The Stereochemical Course of Phosphoric Residue Transfer Catalyzed by Beef Heart Mitochondrial ATPase

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The stereochemical course of phosphoric residue transfer has been determined for beef heart mitochondrial ATPase. When adenosine 5′-(3-thiotriphosphate), stereospecifically labeled with 18O in the γ position, was hydrolyzed in [18O]water in the presence of the ATPase, the product inorganic [18O, 15O, 18O]thiophosphate was chiral. The configuration of the product showed that the hydrolysis had proceeded with inversion at the γ-phosphorus atom. This result suggests that there is a direct, in-line transfer of the phosphoric residue between ADP and water and that there is no phosphoenzyme intermediate.

It is now generally accepted that beef heart mitochondrial ATPase (F1) catalyzes the terminal transphosphorylation reaction of oxidative phosphorylation. When bound to the mitochondrial membrane, F1 serves as a phosphate transfer enzyme in the synthesis of ATP from ADP and P. However, when removed from the membrane and solubilized, F1 becomes a highly active ATPase. The ATPase activity was viewed as an artifact of isolation (Pullman et al., 1960; Penefsky et al., 1960). It has been the operating assumption of most subsequent research on the enzyme that the phosphate transfer sites on membrane-bound F1 are identical with the hydrolytic sites on the solubilized enzyme (reviewed by Penefsky, 1979).

Although it is also generally agreed that an electrochemical potential gradient serves as an energy source for ATP synthesis in oxidative phosphorylation (Mitchell, 1977), the molecular mechanism of ATP synthesis catalyzed by F1 remains to be determined.

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† The abbreviations used are: F1, beef heart mitochondrial ATPase; ATPγS, adenosine 5′-(3-thiotriphosphate); ATPβS, adenosine 5′-(2-thiotriphosphate).

One approach to elucidate the chemical mechanism of the ATP to ADP + Pγ reaction is the determination of the stereochemical course of this phosphoric residue transfer. Does the transfer occur with retention or inversion at the transferred phosphorus atom? Such stereochemical information has been obtained for several systems and reviewed by Knowles (1980). The results suggest strongly that all enzyme-catalyzed phosphoric residue transfer steps occur with inversion of configuration. One-step mechanisms, when the residue is transferred directly between substrates, result in inversion of the final product. Retention of configuration occurs when there is a two-step transfer (usually via a phosphoenzyme) with each step occurring with inversion. The techniques to determine the stereochemistry of phosphoric residue transfer catalyzed by ATPases have been developed recently by Webb and Trentham (1980a and 1980b) and applied to the myosin ATPase reaction (Webb and Trentham, 1980b) and to nucleotidase (Tsai and Chang, 1980). In each case, inversion was found. In this communication, we describe the application of this approach to F1. For this experiment, ATPγS labeled stereospecifically with 18O in the γ position is hydrolyzed in oxygen-17-enriched water, so that the product will be inorganic [18O, 15O, 18O]thiophosphate:

Analysis of the configuration of this product gives the information as to whether the reaction proceeded with inversion or retention (or possibly racemization).

EXPERIMENTAL PROCEDURES

F1 was prepared as described by Knowles and Penefsky (1972). The homogenous enzyme was stored as a suspension (6 mg/ml) in 2 mM ammonium sulfate. [15O]Water (52% enriched) was obtained from Prochem. [3P-3R]βγ-0γγ-18O]ATPγS was prepared as described in Webb and Trentham (1980b). A 31P NMR spectrum of the γ-phosphorus atom was used to determine the 18O content, using the upfield shift in 31P resonance due to 18O substitution for 16O (Cohn and Hus, 1978; Lowe and Sproat, 1978). The two labeled positions contained 18O at 96% enrichment.

The ATPγS was hydrolyzed in the presence of F1, in [15O]water as follows. A solution (1 ml) containing 20 mM [βγ-18Oγγ-32O]ATPγS, 20 mM MgCl2, 20 mM dithiothreitol, 100 mM Tris/HCl, pH 8.0, was evaporated to dryness and the residue was dissolved in [15O]water (1 ml). A sample (100 μl) of the F1 suspension described above was centrifuged at 15,000 × g for 10 min in an Eppendorf 5412 centrifuge at 4°C. The supernatant was removed by carefully touching it with filter paper. The pellet was dissolved in the solution of ATPγS. Hydrolysis was followed by enzymic assay of ADP. After 65 min, the hydrolysis was approximately 80% complete. The [15O]water and inorganic thiophosphate were purified from other components as described by Webb and Trentham (1980b). The inorganic thiophosphate was incorporated stereospecifically into ATPγS so that its configuration could be determined by 31P NMR (Webb and Trentham, 1980a).

For determining the extent of product-water oxygen exchange, unlabeled ATPγS was hydrolyzed as above except in [15O]water. The analysis was as described by Webb and Trentham (1980b).
RESULTS AND DISCUSSION

The hydrolysis of ATP$_3$S catalyzed by F$_1$ proceeds at a rate which is about 20 times slower than that of ATP for the conditions used in this experiment. Thus, ATP$_3$S is a reasonably good substrate for this enzyme. However, to be able to use ATP$_3$S to determine the stereochemistry of phosphoric anhydride, its hydrolysis must occur with little or no oxygen exchange between product and water. To determine the extent of this exchange, unlabeled ATP$_3$S was hydrolyzed in [16O]water. The product inorganic thiophosphate contained only 1 oxygen-18 atom/molecule, showing that there is no exchange.

