A Time-Resolved Fourier Transformed Infrared Difference Spectroscopy Study of the Sarcoplasmic Reticulum Ca^{2+}-ATPase: Kinetics of the High-Affinity Calcium Binding at Low Temperature

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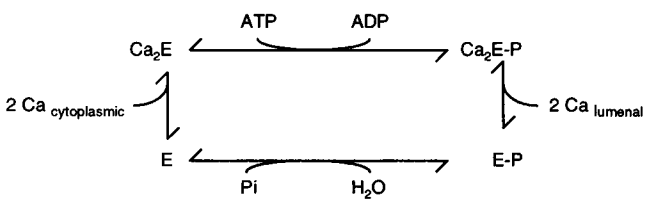
ABSTRACT We have used time-resolved Fourier transformed infrared difference spectroscopy to characterize the amplitude, frequency, and kinetics of the absorbance changes induced in the infrared (IR) spectrum of sarcoplasmic reticulum Ca^{2+}-ATPase by calcium binding at the high-affinity transport sites. 1-(2-Nitro-4,5-dimethoxyphenyl)-*N,N,N',N'*-tetrakis[(oxycarbonyl)methyl]-1,2-ethanediamine (DM-nitrophen) was used as a caged-calcium compound to trigger the release of calcium in the IR samples. Calcium binding to Ca^{2+}-ATPase induces the appearance of spectral bands in difference spectra that are all absent in the presence of the inhibitor thapsigargin. Spectral bands above 1700 cm^{-1} indicate that glutamic and/or aspartic acid side chains are deprotonated upon calcium binding, whereas other bands may be induced by reactions of asparagine, glutamine, and tyrosine residues. Some of the bands appearing in the 1690–1610 cm^{-1} region arise from modifications of peptide backbone carbonyl groups. The band at 1653 cm^{-1} is a candidate for a change in an α-helix, whereas other bands could arise from modifications of random, turn, or β-sheet structures or from main-chain carbonyl groups playing the role of calcium ligands. Only a few residues are involved in secondary structure changes. The kinetic evolution of these bands was recorded at low temperature (−9°C). All bands exhibited a monophasic kinetics of rate constant 0.026 s^{-1}, which is compatible with that measured in previous study at the same temperature in a suspension of sarcoplasmic reticulum vesicles by intrinsic fluorescence of Ca^{2+}-ATPase.

INTRODUCTION

The Ca^{2+}-ATPase of sarcoplasmic reticulum belongs to the P-type ATPase family and represents a model for the study of these enzymes. It catalyzes the active transport of calcium across the sarcoplasmic reticulum (SR) membrane at the expense of ATP hydrolysis.

Enzymes of this group exhibit identical reaction cycles involving the covalent transfer of the γ-phosphate of ATP to an aspartyl residue. The reaction cycle of the Ca^{2+}-ATPase is depicted in Scheme 1. Two calcium ions are transported when one ATP molecule is hydrolyzed. Calcium ion translocation through the membrane is tightly linked to the phosphorylation of the enzyme. Calcium ions bind from the cytoplasmic side of the membrane to sites having a micromolar affinity, whereas they dissociate from the inner side of the reticulum membrane from sites that have a millimolar affinity.



Scheme 1

The Ca^{2+}-ATPase is a 110-kDa monomer. Its structure is known to 14-Å resolution from crystallographic studies (Toyoshima et al., 1993), and a structural model has been deduced from the sequences of several P-type ATPases and other available data (Green and Stokes, 1992). It has been proposed that the high-affinity calcium sites are located in the transmembranous domain of the enzyme, whereas the nucleotidic and phosphorylation sites are situated in the large domain protruding above the membrane on the cytosolic side of the reticulum (see Inesi et al., 1994, for a review).

The binding of calcium at the high-affinity transport sites plays an important role in the activation of the enzyme. It induces a change in the chemical reactivity of the catalytic site, because the enzyme can be phosphorylated by ATP only when the transport sites are occupied by two calcium ions. Calcium binding is known to induce a change in the ellipticity of the enzyme (Girardet and Dupont, 1992), which evidences to a conformational change in the protein, but the structural relations between the high-affinity calcium sites and the catalytic site remain largely unknown. Mutagenesis studies have shown that some amino acids located in the transmembrane helices are crucial for high-affinity calcium binding (see Andersen and Vilsen, 1995, for a review), but the spatial arrangement of these amino acids in a proposed channel-like structure (Inesi, 1987) is

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still unknown. Obtaining detailed structural information about the active sites and their relations is thus necessary for a better understanding of the active transport mechanism.

We report here a study of calcium binding to Ca^{2+}-ATPase monitored by Fourier transformed infrared difference spectroscopy (FTIR). By this method it is possible to detect small absorbance changes between the infrared (IR) spectra of a protein in different conformational states. The absorbance changes can be linked to small structural modifications, including changes of a single atom coordinate (Gerwert et al., 1990; Souvignier and Gerwert, 1992). To reach this sensitivity, difference spectra must be measured without disturbing the sample. This can be achieved by the use of photoactivable caged compounds, which release the substrates of the protein in the IR sample upon application of a UV flash.

Studies at steady state allow the observation of the absorbance difference bands induced by the protein residues that are involved in the enzymatic reactions. Assignment of the bands to these residues can be performed by studying isotopically labeled or mutated proteins (for a review see Gerwert, 1992, 1993). However, the detailed role of the residues during the reactions and the enzymatic mechanism can only be understood by also measuring the reactional kinetics. For example, it is only by measuring kinetics that IR measurements can yield information on the calcium binding mechanism of Ca^{2+}-ATPase, which is known to involve the sequential binding of calcium ions at two different sites (Dupont, 1982).

Previous steady-state studies of high-affinity calcium binding have been reported. The authors have used 1,2-amino-5-[1-hydroxy-1-(2-nitro-4,5-methylene dioxyphenyl)methyl]-phenoxyl-2-(2′amino-5′-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (Nitr-5) (Buchet et al., 1991) or 1-(2-nitro-4,5-dimethoxyphenyl)-*N,N,N′,N′*-tetrakis[(oxycarbonyl)methyl]-1,2-ethanediamine (DM-nitrophen) (Georg et al., 1994) as a caged-calcium compound. We have also used DM-nitrophen, but in contrast to Georg et al., we have used the specific sarco-endo-reticulum calcium (SERCA) pump inhibitor thapsigargin (Sagara et al., 1992) to perform control experiments in the presence of the same calcium concentration as in the calcium binding test. In this way we confirm the main features of the spectral bands induced by calcium binding in the IR spectrum of Ca^{2+}-ATPase. Moreover, we have identified additional bands and we report the disappearance of all of the calcium binding bands in the presence of thapsigargin. Moreover, by studying the calcium binding reaction at subzero temperature, we have been able to measure the kinetics of the reaction, and we have found a rate constant compatible with that observed at the same temperature for a SR vesicle suspension by intrinsic fluorescence measurements (Dupont, 1982). Whereas kinetics measurements were only performed on photoactivable proteins until now, our results demonstrate the possibility of measuring enzyme kinetics by FTIR difference spectroscopy on nonphotoactivable proteins.

MATERIALS AND METHODS

Chemicals

DM-nitrophen was purchased from Calbiochem (La Jolla, CA), and thapsigargin was purchased from Calbiochem or Sigma.

pH evaluation

Glycerol was found to have no effect on the pH of morpholino-2-ethanesulfonic acid (MES) buffer. We have titrated MES solutions in 30% glycerol with KOH at 20, 10 and 4°C and determined the pK variation versus temperature. The MES solution used for IR measurements was adjusted to pH 6.1 at room temperature, and the pH was evaluated to 6.4–6.5 at −8.8°C in the presence of 30% glycerol.

Measurement of DM-nitrophen affinities for calcium

Unphotolyzed DM-nitrophen

Calcium induces the $E \rightarrow E.Ca_2$ intrinsic fluorescence transition of Ca^{2+}-ATPase with a submicromolar affinity. This transition can therefore be used to detect submicromolar free calcium concentrations. We have measured the affinity of the unphotolyzed DM-N by comparing the calcium concentration that can induce the $E \rightarrow E.Ca_2$ fluorescence transition in the presence of the same concentration of either the calcium chelator DM-nitrophen or EGTA. Successive amounts of calcium were added to a solution of 100 μg/ml SR vesicles in 100 mM morpholino-3-propanesulfonic acid (MOPS) KOH buffer at pH 7.2 containing either 100 μM EGTA or 100 μM DM-N. Fluorescence measurements were performed at room temperature, as already published (Troullier et al., 1992).

