Calcium Gradient Dependent Pyrophosphate Formation by Sarcoplasmic Vesicles

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Pyrophosphate Synthesis, Sarcoplasmic Reticulum, Calcium Accumulation

The vesicles of the sarcoplasmic membranes synthesize pyrophosphate from inorganic phosphate. Pyrophosphate synthesis proceeds as long as a calcium gradient is maintained across the vesicular membranes. Pyrophosphate synthesis is inhibited by low concentrations of nucleoside triphosphates.

Introduction

Vesicular fragments of the sarcoplasmic reticulum (SR) accumulate large quantities of calcium from calcium containing media, whereby high concentration gradients are created. The energy which is required to concentrate the calcium ions can be furnished by a great variety of phosphate compounds such as ATP, its natural analogues, NTPs, acetyl phosphate or even *p*-nitrophenyl phosphate. The formation of a phosphorylated intermediate in the transport protein is a common feature of the interconversion of chemical bond energy to osmotic energy. The essential prerequisite for the transfer of the phosphoryl residue of the various energy yielding substrates to the protein is the presence of

Vesicular fragments of the sarcoplasmic reticulum calcium the phosphoryl residue in the protein originating from the energy yielding substrates disappears. In the medium free of calcium, however, protein starts to incorporate inorganic phosphate. Phosphorylation of the calcium transport protein by inorganic phosphate has been recognized as a reaction step in the sequence which leads to continuous synthesis of ATP when the calcium stored inside the vesicles is released in the presence of inorganic phosphate and ADP [1].

The original concept that the existing calcium gradient might provide the energy for the formation of a phosphorylated intermediate whose phosphoryl group can be transferred to ADP was questioned by findings of Kanazawa and Boyer [2], and Masuda and de Meis [3]. They found that the SR membranes could accept inorganic phosphate at low calcium levels on both sides of the membranes, *i. e.* in the absence of a calcium gradient. In analogy to other systems it has been suggested that the formation of ATP is driven mainly by the high affinity of ATP to the enzyme [4, 5]. The osmotic energy present in the gradient and set free during calcium release was assumed to serve for a transient reduction of the affinity of the membranes for ATP causing its release. This kind of mechanism may apply to systems where no intermediary phosphoenzyme appears and which are characterized by a high specifity for ATP [5]. Two findings argue against the application of this concept to energy interconversion in SR membranes. Firstly, the phosphorylated intermediate which is formed in the SR membranes in the presence of a gradient is distinctly different from that formed in the absence of a gradient [8] and secondly, IDP and GDP are phosphorylated in the presence of a gradient although ITP and GTP have a considerably lower affinity to the enzyme than ATP. The results that will be described in the following report show that the membranes are even able to form pyrophosphate by phosphorylation of inorganic phosphate when a calcium gradient is present. Pyrophosphate has a low affinity for the protein and is unable to provide energy for the accumulation of calcium by the SR calcium pump.

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Abbreviation: EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid; NTP, nucleoside triphosphate; PEP, phosphoenolpyruvate.

Methods

The sarcoplasmic vesicles (SR vesicles) were prepared according to the modified procedure of Hasselbach and Makinose [6] from rabbit skeletal muscle.

The SR vesicles were loaded with calcium phosphate in solutions containing 10 mM $MgCl_2$, 10 mM Na phosphate-^{32}P, pH 7.0, 0.5 – 3 mM acetyl phosphate, 0.5 mM $CaCl_2$, and 1 – 4 mg SR protein/ml at 30 °C. Aliquots were taken at appropriate time

intervals and deproteinized by addition of the equal volume of 5% TCA. The radioactive phosphate was removed as its phosphomolybdate complex by extraction with isobutanol benzene according to Avron [7]. The radioactivity remaining in the aqueous extract was determined by liquid scintillation counting. For identification of the radioactive material present in the aqueous solution the loading assay was deproteinized by 5% $HClO_4$. After neutralization with KOH and removal of $KClO_4$ the solution was 5 – 10 fold diluted with water and absorbed on a Dowex 1×8 Cl^- column 0.5×15 cm. Inorganic phosphate was eluted with 0.01 M HCl + 0.05 M KCl. The remaining activity was obtained by elution with 0.05 M HCl + 0.05 M KCl. After concentration by evaporation the material was spotted on PEI – poly(ethylene imine) – impregnated cellulose sheets. The chromatogram was washed with anhydrous methanol, dried, developed for 12 cm with water and dried again. Separation was achieved by 2 M Na formiate + 0.8 M LiCl. The spots were localized by spraying with a solution containing 2 g ammonium molybdate, 14 ccm 60% $HClO_4$, 20 ccm 2 N HCl + 180 ccm water and illuminated for 2 hours under ultraviolet light. Subsequently, an audioradiogram of the chromatogram was made. Pyrophosphate, ATP and ADP containing solutions were used as standards. The nucleotides were localized in UV light.

