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Polarized infrared attenuated total reflectance spectroscopy of the Ca^{2+}-ATPase of sarcoplasmic reticulum

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The mean orientations of the transition dipole moments associated with vibrational modes of the proteins and phospholipids of sarcoplasmic reticulum were determined on dry and hydrated membrane multilayers deposited on germanium or zinc selenide crystals, using polarized infrared attenuated total reflectance spectroscopy (P-IR-ATR). For preservation of the enzymatic activity of the Ca^{2+}-ATPase the films were prepared from solutions containing 0.05 M KCl, 5 mM imidazole (pH 7.4), 0.5 mM $MgCl_2$, 1–10 mM trehalose and dithiothreitol. The anisotropy was highest in dry films containing $\simeq 7.5$ μg protein / cm^2, and sharply decreased with increasing membrane thickness or hydration. The dichroic ratio of the CH_2 vibrations (2923 cm^{-1}) of extracted sarcoplasmic reticulum phospholipids on Ge plate was 1.56, compared with a dichroic ratio of 1.68 obtained on dry films of whole sarcoplasmic reticulum. The dichroic ratios of the amide I band (1650 cm^{-1}) of the Ca^{2+}-ATPase in the Ca_2–E_1 state and in the EGTA and vanadate stabilized E_2–V state were nearly identical (1.60 vs. 1.62). The dichroism of the amide I, amide II and lipid CH_2 vibrations was not affected by changes in the concentration of KCl (25–100 mM) or Ca^{2+} ($\cong 10^{-8}$–10^{-4} M) and by the addition of vanadate (1 mM) or P_i (5 mM) in a calcium-free medium containing 0.5 mM EGTA. The dichroic ratio of the C–C (1033 cm^{-1}) or CO stretching band (1046 cm^{-1}) of trehalose incorporated into SR films was 1.2 on Ge plate; this corresponds to a mean angle of $\simeq 70°$ between the plane of the trehalose ring and the normal of the film plane, suggesting that the trehalose molecules are surprisingly well oriented in the polar headgroup region of the phospholipids. The orientation of the trehalose was not affected by the presence of Ca^{2+}-ATPase.

Introduction

The elucidation of the molecular mechanism of Ca^{2+} translocation in the sarcoplasmic reticulum requires the determination of the associated structural changes of the Ca^{2+}-ATPase. Kinetic data suggest that the Ca^{2+}-ATPase undergoes conformational changes between several states during Ca^{2+} translocation connected with the binding of Ca^{2+} and ATP, the phoshorylation of the enzyme, and the release of the products from the active site [1,2]. Indications of such structural changes have also been derived from studies of the changes in the fluorescence of protein tryptophan [3,4], and of various extrinsic fluorophores incorporated into the Ca^{2+}-ATPase [5–7], and from comparison of the structures of the Ca^{2+}-ATPase crystals obtained in the presence of EGTA and vanadate or lanthanides, respectively [8].

In this report we present observations on the orientations of the principal vibrational modes of the membrane proteins and lipids derived from attenuated total reflectance polarized infrared spectroscopy on oriented multilayers of sarcoplasmic reticulum vesicles. We were primarily interested in the effect of the Ca^{2+}-ATPase on the orientation of the hydrocarbon chains of the membrane phospholipids, and in the influence of Ca^{2+} EGTA, inorganic phosphate and vanadate on the sec-

Abbreviations: ATR, attenuated total reflectance; Brij 36T, polyoxyethylene glycol lauryl alcohol ($C_{12}E_{10}$); Ca^{2+}-ATPase, Ca^{2+} transporting ATPase (EC 3.6.1.38); DTT, dithiothreitol; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; IR, infrared; FTIR, Fourier transform infrared; Mops, 3-(*N*-morpholino)propanesulfonic acid; SR, sarcoplasmic reticulum.

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ondary structure and side chain vibrations of the Ca^{2+}-ATPase.

The polarized infrared spectra provide information on the mean orientation of the transition dipole moment of the amide and side chain groups of proteins, and of the acyl chains of phospholipids. The order parameter of the characteristic vibrational bands is evaluated by measuring the dichroic ratio. The dichroic ratio is influenced by the refractive indices of both the sample and the crystals used as ATR support. Refractive indices of biomembranes are suggested to range from 1.35 to 1.7 [9–18]. Since accurate estimates of the refractive index of the membrane films are usually not available, we determined the order parameter based on the formalism of Flournoy and Schaffer [19] by combining the dichroic ratios of the membrane films measured on two different crystals (i.e., Ge and ZnSe), each of which have distinct, well-defined refractive indices. The refractive index of sarcoplasmic reticulum membrane film obtained by this technique is 1.68 ± 0.15, i.e., it is within the range of values reported in the literature for other biological membranes [9–18].

An extension of these studies to films of different levels of hydration may permit the analysis of changes in the orientation of structural domains of Ca^{2+}-ATPase during Ca^{2+} transport induced by the release of caged Ca^{2+} or caged ATP.

Experimental procedures

Materials

Adenosine 5'-triphosphate, Brij 36T, 1,6-di-*tert*-butyl-*p*-cresol, dithiothreitol, EGTA, imidazole, K-Mops, NADH, magnesium chloride, lactate dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), phospho*enol*pyruvate, bovine serum albumin, trehalose and Trizma base were obtained from Sigma Chemicals, St. Louis, MO. Calcium chloride was obtained from Baker Chemicals, Phillipsburg, NJ, and A23187 from Behring Diagnostics, La Jolla, CA. Sodium vanadate and sodium azide were the products of Fisher Scientific, Fairlawn, NJ. All chemicals were of analytical grade. Trasylol was obtained from Mobay Chemicals New York, NY; glycerol was a product of Aldrich, Milwaukee, WI.

Methods

(a) Isolation of sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles were isolated from rabbit muscles according to Nakamura et al. [20]. The preparations were frozen in liquid nitrogen and stored before use in a medium of 0.3 M sucrose, 10 mM Tris-maleate buffer (pH 7.0) at a protein concentration of 30–40 mg/ml in polyethylene containers at $-70\,^{\circ}C$.

Just before use, 1-ml aliquots of microsome suspensions were thawed and diluted 40-fold with a medium containing 0.1 M KCl, 1 mM $MgCl_2$ and 10 mM imidazole (pH 7.4). The vesicles were centrifuged for 45 min at $92000 \times g$. The pellets were resuspended in the same medium to a final protein concentration of 40–50 mg/ml. After protein determination according to Lowry et al. [21], the solutions were diluted to yield final concentrations of 0.05 M KCl, 0.5 mM $MgCl_2$, 5 mM imidazole (pH, 7.4) and 10 mg sarcoplasmic reticulum protein/ml; when indicated trehalose and dithiothreitol were added to final concentrations indicated in the Legends.

(b) Preparation of oriented sarcoplasmic reticulum multilayers of various thicknesses for infrared spectroscopy. To assess the effect of membrane thickness on the polarization, membrane films containing 2–40 μg protein/cm^2 were prepared.

(i) Thick films (~ 40 μg protein/cm^2). Aliquots of vesicle suspensions (10 mg protein/ml) in a medium containing 50 mM KCl, 0.5 mM $MgCl_2$ and 5 mM imidazole (pH 7.4) and 10 mM DTT were deposited on $\simeq 2.5$ cm^2 surface area on Ge or ZnSe ATR crystals.

(ii) Thin films (~ 7.5 μg protein/cm^2). Aliquots (15 μl) of vesicle suspensions (5 mg protein/ml) in a medium containing 25 mM KCl, 0.25 mM $MgCl_2$, 5 mM imidazole (pH 7.4) 10 mM DTT and 10 mM trehalose were deposited on $\simeq 10$ cm^2 surface area on Ge or ZnSe ATR crystals. The Ca^{2+}-ATPase was stabilized in the Ca_2-E_1 state by the addition of 10^{-4} M Ca^{2+}, in the E_2-V state by 1 mM vanadate and 0.5 mM EGTA and in the E_2-P state by the addition of 5 mM inorganic phosphate and 0.5 mM EGTA.

(iii) Thin films of extracted sarcoplasmic reticulum phospholipids. Phospholipids were extracted from sarcoplasmic reticulum vesicles according to Folch et al. [22]. The solvents (2:1 chloroform/ethanol) were evaporated and the phospholipids were dispersed in a buffer solution containing 25 mM KCl, 0.25 mM $MgCl_2$, 5 mM imidazole (pH 7.4), 10 mM DTT and 10 mM trehalose by vortexing and sonication under N_2 atmosphere at 2–4 °C. The phospholipid concentration was adjusted to 5 mg/ml and aliquots of 15 μl were deposited on $\simeq 10$ cm^2 surface area on Ge or ZnSe ATR crystals.

The films prepared under all three conditions were dried in N_2 atmosphere at 2–4 °C for 1 h under vacuum. After measurement of the infrared spectra the sarcoplasmic reticulum films were rehydrated, desorbed and analyzed for protein and ATPase activity as described below. In control experiments the dried films were stored at 2–4 °C for various times before rehydration and assay of ATPase activity; the data were compared with parallel assays on hydrated specimens of the same preparations.

