Ca\(^{2+}\) Translocation across Sarcoplasmic Reticulum ATPase Randomizes the Two Transported Ions*

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Cytoplasmic Ca\(^{2+}\) dissociation is sequential, and the Ca\(^{2+}\) ions bound to the nonphosphorylated ATPase are commonly represented as superimposed on each other, so that the superficial Ca\(^{2+}\) is freely exchangeable from the cytoplasm, whereas the deeper Ca\(^{2+}\) is not. Under conditions where ADP-sensitive phosphoenzyme accumulates (leaky vesicles, 5°C, pH 8, 300 mM K\(^{+}\)), luminal Ca\(^{2+}\) dissociation is sequential as well, so that the representation of two superimposed Ca\(^{2+}\) ions still holds on the phosphoenzyme, with the superficial Ca\(^{2+}\) facing the lumen freely exchangeable and the deeper Ca\(^{2+}\) blocked by the superficial Ca\(^{2+}\).

Under the same conditions, we have investigated whether a prebuilt Ca\(^{2+}\) order is maintained during membrane translocation. Starting from a prebuilt order on the cytoplasmic side, we showed that the Ca\(^{2+}\) ions cannot be identified after translocation to the luminal side. The same result was obtained starting from a prebuilt order on the luminal side and following the luminal to cytoplasmic translocation. We conclude that the two Ca\(^{2+}\) ions are mixed during ATP-induced phosphorylation as well as during ADP-induced dephosphorylation.

Sarcoplasmic reticulum ATPase is a membranous enzyme that pumps Ca\(^{2+}\) from the cytoplasm of muscle cells into the reticulum lumen, requiring ATP hydrolysis. During its cycle, each ATPase monomer transports two Ca\(^{2+}\) ions (Scheme 1). During transport, the Ca\(^{2+}\) sites change their orientation and affinity, depending on whether the ATPase is phosphorylated. The high affinity transport sites of the nonphosphorylated ATPase are accessible from the cytoplasm, whereas once the ATPase has been phosphorylated the transport sites have lower affinity and are accessible from the lumen. This allows Ca\(^{2+}\) release into the SR\(^{1}\) lumen and is followed by dephosphorylation.

Ca\(^{2+}\) binding to E, the Ca\(^{2+}\)-deprived nonphosphorylated ATPase, has been well characterized. Two Ca\(^{2+}\) ions bind sequentially with high affinity and positive cooperativity (1, 2). In 1982, Dupont (3) showed that the dissociation of one-half of the \(4^{25}\)Ca\(^{2+}\) bound to Ca\(_{E}\) was impaired by the presence of excess \(4^{40}\)Ca\(^{2+}\) in the medium. This was interpreted in 1987 by Inesi (4) as two sites being sequentially accessible from the cytoplasm by the two Ca\(^{2+}\) ions in a crevice with a deep site and a superficial site (Fig. 2). The first ion must reach the deep site to leave the superficial site vacant for the second ion to bind. The Ca\(^{2+}\) bound to the superficial site is freely exchangeable with free Ca\(^{2+}\) in the outer medium, whereas the Ca\(^{2+}\) bound to the deep site is not. Inesi took advantage of the possibility of selectively placing a \(4^{40}\)Ca\(^{2+}\) on top of a \(4^{40}\)Ca\(^{2+}\), to determine whether their dissociation toward the lumen is sequential. He concluded that this was the case and that the first Ca\(^{2+}\) bound to E was the first to be internalized by monitoring the internalization of the Ca\(^{2+}\) ions after phosphorylation. This would correspond to a channel-like structure with a first-in-first-out mechanism for membrane crossing.

The question of whether the dissociation of the Ca\(^{2+}\) ions toward the lumen is sequential or not has been reinvestigated by Hanel and Jencks (5) and Orlowski and Champel (6). Both groups concluded that the two ions cannot be kinetically distinguished, probably because Ca\(^{2+}\) dissociation from the phosphorylated ATPase starts with a rate-limiting deoctusion step. More recently, Forge et al. (7) have shown that under appropriate conditions (pH 8, 300 mM K\(^{+}\), 5°C), the two ions dissociate sequentially from the phosphorylated ATPase, suggesting that Ca\(^{2+}\) dissociation was intrinsically sequential on both sides of the membrane.

