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Ca^{2+} release from caged-Ca^{2+} alters the FTIR spectrum of sarcoplasmic reticulum

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Light-induced Ca^{2+} release from the Ca^{2+} complex of Nitr-5 altered the FTIR spectra of sarcoplasmic reticulum vesicles and purified Ca^{2+}-ATPase preparations. The principal changes seen in difference spectra obtained after and before illumination in the presence of Nitr-5 · Ca^{2+} consisted of an increase in absorbance at 1663 and 1676 cm^{-1} and a decrease in absorbance at 1653 cm^{-1}. The light-induced changes in FTIR spectra were prevented by vanadate or EGTA, indicating that they were associated with the formation of Ca_2E_1 enzyme intermediate. Other light-induced changes in the FTIR spectra at 1600–1250 cm^{-1} were not clearly related to the sarcoplasmic reticulum, and were attributed to photolysis of Nitr-5. The difference absorbance bands are narrow, suggesting that they originate from changes in side chain vibrations, although some changes in secondary structures may also contribute.

Introduction

The Ca^{2+}-ATPase of sarcoplasmic reticulum alternates during Ca^{2+} transport between states of high ($K_D \simeq 10^{-7}$ M) and low ($K_D \simeq 10^{-3}$ M) Ca^{2+} affinity [1,2]. The binding of Ca^{2+} to the high affinity Ca^{2+} sites of the nonphosphorylated enzyme yields the Ca_2E_1 intermediate, accompanied by an increase in intrinsic tryptophan fluorescence [3] and a decrease in the fluorescence of covalently attached FITC [4]. The bound Ca^{2+} modulates the reactivity of the enzyme with ATP and P_i at the catalytic site, some $\simeq 30$ Å away [1], and reciprocally, phosphorylation of the Ca^{2+}-ATPase by ATP affects its Ca^{2+} affinity [1]. Although these observations clearly indicate an effect of Ca^{2+} on the conformation of Ca^{2+}-ATPase, Ca^{2+} did not alter the circular dichroism spectrum of Ca^{2+}-ATPase [5,6], and there are conflicting reports on Ca^{2+}-induced changes in the FTIR spectrum [7–9].

Here we present new evidence indicating that light-induced Ca^{2+} release from the Nitr-5-Ca^{2+} complex [10–12] produces clearly demonstrable changes in the FTIR spectrum of sarcoplasmic reticulum that are associated with the binding of Ca^{2+} to the Ca^{2+}-ATPase.

Experimental procedures

Materials

Adenosine 5′-triphosphate, dithiothreitol, EGTA, imidazole, NADH, magnesium chloride, lactate dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), phospho*enol*pyruvate, bovine serum albumin and Trizma base were obtained from Sigma (St. Louis, MO). Calcium chloride was obtained from Baker Chemical (Phillipsburg, NJ) and A23187 from Behring Diagnostics (La Jolla, CA). Sodium vanadate, sucrose and sodium deoxycholate were the products of Fisher Scientific (Fairlawn, NJ). All chemicals were of analytical grade. Nitr-5 was obtained from Calbiochem (La Jolla, CA), Fluo-3 (calcium indicator) dye from Molecular Probes (Eugene, OR).

Methods

Sarcoplasmic reticulum vesicles were prepared as described earlier [13]. For FTIR measurements the

Abbreviations: SR, sarcoplasmic reticulum; Ca^{2+}-ATPase, Ca^{2+}, Mg^{2+}-stimulated ATPase of sarcoplasmic reticulum (EC 3.6.1.38), Nitr-5, 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 tetrasodium salt; FITC, fluorescein 5′-isothiocyanate; DTT, dithiothreitol.

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sarcoplasmic reticulum suspensions were diluted 10-fold in a ^2H_2O medium containing 0.1 M KCl, 10 mM imidazole (p^{2}H 7.0), 0.5 mM $MgCl_2$ and 1 mM EGTA. After incubation for 2 h in ice the suspensions were centrifuged for 40 min at 80000 × *g*. The sediment was suspended in 10 ml EGTA-free medium, homogenized and centrifuged as above. The final sediment was suspended in 1 ml ^2H_2O medium containing 0.1 M KCl, 10 mM imidazole (p^{2}H 7.0) and 0.5 mM $MgCl_2$. Further additions were made as described in the legends.

Purified ATPase was prepared essentially as described by Meissner et al. [14] by suspending microsome stock suspension (1.77 mg protein/ml) in 0.6 M KCl, 0.3 M sucrose, 20 mM Tris-HCl (pH 8.0), 1 mM EGTA, and 0.6 mg deoxycholate/ml. After gentle stirring for 10 min at 0 °C, the sample was centrifuged at 80000 × *g* for 1 h. The sediment was suspended in ^2H_2O medium containing 0.1 M KCl, 10 mM imidazole (p^{2}H 7.0) and 0.1 mM $MgCl_2$ and sedimented as above. The washing in ^2H_2O medium was repeated once more, followed by resuspension in ^2H_2O medium containing 0.1 M KCl, 10 mM imidazole (p^{2}H 7.0), 0.5 mM $MgCl_2$ and 1 mM Nitr-5 for FTIR measurements.

FTIR spectra were analyzed on a Mattson Cygnus 100 spectrometer using MCT detector and temperature controlled cells (Model TFC-M25; Harrick Scientific, Ossining, NY) equipped with barium fluoride windows and 50 μM spacers. The FTIR spectrometer was modified for photolysis of caged-Ca^{2+} by opening an extra window on the housing and inserting a germanium mirror into the lightpath, that reflected the 360 nm light onto the sample cell. A 500 W high pressure mercury xenon arc lamp (type USH 508SA) operated with an Oriel type 8530 power supply in a type 66011 housing was used as light source for photolysis. The light beam after passing through a water heat filter and a Corion 5840 (CS 7-60) ultraviolet absorbing filter was focused with quartz lenses on the sample cell.

