PHOSPHOPROTEIN FORMATION DURING OSMO-CHEMICAL ENERGY CONVERSION IN THE MEMBRANE OF THE SARCOPLASMIC RETICULUM*

Madoka MAKINOSE
Max-Planck-Institut für Medizinische Forschung, Abteilung Physiologie, 6900 Heidelberg, Jahnstrasse 29, Germany

Received 8 June 1972

1. Introduction

The isolated vesicles of the sarcoplasmic reticulum accumulate actively calcium from the outside solution using the chemical energy of nucleoside triphosphates or acyl phosphates like acetyl phosphate [1–3]. The process of this active calcium accumulation is obviously the result of a series of reaction steps and one of which is the formation of phosphorylated protein [4, 5]. During the calcium uptake, the concentration of ionic calcium inside the vesicles becomes 3000 times (or more) higher than that in the outside milieu of the vesicles.

Recently, it was demonstrated [6–8] that, under specific conditions, the accumulated calcium can be released rapidly from the vesicles and that this release of calcium is coupled with the synthesis of ATP from ADP and PO_4 in the solution. Later, the phosphorylation of ADP during calcium efflux has been reported also by Panet and Selinger [9] although the specific requirements for the calcium release coupled phosphate incorporation obviously were not recognized.

Analysis of these observations, especially the strict stoichiometry between the calcium efflux and the ATP synthesis, suggests that these coupled reactions are the reversal of the active calcium accumulation and the so called "extra ATP-splitting" by the transport ATPase. In this paper, evidence is presented that a phosphoprotein complex is formed as an intermediate of the calcium efflux driven ATP synthesis and that this complex is indistinguishable from that formed during the active calcium accumulation [4, 5].

* *Abbreviations:*
SR: sarcoplasmic reticulum, NTP: nucleoside triphosphate(s), HK: hexokinase, EGTA: ethylene-glyco-bis-(β-aminoethyl)-*N*,*N*′-tetraacetate, AcP: acetyl phosphate, E and E ~ P: free and Phosphorykated enzyme, respectively, Ca_o and Ca_i: calcium (concentration) in- and outside of the vesicles, respectively.

2. Materials and methods

Preparation of SR vesicles, measurement of calcium translocation and phosphorylated protein are carried out as described previously [4]. Synthesized [γ^{32}P] ATP from ADP and [^{32}P] orthophosphate was converted into [^{32}P] glucose-6-phosphate in the presence of glucose and HK. From the radioactivity in the glucose-6-phosphate fraction isolated chromatographically, the amount of the synthesized ATP was calculated.

3. Results and remarks

Fig. 1 shows the typical procedure for observing stimulated calcium efflux and ATP synthesis. The vesicles were loaded with calcium using AcP as energy donator in the presence of magnesium and orthophosphate. After completion of the calcium uptake, an excess of EGTA was added to the medium. Chelation of the calcium ions in the solution by EGTA results in a high and constant concentration gradient of calcium ions across the vesicle membrane and, on the other hand, blocks interaction between AcP and the calcium pump mechanism [10]. However, in spite of an extremely low calcium level in the solution, calcium flows out from the vesicles only with a very low rate. If 2 mM ADP are added to this system, the

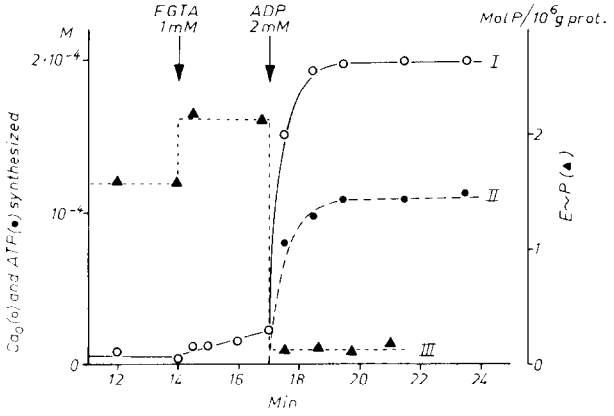

Fig. 1. Labelling of the SR-protein by [^{32}P] orthophosphate and reversal of the SR calcium pump. Reaction mixture contains (mM) 2 AcP, 7 $MgCl_2$, 20 orthophosphate, 100 glucose, 0.2 $CaCl_2$, 0.02 mg/ml HK and 0.5 mg/ml SR protein (pH 7). The mixture for the measurement of the calcium translocation was labelled with [^{45}Ca] $CaCl_2$ (curve I) and those for the measurement of ATP synthesis and phosphoprotein formation with [^{32}P] orthophosphate (curve II and III, respectively). During the pre-incubation time (10 min), the vesicles accumulated calcium added in the medium.

