Properties of a Reconstituted Calcium Pump*

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(Received for publication, September 6, 1974)

1. The translocation of '*'Ca** in vesicles reconstituted with purified Ca** ATPase of sarcoplasmic reticulum and phospholipids was dependent on ATP and varied greatly with the composition of the phospholipids.

2. In contrast to sarcoplasmic reticulum fragments, the reconstituted vesicles were impermeable to '*'C-labeled oxalate, '*'II- or '*'P-labeled ATP, or '*'T**. There was no translocation of phosphate from '*'labeled ATP during Ca** uptake. These results are inconsistent with some current formulations of the mechanism of pump action.

3. Reversal of the Ca** pump and generation of ATP from ADP and P** was observed when vesicles loaded with Ca** were exposed to ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

4. Experiments on the formation of phosphoenzyme with '*'P-labeled ATP showed that most if not all functional ATPase molecules in the reconstituted vesicles were oriented in the same direction, as in the case of sarcoplasmic reticulum fragments.

Sarcoplasmic reticulum, because of its simplicity, is particularly suitable for the study of membrane function and structure. A protein has been isolated from sarcoplasmic reticulum (molecular weight 105,000) which catalyzes Ca**-dependent ATPase activity (1), ADP-ATP exchange (2), and P**-ATP exchange (3). This protein was used to reconstitute phospholipid vesicles that catalyze Ca** translocation (3). Whereas isolated sarcoplasmic reticulum (4) or vesicles reformed from the original components (5) accumulate large quantities of Ca** in the presence of phosphate or oxalate, vesicles reconstituted with excess phospholipids require internally trapped phosphate or oxalate (3, 6). The present paper shows that this requirement of reconstituted vesicles may be explained by an impermeability to anions which has allowed performance of an experiment that has ruled out one proposed mechanism of action of the pump. Another advantage of the reconstitution method is the possibility of varying the phospholipid composition. For example, alteration of the polar group of phosphatidylinositolamine has resulted in marked changes in the Ca** pump and ATPase activity (7). In the present paper, we describe in detail the properties of the reconstituted Ca** pump.

MATERIALS AND METHODS

Materials—Cholic acid and deoxycholic acid were obtained from Schwarz/Mann, Orangeburg, N. Y., and recrystallized as described previously (8). Soybean phospholipids (esoelatin) were obtained from Associated Concentrates, Woodside, N. Y.; '*'CaCl** was from American/Searle Corp., Arlington Heights, Ill.; '*'C-oxalic acid and '*'P** were from ICN, Irvine, Cal.; [*β-3H]adenosine 5'-triphosphate, tet-

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* This work was supported by United States Public Health Service Grant CA-08964 from the National Cancer Institute.
or 0.2 M potassium oxalate, respectively. Liposomes without Ca\textsuperscript{2+}-ATPase were prepared in the same manner to correct for nonspecific binding. Except where specified, partially purified soybean phospholipids were used throughout this study.

**ATP Synthesis Driven by Ca\textsuperscript{2+} Gradient**—Sarcoplasmic reticulum and reconstituted vesicles were loaded with Ca\textsuperscript{2+} using acetyl phosphate as the energy source in 1 ml of a reaction mixture described by Deamer and Baskin (15). After incubation at 23° for 25 min, 0.2-ml solution containing 16 units of hexokinase, 0.25 mM ADP, 5 mM EGTA\textsuperscript{1} were added. After 20 min at 23°, 0.1 ml of 50% trichloroacetic acid was added. Aliquots of the deproteinized solutions were extracted by isobutyl alcohol-benzene and H\textsubscript{2}O-saturated isobutyl alcohol sequentially (16) and the amount of \[^{14}C\]glucose-6-P in the aqueous phase was determined on dried samples in a gas flow counter.

**Vesicles**—The reaction was carried out with 20 to 70 \(\mu\)g of protein of Ca\textsuperscript{2+}-ATPase or reconstituted vesicles in 1 ml of the following reaction mixture: 20 mM Tris-Cl, pH 8.0, 10 mM MgCl\textsubscript{2}, 10 mM ATP, 40 mM K\textsuperscript{+}, (100 \text{ cpm/\mu mole}), and 1.2 mM CaCl\textsubscript{2}. After incubation at 37° for 10 min, the reaction was stopped with 0.1 ml of 50% trichloroacetic acid and aliquots of the deproteinized solutions were extracted (16). \[^{32}P\]ATP in the aqueous phase was determined by counting dried samples in a gas flow counter.