Photolyzed DM-nitrophen

The affinity of the photolyzed DM-N was evaluated using a calcium-specific electrode (Orion 900002; Orion Research AG, Uetikon am See, Switzerland), which is suitable for measuring calcium concentrations in the millimolar range. A 40 mM DM-N solution with and without 40 mM dithiothreitol (DTT) was photolyzed with a xenon UV flash lamp. One hundred percent of the extent of the photolysis was controlled by measuring the UV-visible absorbance spectrum of DM-N. The free Ca^{2+} concentration of a 40 mM MOPS KOH (pH 7.2), 40 mM KCl, 2.5 mM $CaCl_2$ solution was measured before and after the addition of 40 mM photolyzed DM-N, allowing us to determine the amount of calcium chelated by the photolyzed DM-N.

Photolysis of DM-nitrophen for IR experiments

DM-nitrophen was photolyzed during IR experiments by a burst of 140 flashes at 308 nm provided by a XeCl excimer laser (Lambda Physik, Göttingen, Germany). In all experiments, 98–100% extent of photolysis was achieved within 0.42 s, as controlled by the saturation of the amplitude of the IR bands induced upon photolysis.

Preparation of IR samples

The Ca^{2+}-ATPase was prepared in sarcoplasmic reticulum vesicles from rabbit fast skeletal muscles by the technique of Hasselbach and Makinose (1963) as modified by Dupont (1977). Ca^{2+}-ATPase represents about 85% of the total protein in this preparation. The vesicles were suspended in 100 mM KCl, 10% sucrose (w/v), 20 mM MOPS KOH (pH 6.8) and stored in liquid nitrogen until use. The storage medium was rinsed by centrifuging the SR vesicles in 300 mM MES KOH at pH 6. The pellet was resuspended in the reaction medium and centrifuged.

Protein samples were made by placing approximately 1 μl of the pellet between two CaF_2 windows separated by a 6-μm spacer. The windows and the spacer were then tightly sealed in a metal holder. The Ca^{2+}-ATPase concentration in the sample was identical to that in the pellet and could be estimated to about 180–250 mg/ml or 0.7–1.5 mM ATPase. This procedure ensures a sufficient hydration of the samples, a good control of the solute concentrations in the protein samples, and the possibility of preparing medium samples of exactly the same solute composition as the protein samples. We can easily assume that our procedure do not alter the ATPase functions, because this was already shown for samples prepared by the more drastic procedure of dehydration/rehydration (Pierce et al., 1983; Barth et al., 1991). Medium samples without protein were made by collection of the supernatant only.

In the calcium binding condition (+Ca), the composition of the reaction medium was 530 mM MES KOH (pH 6.5) at −8.8°C, 30% glycerol, 30 mM DTT, 30 mM DM-N, 4 mM $CaCl_2$. The excess of DM-N over the Ca^{2+}-ATPase ensures that all of the calcium is chelated by DM-N before the photolysis. About 3–4 mM calcium is released upon 98–100% photolysis of DM-N. This gives a margin sufficient to ensure that, taking all possible sources of variability into account, about 1.4–3 mM high-affinity Ca^{2+}-ATPase binding sites will be saturated. Magnesium was omitted from all of our experiments because DM-N is also a caging group for magnesium, with an affinity in the micromolar range before photolysis (Kaplan and Ellis-Davis, 1988).

Three different control conditions where no calcium binding to the ATPase can occur were performed by adding thapsigargin, EDTA, or calcium to the preceding medium as follows:

Thapsigargin inhibited ATPase (+Ca +thapsi): Thapsigargin (5 mM) was added to ensure that about 0.7–1.5 mM Ca^{2+}-ATPase would be inhibited. As in the +Ca condition, no free calcium is present in the medium before the flash. In that case, thapsigargin inhibits calcium binding in a quasi-irreversible manner (Sagara et al., 1992).

No calcium (0 Ca): EDTA (200 mM) was added to chelate all of the calcium before and after the photolysis of DM-nitrophen. This high concentration ensures that no calcium is bound to 30 mM DM-N before the flash, so that no calcium will be released.

Excess calcium (++Ca): An excess of 35 mM calcium was present to saturate both 30 mM DM-N and the ATPase high-affinity calcium sites before photolysis. About 30 mM calcium is released upon 98–100% photolysis of 30 mM DM-N.

Kinetic IR measurements

Single-beam IR spectra were measured with a 66V IFS Bruker spectrometer (Bruker, Karlsruhe, Germany), using the Bruker OPUS software. The spectrometer was equipped with a globar source, a KBr beam splitter and a HgCdTe detector. All experiments were performed at a temperature of −8.8 ± 0.4°C, which was controlled separately with a thermocouple placed in control samples. No significant heating of the samples was observed upon the flash.

The time scale of spectra recording is shown in Scheme 2.

A single-beam reference spectrum of low noise was recorded just before the flash burst. The first spectrum after the flash was recorded from a single scan measured within 12 ms after the flash. It was used to compute a difference absorbance spectrum regarding the reference spectrum. This spectrum contains the difference absorbance bands due to the photolytic cleavage of DM-N and is referred as the "photolysis spectrum."

In the kinetic experiments the first spectrum after the flash is used as a reference for all of the following spectra to compute difference absorbance spectra, to detect the difference absorbance bands occurring after the photolysis of DM-N. The first spectrum after the flash thus gives the zero time for the kinetic measurements. The following spectra were averaged over 6–1800 scans, giving rise to a time resolution of 0.25 s (until 21 s), 2.5 s (21–62 s), 6.15 s (62–114 s), 12.3 s (114–218 s), 24.6 s (218–632 s), and 73.8 s (632–1255 s), the time interval between two scans being 41 ms.

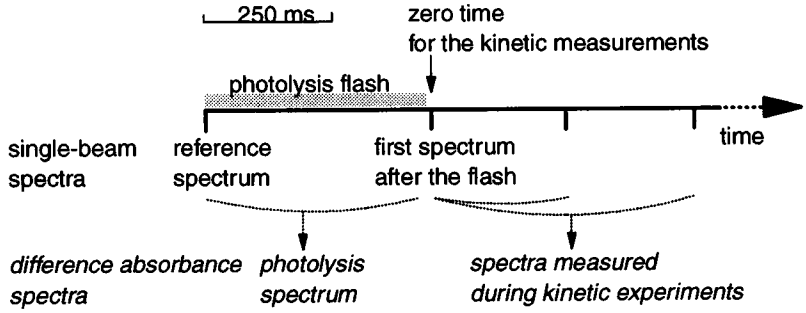
Mathematical treatment of spectra

For each measuring condition as described above (under Preparation of IR Samples), kinetic experiments were performed on four to eight protein and medium samples, and all protein and medium spectra were respectively averaged at each measured time (the resulting spectra are referred as "protein spectra" or "medium spectra").

To eliminate solute bands (see Results), we have subtracted each medium spectrum from the corresponding protein spectrum. As part of the volume of protein samples is occupied by SR vesicles, the volume occupied by the solution is smaller than in medium samples. As a consequence, the bands arising from the solutes have a smaller amplitude in the protein spectra than in the medium spectra and rescaling has been necessary. Another source of amplitude differences between spectra came from the unavoidable variations of the total amount of solution hit by the IR beam from one sample to the other. Before subtraction, the medium spectrum was multiplied by a scaling factor and shifted by an offset so that identical bands in both spectra would have the same amplitude. The scaling factor and offset were determined by minimizing Δ^2 on identical spectral regions, with Δ = (protein spectrum) − (scaling factor × medium spectrum) + offset.

For all spectra in one kinetics, the protein and medium spectra were first rescaled at different time points, and we have retained the values of the scaling factor and offset that were the most representative over the whole kinetics. We have also performed subtraction of spectra with scaling factors differing by 30%, apart from the retained value, to test whether the rescaling procedure could alter the result spectra. We only observed limited amplitude modifications of very few bands, but no significant change in the result spectra. The bands that show a too high sensitivity to the scaling factor were not taken into account.

This last procedure was performed in the same way to rescale the result spectra of +Ca and +thapsi experiments at all time points of the kinetics, to eliminate nonspecific vesicle reactions (see Results).