(c) Preparation of hydrated sarcoplasmic reticulum multilayers for infrared spectroscopy. Aliquots (15 μl) of vesicle suspensions (5 mg protein/ml) in a medium

containing 25 mM KCl, 0.25 mM $MgCl_2$, 5 mM imidazole (pH 7.4), 10 mM DTT and 10 mM trehalose were deposited on the surface of Ge plates (5×2 cm^2). The effect of hydration was monitored by taking successive infrared spectra during drying.

(d) Preparation of microcrystals of Ca^{2+}-ATPase for infrared spectroscopy. The Ca^{2+}-ATPase microcrystals were formed by incubating the sarcoplasmic reticulum (2 mg protein/ml) in a crystallization medium of 0.1 M KCl, 10 mM K-Mops, (pH 6.0), 3 mM $MgCl_2$, 20 mM $CaCl_2$, 5 mM DTT, 25 IU Trasylol/ml, 3 mM NaN_3, 2 μg 1,6-di-*tert*-butyl-*p*-cresol/ml, 40% (v/v) glycerol, and 8 mg Brij 36T/ml at 2°C under nitrogen [23,24]. In order to reach the high protein concentration required for the FTIR measurements (5–10 mg/ml) the microcrystals of Ca^{2+}-ATPase were pooled from eight vials of 1 ml each and centrifuged in an IEC clinical centrifuge at $\simeq 2000 \times g$ for 15 min. The supernatant was discarded and the pellet in ~ 500 μl crystallization medium was transferred to a Beckman microfuge tube and centrifuged twice 5 min. The excess supernatant was removed and the pellet containing microcrystals of Ca^{2+}-ATPase was deposited on the surface of the ZnSe plate (2×5 cm^2). The crystallization was ascertained by negative staining.

(e) FTIR measurements. IR spectra were taken using the Mattson Cygnus 100 FTIR spectrometer with an MCT detector. The attenuated total reflectance accessories consisting of a twin parallel mirror reflectance attachment (model TPMRP), the ATR crystal holder (model IRA), the ATR crystals, and the polarizer (model PSD-J1R) were the products of Harrick Scientific, Ossining, NY. Germanium (Nos. EJ3134 and EJ3131) or zinc selenide (Nos. EM3134 and EM3131) ATR crystals cut with an incidence angle of 45° were used. The dimensions of the ATR crystals were $25 \times 20 \times 3$ mm or $50 \times 20 \times 3$ mm. For each measurement 512 scans were collected and coadded in single beam mode, zero filled once and apodized with triangular function. Each spectrum was ratioed with a background spectrum obtained with the polarizer set at 0° ($A_\parallel$, electrical vector in the horizontal plane transmitted), or at 90° ($A_\perp$, electrical vector in the vertical plane transmitted), using membrane-free clean ATR crystals. The resolution was 2 cm^{-1}.

(f) Evaluation of the dichroic ratio and of the mean angle. The dichroic ratio r is defined by:

$$r = \frac{\Delta A_\parallel}{\Delta A_\perp} \tag{1}$$

r was estimated from the IR ATR spectra. $\Delta A_\parallel$ and $\Delta A_\perp$ are the differences in reflection absorbance spectra between the ATR crystal covered with SR and the clean ATR crystal measured with the polarized infrared light parallel ($\Delta A_\parallel$) and perpendicular ($\Delta A_\perp$) to the normal of the SR film (crystal surface). The $\Delta A_\parallel$ and $\Delta A_\perp$ intensities were estimated from a curve fitting analysis [25], assuming pure Lorentzian band shape.

The order parameter of the transition moment (F) is expressed by:

$$F = \frac{kz - kx}{kz + 2kx} \tag{2}$$

where kz and kx are the absorption indices of the sample along the normal of the ATR plane and parallel to the ATR plane, respectively. Using the equation of Flournoy and Schaffer [19] for the kx and kz indices, and 45° angle of incidence of the IR light into the ATR plate, F becomes:

$$F = \frac{r-2}{r + \frac{1+2P}{1-P}} \tag{3}$$

with $P = (n_2/n_1)^2$, where r is the dichroic ratio as defined in Eqn. 1, and n_2 and n_1 are the refractive indices of the sample and of the ATR crystal, respectively. For Ge: $n_1 = 4$ and for ZnSe: $n_1 = 2.44$. Eqn. 3 is valid only when the thickness of the film is greater than the depth of penetration of the IR light into the film. The critical thickness for sarcoplasmic reticulum membranes is about 15 μg microsomal protein/cm^2. For films thinner than 15 μg microsomal protein/cm^2, Eqn. 3 is only an approximation. Earlier examples of the use of Eqn. 3 can be found in Refs. 17 and 18.

The dichroic ratio data obtained from measurements on two different crystals with different refractive indices were combined, assuming that the refractive indices of the sarcoplasmic reticulum sample was identical on the Ge and ZnSe ATR crystals. After combination of the two data sets, Eqn. 3 becomes:

$$F = \frac{1}{1 + \left(\frac{3}{R-1}\right)\left(\frac{R}{r_a - 2} - \frac{1}{r_b - 2}\right)} \tag{4}$$

with $R = (n_a/n_b)^2$, where n_a and n_b are the refractive indices of the ATR crystal 'a' (Ge), and of the ATR crystal 'b' (ZnSe), and r_a and r_b are the dichroic ratios obtained from the samples on crystal a and on crystal b, respectively. The dichroic ratios r_a and r_b are evaluated in the same manner as r in Eqn. 1. The advantage of using Eqn. 4 instead of Eqn. 3 is to avoid the determination of the refractive indices of the sample.

Finally, the order parameter F is related to the mean angle β between the normal of the ATR plane and the transition dipole moment.

$$F = \tfrac{1}{2}(3\cos^2\beta - 1) \tag{5}$$

It is assumed that the direction of the transition dipole moment vector has an axially symmetric distribution relative to the normal plane. Since the orientation of the sarcoplasmic reticulum membrane is not perfect, one can distribute the membrane normal with a mosaic spread function F_{ms}, again assuming it to be axially symmetric (see for example, Ref. 15). The mosaic spread order can be evaluated from small angle diffraction X-ray data of hydrated sarcoplasmic reticulum samples. An angle of $\pm 15°$ was found [26] corresponding to a distribution function, $F_{ms} = 0.9$. For comparison the mosaic spread order parameter of bacteriorhodopsin is between 1 and 0.9 [15,16].

The mean angle α between the normal of the film plane and the phospholipid hydrocarbon chain axis can be evaluated by assuming that its orientation is axially symmetric and that the transition dipole moment of the CH_2 stretching vibration of the hydrocarbon chains is perpendicularly distributed around the chain axis:

$$F = F_{ms}\cdot\left(\frac{3\cos^2 90° - 1}{2}\right)\cdot\left(\frac{3\cos^2 \alpha - 1}{2}\right) = 0.45\left(\frac{3\cos^2 \alpha - 1}{2}\right) \quad (6)$$

The mean angle γ formed by the normal of the film plane and the peptide carbonyl bond direction can also be evaluated by assuming its axially symmetrical distribution. The transition dipole moment corresponding to the amide I band is oriented at an angle of $15° \pm 3°$ from the C=O bond direction and is in the peptide plane [27].

$$F = F_{ms}\cdot\left(\frac{3\cos^2 15° - 1}{2}\right)\cdot\left(\frac{3\cos^2 \gamma - 1}{2}\right) \quad (7)$$

The Ca^{2+}-ATPase contains a mixture of α-helix (46%), β structure (7%), turns (12%) and random coil (35%) based on circular dichroism spectroscopy [28]. In the case of the Ca^{2+}-ATPase of sarcoplasmic reticulum, Eqn. 7 provides information about the average orientation of the C=O bond, rather than its transition dipole moment.

(g) ATPase activity measurements. ATPase activities were measured by a coupled enzyme system of pyruvate kinase and lactate dehydrogenase [29,30]. The dried films prepared as described under (b) were gently removed by magnetic stirring from their ATR or glass supports with 200 μl buffer containing 0.1 M KCl, 10 mM imidazole (pH 7.4) and 1 mM $MgCl_2$. The supports were rinsed twice and the combined washing fluids were used for determination of ATPase activity and protein concentration. Control suspensions of sarcoplasmic reticulum vesicles were incubated at 2–4 °C for the same time as the dried films before assay of ATPase activity.

(h) Electron microscopy. (i) Analysis of dry SR films (1–30 μg protein/cm^2). Aliquots of vesicle suspension (5–10 mg protein/ml) containing 50 mM KCl, 0.5 mM $MgCl_2$, 5 mM imidazole (pH 7.4) and 10 mM DTT were deposited evenly on the surface of a glass plate (~ 3 cm^2) previously coated with a thin layer of 0.5% parlodion in amyl acetate. The film of SR was dried under nitrogen and vacuum at 2 °C, and fixed with vapors of 2% OsO_4 and 37% formaldehyde in separate containers for 2 h. The fixed dried film was floated off onto water and picked up from underneath with a paper filter. After drying the SR film was carefully removed from the filter paper, embedded in araldite and cross-sectioned at 600 Å thickness using a diamond knife (LKB Ultramicrotome). The samples were placed on 300 mesh copper grids and stained with lead citrate and uranyl acetate [31].

