DYNAMIC INTERCONVERSIONS OF PHOSPHORYLATED AND NON-PHOSPHORYLATED INTERMEDIATES OF THE Ca-ATPase FROM SARCOPLASMIC RETICULUM FOLLOWED IN A FLUORESCEIN-LABELED ENZYME

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1. Introduction

Binding of calcium to the Ca^{2+}-ATPase from sarcoplasmic reticulum induces in the enzyme structural changes which are reflected by enhancement of the intrinsic fluorescence [1], by changes in the availability of SH and carboxyl groups [2,3] and by stabilization against acid inactivation [4]. Kinetic measurements indicated the existence of more than one phosphorylated intermediate of the Ca^{2+}-ATPase and a scheme summarizing the cyclic intermediate interconversions on the basis of all the available information was proposed [5].

We have reported that FITC selectively labels the Ca^{2+}-ATPase from sarcoplasmic reticulum and inhibits Ca^{2+}-uptake coupled to ATP hydrolysis and that the inhibition was prevented by including ATP during the incubation with the inhibitor [6]. The fluorescein-labeled enzyme responded to addition of Ca^{2+} by a rapid quenching of the fluorescence which probably reflects a conformational change. The observation that acetyl phosphate stimulates Ca^{2+}-uptake in a fluorescein-labeled enzyme [7] made it possible to follow conformational interconversions between different phosphorylated intermediates which is reported here and discussed in reference to the scheme in [5].

2. Materials and methods

Fragmented sarcoplasmic reticulum vesicles were prepared as in [8]. Labeled with FITC was done by incubating SRV (1.8–2.1 mg/ml) with 20 μM FITC at room temperature in a medium containing: 0.2 M sucrose, 50 mM Tris–HCl, 50 mM Na-glycine, (pH 9.0) and 100 μM EGTA. The reaction was terminated by removing the unbound FITC on Sephadex G-50 columns as in [6]. Ether treatment of SRV was done essentially as in [9]: membranes (2 mg/ml) were mixed for 15 s in 5 mM Tris–maleate, (pH 7.0) with 20% diethyl ether at room temperature. The suspension was transferred to a Sephadex G-50 column pre-equilibrated with 0.2 M sucrose, 50 mM Tris–HCl (pH 7.0) to remove the ether. This SRV preparation had no Ca^{2+}-uptake activity but retained its Ca^{2+}-ATPase activity. DCCD treatment was done by incubation of SRV with 40 μM DCCD for 1 h as in [3]. Loading of FITC-labeled SRV vesicles with Ca^{2+} was performed by incubating membranes (400 μg/ml) in a medium containing: 20 mM Tris–maleate (pH 7.0) 100 mM KCl, 10 mM $MgCl_2$, 10 mM acetylphosphate, 25 mM K-phosphate and 100 μM Ca for 5 min at 25°C. The membranes were then separated on Sephadex G-50 column pre-equilibrated with 50 mM Tris–HCl (pH 7.0) and 0.2 M sucrose as in [6]. Fluorescence changes were followed in a Perkin Elmer MPF 44 Spectrophotometer as in [6]. The excitation and emission wavelengths were 495 and 525 nm, respectively.

Abbreviations: SRV, sarcoplasmic reticulum vesicles; FITC, fluorescein isothiocyanate; AcP_i, acetylphosphate; DCCD, dicyclohexylcarbodiimide; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid; ^{o}Ca, external Ca^{2+}; ^{i}Ca, internal Ca^{2+}

3. Results

We have shown that conformational changes in response to Ca^{2+} binding can be followed in a Ca^{2+}-ATPase preparation labeled with FITC [6]. Fig.1A demonstrates the rapid fluorescence quenching observ-