Routinely 64 scans were collected, co-added, zero-filled once and apodized with triangular function. The resolution was 4 cm^{-1}. The spectra were recorded before and immediately after 1 min illumination with light of predominantly 360 nm wavelength. The difference absorbance (ΔA) was calculated according to

$$\Delta A = -\log \frac{I \text{ after}}{I \text{ before}}$$

where *I* after and *I* before correspond, respectively, to the spectra taken after and before U.V. illumination. The spectra presented in this report represent the averages of 6–16 difference spectra taken on individual samples under identical experimental conditions.

The effect of Nitr-5 on the ATPase activity was measured using the coupled enzyme assay, as described earlier [15], except that Ca^{2+} and EGTA were not added to the assay solutions. The Ca^{2+} concentration of the assay system was $\simeq 10^{-5}$ M, introduced with the sarcoplasmic reticulum and with other assay components. When the Ca^{2+} concentration was varied the concentration of Nitr-5 was adjusted to optimize the free Ca^{2+} concentration after photolysis.

The free Ca^{2+} concentration of the assay medium was determined using Fluo-3 as Ca^{2+} indicator [16–18]. The fluorescence intensity was measured in an SLM 4800 or in a Varian SF 330 fluorescence spectrometer using light of 495 nm for excitation and 525 nm for emission.

Protein concentration was determined according to Lowry et al. [19]. The total Ca^{2+} concentration was assayed in a Perkin-Elmer atomic absorption spectrometer (Model 3030).

Results

The dissociation constant of Nitr-5 for Ca^{2+} increases from $K_D \simeq 145$ nM to $K_D \simeq 6$ μM [11,12] after photochemical transformation into its nitrosoketone decomposition product (Fig. 1). The light-induced Ca^{2+} release from the Nitr-5-Ca^{2+} complex activates the hydrolysis of ATP by the Ca^{2+}-ATPase (Fig. 2) by increasing the free Ca^{2+} concentration of the medium. The change in free Ca^{2+} concentration can be monitored by the fluorescence of the Ca^{2+}-sensitive fluorophore Fluo-3 (Fig. 3). Addition of Nitr-5 to the dark Ca^{2+}-containing assay medium decreased the free Ca^{2+} concentration, as shown by the decrease in Fluo-3 fluorescence (Fig. 3), and the inhibition of ATPase activity (Fig. 2). Illumination of the system for 1 min with light of 360 nm causes extensive decomposition of the Nitr-5 into nitrosoketone of lower Ca^{2+} affinity, as shown by the increase in Fluo-3 fluorescence (Fig. 3) and ATPase activity (Fig. 2).

The FTIR spectrum of Nitr-5

Nitr-5 (25 mM) suspended in a standard buffer of 0.1 M KCl, 10 mM imidazole (p^{2}H 7.0) and 0.5 mM $MgCl_2$ gave the FTIR spectrum shown in Fig. 4A. The bands at 1584 cm^{-1} and perhaps at 1510 cm^{-1} are tentatively assigned to the C-C stretching vibrations of the aromatic rings, but a contribution by carboxylate groups at 1584 cm^{-1} is also possible. The bands at 1403, 1434, 1457 and 1485 cm^{-1} are presumably associated with $-CH_2$ and CH_3 deformations. Ca^{2+} (5 mM) increased the absorbance of nitr-5 at 1587 and 1620 cm^{-1} (Fig. 4B). The effect of illumination on the FTIR spectrum of Nitr-5 was measured in the absence of dithiothreitol (Fig. 4C) either in a Ca^{2+}-free medium containing 2.5 mM EGTA (sample 1), or at Ca^{2+} concentrations of $\simeq 0.01$ mM (sample 2), 0.25 mM (sample 3) and 2.5 mM (sample 4), respectively. As shown by the difference between the spectra obtained

after illumination and before illumination (Fig. 4C), the conversion of Nitr-5 into 2-nitrosoacetophenone is associated with the following changes in the FTIR spectra. The small band seen at 1640–1630 cm^{-1} at low Ca^{2+} concentration (samples 1 and 2) increased in intensity and shifted to 1655 cm^{-1} at 2.5 mM Ca^{2+} concentration (sample 4). The intense band at 1588 cm^{-1} in the absence of Ca^{2+} (sample 1) shifted to 1601 cm^{-1} at 2.5 mM Ca^{2+} (sample 4) without much change in intensity. As expected, the light-induced conversion of Nitr-5 into 2-nitrosoacetophenone decreased the intensity of the asymmetric and symmetric stretching vibrations of the NO_2 group at 1509 cm^{-1} and at 1341 cm^{-1}, respectively. There were small changes in intensity also at 1522, 1387 and 1365 cm^{-1}.

In the presence of dithiothreitol (3 mM) the light-induced difference spectra of Nitr-5 are simplified (Fig. 4D). The Ca^{2+}-dependent change in the intensity of the band at 1629–1624 cm^{-1} is still observed, but DTT prevented the shift of this band to 1655 cm^{-1} at 2.5 mM Ca^{2+} concentration (Fig. 4D versus Fig. 4C). The large negative band at 1629 cm^{-1} and the positive band at 1578 cm^{-1} in the presence of EGTA + Ca^{2+} (samples 1 and 2; Fig. 4D) is due to the binding of Ca^{2+} released from photolysed Nitr-5 to EGTA. Ca^{2+}-dependent changes were also observed at 1582–1578, 1522 and 1328 cm^{-1}, that may be related to carboxylate groups.

Dithiothreitol protects protein side chain groups from reaction with nitrosoketone, and for this reason most experiments were performed with and without dithiothreitol in the reaction system.