**Phosphorylation of Ca\textsuperscript{2+}-ATPase**—Phosphorylation of Ca\textsuperscript{2+}-ATPase and of reconstituted vesicles by \[^{1-}^3P\]ATP was carried out according to Panet et al. (17). The protein was precipitated with trichloroacetic acid, washed, suspended in 2 ml of 2% Na\textsubscript{2}CO\textsubscript{3} in 0.1 N NaOH, and heated at 100° for 15 min. One milliliter of the solution was dried and counted in a Nuclear-Chicago gas flow counter and another aliquot was used for protein determination.

**RESULTS**

**Effect of Trapping Agents on Ca\textsuperscript{2+} Transport in Reconstituted Vesicles**—Ca\textsuperscript{2+} accumulation in sarcoplasmic reticulum was greatly increased by addition of oxalate or K\textsuperscript{+} (4, 18). These agents were not effective with reconstituted vesicles unless they were present during reconstitution and trapped inside the vesicles. As shown in Table I, relatively large concentrations of these Ca\textsuperscript{2+} sequestering agents were required to yield vesicles with high transport activity. As a rule, liposomes were prepared in the same reconstitution buffer and the values obtained with these liposomes in the Ca\textsuperscript{2+} uptake assays were used to correct for nonspecific Ca\textsuperscript{2+} uptake unrelated to the activity of the enzyme (Table II). When reconstituted vesicles were assayed in the absence of ATP, unexpectedly high values for Ca\textsuperscript{2+} uptake were observed in some experiments. The magnitude of these ATP-independent values increased with the concentration of the potassium buffers used (up to 0.4 M K\textsuperscript{+}) during reconstitution. Data comparing vesicles with 0.1 and 0.4 M K\textsuperscript{+} are shown in Table II. Since increasing the potassium concentration of the assay medium markedly decreased this ATP-independent uptake of Ca\textsuperscript{2+}, it seems likely that it was caused by the potassium gradient created by the differences in the intravesicular and extravesicular ion concentrations. That the small amount of enzyme independent Ca\textsuperscript{2+} uptake in liposomes was also transmembranous was indicated by the fact that it could be decreased considerably by the ionophore A-23187.

**Kinetics of Ca\textsuperscript{2+} Uptake in Reconstituted Ca\textsuperscript{2+}-ATPase Vesicles**—The reconstituted vesicles translocated Ca\textsuperscript{2+} at a rate of 0.2 to 0.6 \(\mu\)moles/mg of protein/min. The extent of Ca\textsuperscript{2+} accumulation was about 1.5 \(\mu\)moles/mg of protein under the conditions of the assay. While there were some variations with different preparations of the enzyme and phospholipids (6), the assay was quite reproducible with the same preparations during the first 8 hours after reconstitution. After 24 hours at 4°, the rate of Ca\textsuperscript{2+} uptake was about half.

The rate of Ca\textsuperscript{2+} uptake increased with the Ca\textsuperscript{2+} concentration up to 0.6 mM Ca\textsuperscript{2+}. A Lineweaver-Burk plot (Fig. 1) yielded a \(K_m\) value of 2.5 \(\times 10^{-4}\) M. As shown in Fig. 2, the pH optimum varied somewhat with the reconstitution buffer used. With K\textsuperscript{+}, the optimum was at 7.8, with potassium oxalate the optimum was at about 7.0.

Although the rate of Ca\textsuperscript{2+} uptake in the reconstituted vesicles was similar to that in sarcoplasmic reticulum, the efficiency as expressed by the Ca\textsuperscript{2+}:ATP ratio was considerably lower. As can be calculated from the data in Fig. 3 which show ATP hydrolysis and Ca\textsuperscript{2+} uptake values measured under identical conditions, the Ca\textsuperscript{2+}:ATP ratios were 0.2 to 0.3 while with sarcoplasmic reticulum values of 2 were obtained in agreement with previous reports (4, 19, 20). However, the efficiency of Ca\textsuperscript{2+} transport is influenced by the composition of proteins and phospholipids, the protein to lipid ratio and the method of reconstitution (6). In recent experiments Ca\textsuperscript{2+}:ATP ratios approaching 2 have been observed with certain preparations of the ATPase and this variability in efficiency is now under further investigation.

