Formation of Adenosine Triphosphate from P<sub>i</sub> and Adenosine Diposphate by Purified Ca<sup>2+</sup>-Adenosine Triphosphatase*  

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SUMMARY

Ca<sup>2+</sup>-ATPase purified from sarcoplasmic reticulum of rabbit muscle forms a phosphoenzyme when exposed to inorganic phosphate in the presence of Mg<sup>2+</sup>. On addition of ADP and Ca<sup>2+</sup> virtually all of the phosphate bound to the enzyme is transferred to form ATP.

It has been shown previously and confirmed by us that (a) the purified ATPase contains one major polypeptide and about 30% phospholipids; (b) on removal of residual detergent by passage through Sephadex the enzyme forms vesicular membranes; and (c) these vesicles are leaky and incapable of accumulating Ca<sup>2+</sup>. Our findings therefore indicate that we have observed ATP formation from ADP and P<sub>i</sub> without the formation of an ion gradient across a membrane. We propose that the energy derived from ion-protein interaction drives the formation of ATP.

The mechanism of ion translocation has become a central theme of the problem of oxidative phosphorylation. According to Mitchell (1) the role of the respiratory chain is to establish a proton gradient which is utilized by the transmembranous oligomycin-sensitive ATPase to generate ATP from ADP and P<sub>i</sub>. We propose that the energy derived from ion-protein interaction drives the formation of ATP.

Considerable advances have been made with ATP-dependent ion translocation systems that are much simpler than mitochondrial phosphorylation. ATP is generated from ADP and P<sub>i</sub> by both the Ca<sup>2+</sup> pump of sarcoplasmic reticulum (3, 4) and the Na<sup>+</sup>K<sup>+</sup> pump of the plasma membrane (5) when they are allowed to operate in reverse, dissipating an ion gradient. Moreover, the ATPases that are involved in these reactions have been obtained in a state of high purity and were shown to interact with ATP<sup>1</sup> to form a phosphoenzyme (6-9). Phosphoenzyme formation could also be observed when sarcoplasmic reticulum vesicles (10) or “microsome” preparations of Na<sup>+</sup>K<sup>+</sup>-ATPase (11, 12) were incubated with P<sub>i</sub> and Ca<sup>2+</sup>. Recently it was briefly reported (13) that ATP was formed stoichiometrically with the Na<sup>+</sup>K<sup>+</sup>-ATPase when P<sub>i</sub>, Na<sup>+</sup>, and ADP were added sequentially to kidney “microsomes” under appropriate conditions.

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Table I
Phosphoenzyme formation with Ca²⁺-ATPase and ³²P
The complete system contained 10 mM maleate (Tris), pH 6.0, 10 mM MgCl₂, 2 mM EGTA, and 4 mM K²⁵Pi (8 × 10⁶ cpm/μmole). Phosphoenzyme formation was measured as described under "Methods."  

| Reaction mixture                                      | Nanomoles of phosphoenzyme/mg of protein |
|-------------------------------------------------------|------------------------------------------|
| Complete system                                       | 2.90                                     |
| Minus Mg²⁺                                            | 0.07                                     |
| Minus EGTA                                            | 1.81                                     |
| Minus EGTA, plus CaCl₂ (1 mM)                         | 0.03                                     |
| Plus (1,2-cyclohexylenedinitrilo)tetraacetic acid (20 mM) | 0                                        |

Fig. 1. Phosphoenzyme formation as a function of pH. The reaction mixture contained 10 mM MgCl₂, 1 mM EGTA, 4 mM K²⁵Pi (5 × 10⁶ cpm/μmole), and 10 mM buffer in a final volume of 2 ml. For pH levels below 5.5, acetate (Tris) buffers were used; for pH 6.0 to 6.5, maleate (Tris) buffers, and for pH above 7, Tris (Cl) buffers were used. The pH values shown in the figure were determined with a glass electrode in the final reaction mixture. The reactions were started by the addition of 0.2 mg of Ca²⁺-ATPase. Phosphoenzyme formation was determined as described under "Methods."  

was released from the native enzyme. This was shown by sedimenting the enzyme after completion of the reaction and by analyzing the supernatant for ATP.

The compound formed from ³²P was in fact ATP as was shown by a variety of tests including adsorption on charcoal and hydrolysis of the pyrophosphate bond in 1 N HCl (Table II). It was also established to establish that the [³²P]ATP was not formed by an exchange of P_i into ATP which may have been either present as a contaminant in ADP or formed by an adenylyl kinase type of reaction. It can be seen from Table II that in the presence of a large excess of hexokinase the formation of acid-labile ³²P was suppressed and instead an acid-stable radioactive compound (presumably glucose 6-phosphate) was formed. The amount of hexokinase added was sufficient to prevent maintenance of ATP for an exchange to take place. This has been previously established in experiments on oxidative phosphorylation (15) and proton pump-driven phosphorylation (2). Moreover, participation of an exchange reaction was monitored by addition of highly radioactive ³²P simultaneously with ADP and CaCl₂ to a phosphoenzyme which was not radioactive. Only small amounts of [³²P]ATP (less than 10% compared to the control) were formed under these conditions. In fact it is likely that some replacement of the phosphate group on the enzyme by ³²P may have taken place during the 1-min incubation.

