The Stereoregional Course of Phosphoric Residue Transfer during the Myosin ATPase Reaction*

Martin R. Webb and David R. Trentham
From the Department of Biochemistry and Biophysics, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104

When adenosine 5'-3-thiotriphosphate), stereospecifically labeled in the γ position with 18O, was hydrolyzed in the presence of myosin subfragment 1 in 18O-enriched water, the product inorganic [18O,17O,16O]thiophosphate was chiral. The configuration of this product showed that the hydrolysis proceeds with inversion at the transferred phosphoric residue. This result suggests a direct, in-line hydrolysis mechanism for the ATPase.

The hydrolysis of MgATP catalyzed by myosin and its fragments obtained by proteolysis occurs via reversible cleavage of ATP followed by rate-limiting release of products. This is supported by quenched flow studies described in Trentham et al. (1976) by the rapid exchange of water oxygens into the γ-phosphoric residue of ATP and P1 (Levy and Koshland, 1959; Bagshaw et al., 1975) and by positional isotope exchange studies (Geeves et al., 1980). No evidence for a phosphoenzyme intermediate has been obtained from oxygen isotope studies (Sartorelli et al., 1966; Trentham, 1977). However, evidence for the involvement of a phosphoenzyme was presented by Kinoshita et al. (1969) but disputed by Wolcott and Boyer (1973).

The steady state intermediates, protein-bound ATP and ADP-P1, interconvert rapidly (relative to product release) in the active site, but the mechanism of this chemical step has not been determined. Important information to elucidate this chemical mechanism is the stereoregional course of phosphoric residue transfer from nucleoside triphosphate to water. Put most simply: does this transfer occur with retention or inversion of configuration at the γ-phosphorus? The techniques to study the stereoregional chemistry of phosphoric residue transfer to species other than water have been developed recently and reviewed by Knowles (1980). In these cases, the oxygens of the transferred phosphoric residue are labeled differently, using three different oxygen isotopes (-P18O-316O) or by using sulfur as an analog of oxygen (-PS16O). The results of the two methods have been compared in the case of glycerol kinase and were in agreement (Pliura et al., 1980). The accumulated data on phosphoric residue transfer suggest strongly that, when the residue is transferred directly between substrates, the configuration is inverted. Retention of configuration occurs when there is a two-step transfer (usually via a phosphoenzyme), with presumably each step occurring with inversion. We have developed a method of determining the stereoregion of phosphoric residue transfer to water (Webb and Trentham, 1980) by configurational analysis of chiral inorganic [18O,17O,16O]thiophosphate. This paper describes the stereospecific hydrolysis of [γ-18O;γ-16O]ATP2S in [18O]water in the presence of myosin and analysis of the product inorganic [18O,17O,16O]thiophosphate. This allows us to answer the problem formulated in Equation 1 as to whether the reaction proceeds with inversion or retention of configuration (or possibly racemization).

\[
\text{ADP} + S_{\text{O}} + \text{H}_2O \rightarrow \text{ADP} + \text{S}_{\text{P}} \text{S} \quad \text{(retention)}
\]

\[
\text{S}_{\text{P}} \text{S} \rightarrow \text{S}_{\text{O}} \quad \text{(inversion)}
\]

The analysis depends on being little or no oxygen exchange between the product and water during the ATP2S hydrolysis. Whether or not oxygen exchange occurs in this reaction was not known and so is investigated.

EXPERIMENTAL PROCEDURES

Myosin and subfragment 1 were prepared from rabbit skeletal muscle by methods based on those described by Weeds and Taylor (1975). Glycerolphosphate dehydrogenase, lactate dehydrogenase, pyruvate kinase, and adenylate kinase (all rabbit muscle), hexokinase (from yeast), glycerol kinase (from Escherichia coli) glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides), phospho-enolpyruvate (monocyclushexammonium salt), NADH, ATP, AMP, ADP, hexose, adenosine, dithiothreitol, dihydroxyacetone, diadename perphosphate, EDTA, and 6-phosphate were from Sigma. 18O-enriched water (50%) was from Yeda, Israel. 18O-enriched water (99%) was from Miles Laboratories, Inc. All other chemicals were of reagent grade.

