Source of the Oxygen in the C-O-P Linkage of the Acyl Phosphate in Transport Adenosine Triphosphatases*

A. Stephen Dahms, Tohru Kanazawa, and Paul D. Boyer

From the Molecular Biology Institute and Department of Chemistry, University of California, Los Angeles, California 90024

SUMMARY

The source of the oxygen in the C-O-P linkage of the acyl phosphate in the Na+, K+-ATPase of porcine kidney and the Ca++, Mg++-ATPase of rabbit muscle sarcoplasmic reticulum has been assessed. Formation of the Na+, K+-ATPase acyl phosphate in presence of ouabain and [32P]Pi results in the C-O-P bridge oxygen remaining nonisotopic. Thus, the mode of entry of Pi into the acyl phosphate is by attack of a carboxylate oxygen on the phosphorus atom and displacement of a hydroxyl group.

When the acyl phosphate of the Ca++, Mg++-ATPase or the Na+, K+-ATPase is formed in the presence of H18O, the C-O-P bridge oxygen also remains nonisotopic. These results show that the acyl phosphate is cleaved by water oxygen attack on the phosphorus atom and displacement of a hydroxyl group.

The phosphoryl oxygens of the Na+, K+-ATPase phosphoenzyme at 15° derived either from [32P]Pi in the presence of ouabain or from ATP in the presence of H18O were observed to undergo exchange with medium water, not accounted for by exchange of Pi oxygens with HOH and incorporation of the Pi into the protein-bound acyl phosphate.

The cleavage of ATP coupled to ion transport by microsomal preparations in presence of Na+ and K+ or by muscle sarcoplasmic reticulum preparations in presence of Ca++ and Mg++ is known to proceed through intermediate phosphorylation of a protein carboxyl group (1-5). Three possibilities warrant consideration for the cleavage of this acyl phosphate intermediate of the transport ATPases. One is by direct attack of a water oxygen on the phosphoryl phosphorus atom to form Pi. A second is by attack of water oxygen on the acyl carbon atom, displacing Pi. A third is by attack of another protein group on the acyl carbon with displacement of Pi, essentially an acyl transfer, followed by subsequent hydrolysis of the acyl derivative.

All three modes of cleavage will, in the usual assay conditions where many molecules of ATP are cleaved per active site, give rise to Pi which derives at least 1 oxygen from water. The first mode of cleavage can be distinguished from the other two by measurement of the source of oxygen in the first Pi formed per active site in ATP cleavage, or by determination of the source of the bridge oxygen of the C-O-P linkage in the acyl phosphate after continued ATP hydrolysis. Cleavage resulting from water attack on the phosphoryl phosphorus atom will, in subsequent reaction cycles, give an acyl phosphate with the bridge oxygen derived from the carboxyl group. Conversely, cleavage by attack of water oxygen or another group on the acyl carbon atom will, in subsequent reaction cycles, give an acyl phosphate with the bridge oxygen derived from water.

In addition to the phosphorylation by ATP, the transport ATPases can, under appropriate conditions (6, 8), be phosphorylated from Pi in the absence of ATP. Phosphorolysis of an acyl linkage or displacement by attack of a carboxylate oxygen on the phosphorus atom of Pi could occur. Again, the mode of phosphorylation can be ascertained by measurement of the source of the C-O-P bridge oxygen in the phosphorylated enzyme.

It is the purpose of this paper to present findings on the source of the C-O-P bridge oxygen in the microsomal and sarcoplasmic reticulum ATPases phosphorylated from either Pi or ATP.

EXPERIMENTAL PROCEDURES

Materials—Electrophorus electricus was obtained from World Wide Aquarium Traders, Marina del Rey, Los Angeles, Calif. 32Pi from various commercial sources was purified before use essentially as described elsewhere (9). Normalized water of approximately 10 atom % excess 18O was obtained from Yeda Research and Development Co., Rehovoth, Israel. KH2PO4 of approximately 80 atom % excess was supplied by Miles Laboratories, Inc.

