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Arrondo, Jose L. R.; Mantsch, Henry H.; Mullner, Nandor; Pikula, Slawomir; Martonosi, Anthony

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Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/S0021-9258(18)48043-X
The Journal of Biological Chemistry, 262, 19, pp. 9037-9043, 1987-07-05

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Vol. 262, No. 19, Issue of July 5, pp. 9037-9043, 1987
Printed in U.S.A.

Infrared Spectroscopic Characterization of the Structural Changes Connected with the $E_1 \rightarrow E_2$ Transition in the Ca^{2+}-ATPase of Sarcoplasmic Reticulum*

(Received for publication, November 14, 1986)

Jose L. R. Arrondo‡ Henry H. Mantsch

From the Division of Chemistry, National Research Council of Canada, Ottawa, Ontario, Canada K1A OR6

Nandor Mullner, Slawomir Pikula, and Anthony Martonosi§

From the Department of Biochemistry and Molecular Biology, State University of New York, Health Science Center, Syracuse, New York 13210

The Ca^{2+}-transporting ATPase (EC 3.6.1.38) of sarcoplasmic reticulum alternates between several conformational states during ATP-dependent Ca^{2+} transport. The E_1 conformation is stabilized by 0.1 mM Ca^{2+} and the E_2 conformation by vanadate in a Ca^{2+}-free medium. Fourier transform infrared spectroscopy reveals significant differences between the two states that indicate differences in the protein secondary structure. The two states and the corresponding spectra can be interconverted reversibly by changing the Ca^{2+} concentration of the medium. The infrared spectral changes indicate the appearance of a new α-helical substructure connected with the $E_1 \rightarrow E_2$ conversion accompanied by small changes in β-turns, while the β-sheet content remains essentially unchanged. There are also differences between the E_1 and E_2 states in the C=O stretching vibrations of the ester carbonyl groups of phospholipids in intact sarcoplasmic reticulum that are not observed under identical conditions in isolated sarcoplasmic reticulum lipid dispersions. These observations imply an effect of proteins on the structure of the interfacial regions of the phospholipids that is dependent on the conformational state of the Ca^{2+}-ATPase. The CH_2- and CH_3-stretching frequencies of the membrane lipids are not affected significantly by the $E_1 \rightarrow E_2$ transition. The Fourier transform infrared spectra of sarcoplasmic reticulum vesicles in the presence of 20 mM Ca^{2+} suggest the stabilization of a protein conformation similar to the E_2 state except for differences in the behavior of COO^- and phospholipid ester C=O groups that may reflect charge effects of the bound Ca^{2+}.

The Ca^{2+}-transporting ATPase (EC 3.6.1.38) is an intrinsic membrane protein that constitutes about 80% of the protein content of isolated sarcoplasmic reticulum vesicles (Martonosi and Beeler, 1983). The predicted structure of Ca^{2+}-ATPase, based on its amino acid sequence consists of an intramembranous, primarily helical domain, and several cytoplasmic domains composed of α-helical, β-sheet, β-turn, and random elements (MacLennan *et al.*, 1985; Brandl *et al.*, 1986). Circular dichroism measurements gave the following composition of secondary structures: 46% α-helix, 8% β-sheet, 13% β-turn, and 33% random coil (Csermely *et al.*, 1987). The dominant α-helical character of the protein is also suggested by Raman spectroscopic studies (Lippert *et al.*, 1981; Williams *et al.*, 1986) in essential agreement with the circular dichroism data. Based on electron microscopy of frozen hydrated Ca^{2+}-ATPase crystals, about ⅓ of the Ca^{2+}-ATPase polypeptide is located within the lipid environment of the membrane, and ⅔ is exposed on the cytoplasmic surface (Taylor *et al.*, 1986a; 1986b). A similar disposition of the Ca^{2+}-ATPase was derived from x-ray and neutron diffraction data (Brady *et al.*, 1981, 1982; Blasie *et al.*, 1985).

During ATP-dependent Ca^{2+} transport the Ca^{2+}-ATPase alternates between several conformations, characterized by different affinities for calcium, ATP, and magnesium, and changes in the reactivity of several functional groups (Inesi and de Meis, 1985). Stabilization of the enzyme in the E_1 conformation by calcium and lanthanides leads to the formation of P1-type crystalline Ca^{2+}-ATPase arrays (Dux *et al.*, 1985). Vanadate and inorganic phosphate in a calcium-free medium shift the equilibrium in favor of the E_2 conformation and promote the formation of P2-type Ca^{2+}-ATPase crystals (Taylor *et al.*, 1986a; 1986b). Previous circular dichroism measurements did not reveal significant differences between these two major conformations in the protein secondary structure of sarcoplasmic reticulum (Nakamoto and Inesi, 1986; Csermely *et al.*, 1987), suggesting that the $E_1 \rightarrow E_2$ transition involves a rearrangement of domains within the ATPase molecule rather than a refolding of polypeptide backbone.

Recent applications of Fourier transform infrared spectrometry (Mendelsohn and Mantsch, 1986; Lee and Chapman, 1986) promise a more penetrating insight into the structural changes connected with the $E_1 \rightarrow E_2$ transition. Infrared spectra of sarcoplasmic reticulum in H_2O or D_2O media show distinct bands in the Amide I region related to α-helix, β-sheet, and random coil structures (Cortijo *et al.*, 1982; Mendelsohn *et al.*, 1984; Lee *et al.*, 1985; Arrondo and Goni, 1985; Arrondo *et al.*, 1985; Muga *et al.*, 1986; Jaworsky *et al.*, 1986). Detailed FT-IR[1] studies of the effect of Ca^{2+}-ATPase on the order and melting characteristics of phospholipids in native

* This work was supported by National Institutes of Health Grant AM 26545, National Science Foundation Grant PCM 84-03679, and the Muscular Dystrophy Association, Inc. Issued as NRCC publication no. 26590. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Acknowledges North Atlantic Treaty Organization for a fellowship. Permanent address: Dept. of Biochemistry, University of the Basque Country, Bilbao, Spain.