(ii) Microcrystals of Ca^{2+}-ATPase. Ca^{2+}-ATPase crystals prepared as described under (d) were fixed with 1% glutaraldehyde for 24 h at 2 °C and pelleted by centrifugation. The crystalline pellet was washed several times, postfixed with 1% OsO_4 for 1 h at room temperature, dehydrated in an ethanol series and embedded in araldite. Thin sections were cut and stained with uranyl acetate and lead citrate [31]. The samples were viewed using a Siemens Elmiskop 102 electron microscope at 60 kV.

Results

The morphology of dried sarcoplasmic reticulum films and of microcrystals of Ca^{2+}-ATPase

The formation of ordered multilayers of sarcoplasmic reticulum membranes is a prerequisite for meaningful measurements of the orientation of transition dipole moments associated with the vibrational groups.

To establish the minimum layer thickness of the sarcoplasmic reticulum films that was consistent both with a reasonable signal to noise ratio of the FTIR spectra and satisfactory order in the membrane layering, membranes of different thicknesses were analyzed for structural regularity by electron microscopy, parallel with the measurements of ATPase activity and FTIR spectra. Fig. 1A shows the cross section of an SR film consisting of several layers of sarcoplasmic reticulum membranes with an average protein density of 1.0 μg protein/cm^2 surface area. The film consists of flattened vesicles of sarcoplasmic reticulum relatively evenly distributed on the surface with the average film-plane coincident with the plane of the support surface. As the film thickness was increased to protein densities of 3, 7, and 30 μg/cm^2 surface area there was a progressively greater disorder in the orientation of the membrane multilayers (Fig. 1B and C) that was also reflected in the dichroic ratios of SR films of

increasing thicknesses (see below). Based on these observations, most of the measurements contained in this report were made at a film thickness of ≃ 7.5 μg protein per cm^2 surface area that provided a reasonable compromise between the ordering of multilayers and the amount of membrane mass required for FTIR spectra of good signal to noise ratio.

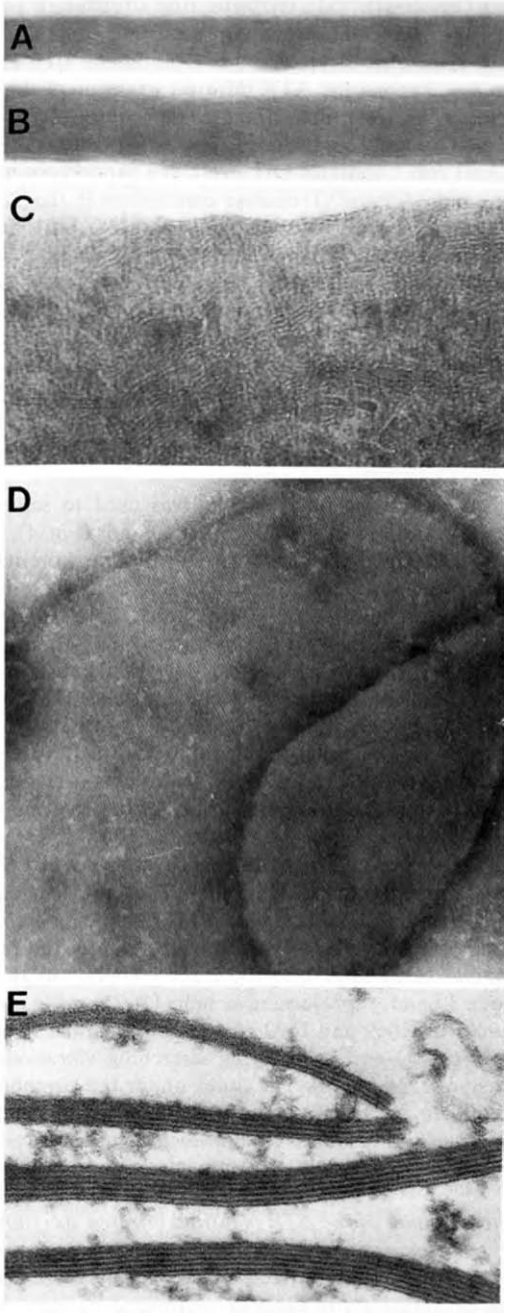


Fig. 1. Electron microscopy of dry sarcoplasmic reticulum films and of microcrystals of Ca^{2+}-ATPase. (A) Cross-section of a fixed and embedded sarcoplasmic reticulum film containing about 1.0 μg protein/cm^2. Magnification: 151200×. (B) Same as A but at a protein density of approx. 3 μg protein/cm^2. Magnification: 151200×. (C) Same as (A), but at a protein density of 30 μg/cm^2. Magnification: 151200×. (D) Negatively stained microcrystals of SR Ca^{2+}-ATPase after two weeks of incubation in the crystallization medium at 2°C under N_2, as described under Methods. Magnification: 56737×. (E) Cross-section of fixed and embedded microcrystals of Ca^{2+}-ATPase illustrating the lamellar structures consisting of 4–6 sheets of Ca^{2+}-ATPase crystals. Magnification: 88276×.

TABLE I

Effect of trehalose and dithiothreitol on the ATPase activity

Microsomes (1–2 mg/ml) were suspended at 2°C in H_2O medium containing 10 mM KCl, 0.1 mM $MgCl_2$ and 10 mM imidazole (pH 7.4), with the indicated additions. Aliquots (50 μl) were taken for the preparation of dried films (≃ 40 μg protein/cm^2) on microscope cover glass surfaces. After drying for several hours the films were rehydrated with 200 μl of H_2O medium containing 0.1 M KCl, 10 mM imidazole (pH 7.4) and 1 mM $MgCl_2$ and washed as described under Experimental procedures. Control vesicle suspensions were kept during the same time at 2–4°C. After protein determination in both control and dehydrated samples [21], ATPase activities were measured as described under Experimentalprocedures. The numbers in parentheses represent the numbers of independent measurements.

Samples additions	ATPase activity (μmol mg^{-1} min^{-1})		
	before drying	after drying	% recovery
no additions	2.63 ± 0.34 (5)	0.61 ± 0.43 (4)	23
10 mM trehalose	2.52 ± 0.41 (4)	1.50 ± 1.00 (3)	60
10 mM trehalose + 1 mM DTT	2.71 ± 0.40 (3)	2.48 ± 0.41 (3)	92
1 mM trehalose + 1 mM DTT	2.89 ± 0.44 (3)	2.45 ± 0.48 (3)	85

Crystalline sheets of Ca^{2+}-ATPase formed in detergent solutions containing 20 mM $CaCl_2$ and 40% glycerol [23,24] can also be deposited on support surfaces, forming well-ordered multilayers consisting of ≃ 5–6 stacked sheets (Fig. 1D and E). The utilization of these highly ordered membranes for FTIR spectroscopy was so far limited by the fact that the crystallization medium required for the preservation of crystals contributes to critical regions of the FTIR spectra.

The effects of drying on the ATPase activity of the Ca^{2+}-ATPase of sarcoplasmic reticulum

The drying of sarcoplasmic reticulum vesicles in surface films results in significant irreversible loss of ATPase activity, indicating denaturation of the Ca^{2+}-ATPase, unless protective substances are added to the suspending media before drying (Table I). The structural change connected with such denaturation caused

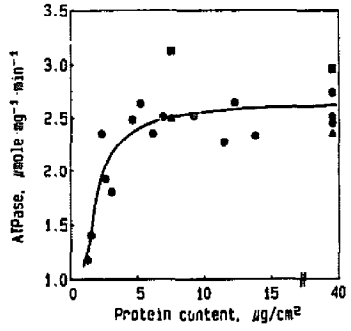


Fig. 2. ATPase activity of sarcoplasmic reticulum vesicles after drying and rehydration. Sarcoplasmic reticulum vesicles (5 mg protein/ml) were suspended in H_2O medium containing 25 mM KCl, 0.25 mM $MgCl_2$, 5 mM imidazole (pH 7.4), 1–10 mM DTT and 10–60 mM trehalose. 5–50 μl of sample was deposited on a glass plate of 4–18 cm^2 surface area corresponding to protein densities of 1.4 to 40 μg protein/cm^2 (●). Data were also obtained on dry films of sarcoplasmic reticulum deposited on ZnSe ATR plate (■) and on Ge ATR plate (▲) after taking their polarized infrared spectra. For other details see Experimental procedures.

a shift of the amide I band from 1649 cm^{-1} to 1654 cm^{-1}. Dithiothreitol (1–2 μmol/mg protein) and trehalose (1–10 μmol/mg protein) nearly completely prevented the loss of ATPase activity (Table I) and reduced the shift of the amide I band, presumably by substituting for H_2O in hydrogen bond formation or by stabilizing some of the hydrogen bonded interactions. The protective effect of trehalose is in agreement with earlier observations of Crowe and his colleagues [32–34]. The recovery of ATPase activity after rehydration was consistently high in films that contained 5–40 μg protein per cm^2 surface area (Fig. 2 and Table I). The recovery of activity decreased sharply in thinner films containing only 2–3 μg microsomal protein/cm^2, that corresponds to two to three layers of sarcoplasmic reticulum (Fig. 2). Addition of trehalose (12 μmol/mg protein) and DTT (1 μmol/mg protein) to the sarcoplasmic reticulum samples before drying of thin films was not sufficient to completely prevent this loss of ATPase activity. To minimize these effects the thickness of the sarcoplasmic reticulum films used for evaluation of dichroic ratios was not less than ~7.5 μg microsomal protein/cm^2, representing approx. seven to eight membrane layers.