To decompose the pyrophosphate formed in the assay 6 units of inorganic pyrophosphatase were added either to the reaction medium or to the deproteinized and neutralized assay medium.

Results and Discussion

Calcium efflux driven ATP synthesis is usually started by the simultaneous addition of ADP and EGTA at final concentrations of 2 mM and 5 mM, respectively, to SR vesicles loaded with calcium phosphate. The synthesized ATP is determined as the radioactivity remaining in the aqueous phase after the radioactive inorganic phosphate has been removed as phosphomolybdate by extraction with isobutanol-benzene [7]. The dependence of the formation of ATP and ITP during calcium efflux on the nucleoside diphosphate concentration is shown by Fig. 1 to illustrate the great difference in the affinities, the protein for ADP and IDP exhibit.

In experiments designed to measure the initial rate of ATP synthesis we observed that already before ATP synthesis was started by ADP addition considerable quantities of radioactive material were present in the aqueous solution after inorganic phosphate had been removed by the Avron procedure. Less than 5% of this radioactivity was found in control assays containing all reagents except SR vesicles. The radioactive material remaining in the aqueous phase was characterized as pyrophosphate by the following experiments.

1.) The active material disappeared with a half time of 3 – 5 min when the deproteinized and neutralized assay was heated to 100 °C after reacidification by addition of an equal volume of 2 M HCl.

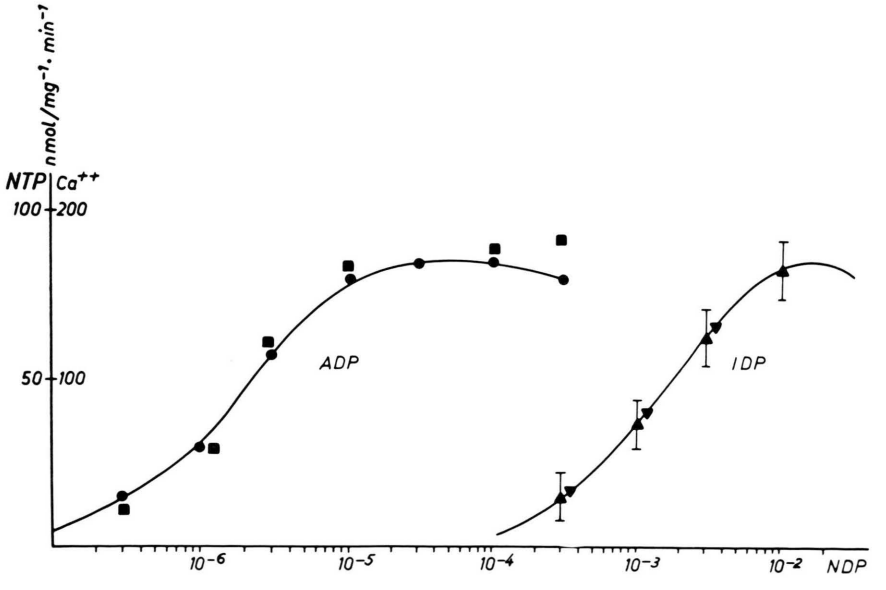

Fig. 1. Dependence on nucleoside diphosphate concentration of calcium efflux, ATP and ITP synthesis. The SR vesicles were loaded with calcium phosphate as described in Methods. The temperature of the assay medium was reduced to 20 °C and a solution containing EGTA, ADP or IDP, 0.05 mg/ml hexokinase and 0.1 M glucose were added to initiate calcium efflux and nucleoside diphosphate phosphorylation. The final concentrations of the nucleoside diphosphates are given on the abscissa. The release of calcium from the vesicles was measured through filters with a pore size of 0.45 μm.
●, ▲ Calcium release;
■, ▲ NTP synthesis.

2.) No light absorbing material could be detected in the deproteinized and neutralized assay which largely excludes the presence of nucleotide bound phosphate.

3.) A very small molecular weight of the radioactive material was indicated by the fact that the radioactive material could not be separated from inorganic phosphate on a Sephadex G 10 column 2×100 cm.

4.) When pyrophosphatase was added to the reaction medium or to the deproteinized and neutralized assay, no radioactivity was found in the aqueous phase after the Avron procedure had been applied.

5.) The radioactive material in the deproteinized assay was absorbed on Dowex 1×8 and inorganic phosphate was separated as decribed under Methods. The radioactivity eluted with 0.05 M HCL and 0.05 M KCL migrates together with pyrophosphate on PEI cellulose thinlayer chromatograms (Fig. 2).

