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MECHANISM OF THE SARCOPLASMIC RETICULUM CALCIUM PUMP. FLUOROMETRIC STUDY OF THE PHOSPHORYLATED INTERMEDIATES

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ABSTRACT

After removal of calcium ions bound to the high affinity sites the sarcoplasmic reticulum calcium pump can be phosphorylated by inorganic phosphate. The intrinsic fluorescence of the protein is used to follow conformational changes of the pump and an intensity change can be observed upon addition of phosphate. This effect is activated by internal calcium ($K^{1}/_{2}$ = 10 mM) and inhibited by external calcium ($K^{1}/_{2}$ = 0.4 µM) and the apparent affinity for phosphate is high (0.2 mM). We conclude that the change observed is linked to the formation of the gradient-dependent phosphorylated intermediate. It is compared with previous results concerning the enzymatic cycle of the pump.

INTRODUCTION

In the sarcoplasmic reticulum vesicles the calcium-dependent ATPase is known to perform an active transport of calcium ions into the internal medium. Under appropriate conditions a reversal of the mechanism can be obtained where the protein catalyses the synthesis of ATP from the osmotic energy of an artificialy made calcium gradient [1,2,3].

One major step of the backward reaction is the formation of a phosphorylated intermediate from inorganic phosphate. Incorporation of phosphate into sarcoplasmic reticulum vesicles occurs independently of the calcium gradient providing the external medium has been emptied of ionised calcium by EGTA [3,4,5]. Distinct species which differ mainly in apparent affinity for Pi appear to be formed in either case. It has been demonstrated that the transfer of phosphate to ADP is only possible in the presence of a calcium gradient [3].

In this report we show that the formation of the gradient-dependent phospho-enzyme is accompanied by a change in the intrinsic fluorescence strength, while no effect can be obtained upon addition of high concentrations of phosphate on unloaded vesicles.

From this we conclude that a conformational change occurs on formation of the gradient-dependent phosphorylated intermediate. This change

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can be related to those observed upon calcium binding or during the turnover cycle and good agreement is obtained with enzymatic schemes proposed elsewhere.

MATERIALS AND METHODS

The preparation is from rabbit white skeletal muscles and has been described by Hasselbach and Makinose [6]. Additional steps have been included and details of preparation and sample conservation has been described elsewhere [7].

The intrinsic tryptophan fluorescence measurements are made with an excitation wavelengh of 296 nm and measured at 330 nm as described previously [8]. The conditions are the following : the vesicles (20 to 30 mg/ml) are diluted to a final concentration of 100 to 200 µg/ml in 2 ml of 20 mM TRIS-maleate pH 7.2, 100 mM KCl in a continuously stirred cuvette. Additional reagents (Mg^{++}, Pi, Ca^{++} or EGTA) are added as described in the figure legends.

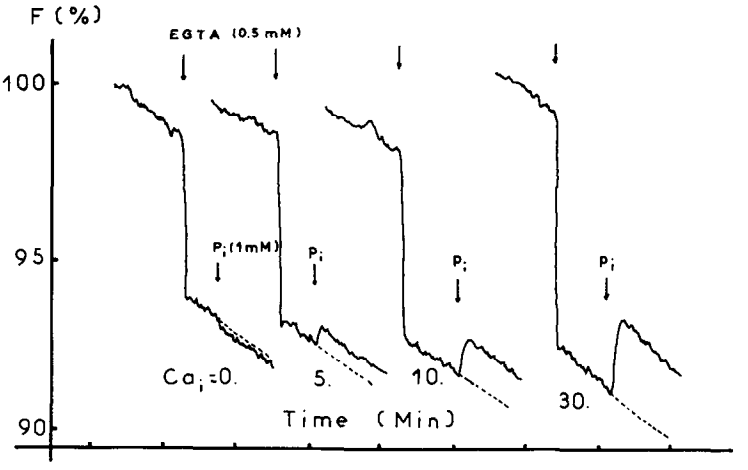
The calcium loaded vesicles are obtained by overnight dialysis in 20 mM TRIS-maleate, 100 mM KCl, 5 mM Mg^{++} and the desired calcium concentration. The amount of calcium stored inside the vesicles has been measured independently in two ways : either by using ^{45}Ca and millipore filtration or by measurement of the proton burst produced by adding, in the presence of EGTA, 20 µM of the calcium ionophore X-537 A. The value obtained varies slightly from one preparation to another since it is dependent on the mean size of the vesicles. The amount of calcium stored for 10 mM Ca^{++} is around 80 nmoles/mg of protein.

The external free calcium concentration is adjusted by a Ca-EGTA buffer taking into account the contaminating calcium added with the vesicles. The free calcium concentration is calculated or measured as described in the reference [8].