Preparation of ATPase Na+, K+ ATPase from porcine kidney outer medulla and the electroplax of the electric eel were prepared by standard procedures.

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† Present address, California State University, San Diego, California.

§ Present address, Department of Biology, University of Osaka, Osaka, Japan.

1 More than 1 oxygen might be incorporated from water into each Pi formed because of exchange reactions catalyzed by the ATPases (7, 8).
Prepared as described previously (8). Endogenous Pi ranged from 0.8 to 3 μmoles per 100 mg of protein. Sarcomplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described by Kanazawa and Boyer (7).

**Determination of 32P Content of Bridge Oxygen of Membrane Acyl Phosphate**—Relatively large scale preparation of the respective phosphoenzymes was carried out as described in the text. For P-O bond cleavage, the acid-washed, protein-bound acyl phosphate was heated in 0.1 N formic acid, pH 3.8, for 15 min at 98°. For C-O bond cleavage, heating for 15 min at 100° was performed in 0.1 N NaOH. Following hydrolysis, the supernatant was made 1 x in HCl and extracted with 4 volumes of isobutyl alcohol-benzene (1: 1, v/v) to remove residual phenol. The phenol lower layer was subsequently extracted with an equal volume of trichloroacetic acid until a constant level of 32P in the supernatant solution was attained; continued small release of 32P represented largely acyl phosphate hydrolysis at 4°.

That the residual 32P present with the precipitated protein was covariantly bound in this and other experiments was to be expected from previous reports of others (1-6). Further evidence for this is given by the observation that the 32P remained with the protein when it was solubilized by 6 x guanidine HCl at pH 2 and reprecipitated by 0.4 M perchloric acid. In addition, conditions for hydrolytic release of 32P from the precipitated protein are those expected to cleave acyl phosphate bonds.

Separate internal standards employed [32P]Pi; addition to denatured protein samples to correct the observed 32O content for small apparent losses of 32O accompanying phosphate isolation, purification, and analysis. Such internal standards were found to be indispensable with the amounts of phosphoprotein conveniently available. Corrections for internal standards ranged from 10 to 12%.

Results given in Table II show that the observed 32O content of Pi resulting from P-O cleavage was identical, within experi-

**RESULTS**

**Methodology for Determination of Source of C-O-P Bridge Oxygen**—The determination of the source of the bridge oxygen is based upon known cleavage patterns of acetyl phosphate in alkaline or acid, resulting in C-O and P-O cleavage, respectively. The Pi isolated from an acyl phosphate cleavage in an appropriate acid medium should contain the 3 phosphoryl oxygens plus an oxygen from medium water. With alkaline cleavage, the 3 phosphoryl oxygens plus the C-O-P bridge oxygen would be present in the Pi. Thus, comparison of the 32O content of the Pi after C-O and P-O cleavage allows calculation of the 32O content of the bridge oxygen. A restriction in the design of experiments, however, is the relatively small amount of acyl phosphate protein available compared to the Pi sample size and 32O enrichment required for accurate analysis.

To test the conditions anticipated to give P-O and C-O cleavage (13, 14), appropriate hydrolyses were carried out with acetyl phosphate in the presence of H32OH. Results obtained with hydrolysis in dilute NaOH or in formate buffer are shown in Table I. They establish clear preferential C-O or P-O cleavage with acetyl phosphate under the conditions used.

For the experiments with acetyl phosphate (Table I), a temperature of 75° was used. The lability of protein-bound acyl phosphate might differ somewhat; hence a temperature of 100° was used to help assure complete cleavage.