As with the nonphosphorylated ATPase, the Ca\(^{2+}\) sites of the phosphorylated ATPase may be represented by a crevice with a deep site and a superficial site accessible from the lumen (Fig. 3). The initial suggestion arising from these sketches is that the first ion to bind to the ATPase is the first to dissociate after transport, as originally proposed by Inesi (4). Because the conditions chosen by Forge et al. (7) have revealed the sequentiality of the luminal dissociation, they appear to be particularly appropriate for reinvestigating whether the Ca\(^{2+}\) ion order is kept during transport. We show that there is mixing of the two Ca\(^{2+}\) ions in the Ca\(^{2+}\)-bound phosphoenzyme; i.e. the first Ca\(^{2+}\) bound to the nonphosphorylated ATPase cannot be identified as being the first or the second to dissociate toward the lumen. This is also shown for the reverse step, i.e. during the ADP-induced dephosphorylation of Ca\(_{E}\)-PMg.

MATERIALS AND METHODS

SR vesicles were prepared and tested as described by Forge et al. (2). All experiments were carried out at 3°C in a cold room. The buffer was 100 mM Tes-Tris, pH 8, plus 1 or 3 mM Mg\(^{2+}\) and 0 or 300 mM K\(^{+}\), as specified in the figure legends. It was prepared with water filtered through a Milli-Q Water Purification System (Millipore Corp., Milford, MA). All salts were added as chlorides. Vesicles were made leaky by an incubation of at least 1 h at 2 mg/ml in 50 mM Tris, 10 mM K\(^{+}\), 2 mM EDTA at room temperature.

Ca\(^{2+}\) Binding and Phosphoenzyme Measurements—Ca\(^{2+}\) binding and phosphoenzyme levels were measured by the filtration technique. Kinetic measurements involving \(4^{40}\)Ca\(^{2+}\) or \(3^{32}\)P]ATP all started with the same incubation and rinsing steps. Vesicles (0.2 mg/ml) were first

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1 The abbreviations used are: SR, sarcoplasmic reticulum; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; FIFO, first-in-first-out; FILO, first-in-last-out.
incubated in the pH 8 buffer. One ml of this suspension was deposited on a filter (Millipore HA 0.45 μm), and the adsorbed vesicles were perfused with 1 ml of 100 μM EGTA to rinse EDTA with a Ca++-deprived buffer. For experiments starting from Ca,E (Figs. 2, 4, and 5), ATPase was converted to this state by manually perfusing the filters for 5 s with 1 ml of 100 μM 45Ca2+ (or 40Ca2+) plus 90 μM EGTA and 3 mM Mg2+. Cytoplasmic isotopic exchanges were performed in the absence of K+ by perfusing 100 μM 4Ca2+ (or 0Ca2+) plus 3 mM Mg2+. For experiments starting from Ca,E-PMg (Figs. 3 and 6), ATPase was converted to this state by manually perfusing the filters for 5 s with 2 ml of 100 μM 4Ca2+ (or 0Ca2+) plus 90 μM EGTA, 100 μM [γ-32P]ATP (or ATP), 3 mM Mg2+, and 300 mM K+. Unless otherwise specified, the kinetic measurements were begun immediately after this step using a rapid filtration system (Bio-Logic, Clai, France). They were carried out by perfusing media containing EGTA, or 4Ca2+, with or without ADP, for various times. All solutions containing 4Ca2+ or [γ-32P]ATP also contained 1 mM [3H]glucose, which allows evaluation of the filter wet volume, usually about 30 μl. 4H and 4Ca and/or 32P retained on the filter were simultaneously measured by scintillation. [γ-32P]ATP or 4Ca2+ contained in the wet volume was subtracted from the total 32P or 4Ca counts to evaluate the phosphoenzyme and the 4Ca2+ bound to the ATPase. The values for the curves plotted in Figs. 3, 5, and 6 are the average of two to six experiments (± S.E.).