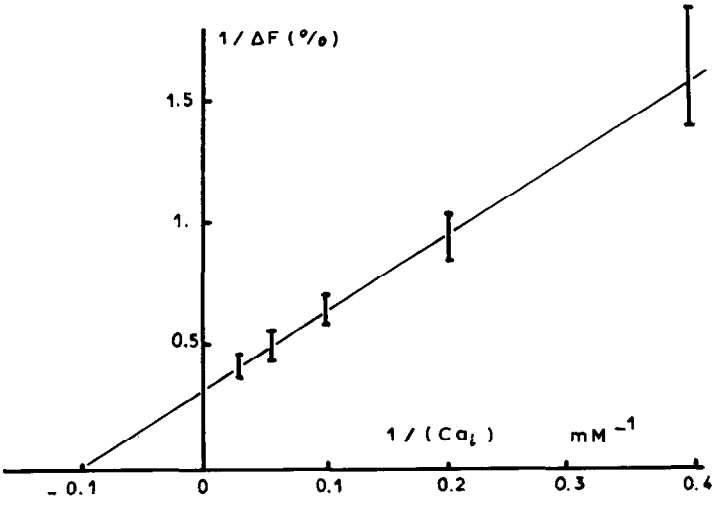
RESULTS

- EFFECT OF Pi ON CALCIUM LOADED VESICLES : INTERNAL CALCIUM CONCENTRATION DEPENDENCE.

Figure 1 shows a record of the fluorescence signal at 330 nm. The concentration of calcium in the medium before addition of EGTA ranges from 5 to 150 µM. After EGTA injection the external free calcium (Ca_e) is reduced to less than 10^{-8} M. There is therefore a complete removal of calcium from the high affinity sites of the pump ($K^1/_2$ = 0.4 µM) and this is followed by a drop of the fluorescence intensity which has been analysed elsewhere [8,9]. After addition of Pi one observes clearly a fluorescence strengh increase and the magnitude of the change is dependent on the amount of calcium stored inside the vesicles (Ca_i). A plot of the inverse of the fluorescence change versus $1/(Ca_i)$ is linear and gives an apparent dissociation constant $K^1/_2$ = 10 mM for the internal calcium. The maximum fluorescence change obtained by this way is ΔF_{max} = 3.3 % (Fig. 2).



1) - Fluorescence record and effect of inorganic phosphate on vesicles incubated in various concentrations of calcium (0 to 30 mM). Mg^{++} concentration in the medium is 5 mM. The effect observed for Ca_i = 0. is due to dilution and is taken into account in the other experiments.



2) - Reciprocal plot of the fluorescence change versus internal calcium concentration.

- EXTERNAL CALCIUM CONCENTRATION DEPENDENCE

It has been proved by several authors that the formation of the phosphorylated intermediates is inhibited by external calcium with a $K^1/_2$ value equal to the dissociation constant of the high affinity sites [2,3,4]. The same effect is observed for the Pi dependent fluorescence change. This is demonstrated in figure 3 where Pi is added to vesicles loaded with 30 mM calcium in the presence of various external calcium con-

centrations. The inhibition constant is $K^{i}_{1/2}$ = 0.5 μM.

- PHOSPHATE CONCENTRATION DEPENDENCE AND EFFECT OF Mg^{++}

The Pi dependent fluorescence change occurs only in the presence of Mg^{++}. As shown in figure 4, Pi and Mg^{++} can be added randomly without any effect on the final state. The apparent affinity for phosphate is around 0.2 mM at 22° and 30 mM internal calcium (Fig. 5). Adding Pi in even high concentrations on unloaded vesicles produces no observable effect (Fig. 4).

DISCUSSION

In this report as in earlier work [8,9] the intrinsic tryptophan fluorescence is used as a tool for the identification of conformational transitions in the calcium pump protein. This is compared with results obtained by other methods on the enzymatic cycle. The largest fluorescence change observed (≃ 4 %) is induced by the binding or release of calcium ions from the high affinity sites. The transition between these two states $E \rightleftharpoons E^{*}$ has been followed by fast techniques [9] and appears to be directly linked to the activation of the calcium dependent ATPase. Single turn-over experiments at low temperatures give the impression that after splitting of ATP the enzyme in the E^{*} state undergoes a transition through the low fluorescence state E before returning to the initial conformation E^{*} [9]. This has been described as follows :