**Source of Bridge Oxygen in Na32P, K+ ATPase Phosphorylated with [32P]Pi**—For these experiments, the enzyme was phosphorylated with [32P]Pi in the absence of ATP. Oubain was added to promote formation of higher amounts of the phosphoenzyme (6). In related studies, phosphoenzyme formation was shown to be complete within 2 s after ouabain addition. The acid-precipitated phosphoenzyme was washed by repeated centrifugation and suspension in 0.3 M trichloroacetic acid until a constant level of 32P in the supernatant solution was attained; continued

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**Table I**

| Source | Bridge Oxygen in Na32P, K+ ATPase Phosphorylated with [32P]Pi |
|--------|---------------------------------------------------------------|
| Formate buffer, 0.1 mM, pH 3.8 | 1.28 | 0.314 | >95% P-O |
| NaOH, 0.2 N | 1.13 | 0.019 | >90% C-O |

Position of bond cleavage

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**Table II**

| Direct loss of Pi oxygen accompanying membrane acyl phosphate formation in Na32P, K+ ATPase |
|---------------------------------------------------------------|
| The reaction mixture contained 7.21 mM [32P, 32O]Pi (73 atom % excess 32O), 5 mM MgCl2, 50 mM imidazole-glycylglycine (pH 7.2), 1 mM ouabain, and 135 γ of porcine outer medullar Na32P, K+ ATPase (specific activity = 13.1 μmoles per hour per mg of protein) in a final volume of 60 ml at 4°. Enzyme and ouabain were added at zero time and 10 s, respectively. The reaction was terminated at 40 s by the addition of 200 ml of 0.4 M perchloric acid containing 20 mM Pi. Phosphoprotein was isolated as described in the text and divided into three portions. Cleavage in presence of 4.9 μmoles of carrier Pi, Pi isolation, and 32O analysis with corrections for internal standards were made as described in the text. Results are averages of the triplicate determinations ± S.D. |

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Position of acyl phosphate bond cleavage

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**Table III**

| Source | Position of acyl phosphate bond cleavage |
|--------|-------------------------------------------|
| Formate buffer, 0.1 mM, pH 3.8 | 1.28 | 0.314 | >95% P-O |
| NaOH, 0.2 N | 1.13 | 0.019 | >90% C-O |

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**Table IV**

| Source | Position of acyl phosphate bond cleavage |
|--------|-------------------------------------------|
| Formate buffer, 0.1 mM, pH 3.8 | 1.28 | 0.314 | >95% P-O |
| NaOH, 0.2 N | 1.13 | 0.019 | >90% C-O |

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**Table V**

| Source | Position of acyl phosphate bond cleavage |
|--------|-------------------------------------------|
| Formate buffer, 0.1 mM, pH 3.8 | 1.28 | 0.314 | >95% P-O |
| NaOH, 0.2 N | 1.13 | 0.019 | >90% C-O |
The reaction mixtures at 15° contained 3 mM [γ-32P]ATP (17 μCi per μmole), 6 mM MgCl₂, 140 mM NaCl, 7.1 mM Pi, 50 mM imidazole-glycylglycine (pH 7.3), 300 mg of electrophor-ATPase (specific activity = 5.2 μmole per min per mg of protein) in a total volume of 8.0 ml of water containing 9.64 atom % 18O. Also, for Experiment 3, 20 mM KCl was present. The reactions were initiated by addition of ATP and quenched after 15 s by the rapid addition of 300 μl of 5.7 M trichloroacetic acid containing 0.1 mM Pi. Cleavage in presence of 4.9 μmole of carrier Pi, Pi were measured by the delta 32P technique. This indicates a phosphate-water exchange at the level of the phosphoryl group. Exchange of H18O with phosphate oxygens of the acyl phosphate was about 30% lower than that expected on the basis of the 18O content of [18O]Pi used. Clearly, the upper limit of the observed atom % excess was well below the expected atom % excess if Pi furnished the bridge oxygen. These data demonstrate that acyl phosphate formation occurs by a direct displacement of a phosphoryl oxygen by the carboxylate oxygen.