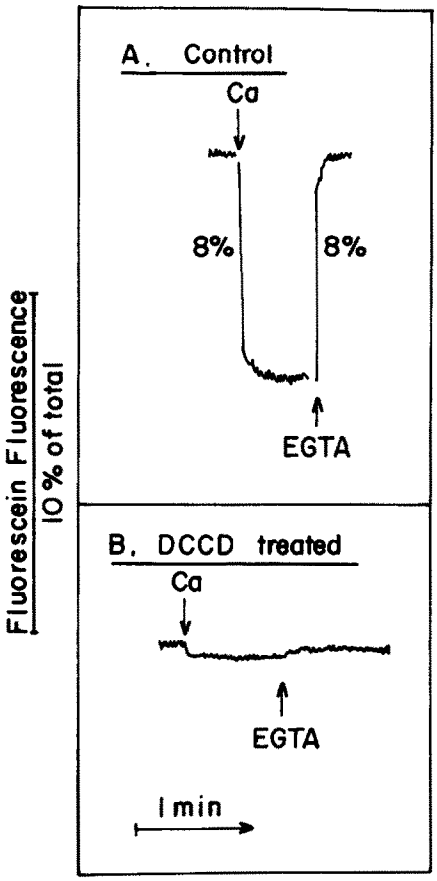


Fig.1. Conformational transition in the unphosphorylated Ca^{2+}-ATPase induced by Ca^{2+}. FITC labeled SRV membranes containing 60 μg protein were suspended in 2.5 ml containing 100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–maleate (pH 7.0) and 100 μM EGTA. $CaCl_2$ (200 μM), and EGTA (600 μM) were added when indicated. The labeling with FITC and the DCCD treatment were done as in section 2.

ed in response to addition of Ca^{2+} which is reversed by removing Ca^{2+} by the complexing agent EGTA. The Ca^{2+} level required for half-maximal change in fluorescence was 0.4 μM (not shown) which is similar to the K_m of the high affinity Ca^{2+}-binding sites of the enzyme. Pretreatment of the SRV with DCCD which inhibits Ca^{2+}-uptake, Ca^{2+}-ATPase and Ca^{2+}-binding to the enzyme as shown in [3] abolished also the fluorescence changes in response to Ca^{2+} (fig.1B).

We have observed that the modification by FITC inhibits specifically the interaction with ATP but does not inhibit the phosphorylation of the enzyme by P_i and only partly inhibits Ca^{2+} uptake with acetyl phosphate as a substrate [7]. Fig.2,3 demonstrate that phosphorylation of the Ca^{2+}-ATPase by acetyl phosphate and by P_i can be followed by quenching of the fluorescence of the fluorescein-labeled enzyme.

Fig.2A shows that in the presence of Ca^{2+} and Mg^{2+}, addition of acetyl phosphate leads to quenching of the fluorescence of ~10–12% lower than the fluorescence level obtained in the presence of Ca^{2+}. The slow fluorescence quenching may reflect the slow rate of phosphorylation by acetyl phosphate [7,18]. Acetyl phosphate has no effect on the fluorescence in the absence of Ca^{2+} (EGTA medium) which is required for the phosphorylation reaction as shown in fig.2H. Fig.2A also shows that elimination of the external Ca^{2+} by addition of EGTA after acetyl phosphate leads to a large transient quenching of the fluorescence (42% of total fluorescence) which slowly decays within several minutes. Addition of the Ca^{2+} ionophore A-23187 results in an immediate enhancement of the fluorescence indicating that the fluorescence quenched state is associated with a Ca^{2+} concentration gradient across the SRV membrane. Fig.2E shows that addition of the Ca^{2+} ionophore before EGTA leads to a fluorescence enhancement and abolishes completely the formation of the fluorescence quenching transient upon addition of EGTA. The fluorescence quenching transient was not created in SRV treated with diethylether which creates leaky vesicles that cannot accumulate Ca^{2+} [9] as shown in fig.2F. Fig.2C shows that oxalate which prevents a large internal increase of the free Ca^{2+} concentration by precipitation of Ca^{2+} oxalate inside the vesicles significantly decreases the fluorescence quenching transient. The reduced fluorescence quenching may be correlated with the smaller Ca^{2+}-concentration gradient across the membrane in the presence of oxalate.