§ To whom all correspondence should be addressed.

[1] The abbreviations used are: FT-IR, Fourier transform infrared; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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sarcoplasmic reticulum and in reconstituted Ca^{2+}-ATPase vesicles (Mendelsohn *et al.*, 1984; Jaworsky and Mendelsohn, 1985; Anderle and Mendelsohn, 1986) reveal interesting selectivity in the interaction of Ca^{2+}-ATPase with certain phospholipid classes.

The focus of this report is the effect of Ca^{2+}, EGTA, and vanadate on the Amide I and II vibrations arising from protein peptide bonds and on the C=O stretching vibrations of the lipid ester carbonyl groups in rabbit sarcoplasmic reticulum vesicles and in isolated sarcoplasmic reticulum lipids. The data indicate distinct differences in protein secondary structures between the E_1 and E_2 conformations of Ca^{2+}-ATPase and a clear relationship between the structure of Ca^{2+}-ATPase and the conformation of the lipid ester carbonyl bond.

EXPERIMENTAL PROCEDURES

Materials

Deuterium oxide was obtained from MSD Isotopes, Merck Chemical Division, St. Louis, MO 63116. EGTA, phosphoenolpyruvate, β-nicotinamide adenine dinucleotide, lactic dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), and adenosine 5′-triphosphate were supplied by Sigma. A23187 was purchased from Behring Diagnostics, and sodium vanadate was obtained from Fisher. All other reagents were of analytical grade.

Methods

Preparation of Sarcoplasmic Reticulum—The sarcoplasmic reticulum vesicles were isolated from predominantly white skeletal muscles of rabbits as described earlier (Jona and Martonosi, 1986), and the preparations were stored before use frozen at −70 °C in 0.3 M sucrose, 10 mM Tris-maleate, pH 7.0, at a protein concentration of 25–38 mg/ml.

About 16–18 h before measurements, 1- to 2-ml aliquots of the microsomes were thawed, centrifuged, and the pellet resuspended in 5 ml of standard medium containing 0.1 M KCl, 10 mM imidazole, pH 7.4, and 1 mM $MgCl_2$ in either H_2O or D_2O. The centrifugation and resuspension steps were repeated twice more. The final sediments were taken up in standard H_2O or D_2O medium to a final protein concentration of 24–33.6 mg/ml.

FT-IR measurements were carried out: (a) without further addition, (b) after the addition of $CaCl_2$ to a final concentration of 0.1 mM, (c) after addition of $CaCl_2$ to a final concentration of 20 mM, and (d) after addition of 1 mM EGTA and 5 mM Na_3VO_4.

Concentrated stock solutions of $CaCl_2$, EGTA, and vanadate were prepared in H_2O or D_2O containing standard medium.

Protein was determined according to Lowry *et al.* (1951).

Extraction of Phospholipids from Sarcoplasmic Reticulum—The preparation of sarcoplasmic reticulum phospholipids was carried out essentially as described by Folch *et al.* (1957). The extracted lipids were dried under nitrogen and suspended in standard H_2O or D_2O medium. After ultrasonic dispersion the liposomes were stored at 2 °C under nitrogen for 1–2 days. Before FT-IR measurements, Ca^{2+} or EGTA and vanadate were added as described for native sarcoplasmic reticulum. The phospholipid P content of the extract was determined after perchloric acid digestion according to Fiske and Subbarow (1925) or Bartlett (1959).

Delipidation of Sarcoplasmic Reticulum—The method of Dean and Tanford (1977, 1978) was used with minor modifications. Microsomes (5 mg of protein/ml) suspended in H_2O or D_2O containing standard medium (pH 7.5) supplemented with 20% glycerol were solubilized with deoxycholate (10 mg/ml). After centrifugation at 59,000 × *g* for 45 min, the supernatant was diluted with equal volume of standard medium, and the Ca^{2+}-ATPase was precipitated with 12.5% polyethylene glycol. Following centrifugation at 33,000 × *g* for 20 min, the pellets were washed with standard medium containing 12.5% polyethylene glycol and were finally resuspended in H_2O or D_2O containing standard medium. FT-IR measurements were carried out after addition of Ca^{2+} or EGTA and vanadate as described for native sarcoplasmic reticulum.

ATPase Assay

ATPase activities were measured either by a coupled enzyme system of pyruvate kinase and lactate dehydrogenase (Dean and Tanford, 1977), or by the analysis of inorganic phosphate liberation from ATP using the Fiske-Subbarow (1925) technique.

(*a*)—In the coupled enzyme assay, the microsomes were preincubated for 5 min at 25 °C in 1 ml of medium I containing 0.1 M KCl, 0.02 M Tris-HCl, pH 7.5, 0.9 mM $CaCl_2$, 5 mM $MgCl_2$, 0.42 mM phosphoenolpyruvate, 0.2 mM NADH, 7.5 IU of pyruvate kinase, and 18 IU of lactate dehydrogenase ± 2 μM A23187; the microsomal protein concentration was 1 μg/ml. After preincubation 1 ml of medium II was added to start the reaction. Medium II contained 0.1 M KCl, 0.02 M Tris-HCl, pH 7.5, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM ATP, and phosphoenolpyruvate, NADH, pyruvate kinase, and lactate dehydrogenase at the same concentrations as in medium I. The preincubation in medium I was required to displace vanadate from the binding site of the Ca^{2+}-ATPase. The absorbance was measured at 340 nm at 25 °C using a Perkin-Elmer Lambda 3-B dual wavelength spectrophotometer. The recorded absorbance was linear up to at least 5–10 min of reaction time. For measurement of the Ca^{2+}-insensitive (basal) ATP hydrolysis, Ca^{2+} was omitted from the medium. The Ca^{2+}-insensitive ATP hydrolysis rate was usually less than 5% of the rate of ATP hydrolysis in Ca^{2+}-containing medium. Data on total ATP hydrolysis rates measured in Ca^{2+}-containing media were corrected for the Ca^{2+}-insensitive hydrolysis rate determined in the same experiment to obtain the rate of Ca^{2+}-stimulated ATPase activity.