Ca2+ dissociation kinetics in Fig. 3A were the same, whether measured in presence of contaminating Ca2+, 1 or 10 mM EGTA, or 1 mM BAPTA. Ca2+ dissociation kinetics proved to be insensitive to the Ca2+ ionophore calcimycin (4%, w/w). Therefore, the curve in Fig. 3A is the average of experiments conducted under these various conditions.

Double Filtration System—Two rapid filtration systems from Bio-Logic (8) were associated to ensure that the phosphorylation reaction would last precisely for a short period of time and therefore be reproducible. Indeed, when manual phosphorylation is performed before kinetics perfusion by a single system, there can be 5–10 s between the beginning of the manual filtration of the phosphorylation medium and the beginning of the perfusion driven by the filtration system; for some experiments, this time lag is too long. To avoid this difficulty, we have coupled two rapid filtration systems face to face and linked them by rails on which the filter holder slides from one system to the other (Fig. 1). At the end of the filtration by the first system, the filter holder is manually moved from the first system to the second system rapidly enough to ensure that there are <1.5 s between the end of the first perfusion and the beginning of the second.

For the experiments described here, the first filtration system was used to deliver phosphorylation substrate during 0.4 s, and the second filtration system was used to perfuse the various experimental media precisely 2 s after the phosphorylation perfusion had started. This ensured that each kinetic measurement started from the same initial state. The experiments shown in Figs. 4 and 5 were carried out with this combined system. All other steps proceeding the use of the double filtration system were done as described above when using a single filtration system.

RESULTS

The Ca2+ Ions Bound at the Transport Sites Are Distinguishable—As mentioned above, the two Ca2+ ions bound on the cytoplasmic side of ATPase are kinetically distinguishable. This is illustrated in Fig. 2 under the conditions used below to test whether the Ca2+ order is kept during transport: leaky vesicles, pH 8, 3°C. The experiment started with 4Ca2+-bound ATPase. 45Ca,E was perfused either with 1 mM EGTA, which induced rapid dissociation of both 45Ca2+ ions, or with 100 μM 40Ca2+ which induced rapid dissociation of only one of the 45Ca2+ ions. This can be visualized as an isotopic exchange at the superficial site of a crevice (Fig. 2). K+ is known to increase the rates of Ca2+ dissociation and exchange (9), so that in this experiment, it was omitted to ensure that the so-called deep 40Ca2+ ion was still blocked after a 10-s perfusion.

Recently, Forge et al. (7) demonstrated similar behavior for...
Ca\textsuperscript{2+} dissociation on the luminal side, i.e. from the phosphorylated ATPase. The experiment required high K\textsuperscript+ to accumulate Ca\textsubscript{2+}-PMg, the ADP-sensitive phosphoenzyme species (10) and low temperatures to stabilize this phosphoenzyme long enough to start the luminal dissociation perfusion before it has spontaneously turned over. Finally, according to de Meis et al. (11), pH 8 and low temperatures induce high affinity for the luminal Ca\textsuperscript{2+} sites making it possible to block the deep Ca\textsuperscript{2+}.

45Ca\textsubscript{2+}-PMg was perfused either with Ca\textsuperscript{2+}-poor buffer (see "Materials and Methods") or with 40Ca\textsuperscript{2+}. The Ca\textsuperscript{2+}-poor buffer induced complete dissociation of 45Ca\textsuperscript{2+} and ATPase dephosphorylation (Fig. 3A), whereas 10 mM 40Ca\textsuperscript{2+} induced partial dissociation of the 45Ca\textsuperscript{2+} bound to the phosphoenzyme without affecting the phosphoenzyme level (Fig. 3B). As with the cytoplasmic side, this can be visualized as an isotopic Ca\textsuperscript{2+} exchange at the most protruding site on the luminal side (Fig. 3).

