**Ca**\(^{2+}\) Release from the Phosphorylated and the Unphosphorylated Sarcoplasmic Reticulum Ca**\(^{2+}\)\-ATPase Results in Parallel Structural Changes**

**AN INFRARED SPECTROSCOPIC STUDY**

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Structural changes of the sarcoplasmic reticulum Ca**\(^{2+}\)\-ATPase occurring in the reaction step involving phosphoenzyme conversion and Ca**\(^{2+}\) release (Ca\(_E\)\(_1\)P \(\rightarrow\) E\(_2\)P) were followed using time-resolved infrared spectroscopy in H\(_2\)O and D\(_2\)O. The difference spectra measured between 1800 and 1500 cm\(^{-1}\) were almost identical to those of Ca**\(^{2+}\) release from the unphosphorylated ATPase (Ca\(_E\)\(_1\) \(\rightarrow\) E), implying that parallel structural changes occur in both steps. This suggests that characteristic structural features of the high affinity Ca**\(^{2+}\) binding sites of Ca\(_E\)\(_1\) are still present in the ADP-sensitive phosphoenzyme Ca\(_E\)\(_1\)-P. In both Ca**\(^{2+}\) release steps at least two carboxyl groups become protonated, each of them experiencing the same strength of hydrogen bonding irrespective of whether or not the Ca**\(^{2+}\) free ATPase is phosphorylated. This suggests that the same amino acid residues are involved and that they are most likely those that participate in high affinity Ca**\(^{2+}\) binding and H\(^+\) countertransport. We propose that during Ca**\(^{2+}\) release from the phosphoenzyme protons from the luminal side have access to these residues. Our results are consistent with only one pair of Ca**\(^{2+}\) binding sites on the ATPase that serves both Ca**\(^{2+}\) translocation and H\(^+\) countertransport.

The sarcoplasmic reticulum (SR) Ca**\(^{2+}\)\-ATPase couples active Ca**\(^{2+}\) transport to the hydrolysis of ATP. A simplified form of its reaction cycle is shown in Fig. 1. Ca**\(^{2+}\) is bound from the cytoplasmic side of the membrane to high affinity binding sites of the ATPase (left-hand step in Fig. 1); ATP then phosphorylates the ATPase (upper step in Fig. 1), which occludes the bound Ca**\(^{2+}\) ions in the protein. Subsequently, the phosphoenzyme converts from the ADP-sensitive to the ADP-insensitive form, and Ca**\(^{2+}\) is released into the SR lumen (right-hand step in Fig. 1). Hydrolytic cleavage of the phosphoenzyme completes the reaction cycle (bottom step in Fig. 1). For reviews see Refs. 1–4.

Effector molecule induced infrared difference spectroscopy has been used by several groups to investigate the reaction cycle of the Ca**\(^{2+}\)\-ATPase (5–12). They disagree as to whether the structural changes induced by Ca**\(^{2+}\) binding to the unphosphorylated ATPase are reversed upon ATPase phosphorylation Ca\(_E\)\(_1\) \(\rightarrow\) Ca\(_E\)\(_1\)-P (or E\(_1\) \(\rightarrow\) P) (11–14) or in the subsequent step of phosphoenzyme conversion and Ca**\(^{2+}\) release Ca\(_E\)\(_1\)-P \(\rightarrow\) E\(_2\)P (7, 8). We have demonstrated recently that the infrared absorbance changes of these two reaction steps can be separated temporally using time-resolved FTIR spectroscopy (9). With the high quality FTIR spectra now obtained in real time, we can unambiguously show that it is the phosphoenzyme conversion and Ca**\(^{2+}\) release step that reverses most of the structural changes induced by Ca**\(^{2+}\) binding to the unphosphorylated ATPase. The resulting implications for the Ca**\(^{2+}\) transport mechanism will be discussed.

**MATERIALS AND METHODS**

**Sample Preparation.—** Samples for time-resolved infrared spectroscopy of the Ca\(_E\)\(_1\)P \(\rightarrow\) E\(_2\)P reaction were prepared as described previously (9) by removal of free water from an SR suspension in a stream of nitrogen. Samples were immediately rehydrated with 20% Me\(_2\)SO in H\(_2\)O or D\(_2\)O. This method resulted in active ATPase samples (5). Approximate concentrations were 0.7 mM ATPase, 300 mM imidazole, pH 7.0, 1 mM CaCl\(_2\), 20 mM glutathione, 20 mM caged ATP, 0.5 mg/ml A23187, 2 mg/ml adenylate kinase, 20% Me\(_2\)SO in approximately 1 \(\mu\)l of sample volume. Approximately 2–3 mM ATP were released per flash.