Fig.2B demonstrates that 10 mM P_i significantly increased the extent of the fluorescence quenching and slowed down the rate of decay ($t_{1/2}$ increased from 134 s to 281 s). In Ca^{2+}-loaded vesicles in the absence of external Ca^{2+}, phosphate readily phosphorylates the enzyme in the presence of Mg^{2+} [10] and in effect these are the conditions used for reversal of the Ca^{2+} pump [11]. The effect of phosphate may be due, therefore, to phosphorylation of the enzyme and it indicates that both phosphorylation and a Ca^{2+} concentration gradient are necessary to create the fluorescence quenching transient. Even without addition of phosphate (2A) low concentrations of phosphate produced by acetyl phosphate hydrolysis are probably sufficient to phosphorylate the enzyme since

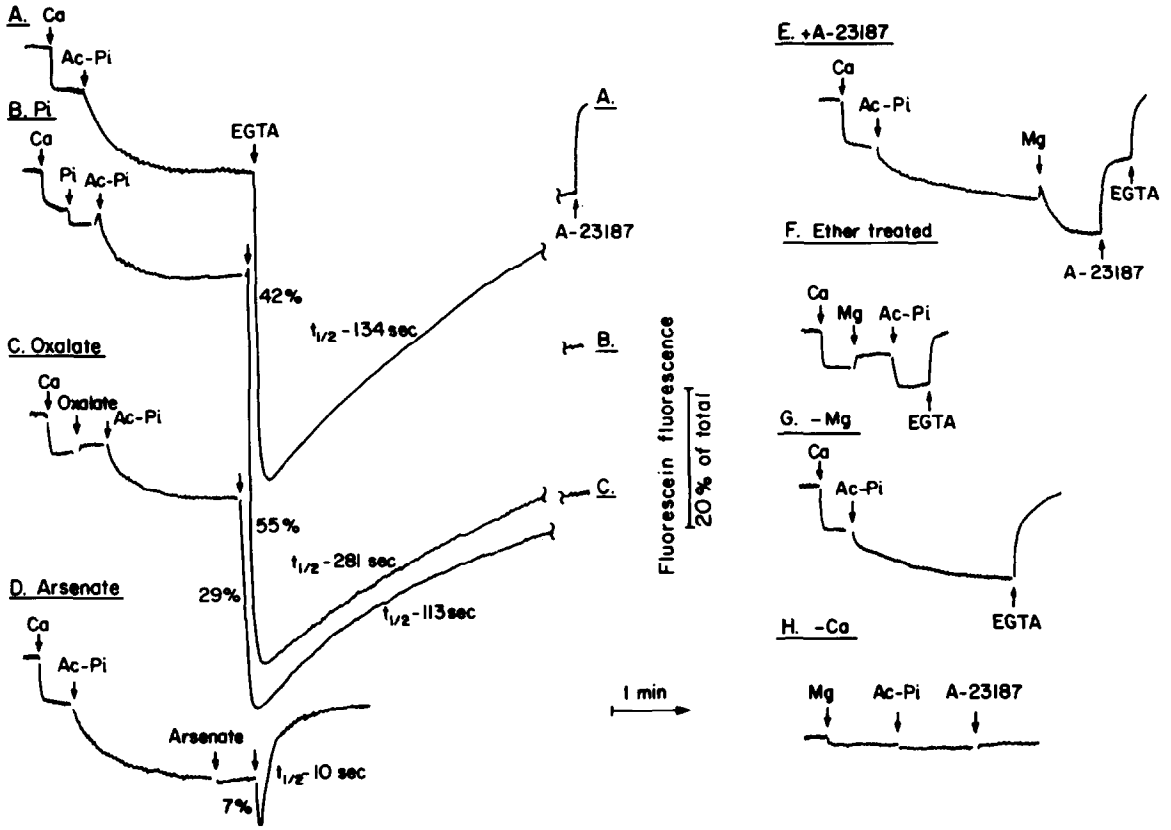
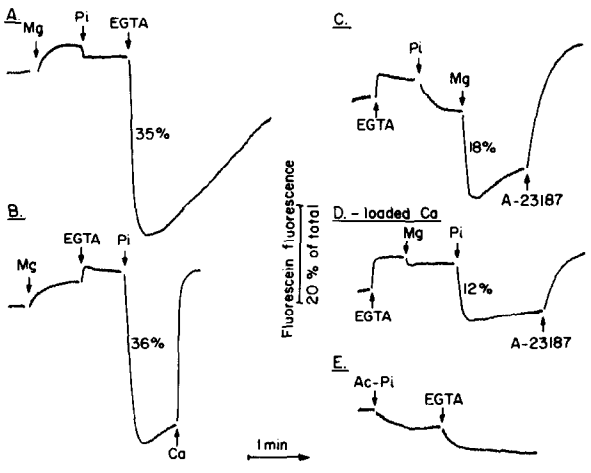