Attenuated total reflectance spectra of dry sarcoplasmic reticulum film

The contributions of proteins, phospholipids, trehalose and DTT to the ATR infrared spectrum of sarcoplasmic reticulum are shown in Fig. 3A and B. Trace 1 corresponds to the ATR infrared spectrum of the dry phospholipids extracted from sarcoplasmic reticulum. The main bands of interest are: 3013 (olefinic C = C-H stretching); 2956 (CH_3 asymmetric stretching); 2924 (CH_2 asymmetric stretching); 2872 (CH_3 symmetric stretching); 2854 (CH_2 symmetric stretching); 1738 (C=O stretching); 1466 (CH_2 scissoring); 1378 (CH_2 symmetric bending); 1242 (PO_2^- asymmetric stretching); 1180 (C–O–C asymmetric stretching); 1090 (PO_2^- symmetric stretching); 1065 (C–O–C symmetric stretching). Trace 2 shows the ATR infrared spectrum of a dry film of sarcoplasmic reticulum. The assignments of the protein bands [35] are: 3294 (amide A); 1654 (amide I); 1545 (amide II); 1515 (tyrosine ring stretching); 1409–1393 (tyrosine OH stretching; aspartate or glutamate CO_2^- symmetric stretching); ~1300 (amide III). Trace 3 in Fig. 3 shows the ATR infrared spectrum of the dry sarcoplasmic reticulum film in the presence of trehalose (2 μmol/mg protein). The broad band centered at 3301 cm^{-1} contains OH stretching superimposed on the amide A band. Trehalose contributes in the 1200–1000 cm^{-1} region with six bands at 1148, 1101, 1075, 1046, 1033 and 992 cm^{-1}; based on comparisons with carbohydrate spectra [36], these presumably arise from C–C–C, C–C–O, C–O–C ring stretching. Trace 4 in Fig. 3 is the ATR infrared spectrum of sarcoplasmic reticulum containing both DTT and trehalose (2 μmol/mg protein each). The contribution of DTT to the sarcoplasmic reticulum spectrum is relatively small, as evidenced by the bands in the 1400–1300 cm^{-1} and in the 1200–900 cm^{-1} regions.

A curve fitting program [25] was used to separate the contributions of the overlapping bands (Fig. 4). The 3100–2800 cm^{-1} region was fitted with four Lorentzian bands located at 2961, 2923, 2873 and 2854 cm^{-1} (Fig. 4A), corresponding, respectively, to the asymmetric CH_3 and CH_2 and to the symmetric CH_3 and CH_2 vibrations. There was a small deviation between the best fit and the experimental curve around 2900 cm^{-1}, that may correspond to the broad Fermi resonance band [37] or to protein and dithiothreitol CH_2 vibrations. The dichroic ratio of the hydrocarbon chain was evaluated by measuring the surface of the most intense 2923 cm^{-1} band (see below). In the 1800–1500 cm^{-1} region, six Lorentzian band shapes were needed to fit the infrared spectrum (Fig. 4B). They are located at 1739, 1669, 1652, 1632, 1543 and 1515 cm^{-1} and correspond, respectively, to the carbonyl group of the phospholipid (1739 cm^{-1}), to the three components of the amide I band, representing α helix (1652 cm^{-1}) and β structures (1669 and 1632 cm^{-1}), to the amide II band (1543 cm^{-1}) and to the ring stretching vibrations of tyrosine (1515 cm^{-1}). The areas under the component bands are in rough agreement with the secondary structures derived from circular dichroism spectroscopy [28].

Determination of the ATR polarized infrared spectrum of sarcoplasmic reticulum film

A typical ATR polarized infrared spectrum of sarcoplasmic reticulum in the Ca^{2+}-stabilized E_1 state (Ca_2

$- E_1$) is shown in Fig. 5. The film (7.5 μg protein/cm^2) was dried on Ge plate from a medium containing 25 mM KCl, 0.25 mM $MgCl_2$, $\approx 10^{-5}$ M $CaCl_2$, 5 mM imidazole (pH 7.4) 10 mM DTT, and 10 mM trehalose. After recording the ATR-IR spectra, the rehydrated film had an ATPase activity of 2.50 $\pm$ 0.25 μmol ATP mg^{-1} min^{-1}, indicating good preservation of structure.

The dichroic ratios of the phospholipid and protein infrared bands were evaluated from their relative polarized intensities measured at two angles using the

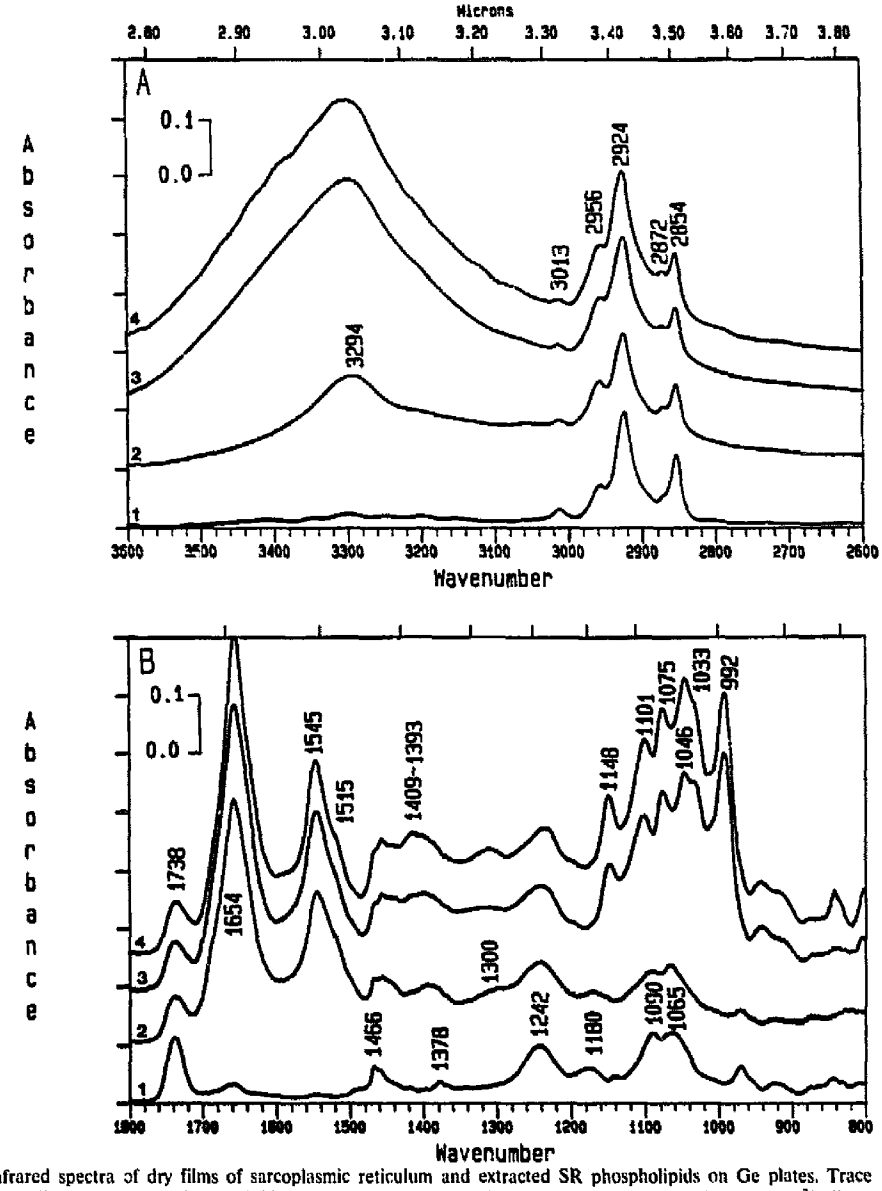


Fig. 3. ATR infrared spectra of dry films of sarcoplasmic reticulum and extracted SR phospholipids on Ge plates. Trace 1: ATR infrared spectrum of a dry film of extracted phospholipids from sarcoplasmic reticulum (approx. 7.5 μg phospholipids/cm^2). Trace 2: ATR infrared spectrum of sarcoplasmic reticulum dry film (approx. 7.5 μg microsomal protein/cm^2). Trace 3: ATR infrared spectrum of dry sarcoplasmic reticulum film (approx. 7.5 μg microsomal protein/cm^2) in the presence of trehalose (approx. 2 μmol/mg protein). Trace 4: ATR infrared spectrum of dry sarcoplasmic reticulum film (approx. 7.5 μg microsomal protein/cm^2) in the presence of trehalose and DTT (each approx. 2 μmol/mg protein). The four dried films were formed from H_2O medium containing 25 mM KCl, 0.25 mM $MgCl_2$, 5 mM imidazole (pH 7.4), with the indicated concentrations of DTT and trehalose. The concentration of extracted phospholipids was 4.7 mg/ml and the protein concentration was 5 mg/ml. (Panel A) 3600–2600 cm^{-1}. (Panel B) 1800–800 cm^{-1}.

curve fitting program described in Fig. 4 to separate the contributions of overlapping bands. These data are shown in Table II (samples A and B). The dichroic ratios measured on thick films (sample A, ~40 μg microsomal protein/cm^2) were greater than those obtained on thinner films (sample B, ~7.5 μg microsomal protein/cm^2). A dichroic ratio of 2 would indicate either a random orientation of the transition dipole moment or that the transition dipole moment is oriented at an angle of 54.7° with respect to the normal of the film plane. Therefore the higher values of dichroic ratios obtained on thick films probably reflect a more random orientation of the transition dipole moment directions compared with thinner films. The reasons for the increase in randomness in thicker films are not very well understood but, as shown in Fig. 1, defects in the alignment of the membrane layers are expected to increase with increased film thickness.