**FTIR Measurements.—** Time-resolved FTIR measurements of the Ca\(_E\)\(_1\)P \(\rightarrow\) E\(_2\)P reaction were performed with a modified Bruker IFS 66 spectrometer as described previously (9). Difference spectra for the reaction were obtained by subtracting a spectrum recorded 3.3–11 s (H\(_2\)O) or 11–19 s (D\(_2\)O) after photolysis of caged ATP from a spectrum recorded between 88 and 146 s (H\(_2\)O and D\(_2\)O) and were normalized as described (9). Spectra were recorded at 1 °C.

Difference spectra of Ca**\(^{2+}\)** release from the unphosphorylated ATPase were obtained from experiments originally monitoring Ca**\(^{2+}\)** binding at 25 °C at pH 7.0 after the release of Ca**\(^{2+}\) from DM-nitrophen (8) but were processed in a different way for a better comparison with the conversion spectra; the spectrum after Ca**\(^{2+}\) release from DM-nitrophen was subtracted from the spectrum before Ca**\(^{2+}\) release. These spectra then represent the absorbance of the Ca**\(^{2+}\)**-free state E minus the absorbance of Ca\(_E\)\(_1\) and thus the Ca**\(^{2+}\)** release reaction from the unphosphorylated ATPase Ca\(_E\)\(_1\) \(\rightarrow\) E. Infrared bands due to the photocleavage reaction were subtracted as described (8).

Difference spectra were normalized to equal protein content by normalizing spectra measured in H\(_2\)O to an amide II absorbance of 0.26 (difference in absorbance between 1546 and 1492 cm\(^{-1}\)) and spectra measured in D\(_2\)O to an amide I absorbance of 0.47 (difference in absorbance between 1706 and 1648 cm\(^{-1}\)).
RESULTS AND DISCUSSION

Fig. 2 shows infrared difference spectra of Ca\(^{2+}\) release from the unphosphorylated (Ca\(_2E_1\) → E, dotted lines) and the phosphorylated ATPase (Ca\(_2E_1\)-P → E\(_2\)P, solid lines) in H\(_2\)O (Fig. 2A) and \(^2\)H\(_2\)O (Fig. 2B). Negative bands are characteristic for the states Ca\(_2E_1\) and Ca\(_2E_1\)-P, positive bands for the Ca\(^{2+}\) free states E and E\(_2\)P. For Ca\(^{2+}\) release from the phosphoenzyme we note that the spectra show the overall transition from Ca\(_2E_1\)-P to E\(_2\)P. The term Ca\(^{2+}\) release therefore refers to the overall Ca\(^{2+}\) transfer from Ca\(_2E_1\)-P to the SR lumen. Putative intermediates in this reaction, such as a Ca\(^{2+}\) form of the ADP insensitive phosphoenzyme Ca\(_2E_2\)-P were not detected in our former experiments and if they exist are only short-lived intermediates (9).

The spectral range shown in Fig. 2 covers the absorption region of the C=O mode of protonated carbonyl groups (1800–1700 cm\(^{-1}\)), the \(\nu_{as}\) COO\(^-\) mode of unprotonated carbonyl groups (1610–1540 cm\(^{-1}\)) in water in the presence of countercations (15), and the amide I (1700–1610 cm\(^{-1}\)) and the amide II (1580–1520 cm\(^{-1}\)) mode of the polypeptide backbone. Other amino acid side chains may contribute to the signals below 1700 cm\(^{-1}\). Thus, the difference spectra are expected to reveal backbone secondary structure changes as well as perturbations of the putative Ca\(^{2+}\) ligating carbonyl groups (4). Their vibrational frequencies are sensitive to the mode by which carboxylate ligands are bound (15–17) (i.e. unidentate, bidentate, and bridging), and their extinction coefficient is relatively high (18, 19), making them ideal reporter groups for events at the Ca\(^{2+}\) binding sites. Therefore, band shifts upon Ca\(^{2+}\) release are expected in the region of the \(\nu_{as}\) COO\(^-\) vibration (around 1570 cm\(^{-1}\)). In addition, protonated carbonyl groups absorb in the 1700–1800 cm\(^{-1}\) region without interference by bands of other groups, which makes the assignment straightforward. Signals in the difference spectra of ATPase partial reactions have been tentatively assigned to Ca\(^{2+}\) release from carbonylate groups (at 1570/1554 cm\(^{-1}\)) and protonation of at least two carbonylate groups (at 1758 and 1710 cm\(^{-1}\)) (7, 10).