The light-induced changes in the difference spectrum of Nitr-5 are of the order of 0.005 absorbance

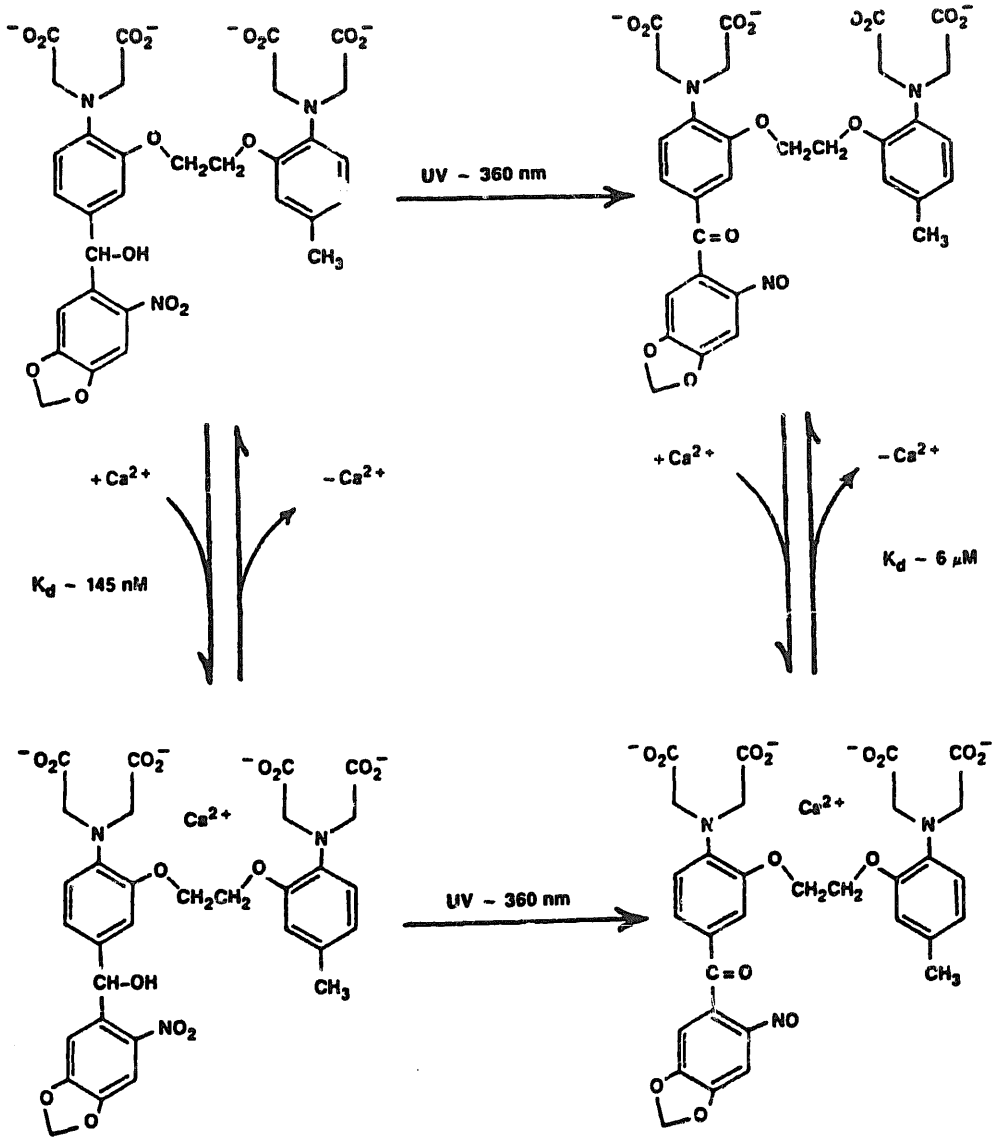

Fig. 1. The structure of Nitr-5. Nitr-5 is converted upon illumination by light of 360 nm into a nitrosoketone with increase in its K_D for Ca^{2+} from 0.145 nM to 6 μM at an ionic strength of 0.1, pH 7.4 and room temperature.

unit or less (Fig. 4C and D). These changes represent the background for the measurements performed in the presence of sarcoplasmic reticulum (see below).

The FTIR spectrum of sarcoplasmic reticulum in the presence of caged-Ca^{2+}

The FTIR spectrum of sarcoplasmic reticulum vesicles suspended in 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 0.5 mM $MgCl_2$, 0.32 mM total Ca^{2+} and 2.5 mM Nitr-5 is shown in Fig. 5. The band at $\simeq 1728\ cm^{-1}$ is due to the C=O stretching vibration of phospholipids. The Amide I band at 1647 cm^{-1} arises mainly from C=O stretching vibrations of the peptide backbone, together with vibrations of protein side chains. The 1575 cm^{-1} region reflects primarily the side chain vibrations of aspartic and glutamic acids.

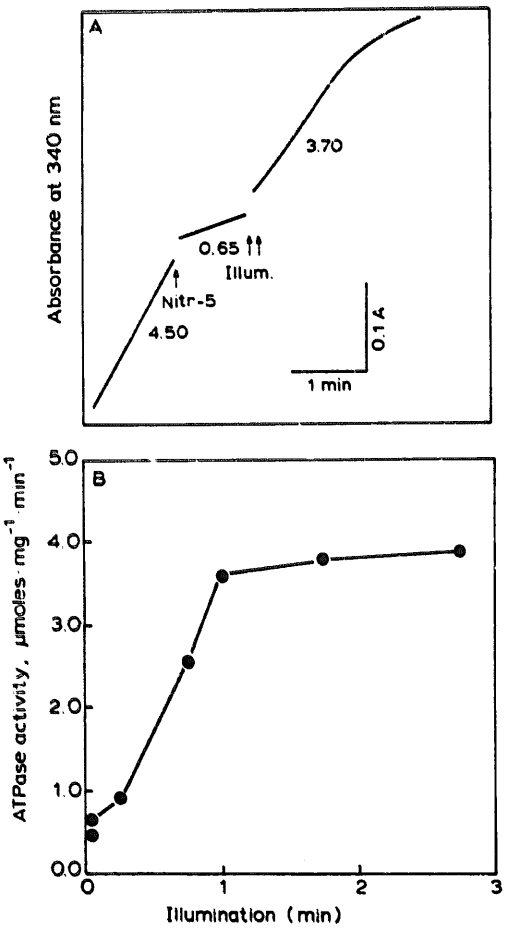

Fig. 2. Effect of Nitr-5 on ATPase activity. (A) ATPase activity was measured at a protein concentration of 5 μg/ml in the coupled assay system [15] at a Ca^{2+} concentration of $\simeq 10^{-5}$ M. At the arrow, Nitr-5 was added to a concentration of 50 μM. At double arrow the sample was illuminated for 1 min with 360 nm light and the assay of activity was resumed immediately afterward. The addition of Nitro-5 and the illumination caused an increase in optical density that was immediately adjusted to the level before Nitr-5 addition. The numbers represent the ATPase activities expressed in μmole mg^{-1} min^{-1}. (B) The effect of illumination time on the ATPase activity of sarcoplasmic reticulum was analyzed in the presence of 50 μM Nitr-5 under the experimental conditions described in (A).