**Role of Deoxycholate in Reconstitution**—The procedure for the isolation of Ca\textsuperscript{2+}-ATPase from sarcoplasmic reticulum (1) depends on the solubilization of the enzyme with deoxycholate. The enzyme remains soluble as long as there is some residual deoxycholate. After passage through Sephadex (1), the enzyme

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1 The abbreviation used is: EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N\textsuperscript{-}tetracetic acid.

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**Table I**

| Buffer used for reconstitution | Concentration | Ca\textsuperscript{2+} uptake  
\(\mu\)moles/min/mg protein |
<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>KP\textsubscript{+}, pH 7.4</td>
<td>0.1</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.30</td>
</tr>
<tr>
<td>K-oxalate, pH 7.4</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.23</td>
</tr>
</tbody>
</table>

**Table II**

| Vesicles | Addition to assay medium | Vesicles reconstituted in  
\(0.1\) M KP\textsubscript{+} | \(0.4\) M KP\textsubscript{+} |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>- ATP</td>
<td>+ ATP</td>
<td>- ATP</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}-ATPase</td>
<td>33.2</td>
<td>52.9</td>
</tr>
<tr>
<td>0.2 M KCl</td>
<td>26.0</td>
<td>69.3</td>
</tr>
<tr>
<td>0.4 M KCl</td>
<td>18.4</td>
<td>61.1</td>
</tr>
<tr>
<td>0.6 M KCl</td>
<td>14.5</td>
<td>54.1</td>
</tr>
<tr>
<td>Liposomes</td>
<td>25.2</td>
<td>11.4</td>
</tr>
<tr>
<td>0.2 M KCl</td>
<td>21.0</td>
<td>10.3</td>
</tr>
<tr>
<td>0.4 M KCl</td>
<td>10.4</td>
<td>11.4</td>
</tr>
<tr>
<td>0.6 M KCl</td>
<td>10.8</td>
<td>7.6</td>
</tr>
</tbody>
</table>
becomes insoluble. We observed that such preparations were unsuitable for reconstitution by the cholate dialysis procedure unless deoxycholate was added. Similar results were obtained with the Ca$^{2+}$-ATPase preparation isolated according to Warren et al. (9). Approximately 1.5 mg of deoxycholate/mg of protein were required during reconstitution to obtain maximal Ca$^{2+}$ transport activity (Fig. 4). In view of the rather critical dependence on deoxycholate, it is not surprising that considerable variability of transport rate and efficiency was encountered in vesicles reconstituted with different enzyme preparations. It is of interest to note that for the reconstitution by the sonication procedure (6), Sephadex-treated enzyme can be used since there is no requirement for either cholate or deoxycholate.

**Reconstitution with Purified Phospholipids**—Sarcoplasmic reticulum contains about 65% phosphatidylcholine and 14%
phosphatidylethanolamine (21, 22). It was surprising therefore to find that phosphatidylethanolamine was quite effective in the reconstitution experiments while phosphatidylcholine alone was virtually inactive. As shown in Fig. 5 (open circles) phosphatidylethanolamine alone was sufficient for the reconstitution of vesicles that were active in Ca\(^{2+}\) translocation, although a slight stimulation of the rate by small amounts of phosphatidylcholine was observed. Slightly different optima of the ratio of the two phospholipids were observed when oxalate was used instead of phosphate as a trapping agent, but again, phosphatidylethanolamine alone was quite effective. This is in curious contrast with the results obtained by the sonication procedure (6) where both phospholipids were required. This points again to differences in the properties of vesicles reconstituted by different methods and to the importance of physical as well as chemical parameters in the success of reconstitutions as was pointed out previously (10). It can also be seen from Fig. 5 that optimal Ca\(^{2+}\) pump activity did not coincide with maximal ATPase activity, indicating another aspect of the efficiency problem which requires further study.

