, relative to that obtained for the initial ATP. This is expected because scrambling causes singly labeled P_i to be formed, as shown in Scheme II. As calculated below,[3] the extent of scrambling is 75%, which is due to the ATP not having reacted to equilibrium in the presence of 3-phosphoglycerate kinase.

Table I: Mass Spectral Data for Trimethyl Phosphate, Derived from the γ-Phosphate of ATP after Cyclization and Hydrolysis, According to Scheme II

	rel peak intensities[a] at		
	141	143	145
[$\alpha\beta$-^{18}O,β-$^{18}O_2$]ATP before treatment with 3-phosphoglycerate kinase[b]	87.3	5.0	7.7
[$\alpha\beta$-^{18}O,β-$^{18}O_2$]ATP after treatment with 3-phosphoglycerate kinase[b,c]	88.5	7.6	3.9

[a] Average for two injections, corrected for natural abundance of oxygen-18. [b] Diluted with unlabeled ATP. [c] The ATP (1.5 mM) was incubated for 30 min with 10 mM phosphoglycerate, 25 mM $MgCl_2$, and 0.09 mg/mL yeast 3-phosphoglycerate kinase, pH 8.0, 20 °C. The resulting ATP after purification by ion-exchange chromatography was diluted with unlabeled ATP.

A feature of the analysis of the mass spectral data is that the intensities of peaks *m/e* 143 and 145 alone were used. The intensity for peak *m/e* 141 is susceptible to P_i impurities so that it is an advantage of this method that this peak is not used directly during the analysis. For the same reason, the extent of dilution of labeled ATP by unlabeled material and the extent of interconversion of the β- and γ-phosphates affect only the intensity of peak *m/e* 141, so that the analysis does not depend on these variables.

These results show that the cyclization and hydrolysis procedure is capable of distinguishing positional isotope exchange of ATP, using small amounts of labeled material (less than 1 μmol). The cyclization by dicyclohexylcarbodiimide

[3] After the treatment with 3-phosphoglycerate kinase, the intensity of peak *m/e* 145 is due to trimethyl phosphate derived from ATP **6** that has not undergone positional isotope rearrangement. Because the isotopic enrichment is only 75.5%, this material gives rise to a family of peaks with 0, 1, and 2 $^{18}O/P_i$ with percent intensities at *m/e* 141:143:145 being 6.0:37.0:57.0, which is 0.4:2.5:3.9 when adjusted to the actual intensity of peak *m/e* 145. The total intensity due to this family of peaks derived from ATP **6** is therefore 0.4 + 2.5 + 3.9 = 6.8. The intensity remaining at *m/e* 143 after subtracting the contribution derived from ATP **6** is 7.6 − 2.5 = 5.1, which is due to trimethyl phosphate derived from ATP **7** that has undergone positional isotope exchange. This material gives rise to a family of peaks with intensities at *m/e* 141:143 being 24.5:75.5, which is 1.7:5.1 when adjusted to the remaining intensity at *m/e* 143. It follows that the total intensity of this family of peaks derived from ATP **7** is 1.6 + 5.1 = 6.8. Therefore, the ratio of ATP **6** to ATP **7** is 6.8:6.8. This represents 75% scrambling since complete scrambling would have shown a ratio of 1:2.

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

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

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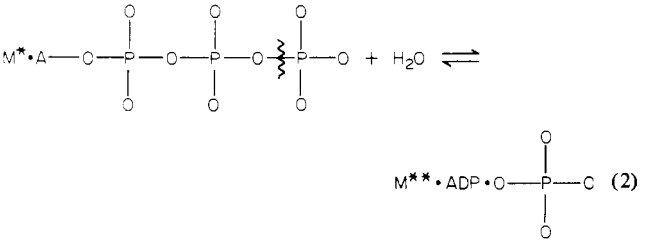
ABSTRACT: During the hydrolysis of MgATP catalyzed by myosin, ATP bound to the protein undergoes a reaction such that the β-nonbridge oxygen atoms exchange position with the βγ-bridge oxygen atom. The extent of this exchange was variable but averaged 45% for ATP that had been bound for 2 s at the myosin 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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