(*b*)—The rate of inorganic phosphate liberation from ATP was determined by incubation of microsomes (0.05 to 0.1 mg of protein/ml) in an assay medium of 0.1 M KCl, 10 mM imidazole, pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM EGTA, 0.45 mM $CaCl_2$ ± 1 μM A23187 at 25 °C for 1–5 min. The reaction was stopped with trichloroacetic acid, and the inorganic phosphate was determined according to Fiske and Subbarow (1925). In the assay of basal (Ca^{2+}-insensitive) ATPase activity, the Ca^{2+} was omitted from the medium.

Fourier Transform Infrared Spectrometry

Infrared spectra of aqueous vesicle suspensions were recorded on a Digilab FTS-15 FT-IR spectrometer using a demountable cell (Harrick Scientific, Ossining, NY) with calcium fluoride windows and 6-μm spacers for samples in H_2O medium or 50-μm spacers for samples in D_2O medium. A tungsten-copper thermocouple was taped directly onto the window and the cell placed into a thermostated cell mount. For each spectrum 512 interferograms were averaged at an optical retardation of 0.5 cm, triangularly apodized, and Fourier transformed to yield FT-IR spectra with a resolution of 2 cm^{-1}. The H_2O or D_2O spectra were digitally subtracted using a spectrum of the medium as reference. In order to separate instrumentally unresolvable infrared band contours, Fourier deconvolution (Mantsch *et al.*, 1986) and Fourier derivation techniques (Moffatt *et al.*, 1986) were applied. Band narrowing by Fourier deconvolution was performed by using Lorentzian bandwidths of 15 cm^{-1} and a resolution enhancement factor of 2.2. Fourier derivation was performed by using a power of 3 and a breakpoint of 0.4. A power of 3 in Fourier derivation corresponds to a smoothed fourth derivative. The ATPase activity of sarcoplasmic reticulum samples was measured before and after recording the FT-IR spectra, and the activities did not change significantly.

RESULTS

The unique advantage of infrared spectrometry is that it allows the simultaneous study of the structure of lipids and proteins of intact biological membranes under near physiological conditions without the introduction of reporter groups. While the strong water absorption in the region 1700–1600 cm^{-1}, where the structurally most significant protein bands are located, presented difficulties in the early applications of infrared spectrometry to biological systems, modern FT-IR spectrometers generate infrared spectra with a signal-to-noise ratio high enough to subtract the water component from the protein spectrum. The problem caused by the H_2O band may also be prevented by the use of D_2O instead of H_2O as solvent; however, under these conditions the replacement of exchangeable hydrogens by deuterium leads to changes in the spectrum that must be taken into account in the analysis and interpretation of the data.

Infrared Spectra of Sarcoplasmic Reticulum Vesicles in H_2O and in D_2O—The spectra of sarcoplasmic reticulum mem-

branes isolated from rabbit muscle are shown in Fig. 1 after solvent subtraction in H_2O (*solid line*) and in D_2O solutions (*dashed line*). Several differences can be seen. The maximum of the Amide I band which is at 1652 cm^{-1} in H_2O buffer has shifted to 1646 cm^{-1} in D_2O. This is due to H-to-D exchange of the amide N-H groups in un- (or less) ordered structures that changes the overall band shape. The Amide II band shifts from 1550 cm^{-1} in H_2O to 1460 cm^{-1} after deuteration; a residual Amide II band that is retained in D_2O medium at 1550 cm^{-1} is due to the slow exchange of amide hydrogens in ordered structures and in domains that are sheltered from solvent exchange. Furthermore, the decrease in the intensity of the Amide II band in a D_2O medium allows one to see other vibrations that are usually hidden by the strong Amide II band in H_2O; among these are the side chain vibrations of amino acids, such as carboxylate and carbimino groups and the C–C stretching vibrations of the phenyl group of tyrosine (Chirgadze *et al.*, 1975).

The Amide I and II modes generate unique band contours in the region of 1800–1500 cm^{-1}. Generally, both the Amide I and II band contours consist of several component bands which overlap each other. Because of the intrinsic widths of these component bands (their half-bandwidth, typically 20–25 cm^{-1}, is larger than the separation between individual bands), they cannot be resolved by increased instrumental resolution. In the past this fact had severely limited the application of infrared spectroscopy to protein conformational analysis. More recently mathematical methods have been developed that allow the computational narrowing of infrared bands that comprise a complex band contour. While this operation is often referred to as resolution enhancement, it does not increase the instrumental resolution but increases the degree to which individual component bands can be visualized. Currently, two such computational procedures are used for band narrowing, Fourier deconvolution and derivation. Fourier deconvolution is an iterative procedure controlled by two adjustable parameters, *i.e.* the bandwidth and a resolution enhancement factor. The Fourier derivation is an alternative and mathematically different method of spectral resolution enhancement that is based on the generation of "non-integer derivative" band profiles; the degree of resolution enhancement is under the control of a single "breakpoint" parameter which can be adjusted. The validity of these procedures was tested on synthetic curves (Kauppinen *et al.*, 1981), and both techniques are now being used extensively to resolve overlapping infrared bands in complex systems, such as lipids (Casal and Mantsch, 1984; Mendelsohn and Mantsch, 1986) and proteins (Lee *et al.*, 1985; Renugopalakrishnan *et al.*, 1985; Byler and Susi, 1986; Olinger *et al.*, 1986; Jaworsky *et al.*, 1986; Haris *et al.*, 1986).