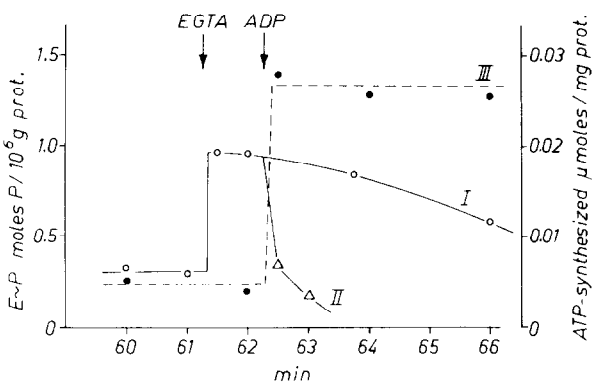

Fig. 2. Phosphoprotein formation in SR membrane in the absence of energy rich phosphoryl substrate. The SR vesicles (0.5 mg/ml) are incubated for 1 hr in a medium containing (mM) 20 histidine, 5 [^{32}P] orthophosphate, 100 KCl, 3 $CaCl_2$, 100 glucose, 7 $MgCl_2$ and 0.02 mg HK per ml (pH 7). At 61 min 15 sec, 10 mM EGTA was added. The EGTA solution used is alkalized previously to neutralize the proton liberated from EGTA at neutral pH. Immediately after the addition of EGTA, phosphoprotein is formed in a significant amount and disappears slowly (curve I). Subsequent addition of 2 mM ADP causes a rapid drop of the phosphoprotein level (curve II) leading to the ATP synthesis (curve III).

calcium is released very rapidly (curve I) and, simultaneously, the system synthesizes ATP from ADP and orthophosphate (curve II). For every two calcium ions released from the vesicles one molecule of ATP is synthesized. Further analysis of these phenomena shows that the presence of calcium in the vesicles and of orthophosphate, ADP and magnesium in the media are essential for the specific activation of the calcium efflux and the coupled ATP synthesis.

Hence, the following reaction scheme of the sarcoplasmic calcium pump can be written

$$2\,Ca_o + ATP + E \overset{Mg}{\rightleftharpoons} Ca_2E\sim P + ADP \qquad \text{(Eq. 1)}$$

$$Ca_2E\sim P \rightleftharpoons 2Ca_i + PO_4 + E \qquad \text{(Eq. 2)}$$

which results in the "overall" reaction

$$2\,Ca_o + ATP \overset{Mg}{\rightleftharpoons} 2Ca_i + ADP + PO_4 \qquad \text{(Eq. 3)}$$

It is obvious that the reverse reaction can take place only in the presence of all reaction products and the activator of the reaction – magnesium.

During and after the accumulation of calcium, orthophosphate in the medium is incorporated into the vesicle protein (fig. 1, curve III). The amount of the phosphoprotein in the membrane increases after the EGTA addition usually up to 30%. The phosphoprotein obtained is stable at acidic pH, decomposed easily in the presence of hydroxyl-amine at pH 5.3, and vanishes promptly after the ADP addition (curve III). The properties of this phosphoprotein are identical with those of the phosphoprotein which is formed by the γ-phosphate transfer from NTP to the membrane protein during active calcium accumulation.

These observations lead to the assumption that the phosphoprotein formed under the described conditions is an energy rich phosphate compound – possibly an acyl phosphate. It is formed as the result of the reversal of Eq. 2. Subsequently, ADP addition leads to dephosphorylation of the complex (Eq. 1) resulting in an ATP synthesis.