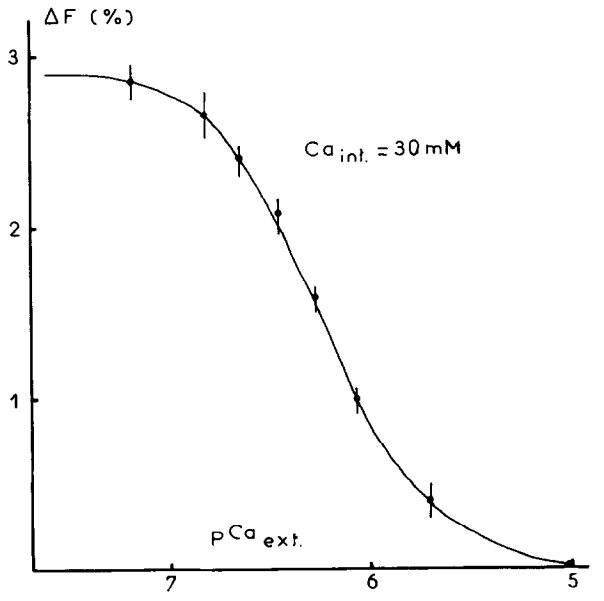
$$Ca_e.E^{*} + ATP \rightleftharpoons Ca_e.E^{***} \sim P + ADP$$

$$Ca_e.E^{***} \sim P \xrightarrow{(a)} E + Pi + Ca_i$$

$$Ca_e + E \xrightarrow{(b)} Ca_e.E^{*}$$

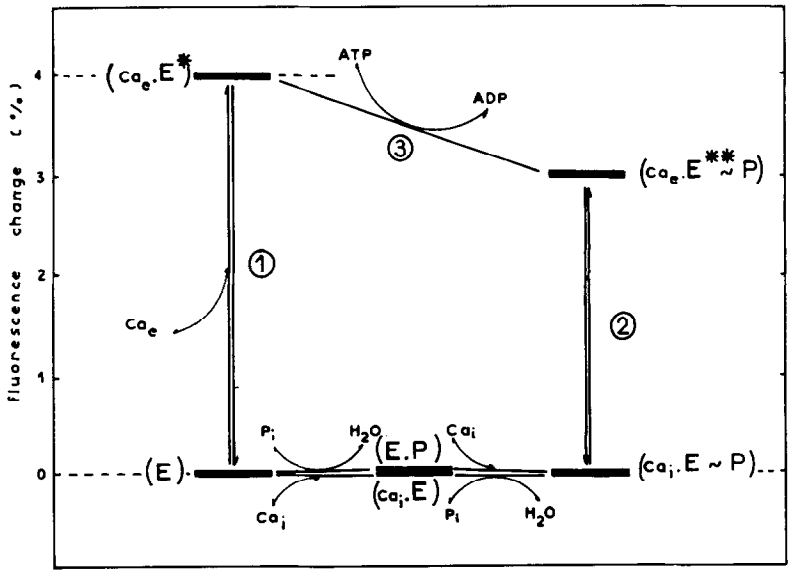
The transition observed here on addition of inorganic phosphate to calcium loaded vesicles is attributed to the reversal of the step (a) giving a stable $E^{***} \sim P$ state.

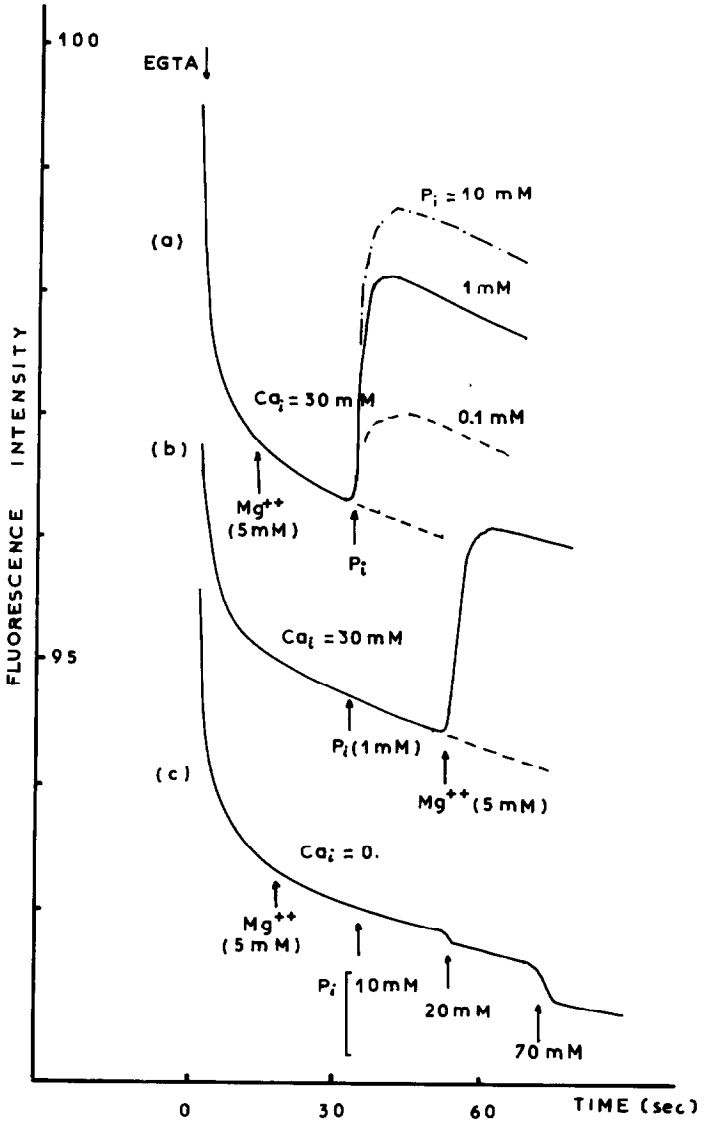
The experimental results indicate that the specificity of the fluorescence change is exactly what is expected for the formation of the gradient dependent phosphorylated intermediate [3] : activation by Ca_i ($K^{1}_{1/2}$ = 10 mM), inhibition by Ca_e ($K^{1}_{1/2}$ = 0.5 μM) and high affinity for phosphate at neutral pH : ($K^{1}_{1/2}$ = 0.2 mM). It appears however that the scheme described above is certainly an oversimplification of the real process and two to three different phospho-enzyme species has been proposed by authors using chemical quench-flow, fluorescent calcium indicators or Pi ⇌ HOH exchange [10-13].