Also of interest is the observation that the δ18O content of the phosphoryl oxygens of the acyl phosphate was about 30% lower than that expected on the basis of the δ18O content of [18O]Pi used. This indicates a phosphate-water exchange at the level of the phosphoryl group. This has been confirmed in other experiments. With longer reaction times in presence of ouabain the δ18O content of the acyl phosphate was less than 0.071 atom % excess. The results show that water oxygen is indicated by the filled oxygen atoms. Such exchange amounted to an average of about 1 atom % per phosphoryl group. One possibility for this δ18O incorporation from water is that a portion of the phosphoenzyme present was formed from medium Pi containing 18O derived by ATP hydrolysis or by Pi = HOH exchange (8). However, control similar experiments with presence of 2P1 indicated that less than 5% of the phosphoenzyme present during ATP hydrolysis arose from medium Pi. This is quite insufficient to account for the observed 18O atom % in the enzyme phosphoryl group. The exchange of 18O from H18O into the Pi formed from either P-O or C-O cleavage has thus occurred prior to formation of medium Pi. Such exchange as observed with myosin ATPase is referred to as intermediate exchange (15).

**Source of Bridge Oxygen in Na⁺, K⁺-ATPase Phosphorylated by ATP in Presence of H18O—**Phosphorylation of sarcoplasmic reticulum vesicles was carried out in H18O with ATP as substrate as described in Table IV. The observed incorporation of 18O into the P1 from either P-O or C-O cleavage was less than 0.001 atom % excess. The results show that water oxygen was not incorporated into either the C-O-P or O-C-P bridge oxgens of the acyl phosphate. Measurements of the steady state level of the phosphoryl enzyme and Ca²⁺-dependent P1 liberation indicate that the phosphoryl enzyme turned over at least 20 times during the reaction period.

**DISCUSSION**

The results demonstrate that with the microsomal and the sarcoplasmic reticulum ATPases, the acyl phosphate intermediate is cleaved by water as shown in Equation 1. Incorporation of water oxygen is indicated by the filled oxygen atoms.

\[
\text{Enzyme} \rightarrow \text{C-O-P} + \text{HOP} + \text{H} + \text{HPO}_4^{-} \tag{1}
\]

The data rule out the possibility of formation of any internal

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**Table III**

| Experiment | Position of acyl phosphate bond cleavage | δ18O released | Observed atom % excess δ18O in isolated P1 | Mole | 18O |
|------------|----------------------------------------|---------------|------------------------------------------|------|-----|
| 1          | P-O                                   | 41            | 0.16                                     | <0.001 |
|            | C-O                                   | 39            | 0.14                                     | <0.001 |
| 2          | P-O                                   | 32            | 0.14                                     | <0.001 |
|            | C-O                                   | 32            | 0.13                                     | <0.001 |
| 3a         | P-O                                   | 122           | 0.053                                    | <0.001 |
|            | C-O                                   | 123           | 0.048                                    | <0.001 |

* Calculated from δ18P released from 82 to 85% of protein-bound 18P was released.
* Corrected for internal standards as described in the text.
* Twice as much protein and ATP were used.

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**Table IV**

| Experiment | Position of acyl phosphate bond cleavage | Observed atom % excess in isolated P1 | 18O |
|------------|----------------------------------------|--------------------------------------|-----|
| 1          | P-O                                   | <0.001                               | <0.001 |
|            | C-O                                   | <0.001                               | <0.001 |
| 2          | P-O                                   | <0.001                               | <0.001 |
|            | C-O                                   | <0.001                               | <0.001 |

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The results show no experimentally significant differences between the δ18O content of the P1 derived by P-O or C-O cleavage. Within the experimental error of 5 to 10%, all δ18O present could be accounted for by that derived from the phosphoryl oxygens. Thus, no measurable exchange of the carboxyl group oxygens with water occurred during catalysis.