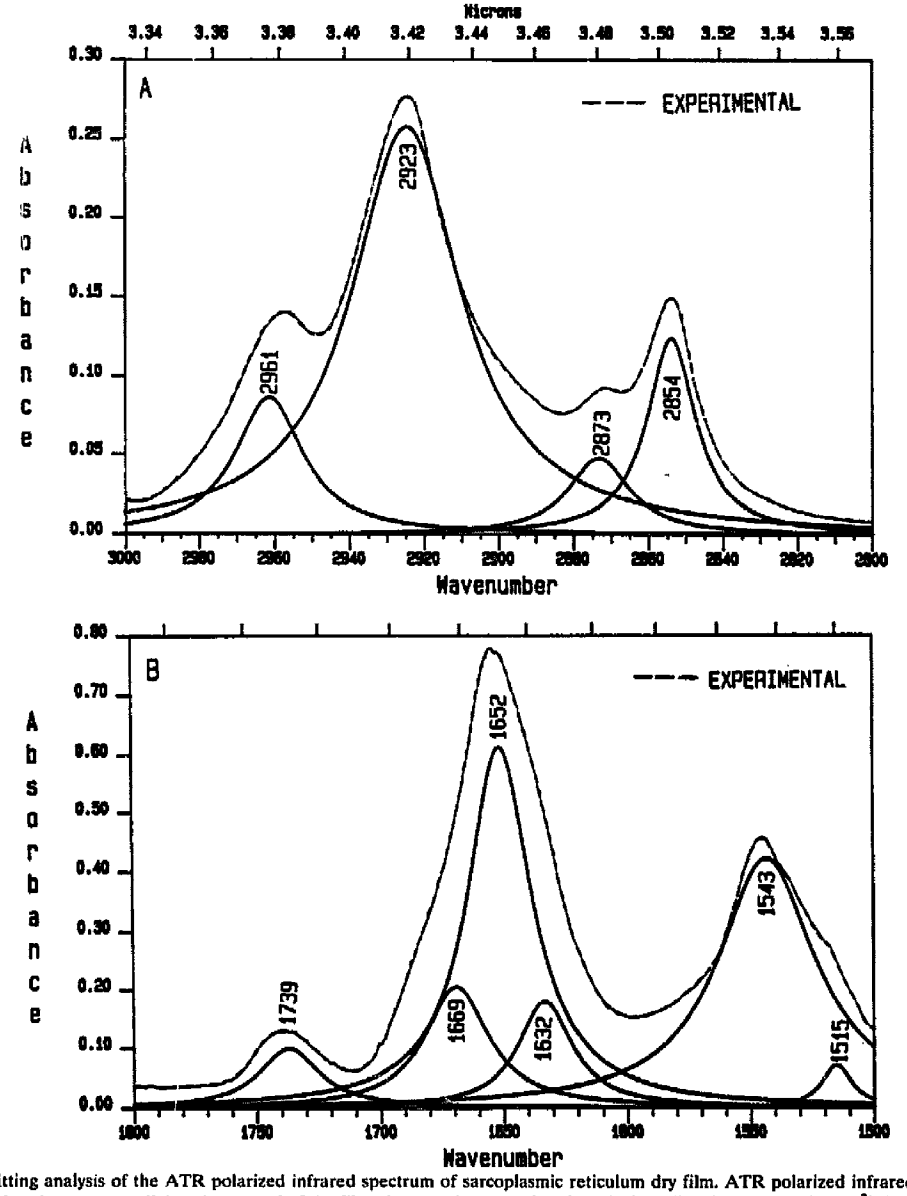


Fig. 4. Curve fitting analysis of the ATR polarized infrared spectrum of sarcoplasmic reticulum dry film. ATR polarized infrared spectrum was obtained with electric vector parallel to the normal of the film plane on dry sarcoplasmic reticulum films (40 μg protein/cm^2) deposited on ZnSe plates in the Ca_2-E_1 state. For medium compositions and film preparation, see Methods. (Panel A) 3000–2800 cm^{-1}. (Panel B) 1800–1500 cm^{-1}.

The ATR polarized infrared spectra of the Ca^{2+}-ATPase of sarcoplasmic reticulum in its various stable conformations

During Ca^{2+} transport and ATP hydrolysis, the Ca^{2+}-ATPase of sarcoplasmic reticulum goes through a series of conformational changes [1,2,38–41]. The binding of Ca^{2+} at low concentration (10^{-4} M) stabilizes the Ca_2-E_1 state, while the addition of vanadate and EGTA promotes the transition to the E_2-V state; these transitions can be readily differentiated using fluorescence spectroscopy [7]. The dichroic ratios of sarcoplasmic reticulum films formed from solutions that are

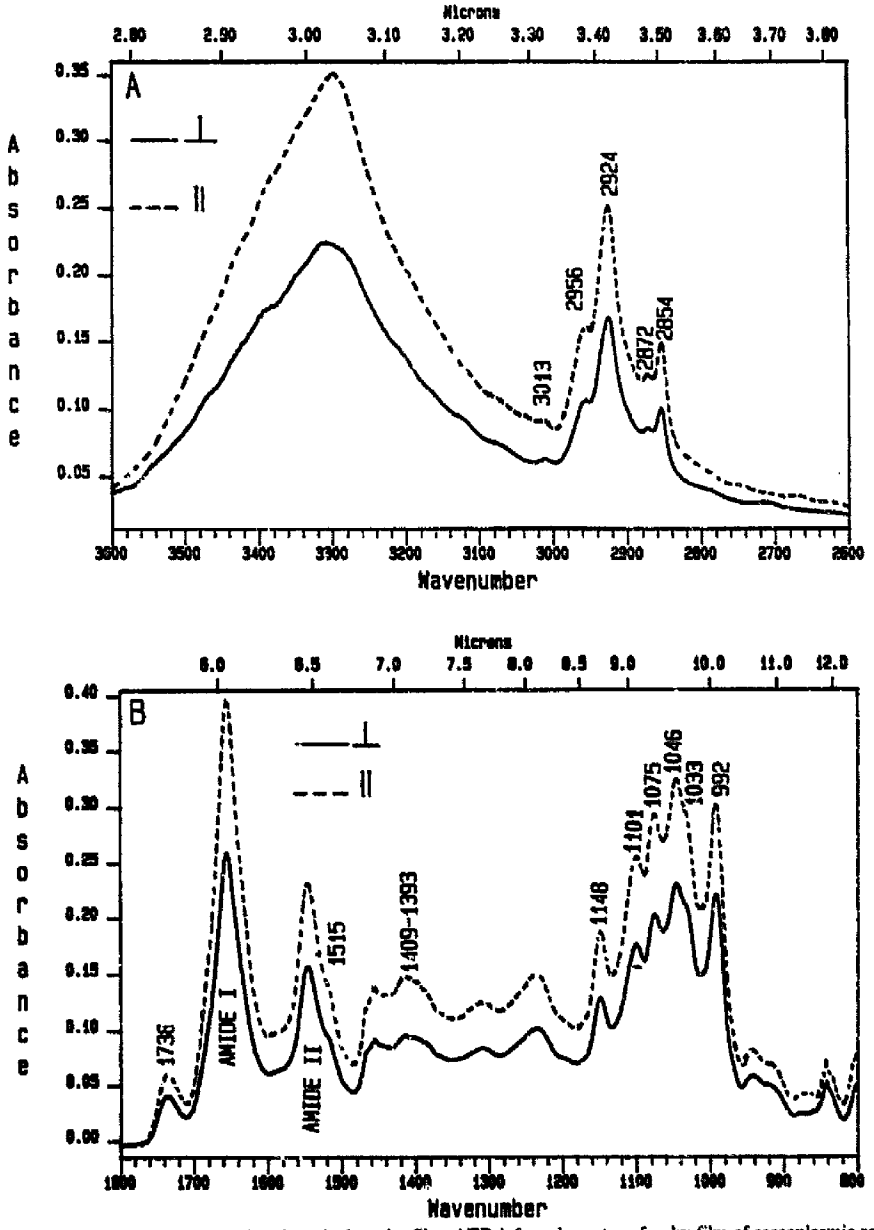


Fig. 5. ATR polarized infrared spectra of sarcoplasmic reticulum dry film. ATR infrared spectra of a dry film of sarcoplasmic reticulum (approx. 7.5 μg microsomal protein/cm^2) in the Ca_2-E_1 state on Ge plate. For medium compositions and film preparation, see Methods. The dashed line and the full line correspond to the electric vector of transmitted light polarized parallel and perpendicular to the normal of the film plane, respectively. (Panel A) 3600–2600 cm^{-1} (Panel B) 1800–800 cm^{-1}.