Table II
Formation of ATP by Ca²⁺-ATPase and its utilization by hexokinase
Phosphoenzyme was formed in the reaction mixture as described under "Methods" with 1.5 mg of Ca²⁺-ATPase. After incubation for 1 min at 25°, 0.2 ml of CaCl₂ (80 mM in Experiment 1 and 20 mM in Experiment 2) and 0.2 ml of a solution containing 60 mM ADP-33 mM glucose with or without 80 units of hexokinase were added simultaneously. After further incubation for 1 min at 25° the reaction was terminated by addition of 0.4 ml of cold 60% trichloroacetic acid. To an aliquot (1.2 ml) of the deproteinated solution, 0.11 ml of concentrated HCl was added, and the solution was heated at 100° for 7 min. To another aliquot of the deproteinated solution, 0.11 ml of H₂O was added. Both solutions (with and without acid hydrolysis) were analyzed for phosphate esters by isobutyl alcohol-benzene extraction as described (15).

| Treatment of product | Organic ³²P formed |
|----------------------|--------------------|
|                      | -Hexokinase | +Hexokinase |
| Experiment 1         |             |             |
| None                 | 0.54        | 0.49        |
| Boiled 7 min in 1 N HCl | <0.05    | 0.40        |
| Experiment 2         |             |             |
| None                 | 0.71        | 0.92        |
| Boiled 7 min in 1 N HCl | 0.05    | 0.89        |

* Phosphoenzyme production determined in separate samples was 0.94 nmole/mg of protein in Experiment 1 and 1.0 in Experiment 2.
Finally, an experiment was performed that showed that the energy for ATP formation was not originally stored in the isolated enzyme, e.g., in the form of a thiolester. The enzyme was re-isolated after it had been incubated under the above described conditions that give rise to ATI. The re-precipitated enzyme catalyzed ATP formation as well as the control enzyme that had not been exposed to 1 cycle of ATP formation.

**DISCUSSION**

A number of questions arise with the demonstration of ATP formation with a purified ATPase. What is the mechanism of phosphoenzyme formation? Where is the energy derived for the formation of ATP? It has been shown in several laboratories that the phosphoenzyme of Na⁺K⁺-ATPase (16, 17) as well as of Ca⁺⁺-ATPase (18) can be digested to a polypeptide containing an aspartyl phosphate group. It has been known for many years (19) that acyl phosphates of this type can transphosphorylate phosphate to ADP in the presence of the appropriate catalysts. In fact, the free energy of hydrolysis of free acyl phosphates is several kilocalories higher than that of ATP. Unfortunately nothing is known about the free energy of hydrolysis of the acyl phosphate formed from 1, which is buried in the hydrophobic regions of the protein. Judging from the relatively low concentrations of phosphate required for phosphoenzyme formation, it is likely that the values of the free energy of hydrolysis of the acyl group in the protein are much lower than those of acyl phosphates in an aqueous medium. Moreover, there is no direct evidence that the acyl phosphate is an intermediate during catalysis; it might be formed during denaturation or proteolytic digestion of the protein. Therefore, the problem of the energetics is more sharply focused in the experiment demonstrating formation of ATP that can be utilized by hexokinase while the ATPase functions as a native enzyme. Even if the acyl phosphate were not the true intermediate, the free energy of hydrolysis of the terminal pyrophosphate bond must be derived from the reagents that are present.

There are two aspects of these findings that distinguish them from previous ones. We have described ATP formation from ADP and P; by an enzyme that has been removed from the original membrane by solubilization with deoxycholate. As previously pointed out by MacLennan (14) and repeatedly confirmed in our laboratory, these preparations cannot accumulate Ca⁺⁺. Moreover we have observed phosphoenzyme and ATP formation in the presence of 3% Tween 80 which prevents ion accumulation into phospholipid vesicles. Thus the formation of ATP does not take place by dissipation of an ion gradient across a membrane. The second point which should be emphasized is that we have shown that we are not dealing with ATP tightly bound to the enzyme but that it is free in solution and available to hexokinase. This is an important consideration in the energetics of the reaction. We propose that the energy for ATP formation is derived from the interaction of Ca⁺⁺ with the protein. It thus appears that the problem of ion-dependent ATP formation has now become amenable to a physicochemical approach to protein structure and ion interaction. However the problem of ATP-driven ion translocation is a much more complex one involving directional movement across the membrane and will be more difficult to approach experimentally.

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