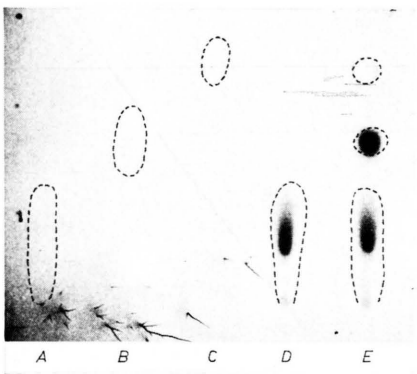

Fig. 2. Separation of pyrophosphate, ATP and ADP on PEI cellulose visualized by audioradiography and molybdate staining. Pyrophosphate formation occurred at 30 °C in the described assay medium. After 30 min the temperature of the assay was reduced to 20 °C and ADP+EGTA were added at final concentrations of 1 mM and 5 mM, respectively. Thinlayer chromatography was performed after the removal of inorganic phosphate by ion exchange chromatography. – – – Molybdate staining; dark spots audioradiographic tracing; A, B and C nonradioactive pyrophosphate; ATP and ADP as marker substances; D pyrophosphate formed in the incubation medium; E pyrophosphate formed in the incubation medium+ATP formed during calcium release after the addition of ADP.

The results in Table I reveal that pyrophosphate is formed only when a calcium gradient is created during AcP supported calcium transport. When the formation of a gradient is prevented either by the application of the calcium ionophore X 537 A or by sonic disruption of the SR vesicles or by omitting calcium from the assay, only very small quantities of pyrophosphate are formed.

Table I. Pyrophosphate formation during calcium accumulation.

Preparations	Conditions	Pyrophosphate formation [nmol/mg protein·30 min]
native vesicles	complete assay	5.6
sonicated vesicles	complete assay	0
native vesicles	no acetyl phosphate	0.15 – 0.5
native vesicles	calcium omitted	0.3 – 0.65
native vesicles	complete assay +0.1 mM X537A	0.55 – 1.1
native vesicles	complete assay +pyrophosphatase	0.15

Complete assay: 10 mM Mg^{2+}, 3 mM acetyl phosphate; 10 mM $^{32}P_i$, 0.5 mM Ca^{2+}; 30 °C.

Fig. 3 shows that pyrophosphate production increases linearly with time approaching after 10 – 20 min an optimal level. In some experiments the pyrophosphate level starts to decline during the experiment. This decline must be attributed to the exhaustion of acetyl phosphate in the medium resulting in a decline of the calcium gradient and, furthermore, due to the presence of a weak pyrophosphatase activity in the preparations. Under the

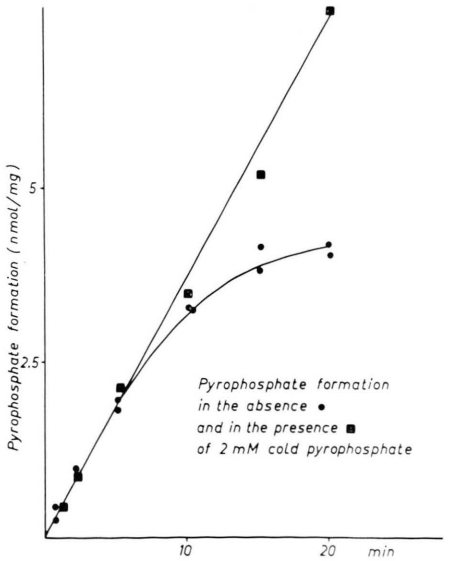

Fig. 3. Time course of pyrophosphate formation. ■ 2 mM nonradioactive pyrophosphate were added to the complete assay; ● no addition of pyrophosphate. The reaction was performed in the described medium at 30 °C.