The observed atom per cent excess of δ18O reported in Table III when corrected for carrier P1; addition shows that a relatively large amount of water oxygens was incorporated into the phosphoryl oxygens of the acyl phosphate. Such exchange amounted to an average of about 1 atom % per phosphoryl group. One possibility for this δ18O incorporation from water is that a portion of the phosphoenzyme present was formed from medium Pi containing 18O derived by ATP hydrolysis or by Pi = HOH exchange (8). However, control similar experiments with presence of 2P1 indicated that less than 5% of the phosphoenzyme present during ATP hydrolysis arose from medium Pi. This is quite insufficient to account for the observed 18O atom % in the enzyme phosphoryl group. The exchange of 18O from H18O into the Pi formed from either P-O or C-O cleavage has thus occurred prior to formation of medium Pi. Such exchange as observed with myosin ATPase is referred to as intermediate exchange (15).

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The results demonstrate that with the microsomal and the sarcoplasmic reticulum ATPases, the acyl phosphate intermediate is cleaved by water as shown in Equation 1. Incorporation of water oxygen is indicated by the filled oxygen atoms.

\[
\text{Enzyme} \rightarrow \text{C-O-P} + \text{H} + \text{HPO}_4^{-} \tag{1}
\]

The data rule out the possibility of formation of any internal
acyl-X derivatives in the membrane as a mode of utilization of the acyl phosphate for active transport. Also, they rule out attack of water on the acyl carbon in preference to the phosphoryl phosphorus atom.

The results on the phosphorylation of the microsomal ATPase from P_i in presence of ouabain demonstrate that this occurs by direct displacement of a phosphate oxygen by the enzyme carboxylate, essentially a reversal of Equation 1. The active site in some manner may increase the nucleophilicity of the carboxylate oxygen, increase the susceptibility of the phosphorus oxygen to nucleophilic attack, or favor departure of the OH--leaving group. The formation of a substantial equilibrium amount of an acyl phosphate from P_i without phosphorolysis of a pre-existing covalent bond points to conformational or other factors favoring existence of the phosphorylated form. Such a capacity, promoted by ouabain binding, may be germane to suggested conformational coupling for oxidative phosphorylation (16).

Mention should be made that the experimental limitations are such that the 31P analyses by themselves do not establish that the enzyme-COOH group is the sole source of the C-O-P bridge oxygen. They do establish that it is the major source and, within experimental limitations, could be the only source. The high probability that the enzymic reactions involved in the phosphorylation are completely specific as to position of bond cleavage and formation allow the conclusions made herein.

The demonstration in these studies of a preferential exchange of water oxygens with P_i released from the phosphorylated Na^+,K^+-ATPase is quite analogous to the "intermediate" exchange observed in hydrolysis by myosin and actomyosin ATPases (15). Previous studies from this laboratory showed considerable exchange capacity of the Na^+,K^+-ATPase but only with P_i oxygens of the medium (8). The essential difference appears to be that the earlier studies were conducted at 37\(^\circ\) whereas the present results were obtained at 15\(^\circ\). Temperature-dependent changes in mechanism of the Na^+,K^+-ATPase have been proposed previously by Kanazawa et al. (17). Conformational changes in the 14 to 30\(^\circ\) region are known to occur which have been invoked to explain biphasic Arrhenius plots of Na^+, K^+-ATPase activity (18). Rate-limiting steps in ATP hydrolysis at the lower temperature may include release of P_i from the enzyme form as depicted by Step 2 of Equation 2.

\[
\text{Enzyme-PO}_2 + \text{HOH} \xrightleftharpoons{(1)} \text{enzyme-HOPO}_4^- \xrightleftharpoons{(2)} \text{enzyme + HOPO}_4^-
\]

Dynamic reversal of Step 1 during ATP hydrolysis and prior to P_i release would give rise to the "intermediate" exchange. Reversal of both steps of Equation 2 would give rise to "medium" exchange. Both types of exchange may thus logically occur by dynamic reversal of covalent bond formation.

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