\[\beta_y;\gamma-18O,17O \text{ATP2S} \text{ was prepared by the method of Richard and Frey (1978), outlined in Scheme 1. ([\beta_y;\gamma',\gamma'']18O)] \text{JAMP2S} \text{ was prepared by a modification of the method of Murray and Atkinson (1968). Thio-phosphoryl chloride (4 mmol) was added to adenosine (1 mmol) and dissolved in triethylphosphate (10 ml) at 0°C. The mixture was left at 0°C overnight. Excess thiophosphoryl chloride was then removed by rotary evaporation and [18O]water (1 ml) was added. The solution was left at room temperature for 1 h before dilution with unlabeled water. The [18O]JAMP2S was purified by ion exchange chromatography on a column of DEAE-cellulose (40 X 3 cm in diameter), with elution by a gradient (1:21) of triethylammonium bicarbonate from 10 to 400

*\text{This work was supported by Grant AM 23030 from the National Institutes of Health, and grants from the Muscular Dystrophy Association of America and the Whitehall Foundation. \text{18P} NMR spectra were obtained at the Middle Atlantic NMR Facility, which is supported by National Institutes of Health Grant RR 542 at the University of Pennsylvania. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.}

† Postdoctoral fellow of the Muscular Dystrophy Association of America.
The methoxymethylidene-AMP was prepared as follows. AMP, as the free acid (400 μmol), was stirred with 75% (v/v) trimethyl orthoformate, 20% dimethylformamide (20 ml) containing 0.1 M HCl (50 μl). After several hours, the solid AMP had dissolved and the product was obtained by rotary evaporation. The residue was removed by rotary evaporation and the product was dissolved in dimethylformamide. Tri-n-octylamine (1.6 mmol) which was sufficient to ensure complete solubilization in dimethoxyformamide. The solution was incubated for 30 min at room temperature: 5 mM ATP, 6 μM MgCl₂, 50 mM glucose, 5 units.ml⁻¹ of hexokinase, purified as described above and converted to the diphosphate by product on polyethyleneimine-cellulose plates, eluted with 1 M KCl, formate, 25% dimethylformamide (20 ml) containing HCl (50 μl). After 20 min, the pH was raised to 10.5 by 5 mM NaOH and the solution was incubated at 50°C for 30 min. The product ATP₈S after purification by ion-exchange chromatography was obtained in 45% yield from [³²P]AMP(S). ATP₈S was assayed spectrophotometrically using glycerol kinase and glycerolphosphate dehydrogenase. The assay solution (2.5 ml) contained the following: 200 μM Tris buffer, pH 8.0, 20 mM MgCl₂, 0.15 mM NADH, 1 mM dihydroxyacetone, 5 units.ml⁻¹ of glycerol kinase, and 10 units.ml⁻¹ of glycerolphosphate dehydrogenase. The concentration of ATP₈S in an added aliquot was determined spectrophotometrically at 340 nm following the oxidation of NADH.

\[ \text{Glycerol phosphate dehydrogenase} \rightarrow \text{Glycerol 3-phosphate} + \text{NAD}^{+} \]

Any ATP gives rise to a rapid reaction, so that its concentration can be determined separately from the slower reacting ATP₈S. ³¹P NMR analysis was used to determine the ²³O content and distribution in the ATP₈S and its precursors. A ³¹P NMR spectrum of the ATP₈S showed no observable B isomer (Shue and Frey, 1977; Jaffe and Cohn, 1978). It follows that the ²³O in the product ATP₈S is stereospecifically attached. The ²³O enrichment of the AMP(S) was 75% in the two labeled positions as determined by ³¹P NMR using the upfield shift in ³¹P resonance due to ²³O (Cohn and Hu, 1978; Low and Spruyt, 1978). The product ATP₈S had the same ²³O enrichment showing that there was no loss of isotope during the synthesis.