A Double Filtration System for Phosphorylating Having Bound the Ca\textsuperscript{2+} Ions in a Specific Order—To follow either Ca\textsuperscript{2+} ion during its translocation across the membrane, we had to first place a 40Ca\textsuperscript{2+} on top of a 45Ca\textsuperscript{2+}, or vice versa, on the cytoplasmic side; then phosphorylate ATPase; and finally induce luminal Ca\textsuperscript{2+} dissociation from the phosphoenzyme by perfusing either 40Ca\textsuperscript{2+} or EGTA. This required that the luminal perfusion started during the first cycle of ATPase; otherwise, the prebuilt Ca\textsuperscript{2+} order on the cytoplasmic side would have been lost due to turnover before the dissociation experiments had started. Therefore, starting with a 40Ca\textsuperscript{2+} on top of a 45Ca\textsuperscript{2+}, we first studied the turnover of the deep 45Ca\textsuperscript{2+} while perfusing a phosphorylating medium during various times to determine an optimal duration for phosphorylation. The level of bound 45Ca\textsuperscript{2+} slowly decreased during perfusion of the phosphorylation substrate, showing that there would be <20% loss of bound Ca\textsuperscript{2+} if a luminal dissociation experiment was started 2 s after the phosphorylation perfusion had started (data not shown).

To control the decrease in bound Ca\textsuperscript{2+} during preliminary phosphorylation, we had to switch from manual to electronically controlled phosphorylation. Therefore, we associated two rapid filtration systems in a so-called double filtration system, with the first system perfusing the phosphorylation medium and the second starting the luminal perfusion 2 s after phosphorylation perfusion was triggered (see "Materials and Methods"). This ensured starting the dissociation kinetics from the same initial state repeatedly. Covalent phosphorylation was checked upon phosphorylation with [γ-32P]ATP followed by acid quenching by the second system (120 mM perchloric acid, 15 mM P\textsubscript{i}, data not shown).

We also checked that the phosphoenzyme formed within 2 s was ADP-sensitive by measuring the dephosphorylation kinetics induced by a mixture of ADP and EGTA, as shown in Fig. 4. The ratio of the initial level of bound 45Ca\textsuperscript{2+} to the initial phosphoenzyme is compatible with all ATPase being in Ca\textsubscript{2+}-PMg. The ADP plus EGTA mixture induced complete Ca\textsuperscript{2+} dissociation and dephosphorylation confirming that the phosphoenzyme formed was entirely the Ca\textsubscript{2+}-PMg species. In addition, ADP-induced dephosphorylation was too fast to be accurately measured by the filtration technique, even at 3 °C, in agreement with other quench flow measurements (12). Ca\textsuperscript{2+} dissociation that followed ADP-induced dephosphorylation can be compared to cytoplasmic Ca\textsuperscript{2+} dissociation (Fig. 2). In Fig. 4, Ca\textsuperscript{2+} dissociation is faster than in Fig. 2 because of the presence of K\textsuperscript+ and ADP.

The Order Created on the Cytoplasmic Side Is Lost after Translocation—Suppose that we have superimposed a 40Ca\textsuperscript{2+} on a 45Ca\textsuperscript{2+}, as in Fig. 2. If during transport, the Ca\textsuperscript{2+} ions crossed the membrane in a single file, we would expect to have
after membrane crossing, a $^{45}$Ca$^{2+}$ at the superficial luminal site on top of a $^{40}$Ca$^{2+}$. Because this superficial $^{45}$Ca$^{2+}$ is freely exchangeable with the medium, it should display the same dissociation kinetics toward the lumen, whether its dissociation is induced by EGTA or by $^{40}$Ca$^{2+}$. Conversely, if during transport the Ca$^{2+}$ order was inverted, the $^{45}$Ca$^{2+}$ would be blocked by the $^{40}$Ca$^{2+}$ at the superficial luminal site and thus perfusion with $^{40}$Ca$^{2+}$ would not induce noticeable radioactive Ca$^{2+}$ dissociation, at variance with perfusion with EGTA. The same reasoning holds starting with a $^{45}$Ca$^{2+}$ superimposed on a $^{40}$Ca$^{2+}$ on the cytoplasmic side. Therefore, if during transport the Ca$^{2+}$ order was kept or inverted, we would expect to observe superimposed EGTA- and $^{40}$Ca$^{2+}$-induced dissociation kinetics in one of the two cases described above. We demonstrate below that this is not the case.