Fig.2. Conformational transitions induced by phosphorylation and a Ca^{2+} concentration gradient. FITC-labeled SRV membrane were incubated in 2.5 ml as in fig.1, but in E–G, $MgCl_2$ was eliminated. $CaCl_2$ (200 μM) EGTA (600 μM), acetyl phosphate (9 mM), Na-phosphate (10 mM), Na-arsenate (10 mM), K-oxalate (5 mM), $MgCl_2$ (5 mM) and the Ca^{2+} ionophores A23187 (2.5 μg) were added when indicated. Ether-treatment SRV were prepared as in section 2.

the affinity for phosphate is increased when a Ca^{2+} concentration gradient is created across the membrane [12].



Arsenate which uncouples ATP formation by reversal of the Ca^{2+} pump [13] very markedly inhibited the fluorescence quenching transient by accelerating the decay of the fluorescence quenched state ($t_{1/2}$ 10 s compared to $t_{1/2}$ 134 s in the control) as shown in fig.2D. Arsenate also induced a fast restoration of the fluorescence when added at any time after creation of the fluorescence quenched state (not shown). The accelerated decay could be due to formation of a labile arsenylated enzyme intermediate which is rapidly hydrolyzed as will be discussed below.

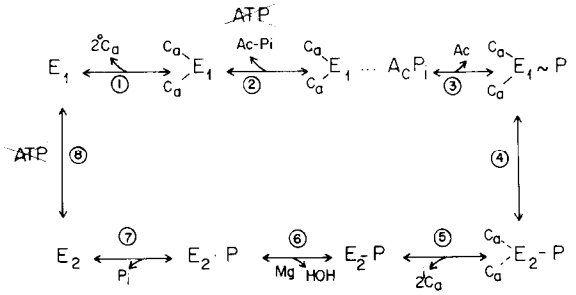
Fig.3. Conformational transition in Ca^{2+}-loaded vesicles. FITC-labeled SRV were loaded with Ca^{2+} in the presence of acetyl phosphate as in section 2. Vesicles (50 μg) were incubated in 2.5 ml containing 100 mM KCl, 50 mM Tris–maleate (pH 7.0) and 50 μM $CaCl_2$. $MgCl_2$ (5 mM), Na-phosphate (10 mM), EGTA (500 μM), $CaCl_2$ (1 mM), acetyl phosphate (9 mM) and A23187 (1 μg) were added where indicated.

In the absence of Mg^{2+} acetyl phosphate leads to a slower and smaller fluorescence quenching (2E, 2G) and the fluorescence quenched transient is not created upon addition of EGTA. This observation also indicates that the fluorescence quenching transient involved formation of a Ca^{2+} concentration gradient since Ca^{2+} accumulation will take place only in the presence of Mg^{2+}. However, phosphorylation of the Ca^{2+}-ATPase takes place also in the absence of Mg^{2+} at a reduced rate [14] and that may be reflected by the slow quenching observed following the addition of acetyl phosphate. Addition of Mg^{2+} after acetyl phosphate (2E) caused a further quenching probably associated with Ca^{2+} translocation and release inside as discussed below. Subsequent addition of the Ca^{2+} ionophore A23187 which can release the internal accumulated Ca^{2+} reverses this quenching.