\[ \text{(Py-²³O;²³O)} \text{ATP₈S, 25 mM MgCl₂, 25 mM dithiorthietol, 100 mM Tris, pH 8.0, and 50 mM KCl was evaporated to dryness and the residue was taken up in [²³O]water (1 ml). Subfragment 1 (5 mg in 50 μl) was added and the hydrolisis was monitored by enzymic assay of ADP. After 45 min, the reaction was 80% complete. In a control reaction in the absence of protein, there was no observable hydrolisis.} \]

\[ \text{The solution was frozen rapidly in dry ice/isopropyl alcohol and the [²³O]water was recovered by vacuum sublimation. The residue was dissolved in 1 N HCl (1 ml) to ensure that no enzymic activity remained. The acid was neutralized and the inorganic [²³O]³¹P ATP₈S was purified by ion exchange chromatography on a column of DEAE-cellulose (15 × 1.5 cm in diameter). Elution was by a gradient (200 ml) of triethylammonium bicarbonate (10 to 250 mM). The inorganic thiophosphate was assayed by detecting the thiophosphate with 5,5'-dithiobis-(2-nitrobenzoic acid). Buffer was removed by rotary evaporation of several batches of methanol from the product. The thiophosphate was incorporated into ATP₈S for NMR analysis as previously described (Webb and Trentham, 1980).} \]

For determining the extent of product-water oxygen exchange, unlabeled ATP₈S was hydrolyzed as above, except in [²³O]water. The purified product as its triethylammonium salt (20 mM) was converted in methanol to S-methyl thiophosphate by addition of a 20-fold excess of methyl iodide. This derivatization enabled a sharp ³¹P NMR signal to be obtained and hence allowed the ²³O content to be determined.

\[ \text{Dihydroxyacetone + ATP₈S → Glycerophosphate dehydrogenase} \]

3²³O ATP₈S was hydrolyzed in the presence of subfragmen 1 in [²³O]water as follows. A solution (1 ml) containing 20 mM [²³O;²³O;³²P]ATP₈S, 25 mM MgCl₂, 25 mM dithiothreitol, 100 mM Tris, pH 8.0, and 50 mM KCl was evaporated to dryness and the residue was taken up in [²³O]water (1 ml). Subfragment 1 (5 mg in 50 μl) was added and the hydrolisis was monitored by enzymic assay of ADP. After 45 min, the reaction was 80% complete. In a control reaction in the absence of protein, there was no observable hydrolisis.
Routine $^{31}$P NMR spectra were recorded at 24.3 MHz on a Varian NV 14 spectrometer modified to operate in the Fourier transform mode and equipped with a multinuclear probe and with quadrature phase detection.

$^{31}$P NMR spectra to determine $^{18}$O content and distribution were recorded at 145.7 MHz on a Bruker WH 360/180 spectrometer, equipped with a dueterium field lock and operating in the Fourier transform mode. The sample solution (1.5 ml) was in a 1-cm diameter sample tube (Wilmsd Glass Co.) and was maintained at 22°C. Spectral width was 1000 Hz, with a pulse width of 15 μs and an acquisition time of 8.2 s. A sensitivity enhancement exponential function gave a line broadening of 0.1 Hz.

RESULTS AND DISCUSSION

To determine whether any product water-oxygen exchange occurred during ATPyS hydrolysis in the presence of subfragment 1, unlabeled ATPyS was hydrolyzed in $^{18}$O-enriched water. A $^{31}$P NMR spectrum of the product inorganic thiophosphate (with a signal to noise ratio of 20) showed that only 1 $^{18}$O atom per molecule had been incorporated into the product. Thus, no exchange occurs. This is probably because the cleavage of ATPyS is the rate-limiting step of the catalytic mechanism.