3) - Inhibition of the Pi induced fluorescence change by external calcium. The different calcium concentrations are obtained in varying the Ca-EGTA ratio.

If we try to reconcile our data with these results we arrive at the following relation between the fluorescence level and the enzymatic cycle :

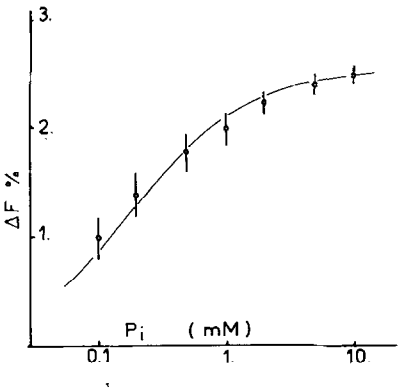




4) - Effect of the various substrates on the fluorescence change. The traces (a) and (b) demonstrate that Pi and Mg^{++} are necessary and that the sequence of injection is unimportant. In (c) the vesicles have been incubated in a control medium without calcium. Pi is added up to a concentration of 70 mM without effect (except dilution). Mg^{++} concentrations up to 50 mM have also been tested with no more success. The trace have been arbitrarily shifted on the vertical axis for clarity.

- Transition (1) is the calcium dependent conformational change described earlier [8,9].

- (2) is the change produced by the addition of Pi on calcium loaded vesicles. It corresponds to the fluorescence drop observed at the



5) - Phosphate concentration dependence of the fluorescence change. The curve drawn through the points is a fit calculated from $\Delta F = (2.6\ \%)\ \frac{(Pi)}{0.2 + (Pi)}$, giving a reasonable agreement with a single phosphate binding site.

beginning of the single turn-over experiment, when the pump is working in the forward direction [9].

- The small difference (3) has been introduced in the reference [9] in view of turn-over experiments at high temperature when transition (2) is rate limiting. It is also observed at any temperature in the presence of high internal calcium.

The Pi $\rightleftharpoons$ HOH exchange occurs in the absence of calcium by the reaction E + Pi $\rightleftharpoons$ HOH + E.P [10], and the E.P state is probably the gradient independent phospho-enzyme described by Beil et al [3]. Ca_i.E $\sim$ P is a common intermediate which can be reached by two different routes since it is assumed that Ca_i and Pi can bind independently to the enzyme [13].

If we follow the results of Ikemoto [12] the states E.P and E $\sim$ P are of low affinity for calcium while E$^{::::}$ $\sim$ P is the high affinity form of the phospho-enzyme. It is likely that the low and high affinity species represent respectively the internal and external form of the calcium binding sites and the asymmetric effect of calcium ions on either side of the membrane reflects this difference of affinity. Then the main transitions (1) and (2) observed in fluorescence belong to the same kind of transition between two main states of the enzyme. The external form corresponds to the high fluorescence level while the internal and low affinity species has a lower fluorescence.

An interesting point concerns the accessibility of the calcium site from the external space when the enzyme is in the state E$^{::::}$ $\sim$ P. If calcium ions exchange freely one should observe a transient release of calcium in the external medium after addition of Pi to calcium loaded ve-

sicles. But the possibility remains that the ions are occluded as long as the protein is in the $E^{::} \sim P$ state and that calcium ions are not really exchangeable with the outer medium before addition of ADP. This point is open and it is directly related to the Ca-Ca exchange process observed by Makinose [14]. It is likely that this Ca-Ca exchange occurs partly via the transition : $Ca_i.E \sim P \rightleftharpoons Ca_e.E^{::} \sim P$.

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