TABLE II

Dichroic ratios and mean angles of orientation of the transition dipole moments of sarcoplasmic reticulum films relative to the normal of the membrane plane

Dichroic ratios were estimated from curve fitting analysis of the ATR polarized infrared spectra of thin sarcoplasmic reticulum films (40 and 7.5 μg protein/cm^2) as described under Experimental procedures and in Legends to Figs. 4–6. The standard deviations were between 0.05–0.12, except for the lipid carbonyl, that was between 0.15–0.24. The orientation factor was evaluated from the dichroic ratio data obtained from Ge and ZnSe support using Eqn. 4. The mean angle was evaluated according to Eqn. 5 and the estimated range of uncertainty is 1–2°. The dichroism of the amide I band was evaluated from the sum of the three component bands.

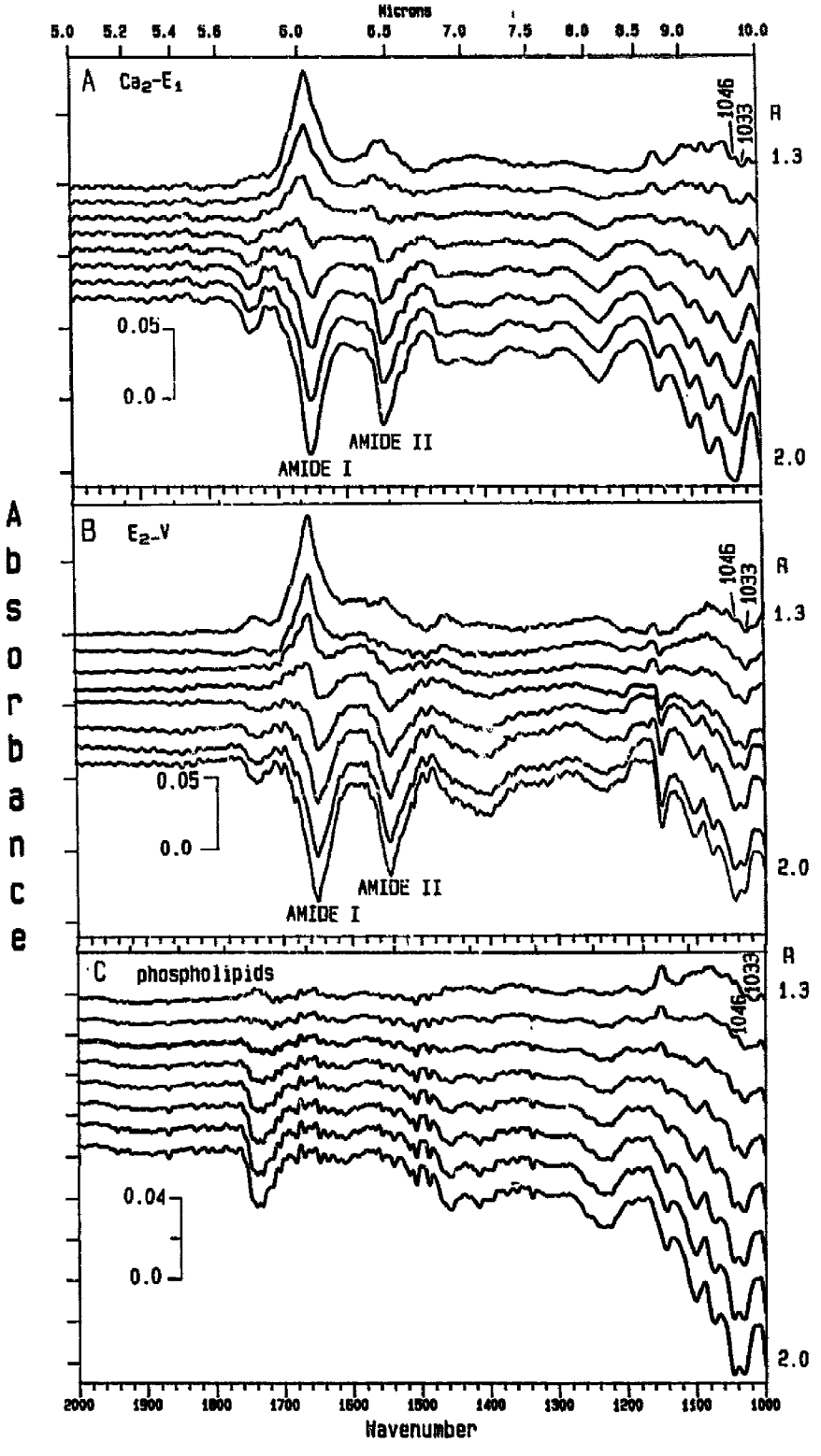
Samples	Infrared bands (cm^{-1})	Attribution	Dichroic ratios		Mean angle (degrees)
			from Ge support	from ZnSe support	
A Thick film of SR					
= 40 μg/protein/cm^2)	2923	$-CH_2$-lipids	1.97	1.80	55
stabilized in the	1739	-C=O lipids	2.14	1.77	53
Ca_2-E_1 state	1650	amide I protein	1.90	1.74	56
	1543	amide II protein	1.82	1.71	57
B Thin film of SR					
(= 7.5 μg protein/cm^2)	2923	$-CH_2$-lipids	1.67	1.48	59
stabilized in the	1739	-C=O lipids	1.40	1.21	64
Ca_2-E_1 state	1650	amide I protein	1.60	1.36	60
	1543	amide II protein	1.57	1.32	60
C Thin film of SR					
(= 7.5 μg protein/cm^2)	2923	$-CH_2$ lipids	1.68	1.40	58
stabilized in the	1739	-C=O lipids	1.45	1.20	63
E_2-V state	1650	amide I protein	1.62	1.31	59
	1543	amide II protein	1.52	1.27	61
D Thin film of extracted					
phospholipids from	2923	$-CH_2$-lipids	1.56	1.33	61
sarcoplasmic reticulum	1739	-C=O lipids	1.37	1.35	66

expected to stabilize the Ca_2-E_1, the E_2-V or the E_2-P states of the Ca^{2+}-ATPase were determined by difference ATR polarized infrared spectroscopy on thin films of sarcoplasmic reticulum containing 7.5 μg protein/cm^2 (Figs. 6A, B and Fig. 7). Films made from extracted sarcoplasmic reticulum phospholipids were subjected to the same analysis (Fig. 6C).

The difference between the ATR infrared spectra of sarcoplasmic reticulum recorded with electric vector polarized parallel($\parallel$) and perpendicular($\perp$) to the normal of the membrane plane provides a qualitative measure of the dichroism of the infrared bands (Fig. 6). Subtraction factors (R) ranging from 1.3 to 2 were used in Fig. 6 to generate the successive traces. By convention, positive bands in the difference spectrum ($\Delta I_\parallel$ minus $R\Delta I_\perp$) indicate that the transition dipole moment direction is oriented toward the normal of the film plane, while negative bands imply an orientation within the film plane. For example, the trehalose bands located at 1033 and 1046 cm^{-1} (tentatively attributed to C–C and C–O stretching ring vibrations) are always negative (Fig. 6A and B), suggesting that their transition dipole moments are oriented toward the plane of the film surface. By contrast, the amide 1 band is still positive when the other bands are negative (Fig. 6A and B), suggesting that the mean transition dipole moments of the protein C = O groups are closer to the normal of the sarcoplasmic reticulum film plane. The subtraction factors used to cancel the intensity of the various bands should approximate their respective dichroic ratios and are expected to be similar to the values given in Table II.

The dichroic ratios of the CH_2 groups of the phospholipids extracted from sarcoplasmic reticulum (Fig. 6C and Table II, sample D) are slightly smaller than those obtained from dry films of whole sarcoplasmic

Fig. 6. Infrared-ATR dichroic difference spectra ($\Delta I_\parallel$ minus $R\Delta I_\perp$) of dry films of sarcoplasmic reticulum and extracted SR phospholipids. (Panel A) Sarcoplasmic reticulum dry thin film (approx. 7.5 μg microsomal protein/ml) stabilized in the Ca_2-E_1 state with approx. 10^{-5} M Ca^{2+}. (Panel B) Sarcoplasmic reticulum dry film (approx. 7.5 big microsomal protein/ml) stabilized in E_2-V state with 0.5 mM EGTA and 1 mM vanadate. (Panel C) Dry film of extracted sarcoplasmic reticulum phospholipids. The subtraction factor R was varied from 1.3 (top trace in each panel) to 2.0 (bottom trace in each panel) with 0.1 unit increments. The compositions of media and the preparation of the dry film on Ge ATR plate are described under Methods.