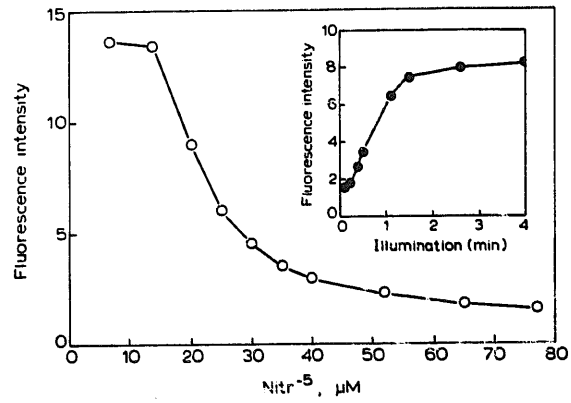

Fig. 3. Changes in free Ca^{2+} concentration monitored by Fluo-3 upon addition of Nitr-5 to sarcoplasmic reticulum. To sarcoplasmic reticulum vesicles (100 μg/ml) suspended in 0.1 M KCl, 0.5 mM $MgCl_2$ and 10 mM imidazole (pH 7.0), Fluo-3 (0.1 μM) was added as Ca^{2+} indicator and the fluorescence was monitored at 525 nm using light of 495 nm for excitation. The decrease in free Ca^{2+} concentration caused by Nitr-5 at concentrations indicated on the abscissa is recorded as a decrease in Fluo-3 fluorescence. At 77 μM Nitr-5 concentrations the free Ca^{2+} concentration was less than 10 nM. Insert. The above system containing 77 μM Nitr-5 was illuminated with a 500 W high pressure mercury xenon arc lamp for times ranging between 5 and 455 s and the fluorescence of Fluo-3 was measured. The Ca^{2+} release was nearly complete after 60 s of illumination. The recovery of fluorescence is partial due to photodecomposition of Fluo-3 ($\simeq 8\%$ in one minute) and the retention of significant Ca^{2+} affinity ($K_D = 6\ \mu M$) by the nitrosoketone photolysis product of Nitr-5.

The residual Amide II band at 1548 cm^{-1} is assigned to C=N stretching and N-H deformation in regions of the molecule that did not exchange with ^2H, while the small peak at 1515 cm^{-1} may be due to the ring stretching of tyrosine. The 1459 cm^{-1} band is a composite of HO^2H and CH_2 deformations in phospholipids and proteins.

Illumination of the system containing sarcoplasmic reticulum vesicles and caged-Ca^{2+} produces significant changes in the FTIR spectra throughout the spectra range shown in Fig. 6A and B. Similar changes were observed using purified Ca^{2+}-ATPase (Fig. 7). The difference spectra indicated by the solid lines in Figs. 6 and 7 represent Ca^{2+}-induced changes in the vibrations of sarcoplasmic reticulum proteins, together with changes caused by the photolysis of Nitr-5. The separation of the contribution of proteins and Nitr-5 to the observed spectra is possible by comparison with the FTIR spectra obtained in the absence of sarcoplasmic reticulum (Figs. 6 and 7, broken lines) or in the presence of sarcoplasmic reticulum, but with vanadate (Fig. 8) or EGTA (Fig. 9) added to prevent the binding of released Ca^{2+} to the Ca^{2+}-ATPase.

The light-induced changes in the spectra at 1676–1677, 1663 and 1653 cm^{-1} and some of the changes at 1632–1633 cm^{-1} are due to Ca^{2+}-induced changes in sarcoplasmic reticulum proteins; these changes are ob-

served both in the absence (Fig. 6A) and in the presence (Fig. 6B) of 3 mM dithiothreitol. The peaks at 1750, 1705 and 1644 cm^{-1} (Fig. 6A and Fig. 7) were observed only in the absence of dithiothreitol and may reflect the reaction of the nitrosoketone photoproducts of Nitr-5 with the protein.

Essentially identical changes were seen after photolysis of caged-Ca^{2+} in systems containing sarcoplasmic reticulum vesicles (Fig. 6A and B) or purified Ca^{2+}-ATPase (Fig. 7). Therefore the Ca^{2+}-induced changes in both systems are attributable to the formation of $Ca_2 \cdot E_1$ complex of the Ca^{2+}-ATPase.

The changes in intensity between 1600 and 1300 cm^{-1}, best seen in Fig. 6B, coincide with bands of the Nitr-5 photoproducts (Fig. 4C,D) and are not related to sarcoplasmic reticulum. The band associated with the photolysis of Nitr-5 at 1595–1600 cm^{-1} decreased in intensity in sarcoplasmic reticulum samples illuminated without dithiothreitol (Fig. 6A and 7). Decreased photolysis due to the absorption of light by the sarcoplasmic reticulum cannot fully explain this difference, since the change in intensity is not proportional in the various bands related to photolysis. It is more likely that the 2-nitrosoacetophenone photoproduct reacts