The $^{40}$Ca$^{2+}$ dissociation experiments starting with a $^{40}$Ca$^{2+}$ on a $^{45}$Ca$^{2+}$ are shown in Fig. 5A and those starting with a $^{45}$Ca$^{2+}$ on a $^{40}$Ca$^{2+}$ in Fig. 5B. The phosphoenzyme level measured in a parallel experiment without radioactive Ca$^{2+}$ ions is shown in Fig. 5C.

In Fig. 5C, the initial phosphoenzyme level corresponded to full phosphorylation; it was stable during the $^{40}$Ca$^{2+}$ perfusion, whereas EGTA perfusion induced dephosphorylation of ATPase. Prior to start of the kinetics or after 10 s of perfusion, ATPase was manually perfused with a mixture of ADP plus EGTA to check that there was negligible ADP- insensitive phosphoenzyme. These measurements confirmed that during both EGTA and $^{40}$Ca$^{2+}$ perfusions, the phosphoenzyme was ADP sensitive, thus, in the Ca$^{2+}$-PMG form, exchanging its superficial Ca$^{2+}$ with that of the medium during the $^{40}$Ca$^{2+}$ perfusion.

Fig. 5A shows luminal Ca$^{2+}$ dissociation from phosphorylated ATPase, having previously bound one $^{40}$Ca$^{2+}$ on top of one $^{45}$Ca$^{2+}$ on the cytoplasmic side. EGTA induced complete dissociation of $^{45}$Ca$^{2+}$ with a rate constant in good agreement with Fig. 3 and with corresponding dephosphorylation shown in Fig. 5C. In contrast, $^{40}$Ca$^{2+}$-induced biphasic dissociation of the bound $^{45}$Ca$^{2+}$, whereas the phosphoenzyme level remained constant (Fig. 5C). $^{40}$Ca$^{2+}$-induced $^{45}$Ca$^{2+}$ dissociation did not follow the first-in-first-out (FIFO) or the first-in-last-out (FILO) pattern described above. $^{45}$Ca$^{2+}$ kinetics were not superimposed with the EGTA-induced dissociation, as expected if the transport mechanism had kept the Ca$^{2+}$ order (FIFO mechanism), nor were the kinetics stable, as expected if the transport mechanism had reversed the Ca$^{2+}$ order (FILO mechanism). Instead, part of the initially bound Ca$^{2+}$ was exchanged, as if there had been mixing during transport: part of the ATPases had lost their $^{45}$Ca$^{2+}$, as in a FIFO process and the other part had kept their $^{45}$Ca$^{2+}$, as in a FILO process. This mixing is confirmed by the asymmetrical experiment described below (Fig. 5B).

The experiment was repeated (Fig. 5B), inverting $^{45}$Ca$^{2+}$ and $^{40}$Ca$^{2+}$ during the cytoplasmic superimposing of the two Ca$^{2+}$ and perfusing $^{45}$Ca$^{2+}$ during phosphorylation. Again the EGTA- and $^{40}$Ca$^{2+}$-induced $^{45}$Ca$^{2+}$ dissociations were not superimposed, and $^{40}$Ca$^{2+}$ was not blocked by 10 mM $^{40}$Ca$^{2+}$. Again, this does not correspond to a pure FIFO or to a pure FILO mechanism. Apart from an additional stoichiometry in Fig. 5B, the experiments shown in Fig. 5A and B show similar results, suggesting that whatever the order created on the cytoplasmic side, it is lost during Ca$^{2+}$ transport toward the lumen (Scheme 2).

In Fig. 5B, the initial level was higher than in Fig. 5A. It should be pointed out here that from a procedural point of view, the experiments shown in Fig. 5A and B, are slightly different. Namely, in the experiment described in A, radioactive Ca$^{2+}$ was added at the beginning and rinsed twice before the luminal perfusion has started, whereas in the experiment described in B, radioactive Ca$^{2+}$ was added during the isotopic exchange and the phosphorylation steps, and thus there was no rinsing before the luminal perfusion started. This could induce nonspecific binding which would appear in Fig. 5B.