Microns
5.0 5.2 5.4 5.6 6.0 6.5 7.0 7.5 8.0 8.5 9.0 10.0
A Ca_2-E_1
1046
1033
R
1.3
0.05
0.0
AMIDE II
AMIDE I
2.0
B E_2-V
1046
1033
R
1.3
0.05
0.0
AMIDE II
AMIDE I
2.0
C phospholipids
1033
1046
R
1.3
0.04
0.0
2.0
Absorbance
2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000
Wavenumber

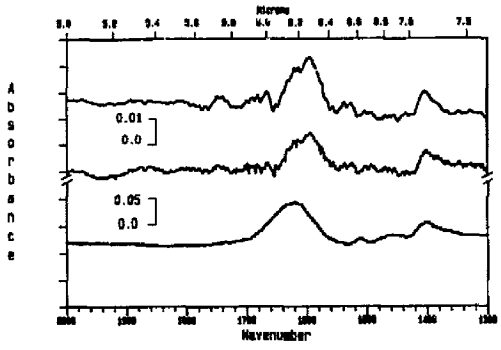


Fig. 7. ATR polarized infrared difference spectra of sarcoplasmic reticulum dry thin films (E_2-V minus $Ca_2 \cdot E_1$). Top trace: difference ATR polarized infrared spectrum (electric vector perpendicular to the film plane, transmitted) between sarcoplasmic reticulum films (approx. 7.5 μg microsomal protein/cm^2) in the E_2-V and Ca_2-E_1 states. Middle trace: difference ATR polarized infrared spectrum (electric vector parallel to the film plane) between sarcoplasmic reticulum films (approx. 7.5 μg microsomal protein/cm^2) in the E_2-V and Ca_2-E_1 states. Bottom trace: non-polarized difference ATR infrared spectrum of films of extracted sarcoplasmic reticulum phospholipids in E_2-V medium (5 mM EGTA and 10 mM vanadate) minus the same phospholipids in Ca_2-E_1 medium (no EGTA and vanadate). For other details, see Methods.

reticulum (Table II, samples A–C), but the mean angle of the transition dipole moment of CH_2 groups does not vary greatly. Using Eqn. 6 with a mosaic spread of 0.9 and the dichroic ratios given in Table II, the mean angles between the phospholipid hydrocarbon chain axis and the normal of the membrane plane are 43° for the films of extracted lipids and 46° for the films of sarcoplasmic reticulum. With a mosaic spread of 1.0 the calculated mean angle for the extracted phospholipid would change to 44°. Typical values for pure dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine range from 20 to 34° [9,42]. A random orientation would give a mean angle of 54°. This suggests that the disorder of hydrocarbon chains is greater in the complex mixture of sarcoplasmic reticulum lipids than in pure phospholipids. The Ca^{2+}-ATPase may also contribute to the disorder of the lipid CH_2 group orientation in sarcoplasmic reticulum films, although variations in the mosaic spread may be in part responsible for the observed differences.

The intensities of the difference spectra of trehalose bands located at 1033 and 1046 cm^{-1} were similar in extracted phospholipids (Fig. 6C) and in sarcoplasmic reticulum (Fig. 6A and B), suggesting that the orientations of trehalose molecules were not affected significantly by the presence of the Ca^{2+}-ATPase.

The dichroic ratios of sarcoplasmic reticulum films formed from solutions that are expected to stabilize the Ca_2-E_1 or the E_2-V states were similar (Table II, samples B and C and Fig. 6A and B). The slight difference between the dichroic ratios of the CH_2 groups on ZnSe crystals is of doubtful significance, since the corresponding difference was much smaller and of opposite sign in the data collected on Ge crystals. There were also slight differences in the dichroic difference spectra between sarcoplasmic reticulum films stabilized in Ca_2-E_1 and E_2-V states in the region of the amide I and amide II bands at 1550 and 1600 cm^{-1} (Fig. 6A and B). This difference was further investigated by comparing the difference ATR polarized infrared spectra of the sarcoplasmic reticulum in the Ca_2-E_1 and in the E_2-V states with the subtraction factor so adjusted that the main phospholipid and protein bands were cancelled (Fig. 7). Two positive bands were identified in the difference ATR polarized infrared spectra at 1610 and 1400 cm^{-1} (Fig. 7, two top traces). These bands belong to EGTA and there are no significant differences between Ca_2-E_1 and E_2-V states in the dichroic ratios of protein vibrations. Using Eqn. 7, the dichroic ratios given in Table II yield 61° and 60° for the mean angles of the protein C=O bond direction relative to the normal of the membrane plane, in the Ca_2-E_1 and in the E_2-V states, respectively.

Inorganic phosphate (5 mM) in the absence of calcium (0.5 mM EGTA) stabilizes the Ca^{2+}-ATPase of sarcoplasmic reticulum in the E_2-P state [40]. The difference ATR polarized infrared spectrum of Ca^{2+}-ATPase stabilized in the E_2-P state (not shown) was identical with those obtained in the E_2-V (Fig. 6B) and the Ca_2-E_1 (Fig. 6A) states, except in the 1200–1000 cm^{-1} region; the small differences in this region are attributed to the P–O vibrations of the inorganic phosphate.

Parallel polarized ATR infrared difference spectra of sarcoplasmic reticulum films made from solutions containing ~ 10^{-5} M versus 10^{-4} M Ca^{2+} and 25 mM vs. 100 mM KCl were featureless (not shown), implying that there was no significant difference in the infrared dichroism upon changing the Ca^{2+} concentration from 10^{-5} M to 10^{-4} M, or the KCl from 25 mM to 100 mM.

Influence of hydration on the orientation of the hydrocarbon chain

Polarized infrared spectra were taken successively during drying of a wet film of sarcoplasmic reticulum in the dry chamber of the FTIR spectrometer. The dichroic ratio of the phospholipid CH_2 band (2923 cm^{-1}) of fully hydrated sarcoplasmic reticulum deposited on germanium plate varied between 1.85–1.95, and decreased during drying to values between 1.60–1.69 (Fig. 8). This change in dichroic ratio suggests that the mean orientation order of the hydrocarbon chains and/or the relative population size of oriented versus random phospholipids increases during drying.

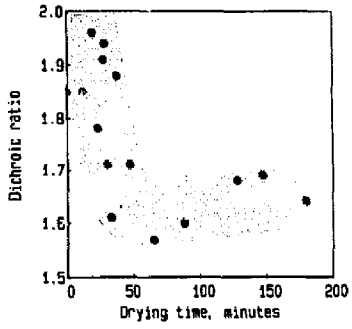


Fig. 8. The effect of hydration on the dichroic ratio of the $-CH_2$ group of the sarcoplasmic reticulum phospholipids. Dichroic ratios of the $-CH_2$ groups of hydrated sarcoplasmic reticulum film (approx. 7.5 μg protein/cm^2) on Ge plate in the Ca_2-E_1 state. For compositions of the medium and preparation of wet films, see Methods. The zero time indicated in the abscissa corresponds to the time when the wet sarcoplasmic reticulum film was placed in the IR spectrometer chamber. Successive dichroic ratios were determined during the drying of the sarcoplasmic reticulum film in the spectrometer.

Polarized infrared spectra of microcrystals of Ca^{2+}-ATPase

Microcrystals of Ca^{2+}-ATPase develop in detergent-solubilized sarcoplasmic reticulum in media containing 20–40% glycerol and 20 mM Ca^{2+} [23,24,43]. The high (40%) glycerol concentration favors the formation of large extended crystalline sheets (Fig. 1D and E) consisting of four to six membrane layers (Fig. 1D and E, and Ref. 43). ATR infrared spectra of microcrystals of Ca^{2+}-ATPase recorded on ZnSe crystals with electric vector polarized parallel (full line) or perpendicular (dashed line) to the normal of the ATR plane are shown in Fig. 9. The dichroic ratio of the amide II band of the Ca^{2+}-ATPase microcrystals was

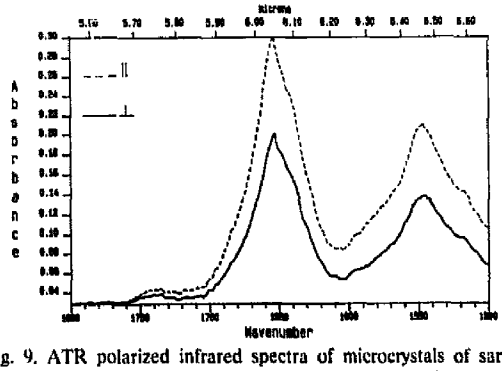


Fig. 9. ATR polarized infrared spectra of microcrystals of sarcoplasmic reticulum. ATR infrared spectra of microcrystals of sarcoplasmic reticulum (approx. 6 μg protein/cm^2) on ZnSe plates. For medium compositions and preparation of microcrystals of Ca^{2+}-ATPase, see Methods. The dashed and full lines correspond to the electric vector of the transmitted light polarized parallel and perpendicular to the normal of the film plane, respectively. The spectra were corrected for solvent contribution.