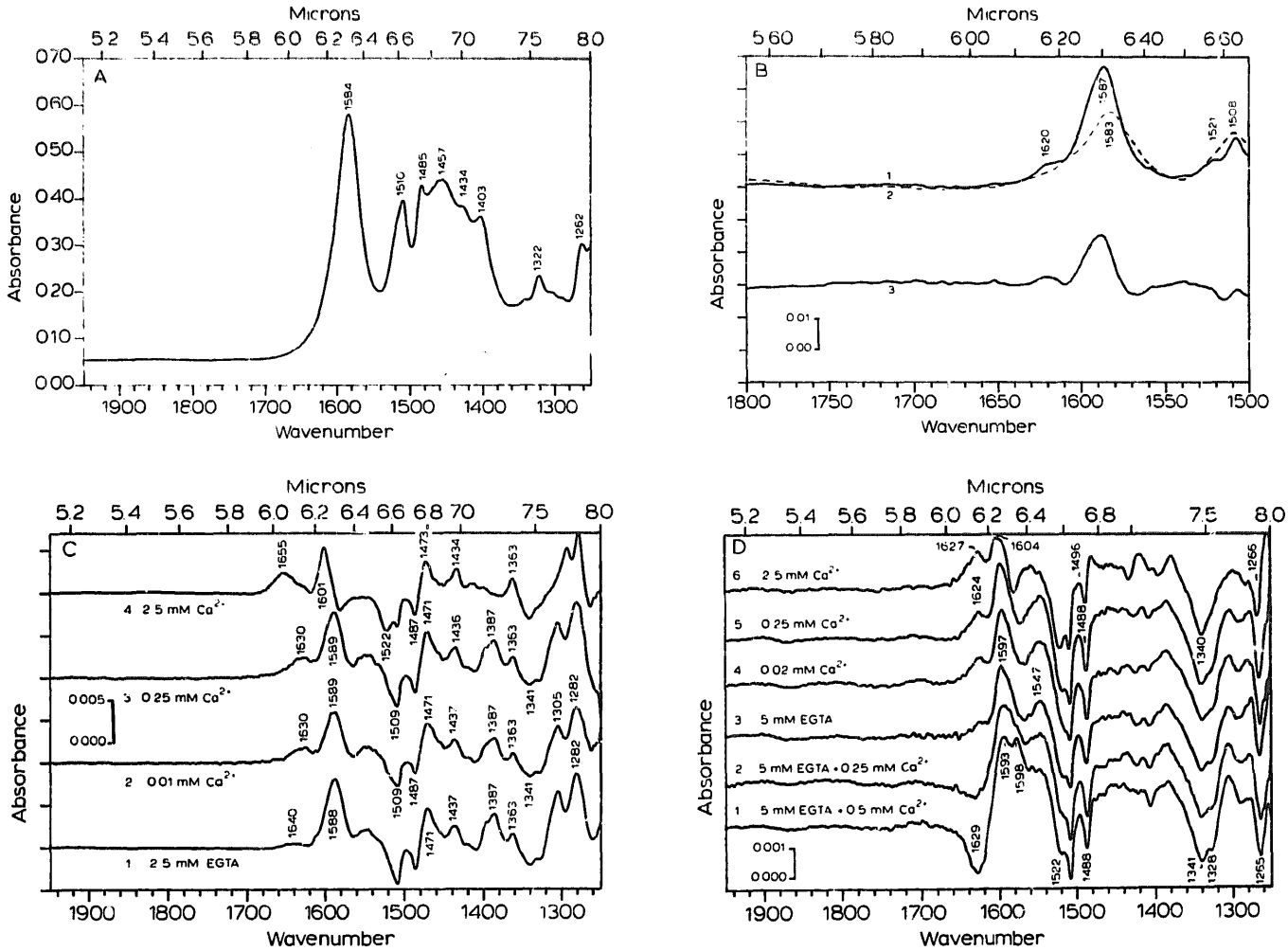

Fig. 4. The FTIR spectrum of Nitr-5. (A) The FTIR spectrum of Nitr-5 (25 mM) was measured in ^2H_2O medium of 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 0.5 mM $MgCl_2$ and 5–10 μM $CaCl_2$. The spectrum was corrected by subtraction of the buffer contribution. The wave numbers of maxima are marked. (B) The FTIR spectrum of Nitr-5 (2.5 mM) was measured in a ^2H_2O medium containing 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 5 mM DTT and either 5 mM Ca^{2+} (line 1) or 0.025 mM Ca^{2+} (line 2). Line 3 in the bottom part of the figure is the difference spectrum of line 1 minus line 2. (C) Difference FTIR spectra of Nitr-5 (2.5 mM) were obtained at various Ca^{2+} concentrations in the absence of DTT, by deducting the spectra taken before illumination from the spectra taken after illumination at 360 nm for 1 min. Buffer composition was the same as in (A) except that the Ca^{2+} concentration was varied by the addition of 2.5 mM EGTA to sample 1, 0.25 mM $CaCl_2$ to sample 3 and 2.5 mM $CaCl_2$ to sample 4. No addition was made to sample 2, which contained ≈ 10 μM Ca^{2+}. (D) Difference FTIR spectra of Nitr-5 (1.5 mM) in ^2H_2O medium containing 3 mM DTT, 100 mM KCl, 10 mM imidazole (p^2H 7.0), 0.5 mM $MgCl_2$ with the indicated additions of Ca^{2+} or EGTA. The spectra taken before illumination were deducted from the spectra taken after 1 min illumination at 360 nm.

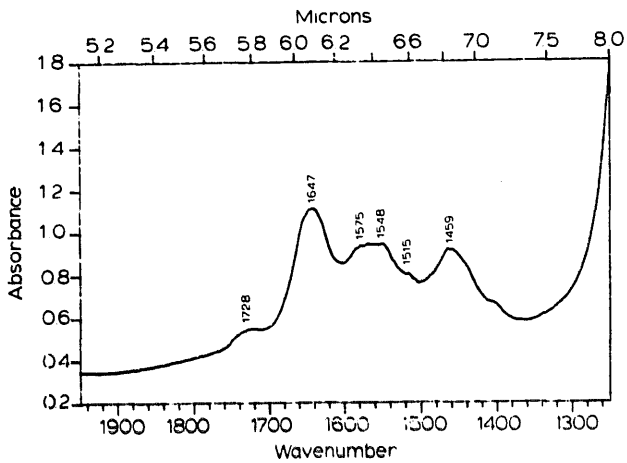

Fig. 5. FTIR spectrum of sarcoplasmic reticulum containing Nitr-5. Sarcoplasmic reticulum vesicles (40 mg protein/ml) were suspended in a ^2H_2O medium of 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 0.5 mM $MgCl_2$, 2.5 mM Nitr-5 and about 0.32 mM total Ca^{2+}. The spectra were taken at 22 °C. The wavenumbers of the principal bands are indicated.