In [17O]water, the hydrolysis of ATP$_3$S, stereospecifically labeled in the $\gamma$ position with 17O, will proceed as in Equation 1. The product inorganic thiophosphate will be chiral and the $R$ and $S$ enantiomers can be distinguished by 31P NMR after their enzymic incorporation into ATP$_3$S. Apart from ATP$_3$S species containing 17O, which are not visible in the 31P NMR spectrum of the $\beta$-phosphorus, due to the quadrupole moment of $^{17}$O (Tsai, 1979), the following species are formed:

\[
\begin{align*}
S & : O \quad P - O - P - O - P - O - P - O - \quad S\\
\quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad 1\\
\quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad 2
\end{align*}
\]

These can be distinguished by the 31P NMR spectrum of the $\beta$-phosphorus (Webb and Trentham, 1980a).

In practice, the isotopic enrichments are well below 100% so that other peaks are in the spectrum. The estimated isotopic enrichments of the 16O in ATP$_3$S and the 17O in water in the F$_1$-catalyzed hydrolysis are shown in Table I. The spectrum of the $\beta$-phosphorus atom of the ATP$_3$S derived from the inorganic thiophosphate product is shown in Fig. 1. This figure also shows the assignments of the peaks to the various 16O-labeled species (Webb and Trentham, 1980a). Qualitatively, there is an excess of bridging 17O over nonbridging, indicating the major pathway is inversion. Indeed, the calculation below shows that the observed spectrum is consistent with 100% inversion.

Table II shows the peak intensities calculated from the spectrum shown in Fig. 1, taking into account peak overlap. These data show that the 17O content is less than that predicted from the 16O contents in Table I. This discrepancy is almost certainly due to loss of isotope during the analytical procedure, which has been observed previously (Webb and Trentham, 1980b). The first step of this procedure is the incorporation of inorganic thiophosphate into ATP$_3$S using glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase. An intermediate is the unstable species glycerate 1-thiophosphate-3-phosphate, which hydrolyzes readily back to inorganic thiophosphate. Loss of isotope occurs on incorporation of this new inorganic thiophosphate into product ATP$_3$S.

The observed 17O content is consistent with 10% hydrolysis of glycerate 1-thiophosphate-3-phosphate. Based on this figure, the peak intensities calculated for inversion and retention are shown in Table II. Those figures for inversion correspond, within the sensitivity of the measurement, to those observed. In a separate, similar experiment, ATP$_3$S stereospecifically labeled with 16O to an extent of 75% was hydrolyzed in 38% enriched [17O]water in the presence of F$_1$. In this experiment also, analysis of the product inorganic thiophosphate confirmed that the hydrolysis had proceeded with inversion at the transferred phosphorus atom. Since it is likely that the chemical mechanisms of ATP and ATP$_3$S hydrolysis are 4.

This hydrolysis is probably due to the reaction time being too long, because the reaction was not followed by 31P NMR, in turn because the spectral peaks were very broad.

TABLE I

| Composition | $^{16}$O | $^{17}$O | $^{18}$O |
|------------|--------|--------|--------|
| $^{16}$O of ATP$_3$S* | 5      | 0      | 95     |
| $^{17}$O of water* | 21     | 47     | 32     |

* Determined by 31P NMR. See text for details.

**TABLE II**

| Relative peak intensities | Unlabeled | $^{16}$O (bridging) | $^{17}$O (non-bridging) | $^{18}$O |
|--------------------------|----------|---------------------|------------------------|--------|
| Estimated if retention*   | 22       | 25                  | 42                     | 11     |
| Estimated if inversion*   | 22       | 42                  | 25                     | 11     |
| Observed                  | 22       | 41                  | 23                     | 14     |

* Calculated assuming that there was 10% hydrolysis of glycerate 1-thiophosphate-3-phosphate during the analytical procedure, as described in the text.

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1 However, the hydrolysis of the 16O-labeled ATP$_3$S was a further 5 times slower, probably due to inhibition by an unidentified impurity present in this nucleotide preparation. This impurity co-chromatographs with the ATP$_3$S on DEAE-cellulose and is visible in the 31P NMR spectrum. Such an impurity was present in a previous preparation and caused almost complete inhibition of the myosin ATPase.

2 Calculated from manufacturer's data sheet, assuming a 10% dilution due to residual unlabeled water in the protein and substrate.
similar, this result constitutes strong evidence against the possibility that a phosphoenzyme or other phosphorylated compound serves as an intermediate in the reaction pathway. It is likely also that synthesis during oxidative phosphorylation occurs via a reversal of ATP hydrolysis catalyzed by F₁. A possibility that has been proposed is that ATP synthesis occurs via the intermediate phosphorylation of AMP by Pᵢ (Vambutas and Bertch, 1976; Tiefert et al., 1977; Beyeler and Bachofen, 1978), followed by transfer of this phosphoric residue to give the final ATP. However, evidence has been presented against such a mechanism and in favor of one in which ADP is the primary phosphoric residue acceptor in both oxidative phosphorylation (Hill and Boyer, 1967; Colli and Pullman, 1969) and photophosphorylation (Vinkler et al., 1978). Our results also indicate AMP is not the primary acceptor in oxidative phosphorylation as retention of configuration is predicted for such a mechanism.

Indeed, the most likely mechanism for ATP hydrolysis is direct, in-line displacement of ADP by a water oxygen in a single characterizable step.

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