Scheme 2

Kinetic analysis

We have analyzed the kinetic evolution of the IR spectral bands by calculating the difference between the absorbance at the peak and at one isobestic point, to correct the kinetics from baseline instability. The calculation led to the same result, regardless of which of the two isobestic points was used.

RESULTS

Characterization of DM-nitrophen properties and reactions

Affinities of unphotolyzed and photolyzed DM-nitrophen for calcium

DM-nitrophen is a calcium chelator consisting of a photolabile group linked to an EDTA molecule (Ellis-Davis and Kaplan, 1988). The DM-N molecule and its photolysis reaction are represented on Fig. 1. The EDTA moiety of the molecule is split into two parts upon photolysis by a UV flash, and the affinity of the photoproducts for calcium is drastically reduced. The free calcium concentration before and after the flash is determined by the balance of calcium and DM-N concentrations and the affinities of the unphotolyzed and photolyzed DM-nitrophen for calcium. To optimize the calcium concentration increase and the percentage of ATPase molecules undergoing the calcium binding reaction, we have measured both affinities.

We have measured the apparent dissociation constant of the unphotolyzed DM-N with calcium by comparing the concentration of calcium that can induce the E to $E.Ca_2$ intrinsic fluorescence transition of Ca^{2+}-ATPase in the presence of 100 μM EGTA or in the presence of 100 μM DM-N. Half of the fluorescence change occurred with the addition of 55 μM calcium in the presence of EGTA as compared to 73 μM in the presence of DM-N. This shows that DM-N has a higher affinity for calcium than does EGTA. At a given amplitude of the transition, the free calcium concentration was evaluated from the EGTA experiment, assuming a dissociation constant of EGTA with calcium of 100 nM at pH 7.2. The free calcium concentration in the presence of DM-N was plotted as a function of the total calcium, and the curve was fitted with an equilibrium expression, giving an apparent dissociation constant of DM-N with calcium in the range 5–20 nM. This is in agreement with the value reported by Kaplan and Ellis-Davis of about 5 nM at pH 7.1 (Kaplan and Ellis-Davis, 1988). This dissociation constant is slightly lower than that of EDTA with calcium (40 nM). As a precaution we have assumed that the dissociation constant of DM-N is equivalent to that of EDTA in choosing the calcium and DM-N concentrations in IR experiments, and we have assumed that the pH and ionic strength dependence of the DM-N apparent dissociation constant with calcium would be similar to those of EDTA. The variation induced by the different ionic strengths in the IR measurements (0.4 M) was evaluated by the method of Smith and Miller (1985) and found to be within the range of error that can be induced by the use of different sets of absolute affinity constants at a given ionic strength. We have also assumed that EDTA and DM-N would have a behavior similar to that of EGTA, which undergoes only limited change of affinity for calcium in the presence of glycerol and as a function of temperature (Dupont, 1982). With these assumptions, the apparent dissociation constant of DM-N with calcium is lower than that of Ca^{2+}-ATPase at all pH values between 6 and 7. This fact and the high ratio of concentration of unphotolyzed DM-N/high-affinity calcium binding sites in the IR experiments allow the Ca^{2+}-ATPase to be mostly free of calcium before photolysis, the calcium being bound to the DM-N.

We have evaluated the affinity of photolyzed DM-N for calcium by using a calcium-specific electrode to measure the free calcium concentration in a solution of known total calcium concentration and photolyzed DM-N. Six different measurements gave a lower estimate of 12 mM for the apparent dissociation constant of the photolyzed DM-N with calcium at pH 7.2. This is slightly higher than the estimation of 3 mM reported by Kaplan and Ellis-Davis at pH 7.1 (Kaplan and Ellis-Davis, 1988). This value is four orders of magnitude higher than the apparent dissociation constant of Ca^{2+}-ATPase and ensures that, in the IR measurements, about 4 mM calcium liberated by the photolysis of DM-N will saturate about 1.4–3 mM of high-affinity transport sites instead of binding to about 30 mM photolyzed DM-N. The measured apparent dissociation constant of the photolyzed DM-N should be even higher at the lower pH of the IR experiments, and we consider it a reasonable assumption that the modifications of this constant that could

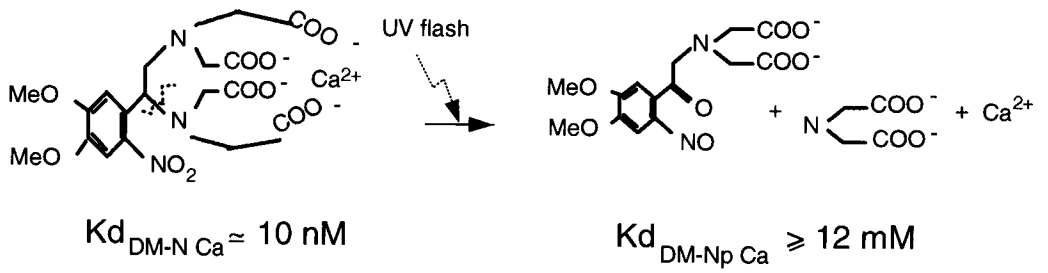


FIGURE 1 Photolysis reaction of DM-nitrophen. The dissociation constants of unphotolyzed and photolyzed DM-nitrophen for calcium (DM-N and DM-Np, respectively) were measured at pH 7.2, at room temperature as described in Materials and Methods.

be induced by the different ionic strength, the presence of glycerol, and the low temperature in the IR experiments would not be of such a magnitude as to impair high-affinity binding to the Ca^{2+}-ATPase.

IR spectrum of DM-nitrophen photolysis

The IR absorbance changes induced by the photolysis of DM-N are observed in the first spectrum after the flash and do not evolve further in the following spectra. The photolysis spectra from protein samples in calcium binding and control conditions are represented in Fig. 2. The positive bands in the photolysis spectra are due to the molecular vibrations of the photolysis products, whereas the negative bands come from the unphotolyzed DM-N chemical bonds. The negative bands at 1332 and 1525 cm^{-1} can be clearly assigned to the symmetrical and asymmetrical stretching vibration of the NO_2 group of unphotolyzed DM-N (Colthup et al., 1990). The photolysis of DM-N is thus achieved within 12 ms after the flash, which is the time necessary to measure the first spectrum after the flash. This observation is in agreement with the fact that the photolysis of DM-N has been shown to be a very fast reaction (half-time not larger than 180 μs at 21°C, pH 6.9, in the presence of 130 mM KCl; McCray et al., 1992).

The photolysis spectrum is essentially the same in the presence or absence of vesicles (not shown), which shows that the presence of the SR vesicles does not interfere with the photolysis reaction. Only a small negative band at 1720 cm^{-1} and a small positive band at 1740 cm^{-1} can be observed only in the presence of vesicles. These bands do not undergo further change during the measurements, and their frequencies indicate that they may be due to lipid CO groups.

Thapsigargin also has no influence on the photolysis reaction, as shown by the complete similarity between +Ca and +Ca +thapsi spectra, which only differ by the presence of thapsigargin.

The different calcium concentrations in the different conditions affect the photolysis spectrum to some extent, but most of the bands are found to be identical in the 0 Ca, +Ca/+Ca +thapsi, and ++Ca conditions. The differences are observed at 1589–1586, 1415–1330, and around 1200 cm^{-1}. They can be attributed to the EDTA part of the DM-N molecule, because they are also observed in the difference between EDTA-Ca and EDTA spectra measured in the same conditions (not shown), in agreement with the results of Georg et al. (1994). The negative band at 1586–1589 cm^{-1} can be attributed to the asymmetrical stretching vibration of the COO^- group of DM-N, which is differently saturated by calcium before the flash, as well as to the differences around 1415 cm^{-1} (Colthup et al., 1990).

IR spectra induced by post-photolysis reactions of DM-nitrophen photoproduct

To test whether reactions of the DM-N photoproduct were occurring after the photolysis, we have performed kinetic measurements on samples containing only the reaction medium. The spectra recorded on medium samples between 923 and 1255 s after the flash when no further reaction occurs are shown on Fig. 3 *a* for the calcium binding and control conditions. Under all conditions used, we have observed the appearance of the same broad bands on the entire 1750–1000 cm^{-1} range.