As seen in Fig. 2, spectra of Ca\(^{2+}\) release are remarkably similar irrespective of whether or not the ATPase is phosphorylated (compare solid and dotted lines in Fig. 2). However, some differences are observed between the two types of Ca\(^{2+}\) release spectra, which we discuss first before turning to the implications of the similarities.

Differences between the Ca\(_2E_1\)-P → E\(_2\)P and the Ca\(_2E_1\) → E spectra are expected because release from the unphosphorylated ATPase is to the cytoplasm, whereas release from the phosphoenzyme is toward the SR lumen. Furthermore, the Ca\(^{2+}\) binding sites of Ca\(_2E_1\) and Ca\(_2E_1\)-P are different in that the binding site of Ca\(_2E_1\) is accessible from the cytoplasm, whereas the bound Ca\(^{2+}\) in Ca\(_2E_1\)-P is occluded and the affinity for Ca\(^{2+}\) may be different in the two enzyme states. Totally unrelated spectra may be expected if Ca\(^{2+}\) release proceeds from binding sites that are different or different in structure in Ca\(_2E_1\) and Ca\(_2E_1\)-P, i.e. if ATPase phosphorylation moves the Ca\(^{2+}\) ions from one set of binding sites to another or if it changes the structure. However, there is only one major difference between the two types of release spectra, found between 1650 and 1620 cm\(^{-1}\), and some subtle differences at 1689 cm\(^{-1}\) (H\(_2\)O), 1580 cm\(^{-1}\) (\(^2\)H\(_2\)O) and 1555 cm\(^{-1}\) (H\(_2\)O and \(^2\)H\(_2\)O). They point to structural differences between E and E\(_2\)P as well as between Ca\(_2E_1\) and Ca\(_2E_1\)-P, which are associated with the Ca\(^{2+}\) release reactions. In addition, the different conditions under which the two types of experiment were performed may also contribute to the differences: (i) A regulatory ATP molecule is bound to the two forms of the phosphoenzyme (7, 9), which are monitored after ATP release but not to the unphosphorylated ATPase, because there is no ATP present in these experiments. (ii) Experiments with the phosphorylated ATPase were done in the presence of Me\(_2\)SO, A23187, and adenylate kinase, none of which were present in the experiments with unphosphorylated ATPase. The buffer used in the latter experiments was MOPS containing KCl at 25 °C, whereas imidazole without KCl at 1 °C was used in the former.

Given the number of factors that could result in differences in the Ca\(^{2+}\) release spectra, the overall similarity of the spectra is striking. Nearly all positive and negative bands are found at approximately the same wavenumber in both types of Ca\(^{2+}\) release spectra, and this holds for H\(_2\)O and \(^2\)H\(_2\)O. As each of the bands corresponds to a particular change in structure and interaction, we conclude that parallel secondary structure changes and parallel alterations of amino acid side chain interactions take place in the two Ca\(^{2+}\) release reactions Ca\(_2E_1\)-P → E\(_2\)P and Ca\(_2E_1\) → E. To put it differently, during enzyme turnover most of the structural changes induced by Ca\(^{2+}\) binding to the unphosphorylated ATPase are reversed upon phosphoenzyme conversion and Ca\(^{2+}\) release to the SR lumen, thus confirming our previous interpretation (7, 8).