**Reversal of Pump**—Sarcoplasmic reticulum vesicles loaded with Ca\(^{2+}\) catalyze the formation of ATP from ADP and Pi when the Ca\(^{2+}\) is released by addition of EGTA (23, 24). ATP generation was also observed when Ca\(^{2+}\) was released from Ca\(^{2+}\)-loaded reconstituted vesicles. As shown in Fig. 6, the amount of ATP formed increased with the Ca\(^{2+}\) concentration in the loading medium up to 0.2 mM Ca\(^{2+}\). Since under these conditions only between 40 and 50% of the Ca\(^{2+}\) input was released on addition of EDTA, the corrected value for the Ca\(^{2+}\)-ATP ratio was about 0.3 which was about the same as observed in these vesicles for the Ca\(^{2+}\) uptake.

**P\(_{i}\)-ATP Exchange by Enzyme and Reconstituted Vesicles**—It has been previously shown that both the isolated Ca\(^{2+}\)-ATPase and the reconstituted vesicles catalyze a Ca\(^{2+}\)-dependent P\(_{i}\)-ATP exchange reaction (3). We have examined this reaction more closely and found that optimal conditions require higher Ca\(^{2+}\) and P\(_{i}\) concentrations than previously employed. Table III shows that under these conditions, the P\(_{i}\)-ATP exchange activity of Ca\(^{2+}\)-ATPase was considerably higher than previously reported values. The reaction catalyzed by either the soluble Ca\(^{2+}\)-ATPase or the reconstituted vesicles was inhibited by chlorpromazine and by a sulfhydryl reagent, p-hydroxymercuriphenylsulfonate. However, neither A-23187 which abolished Ca\(^{2+}\) uptake in the reconstituted vesicles nor nigericin which slightly stimulated it had any effect on the exchange activity. The P\(_{i}\)-ATP exchange activity was much lower in the reconstituted vesicles partially due to some inactivation of the enzyme by cholate during reconstitution.

**Table III**

**P\(_{i}\)-ATP exchange in soluble Ca\(^{2+}\)-ATPase from sarcoplasmic reticulum and the reconstituted Ca\(^{2+}\)-ATPase vesicles**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Soluble Ca-ATPase</th>
<th>Reconstituted vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles [(^{32})P]ATP/min/mg protein</td>
<td>nmoles [(^{32})P]ATP/min/mg protein</td>
</tr>
<tr>
<td>None</td>
<td>217</td>
<td>43</td>
</tr>
<tr>
<td>EGTA (4 mM)</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Chlorpromazine (200 µg)</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Chlorpromazine (400 µg)</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>A-23187 (2 µg)</td>
<td>215</td>
<td>50</td>
</tr>
<tr>
<td>A-23187 (10 µg)</td>
<td>200</td>
<td>46</td>
</tr>
<tr>
<td>Nigericin (4 µg)</td>
<td>197</td>
<td>49</td>
</tr>
<tr>
<td>p-Hydroxymercuriphenylsulfonate (0.1 mM)</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

**Fig. 6.** Net synthesis of ATP by a reversal of the Ca\(^{2+}\) gradient. Ca\(^{2+}\) uptake was carried out in 1-ml reaction mixture containing 4 mM acetyl phosphate, 5 mM MgCl\(_{2}\), 0.1 mM glucose, and carrier-free \[^{32}\]P\(_{i}\). Vesicles reconstituted in 0.4 M potassium phosphate were first concentrated by centrifuging at 165,000 \(\times\) g for 2 hours. The aliquot of the concentrated vesicles used in the experiment contained 1 mg of protein and introduced an amount of phosphate into the medium so that the final concentration of phosphate in the Ca\(^{2+}\) loading medium was 30 mM. After incubation for 20 min at 23\(^\circ\), 0.2 ml of a solution containing 16 units of hexokinase, 0.25 mM ADP, 5 mM EGTA was added. After further incubating for 20 min the reaction was terminated by the addition of trichloroacetic acid and \[^{32}\]P-glucose 6-phosphate was extracted.
(ATPase activity of the reconstituted vesicles was only half of that of the soluble enzyme).

**Ion Movements in Reconstituted Vesicles**—The requirement for phosphate or oxalate during reconstitution and the ineffectiveness of these anions when added after reconstitution suggested that in contrast to sarcoplasmic reticulum (25) the reconstituted vesicles are impermeable to oxalate or phosphate. As shown in Fig. 7, $[^{14}C]$oxalate was taken up into sarcoplasmic reticulum together with Ca$^{2+}$ as shown previously (25), but there was no entry of oxalate with the reconstituted vesicles. Similar experiments were carried out with $[^{38}P]$ and $[^{32}P]$ATP as well as with vesicles loaded with phosphate instead of oxalate. The experiment with $[^{32}P]$ATP is of particular significance because it not only eliminates the terminal phosphate of ATP as a possible co-ion of Ca$^{2+}$, but it is also inconsistent with one of the popular proposals for the mode of action of the Ca$^{2+}$ pump as will be discussed later.