In $[^{18}$O]water, the hydrolysis of the ATPyS, stereospecifically labeled with $^{18}$O in the γ-position will give rise to inorganic $[^{18}$O,$^{18}$O,$^{18}$O]thiophosphate, as in Equation 1. Enantiomeric analysis of this thiophosphate is by its incorporation into ATPβS. The R and S enantiomers give different isotope labeling patterns in ATPβS which can be distinguished by the $^{31}$P NMR spectrum of the β-phosphorus (Webb and Trentham, 1980). Thus:

\[
\text{Enantiomer 1: } R \xrightarrow{\text{hydrolysis}} \text{R-AMP} \xrightarrow{\text{degradative cleavage}} \text{AMP} + \text{P}_{\text{γ-P-PO}_3} \\
\text{Enantiomer 2: } S \xrightarrow{\text{hydrolysis}} \text{S-AMP} \xrightarrow{\text{degradative cleavage}} \text{AMP} + \text{P}_{\text{γ-P-PO}_3}
\]

Apart from these ATPβS species, there are ATPβS molecules that contain $^{18}$O covalently bound to the β-phosphorus. These are not visible in the $^{31}$P NMR spectrum due to the quadrupole effect (Tsai, 1979). In practice, the lack of isotopic purity gives rise to other peaks in the spectrum as quantified below. $[^{18}$O,$^{18}$O]ATPβS (1) and $[^{18}$O,$^{18}$O]ATPβS (2) can be distinguished by the NMR spectrum of their β-phosphorus atoms. This is known from the spectrum of a mixture of unlabeled ATPβS (9%), $[^{18}$O,$^{18}$O]ATPβS (21%), and $[^{18}$O,$^{18}$O]ATPβS (49%) due to ATPβS, labeled to an extent of 70% with $^{18}$O in the β- and γ-positions; (b) ATPβS derived from inorganic $[^{18}$O,$^{18}$O,$^{18}$O]thiophosphate product of the subfragment 1-catalyzed hydrolysis of (3P−R)$[^{18}$O,$^{18}$O,$^{18}$O]ATPβS in $^{18}$O-enriched water.

The spectrum of the ATPβS derived from the myosin hydrolysis product in Fig. 1b shows that there is an excess of species 1 over 2. Hence, there was an excess of the R enantiomer of inorganic thiophosphate, which means that myosin subfragment 1 catalyzed the hydrolysis of ATPβS with inversion (Equation 1). The calculation below indicates that nearly all of species 2 arises because of the lack of isotopic purity.

From the estimated isotopic enrichments (75% for $^{18}$O in ATPβS and 45% for $^{18}$O in water, which also contains 12% $^{16}$O), the ratio of each isotopically labeled ATPβS species can be calculated assuming complete stereospecificity. This leads to calculated, relative spectral intensities for the β-phosphorus of unlabeled ATPβS, $[^{18}$O,$^{18}$O]ATPβS, $[^{18}$O,$^{18}$O]ATPβS, and $[^{18}$O)$[^{18}$O]ATPβS being 38:37:21:4. The observed values were 39:34:22:5 (after making a small correction for peak overlap).

The fact that the (3P−R)$[^{18}$O,$^{18}$O,$^{18}$O]ATPβS gives rise to $^{18}$O incorporation in the hydrolysis solution with $[^{18}$O]water from the protein solution and from residual water in the substrate/buffer.

This figure takes into account dilution of the $[^{18}$O]water in the hydrolysis solution with $[^{18}$O]water from the protein solution and from residual water in the substrate/buffer.

The small difference between the calculated and observed ratios may be due to incomplete stereospecificity of the myosin ATPase reaction. If the product inorganic thiophosphate contained the R and S enantiomers in a ratio of 18:1, the ratio of ATPβS species would have been 38:35:23:4. However, a more likely explanation for the difference is that, during the incorporation of inorganic thiophosphate into ATPβS, there was partial hydrolysis of the intermediate, glyceraldehyde phosphate dehydrogenase in the incorporation procedure (Webb and Trentham, 1980) is rate-limiting.