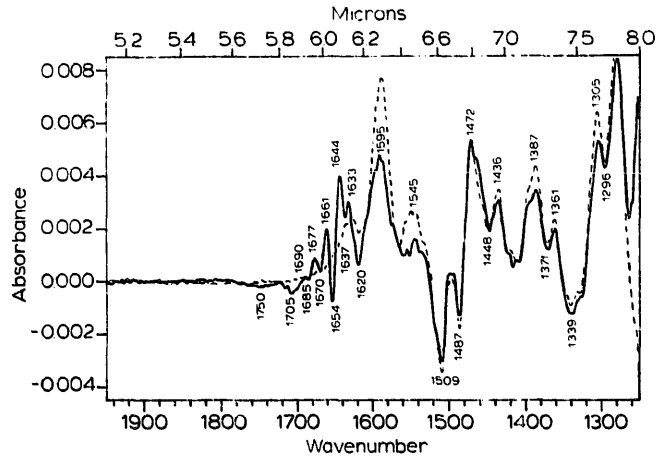

Fig. 7. Difference FTIR spectrum of purified Ca^{2+}-ATPase in the presence of Nitr-5. Purified Ca^{2+}-ATPase preparations (27 mg protein/ml) were suspended in a ^2H_2O medium of 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 0.5 mM $MgCl_2$ and 1 mM Nitr-5, at a total Ca^{2+} concentration of ≈ 0.1 mM. The spectrum shown is the average of eight difference spectra taken after – before illumination for 1 min (solid line). As control the same experiments were also performed in the absence of sarcoplasmic reticulum (broken line).

with the sarcoplasmic reticulum causing a decrease in its absorption at 1595–1600 cm^{-1} without interfering either with the Ca^{2+}-induced changes in the Amide I region of the sarcoplasmic reticulum spectrum or with the activation of ATP hydrolysis by Ca^{2+}. Inclusion of 3 mM dithiothreitol in the reaction system containing sarcoplasmic reticulum prevented the decrease in the intensity of the 1600 cm^{-1} band (Fig. 6B).

The effect of vanadate

Vanadate interacts with the Ca^{2+}-ATPase in the E_2 conformation reducing its affinity for Ca^{2+} [3,4]. Therefore vanadate is expected to inhibit the Ca^{2+}-induced structural change of the Ca^{2+}-ATPase during Ca^{2+} release from photolysed Nitr-5. Indeed, 2 mM vanadate (Fig. 8) completely prevented the appearance of the bands at 1676, 1663 and 1653 cm^{-1}, that are induced by Ca^{2+} binding to the Ca^{2+}-ATPase in vanadate-free systems (Fig. 6 and 7). The bands at 1602, 1578, 1519, 1509, 1487, 1339 and 1264 cm^{-1} were still observed in the presence of vanadate (Fig. 8), indicating that vanadate did not interfere with the photolysis of Nitr-5.

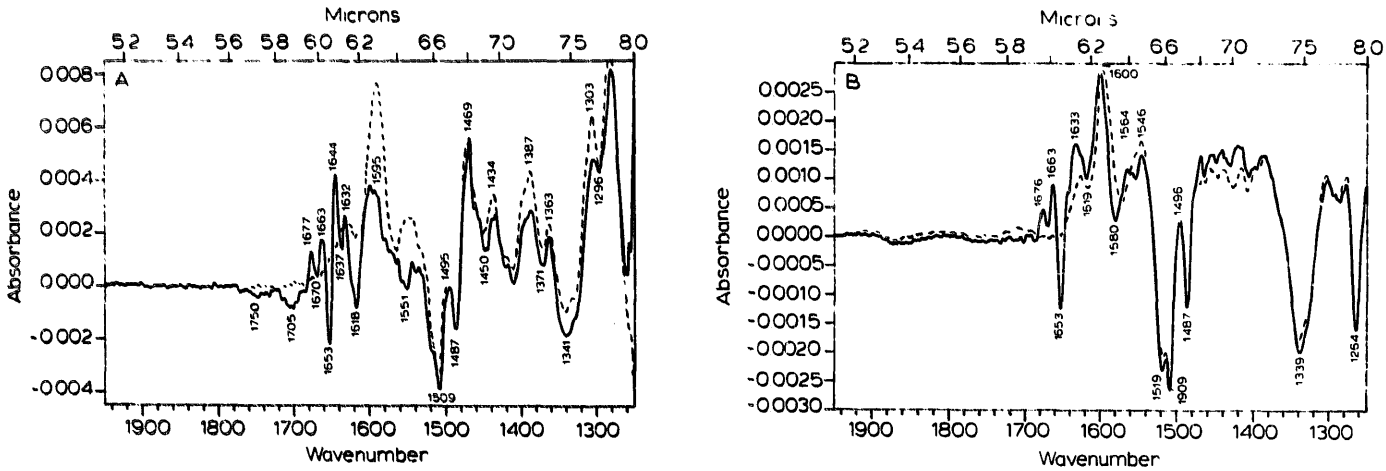

Fig. 6. Difference FTIR spectra of sarcoplasmic reticulum after – before illumination in the presence of Nitr-5-Ca^{2+} complex. (A) Sarcoplasmic reticulum vesicles (40 mg protein/ml) were suspended in 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 0.5 mM $MgCl_2$, 2.5 mM Nitr-5 and ≈ 0.32 mM total Ca^{2+} (Fig. 6A, top panel). FTIR spectra were taken before and after illumination with 360 nm light for 1 min. The spectra (solid lines) represent the averages of six difference spectra (after – before illumination). As control the same experiment was performed in the absence of sarcoplasmic reticulum (broken lines). (B) The conditions were similar to those in (A) except that the concentration of Nitr-5 was 1.5 mM and the medium also contained 3 mM dithiothreitol. The spectra represent the average of sixteen individual difference spectra taken in different samples (Fig. 6B, bottom panel). Control measurements without sarcoplasmic reticulum are indicated by broken lines.