The Order Created on the Luminal Side Is Lost When Reversing the Cycle toward the Cytoplasmic Side—In the results presented above, the important step for Ca$^{2+}$ reorganization was the phosphorylation step, when the transport sites change their orientation from the cytoplasmic to the luminal side. We next examined the dephosphorylation step during the reverse cycle, when the transport sites change from luminal to cytoplasmic orientation. The experimental procedure was to pro-
duce a specific order on the phosphorylated ATPase and then to perfuse ADP plus EGTA to dephosphorylate and dissociate Ca$^{2+}$ during the same perfusion, or to perfuse ADP plus 40Ca$^{2+}$ to dephosphorylate and exchange Ca$^{2+}$ on the cytoplasmic side. We first checked that mixtures of ADP plus 40Ca$^{2+}$ or ADP plus EGTA induced the same rapid dephosphorylation of Ca$_E$-PMg, too fast to be measured by the filtration technique (data not shown).

Cytoplasmic Ca$^{2+}$ dissociation kinetics were induced by perfusing 1.5 mM ADP plus 1.5 mM 40Ca$^{2+}$, i.e. 1 mM free ADP plus 1 mM free 40Ca$^{2+}$, or 1 mM ADP plus 1 mM EGTA (Fig. 6). When two 45Ca$^{2+}$ ions were initially bound to the phosphorylated ATPase, the ADP plus EGTA mixture induced dissociation of both 45Ca$^{2+}$ ions and the ADP plus 40Ca$^{2+}$ mixture induced dissociation of only one 45Ca$^{2+}$ ion (Fig. 6A). The difference between 45Ca$^{2+}$- or EGTA-induced Ca$^{2+}$ dissociations was observed whether the dissociation experiments were started from Ca$_E$-PMg in presence of ADP or from Ca$_E$ (Figs. 2 and 6A).

In Fig. 6B, a specific order was first produced on the luminal side via an isotopic exchange on the phosphoenzyme, as described in Fig. 3B, i.e. a 40Ca$^{2+}$ on top of a 45Ca$^{2+}$. When Ca$^{2+}$ dissociation was induced by EGTA after this exchange, the kinetics started from one 45Ca$^{2+}$ bound per ATPase and resulted in the loss of this 45Ca$^{2+}$ ion. When the ADP plus 40Ca$^{2+}$ perfusion was performed, the kinetics resulted in the loss of part of this 45Ca$^{2+}$ ion. Note the different scales in Fig. 6, A and B. Thus, the radioactive 45Ca$^{2+}$, which was bound at the deepest site on the luminal side was neither completely dissociated nor completely blocked after ATPase dephosphorylation. As observed for the cytoplasmic-to-luminal transport, during reverse reorientation of the transport sites leading to luminal-to-cytoplasmic transport, the prebuilt Ca$^{2+}$ order was lost (Scheme 3).

**DISCUSSION**

The aim of this work was to study a particular feature of Ca$^{2+}$ ion transport, namely the putative FIFO mechanism. Inesi (4) showed that two different Ca$^{2+}$ isotopes can be superimposed in the cytoplasmic sites and Forge et al. (7), using specific conditions, demonstrated that the same superimposition was possible in the luminal sites. There is some controversy about the luminal sequentiality, because Inesi (4) found that luminal dissociation was sequential, whereas Hanel and Jencks (5) and Orlowski and Champeil (6) found that the Ca$^{2+}$ ions were not distinguishable during luminal dissociation. This controversy indicates that under the usual conditions the sequentiality is difficult to investigate and may depend critically on a number of factors.

Starting from this observation, Forge et al. (7) defined a set of conditions that allow accumulation of Ca$_E$-PMg, the ADP-sensitive phosphoenzyme, stabilizing this phosphoenzyme long enough to start the luminal dissociation perfusion before it has significantly turned over, and finally to ensure high affinity for the luminal Ca$^{2+}$ sites (300 mM K$^+$, pH 8, and 5°C). These conditions were used here to identify, during its luminal dissociation, a Ca$^{2+}$ ion that had been specifically bound to one or...
the other cytoplasmic site. We interpret the data presented here as resulting from a mixing of the two Ca\(^{2+}\) ions occurring after binding and during translocation.