The possibility of a primary proton pump was also considered in view of its operation in mitochondrial ion movements (26). Measurements of H$^+$ movements were performed similar to the procedure described for the measurements of H$^+$ movements in submitochondrial particles (27) at pH 6.3 where ATP hydrolysis does not give rise to a release of protons. At this pH the reconstituted vesicles still translocated Ca$^{2+}$ at about half maximal rate (see Fig. 2). No H$^+$ movement could be detected during Ca$^{2+}$ translocation.

It is therefore most likely that the monovalent cation (e.g. K$^+$) which is present in the vesicles reconstituted with oxalate or phosphate serves as counter ion for Ca$^{2+}$. A direct test for this possibility was complicated by the observation that the enzyme was unstable unless either Na$^+$, K$^+$, or NH$_4^+$ ions were present as shown in Table IV. Participation of K$^+$ in the mechanism of Ca$^{2+}$-ATPase (28) and Ca$^{2+}$ translocation has been suggested previously (29, 30). A 30 to 50% stimulation of Ca$^{2+}$ uptake in reconstituted vesicles by valinomycin and nigericin which was noted previously (3) and repeatedly confirmed in the present work is in line with the proposition that K$^+$ serves as a counter ion.

**Orientation of Ca$^{2+}$-ATPase in the Reconstituted Vesicles**—In order to investigate the orientation of Ca$^{2+}$-ATPase in the reconstituted vesicles it is essential to establish first that the incorporation of Ca$^{2+}$-ATPase into the vesicles is complete. Fig. 8 shows that this is the case in the vesicles of

Table IV

<table>
<thead>
<tr>
<th>Solutions for preincubation</th>
<th>ATPase activity</th>
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<tbody>
<tr>
<td></td>
<td>$\mu$moles ATP hydrolyzed/10 min</td>
</tr>
<tr>
<td>0.2 M K+ oxalate</td>
<td>2.06</td>
</tr>
<tr>
<td>0.2 M Tris-oxalate + 0.15 m M KCl</td>
<td>0.39</td>
</tr>
<tr>
<td>0.2 M Tris-oxalate + 0.15 m M NaCl</td>
<td>2.01</td>
</tr>
<tr>
<td>0.2 M Tris-oxalate + 0.15 m M NH$_4$Cl</td>
<td>1.73</td>
</tr>
</tbody>
</table>

Fig. 8. Sucrose gradient profiles of reconstituted Ca$^{2+}$-ATPase vesicles, liposomes, and Ca$^{2+}$-ATPase. Phospholipids (25 $\mu$moles/ml) were reconstituted with or without Ca$^{2+}$-ATPase as described. Immediately before centrifugation, Ca$^{2+}$-ATPase was added to the sample containing liposomes and 1.5 ml of the mixture and reconstituted vesicles were each applied to separate tubes on a 10-ml sucrose gradient consisting of 20 to 60% sucrose containing 50 mM N-tris(hydroxymethyl)methylglycine (Tricine)-KOH, pH 8.0, and 0.4 M KCl. After centrifugation in a Beckman SW 41 rotor for 24 hours at 35,000 rpm, 0.5-ml fractions were collected from the top. Ca$^{2+}$ uptake activity was measured at 23$^\circ$ and Ca$^{2+}$-ATPase activity was measured at 37$^\circ$ as described.
high Ca\(^{2+}\) transport activity. It can be seen that without reconstitution the enzyme was well separated from the liposomes which showed some nonspecific uptake of \(\Delta^{4}\text{Ca}^{2+}\) (see Table II). After reconstitution, the Ca\(^{2+}\) uptake activity peak coincided with the peak of ATPase activity. Less than perfect coincidence had been observed with reconstituted vesicles which had low Ca\(^{2+}\) uptake activity (0.1 to 0.2 \(\mu\)moles of Ca\(^{2+}/\min/mg\) of protein).