Changes in the Infrared Spectra of Sarcoplasmic Reticulum Connected with the Transition between the E_1 and E_2 Conformations of the Ca^{2+}-ATPase—The Ca^{2+}-ATPase of sarcoplasmic reticulum is stabilized in the E_1 conformation by 0.1 mM $CaCl_2$ and in the E_2 conformation by 1.0 mM EGTA + 5 mM vanadate. Fig. 2*A* shows the spectra of sarcoplasmic reticulum dispersed in D_2O medium in the two conformational states. The naked eye is unable to detect significant differences between the two spectral curves. However, subtle differences became apparent after suitable data processing, as evident from Fig. 2, *B* and *C*, which show the same spectra after band narrowing, respectively, by Fourier deconvolution or Fourier derivation. In each case the *solid line* corresponds to sarcoplasmic reticulum in the presence of 0.1 mM Ca^{2+} (E_1

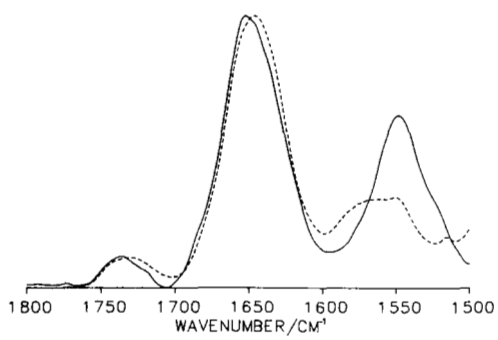

FIG. 1. **Infrared spectra of sarcoplasmic reticulum in H_2O and D_2O.** *Solid line*, spectrum of sarcoplasmic reticulum vesicles (24 mg of protein/ml) in H_2O medium containing 0.1 M KCl, 10 mM imidazole, pH 7.4, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$, after subtraction of the spectra of the H_2O medium without sarcoplasmic reticulum. The spectra were recorded at 10 °C in a CaF_2 cell of 6 μm thickness and corrected for water vapor. *Broken line*, spectra of sarcoplasmic reticulum vesicles (24 mg of protein/ml) in a D_2O medium of otherwise identical composition, measured at 10 °C in a BaF_2 cell of 50 μm thickness, after subtraction of the spectrum of D_2O medium without sarcoplasmic reticulum and correction for water vapor. In both cases the recording started 1 h after addition of Ca^{2+} to the sarcoplasmic reticulum vesicles dispersed in the appropriate standard medium.

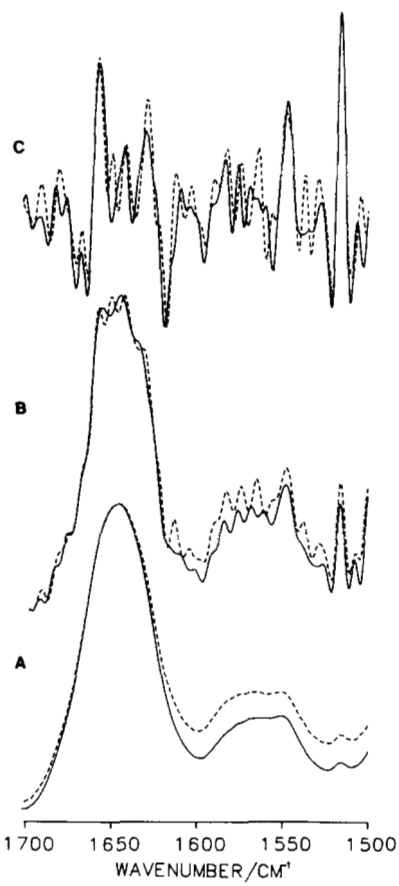

FIG. 2. **Original, deconvolved, and derivative spectra of sarcoplasmic reticulum in D_2O medium in the E_1 and in the E_2 states.** *A*, to sarcoplasmic reticulum vesicles (24 mg of protein/ml) suspended in a standard medium of 0.1 M KCl, 10 mM imidazole, pH 7.4, and 1 mM $MgCl_2$, either 0.1 mM $CaCl_2$ (*solid line*) or 1 mM EGTA and 5 mM monovanadate (*broken line*) were added to stabilize the E_1 and E_2 conformations, respectively. The spectra were taken at 10 °C about 1 h after the additions in a 50-μm BaF_2 cell. From both spectra the spectrum of D_2O medium and the contribution of water vapors were subtracted. *B*, same spectra as in *A* after resolution enhancement by Fourier deconvolution, using a band narrowing factor of 2.2. *C*, same spectra as in *A* after band narrowing by Fourier derivation using a power of 3 and a breakpoint of 0.4.

state), and the *dashed line* in the presence of 1 mM EGTA + 5 mM vanadate (E_2 state). Our practice in assessing the validity of resolution-enhanced band profiles is to generate them independently by two different procedures and compare the results. Only peaks that show up in both procedures are considered. Thus, from a comparison of the resolution-enhanced spectra we are confident that all spectral features observed as individual bands in Fig. 2*B* or as shoulder bands in Fig. 2*C* are real and represent specific conformational structures. As can be seen from Fig. 2*B*, the degree of band narrowing is less pronounced upon deconvolution; however, the intensities of the component bands (integrated band areas) are preserved. Upon derivation (Fig. 2*C*) sharper peaks are obtained, but the true line shape is lost, and the peak intensity becomes dependent on the width of the original bands. For example, the peak at 1516 cm^{-1} has the greatest intensity in the derivative spectra because it is narrower than the other bands, although it is only a small peak in the original spectrum (see Fig. 1). The advantage of derivation over deconvolution is that it allows a more precise determination of the position of component bands that appear only as shoulder bands in the deconvolved spectra.

The region 1800–1500 cm^{-1} contains bands coming from the peptide backbone of the protein, from the amino acid side chains, and from the lipid moieties of sarcoplasmic reticulum. The following regions have been chosen to analyze the conformational differences in sarcoplasmic reticulum between the E_1 and E_2 states: (*a*) 1700–1620 cm^{-1}, which corresponds to the Amide I band, that is most sensitive to conformational changes, (*b*) 1565–1535 cm^{-1}, which contains the Amide II band, (*c*) 1620–1560 cm^{-1}, with bands related to amino acid side chain vibrations, and (*d*) 1760–1700 cm^{-1} that gives information about the phospholipid headgroup.