Mg\(^{2+}\) at the Catalytic Site—in 1987, Inesi (4) proposed a FIFO mechanism for Ca\(^{2+}\) translocation on the basis of the following evidence: phosphorylation of ATPase with two bound \(^{45}\)Ca\(^{2+}\) ions induced biphasic Ca\(^{2+}\) internalization, suggesting that the two ions dissociate sequentially, whereas phosphorylation of ATPase with a \(^{40}\)Ca\(^{2+}\) bound on top of a \(^{45}\)Ca\(^{2+}\) induced fast monophasic luminal Ca\(^{2+}\) dissociation, suggesting that the deep \(^{45}\)Ca\(^{2+}\) was the first to dissociate. However, these experiments were conducted under conditions that probably lead to phosphorylation by both Ca\(\text{ATP}\) and Mg\(\text{ATP}\). Because a phosphoenzyme with Ca\(^{2+}\) at its catalytic site releases Ca\(^{2+}\) much more slowly than a phosphoenzyme with Mg\(^{2+}\) at its catalytic site (13), the two phases could be attributed to Ca\(^{2+}\) dissociation from Ca\(\text{E-PMg}\) (rapid phase) and to Ca\(^{2+}\) dissociation from Ca\(\text{E-PCa}\) (slow phase), rather than to sequential dissociation of the two Ca\(^{2+}\) ions from a unique phosphoenzyme.

Being aware of this difficulty, we used the filtration technique which allows, under our conditions, rinsing the phosphoenzyme and therefore the possibility that two ions are occluded and need to exchange positions, as if their binding to the amino acid residues and the local structure were becoming loose.

Site-directed mutagenesis results show six charged residues belonging to the putative membrane helices M4, M5, M6, and M8 as critical residues for Ca\(^{2+}\) transport (14), and among them five were critical for Ca\(^{2+}\) occlusion (15, 16). The possibility that the two Ca\(^{2+}\) ions can be superimposed in a channel formed by M4, M5, M6, and M8 is still discussed in the literature (17–20).

Recently, Mészáros and Bak (21, 22) and Jencks and co-workers (23, 24) proposed that the cytoplasmic and luminal sites could be distinct; i.e. there are four Ca\(^{2+}\) sites on the ATPase. Making this assumption and given our results, there would be during phosphorylation and dephosphorylation a transfer of the Ca\(^{2+}\) ions from one pair of sites to the other, during which the Ca\(^{2+}\) ions would be randomized (Scheme 4A). Martonosi (25) proposed a structural model accounting for four possible sites with two channels, including the above described channel and an additional channel, formed by M2, M3, M4, and M5. Since each Ca\(^{2+}\) ion in this model crosses the membrane in its own channel, it does not allow the mixing observed in our results. This model to be correct should include, at least during phosphorylation and dephosphorylation, a different arrangement of the six helices mentioned above, i.e. in a tubular hexagonal structure allowing the Ca\(^{2+}\) ions to mix (Scheme 4B). In this context, note that the representation of the two Ca\(^{2+}\) ions as being superimposed is a choice of one possible structure (the simplest one) among others to interpret the sequential dissociation. The only requirement derived from experiment is that the Ca\(^{2+}\) sites are not independent.

Because the two Ca\(^{2+}\) ions are thought to be randomized during their translocation, it appears necessary to include a major structural change involving the Ca\(^{2+}\) sites. Starting from an ordered state for the Ca\(^{2+}\) ions, the chemical reaction (phosphorylation or dephosphorylation) must lead to another ordered state for the Ca\(^{2+}\) ions through a transient state where the Ca\(^{2+}\) coordinations become loose and allow randomization. Because phosphorylation and dephosphorylation reactions are very rapid, this transient state may have not been seen in structural studies (for references, see Ref. 25).
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SR-ATPase Randomizes Ca^{2+} Ions during Transport