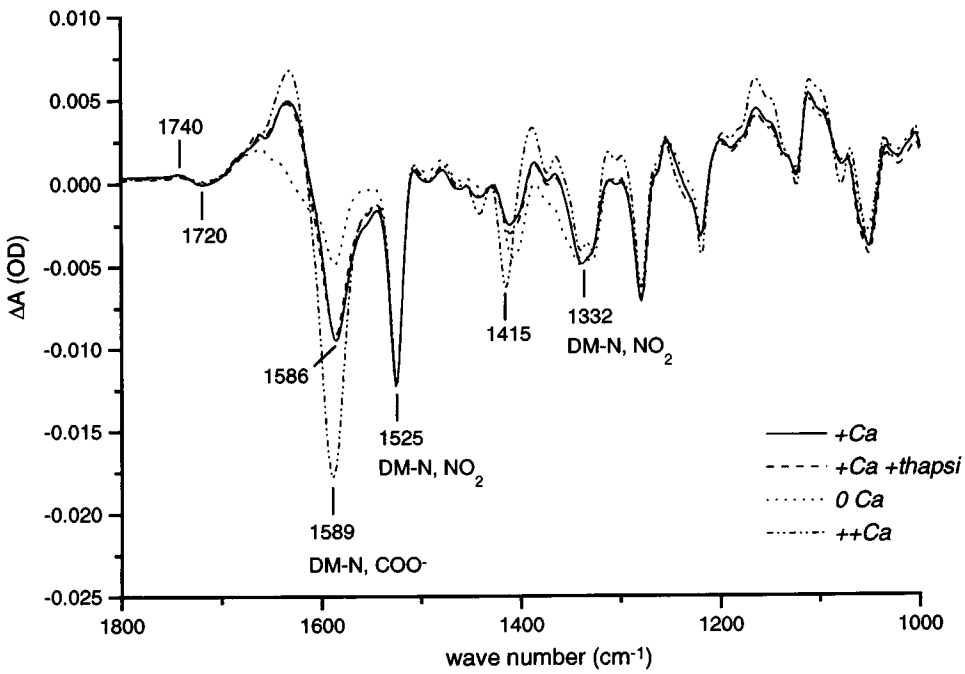


FIGURE 2 IR spectrum of DM-nitrophen photolysis. Kinetic measurements were performed on protein samples as described in Materials and Methods and in Scheme 2. The temperature was −8.8 ± 0.4°C. The figure shows the average of the first spectrum after the flash measured on four to eight protein samples. The spectra are rescaled so that the amplitude at 1525 cm^{-1} is identical. The indications on the figure refer to the different measuring conditions, which are described in Materials and Methods. Before the flash, the DM-nitrophen is free of calcium in 0 Ca conditions, partially saturated by calcium in +Ca and +Ca +thapsi conditions, and fully saturated by calcium in ++Ca conditions. The difference between the spectra arise from the different calcium concentrations.

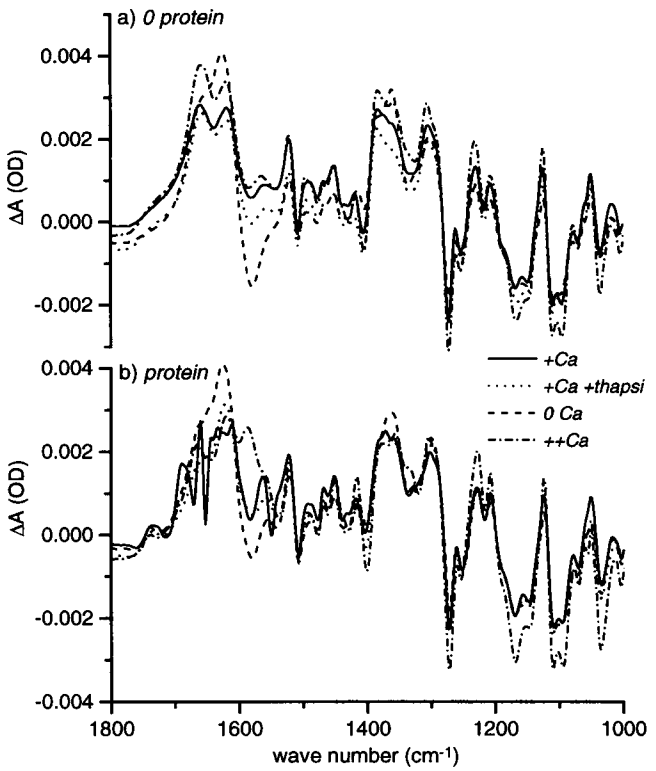


FIGURE 3 IR spectra induced by post-photolytic reactions of the DM-nitrophen photoproduct. Kinetic measurements were performed on medium and protein samples as described in Materials and Methods and in Scheme 2. The temperature was -8.8 ± 0.4°C. The spectra shown on the figure are averaged over the time period between 923 and 1255 s after the flash for four to eight medium samples (*a*) and protein samples (*b*), as all reactions are completed. The indications on the figure refer to the different measuring conditions, which are described in Materials and Methods. Similar bands induced by reactions of the DM-N photoproduct arise in all conditions over the 1750–1000 cm^{-1} range, in the absence and presence of SR vesicles.

The fact that most of these bands are similar under all conditions proves that they are not sensitive to the presence of calcium, EDTA, and thapsigargin. These bands are thus produced by reactions of the DM-N photoproduct occurring much more slowly than the photolysis reaction. The bands exhibit two different kinetics, the fastest having a rate constant of about 0.08–0.1 s^{-1}, and the slowest 0.002–0.01 s^{-1} (not shown).

The most reasonable interpretation is that these bands are due to reactions of the photoproduct of DM-N with DTT, which is present in all of our experiments.

The photolysis of the majority of available caged compounds, including DM-N, gives rise to a 2-nitrosoacetophenone (Kaplan et al., 1978; Walker et al., 1988; McCray and Trentham, 1989; McCray et al., 1992), which contains a very reactive nitroso group (NO; see Fig. 1). The SH group reacts very easily with the NO function to produce nitrosothiols (Williams, 1985). Therefore, caged compounds are generally used together with thiol compounds, which protect the biological material by reacting with the NO group. Inhibition of Na/K-ATPase activity by the photoproduct of caged ATP has been reported by Kaplan et al. (1978). These authors observed a complete protection of the protein upon the addition of a stoichiometric amount of glutathione.

The reaction of the photoproduct of caged ATP with DTT was studied by Walker et al. (1988) by UV-visible spectroscopy, and the authors observed two phases: a fast reaction involving the nitroso group and a slower reaction involving the ketone group of the photoproduct. The rate constant of these two phases is smaller than 500 s^{-1} (21°C, pH 6.3). They are therefore much slower than the photolytic cleavage of DM-N, which has a rate constant of 3800 s^{-1} under similar conditions (McCray et al., 1992). We have used a stoichiometry of 1 DTT/1 DM-N in all experiments, as recommended by Walker et al. (1988), and we indeed observe two different kinetics evolutions of the "photoproduct bands." We therefore assume that they are very probably induced by reactions similar to those described by Walker et al.; their reaction velocity is probably decreased by the low temperature and possibly the presence of glycerol. It must be noted that DTT has no effect on Ca^{2+}-ATPase in the absence of both Ca^{2+} and ATP (Daiho and Kanazawa, 1994).

High-affinity calcium binding to Ca^{2+}-ATPase

Identification of the IR bands induced by calcium binding to Ca^{2+}-ATPase

In the presence of SR vesicles, we have also observed the bands induced by reactions of DM-N photoproduct with DTT as described above. This can be seen in Fig. 3 *b*, where the protein spectra measured at stability and averaged between 923 and 1255 s after the flash are shown below the medium spectra for comparison.