Buchet, Martinosi, and co-workers have reached a different conclusion (11–14) by focusing attention on a positive band at 1650 cm\(^{-1}\) and its negative side bands in ATP-induced difference spectra. These spectra were assigned to the Ca\(_2E_1\) → Ca\(_2E_1\)-P transition, which is supported by the lack of the E\(_2\)P marker bands at 1750, 1616 (shoulder), and 1552 cm\(^{-1}\) (7).
Buchet, Martonosi, and co-workers suggest that the bands near 1650 cm$^{-1}$ in the Ca$_2$E$_1$ → Ca$_2$E$_1$-P spectra represent the reversal of structural changes induced by Ca$^{2+}$ binding E → Ca$_2$E$_1$. We have shown (7, 9) that the ATP-induced 1650 cm$^{-1}$ band is already present upon nucleotide binding and increases further in intensity upon phosphorylation as well as upon phosphoenzyme conversion and Ca$^{2+}$ release. It is tempting to speculate that this band represents a structural transition proceeding stepwise in one direction in these consecutive reactions. Therefore, it seems difficult to assign the reversal of Ca$^{2+}$-induced changes observed near 1650 cm$^{-1}$ to only one of these steps. On the other hand, the intensities at 1650 cm$^{-1}$ in Fig. 2 approximately match, thus arguing in favor of a reversal of the associated conformational changes in the conversion and Ca$^{2+}$ release step, in line with most other changes as discussed above.

The similarity of the spectra of Ca$^{2+}$ release from the phosphorylated and the unphosphorylated ATPase sheds some light on the Ca$^{2+}$ binding sites of the different enzyme states. Nearly all of the negative bands and minima in Fig. 2, which are characteristic for the Ca$^{2+}$-loaded forms Ca$_2$E$_1$ and Ca$_2$E$_1$-P, are found in both Ca$^{2+}$ release spectra. Therefore, characteristic structural features of the high affinity Ca$^{2+}$ binding sites of Ca$_2$E$_1$ are also present in Ca$_2$E$_1$-P, comprising elements of secondary structure and aspects of the Ca$^{2+}$ binding mode (see above). This suggests that after phosphorylation Ca$^{2+}$ remains bound to the same sites in Ca$_2$E$_1$-P as it was in Ca$_2$E$_1$, in agreement with site-directed mutagenesis studies (4), but in contrast to the model of Jencks and co-workers (20).

Following the same line of argument, structural features of the free Ca$^{2+}$ binding sites of E and E-P are very similar. In particular, in both Ca$^{2+}$ release reactions signals of at least two protonated carboxyl groups are observed (bands at 1758 and 1710 cm$^{-1}$). In principle, these signals may originate from the protonation of carboxyl groups or from a change in the environment of already protonated carboxyl groups. The latter seems to be unlikely, because it would lead to a shift in the vibrational frequency and therefore to negative and positive bands in the 1700–1800 cm$^{-1}$ region of the difference spectrum, of which the negative bands are not observed. Therefore, we think that at least two carboxyl groups of E and E-P become protonated in both Ca$^{2+}$ release reactions. They are first discussed for the unphosphorylated ATPase.

Ca$^{2+}$ binding to the unphosphorylated ATPase E → Ca$_2$E$_1$ is accompanied by H$^+$ release, and the protonated residues have been associated with the Ca$^{2+}$ binding sites and proton countertransport (3, 21–27) (for a contrasting view see Ref. 28). They are probably direct Ca$^{2+}$ carriers. At pH 7.0 and 25 °C, the conditions for recording the Ca$_2$E$_1$ → E spectra, there seems to be either an equilibrium of species holding one or three protons at the Ca$_2$E$_1$ binding sites (22) or the predominant species holds two protons (27). Proton ejection during enzyme turnover has been observed at 25 °C and pH 6–8 (23, 25).

It has been proposed (29) that the Ca$^{2+}$ binding sites become accessible for high affinity binding from the cytoplasm only after a conformational change of the Ca$^{2+}$ free enzyme, which switches the accessibility from the lumen to the cytoplasm. However, several partial reactions involving the cytoplasmic high affinity sites are unaffected by 20–40 mM luminal Ca$^{2+}$ (26, 30, 31), indicating that such a transition may not occur.