![Fig. 1. $^{31}$P NMR spectra.](image-url)
Stereochemistry of Myosin ATPase

the R enantiomer of inorganic $[^{18}\text{O}]$thiophosphate means that the hydrolysis proceeds with inversion of configuration. It is probable that the chemical mechanisms of ATP and ATP$_y$S hydrolysis are similar, so that the most likely mechanism for ATP hydrolysis is direct, in-line displacement of ADP by a water oxygen. The results described here, together with the evidence cited above, point toward phosphoric residue transfer during ATP hydrolysis being a single characterizable step that is reversible and rapid, relative to the overall myosin ATPase catalytic center activity.

This result is strong evidence against a phosphoenzyme being an intermediate on the reaction pathway. Tsai and Chang (1980) have shown that nucleotidase catalyzes the hydrolysis of AMP$\beta$, also with inversion of configuration. It will be of great interest to see whether, in the sarcoplasmic reticulum Ca-ATPase for which there is strong evidence for a phosphoenzyme intermediate (Yamamoto and Tonomura, 1968), the hydrolysis of ATP$_y$S proceeds with retention of configuration. The question of a phosphoenzyme intermediate in the mitochondrial ATPase remains open. The application of this stereochemical approach is likely to provide evidence for or against phosphoenzyme intermediates in nucleoside triphosphate-requiring, energy-transducing systems.

Acknowledgment—We would like to thank Dr. P. Frey for helpful discussions, particularly with regard to the synthesis of ATP$_y$S.

REFERENCES

Bagshaw, C. R., Eccleston, J. F., Trentham, D. R., Yates, D. W., and Goody, R. S. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 127-135

Bagshaw, C. R., Trentham, D. R., Wolcott, R. G., and Boyer, P. D. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2592-2596

Cohn, M., and Hu, A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 200-203

Eckstein, F. (1975) Angew. Chem. Int. Ed. Engl. 14, 160-166

Eckstein, F., and Goody, R. S. (1976) Biochemistry 15, 1685-1691

Geeves, M. A., Webb, M. R., Midelfort, C. F., and Trentham, D. R. (1980) Biochemistry, in press

Jaffe, E. K., and Cohn, M. (1978) Biochemistry 17, 652-657

Kinoshita, N., Kubo, S., Ohnishi, H., and Tonomura, Y. (1969) J. Biochem. (Tokyo) 65, 285-301

Knowles, J. R. (1980) Annu. Rev. Biochem. 49, 877-919

Levy, H. M., and Koshland, D. E. (1969) J. Biol. Chem. 234, 1102-1109

Lowe, G., and Sproat, B. S. (1978) J. Chem. Soc. Chem. Commun. 595-596

Murray, A. W., and Atkinson, M. R. (1968) Biochemistry 7, 4023-4029

Pliura, D. H., Schomburg, D., Richard, J. P., Frey, P. A., and Knowles, J. R. (1980) Biochemistry, 19, 325-329

Richard, J. P., and Frey, P. A. (1978) J. Am. Chem. Soc. 100, 7757-7758

Sartorelli, L., Froman, H. J., Benson, R. W., and Boyer, P. D. (1966) Biochemistry 5, 2877-2884

Sheu, K.-F. R., and Frey, P. A. (1977) J. Biol. Chem. 252, 4445-4448

Trentham, D. R. (1977) Biochem. Soc. Trans. 5, 5-22

Trentham, D. R., Eccleston, J. F., and Bagshaw, C. R. (1976) Q. Rev. Biophys. 9, 217-281

Tsaï, M.-D. (1979) Biochemistry 18, 1468-1472

Tsaï, M.-D., and Chang T.-T. (1980) J. Am. Chem. Soc., in press

Webb, M. R., and Trentham, D. R. (1980) J. Biol. Chem. 255, 1775-1779

Weeds, A. G., and Taylor, R. S. (1975) Nature 257, 54-56

Wolcott, R. G., and Boyer, P. D. (1973) Biochim. Biophys. Acta 303, 292-297

Yamamoto, T., and Tonomura, Y. (1968) J. Biochem. (Tokyo) 64, 137-145