1.46 ± 0.15. This value is closer to the dichroic ratio of thin SR films (1.32) than to the corresponding value of thick SR films (1.71) (Table II). The intensity of the amide I band could not be determined accurately, and for this reason the dichroic ratio associated with the amide I vibration was not evaluated. The band centered at 1739 cm^{-1} corresponding to the native phospholipids is still present in the infrared spectrum (Fig. 9), indicating that the solubilization with detergents was not sufficient to remove completely the native phospholipids. Comparison of the intensity ratios of the phospholipid carbonyl band (1738 cm^{-1}) and the amide II band (1545 cm^{-1}) in films made from microcrystals of Ca^{2+}-ATPase and from native sarcoplasmic reticulum suggest that $18 \pm 4\%$ of the native phospholipid content was still retained in the Ca^{2+}-ATPase microcrystals.

Discussion

Effects of drying on the structure and ATPase activity of the Ca^{2+}-ATPase of sarcoplasmic reticulum

Addition of polyhydroxy compounds such as trehalose, or SH compounds such as dithiothreitol to suspensions of sarcoplasmic reticulum vesicles provided nearly complete protection against loss of ATPase activity during drying into thin films. Omission of these protective agents resulted in irreversible inhibition of Ca^{2+}-ATPase activity (Table I), in accord with previous reports [32,33]. The protective effect of polyhydroxy compounds was also observed during other types of perturbations such as pressure treatment [29] and solubilization by detergents [23], particularly in media of low Ca^{2+} concentration. The degree of protection by polyhydroxy compounds depends on the thickness of the sarcoplasmic reticulum membrane film. Trehalose, even at a high concentration (12 μmol/mg microsomal protein), was not able to preserve full ATPase activity in very thin films containing only one to two layers. Addition of higher trehalose concentration was impractical due to its contribution to the infrared spectrum. The dichroic ratios of the amide I and amide II protein bands and of the C=O and CH_2 phospholipid bands are higher in thick films of sarcoplasmic reticulum (~ 40 μg protein/cm^2) than in thin films (~ 7.5 pg protein/cm^2) (Table II), suggesting differences in the orientations of the Ca^{2+}-ATPase and of the phospholipids.

These observations imply that the first layers of sarcoplasmic reticulum in direct contact with the surface may contain denatured Ca^{2+}-ATPase. Traces of metal ions on the crystal surface or deformation of ATPase molecules in contact with the Ge, ZnSe, or Si surface may contribute to this loss of ATPase activity. The possibility must also be considered that more water molecules may be trapped in the thicker layers,

assuring better preservation of ATPase activity. This possibility gains particular importance in view of the fact that decreasing the water content of the sarcoplasmic reticulum introduces changes in the structure of the membrane [44–46]. An argument against this interpretation is the observation that the infrared spectra of thick dry films continuously monitored in the dry atmosphere of the FTIR spectrometer did not indicate changes consistent with removal of significant amount of H_2O.

According to Crowe and his colleagues [32–34,47,48], the interaction between sugars, such as trehalose, and dry phospholipids may be due to the formation of hydrogen bonds between the hydroxy groups of the sugar and the polar groups of the dry phospholipids, mimicking the effect of water. The protective effect of dithiothreitol probably involves SH groups in similar hydrogen bonded interactions, but in addition, DTT also provides protection against oxidation of protein SH groups and phospholipids.

Evaluation of dichroic ratios and orientation parameters

The orientation order parameter F was estimated by means of equation 4, assuming that the axial distribution of the transition dipole moments with respect to the normal of the film plane is symmetrical and that the refractive index of the dry film is the same on the Ge and ZnSe ATR supports. The use of Eqn. 4 instead of Eqn. 3 presents two advantages. First, the determination of the order parameter F can be made without guessing the refractive index of the sample. Second, the order parameter F is determined by two sets of data obtained on two different supports, which provides greater reliability. After the determination of the order parameter, the refractive index of each corresponding infrared band can be estimated using Eqn. 3, allowing a check on the consistency of the dichroic ratios. A mean refractive index value of 1.68 ± 0.15 was estimated from all the data obtained on sarcoplasmic reticulum dry thin films. This value corresponds to the upper limit of the typical range of refractive indices of biomembranes (1.35 to 1.70) reported by several authors [9–18]. This result is important, since some of the assumptions made in establishing Eqn. 4 are difficult to validate on very thin films (< 15 μg protein/cm^2), where the refractive index of the air and of the bulk medium must also be taken into account [42].

Orientation of the CH_2 groups of sarcoplasmic reticulum phospholipids

The dichroic ratio of the CH_2 symmetric stretching vibration measured in films of extracted sarcoplasmic reticulum phospholipids deposited on germanium ATR plate was 1.56 (Table II); the corresponding mean angle between the phospholipid axis and the normal of the plane of the film determined by Eqn. 6 was 43°. For comparison a typical value for the dichroic ratio of pure dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine films is 1.1 and the corresponding mean angle between the phospholipid axis and the normal of the film plane is 26–33° [18,42]. The dichroic ratio of the symmetric CH_2 vibration of wet DPPC film on Ge support increases from 1.1 to about 1.4 during gel to liquid crystalline lipid phase transition [49], due to an increase in the disorder of the hydrocarbon chains. The relatively high values obtained on dry films of sarcoplasmic reticulum lipids ($= 1.56$) indicates a still greater disorder of the hydrocarbon chains, presumably because of the presence of highly unsaturated fatty acyl chains in a variety of lipid classes.

The main lipid phase transition of the sarcoplasmic reticulum shifts from below 0°C to 15°C during transition from the hydrated to the dry state [46]. The dichroic ratio of the phospholipid CH_2 groups measured in sarcoplasmic reticulum films deposited on Ge plates varies from ~ 1.90 in the hydrated state to ~ 1.65 during drying at room temperature. Since the lipid phase transition of the sarcoplasmic reticulum is relatively broad, some of this change may reflect a change in the ratio of phospholipids that are in the liquid crystalline and gel states. The most important contribution to the change in the dichroic ratio of the CH_2 vibrations during drying is likely to be, however, the transition from spherical vesicles in the hydrated state to the flattened lamellae in the dry state, that must involve a significant increase in the polarity of average acyl chain orientation.

The dichroic ratios of the CH_2 symmetric vibrations in sarcoplasmic reticulum membranes deposited on Ge crystals are 1.67 in the Ca_2-E_1 and 1.68 in the E_2-V state; these values are slightly higher than the dichroic ratio of 1.56 measured in extracted sarcoplasmic reticulum phospholipids, suggesting an increase in the disorder of the hydrocarbon chains when the Ca^{2+}-ATPase is present. The reported dichroic ratios of the CH_2 symmetric vibrations in membrane films of Na^+/K^+-ATPase on germanium plates were 1.60, 1.77 and 1.83 in media containing Tris, K^+ or Na^+, respectively [9]. They are in the same range as our values (1.67–1.68) on sarcoplasmic reticulum films. The unexpectedly high dichroic ratios of the CH_2 vibrations of the phospholipids in ion transport membranes may reflect the influence of the high concentration of ion transport ATPase proteins on the orientation of membrane phospholipids. Since the mosaic spread will moderately affect the calculated values of mean angles, but its precise contribution under the conditions of these experiments was unknown, the exact magnitude of the effect of Ca^{2+}-ATPase on the ordering of the lipid phase cannot be determined from our data.

Effects of ligands on the conformation of Ca^{2+}-ATPase

The dichroic ratios of the amide I and amide II protein bands corresponding to the Ca2-E_1, E_2-P and E_2-V states were similar. This implies that if there are any changes in the orientations of the vibrational modes of amide I and amide II induced by low calcium concentration (Ca^{2+}-E_1 state) or by addition of EGTA and either vanadate (E_2-V state) or Pi (E_2-Pi state), it cannot be readily detected by ATR polarized infrared spectroscopy. The dichroic ratios of the amide I band of the Na^+/K^+-ATPase were also similar (1.63–1.64) in Tris, Na^+ or K^+ media [9].

Orientation of trehalose

The 1033 and 1046 cm^{-1} trehalose bands were tentatively assigned to the C–C and C–O ring stretching vibrations by comparison with cellulose [50,51] and other sugars [36]. The dichroic ratio of 1.2 corresponds to a mean angle of $\sim 70°$ between the transition dipole moment of these groups and the normal of the sarcoplasmic reticulum film. Assuming that the mean distribution of the transition moment of the C–C and C–O bonds is within the plane of the ring, the 'plane' of the trehalose ring is at an angle of $\sim 70°$ to the normal of the sarcoplasmic reticulum film plane. This would imply that most of the trehalose molecules are located within the polar headgroup region of the phospholipids and do not penetrate significantly into the hydrocarbon chains. The similar polarized spectra of trehalose in films made from extracted sarcoplasmic reticulum lipids and from sarcoplasmic reticulum membranes indicate that the orientation of the trehalose molecules is not affected significantly by the presence of the Ca^{2+}-ATPase.

Acknowledgements

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