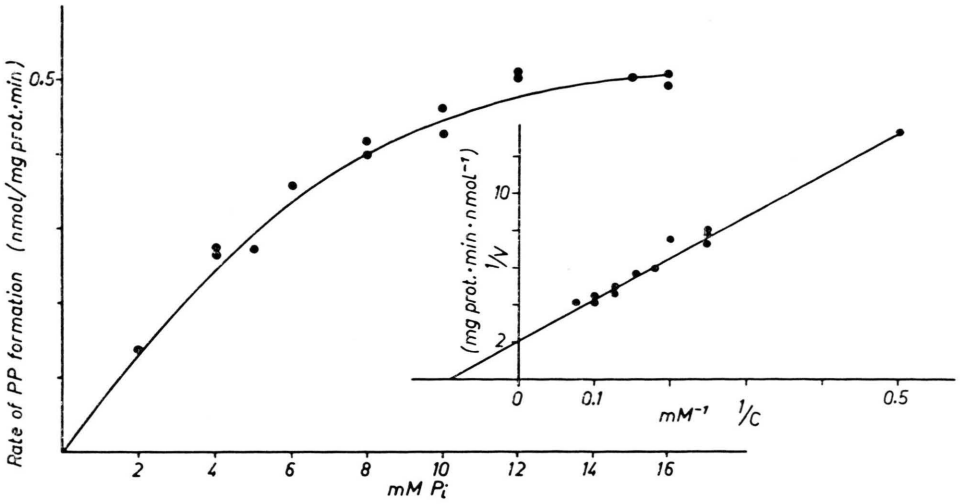

Fig. 4. Dependence on phosphate concentration of the rate of pyrophosphate formation. The reaction was performed at 30 °C in the described medium containing phosphate at concentrations as given in the abscissa.

prevailing conditions 0.5 – 3 μm acetyl phosphate are used up by 1 – 4 mg protein/ml present in the assay during 5 – 20 min, whereafter pyrophosphate production is limited by the slowly declining store of accumulated calcium phosphate of 100 – 500 nmol/mg protein. The presence of a pyrophosphate hydrolyzing activity inferes from the observation that after the addition of unlabelled pyrophosphate production proceeds nearly linearly for 20 min. Due to the added cold pyrophosphate the enzymatic hydrolysis of the newly formed radioactive pyrophosphate is largely repressed.

As to the mechanism of how two molecules of inorganic phosphate are linked by the SR membranes, the dependence of the reaction of phosphate has some bearing. As show by Fig. 4 the rate of pyrophosphate formation increases in the millimolar range of phosphate concentration approximating a maximum rate of 0.5 nmol/ml protein·min. Evidently, the affinity of the enzyme for phosphate is quite low. The value of 80 M^{-1} obtained from the Lineweaver Burk plot (Inset Fig. 4) in the order of the affinity which the enzyme exhibits when it accepts phosphate in the absence of a gradient. Since however, under these conditions a calcium gradient exists which increases the affinity of the enzyme for phosphate to approximately 2000 – 3000 M^{-1}, we must assume that two phosphate binding sites with quite different affinities are involved in pyrophosphate formation.

A further observation relevant to the mechanism of pyrophosphate formation is the dependence of the reaction on the substrate used for the formation of the calcium gradient. Table II shows that acetyl phosphate is the most suitable substrate followed by *p*-nitrophenyl phosphate and low concentrations of ATP. When 3 mM ATP or ITP were used no pyrophosphate production could be detected. The fact that pyrophosphate synthesis only proceeds optimally if the reaction is supported by the weak substrates of the enzyme suggests that those substrates and their splitting products having a high affinity for the enzyme prevent phosphate from being bound and linked together. Presumably, this competition takes place at the site to which phosphate has a low affinity. This observation supports the notion that pyrophosphate formation occurs in two steps. At first, a gradient dependent phosphoprotein is form-

Table II. Pyrophosphate formation supported by different energy yielding substrates. 30 °C.

Substrate	Concentrations [mM]	Pyrophosphate [nmol/mg protein·30 min]
acetyl phosphate	3	6.1 ± 0.5
pNPP	3	0.8
ATP	3	0
ATP	0.1	
+PEP	3	0.3 + 4 nmol ATP
ITP	3	~0.1

ed, subsequently its phosphoryl residue reacts with the phosphate group bound to the low affinity site. It may be suggested that the site to which phosphate is bound in the gradient dependent phosphoprotein is most likely identical with the site which accepts the phosphoryl group of ATP or other energy yielding substrates in the presence of calcium ions. The second site from which inorganic phosphate is prevented from being bound by di- and triphosphates of adenosine or inosine is the site to which the nucleotides have a low affinity. Such a site has been postulated to explain the activation of the calcium dependent ATPase occuring at concentrations of ATP between 0.1 and 5 mM [10, 11]. Recently, Dupont [12] furnished some additional indirect evidence for the existence of such a site. The presented experiments do not allow to decide if the two phosphate binding sites belong to the same enzyme molecule or if in a dimeric structure each molecule has only one binding site for phosphate but either a high or low affinity site. If we assume that the membrane preparations contain no or only little inactive material, the maximal value of phosphorylation of 4 nmol/mg [4, 9] favours the assumption that a dimeric protein unit might be involved in pyrophosphate formation, since the concentration of monomeric units of a molecular weight of 120 000 [1, 13] would be 8 nmol/mg.

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