To observe only the bands that are produced by SR vesicle reactions and to eliminate the bands due to DM-N photoproduct reactions, we have subtracted the medium spectra from the protein spectra. The whole procedure is presented completely only for the calcium binding experiment (+Ca) in Fig. 4 *a*, where the protein, medium, and difference spectra are shown. The protein spectrum and the difference spectrum show many sharp bands (labeled by arrowheads), which are absent from the medium spectrum and therefore arise from SR vesicle reactions. These bands may be induced by calcium binding to Ca^{2+}-ATPase. Nevertheless, they may also be induced by nonspecific calcium binding to the lipids and the protein (nonspecific cation binding has been reported on native SR vesicles; Forge et al., 1993a, and references therein) and DM-N photoproduct reacting with the protein and/or the lipids. To explore these nonspecific effects, we have performed three different control experiments in which no change in the high-affinity calcium binding to the ATPase can occur, and where the final concentration of released calcium is very different 1) in the absence of calcium (0 Ca), 2) in the presence of an excess of calcium before the flash (5 mM) and the release of

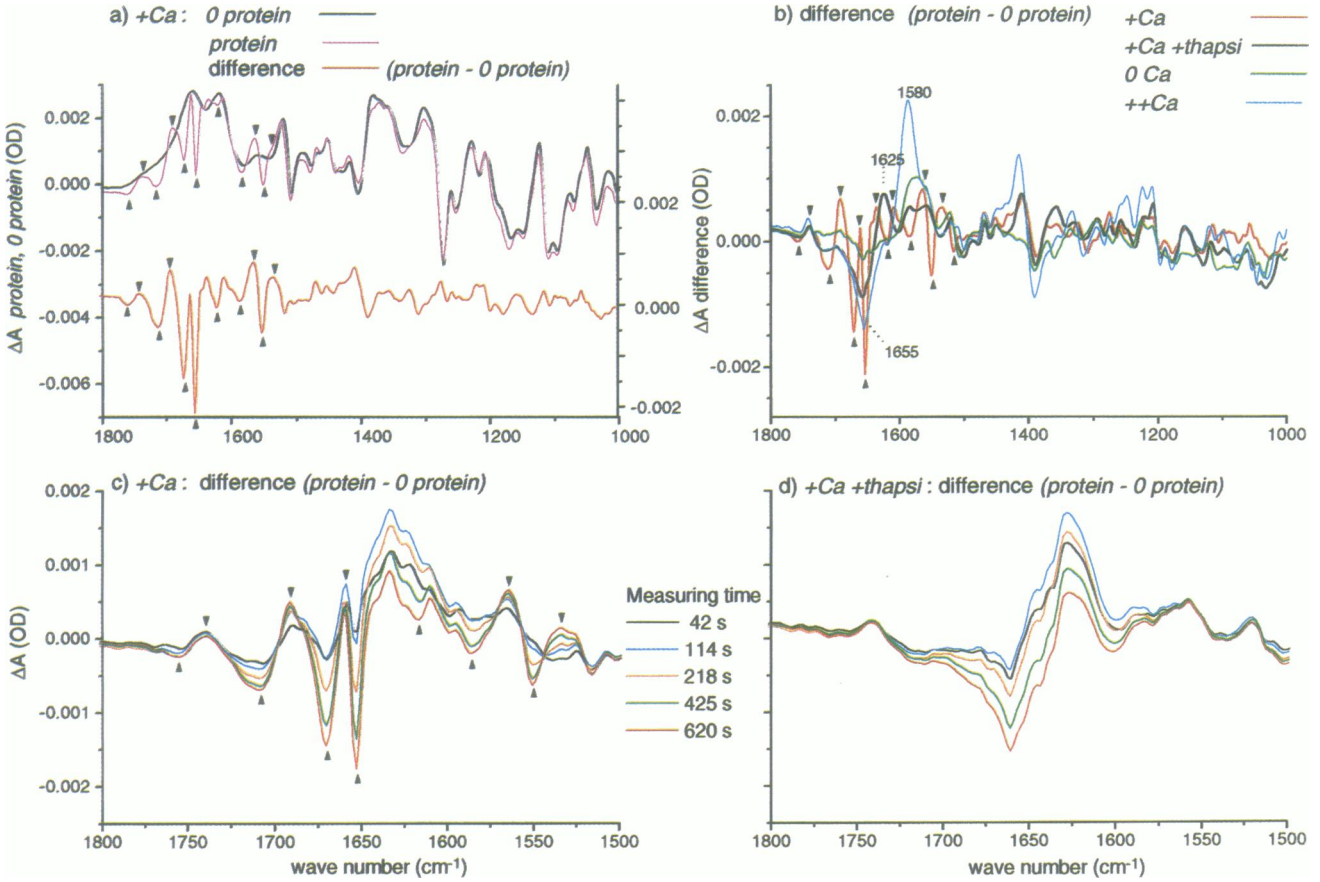


FIGURE 4 Identification of the IR bands induced by calcium binding on Ca^{2+}-ATPase. Kinetic measurements were performed on protein and medium samples as described in Materials and Methods and in Scheme 2. The temperature was -8.8 ± 0.4°C. +Ca represents the calcium binding experiment, whereas 0 Ca, ++Ca, and +Ca +thapsi are controls. The calcium concentration is identical in +Ca +thapsi and +Ca experiments, which differ only by the presence or absence of thapsigargin. 0 Ca is a control in the absence of calcium, and ++Ca is a control in the presence of an excess of calcium before the flash. (*a*) Average of the spectra measured between 923 and 1255 s after the flash on protein (+ protein) and medium (0 protein) samples are redrawn from Fig. 3 for the +Ca conditions. The difference spectrum (protein − 0 protein) shown below was computed as described in Materials and Methods. (*b*) This figure shows the difference (protein − 0 protein) spectra for all conditions. The difference spectra make it possible to observe the bands due to Ca^{2+}-ATPase and/or lipid reactions with calcium and DM-N photoproduct. Arrowheads show bands that are present in the +Ca spectra and absent in all control spectra. These bands are therefore induced by high-affinity calcium binding to Ca^{2+}-ATPase. (*c, d*) The difference (protein − 0 protein) spectra were computed at every measuring time of the kinetics as described in Materials and Methods. The spectra corresponding to the indicated times are shown for the +Ca and +Ca +thapsi experiments. The appearance of two similar positive (1625 cm^{-1}) and negative (1655 cm^{-1}) large bands can be observed in both experiments. These bands are superimposed on the calcium binding bands in the +Ca spectra and are due to a DM-N photoproduct reaction with the vesicles.

a higher concentration of calcium (30 mM, ++Ca), and 3) in a calcium concentration similar to that of the +Ca experiment, but in the presence of thapsigargin, which specifically inhibits high-affinity calcium binding to Ca^{2+}-ATPase (+Ca +thapsi).

The same difference procedure was performed for control conditions, and the difference spectra obtained are shown on Fig. 4 *b* together with the +Ca difference spectrum for comparison. As the labeled bands of the +Ca difference spectrum do not appear in any of the three control experiments, they are clearly induced by calcium binding at the high-affinity sites of the Ca^{2+}-ATPase and not by any nonspecific calcium binding or reaction of the photoproduct with the vesicles.

With the exception of the calcium binding bands, the difference spectra of all conditions contain mainly small noisy bands and three clearer bands at 1655–1625, 1580, 1400 and to some extent 1200 cm^{-1}:

The bands around 1400 and 1200 cm^{-1} depend on the presence of calcium but not on the presence of thapsigargin. This suggests that they could be induced by nonspecific calcium binding.

A broad band at about 1580 cm^{-1} appears in the ++Ca controls but not in the 0 Ca, +Ca, and +Ca +thapsi experiments, suggesting that it is induced by low-affinity calcium binding occurring at high calcium concentration.

A negative band at 1655 cm^{-1} and a positive band at 1625 cm^{-1} appear with a slow kinetics in all controls. These two bands appear in the 0 Ca experiments, where the flash only induces the appearance of photolyzed DM-N without modifying the calcium concentration. On the other hand, the amplitude of these bands increases in the 0 Ca, +Ca

+thapsi, and ++Ca experiments in this order, namely with the free calcium concentration after the flash (not shown). This indicates that they are probably induced by DM-N photoproduct reactions with the vesicles and that calcium interferes with the reaction, probably through low-affinity binding. The two bands can be more clearly observed on spectra measured at different times, obtained by performing the normalization and subtraction procedure over the duration of the kinetics, as shown on Fig. 4 *d* in the case of the +Ca +thapsi control experiments. They also appear in the calcium binding experiments (+Ca) as shown in Fig. 4 *c*. They look very similar to the thapsigargin control and are superimposed on the high-affinity calcium binding bands, impairing their resolution.