The Amide I Region (1700–1620 cm^{-1})—Fig. 3 shows the resolution enhanced infrared spectra of sarcoplasmic reticulum in D_2O in the presence of 0.1 mM calcium that stabilizes the E_1 state (*solid line*), 1 mM EGTA + 5 mM vanadate that stabilizes the E_2 state (*broken line*), or 20 mM calcium without definite conformational assignment (*dotted line*). The Amide I bands observed in the 1700–1620 cm^{-1} region of the infrared spectra of Ca^{2+}-ATPase in the E_1 and E_2 conformations are given in Table I, along with their assignment to specific secondary substructures. Whereas the spectra obtained in the presence of 5 mM vanadate or 20 mM calcium are very similar, there are differences between these and the spectrum obtained in the presence of 0.1 mM calcium. The most important of these differences is the presence of a peak at 1650 cm^{-1} in the E_2 state stabilized by vanadate, that is absent in the E_1 state, stabilized by 0.1 mM $CaCl_2$. Surprisingly, the 1650 cm^{-1} peak reappears when the [Ca^{2+}] is raised to 20 mM. The binding of Ca^{2+} to the low affinity Ca^{2+}-binding sites of the Ca^{2+}-ATPase apparently stabilizes a conformation that is similar to the E_2 state induced by vanadate.

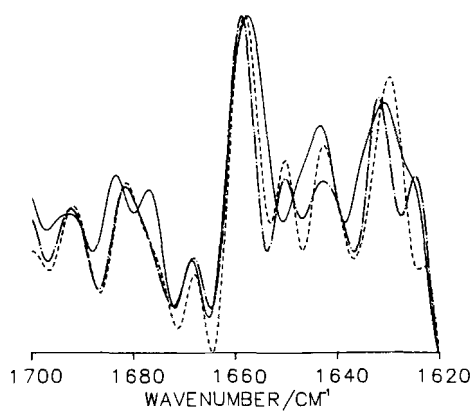

FIG. 3. **Fourier derivative infrared spectra of sarcoplasmic reticulum in the various conformations stabilized by Ca^{2+} or vanadate (Amide I region).** To sarcoplasmic reticulum vesicles (24 mg of protein/ml) suspended in standard D_2O medium, 0.1 mM $CaCl_2$ (*solid line*) or 1 mM EGTA and 5 mM sodium vanadate (*broken line*) were added to stabilize the E_1 and the E_2 conformations, respectively. In a third sample of sarcoplasmic reticulum, the Ca^{2+} concentration was raised to 20 mM (*dotted line*). Although conformational assignment under the latter condition is not entirely established, kinetic considerations suggest that saturation of the low affinity binding sites of Ca^{2+}-ATPase by 20 mM Ca^{2+} would stabilize the E_2 state. The spectra were taken at 10 °C and corrected for the contributions of the D_2O medium and water vapor. Band narrowing by Fourier derivation was carried out as described under "Experimental Procedures."

The peak at 1650 cm^{-1}, specific of the E_2 state, occurs in the Amide I region characteristic for α-helices and probably represents a new α-helical structure that may arise from the rearrangement of other structures during the $E_1 \rightarrow E_2$ transition. The appearance of the new 1650 cm^{-1} band in the E_2 state may occur at the expense of the 1657 cm^{-1} or the 1643 cm^{-1} bands.

In the spectra of both the E_1 and E_2 conformations, the band at 1657 cm^{-1} can be assigned to α-helices, and the bands at 1630 and 1682 cm^{-1} to antiparallel β-sheet structures (Susi *et al.*, 1967; Cortijo *et al.*, 1982; Mendelsohn *et al.*, 1984; Arrondo *et al.*, 1985; Lee *et al.*, 1985; Jaworsky *et al.*, 1986). The infrared bands at 1668 and 1692 cm^{-1} are attributable to turns (Krimm and Bandekar, 1980). There is one additional band in the spectrum of the E_1 state of Ca^{2+}-ATPase at 1677 cm^{-1} which is absent in the spectrum of the E_2 state and which most likely also represents turns. Alternatively, the peak at 1677 cm^{-1} may correspond to the high frequency component of a β-sheet structure (Susi *et al.*, 1967); this is, however, less likely since there is no doubling of the corresponding low frequency component at 1630 cm^{-1} that is much stronger in intensity. In the spectra obtained in the presence of 20 mM calcium or 5 mM vanadate, the two peaks at 1683 and 1677 cm^{-1} have apparently merged into one composite peak due to a change in the position or a decrease in the intensity of one of the bands. It is more likely that the band representing turns is affected. The band at 1643 cm^{-1} is assigned to un- (or less) ordered structures. In H_2O medium (not shown), the band assigned to unordered structures overlaps with the α-helix band, giving rise to a composite band at 1654.5 cm^{-1}.

The Amide II Region (1565–1535 cm^{-1})—The Amide II and Amide I bands represent different vibrational modes; whereas the Amide I mode is a C=O stretching vibration, the Amide II mode is mainly a N–H bending vibration (Susi, 1969, 1972; Mendelsohn and Mantsch, 1986). Despite some attempts to

TABLE I

Characteristic Amide I bands from the infrared spectra of Ca^{2+}-ATPase in the E_1 and E_2 conformations and their assignment

Frequencies (in cm^{-1}) are from resolution-enhanced infrared spectra in D_2O buffer and are rounded off to the nearest integer. The symbols α, β, T, and R stand for α-helices, β-structures, turns, and random structures, respectively.