Fig.3 demonstrates that the fluorescence quenching transient can be induced in Ca^{2+}-loaded vesicles in the absence of acetyl phosphate by P_i in the presence of Mg^{2+}. In this experiment SRV have been preloaded with Ca^{2+} in the presence of acetyl phosphate which was removed by passing the vesicles through a Sephadex G-50 column [15]. Addition of Mg^{2+}, phosphate and EGTA induced a large fluorescence quenching transient (fig.3A–C). The fluorescence quenching transient was abolished by dissipation of the Ca^{2+} gradient by the Ca^{2+} ionophore (3C) and by external Ca^{2+} (2B). A smaller but significant fluorescence quenching was observed in control vesicles which have not been loaded with Ca^{2+} (3D) indicating the presence of internal trapped Ca^{2+} inside the vesicles. Acetyl phosphate failed to induce a similar fluorescence quenching in the absence of Mg^{2+} as shown in fig.3E but in the presence of Mg^{2+} induced a similar fluorescence quenching transient upon EGTA addition as in the control vesicles (not shown). These experiments strongly indicate that the large fluorescence quenching transient reflect a phosphorylated intermediate conformation of the enzyme which is stabilized when a Ca^{2+} concentration gradient is induced across the membrane. The nature of this conformational transition is discussed below.

4. Discussion

We have demonstrated that FITC selectively labels the Ca^{2+}-ATPase from SRV probably by reacting with a single lysine group at the ATP binding site of the enzyme [6,7]. Since FITC specifically inhibits the interaction with adenine nucleotides but does not abolish completely Ca^{2+}-uptake with acetyl phosphate as a substrate (50% inhibition, [7]) the fluorescein-labeled enzyme can be phosphorylated and accumulate Ca^{2+}. This finding and the location of the bound fluorescein at the active site of the enzyme make it a sensitive assay for following directly different phosphorylated intermediates which have been predicted before. A scheme summarising the partial reactions and different conformations of the enzyme during the turnover cycle was described in [5] and modified to describe the fluorescein labeled ATPase as shown in the scheme below:



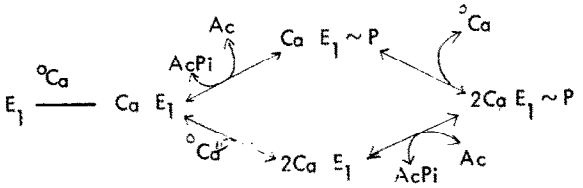
Scheme 1

According to this scheme the unphosphorylated enzyme can exist in either the E_1 conformation which binds Ca^{2+} with a high affinity at the outer side of the membrane and is phosphorylated by ATP or in the E_2 conformation which is the Ca^{2+} releasing form presumably having low affinity Ca^{2+} binding sites facing inwards which can be phosphorylated by P_i.

The scheme distinguishes between at least 3 states of phosphorylated intermediates: 2 Ca–$E_1 \sim P$; 2 Ca–E_2–P; E_2–P. In the FITC modified ATPase the effect of ATP on reactions 2 and 8 is probably blocked. The phosphorylation reaction 2 with acetyl phosphate which is very slow also in the unmodified enzyme probably becomes rate limiting as indicated by the slow rate of quenching after acetyl-phosphate addition (fig.2) and by the time lag in Ca^{2+}-uptake [7,16].

Induction of a Ca^{2+} concentration gradient across the SRV membrane by elimination of the external Ca^{2+} after Ca^{2+} loading followed by phosphorylation by P_i (fig.2,3) should according to this scheme shift the equilibrium 4 towards accumulation of 2 Ca–$E_1 \sim P$ and in an unmodified enzyme in the presence of ADP these conditions would lead to ATP formation. How-

ever, in the FITC modified enzyme in the absence of a phosphoryl acceptor a large accumulation of 2 Ca–$E_1 \sim P$ could be predicted. The highly quenched state may represent therefore accumulation of this intermediate. However, the fast fluorescence changes in response to EGTA (2A,B, 3A) and to Ca^{2+} (3B) which according to scheme 1 include the partial reactions 1–5 are not in line with the slow rate of reaction 2 and it may suggest Ca^{2+} binding to the phosphorylated intermediate. This interpretation may be related to the sigmoidal Ca^{2+} dependence for Ca^{2+} uptake with acetyl phosphate reported in [17]. A possible scheme in line with these results suggesting sequential Ca^{2+} binding before and after phosphorylation of the enzyme is described below:



Scheme 2

According to this scheme Ca–$E_1 \sim P$ should be the intermediate accumulated when a Ca^{2+} gradient exists across the membrane in the absence of a phosphoryl acceptor.

The small fluorescence quenching obtained upon addition of acetyl phosphate in the presence of Ca^{2+} and in the absence of Mg^{2+} (fig.2E,G) is compatible according to this scheme with accumulation of the E_2–P intermediate. Under these conditions no accumulation of Ca^{2+} can take place and the low internal Ca^{2+} concentration would shift reactions 4–5 to E_2–P. Addition of Mg^{2+} would initiate a rapid hydrolysis of E_2–P and Ca^{2+} uptake and this will create a complex equilibrium between several phosphorylated and non-phosphorylated intermediates and it is difficult to estimate their relative concentrations from our results.

4.1. *Mechanism of arsenate inhibition*

It was reported before that arsenate stimulated Ca^{2+} efflux from Ca^{2+}-loaded vesicles in the absence of ADP and inhibited ATP formation by reversal of the Ca^{2+} pump but had no effect on Ca^{2+}-uptake [13]. Fig.2D demonstrated that arsenate largely accelerated the decay of the fluorescence quenched state which was tentatively suggested to be 2 Ca–$E_1 \sim P$ or Ca–$E_1 \sim P$. These two observations suggest that arsenate binds to the enzyme at the phosphate binding site forming an arsenylated intermediate which by reversal of the reactions 7–4 will catalyse Ca^{2+} efflux. This interpretation may suggest that 2 Ca–$E_1 \sim P_i$ is more susceptible to hydrolysis than E_2–P_i and 2 Ca–E_2–P_i.

5. Conclusions

(i) Dynamic interconversions between different conformational states of the phosphorylated and dephosphorylated intermediates can be followed in a fluorescein-labeled Ca^{2+}-ATPase from sarcoplasmic reticulum.

Addition of Ca^{2+} leads to fluorescence quenching which probably reflects Ca^{2+} binding to the external high affinity sites and accumulation of the E_1 state by shifting of reactions 8 and 1 towards 2 Ca–E_1. Phosphorylation of the enzyme under different conditions creates an additional quenching of the fluorescence. At least two different conformations of the phosphorylated enzyme can be distinguished – a large fluorescence quenching state can be created in SRV phosphorylated by either acetyl phosphate or by P_i by induction of a Ca^{2+} concentration gradient across the membrane (internal Ca^{2+} and no external Ca^{2+}) which was suggested to reflect a Ca–$E_1 \sim P$ or a 2 Ca–$E_1 \sim P$ conformation. In the presence of Ca^{2+} and in the absence of Mg^{2+} phosphorylation by acetyl phosphate creates a smaller quenching which may represent accumulation of E_2–P. A rough estimation of the relative fluorescence levels of the intermediates referring to the fluorescence level in a Ca^{2+} free medium as 100% would be:

E_1, 92%; E_2, 100%; E_2–P, $\leqslant$ 84%; Ca–$E_1 \sim P$ (or 2 Ca–$E_1 \sim P$) ~30%.

The effects of increasing the membrane permeability to Ca^{2+} by the ionophore A-23187 and by an ether treatment and the effects of Mg^{2+}, oxalate, phosphate and arsenate have been discussed and are in line with the suggested scheme.

(ii) The effect of arsenate is compatible with formation of an arsenylated intermediate which is hydrolysed at the 2 Ca–$E_1 \sim$ Asi conformation.

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