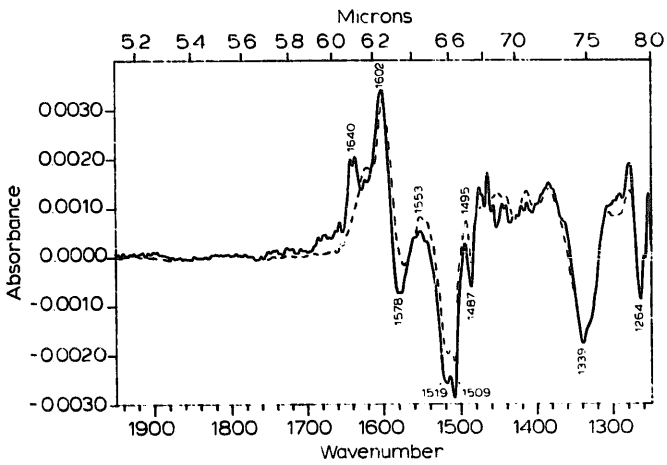

Fig. 8. Difference FTIR spectra of vanadate-treated sarcoplasmic reticulum after illumination in the presence of Nitr-5-Ca^{2+} complex. Sarcoplasmic reticulum vesicles (40 mg protein/ml) were suspended in 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 0.5 mM $MgCl_2$, 1.5 mM Nitr-5, 3 mM DTT, ≈ 0.32 mM Ca^{2+} and 2 mM vanadate. The spectrum indicated by the solid line represents the average of twelve difference spectra (after – before illumination). As control (broken line) the same experiment was performed in the absence of sarcoplasmic reticulum (average of twelve difference spectra).

The composite band at ≈ 1640 cm^{-1} (Fig. 8) may contain some contribution from the 1633 cm^{-1} band of sarcoplasmic reticulum shown in Figs. 6 and 7.

The effect of EGTA on the FTIR spectra of sarcoplasmic reticulum

Chelation of the Ca^{2+} released during photolysis of Nitr-5 by 0.5–1.5 mM EGTA prevents the Ca^{2+}-induced changes in the FTIR spectra of Ca^{2+}-ATPase at 1676 and 1663 cm^{-1} (Fig. 9A). In the spectra range of 1650–1500 cm^{-1} the EGTA contributes significantly to the light-induced changes in the FTIR spectra. These effects were further analyzed in Fig. 9B in a simplified system containing only 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 5 mM dithiothreitol, 2.5 mM EGTA with or without 5 mM $CaCl_2$.

The FTIR spectrum of EGTA in the absence of Ca^{2+} is characterized by a large band at 1628 cm^{-1} (Fig. 9B). Chelation with Ca^{2+} decreases the intensity at 1628 cm^{-1} with the formation of a new band at 1585 cm^{-1} and two small bands at 1618 and at 1740 cm^{-1} (Fig. 9B). The positive band at 1580 cm^{-1} observed after photolysis of the Nitr-5-Ca^{2+} complex in the presence of EGTA (Fig. 9A) is due to the formation of the EGTA-Ca^{2+} chelate, while the negative band at ≈ 1630 cm^{-1} represents the corresponding decrease in the concentration of unliganded EGTA (Fig. 9A).

Discussion

The conformational changes usually associated with Ca^{2+} transport do not produce readily identifiable changes in the shape of Ca^{2+}-ATPase determined by electron crystallography [20–22], or in the distances between intramolecular markers analyzed by fluorescence energy transfer [2]. The secondary structure content of the Ca^{2+}-ATPase determined by circular dichroism spectroscopy [5,6] also remains largely unchanged. Therefore the energy transduction during Ca^{2+} translocation was attributed to hinge-bending and single strand motions, stretching or rotation of helices or deformation of β sheets that may alter the