E_1 state	E_2 state
1630 (β)	1630 (β)
1643 (R)	1643 (R)
	1650 (α)
1657 (α)	1657 (α)
1668 (T)	1668 (T)
1677 (T/β)	
1683 (β)	1682 (β)
1692 (T)	1692 (T)

correlate the Amide II band with the same structural patterns as established for the Amide I band, no conclusive experimental results have been obtained. The position of the Amide II band is sensitive to deuteration, shifting from around 1550 cm^{-1} to the so-called Amide II′ frequency at 1450 cm^{-1}. The Amide II′ band overlaps with the H–O–D bending vibration, so it has no value in the analysis of conformational changes. Fig. 1 shows clearly the decrease in Amide II intensity upon deuteration (H → D exchange of the N-H groups). However, the remainder of the Amide II band at 1550 cm^{-1} is a clue to the accessibility of solvent to the peptide backbone. Hydrophobic environments or tightly ordered structures (α-helix or β-sheet) prevent the amide H from being exchanged. In Fig. 4 the residual Amide II bands of the two conformational states (E_1 and E_2) of sarcoplasmic reticulum are compared. It is evident that in D_2O medium only the peak at 1550 cm^{-1} is left in the E_1 state, whereas there is a second Amide II peak at 1537 cm^{-1} in the E_2 state. This suggests that at least some of the groups which were accessible to H → D exchange in the E_1 conformation are protected in the E_2 state. The vanadate-induced changes characteristic of the E_2 state are reversed on addition of 0.1 mM $CaCl_2$ that restores the spectral characteristics of the E_1 state.

Amino Acid Side Chain Vibrations (1620–1560 cm^{-1})—In the region 1800–1500 cm^{-1}, in addition to the protein bands attributed to the peptide backbone, some bands due to side chain vibrations are also seen (Chirgadze *et al.*, 1975). Little is known about these vibrations and their conformational sensitivity. The 1516 cm^{-1} band due to the C–C stretching of the phenolic tyrosine ring (Fig. 2, *B* and *C*) is apparently not affected by protein conformational changes. It is interesting to note that in the region 1620–1560 cm^{-1}, where the side chain vibrations of charged amino acids occur, the spectrum of the 0.1 mM calcium sample is closer to that of the 20 mM calcium sample than to that of the sample in 5 mM vanadate. It is plausible to assume that the charged residues (*i.e.* the $-COO^-$ groups) are more sensitive to the type of cation bound than to changes in protein conformation. The bands due to sarcoplasmic reticulum protein side chain vibrations in H_2O and in D_2O media are similar. Some bands around 1700 cm^{-1} may correspond to the nonionized forms of the amino acids.

Phospholipid Headgroup Vibrations (1760–1700 cm^{-1})—The region 1760–1700 cm^{-1} contains vibrations from the ester carbonyl groups of the membrane phospholipids. In model systems two bands can be distinguished in this region that correspond to the *sn*-1 and *sn*-2 C=O groups (Bush *et al.*, 1980; Levin *et al.*, 1982). In natural membranes the interpretation may be less straightforward, since several phospholipid classes with different acyl chains are normally present and even some amino acid side chain vibrations can be encountered in this region. Fig. 5*A* shows the deconvolved spectra of sarcoplasmic reticulum in the presence of 0.1 mM calcium (*solid line*), 20 mM calcium (*dotted line*), and 5 mM vanadate (*broken line*) in H_2O medium. In all three cases there are two peaks at 1741 and 1731 cm^{-1} that can be assigned to the *sn*-1- and *sn*-2-ester carbonyls, respectively. The ratio between the 1741 and 1731 peaks is greater than 1 in the presence of 0.1 mM calcium (E_1) and less than 1 in the presence of vanadate (E_2). There are two additional peaks at 1750 cm^{-1} and 1717 cm^{-1} in the 5 mM vanadate and in the 0.1 mM calcium samples that are not seen in the 20 mM calcium spectrum.

In order to establish whether the differences observed in Fig. 5*A* are due to the protein or to a direct effect of calcium on the phospholipids, the phospholipids were extracted from sarcoplasmic reticulum and their spectra compared in the presence of 0.1 mM calcium, 5 mM vanadate, or 20 mM calcium (Fig. 5*B*). The 1741 cm^{-1} peak is observed more or less in the same position; however, in the 20 mM Ca^{2+} spectrum (and to a lesser extent in the 0.1 mM Ca^{2+} spectrum) the 1731 cm^{-1} peak has split in two. The bands at 1750 cm^{-1} and at 1717 cm^{-1} which are clearly seen in the spectra of sarcoplasmic reticulum in the presence of 0.1 mM $CaCl_2$ or 5 mM vanadate (Fig. 5*A*) become weak shoulders in the corresponding lipid extracts (Fig. 5*B*).

From these data it is clear that there is an influence of the protein on the ester carbonyl groups of phospholipids, since the lipids exhibit different infrared spectra in the presence or in the absence of protein. It is not possible to attribute this to a direct effect of Ca^{2+} on phospholipids, since no large differences are seen under identical conditions in the extracted lipid. The splitting found in the lipid extract at high calcium concentration is not observed when there is protein

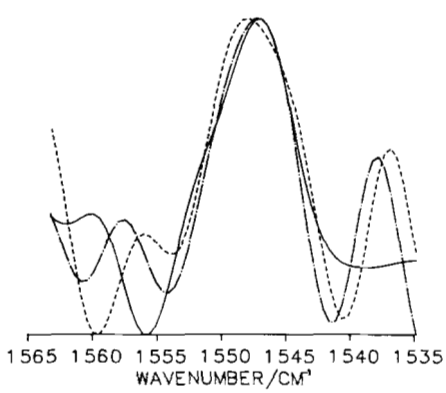

FIG. 4. **Fourier derivative spectra of sarcoplasmic reticulum in D_2O in the various conformations stabilized by Ca^{2+} or vanadate (Amide II region).** The samples and conditions are identical to those described in Fig. 3.