The Ca$^{2+}$/H$^+$ exchange at the high affinity sites for Ca$^{2+}$ is expected to be detected in the infrared difference spectrum, which was recorded under similar conditions (see above). As the most straightforward interpretation of the protonation signals of carboxyl groups seen in the Ca$_2$E$_1$ → E spectra, we therefore suggest that these carboxyl groups are those that participate in the Ca$^{2+}$/H$^+$ exchange and tentatively assign them to residues within the high affinity binding sites. The high affinity binding sites are thought to be located in the transmembrane region of the Ca$^{2+}$-ATPase (4) and to be in a hydrophobic environment (32). Consistent with this we find that one of the protonated carboxyl groups has a vibrational frequency characteristic of a nonhydrogen-bonded carboxyl group (1758 cm$^{-1}$) (8, 10). The frequency of the second protonated carboxyl group (1710 cm$^{-1}$) indicates that it is hydrogen-bonded (8, 10). This, however, does not necessarily mean that it is in a hydrophilic environment, because the hydrogen bond donor may be a protein residue in an otherwise hydrophobic protein environment.

Ca$^{2+}$ uptake is paralleled by luminal alkalinization, which has been attributed to proton binding to E-P (33–36) (for a different view see Ref. 37). Because this has been observed at 4 °C, pH 6.0–6.4 (33, 38) and at 17–25 °C, pH 7.2 (23, 36), conditions not far removed from the 1 °C, pH 7.0 used to record the Ca$_2$E$_1$-P → E-P spectra, we suggest that the protons responsible for the protonation signals of E-P in these spectra (Fig. 2) are those that cause luminal alkalinization and therefore originate from the lumen. Protonation of protein residues of E-P has been found to increase the stability of the phosphoenzyme (39) and to ensure the low affinity for Ca$^{2+}$ (40, 41). It may well be that the protonated carboxyl groups that we detect in the conversion spectra mediate both effects.

The C=O bands of the two (or more) protonated carboxyl groups of E and E-P are observed at the same characteristic wavenumbers in the difference spectra, clearly indicating that each group experiences a hydrogen bonding strength that is the same in E and E-P. This coincidence strongly suggests that the same amino acid residues become protonated in the two enzyme states. For E, they have been associated with the high affinity binding sites (see above). For E-P, we therefore propose that residues that form the high affinity sites of Ca$_2$E$_1$ become protonated when Ca$^{2+}$ is released from the phosphoenzyme Ca$_2$E$_1$-P → E-P. This requires that luminal protons have access to these residues during this reaction via a proton conducting network or a channel. In the latter case Ca$^{2+}$ would probably also have access to these residues, which then would be involved in high as well as low affinity binding of Ca$^{2+}$.

The above line of argument concludes from the identical positions of the two bands in both Ca$^{2+}$ release reactions that the same two carboxyl groups are involved in proton binding to E and E-P and locates them in the high affinity Ca$^{2+}$ binding sites of Ca$_2$E$_1$. They are thought to be those Ca$^{2+}$ ligands that are involved in the H$^+/Ca^{2+}$ exchange. Pursuing this, one might look for possible candidates that meet the criterion of participating in both, Ca$^{2+}$ and H$^+$ binding. Site-directed mutagenesis studies have identified several carboxyl groups that are thought to be Ca$^{2+}$ ligands of the high affinity binding site: Glu$^{309}$, Glu$^{771}$, Asp$^{800}$, and perhaps Glu$^{908}$ (4). Of these, only Glu$^{309}$ and Glu$^{771}$ seem to be crucial for phosphoenzyme hydrolysis E-P → E, and this has been explained with their possible role in proton binding to E-P and proton countertransport (4). Because these residues seem to bind protons in the E-P state and Ca$^{2+}$ in Ca$_2$E$_1$ and Ca$_2$E$_1$-P, they are likely candidates for the carboxyl groups that give rise to the protonation signals in the Ca$_2$E$_1$ → E and Ca$_2$E$_1$-P → E-P spectra. As a working hypothesis, we therefore tentatively assign the two protonation signals in both Ca$^{2+}$ release spectra to Glu$^{309}$ and Glu$^{771}$. A definite assignment would require studies with site-directed mutants, which are presently not available in the quantities needed for infrared experiments.
CONCLUSIONS

The simplest model consistent with our data would assume only one pair of Ca$^{2+}$ binding sites on the ATPase, serving both Ca$^{2+}$ translocation and H$^+$ countertransport. However, more complicated models with two pairs of binding sites cannot be ruled out (reviewed in Refs. 2 and 13). Our results then require that residues of the high affinity binding sites have access to luminal protons at some stage of the Ca$^{2+}$ release reaction $\text{Ca}_2E_1\Pi \rightarrow E_2\Pi$.

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