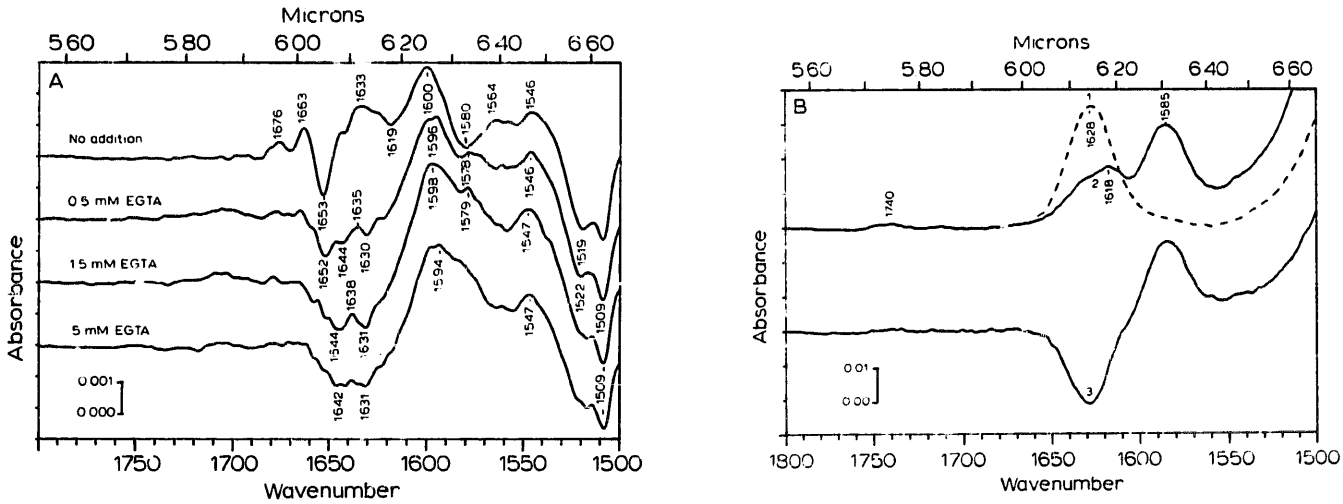

Fig. 9. Difference spectra of sarcoplasmic reticulum after illumination in the presence of Nitr-5 at varying concentrations of Ca^{2+} and EGTA. (A) Sarcoplasmic reticulum vesicles (40 mg/ml) were suspended in 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 0.5 mM $MgCl_2$, 1.5 mM Nitr-5, 3 mM DTT, 0.32 mM total Ca^{2+} and EGTA at the indicated concentrations. The spectra represent the average of sixteen difference spectra obtained by deducting the spectra taken before illumination from the spectra taken after illumination for 1 min at 360 nm. (B) The FTIR spectra of EGTA (2.5 mM) were measured in ^2H_2O medium containing 0.1 M KCl, 10 mM imidazole (p^2H 7.0), 5 mM DTT and 0.025 mM Ca^{2+} (line 1, dashed) or 5 mM Ca^{2+} (line 2, solid line). The bottom line (3) represents the difference between the two spectra (line 2 minus line 1).

rotational isomerization and ionization of side chain groups, but do not produce large changes in the secondary structure content or in the overall shape of the ATPase molecule.

As previous studies using simple FTIR spectroscopy yielded conflicting data about the structural changes associated with Ca^{2+} binding to the Ca^{2+}-ATPase [7–9], we used difference FTIR combined with flash photolysis of caged Ca^{2+} to study the effects of Ca^{2+} on sarcoplasmic reticulum in the absence of ATP. The use of caged Ca^{2+} permitted measurements on the same sample before and after Ca^{2+} release by photolysis, increasing the sensitivity and reproducibility of the detection of small differences.

The highly reproducible Ca^{2+}-induced changes in the FTIR spectra of sarcoplasmic reticulum Ca^{2+}-ATPase at $\simeq$ 1676, 1663 and 1653 cm^{-1} provide clear evidence for a structural change associated with the $E \rightarrow Ca_2E_1$ transition. The Ca^{2+}-induced spectral changes were prevented either by vanadate that stabilizes the Ca^{2+}-ATPase in the E_2V conformation characterized by low affinity for Ca^{2+}, or by EGTA that binds the Ca^{2+} released from photolysed Nitr-5; therefore the changes in the FTIR spectra caused by photolysed of caged Ca^{2+} are clearly associated with Ca^{2+} binding to the Ca^{2+}-ATPase.

The molecular interpretation of the structural changes requires further studies. Changes in the ionization of protein side-chain groups associated with Ca^{2+} binding, deformation of existing structures or formation of new secondary structures may contribute to the observed effects. The narrow band width of the peaks seen in the light-induced difference spectra favor the participation of side chain groups.

Difference spectra of sarcoplasmic reticulum produced by photolysis of caged ATP [23] show an increase in absorbance at 1676, 1650 and 1624 cm^{-1} and a decrease in absorbance at 1662 and 1640 cm^{-1} associated with the formation of E ~ P intermediate and the translocation of Ca^{2+}. The ATP-induced band at 1676 cm^{-1} [23] coincides in sign and in position with the band induced by Ca^{2+} release from caged Ca^{2+} (Figs. 6 and 7). The positive band produced by photolysis of caged-ATP at 1650 cm^{-1} [23] coincides with the negative band induced by Ca^{2+} at 1653 cm^{-1}, while the negative bands induced by ATP at 1662 and at 1640 cm^{-1} are matched by the positive bands at 1663 and at 1633–1644 cm^{-1} induced by Ca^{2+}. These differences suggest that the spectral changes associated with the formation of the Ca_2E_1 intermediate are related to different, perhaps opposite, structural transitions from those seen during ATP hydrolysis.

The Ca^{2+} translocation from the outside to the inside surface of the sarcoplasmic reticulum vesicles energized by ATP hydrolysis [1] occurs with a decrease in the Ca^{2+} affinity of the Ca^{2+}-ATPase, that causes the release of Ca^{2+} from the enzyme into the vesicle interior:

$$Ca_2E_1 + ATP \leftrightarrows E_2 + 2Ca_i + ADP + P_i$$

Therefore the $E_2 \rightarrow Ca_2E_1$ transition induced by the release of Ca^{2+} from the Nitr-5-Ca^{2+} complex can be viewed as the reversal of the reaction sequence induced by ATP in the presence of Ca^{2+}.

Arrondo et al. [7] compared the deconvolved FTIR spectra of sarcoplasmic reticulum taken in the presence of 0.1 mM Ca^{2+} (Ca_2E_1 state) with the spectra taken in the presence of 1 mM EGTA and 5.0 mM monovanadate (E_2V state). The small differences between the spectra of Ca^{2+}-free and Ca^{2+}-bound enzymes compared with relatively large sample to sample variations may explain that the data of Arrondo et al. [7] could not be confirmed by Villalain et al. [8] and by Buchet et al. [9]. Nevertheless, the Ca^{2+}-induced positive bands at 1663, 1644 and 1632 and the negative bands at 1653 cm^{-1} shown in Figs. 6 and 7 agree in sign with the earlier data of Arrondo et al. [7]; these small changes were at the limit of detection in the earlier studies [7], but the use of difference FTIR spectroscopy coupled with caged Ca^{2+} photolysis made them easily observable.

The identification of protein side-chain groups that are involved in the Ca^{2+}-induced changes is in progress using chemical modification by dicyclohexylcarbodiimide [24,25] and cyclopiazonic acid [26,27], that are known to influence Ca^{2+} binding to the Ca^{2+}-ATPase.

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