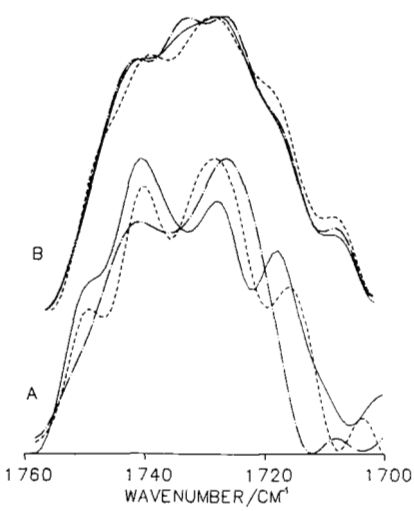

FIG. 5. **Infrared spectra of sarcoplasmic reticulum (A) and of the extracted sarcoplasmic reticulum lipids (B) in H_2O medium under various experimental conditions after Fourier deconvolution (C=O stretching modes).** *A*, sarcoplasmic reticulum vesicles (24 mg of protein/ml) were suspended in standard H_2O medium and supplemented with 0.1 mM $CaCl_2$ (*solid line*), 1 mM EGTA, and 5 mM sodium vanadate (*broken line*) or 20 mM $CaCl_2$ (*dotted line*). Spectra were taken at 8.8–10 °C and the spectrum of H_2O medium subtracted. *B*, sarcoplasmic reticulum lipids were extracted as described under "Experimental Procedures" and suspended in standard H_2O medium to a final concentration of 53 mg/ml. Additions were made and spectra recorded as described under *A*. Band narrowing by Fourier deconvolution was carried out as described under "Experimental Procedures."

in the system. Although some buffering of calcium by proteins may occur, the differences observed between the E_1 and E_2 states in sarcoplasmic reticulum in the vibrations assigned to the ester carbonyl bonds most probably reflect the influence of protein on phospholipids, and this influence is different when the Ca^{2+}-ATPase is in the E_1 as compared with the E_2 state. The precise nature of the conformation-dependent effect of Ca^{2+}-ATPase on the ester carbonyl vibration of phospholipids is not known, but it is likely to involve the stalk region (Brandl *et al.*, 1986) of the Ca^{2+}-ATPase that is close to the water-lipid interface.

The PO_2-stretching vibrations are not changed, which is consistent with the proposition that the differences in the carbonyl ester stretching vibrations are not due to a direct effect of the cations on the phospholipids, but reflect an influence of the protein.

The infrared spectra of delipidated sarcoplasmic reticulum (not shown) indicate gross structural changes (decrease of α-helix content and increase of β-sheet and unordered structures) connected with the removal of phospholipids. Based on the intensity of Amide II band the H → D exchange is more pronounced in native than in delipidated sarcoplasmic reticulum.

The Acyl Chains of Phospholipids—Another spectral region of interest is the 3000–2800 cm^{-1} range where the bands due to the methyl- and methylene-stretching vibrations are seen; these are related to the conformational arrangement of the acyl chains (Casal and Mantsch, 1984). In native sarcoplasmic reticulum membranes at 8–10°C, the acyl chains are in the liquid crystalline state, which is thought to be necessary to sustain the enzymatic activity of the Ca^{2+}-ATPase. There is no difference in the CH_2- or CH_3-stretching band frequencies between the E_1 or E_2 states.

Temperature-dependent Changes in the Structure of Sarcoplasmic Reticulum—Proteins undergo a temperature-induced denaturation that involves a major rearrangement of the tertiary structure. A nearly complete H → D exchange is observed in D_2O medium upon denaturation (Englander and Kallenbach, 1984). This is also evident from the loss of the Amide II band at 1550 cm^{-1} upon thermal denaturation (Fig. 6*A*). Furthermore, new peaks appear in the Amide I region of the infrared spectra that indicate a loosening of structures. After thermal denaturation of the sarcoplasmic reticulum in D_2O, the new peaks at 1619 and 1686 cm^{-1} dominate the Amide I band, and there is a decrease in the intensity at 1658 and 1630 cm^{-1}. The broad peak centered at 1648 cm^{-1} probably represents primarily nonordered structures together with some remnants of the secondary structures that were present in the native protein before thermal denaturation.

Fig. 6*B* compares the spectra of sarcoplasmic reticulum in D_2O in the presence of either 0.1 mM calcium or 1 mM EGTA + 5 mM vanadate after thermal denaturation. In the Amide I region, the two spectra are similar with peaks located at 1689, 1648, and 1619 cm^{-1}; the Amide II is completely exchanged in both cases. In the 1760–1700 region, two peaks are visible in the presence of 0.1 mM calcium, while the 5 mM vanadate sample has only one broad band. The midpoint of denaturation temperature is 42 °C in the E_1 and 50 °C in the E_2 state. Therefore, 1 mM EGTA and 5 mM vanadate slightly stabilize the Ca^{2+}-ATPase against thermal denaturation. Under similar conditions vanadate also increased the stability of Ca^{2+}-ATPase against inactivation by 1–2 kbar pressure (Varga *et al.*, 1986) suggesting a more compact structure in the E_2 than in the E_1 state. The similar profiles of thermally denatured sarcoplasmic reticulum in the presence of 0.1 mM calcium or 1 mM EGTA + 5 mM vanadate show that the two different conformational states (E_1 and E_2) can only exist when the native tertiary structure is present.