It must be noted that the presence of thapsigargin does not induce the appearance of any specific bands in the difference spectra compared to the other controls. We have thus assumed that thapsigargin has no effect other than the inhibition of high-affinity calcium binding. To observe only the high-affinity calcium binding bands and to improve their spectral and kinetic resolution in the spectral range of the large 1655–1625 cm^{-1} bands, we therefore subtracted the +Ca +thapsi difference spectra from the +Ca difference spectra over the duration of the kinetics. The resulting spectra, reflecting only high-affinity calcium binding, are shown in Fig. 5, and the bands are listed in Table 1. The direct subtraction of +Ca +thapsi protein spectra from the +Ca protein spectra is equivalent to this last procedure and indeed gives identical high-affinity calcium binding spectra (not shown).

Previous IR spectroscopy studies of calcium binding to ATPase have been performed by Buchet et al. (1991) and Georg et al. (1994), who used Nitr-5 and DM-N, respectively, as the caged calcium compound. The authors measured the difference at steady state between the IR spectra before and after the flash. In this way they determined the photolysis spectrum superimposed on the calcium binding bands and probably on bands arising from reactions of the photoproduct. Buchet et al. (1991) observed some calcium binding bands similar to those discussed here, although the photolysis spectrum of Nitr-5 prevented a good resolution of the bands in the 1600–1500 cm^{-1} range. To observe only

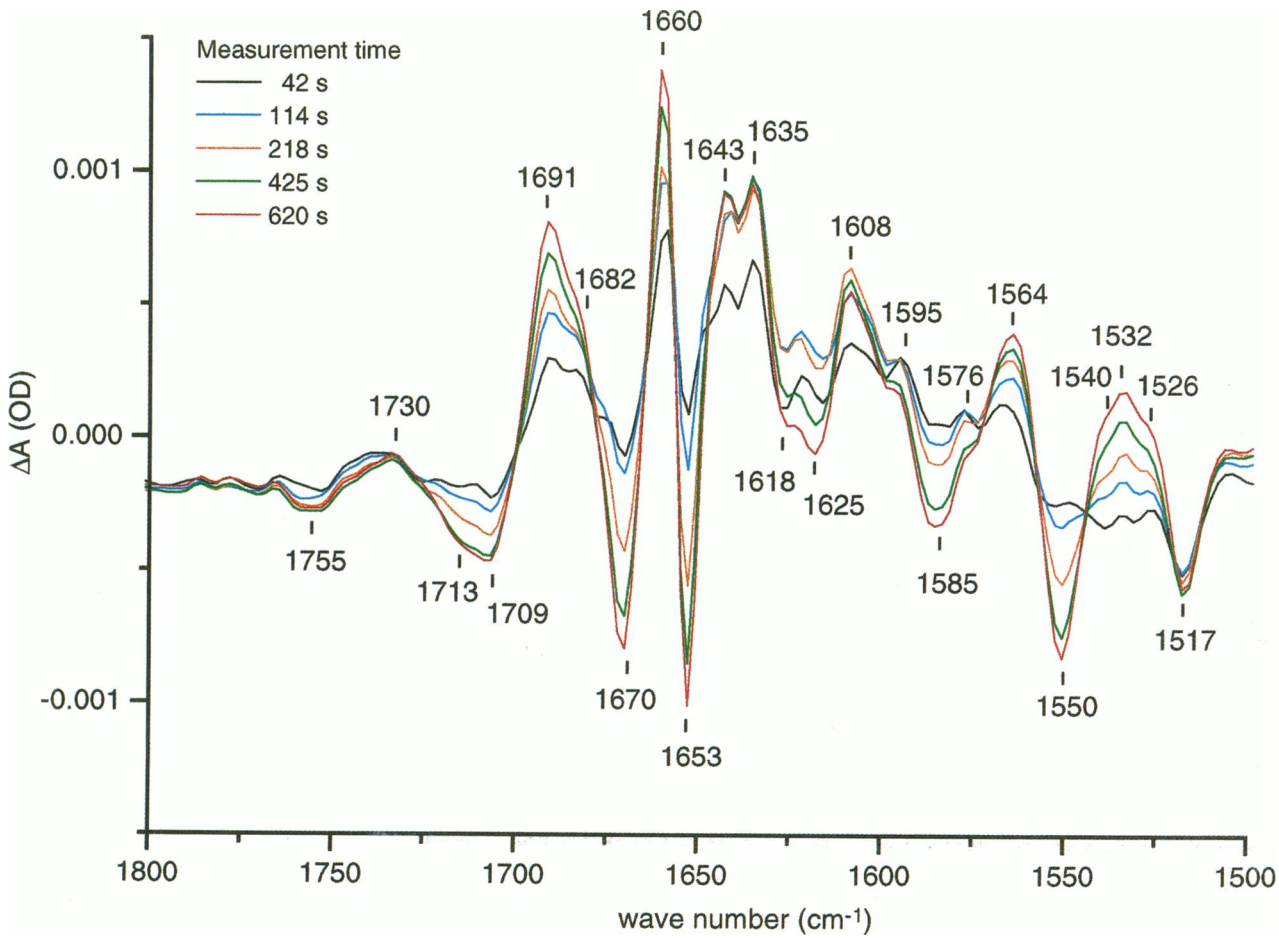


FIGURE 5 IR difference spectrum induced by high-affinity calcium binding to Ca^{2+}-ATPase. The +Ca +thapsi difference spectra (see Fig. 4 *d*) were substracted from the +Ca difference spectra (see Fig. 4 *c*) over the whole kinetics duration as described in Materials and Methods. The result spectra are shown for the indicated measuring times. This procedure makes it possible to observe only the bands induced by high-affinity calcium binding on the Ca^{2+}-ATPase. The baseline of the spectra exhibits a bell-like shape from 1700 to 1560 cm^{-1}.

TABLE 1 Tentative assignment of the IR bands induced by high-affinity calcium binding to Ca^{2+}-ATPase protein structures

	Amino acid side-chain absorbance frequencies		Protein backbone absorbance frequencies		
			All proteins	Ca^{2+}-ATPase	
Ca^{2+} binding bands	Asp, Glu*	Other amino acids*	assignment to secondary structures#§	Lee and Chapman (1986)	Villalain et al. (1989)
−1755					
+1730					
−1713	COOH				
−1709					
+1691			Amide I: β	1690 t	1690 t
+1682		Asn CO	t, β	1681 β	1679 β
−1670		Gln CO	t		1667 t
		Arg CN_3H^{5+}as			
+1660			t, α		
−1653			α	1657 α	1657 α
+1643			α, random	1643 α	1646 α
+1635		Arg CN_3H^{5+} s	β	1630 β	1634 β
−1625		Asn NH_2	β		1623 βt
		Lys NH^{3+} as			
−1618					
+1608		Gln NH_2			
+1595					
−1585					
+1576	Asp COO^- as				
+1564	Glu COO^- as		Amide II: β		
−1550			α, random		
+1540					
+1532			β	1531 β	
+1526		Lys NH^{3+} s			
−1517		Tyr-OH ring			

*Venyaminov and Kalnin (1990a).
#Goormaghtigh et al. (1994).
§Venyaminov and Kalnin (1990b).

the calcium binding bands, Georg et al. subtracted combined control spectra measured in the absence of calcium and in the presence of excess calcium from the spectrum containing the calcium binding bands. By using thapsigargin we have been able to design a more rigorous control condition with a calcium concentration identical to that in the calcium binding experiment. We observe the same main calcium binding bands as Georg et al. In addition, we observe a difference in the amplitude of the 1660 cm^{-1} band and additional bands at 1691, 1625, 1595, 1576, 1540, and 1526 cm^{-1}. Because the kinetic evolution of these bands is in agreement with that of all the other bands (see below), we assume that these bands are indeed induced by high-affinity calcium binding. These deviations may thus be due to the different control procedure used by Georg et al. or the different experimental conditions used by these authors.

Kinetic measurements of the IR bands induced by calcium binding

The kinetics of the high-affinity calcium binding bands were analyzed from the set of spectra, some of which are shown in Fig. 5. No signal change could be observed at the frequencies of the DM-N/DTT photoproduct bands (Fig. 3), showing that these bands were correctly subtracted. Ninety percent of the amplitude of the large bands of DM-N photoproduct reaction with the vesicles (Fig. 4, *c* and *d*) was compensated over the whole kinetics. Remaining drifts of about 10% of the amplitude of the large bands were observed at the isobestic points of the high-affinity calcium binding spectra (Fig. 5) situated in the spectral range of the large bands, between 1660 and 1580 cm^{-1}. These drifts were corrected as described in Materials and Methods.