The reaction scheme implies that the phosphoprotein is formed from PO_4 and E independently of the presence of energy rich phosphate compounds. The experiment illustrated in fig. 2 shows that this is actually the case. The vesicles were incubated for

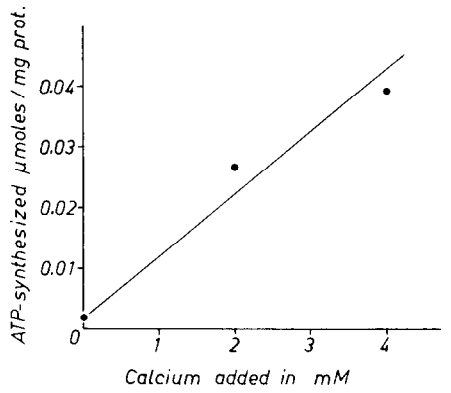

Fig. 3. ATP-synthesis by SR membrane in varied calcium concentrations. Reaction mixtures consist of the same components as described in fig. 2 except the concentration of calcium added. After 120 min incubation, together with 2 mM ADP, 6 mM EGTA is added to the mixtures containing 0 and 2 mM calcium, and 12 mM EGTA to the mixture containing 4 mM calcium. 5 min later, the reaction is stopped by the addition of perchloric acid and the amount of synthesized ATP is determined by measuring the glucose-6-phosphate formed in the system.

120 min in media containing magnesium, [^{32}P] orthophosphate and calcium, but no NTP or AcP. During this incubation time, in all likelihood, the calcium concentration inside the vesicles has become equal to that in the solution. If an excess of EGTA is added to the system, a significant amount of orthophosphate is incorporated into the vesicle protein. The formed phosphoprotein is stable at acidic pH and sensitive to hydroxylamine. It disappears instantaneously after addition of ADP leading to the formation of ATP from ADP and orthophosphate in the media. Obviously, the phosphorylation has the same properties as all other phosphoproteins described [4, 5]. The amount of ATP synthesized after EGTA addition increases when the calcium concentration in the incubation media is raised (fig. 3).

These observations show clearly that the condition required for the formation of the energy rich phosphoprotein in the reverse process is not the high calcium ion concentration in the vesicles itself but the gradient of the calcium concentration across the membrane. Obviously, the energy for the formation of the energy rich phosphate bond in the membrane can be supplied by the osmotic energy of the calcium ions.

If one assumes the standard free energy of the phosphate bond formed in the SR protein to be 7000 cal and translocation of two calcium ions to be required for one molecule ATP synthesis (see fig. 1), the ratio Ca_i/Ca_0 should be higher than 6000 in order to phosphorylate 50% of the active sites in the membrane Under the conditions given in fig. 2, the ratio Ca_i/Ca_0 after the EGTA addition is ~20,000. Hence, ample energy is available.

The simplicity of the experiments presented demonstrates clearly that the SR membrane possesses a mechanism which converts osmotic energy of ions directly into the chemical energy independentl of any high energy substrate.

The experimental results described in this paper have been presented at the 39. Meeting of the German Physiological Society in Erlangen, April 1972 and partially at the Meeting of "International Conference on Biological Membranes" at Gargnano, Italy, June 1971.

Acknowledgement

I wish to thank Ms. A. Hommel for the excellent technical assistance.

References

[1] W. Hasselbach and M. Makinose, Biochem. Z. 333 (1961 518; ibid. 339 (1963) 94.
[2] M. Makinose and R. The, Biochem. Z. 343 (1965) 383.
[3] L. de Meis, J. Biol. Chem; 244 (1969) 3733.
[4] M. Makinose, Second International Biophysics Congress (IOPAB), No. 276, Vienna (1966); European J. Biochem 10 (1969) 74.
[5] T. Yamamoto and Y. Tonomura, J. Biochem. (Tokyo) 64 (1968) 558.
[6] M. Makinose, Pflügers Arch. 319 (1970) R116; FEBS Letters 12 (1971) 269.
[7] W. Barlogie, W. Hasselbach and M. Makinose, FEBS Letters 12 (1971) 267.
[8] M. Makinose and W. Hasselbach, FEBS Letters 12 (1971) 271.
[9] R. Panet and Z. Selinger, Biochim. Biophys. Acta 235 (1972) 34.
[10] Z. Friedman and M. Makinose, FEBS Letters 11 (1970) 69.