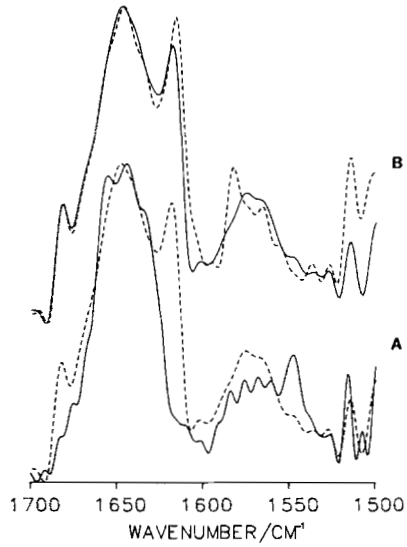

FIG. 6. **The effect of thermal denaturation on the infrared spectra of sarcoplasmic reticulum in D_2O.** *A*, sarcoplasmic reticulum vesicles (24 mg of protein/ml) were suspended in a solution of 0.1 M KCl, 10 mM imidazole, pH 7.4, 1 mM $MgCl_2$ in D_2O; 0.1 mM $CaCl_2$ was added, and the spectrum was recorded ≃1 h later at 10 °C (*solid line*). Thermal denaturation was induced in a different sample of identical composition by stepwise increase of temperature to 85 °C (*broken line*). Deconvolved spectra (see "Experimental Procedures") are presented after subtraction of the contribution of the D_2O medium. *B*, sarcoplasmic reticulum vesicles were thermally denatured in standard D_2O medium containing either 0.1 mM $CaCl_2$ (*solid line*) or 1 mM EGTA and 5 mM sodium vanadate as described under *A*. Data treatment was carried out as under *A*.

DISCUSSION

The FT-IR spectra of sarcoplasmic reticulum stabilized by 0.1 mM Ca^{2+} in the E_1 conformation and by vanadate in the E_2 conformation show significant differences. These differences indicate changes in the secondary structure of the protein and in the conformation of the phospholipid ester carbonyl group related to the transition between the E_1 and E_2 states. The -CH_2 and -CH_3 stretching vibrations of the acyl chains of phospholipids are not affected by the transition, and there are no clearly identified changes in vibrations related to amino acid side chains in the sarcoplasmic reticulum proteins. Since the Ca^{2+}-ATPase constitutes at least 80% of the protein content of sarcoplasmic reticulum, the Ca^{2+} and vanadate induced changes in protein secondary structure are likely to occur in the Ca^{2+}-ATPase rather than in the accessory proteins of sarcoplasmic reticulum. Nevertheless, conclusive evidence on this point is not available and may be very difficult to obtain. Reconstituted vesicle preparations containing purified Ca^{2+}-ATPase have been widely used, but recent x-ray and neutron diffraction data indicate that the disposition of Ca^{2+}-ATPase in reconstituted vesicles is very different from that in the native sarcoplasmic reticulum (Herbette *et al.*, 1983); therefore, it is difficult to relate with confidence structural information obtained on reconstituted Ca^{2+}-ATPase vesicles to the native membranes. Ca^{2+}-induced changes in the secondary structure of calsequestrin have been reported (for review see MacLennan *et al.*, 1983); however, a recent Raman spectroscopic study (Williams and Beeler, 1986) casts

some doubt on these observations. In any case, calsequestrin is a minor component representing less than 5% of the protein content of sarcoplasmic reticulum used in these studies; therefore, it is unlikely to affect significantly the FT-IR spectrum. Based on these considerations, it seems justified to assume that the observed changes in protein secondary structure involve the Ca^{2+}-ATPase.

The E_1 conformation stabilized by 0.1 mM Ca^{2+} contains less α-helix than the E_2 conformation stabilized by EGTA + vanadate. This is in essential agreement with earlier observations of Arrondo *et al.* (1985) that Ca^{2+} decreases the helix content of sarcoplasmic reticulum, compared with an EGTA-containing, *i.e.* Ca^{2+}-free, system. There is no detectable difference in β-sheet and only a slight difference in β-turn content between the E_1 and E_2 conformations. These data represent the first clear indication that changes in the secondary structure of Ca^{2+}-ATPase accompany the previously observed rearrangement of structural domains within the ATPase molecule connected with the $E_1 \rightarrow E_2$ transition (Dux *et al.*, 1985).

Previous circular dichroism studies did not indicate differences in the secondary structure composition between the E_1 and E_2 states in sarcoplasmic reticulum (Nakamoto and Inesi, 1986; Csermely *et al.*, 1987). It is difficult to list the specific reasons that may contribute to these differences between circular dichroism and FT-IR data. It is likely, however, that the greater information content, better signal-to-noise ratio, less interference by light scattering, and more sophisticated data analysis by Fourier transform infrared spectroscopy permitted the resolution of structural differences between the E_1 and E_2 states that remained undetected by circular dichroism, although the magnitude of the structural differences seen by FT-IR is difficult to quantitate.

Surprisingly, 20 mM Ca^{2+} produced similar changes in the vibrational modes assigned to protein secondary structure as vanadate, suggesting stabilization of the E_2 conformation at high Ca^{2+} concentration. Based on kinetic studies the Ca^{2+}-ATPase in the E_2 conformation has low affinity sites for Ca^{2+} (Inesi and de Meis, 1985). Therefore, it is plausible to assume that Ca^{2+} at concentrations sufficient to saturate low affinity binding sites ($\simeq$20 mM) would shift the conformational equilibrium in favor of the E_2 form. Vanadate achieves the same effect by stabilizing an E_2 type intermediate at a different stage of the Ca^{2+} transport cycle. Such interpretation is supported by recent observations of Varga *et al.* (1986) that the irreversible denaturation of Ca^{2+}-ATPase at pressures of 1000–2000 atm can be prevented or slowed either by vanadate in a calcium-free medium or by high Ca^{2+} concentration; at similar pressures sarcoplasmic reticulum vesicles stripped from Ca^{2+} by EGTA completely and irreversibly lose their enzymatic activity within minutes. These observations also imply that the mixture of enzyme conformations that exist in Ca^{2+}-free media, although usually considered to be predominantly E_2, differs from the E_2 conformation stabilized in a Ca^{2+}-free system by vanadate.

There are significant differences between spectra obtained in the E_1 and E_2 state in the intensity ratio of the 1741 cm^{-1} and 1731 cm^{-1} bands that are assigned to the vibrational modes of the *sn*-1- and *sn*-2-ester carbonyl groups of phospholipids. This observation suggests a relationship between the conformation of the protein and that of the interfacial region of phospholipids. The methylene and methyl vibrations associated with the acyl chains of the phospholipids do not show such sensitivity.

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