As the reconstituted vesicles are impermeable to ATP, only those enzyme molecules having the correct orientation are capable of being phosphorylated by ATP. Any significant extent of incorrect orientation of the Ca\(^{2+}\)-ATPase would cause a decreased formation of phosphorylated protein compared to the free enzyme. Table V shows that the amount of phosphorylated protein formed by the enzyme without reconstitution and the reconstituted vesicles were the same indicating the incorporated functional Ca\(^{2+}\)-ATPase was oriented unidirectionally and in the same direction as in sarcoplasmic reticulum. However, we cannot rule out the possibility that some inactive ATPase molecules are oriented randomly.

**DISCUSSION**

The reconstitution of a Ca\(^{2+}\) pump with the purified ATPase has unambiguously established the role of this enzyme in the translocation of the divalent cations, although considerable indirect evidence for its participation has been available for many years. Vesicles formed with pure phospholipids are active in the Ca\(^{2+}\) transport process, but it must be remembered that the ATPase preparation (1) still contains about 25% phospholipids. Since we have used about 50 to 200 \(\mu\)moles of phospholipids per mg of protein, the percentile contribution of the enzyme-associated phospholipids is negligible. Yet in view of the firm association between the enzyme and endogenous phospholipids, this contribution cannot be ignored. It is therefore of considerable importance that Warren et al. (9) have recently established that a preparation of Ca\(^{2+}\)-ATPase in which all phospholipids had been replaced by dioleyl phosphatidylcholine was functional in reconstitution of a Ca\(^{2+}\) pump. Furthermore, the same investigators were able to reconstitute active vesicles from these enzyme preparations with dioleyl phosphatidylcholine and obtained considerable enhancement of Ca\(^{2+}\) uptake by externally added oxalate.

**TABLE V**

Formation of phosphorylated protein in reconstituted Ca\(^{2+}\)-ATPase vesicles

A suspension of asolectin (40 \(\mu\)moles/ml) in 0.4 M KP\(_4\), pH 7.4, was prepared by sonication. To this suspension was added either potassium cholate (reconstituted Ca\(^{2+}\)-ATPase vesicles). The phospholipid to protein ratio, the similarity to the native vesicles can be greatly increased. However, we have shown in this paper that the impermeability of our reconstituted vesicles permitted the design of an experiment that could not have been performed with sarcoplasmic reticulum. We found that Ca\(^{2+}\) translocation can take place without simultaneous movement of oxalate or phosphate and that the terminal phosphate of ATP does not enter the vesicles. The latter observation appears to rule out a model of Ca\(^{2+}\) translocation proposed by several investigators (15, 31, 32) which involves a rotating carrier that releases the phosphate of the phosphoenzyme on the inside of the vesicles. It is clear from our experiment with \(\gamma\)-\(\text{P}\)ATP that at least in the reconstituted system the \(P_i\), from the phosphoenzyme is released to the outside and not to the inside of the vesicles. The generation of ATP by these vesicles from externally added ADP and \(P_i\) also speaks against a translocation of phosphate from one side of the membrane to the other. In addition, we have observed that reconstituted vesicles like the soluble enzyme and the sarcoplasmic reticulum can be phosphorylated by inorganic phosphate from the outside.

We have explored the possibility that a channel mechanism is operative in the Ca\(^{2+}\) pump. The approach used in a previous study which showed that a rhodopsin proton pump (33) was operative well below the transition temperature of the phospholipid, was not successful with the Ca\(^{2+}\) ATPase because of the low pump activities we have encountered in reconstitutions with synthetic phospholipids. Since Warren et al. (9) have reconstituted active pumps with synthetic phospholipids we shall summarize the temperature studies after the cause of the divergent results has been elucidated.

**REFERENCES**


Their results differ however from those presented in this paper since we did not obtain very active Ca\(^{2+}\) uptake in vesicles reconstituted with either soybean phosphatidylcholine or synthetic dioleoyl phosphatidylcholine (kindly supplied by Dr. J. Metcalfe) and we failed to observe a stimulation of Ca\(^{2+}\) uptake activity by externally added oxalate. These differences may be indicative of some minor variations in the reconstitution procedure and should be explored further.
30. Carvalho, A. D., and Leo, B. (1967) J. Gen. Physiol. 50, 1327-1352
Properties of a reconstituted calcium pump.
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