The kinetics of some representative bands are shown on Fig. 6. All of the kinetics could be fitted with single exponential curves of similar rate constant. The mean rate constant is equal to 0.0026 s^{-1} with a standard deviation of 27%.

Calcium binding in our experiments is slower than the fastest photoproduct reaction we have observed, which was achieved within 40 s. Therefore we can conclude that the velocity of the high-affinity calcium binding reaction in our experiments was not limited by diffusion, in spite of the high viscosity of the IR samples.

DISCUSSION

We have identified the infrared absorbance changes induced by calcium binding at the high-affinity transport sites of

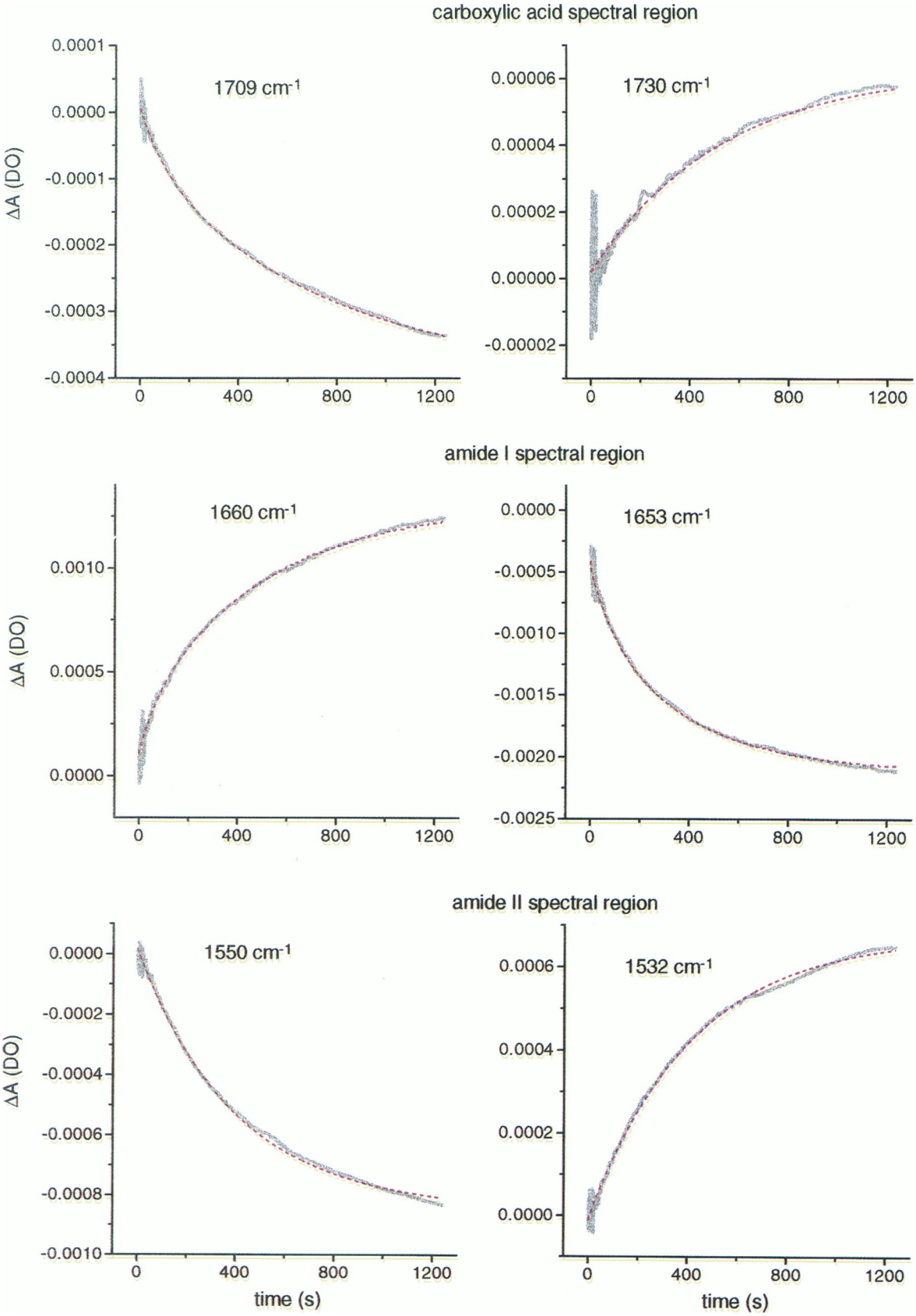


FIGURE 6 Kinetics of the calcium binding bands. The kinetics of the calcium binding bands were analyzed from the set of spectra shown on Fig. 5, as described in Materials and Methods. Six representative curves taken from three different spectral regions are shown, of the 23 bands analyzed. All of the kinetics can be fitted with single exponential curves of rate constant 0.0026 ± 0.0007 s^{-1}.

Ca^{2+}-ATPase. By using thapsigargin, a specific inhibitor of the SERCA pumps that is known to inhibit calcium binding to Ca^{2+}-ATPase quasi-irreversibly (Sagara et al., 1992), we have performed a control with the same calcium concentration as in the calcium binding experiments. We have used the spectra measured in the presence of thapsigargin to correct the calcium binding spectra from a band that may be induced by a DM-nitrophen photoproduct reaction with the vesicles. Using this procedure, we confirm the main features of the calcium binding bands obtained by Georg et al. (1994), who used a combination of control spectra measured in the presence of different calcium concentrations. However, we have observed additional calcium binding bands, and we report the absence of all bands when the Ca^{2+}-ATPase is complexed with the inhibitor thapsigargin. Moreover, we have measured the kinetics of appearance of the calcium binding bands at low temperature.

The calcium binding bands we have observed are listed in Table 1, along with the usual absorbance frequencies of amino acid side chains and protein secondary structures. Absorbance parameters of amino acid side chains showing significant absorption intensity in the 1800–1480 cm^{-1} region were determined in aqueous solution by Venyaminov and Kalnin (1990a). The absorbance maxima determined by these authors may be shifted by interactions with the protein environment, especially hydrogen bonding. Known spectral ranges of such shifts are indicated by vertical lines (Colthup et al., 1990). Absorbance frequencies of the different types of secondary structures are known from the analysis of the IR absolute absorbance spectrum in the amide I and to a less extent in the amide II regions (see Surewicz et al., 1993; Goormaghtigh et al., 1994, for a review). The usual absorbance frequencies of the carbonyl (amide I, 1690–1610 cm^{-1}) and CNH vibration (amide II, 1570–1510 cm^{-1}) of the peptide bond in the different secondary structures are indicated in the last columns of Table 1, together with the amide bands observed by Lee and Chapman (1986) and Villalain et al. (1989) in the IR spectrum of Ca^{2+}-ATPase.

Involvement of protein backbone in calcium binding

Movements of the protein backbone are linked to modifications in hydrogen bonding and the bond angle of peptide groups. Such modifications produce a shift of the absorbance frequency of the carbonyl and CNH vibrations of the peptide unit, which induce the appearance of couples of positive and negative bands when studied by difference spectroscopy. We indeed observe several positive and negative bands in both amide I and II regions.

The bands at 1653 and 1660 cm^{-1} very probably arise from main-chain carbonyl vibrations, because no other protein structure is known to absorb at these frequencies. The frequency 1653 cm^{-1} corresponds to the carbonyl absorbance frequency, most likely of α-helical structures (Goormaghtigh et al., 1994; Venyaminov and Kalnin, 1990b). The bands at 1660 and 1643 cm^{-1} could also be induced by movements of α-helices or of turn and random structures, respectively (Goormaghtigh et al., 1994; Venyaminov and Kalnin, 1990b).

Some amino acid side chains are known to absorb in the same spectral range as the amide II band, but it seems likely that some calcium binding bands in this region arise from the protein backbone. The band at 1564 and at 1550 cm^{-1} could be induced by β and α or random structures, respectively, or by the asymmetrical stretching vibration of COO^- side chains. The band at 1532 cm^{-1} is likely an amide II band. This frequency has been assigned to β-sheets (Venyaminov and Kalnin, 1990a,b; Goormaghtigh et al., 1994).

The largest amide I absorbance change upon calcium binding occurs at 1653 cm^{-1} and represents about 0.2% of the total protein absorbance at this frequency. A secondary structure analysis of Ca^{2+}-ATPase has shown that about 23% of the peptide groups of the enzyme absorb around this frequency (1657 cm^{-1} band; Villalain et al., 1989; see Table 1), which correspond to about 230 peptide groups. This shows that the amplitude of the calcium-induced absorbance change at 1653 cm^{-1} is on the same order of magnitude as the absorbance of the carbonyl of a single peptide unit. The same evaluation applies to the other bands in the 1690–1610 cm^{-1} (amide I) and 1570–1510 cm^{-1} (amide II) ranges. If these bands are indeed peptide backbone bands, we believe that each of them most probably represent absorbance change of a single peptide unit rather than smaller absorbance changes of several peptide groups adding together at the same frequency. Indeed, this last situation would result from small shifts of broad absorbance bands, which would in turn induce difference bands of larger width than observed here. It seems unlikely to us that the narrow bands we observe may mask broader bands. If we assume that all of the bands in the amide I and II regions are peptide backbone bands, calcium binding involves a maximum number of about 10–15 carbonyl and CNH groups of the main chain. This number can be underestimated if some very narrow bands are hidden by others. This could be the case for the larger bands we have observed, but this would not significantly modify our evaluation. This shows that calcium binding probably induces only limited secondary structure changes of the enzyme, as already suggested by CD studies (Girardet and Dupont, 1992).

The structural model of Ca^{2+}-ATPase proposes that the transmembranous domain is made of 10 α-helices, whereas the cytosolic domain also contains β-sheet structures. Different kinds of experimental results suggest that the transport sites lie in the membranous part of the protein and would thus be distant from the catalytic site, which is located within the cytoplasmic domain of the enzyme (see Inesi et al., 1994, for a review). On the other hand, it is known that Ca^{2+}-ATPase undergoes some secondary structure change upon calcium binding, which is related to a change in the reactivity of the phosphorylation site.

The band at 1653 cm^{-1} corresponds to the modification of a peptide group most likely located in an α-helix. This

bond could be involved in some movements of an α-helix that could occur in the vicinity of the binding sites or in other putative helical parts of the protein such as the stalk between the membranous and the cytoplasmic domain, as proposed by Inesi et al. (1994).

Other bands in our spectra could be induced by modifications of β-sheets, turns, or random structures (Surewicz et al., 1993; Goormaghtigh et al., 1994). According to the structural model, modifications of β-sheet structures upon calcium binding would be situated in the cytoplasmic domain. X-ray diffraction studies showing that high-affinity calcium binding induces changes in the electron density profile corresponding to the cytoplasmic domain of the ATPase would also be in favor of this hypothesis (DeLong and Blasie, 1993). Another hypothesis is that the membranous domain contains structures other than α-helices. This has already been suggested by Andersen and Vilsen (1994) on the basis of mutagenesis studies showing that the putative position of Asn796, Thr799, and Asp800 in a channel-like structure is not compatible with their position on an α-helix.

One of the band we observe in the amide I region could be induced by a carbonyl group of the main chain playing the role of a calcium ligand. McPhalen et al. (1991) have analyzed the structure of the calcium sites of 17 crystallized proteins and found that all of the sites have at least one peptide carbonyl oxygen atom ligand in their calcium coordination sphere. Amide I bands that are assigned to turn structures are candidates for such bonds, because such ligands are generally not part of a regular secondary structure (Glusker, 1991).

Water molecules are also often found in the coordination sphere of calcium ions (McPhalen et al., 1991). In this regard, it may be noted that some bands in the 1620–1680 cm^{-1} amide I region could be induced by the OH vibration of a water molecule (Schiöberg and Luck, 1979).

Involvement of amino acid side chains in calcium binding

Among all possible absorbing groups of a protein, only COOH groups induce bands above 1700 cm^{-1} because of the carbonyl stretching vibration (Venyaminov and Kalnin, 1990a). Therefore, the calcium binding bands we observe between 1800 and 1700 cm^{-1} are very probably induced by reactions of aspartic or glutamic acid side-chain COOH groups. The bands between 1800 and 1735 cm^{-1} probably correspond to COOH that are not hydrogen bonded, whereas the bands between 1730 and 1705 cm^{-1} are likely induced by hydrogen-bonded COOH (Colthup et al., 1990). The large width of the 1709–1713 cm^{-1} band indicates that thinner bands are probably overlapping at these frequencies. Some of the negative COOH bands very likely correspond to deprotonations, the absorbance of the COO^- group being shifted in the 1610–1540 cm^{-1} range. Couples of positive and negative bands, involving the positive 1730 cm^{-1} band, could be induced by a small frequency shift of the COOH absorbance upon changes in the hydrogen bonding strength on the carbonyl group.

In agreement with these observations, we observe several positive bands in the absorbance region of the COO^- asymmetrical stretching vibration (1610–1540 cm^{-1}; Venyaminov and Kalnin, 1990a). COO^- vibration frequency depends on the number of coordinations of the oxygen atoms with divalent metal ions, as evidenced by Nara et al. (1994) in the case of parvalbumin. Different calcium binding bands in the 1610–1540 cm^{-1} range in our spectra could arise from the coordination of COO^- groups with one or both of the bound calcium ions (Tackett, 1989).

Other amino acid side chains showing significant absorption in the amide I region are asparagine, glutamine, and arginine (see Table 1). Some of the calcium binding bands we observe may be due to these residues. The band at 1517 cm^{-1} occurs at the limit of the amide II region, but also at the exact tyrosine ring absorbance frequency.

These results are relevant to mutagenesis studies performed on Ca^{2+}-ATPase. The mutation of tyrosine 763 has been shown to induce uncoupling between calcium transport and ATP hydrolysis, and it has been suggested that the tyrosine side chain could play the role of a cytoplasmic gate for the calcium binding sites (Andersen and Vilsen, 1995). Moreover, the mutations of glutamate 309, 771, 908; aspartate 800; asparagine 796; and threonine 799, which are proposed to be located in the transmembranous domain of Ca^{2+}-ATPase, abolish the calcium dependence of the phosphorylation of Ca^{2+}-ATPase by ATP or P_i Clarke et al., 1989). The authors have proposed that these residues provide ligands for one or both calcium-binding sites. On the other hand, kinetic studies of Ca^{2+}-ATPase have shown that calcium binding at the high-affinity sites is preceded by the dissociation of two or three protons (Wakabayashi et al., 1990; Forge et al., 1993b). Our data show that some aspartic and/or glutamic acid side chains play a role in calcium binding by undergoing a deprotonation.

We have observed that all of the IR bands induced by calcium binding undergo a monophasic kinetic evolution. At the pH of the experiment, this step very probably coincides with the slow rate-limiting proton dissociation step, which has been evidenced at room temperature at acidic pH by Forge et al. (1993b). A pH-driven conformational change of the enzyme preceding calcium binding has been proposed by Pick and Karlish (1982) and Wakabayashi et al. (1990). Calcium binding has also been shown to be monophasic at the pH we have used at 0°C (Nakamura, 1989).

The calcium binding rate in our experiments (0.0026 s^{-1}) is about four times slower than the rate of calcium binding observed at the same temperature in vesicular suspension by measuring the intrinsic fluorescence of Ca^{2+}-ATPase (0.012 s^{-1}, pH 7.2, 5 mM magnesium; Dupont, 1982). The slower rate we have observed can be nearly entirely accounted for on one hand by the lower pH we have used and on the other hand by the absence of magnesium in the IR experiments. A reduction in the rate of calcium binding by

a factor of 3 was demonstrated at 20°C by Forge et al. (1993b) at pH 6 without magnesium compared to pH 7 in the presence of magnesium. This reduction is due to the occurrence at acidic pH of slow limiting steps involving the dissociation of protons, which are progressively eliminated as pH and magnesium concentration are increased. Our results demonstrate the suitability of the low-temperature method to measuring enzyme kinetics on a nonphotoactivable protein by FTIR spectroscopy.

The structural information available from FTIR spectroscopy, in combination with the possibility of measuring enzyme kinetics, opens new possibilities to obtain detailed insight into the mechanism of coupling between ATP hydrolysis and the translocation of calcium. The analysis of the IR bands induced by calcium binding shows that most bands should be affected by point mutations of the enzyme, allowing us to understand the role of individual amino acids in the reaction mechanism of the enzyme. Future studies using other caged compounds and studies of mutants should therefore help us to understand the chemical and structural basis of ionic translocation and specificity, energy changes in the acylphosphate bond, and relations between the catalytic and transport sites.

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